Chapter 5

The Structure and Function of Chromatin

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INTRODUCTION

Biochemical and genetic studies have produced a vast fund of knowledge concerning gene action and regulation in prokaryotes. In these organisms the DNA is exposed rather nakedly to the world, protected primarily by the cell membrane. In eukaryotes the DNA seems far better shielded, being enmeshed in histone and nonhistone proteins and sequestered behind both the cell and the nuclear membrane. These differences have led to a considerable degree of caution in the application of this knowledge of prokaryotes to problems of gene regulation in eukaryotes, and rightly so. There are, however, several observations which suggest that higher organisms may have picked up a number of fundamental genetic tricks from their lowly predecessors. It has frequently been suggested that eukaryotes must do things differently from prokaryotes, until proven otherwise. It may be prudent to reverse this line of thought and suggest that they do things the same until proven different. The following similarities suggest this. (1) The basic genetic dogmas concerning DNA replication, transcription, and translation are similar. (2) The genetic code is the same. (3) Both systems appear to make liberal use of cyclic AMP as a basic mediator for humoral or diffusible signals. (4) In both systems DNA synthesis may be controlled at membranes. (5) Both make use of different types of RNA polymerase and RNA polymerase cofactors. (6) Recent studies of polylysine binding to chromatin suggest the eukaryotic DNA may not be so thoroughly

enmeshed in protein as once thought. (7) The visualization of genes in action by electron microscopic techniques intimates that genes are spaced and read in a similar manner. And finally, (8) merely because the clustering of related genes is unusual in higher organisms is no reason in itself to totally discard the promoter-operator-repressor concept as a way of regulating single structural genes. This system has provided an immense amount of data concerning the manner in which proteins interact with specific DNA sequences to control the attachment and utilization of RNA polymerase. It is hard to imagine that eukaryotes, being presented with such a superb mechanism for controlling DNA transcription, would totally discard it and opt for something different. It is far more likely that they would build on to this solid foundation.

In this review I will take the preceding thoughts as license to draw freely on the lessons from prokaryotes in order to set up some fundamental principles and ways of doing things that may have relevance to higher organisms. Despite this, it is obvious that eukaryotes have many characteristics that are unique, such as chromosomes, nuclei, mitosis, meiosis, histones, repetitious DNA, heterodisperse nuclear RNA, and heterochromatin, to name a few. These are the features that make them so intriguing.

COMPOSITION OF CHROMATIN

The DNA of higher organisms is coated with histone and nonhistone proteins. The result is a deoxyribonucleoprotein (DNP) fiber which forms the basic unit of chromosome structure. Throughout this review this DNP fiber will be referred to as chromatin. Chromatin derived from metaphase chromosomes contains 13–17% DNA, 8–15% RNA, and the remaining 68–79% is protein.[96,330,331,428,585,588] One-half to three-fourths of the protein is extractable in acid and composed predominately of histones. Chromatin that is isolated from interphase cells contains less RNA (3–4%)[585,292] and thus proportionally more DNA (25%). Most of the chromatin that has been used in studies of template activity has been sheared to make it soluble in dilute salt solution. This process appears to remove some of the chromosomal proteins with the result that the percentage of DNA in sheared interphase chromatin rises to 35%.[43,585]

Metaphase vs. Interphase Chromatin

Because of the interest in the role of histones in chromosome condensation, the properties of histones isolated from metaphase chromosomes and from interphase chromatin have been compared. These studies have shown

that the content and types of histones in these two tissues are similar,[126,585] although the arginine-rich cysteine-containing histone III fraction may have more S-S interlinking in metaphase chromosomes than in interphase chromatin. This may also be true for some of the nonhistone proteins.[585] This could play a role in chromatin condensation during mitosis. A second difference was the observation that if chromatin from these two sources is extracted with 0.2 M HCl, significantly more acid-soluble nonhistone protein is extracted from metaphase chromosomes than from interphase chromatin.[585] This protein is not removed from the chromosomes by 0.2 N H_2SO_4. The role of this acid-soluble, but nonhistone protein present in metaphase chromosomes is unknown.*

A third distinction between metaphase and interphase chromatin concerns their content of RNA. A large amount of RNA on metaphase chromosomes appears to be due in large part to the adherence of ribosomal RNA to the surface of chromosomes[126a,292,330,428,588] following the breakdown of the nucleolous during mitosis. This problem is avoided in the isolation of interphase chromatin. In one report no RNA was found on metaphase chromosomes isolated in hexylene glycol.[769] The significance of the small amount of RNA in interphase chromatin is controversial (see the section on chromosomal RNA).

The possible role of repressorlike proteins in chromosome condensation is discussed later.

CHROMATIN STRUCTURE

Size of the Chromatin Fiber—Electron Microscopy

One might suppose that the question of the diameter of the chromatin fiber should be a straightforward problem with a straightforward answer. Unfortunately, this is not the case. Estimates of the size of the basic fiber range from 30 Å to over 250 Å. Fortunately, there is at least some degree of consistency in that the estimates tend to fall into four groups of over 300 Å, 250 Å, 100 Å, and 30–50 Å, and recent studies of the effect of various agents and methods of fixation are finally allowing a reasonably clear idea of how these different sizes relate to each other.

Over 300 Å

The water-spread technique for examination of chromosomes was first used by Gall in 1963.[244] Prior to that time a number of investigators had

* Much of it may be an artifact of preparation.[157]

used other techniques, such as touching the copper grid to various types of chromatin preparations. Many of the early estimations suggested a chromatin diameter of between 300 and 600 Å or more.[192,244,246,297,504,563,564,709] It is now apparent that most of these higher estimates were the result of poor fixation, excessive clumping of fibers, or contamination of the fibers resulting from prolonged exposure to the electron beam.[246]

250 Å Fiber

So many observers have reported that the size of the chromatin fiber in water spread preparations is around 250 Å (200–300 Å)[1,38,201–206,246,390,418,564,606,757–762] that this has frequently been referred to as *the* basic chromatin fiber. In these preparations unfixed chromosomes or chromatin fibers were allowed to spread on a surface of distilled water. They were then picked up on grids, dehydrated in ethanol, and critical point dried.[13] The absence of prefixation and the exposure to ethanol appear to be important variables.

100 Å Fiber

Despite the frequent appearance of the 250 Å fiber in water-spread preparations, there are many conditions in which a smaller 100 Å fiber has been repeatedly observed. These conditions include the observation of fixed and embedded chromatin by thin-section electron microscopy,[390,567,568,606,759,774] the examination of water-spread chromatin that has been prefixed in formaldehyde[604,758,759] or treated with chelating agents,[567] and the stretching of chromatin fibers.[205,390,757] Various interpretations have been placed on this observation. Wolfe and Grim[759] have suggested that there is a basic 100 Å fiber which swells or accumulates nuclear proteins during water spreading to attain a diameter of 250 Å. Ris[565–569] has proposed that the 250 Å fiber is composed of two 100 Å fibers as the result of a single fiber folding back upon itself, or two fibers adhering to each other. He has suggested that chelating agents, and fixatives which act like chelating agents, allow enough separation so the 100 Å fiber can be seen. The frequency with which the 250 Å fibers branch into two parts (Fig. 1) supports this proposal. In reality both interpretations have validity since the studies of Solari (see below) have shown that some of this variability in width is the result of ethanol fixation and the accumulation of proteins during water spreading.

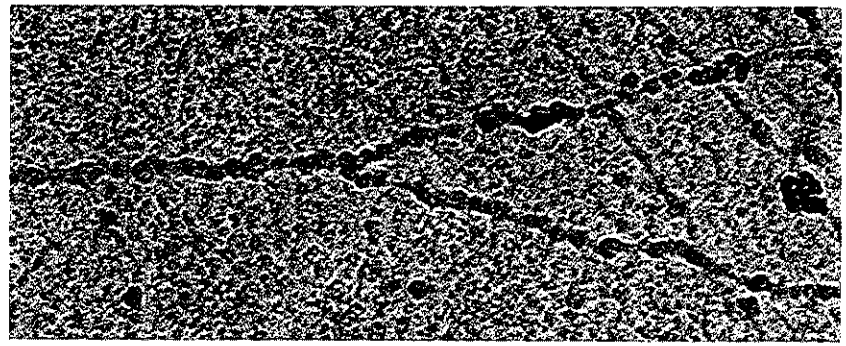

Fig. 1. A water-spread preparation of mouse mitotic chromosomes showing a bifurcation of the chromatin fiber. The diameter of the fibers distal to the fork is not significantly less than proximal to the fork, indicating the ease with which two fibers can meld into one. × 124,000.

30-50 Å Fiber

Although there is much evidence to suggest a basic 100 Å fiber, there is equally strong evidence for a second fiber size of 30–50 Å. This is inherently believable since electron microscopy of DNA shows a 20 Å fiber and when a preparation of purified chromatin is sprayed on a grid and air dried without exposure to ethanol, its width is 30 Å.[787] The 30 and 100 Å fibers seem to be interconvertible, depending on the degree of coiling[277,390,510] (see below).

The 30 Å fiber can be seen under the following conditions. Exposure of sea urchin sperm chromatin to EDTA,[639–641] stretching of the larger chromatin fiber,[56,205,390] high voltage electron microscopy of metaphase chromatin,[122] shearing of purified chromatin,[277,259] and careful examination of enlarged pictures of embedded and thin-sectioned interphase and metaphase chromatin.[55,390,604] In an interesting study of chromatin fibers, Solari[642] showed that in EDTA-treated chicken erythrocyte chromatin freed of hemoglobin, water spread, and then examined by negative staining without exposure to ethanol, the mean fiber width was 37 Å ± 13 Å. If this chromatin was treated with ethanol, and critical point dried in amyl–acetate, an integral part of all water-spreading techniques, the mean fiber diameter rose to 138 ± 48 Å. If the nuclei were floated on a surface containing hemoglobin, the mean fiber diameter was 313 ± 64 Å. Observations such as these point out the potentially important role of technical variables in the study of chromatin fibers, and raise the intriguing possibility that the 30 Å fiber may be the true width of most chromatin and the higher estimates artifacts of

preparation. The major alternative to this is that the higher values represent a true physiologic state of increased coiling of the 30 Å fiber. Although most evidence favors the latter explanation, this problem begins to resemble the uncertainty principle in physics which questions the validity of some constants, since the very processes involved in taking the measurement may alter the parameters measured.

The Genetically Active Fiber

The problems of transcription would seemingly be simplified if genetically active chromatin was the simple 30–50 Å fiber rather than the supercoiled and more complex 100 or 250 Å fiber. There are several lines of evidence suggesting that this is the case. Perhaps the most convincing is the demonstration that the width of the chromatin fiber in the loops of lampbrush chromosomes, which are vigorously synthesizing RNA, is 30–50 Å,[458,459] and in preparations of amphibian nucleoli 125 Å granules representing RNA polymerase can be seen aligned on a small DNP fiber about 50 Å in width.[459] These preparations are prefixed in formaldehyde, thus avoiding the problems of subsequent exposure to ethanol.[642] Another source of genetically active chromatin is *Tetrahymena*. The DNA of the macronuclei of this protozoan contains histones similar to those of calf thymus.[398] Nonhistone proteins are also present.[277] Despite the possession of chromatin, this organism is similar to bacteria in showing rapid growth, having no chromosomal structures in the amicronucleolate strain, and undergoing no differentiation. These properties in association with its small DNA content suggest that a significant part of its genome is genetically active.[277] This is confirmed by DNA-RNA hybridization studies which imply activity of some 38% of the genome.[277] Electron microscope studies of *Tetrahymena* nuclei lysed directly on grids show long uniform fibers 30 Å in width and none larger than 100 or 250 Å.[277]

The observation that 110 Å chromatin fibers change to fibers of much smaller diameter (30–40 Å) in the region of the replication band in the macronucleus of *Euplotes*[377,562] suggests that a reduction to the basic 30 Å fibers may occur with both transcription and replication.

Relationship Between the 30 and 100 Å Fiber

On the basis of examination of high magnification views of Epon-embedded and thin-sectioned material, Davies[180] reported a hollow rod 150 Å in diameter in chicken erythrocyte nuclei, and Lampert and Lampert[390]

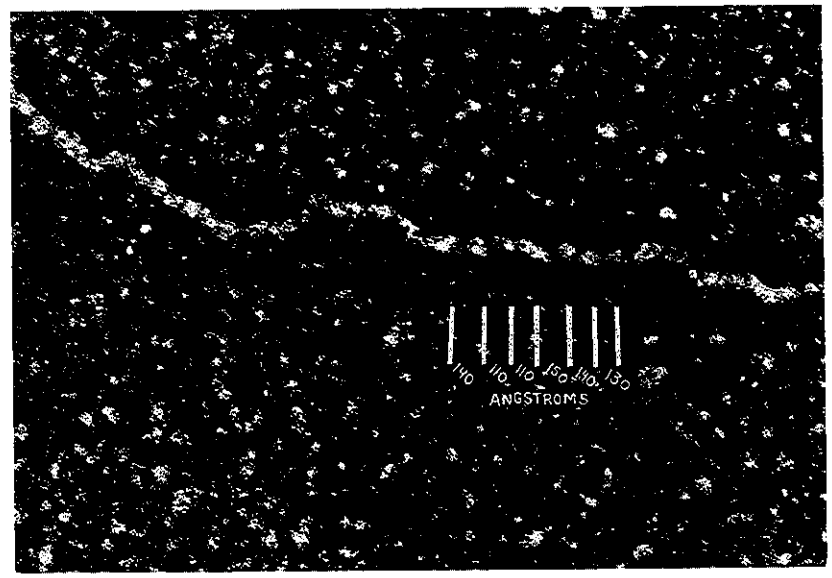

Fig. 2. Supercoiling of the 30 Å nucleohistone fiber. The apparent coiling has a repeat distance of 100–150 Å. Fibers which appear to have been stretched during mounting may show a larger repeat distance or no coiling at all. (Courtesy of Dr. Jack Griffith, *J. Mol. Biol.*, in press).

have suggested a 70–80 Å hollow tube formed by the secondary coiling of the basic DNP fiber. They further suggested that a 200–300 Å fiber was formed by the tertiary coiling of the 70–80 Å fiber. A fine structural demonstration of the relationship between the 30 and 100 Å fiber is the electron micrography by Griffith[277] (Fig. 2) of a 100 Å thick chromatin fiber from chicken erythrocyte nuclei which is formed by the coiling of a basic 30 Å DNP fiber. The distance between successive coils was 100–150 Å. This conforms remarkably well with X-ray diffraction data (see below).

X-Ray Diffraction

One of the principal alternatives to electron microscopy for the study of chromatin structure is the use of X-ray diffraction.[224,419,509,510,555,746–748,786] A feature of the diffraction pattern of chromatin that is not present in DNA or histone alone is a series of low-angle diffraction rings at 105, 55, 35, 27, and 22 Å (Fig. 3). Numerous interpretations have been placed on these results. Pardon *et al.*[510] suggest that a 30 Å DNP fiber is coiled into a superhelix with an intercoil distance (pitch) of 120 Å and a diameter

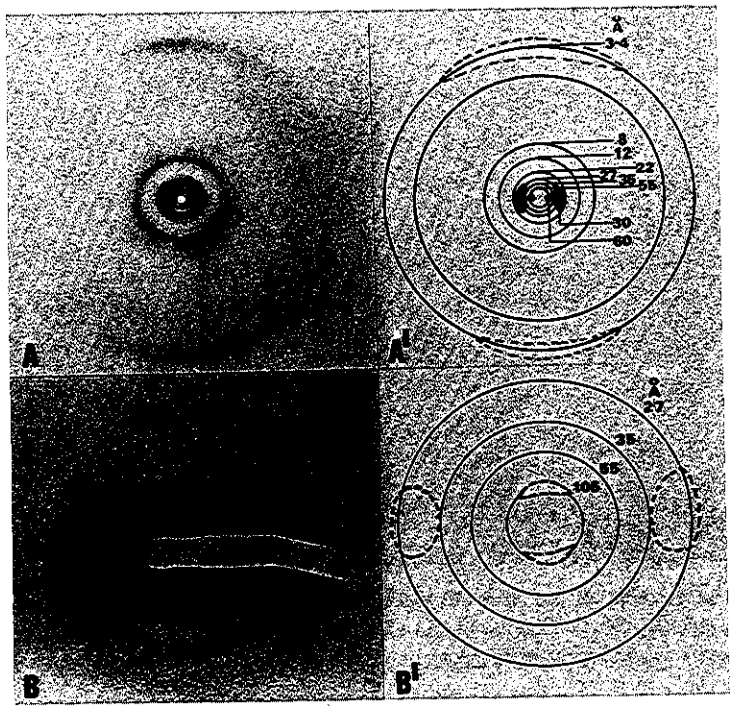

Fig. 3. X-Ray diffraction patterns of native DNP. A and A′ illustrate the high angle diffraction pattern from calf thymus DNP at 98% relative humidity. B and B′ illustrate the low-angle diffraction pattern in which the rings from the supercoiled DNP fiber are recorded.
The rings can be divided into three groups. (1) Reflections from the DNA component including the 3.4 Å ring arising from the base stacking repeat distance, the 8 and 12 Å rings arising from the third and second layer lines in the DNA pattern, and a 30 Å ring arising from the intermolecular side-by-side packing. (2) The 60 Å ring represents lipid contaminant (in A only). (3) The DNP supercoil rings including 22, 27, 35, 55, and 105 Å. (From Richards and Pardon,[555] by permission.)

of 100 Å. Further evidence for such a structure was provided by studies of stretched and unstretched chromatin (Fig. 4). At 92% relative humidity, in unstretched chromatin, the low angle rings at 105, 55, 35, 27, and 22 Å were present and the reflections characteristic of pure DNA were poorly oriented as would be expected if the DNA was secondarily coiled. When the chromatin was stretched there was improved orientation of the DNA reflections and loss of the low-angle rings. Upon further wetting and relaxation, the pattern returned to its previous poor orientation and the low-angle

rings reappeared. Further studies also showed that chromatin which has been reconstituted by mixing undenatured histones with DNA shows the same diffraction pattern as native chromatin.[555]

The low-angle diffraction rings can also be made to disappear in a salt range of 1.0–1.3 M NaCl[53,555] when 80% of the histones have been removed. The fact that most of the lysine-rich histones are dissociated before this salt range, plus the observation that reconstitution experiments using lysine-rich F_1 histone alone do not give the supercoil configuration, suggests that F_1 histones are not involved in forming the supercoil.[555]

On the basis of X-ray diffraction studies of DNA, combined with protamine (nucleoprotamines), it has been suggested that the protamine wraps around the DNA molecule in either the small groove or in both grooves.[224,746] This proposal in terms of DNA and poly-L-arginine is illustrated in Fig. 5. Even if it should be incorrect in some details, this picture allows an appreciation of the relative size of the protamine and histone molecules in relation to the large and small groove of DNA. In this proposal all arginines of protamine would be combined with phosphate groups of DNA, and nonbasic residues would be introduced as loops. A single nonbasic amino acid cannot form a loop. It would only be possible to form such loops with two adjacent nonbasic residues. It is of interest that sequence analysis of protamines[14,15] shows the nonbasic amino acids occurring mostly in pairs.

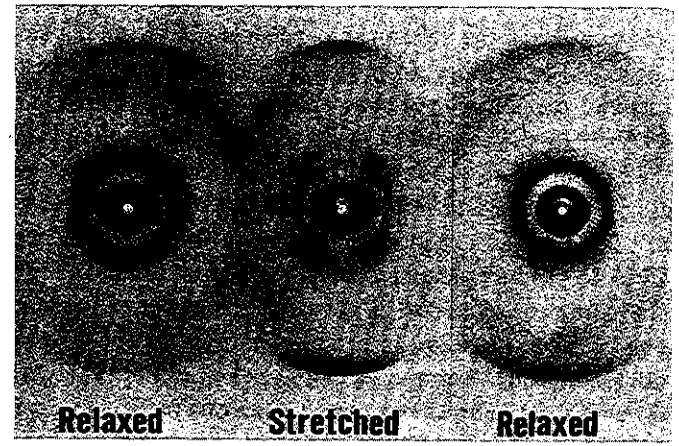

Fig. 4. X-Ray diffraction patterns of relaxed, stretched, and relaxed calf thymus DNP at 92% relative humidity. The low-angle rings representing the supercoiling of the DNP fiber disappear in the stretched fiber and reappear in the relaxed fiber. (From Pardon *et al.*[510] by permission.)

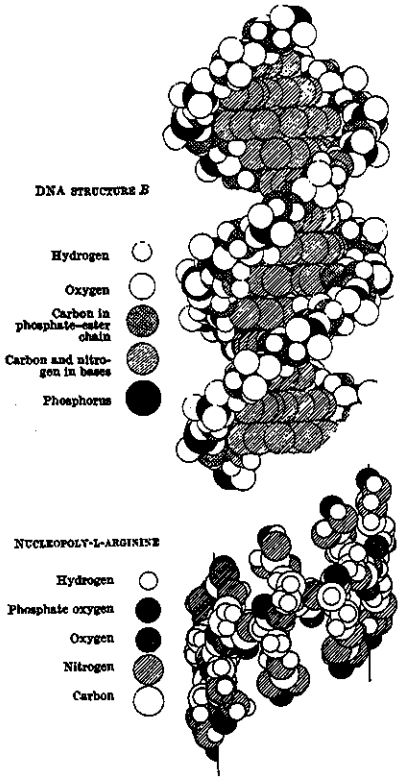

Fig. 5. Diagrammatic illustration of the relative size of the large and small grooves of DNA and basic proteins (polyarginine). In this illustration the polyarginine has been wrapped around the DNA helix. (From Feughelman *et al.*[224] by permission.)

Several models of the relationship between DNA and histones have been proposed. These range from the suggestion that the histones are wrapped around the DNA in a manner similar to that envisioned for the protamines[224,746] to the suggestion that histone may lie diagonally across the large (18 Å) or small (12 Å) groove of two different DNA molecules, thus holding them in register in a chromatin fiber composed of two DNA molecules.[748,786] The most popular proposal is that the histones interact with the DNA to produce a supercoiled 100 Å chromatin fiber.[510,555] Knowledge of the complete sequence of some of the histones has allowed the introduction of more precision into considerations of the relationship of histones to DNA. For example, arginine-rich histone IV (F2a1) is 102 residues in length. Its complete sequence is shown in Fig. 6. From the N-terminal to the 45th residue the molecule is very basic; the remaining portion is relatively acidic and hydrophobic and contains two zones at residues 57 to 74 and 80 to 90 which are probably in an α helix configuration. On the basis of model building and X-ray diffraction studies, Richards and Par-

don[555] (Fig. 7), have suggested that the basic portion is extended and lies in the large groove of the DNA occupying four to five turns of the helix, while the remainder of the molecule forms two loops extending away from the DNA with some reattachment to the large groove at the basic residues 77 to 79, 91 to 92, and 95. The latter may serve to help form the supercoiling.

Despite the increasing precision of these models, many further studies will be necessary to provide a complete and convincing picture of how the different histones relate to DNA and to each other.

Other Evidence for Secondary Coiling

Comparative studies of DNA and DNP by sedimentation velocity,[263,787] flow birefringence, and flow dichroism[488,787] indicate that there is a significant shortening of DNA in chromatin. Both an ordered coiling or a disordered folding could give this result.

Alteration of DNA Structure in Chromatin

Because of its relevance to the ability of proteins to either repress or derepress genetic activity, the question of whether the interaction between chromosomal proteins and DNA results in a change in the structure of DNA is of some importance. Optical rotary dispersion and circular di-

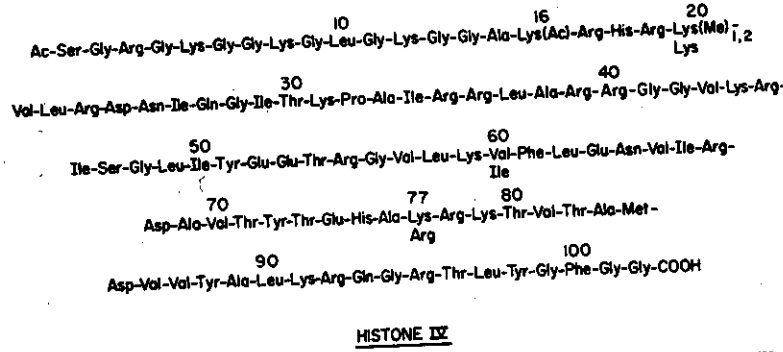

Fig. 6. Comparison of the amino acid sequences of calf thymus and pea seedling histone IV. The continuous sequence is that of the calf histone, with the pea histone residues that are different shown below at 60 and 77. The 20 Lys is methylated (mono- or dimethyl lysine) in calf histone but is unmethylated in pea histone. In calf histone residue 16 in acetylated to the extent of 50% and this is the only lysine so changed. Only about 6% of the pea histone molecules are acetylated and the affected residues include 16 and at least one of three others—5, 8, and 12. (From Delange *et al.*[188] by permission.)

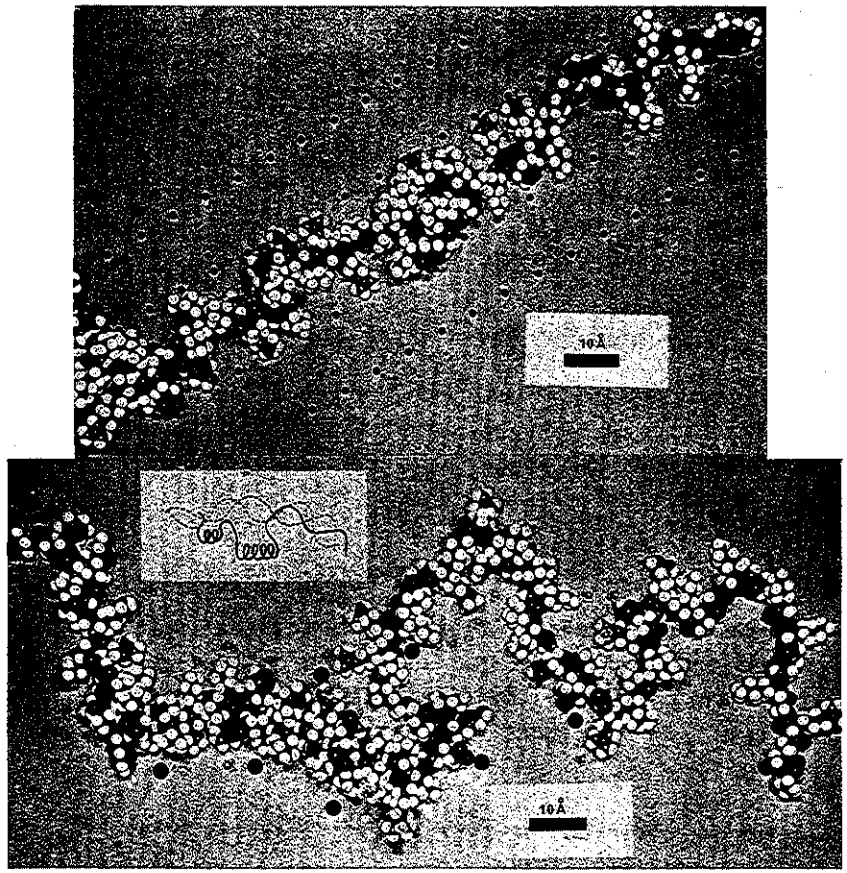

Fig. 7. Model building of histone f2a1 (arginine-rich). Above: The basic region of the histone displayed on the phosphate groups of supercoiled DNA. The maximum bonding of histone to DNA in this configuration is 60%. Below: A configuration of the molecule with two α-helix regions, and a possible configuration of the molecule attached to DNA. (From Richards and Pardon[555] by permission.)

chroism studies of chromatin and DNA complexed with specific histones or with poly-L-lysine suggest that the DNA in chromatin may have a larger tilting of the bases,[124,223,617,707] or a different dimension of the helix.[223] Changes in the DNA conformation due to a supercoiled tertiary structure rather than as a direct result of the protein were not excluded.[124,617] On the basis of their studies of various DNA-protein complexes, Tuan and Bonner[707] concluded that neither lysine-rich histones nor nonhistone proteins were responsible for the altered conformation of DNA in chromatin.

Alteration of Histone Structure in Chromatin

The converse situation of alteration of the structure of histone upon binding with DNA also seems to occur. On the basis of circular dichroism studies of histone and histone-DNA complexes, Wagner[716] concluded there was an increase in the helical content of the arginine-rich histone F2a1 from 10 to 23% on interaction with DNA. It also changed its configuration on interaction with another polyanion, polyvinylphosphate (PVP). Similar studies of lysine-rich histones showed no change.[501]

Studies of the change in conformation of histones upon binding with DNA have also been carried out by determining alterations in proton magnetic resonance.[51] These results suggest that the amino half of histone F2b, which is rich in basic residues, becomes stabilized by interaction with DNA while the carboxyl half, which is rich in acidic residues, becomes more labile. By contrast, the entire lysine-rich F1 histone was immobilized by its interaction with DNA. This suggests that while part of the F2b histone is free for other interactions, this is not the case for histone F1.

The possible role of alteration in the structure of nonhistone proteins upon their interaction with DNA is discussed later.

How Much DNA is Covered with Protein?

The above electron microscopy and X-ray diffraction studies, in conjunction with biochemical observations which indicate an approximate equality of histone basic groups and DNA phosphate groups, leave the impression that the DNA of chromatin is entirely ensheathed in protein. Three lines of evidence reported by Clarke and Felsenfeld[120] tend to suggest that this is not the case.

Digestion of Chromatin with Deoxyribonuclease

If calf thymus chromatin is digested with deoxyribonuclease, approximately 54% of the DNA is rendered acid soluble and 46% is protected by protein and resistant to the enzyme.* The fact that only 1.5% of the chromatin proteins are released suggests that the digested regions were uncovered. The mean molecular weight of the protected regions was 105,000 daltons, equivalent to approximately 175 base pairs, significantly less than the length of an average gene. The digested material had the same base composition as the undigested DNA, indicating that AT- or GC-rich regions

* See also Ref. 463a.

were not involved in the binding of protein to the protected regions. There was also no difference in renaturation kinetics, indicating the protein was not preferentially bound to repetitious (or nonrepetitious) regions.

Titration with Polylysine

The extent of naked DNA in chromatin can also be studied by utilizing a model based on the observation that polylysine binds tightly to DNA, and when all the binding sites are covered with either endogenous protein or polylysine, the DNA precipitates.[120] When purified calf thymus DNA is combined with polylysine, it precipitates at a lysine–nucleotide molar ratio of 1:1. However, when lysine is combined with calf thymus chromatin it precipitates at a molar ratio of 0.51:1, suggesting that 50% of the sites are already occupied but that the other 50% are free. At this ratio there is no significant displacement of endogenous chromosomal proteins. The use of poly-*d*-lysine, which is resistant to pronase, allows the isolation of the naked DNA. This is accomplished by complexing it with poly-*d*-lysine, removing the chromosomal proteins with pronase, and digesting the exposed DNA with DNase.

Utilizing a similar technique but titrating at higher chromatin concentrations, and using chromatin with a somewhat higher protein content, Itzhaki[336] concluded that 38% of the chromatin of DNA was free for polylysine binding.

Exchange of Chromosomal Proteins

To study the possibility that some of their findings were the result of the exchange of chromosomal proteins from one site to another, Clarke and Felsenfeld mixed pure tritium-labeled calf thymus DNA with unlabeled calf thymus chromatin. If exchange occurred it would be possible to recover some tritium counts in the DNA-protein pellet. Under the conditions of the DNase studies there was only 1.3% exchange of protein. At higher salt concentrations (0.8 M NaCl) the protein is freely exchanged.

Interpretation

Although these results seem to clearly indicate that only about half of the DNA is protected by protein, the meaning of these results is far from clear. Widely diverging interpretations can be suggested. These range from the conclusion that previous studies, which have implicated histones or nonhistone proteins as repressors of gene activity, are incorrect,[212] to the opposite extreme that these findings do not significantly change any of the

previous conclusions about chromatin function. The crux of the matter lies in the question: What does the tightly bound protein cover? If it covers all the promoter regions, then histones and nonhistone proteins will still have to be removed or inactivated to allow transcription, and the previous conclusions about the role of chromosomal proteins are still valid. If all promoter regions are in the free parts, then chromosomal proteins do not have to be removed to use the genes. Based on the knowledge that repressor proteins in *E. coli* bind to promoter regions, this is extremely unlikely. An intermediate ground of some genes covered and some uncovered somehow seems equally unsatisfactory.

These results are not inconsistent with the 1:1 ratio of histone basic residues to DNA phosphate groups in view of the results of sequence data which suggest that only about half of the molecule of most histones is suitable for binding to DNA. If the conclusions that upwards of 80% of the DNA of mammals is junk is valid, these results are interesting in that they indicate that even if DNA is not used, it must still be covered with proteins, possibly in part to protect the chromosomes from nuclease attack.

Finally, the observation that bacterial DNA is associated with some nonhistone proteins,[548] and eukaryotic DNA is less associated with protein than originally thought, again suggests that the prokaryote and eukaryote systems may not be all that different.

The Packing Ratio

The packing ratio refers to the ratio of the length of a segment of DNA to the length of the chromatin fiber it is packed in. It is obviously dependent upon whether the chromatin is in the form of a 30, 100, or 250 Å fiber. Utilizing simple model building with wire and calipers, it can be seen that the packing ratio of the 30 Å fiber is 1:1; that of a 100 Å fiber produced by a supercoiled 30 Å fiber with an intercoil (pitch) distance of 120 Å is 2.1:1, and that of a 100 Å fiber produced by a supercoil of a 30 Å fiber with the minimal possible intercoil distance of 30 Å, is 7.5:1. With maximal tightness of the coiling, four lengths of 100 Å fiber can be coiled into one length of a 250 Å fiber. Given these figures, if we accept the model of the 100 Å fiber as a coiled 30 Å fiber with a 120 Å pitch, then the packing ratio of a 250 Å fiber made up of two parallel 100 Å fibers would be 4.2:1, and that of a 250 Å fiber made up of a tightly coiled 100 Å fiber would be 4×2.1 or 8.4:1. If we discard the 120 Å pitch model and opt for maximum coiling of the 30 Å DNP fiber into the 100 Å fiber, then the maximum possible packing ratio for the 250 Å fiber is $7.5 \times 4 = 30{:}1$.

Returning to the 120 Å pitch model, with a packing ratio for the 250 Å fiber of 8.4:1, we can ask if this is adequate to account for the degree of packing seen in a metaphase chromosome. If we take an average human number 13 chromosome 5.8 μ long by 0.8 μ thick (uncoiled),[498] and containing DNA that is 32,800 μ long,[21] we can make some crude calculations. Observations of whole-mount preparations of human chromosomes provide a rough estimate that there may be approximately 100 fibers in a cross section of an *undispersed* chromatid. Since the fibers are looped and folded and show no preferential longitudinal orientation,[149,150,152] this would represent $5.8/0.8 \times 100$ or 725 fibers on cross section from telomere to telomere. This calculation suggests a total lengthwise fiber length of 100×5.8 μ or 580 μ, and a total transverse fiber length of 725×0.8 or 580 μ for a total chromatin fiber length of 1160 μ with a packing ratio of 32,800/1160 or 28:1. If the coiling of the chromatid itself is taken into consideration, one could just about get 32,800 μ of DNA into the number 13 chromosome with a 250 Å chromatin fiber that had a packing ratio of 8.4:1. These calculations present one extreme.

The other end of the scale has been provided by results based on quantitative electron microscopy. With this technique, DuPraw and Bahr[205] suggested a packing ratio of 50:1 to 100:1 for the 250 Å fiber, and recently Bahr[21] has increased it still further. With the same number 13 chromosome discussed above, he suggests it contains only 218 μ of chromatin fiber with a packing ratio of 150:1. To accommodate a packing ratio of this degree it is necessary to give the chromatin fiber yet a third supercoil. The disturbing feature about this is that to do so requires a chromatin fiber 455 Å in diameter, somewhat above the usual range, and even this requires an effective DNA diameter of only 14.4 μ.[21] The main exit from this dilemma is the suggestion that possibly in the quantitative technique the length of chromatin fiber in the chromatid is underestimated. Since both extremes are potentially capable of packing the DNA into the chromosome, perhaps the truth lies somewhere between the second and third coil of the basic 30 Å fiber.

A Model of Chromatin Structure

Figure 8 presents a summary of the above considerations of chromatin structure based on electron microscopy, X-ray diffraction, and other evidence. It progresses from a DNA helix of 20 Å diameter to an uncoiled 30 Å DNP fiber, to a 100 Å fiber produced by supercoiling. To this point there is a reasonable degree of agreement. To cover all alternatives, the

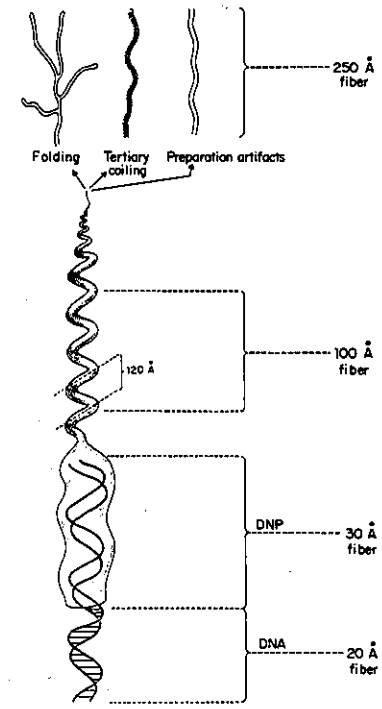

Fig. 8. The structure of the deoxyribonucleoprotein fiber. At the bottom is the 20 Å DNA fiber. When covered with histone and nonhistone proteins, it forms the basic 30 Å DNP fiber of genetically active chromatin. The scale of the diagram is then changed to illustrate the formation of the 100 Å fiber by supercoiling of the 30 Å fiber with a 120 Å pitch distance. The scale is then changed again to illustrate the three ways in which the 250 Å fiber might be formed: (a) by folding, (b) by coiling again, or (c) by adsorbing proteins or other artifacts.

250 Å fiber may result from two 100 Å fibers folded upon themselves; it may arise from an additional supercoil of the 100 Å fiber; or it may in part be an artifact of fixation and exposure to exogenous protein. Perhaps the last is least likely since it would imply the 100 and 250 Å fiber had the same packing ratio, and significantly higher radios than this must be attained to get the DNA packed into a chromosome.

Direct Visualization of Gene Action

Utilizing a technique which involves centrifuging chromatin or DNA through a sucrose-formalin solution directly onto an electron microscope grid, Miller[457–461] has produced some remarkable photographs of genes in action. A study of the nucleolar chromatin produced by gene amplification during the pachytene stage of meiosis in *Xenopus* and *Triturus* provided the following observations.

1. Nucleolar gene amplification results in circles of nucleolar chromatin ranging from 35 to 5000 μ in length and containing from 8 to 1000 sets of nucleolar genes.

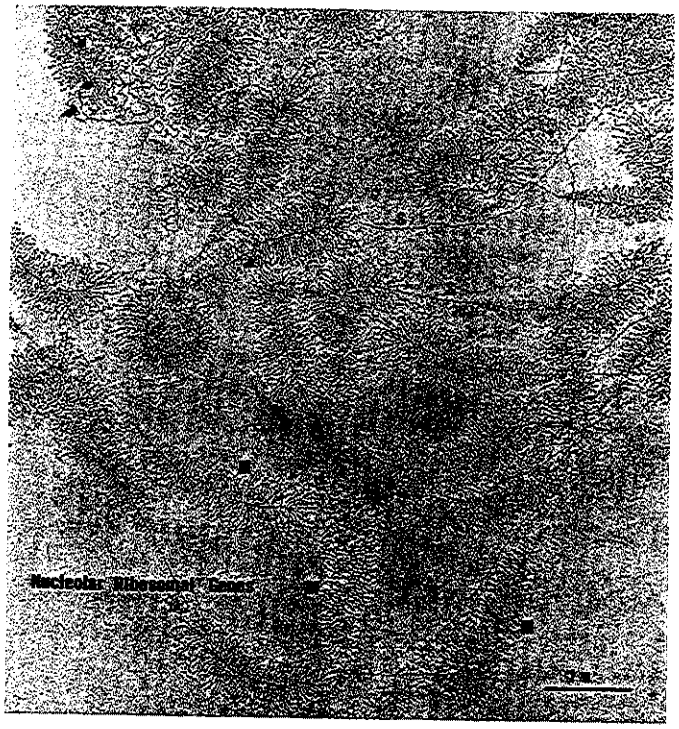

Fig. 9. Nucleolar ribosomal genes from *Triturus viridescens* oocyte. The ribosomal RNA genes are repeated along the nucleolar DNA and can be visualized because approximately 100 RNA polymerase molecules are simultaneously transcribing each gene. The matrix (M) is composed of ribosomal RNA in progressive stages of completion. Spacer DNA (S) between the genes is not transcribed. × 25,000 (From Miller and Beatty, *Science* **164**: 955, 1969, by permission.)

2. During transcription the chromatin appears as a 30–50 Å core along which are arranged matrix and matrix-free regions (Figs. 9 and 10). The matrix consists of about 100 fibers attached at one end to the core. There is a polarity to the fibers such that at one end of the matrix they are short and progressively increase in size toward the opposite end. The direction of the gradient is the same in successive matrices.

3. Results of staining and enzyme digestion suggest that each fiber is an RNA molecule which is coated with protein immediately after transcription. Since the length of the completed ribonucleoprotein fiber is 0.5 μ, and contains 6 μ of RNA, the packing ratio of RNA into RNP is 12:1.

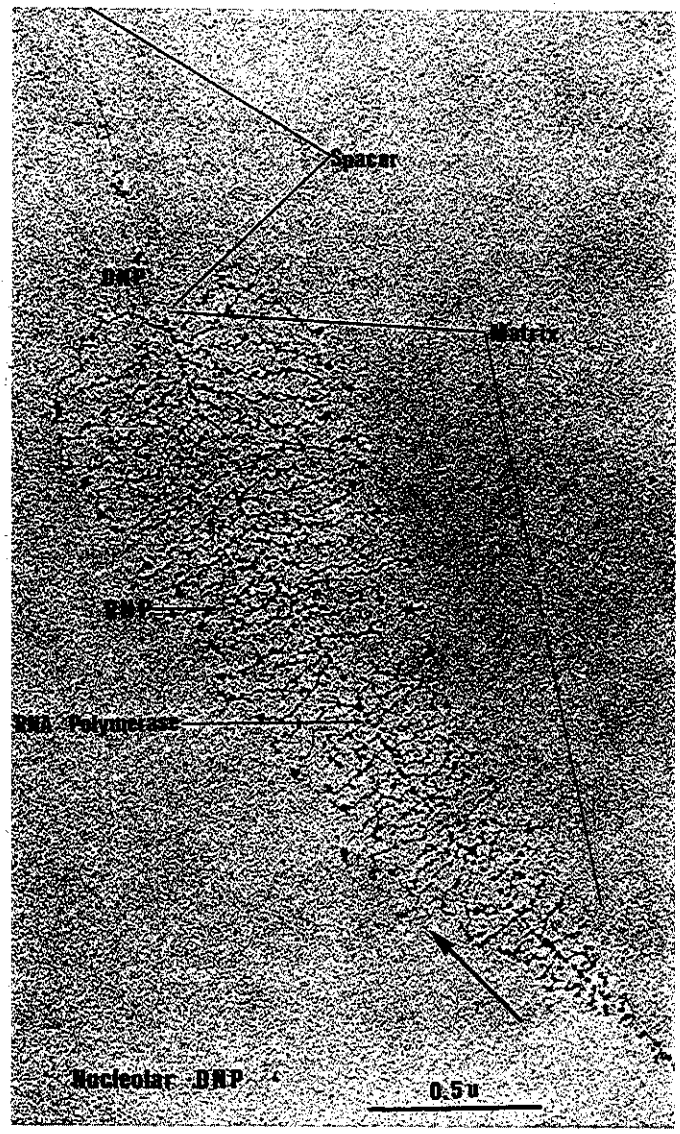

Fig. 10. Enlargement of a single *Triturus* nucleolar gene. The RNA polymerase molecules can be seen attached to the DNA and transcribing ribosomal RNA. The arrow indicates the direction of transcription. Some RNA polymerase molecules may remain on the non transcribed spacer portion of the DNA. (From Miller and Beatty, *J. Cell Physiol.* **74**: Suppl. 1, 225, 1969, by permission.)

4. The matrix length averaged 2.5 μ and the length of the spacer was usually about one-third of this, but some spacers were up to 10 times or more the length of the matrix.

5. At the point of attachment of the RNP to the core there was a 125 Å granule. The size and location is consistent with this being RNA polymerase. Similar granules were present on the spacer regions even though they were not transcribing RNA. Miller suggests that the RNA polymerase may continue to travel the core after the gene is transcribed and it may thus encounter the following sequence: initiate-read-terminate-travel-initiate. The presence of up to 100 RNA polymerase molecules, each with its separate newly synthesized RNP molecule, indicates that the genes can be simultaneously transcribed to produce many RNA copies. This, combined with

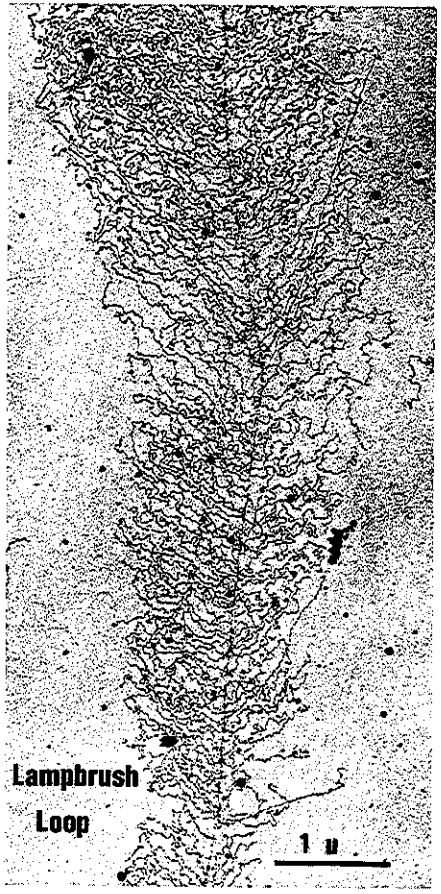

Fig. 11. A lampbrush loop from a *Triturus viridescens* oocyte, showing the central DNA axis with a gradient of RNP fibrils. (From Miller and Beatty, *J. Cell Physiol.* 74: Suppl. 1, 225, 1969, by permission.)

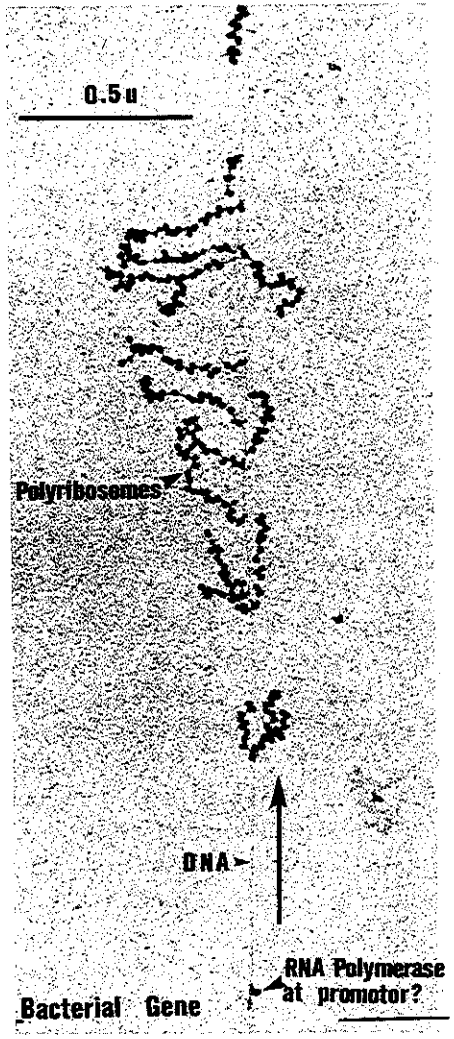

Fig. 12. Visualization of transcription of an *E. coli* structural gene. As the RNA is synthesized, ribosomes immediately attach to it to form polysomes. The arrow indicates the direction of transcription. The single distal RNA polymerase molecule may be attached at or close to the promoter site. (From Miller *et al.*[461] by permission.)

an amplification of rRNA genes of 2.34×10^6 [535] results in a more than adequate ability to produce large amounts of ribosomal RNA.

Similar studies with lampbrush chromosomes[458,459] also show a gradient of RNP molecules along a considerably longer matrix (50 μ) (Fig. 11).

Upon examination of *E. coli* DNA from freshly lysed cells[461] a different type of configuration was seen. In this case the RNA fibers were associated with ribosomes to form polysomes (Fig. 12). These also showed a gradient of increasing size distal to the initiation point, with the longest containing

40 ribosomes. The fact that the ribosomes became attached very close to the RNA polymerase and that all the polysomes were attached at one end to the DNA, clearly shows the intimate coordination of transcription and translation in prokaryotic cells. Extrapolation along the gradient frequently showed a single RNA polymerase molecule at the most proximal site (Fig. 12). This may represent the promotor site. Ribosomes do not associate with ribosomal RNA, since it is not transcribed. Because of this, the ribosomal sites could be distinguished from the structural genes, allowing the observation that the 16*S*–23*S* cistrons were widely spaced in the genome. A final observation of considerable interest was that a large part of the genome was not being transcribed, suggesting that a significant portion of the genome may be rarely or possibly never used.

Secondary Structure of Chromatin in Relation to Genetic Activity

Selecting some of the above observations, such as the existence of a 30 Å DNP fiber in genetically active chromatin, a supercoiled 100 Å fiber in most interphase and metaphase chromatin, and the apparent contribution of histones to both the formation of the supercoiling and genetic repression, it is not unreasonable to suggest that when the DNP fiber is in the supercoiled 100 Å configuration, RNA polymerase is unable to move along the DNA and transcription is inhibited.[346] This potentially allows a mechanism of gene activation which would involve relaxation of the supercoil. This could occur simply by loss of the α-helix in the acidic portion of the histone molecule, resulting in the release of the secondary attachment sites and thus the supercoiling.[555] This would be both consistent with the observation that transcription of chromatin can occur despite the fact that significant amounts of histone are still attached to it,[381] and with the suggestion that interaction between nonhistone proteins and the acidic portion of the histone molecules may be responsible for gene activation.[188] In contrast to this model, others have suggested that the supercoil might not serve as any impediment to the movement of RNA polymerase along the chromatin.[84a]

HISTONES

Interest in histones has been intense since the demonstration by Huang and Bonner[325] that they appear to play a role in repressing the genetic activity of DNA. Space does not allow a thorough discussion of the extensive

TABLE I. The Histones[a]

	Lysine-rich		Moderately lysine-rich		Arginine-rich	
Terminology						
Rasmussen *et al.*[351]	Ia	Ib	IIb1	IIb2	III	IV
Johns and Butler[247]	f1	f1	f2a2	f2b	f3	f2a1
Iwai *et al.*[337]	Alanine-rich, very lysine rich		Leucine-rich intermediate	Serine-rich Slightly lysine-rich	Glutamic-rich Arginine-rich	Glycine-rich Arginine-rich (GAR)
Panyim and Chalkley[507]	1	1	4	3	2	5
Biochemistry						
Lysine–arginine ratio	22[90]		2.5[338]	2.5[337]	0.8[233]	0.7[632]
Percent total histone[507]	20		18	24	20	18
Molecular weight	21,000[90]		13–15,000[317]	13,774[337]	13–15,000[317]	11,282[632]
Number of residues[189]	@ 212		136–145	125	130–145	102
N-Terminal[188,235,637,550]	Acetyl-serine	Acetyl-serine	Acetyl-serine	Proline	Alanine	Acetyl-serine
C-terminal[235,637]	Lysine	Lysine	Lysine	Lysine	Alanine	Glycine
Probable DNA binding site[90,188,337,487]	C-Terminal portion			N-Terminal portion	C-Terminal	N-Terminal portion
Sulfhydryl groups[90,138,234,337]	None	None	None	None	Present	None
Acetyl groups[188,190,261,337,487,636,711]	None		±	None	Two	Several
Methylated groups[188,190,237,487]	–		–	None	One	20 lys in cth
Substrate for protein kinase phosphorylation[450]	+++		±	+++	+	+

[a] Predominately calf thymus histone.

literature, which has been covered in a number of reviews.[44,85,189,217,536a,666] The terminology and biochemical features of the major histone fractions are shown in Table I. The following is a summary of some of the important aspects of histone biochemistry as they relate to chromatin structure and function.

Evidence that Only a Portion of the Histone Molecule Binds to DNA

The early observation that there was a one-to-one ratio of histone basic groups to DNA phosphate groups implied that the entire length of the histone was bound to DNA and suggested that the basic residues should be evenly spread throughout the molecule. This idea had to be abandoned after several of the histones were sequenced. Such data are now available on arginine-rich histone IV[188,487] (Fig. 6), lysine-rich histone I,[90] and moderately lysine-rich histone, histone IIb2.[337] On the basis of these results several generalizations can be made. The most important is the observation that the basic residues tend to be concentrated in the C-terminal half in histone I and in the N-terminal half in histones IIb2 and IV. The portion which is rich in basic residues also tends to be rich in proline residues. Since proline allows free rotation of the polypeptide chain, this suggests that the regions containing a combination of basic amino acids and proline are probably those that can bind to DNA. The opposite ends tend to be more acidic and hydrophobic. It has been suggested that this type of configuration allows a portion of the histone to bind to DNA and leaves another portion free to interact with other molecules such as nonhistone proteins, thus providing some control over repression and derepression.[188]

Histones Are Extremely Conservative Molecules

A remarkable feature of the studies by Delange *et al.*[188] on calf and pea histone IV was the fact that they differed by only two amino acids, pea 60 isoleucine → calf valine, and 77 lysine to arginine. Even these are conservative substitutions and do not involve a significant change in the type of amino acid. Another difference was the presence of methylated lysine at position 20 in the calf while in the pea this lysine was unmethylated (Fig. 6). Studies of other aspects of the rest of the histones of calf and pea suggest that they are also remarkably similar.[233–235]

This degree of conservatism in base sequence between such widely different species is unique among proteins. It suggests that every position

along the entire molecule is used in an essential function and that this is true not only of the DNA binding site but also the site that may interact with molecules other than DNA. This degree of similarity, spanning such a breadth of evolutionary diversity, also takes the air out of proposals that within a species there may be meaningful variations in the types of histones in different organs. Although some such differences may exist, it is unlikely that significant alterations in sequence are involved, and unlikely that they have anything to do with organ-specific gene activity.

Microheterogeneity of Histones

The electrophoretic pattern of calf histone III shows a complex pattern of multiple bands.[234] This has probably contributed in part to the thought that there might be significant differences in the histones of different tissues. However, Frambrough and Bonner[234] have shown that these bands can all be changed to a single band when the disulfide bonds between electrophoretically identical subunits are reduced in β-mercaptoethanol. Each monomer of calf thymus histone III was found to have two cysteinyl residues (pea bud histone III has only one cysteinyl per molecule and thus could only form dimers).

Other forms of heterogeneity can be explained by differences in acetylation and methylation. For example, histone IV contains the modified amino acids e-*N*-acetyl lysine at position 16 and e-*N*-methyl lysine at position 20.[188,487] Since acetyl lysine was present in only half of the molecules, and the 20-lysine could contain either 0, 1, or 2 methyl groups, this allowed a microheterogeneity of six possible species of histone IV without altering the basic amino acid sequence.[487]

Histone V

A further type of heterogeneity involves a specific histone found in nucleated erythrocytes.[480,657] Studies of this fraction (histone fraction V) indicate that in the chicken some molecules have glutamine and other molecules have arginine at position 15.[275] The many similarities between histone V and I, and the fact that the amount of V+I in nucleated erythrocytes equals the amount of I in other tissues, suggest that V may be an evolutionary precursor of I.[275] An intriguing aspect of this work is the observation that some chickens had only type Vb with arginine at position 15, suggesting a situation in which there is one polymorphic gene. There are some problems reconciling this with the observation (see below) that there may be several hundred copies of the histone genes.[361]

Lysine-Rich Histones

The greatest degree of microheterogeneity is seen in the lysine-rich histones. This is indicated by reports of variations in electrophoretic pattern[89,366,507] and amino acid composition[88,367] in different species, organs, and developmental stages.[88,665] A minor histone in this class has also been reported to be present in nondividing tissues and absent in rapidly dividing ones.[508] The limited variation in amino acid composition of the subfractions suggests that some of the heterogeneity, like that of histone V, may be due to a small number of base substitutions. Further studies of this are of some importance in terms of the proposal that there may be several hundred copies of some histone genes.[361] In the presence of many gene copies, some microheterogeneity would be understandable and would imply that a completely effective correction mechanism, of the master-slave type, was not operating.

Recent studies have clearly shown that some of the microheterogeneity of lysine-rich histones that occurs in rapidly dividing tissues,[615] and in various organs and species[345] can be ascribed to variations in the degree of phosphorylation. Thus some or all of the microheterogeneity that occurs in histones can be ascribed to changes that are exclusive of the alteration of primary structure.

Histone Acetylation, Methylation, and Phosphorylation

It is clear that some of the microheterogeneity in the various histone fractions can be due to variations in acetylation, methylation, and phosphorylation. It has been suggested that such secondary alterations in histone structure may also play a role in altering the ability of histones to act as repressors or to bind to DNA.[7,9,392,541] In view of the observations from *in vitro* studies that (1) histones which are fully acetylated at the N-terminal residues are just as effective as nonacetylated histones in suppressing RNA synthesis,[121] (2) acetylation of lymphocyte histones in response to PHA (phytohemagglutinin) may be a nonspecific effect,[425] (3) in *Drosophila* polytene chromosomes, acetylation does not preferentially occur at regions of puff formation,[123] and (4) acetylation does not seem to significantly alter the association of histones with DNA,[9,121] acetylation may only be a nonspecific event during derepression.[217] The role of methylation is also unclear.

The possibility that phosphorylation may play some role in regulating the effect of histones is perhaps more convincing. One or more histone kinases occur and Langan[393] has shown that a liver histone kinase undergoes

an 8 to 20-fold increase in activity after injection of cyclic AMP. This appears to result in phosphorylation of a specific serine in lysine-rich histone. Glucagon also causes up to a 20-fold increase in phosphorylation of rat liver histone I[392] and when phosphorylated, this fraction is less effective in producing conformation changes in DNA.[2] This may provide a mechanism by which hormones can produce a nonspecific alteration in the level of gene activity.

Histone Messenger RNA and Repetitious DNA

The first clear demonstration that histones were synthesized in the cytoplasm of mammalian cells was provided by Robbins and Borun.[570] Using HeLa cells they showed a precise correlation between initiation of histone synthesis and DNA synthesis. When synchronized cells, at the peak of histone synthesis, were pulse labeled with ^{14}C-tryptophan (histones contain no tryptophan) and ^{3}H-lysine, and then chased with unlabeled amino acids, it was possible to demonstrate a migration of lysine-labeled proteins from the cytoplasm to the HCl-soluble (histone) fraction in the nucleus. There was a concomitant loss of labeled tryptophan from the HCl-insoluble fraction of the nucleus. These observations, suggesting that histones were synthesized in the cytoplasm, were corroborated by studies of the cytoplasmic polysomes. During the S period there was a disproportionate incorporation of ^{3}H-lysine (compared to ^{14}C-tryptophan) on small polysomes which was not present in cells labeled during the G_1 period. The addition of cytosine arabinoside, a compound known to inhibit DNA and histone synthesis, resulted in a disappearance of some of these small polysomes. Thus, whatever signals exist to cause histone synthesis to be intimately linked to DNA synthesis, they must exert their effect through the standard pathway of protein synthesis by cytoplasmic polysomes.

The isolation of presumptive 9*S* histone messenger RNA from cytoplasmic polysomes of sea urchins allowed Kedes and Birnstiel[361] to perform some interesting RNA-DNA hybridization studies. When this RNA was annealed to sea urchin DNA, the kinetics of the reaction led to the conclusion that it was hybridizing to moderately repetitious sequences (average *Cot* of 15–40) estimated to be on the average 400-fold repetitious. By way of a control, the higher molecular weight RNA of 23*S* or greater hybridized only to minimally repetitious DNA (average *Cot* of 5000). On the basis of buoyant density studies the DNA hybridizing to the 9*S* RNA was distinctly heavy or GC-rich. This is consistent with the theoretical base composition of histone genes based on their amino acid composition and the genetic

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code. In these studies the thermal stability of the 9*S* RNA-DNA hybrids and the fact that the hybridizing DNA had a specific buoyant density, tended to rule out the possibility that the RNA was nonspecifically hybridizing to DNA in general (cf. the section on hemoglobin messenger RNA).

An additional aspect of these studies was of some interest. Since histones are extremely conservative molecules, the DNA base sequence of histone genes of different species should be quite similar. This was demonstrated by the fact that sea urchin 9*S* RNA also hybridized well to *Xenopus* and *Rhynchosciara* DNA.[361] However, the efficiency of the hybridization was less than with the homologous system. This is understandable since each amino acid has several potential codons. Thus, this system demonstrated that it is possible to have base sequence divergence while maintaining the amino acid sequence unchanged.

Implications

The observation that some proteins, like the histones, may be synthesized by a large number of genes, but still have a relatively invariant amino acid sequence, raises some very intriguing questions. Namely, how is this possible? A fundamental tenet in the field of gene duplication[490] is that once a gene is duplicated, one of the two is freed from selective pressure and may undergo base substitutions to either degenerate or take on a new function. What mechanism operates to allow selection to simultaneously operate on a series of several hundred gene copies and keep them all out of mutagenic mischief? There are several possibilities.

1. The first is that a master-slave type of mechanism is at work in which all the repetitious copies are periodically matched against a master copy and any mutations they have picked up are corrected. For reasons discussed later, I feel this is unlikely. A variant of this is the possibility that a few histone genes undergo gene amplification each generation. The fact that sperm DNA was used rules this out.

2. The second is that a single class of mutant histone molecules may be lethal, despite the fact that the ratio of mutant to normal molecules may be one to several hundred. The extreme conservatism of histones, indicating that each amino acid position is vital, lends some credulity to this. For example, with several thousand mutant molecules dispersed on chromatin throughout the genome, it is possible that a mutant lodged at a critical gene might respond incorrectly to derepression and thus be lethal. If this were the case, the combination of the fact that almost all positions

on the histone molecule are invariant, plus the 400-fold redundancy of histone genes, might make histone lethals a small but important class of lethal mutations.

3. The third possibility is that the selective pressure is toward maintaining a given pool of say 50–100 normal histone genes, and the other 200 or so is the obligatory mutational load. These other abnormal genes would be sufficiently similar in base sequence to hybridize to histone messenger RNA, but their gene products would be sufficiently abnormal that they would not be isolated as chromosomal proteins.

4. Finally, the possibility exists that the data, which at present seem convincing for the existence of multiple histone genes, may at some future date be shown to be artifactual, and in reality there may be only a single functioning gene for each type of histone, plus a series of similiar but nonfunctioning copies.

Similar Function of All the Histone Classes

Since the original demonstration by Huang and Bonner[325] that histones were effective in suppressing the template activity of DNA, a number of studies have suggested that either lysine-rich[25,280,312,326] or arginine-rich[10,308,315] histones were more effective template inhibitors. Other studies have shown that as histones are selectively removed, lysine-rich first and arginine-rich last, it is only with the removal of the arginine-rich histones that a significant increase in template activity occurs.[329] These findings have suggested that the different histone fractions may have significantly different functions and that arginine-rich histones may be the most effective repressors.

Some more recent observations, however, tend to indicate that these differences are not all that striking. The inability of different investigators to agree on whether lysine-rich or arginine-rich histones are more effective template restrictors suggests that differences in technique may be important.[11] This conclusion is supported by the observation[348] that both fractions can cause complete precipitation of DNA but only at a narrow peak in the histone–DNA ratio (0.8 for F1 and 1.5 for F3). Depending on the ratio used, either lysine-rich or arginine-rich histones could seem the best inhibitor.

Even more convincing are the studies of Smart and Bonner.[626–629] In previous experiments on the effect of histone removal on template activity, the histones have been removed in the order of lysine-rich first, then moderately lysine-rich, and finally arginine-rich. Utilizing different concentrations

of sodium deoxycholate, it was possible to change the order to moderately lysine-rich first, then arginine-rich, and finally lysine-rich. Since the effect on many different parameters was the same, regardless of the order of histone removal, it is difficult to say that one class is more important in a certain function than another.

Having made this statement, it is perhaps well to qualify it somewhat. There are a number of properties of the lysine-rich histones that suggest they may play a role that is somewhat different than that of the rest of the histones. These properties include the observation that they have a very low helix content,[54] they are more susceptible to amino acid substitution, and X-ray diffraction studies have suggested they may not be involved in maintaining the supercoiled structure of the DNP fiber.[476,555] Also pertinent is the observation of Koslov and Georgiev[381] that RNA synthesized on chromatin had a short chain length, but after the removal of lysine-rich histone the chains were as long as those synthesized on DNA. They suggested that lysine-rich histones may prevent the movement of RNA polymerase along the DNA. They also observed that the DNA did not have to be naked for RNA synthesis. Their DNP minus the F1 histones had a protein to DNA ratio of 1:1 and served as a good template.

An additional qualifier is the possibility that a shift between SH and S-S in histone III may allow it to play a role in chromatin condensation.[585]

Histones of Heterochromatin and Euchromatin

If the histones themselves were important in determining whether a given segment of chromatin was genetically active or inactive, there should be significant differences in the histone to DNA ratio, or in the ratio of lysine-rich to arginine-rich histones, or in the electrophoretic pattern of histones of genetically active euchromatin compared to inactive heterochromatin. With the possible exception of minor variations in the SH-containing histones,[271] none of these predictions are true.[126,506]

Histones as Generalized, Nonspecific Repressors

The search for heterogeneity of histones in different species and in different organs has been motivated by the possibility that they may act as specific repressor molecules. However, the observation that (1) in relation to amino acid substitutions they are extremely conservative molecules, (2) the degree of their heterogeneity is severely limited and what little heterogeneity exists can be explained by a small number of base substitutions or

by modifications that are independent of sequence, (3) the function of the different classes is basically the same, and (4) the histones of genetically active and inactive chromatin are very similar, speak against a role as specific repressors. The situation was succinctly summarized by Hnilica[311] when he said, "the biological role of histones appears to be rather passive, with the histones being the tools for the regulation, rather than the regulators themselves." This moves the battleground of specificity into another arena, with the nonhistone proteins being the most likely eventual victors.

NONHISTONE PROTEINS

Introduction

On the basis of biochemical studies Miescher[454] and Kossel[382] demonstrated the presence of DNA and protamines or histones within nuclei. The assumption that these were the two primary components of nuclei persisted until the early 1940's when Mayer and Gulick[442] and the Stedmans[656] described an acidic nuclear protein, soluble in alkali, precipitated in acid, and containing tryptophan. The Stedmans called this chromosomin. Mirsky and Pollister[464] noted that after histones were removed from a chromatin preparation, a considerable amount of protein remained. This was termed nonhistone protein or residual protein.[465,466]

Methods of Isolation

If the whole nuclei are used, the soluble nuclear proteins must be thoroughly washed out with dilute NaCl[465,466] or tris buffer.[260,724] Histones may be removed with 0.1 M HCl, and DNA removed with DNase,[198] or by centrifugation after solubilization of the nonhistone proteins with alkali,[732] deoxycholate,[516] sodium dodecyl sulfate,[218] or phenol.[614] Some more gentle methods allow the nonhistone proteins to be solubilized without being denatured. One of these involves the solubilization of chromatin in 1 or 2 M NaCl, followed by precipitation of histones and DNA by decreasing the salt concentration to 0.14 M. This leaves much of the nonhistone material still in solution.[260,391,725] In other techniques the nonhistone proteins may be solubilized in 4 M cesium chloride and separated from DNA by centrifugation,[36] or solubilized in urea-salt and the nonhistones selectively precipitated with polyethylene sulfonate,[383] or separated from histones by polyacrylamide gel electrophoresis.[609]

Residual Proteins

Because of the many different methods that have been used to isolate nonhistone proteins, the fractions studied by different investigators are not always the same. The term residual protein has frequently been used in connection with studies of nonhistone chromosomal proteins. To Busch and Steele[86,658,659] and others,[781,782] residual proteins are the residue after nuclei have been successively extracted with 0.14 M NaCl 2 M NaCl, and dilute NaOH, to remove first nuclear sap proteins, then DNA, histones, and some nonhistone proteins, and finally to remove the alkali-soluble proteins. It is understandable that what is left is truly a residual protein and appears to consist of a collagenlike protein,[659] nuclear membranes, and nucleolar material. To Dounce and Hilgartner,[198,427] residual proteins are those which remain after nuclei are treated with 0.14 M NaCl, 0.1 H NCl (to remove histones), alcohol-ether (to remove lipids), and DNase. This residuum would contain alkali-soluble and insoluble nonhistone proteins. To Mirsky,[8,465,466] residual proteins are those which remain after thorough extraction of the nuclei with 0.14 M NaCl and 1 M NaCl. In more recent studies, the nonhistone proteins are all those chromatin proteins which are left after histone and DNA have been removed[218] or those which remain in solution after histones and DNA are precipitated from 1 M NaCl.[260,370,374] It is important to bear in mind these many variations on a theme when comparing different studies.

Protein Synthesis for Residual Proteins

Wang and others have reported amino acid incorporation into residual proteins that was dependent on the presence of DNA, but independent of ribosomal particles and soluble enzymes and was unaffected by RNase. This was observed with crude nuclear residue,[517,518] and with isolated calf thymus[478,518,723] and rat liver[607,725,783] nuclear residual proteins. Although one possible explanation is the direct involvement of DNA in nuclear protein synthesis, the true significance of this amino acid incorporation remains to be clarified.[725]

Two Classes of Nonhistone Proteins

Although yet to be proven, the theme that will be developed in the next two sections is that there are two major classes of nonhistone proteins; (1) proteins with enzymic and structural functions, and (b) regulatory proteins. Evidence will be marshaled to suggest that the enzymic and structural type form the bulk of the nonhistone proteins and although there are some variations they are probably on the average more similar than different in various organs. By contrast, the nonhistone regulatory proteins are individually present in minute amounts (analogous to the lac and lambda repressors of prokaryotes), but will show greater variability. Electrophoresis of nonhistone proteins will demonstrate primarily the enzymic and structural proteins. Although it is possible that many different regulatory proteins with the same molecular weight may produce a few electrophoretically visible bands, in general, techniques other than electrophoresis will be necessary to examine these proteins.

Enzymic and Structural Functions—Organ and Species Similarity

The nonhistone proteins are best electrophoresed in SDS-polyacrylamide gels. The proteins are solubilized under these conditions and migrate according to their molecular weights. Using this technique, from 13 to 30 bands can be visualized[157,218,424,685] ranging in molecular weight from 5000 to 100,000 daltons. Although there are some differences in a few bands, the patterns of nonhistone proteins from different organs and species are generally similar, with predominately quantitative rather than qualitative differences.[218] Pea bud nonhistone proteins had half of the bands present in calf thymus nonhistone proteins.[218] This type of observation suggests that the major electrophoretically visible bands represent DNA binding enzymes and structural proteins that are necessary for general running and maintenance of the genetic machinery.

The following types of proteins and enzymes have been identified as occurring within the nonhistone proteins: DNA polymerase,[322,486,515,726] DNase,[486,521] RNA polymerase,[737] a neutral protease,[240–242] protein phosphokinase,[370,371,391] methylase,[608] terminal DNA-nucleotidyl transferase,[727] DNA nucleosidase,[42] ATPase,[725] maleate dehydrogenase,[725] lactate dehydrogenase,[725] glutamate dehydrogenase,[725] and glutamate oxylacetate transaminase.[725] It is not clear why some of the latter should be DNA binding enzymes associated with acidic nuclear proteins, but it is possible that they, as well as all the others mentioned, may be relatively easily dissociable from the chromatin and in an equilibrium with the protein of the nuclear and cytoplasmic sap. To date none of these enzymes have been clearly correlated with the bands seen by SDS gel electrophoresis.

Regulatory Functions—Organ and Species Dissimilarity

Since genes must be regulated, and (1) the histones do not possess the heterogeneity to do so, (2) chromosomal RNA is probably not involved, (3) the electrophoretically distinguishable nonhistone proteins are probably nonspecific DNA binding enzymes, (4) the prokaryotes utilize trace amounts of acidic proteins to do the job, it is quite likely that eukaryotes also use trace amounts of nonhistone proteins to provide for gene regulation. Is there any evidence that this is the case? As pointed out by Elgin *et al.*,[217] to demonstrate this point it is necessary to show that (1) the effect is not simply the result of precipitation of the DNA or histone, (2) the nonhistone protein binds to DNA, (3) the binding is specific, (4) the effect occurs at the normal salt concentration of the nucleus (0.2 M[395]), (5) the effect is not being mediated by a direct effect on RNA polymerase, and (6) proteases have not been introduced into the system. Although all of these conditions have not been satisfied, a number of experiments suggest that some nonhistone proteins possess some of these characteristics.

Positive or Negative Control

In prokaryotes some proteins exert positive control (gene activation) and some exert negative control (gene repression). A similar diversity of effect may be present in eukaryotes. In the case of positive control one might anticipate that a regulatory protein could recognize and bind to a specific base sequence and inhibit the repressive effect of the histones, or activate transcription of certain promoters. In the case of negative control it could act in the same manner as lac and lambda repressors by binding to promoter sites and preventing transcription by RNA polymerase.

Reconstitution Experiments

Studies of Gilmour and Paul[267,522] have suggested that the nonhistone proteins may be supplying the specificity for gene regulation. When chromatin was reconstituted from DNA, histone, and nonhistone proteins, by slowly dialyzing away salt and urea, the resulting nucleoprotein had template activity similar to native chromatin. If the nonhistone proteins were left out, the histones effectively suppressed all template activity. Gilmour and Paul noted that bovine serum albumin was also able to inhibit the complete repression of template activity, but by competition hybridization experiments the RNA synthesized did not resemble that of native chromatin. Other studies of the rate of RNA synthesis have also indicated that the

nonhistone proteins can alter the inhibitory effect of histone on template activity. In some cases this was observed when the acidic proteins were added to chromatin.[238,729] In other reports an effect was seen only if the nonhistone proteins and histones were first exposed to each other and then added to the DNA[115,391,394,649,728] or chromatin.[686] Some of this is certainly related to the observation that in simple mixtures, nonhistone proteins combine with and precipitate histones.[391,725,728,731] However, a variation of the effect, depending upon the type of histone used, suggests that factors other than the simple interaction between histones and nonhistone proteins may be involved.[728]

Wang[729] observed a two to threefold increase in template activity when nonhistone proteins were added to a chromatin-RNA polymerase reaction mixture. The RNA synthesized by the activated chromatin had a greater U + G content, showed a change in nearest neighbor frequencies, and more closely resembled DNA-like RNA. RNA from untreated chromatin hybridized to 4% of denatured rat liver, while RNA synthesized by a nonhistone-activated chromatin hybridized to 8%. RNA isolated from the nonhistone fraction failed to stimulate RNA synthesis, and controls ruled out the possibility that nucleases or proteases were added to the system. This type of study suggests that nonhistone proteins are capable of activating additional transcription sites on chromatin and that the effect is not due to an RNA component.

Selective Removal of Histones

The above studies suggest positive control. Some evidence for negative control has been provided by studies of Spelsberg and Hnilica[650–652] in which chromatin was treated with increasing concentrations of salt until all of the histones were extracted. Despite this, significant template restriction remained in the treated chromatin. This phenomenon was also reported by Gilmour and Paul,[267] although in a similar experiment Marushige and Bonner[436] found no significant template restriction after histone removal and nonhistone retention, and they found no restriction of template activity when nonhistone proteins were reconstituted to DNA. The latter could be explained if some interaction with histones is necessary for the nonhistones to be completely effective,[649] or if the sodium dodecyl sulfate used to isolate the nonhistone proteins also denatured them. In a study by Georgiev *et al.*[259] 0.4 M NaCl removed only F1 histones and much nonhistone protein. This resulted in a significant increase in template activity of Ehrlich ascites cell chromatin.

Specificity

A demonstration that some nonhistone proteins possess the capability of associating only with homologous or near homologous DNA has been demonstrated by Kleinsmith *et al.*[374] Utilizing DNA-cellulose chromatography, they isolated DNA binding proteins from the nonhistone fraction of rat liver. Among these were some that would bind to rat liver DNA but not to heterologous salmon sperm or *E. coli* DNA.

A different indication of specificity has been demonstrated by Gilmour and Paul.[268] They utilized nonhistone proteins isolated from different organs of the rabbit to reconstitute chromatin. Competition hybridization experiments showed that these chromatins synthesized RNA molecules that were similar to the RNA made by the native chromatin from the same organs. The type of crossover hybridization experiments that this required suggest that nonhistone proteins have the capability of supplying the tissue-specific control of chromatin transcription. Similiar results have been obtained by Spelsberg *et al.*[650–652]

Nonhistone Phosphoproteins

One of the more gentle techniques of isolating nonhistone proteins involves their retention in solution after precipitation of DNA and histones by dilution of a 1 M NaCl extract of chromatin to 0.14 M NaCl. Phosphoproteins can be selectively removed from this solution by adsorbing them to a calcium phosphate gel.[260,370,391] SDS-Acrylamide gel electrophoresis of these proteins shows a certain degree of organ specificity.[540] The proteins are phosphorylated at serine and threonine residues[372,373] and there is rapid turnover of these phosphate groups[370,371,391] even though they do not act as a storage form of high-energy bonds. These proteins are effective in counteracting the ability of histones to inhibit template activity.[391] Among them are some which possess protein kinase (phosphorylation) activity.[370] Thus these phosphoproteins possess the capability of altering template activity, and that ability may be potentially controlled by variations in the degree of their phosphorylation.

Nonhistone Proteins in Euchromatin

In a remarkable show of unanimity, every study in which the subject has been investigated has agreed that there is an increase in the amount of nonhistone protein[5,80,197,238,306,307,360,785] or phosphoprotein[372] in euchromatic or genetically active chromatin compared to condensed, or heterochromatic, or genetically inactive chromatin. This can be visually appreciated in a rather striking manner by the studies of Zirkin[785] on spermiogenesis in the frog. Utilizing alkaline fast green for histones, and acid fast green for nonhistones, he showed that the diffuse chromatin of the early spermatids was well stained by both, but the condensed chromatin of the mature sperm failed to stain for nonhistone proteins (Fig. 13).

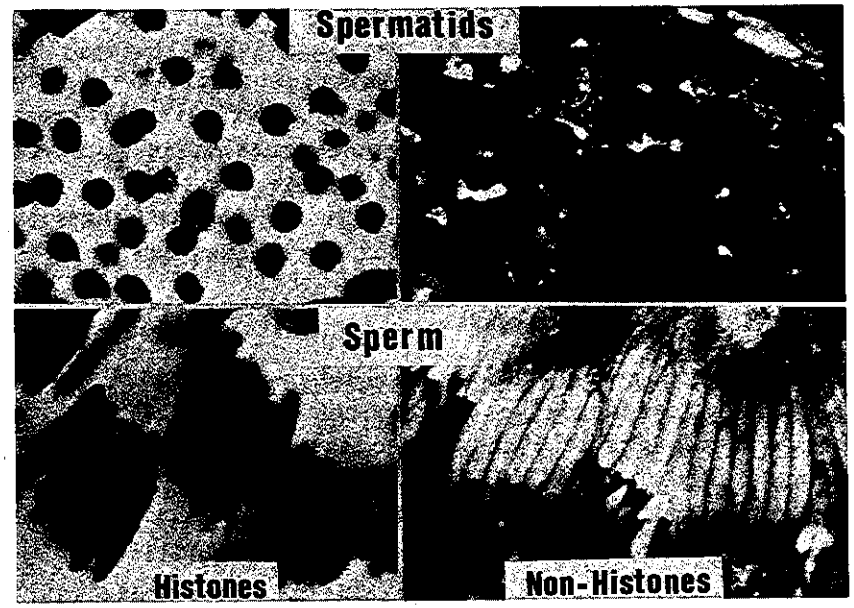

Fig. 13. The content of histones and nonhistone proteins in diffuse and condensed chromatin during spermatogenesis of the leopard frog, *Rana pipiens*. The histones are stained with alkaline fast green and the nonhistones with acid fast green. It can be seen that the histones are present in both the diffuse chromatin (spermatids) and condensed chromatin (sperm), while the nonhistones are present in diffuse but absent in condensed chromatin. (From Zirkin,[785] by permission.)

One look at Miller's pictures (Figs. 9–12) of RNA polymerase molecules densely strung along the core of nucleolar or lampbrush chromatin is enough to convince one that some of the nonhistones in genetically active chromatin must be RNA polymerase. In this respect it is a little disturbing not to see greater differences in the size of some of the nonhistone bands in chicken erythrocyte (inactive) compared to chicken liver (active),[218] between isolated heterochromatin and euchromatin[157] and between interphase and metaphase[157] chromatin. The subject obviously needs further investigation.

Synthesis of Nonhistone Proteins

Three additional features of the nonhistone proteins are based on the observations that they: (1) have a high rate of turnover, (2) are synthesized throughout the cell cycle, and (3) show an increased rate of synthesis in response to some hormones. Although these features are consistent with a possible regulatory role for the nonhistone proteins, the same features may be seen with enzymes which have no apparent regulatory function.[28,375]

High Rate of Turnover

The residual protein fraction as isolated by a number of different techniques has consistently been noted to show a high rate of amino acid incorporation,[87,91,187,316,317,658] and this rate has been noted to correlate with the rate of RNA synthesis.[316,317] This synthesis is sensitive to puromycin, actinomycin, and RNase,[187,316] indicating that at least a portion of the synthesis of nonhistone protein is by the usual ribosome pathway.

Relation to DNA Synthesis

Although there has been a great deal of investigation of the correlation between DNA and histone synthesis, there have been relatively few studies of nonhistone protein synthesis during the cell cycle. An early autoradiographic study of *Tradescantia* (liverwort) and *Allium cepa* (onion) by De,[183] in which incorporation into histone or nonhistones was based on the nuclear grain counts before and after 0.01 N HCl extraction, concluded that predominately histones were synthesized during the S period and considerable amounts of nonhistone were synthesized during the G_2 period. A more precise autoradiographic distinction between cytoplasmic, nuclear sap, and true chromosomal protein synthesis was possible in a study by Cave[110] of the incorporation of ^{3}H-thymidine, ^{3}H-lysine, and ^{3}H-tryptophan into the giant chromosomes of *Chironomus thummi*. These studies showed that the synthesis of the nonhistone proteins was not keyed to DNA synthesis. The incorporation of ^{3}H-tryptophan into the giant chromosomes proceeded equally well whether or not there was concurrent DNA replication. Some studies have been reported using isolated chromatin from synchronized cells, in which the nonhistone proteins were taken as the H_2SO_4 insoluble fraction from purified chromatin. A report by McClure and Hnilica[448] using Chinese hamster cells concluded there was an inverse relation between the synthesis of histone (S phase) and nonhistone proteins (G_1, G_2, and less in S). A similar study by Stein *et al.*,[664] but using HeLa cells, demonstrated

continued active synthesis of nonhistone proteins throughout the cell cycle, with two peaks during the S phase. There was also a significant amount of nonhistone protein synthesis during mitosis.[662] This is of particular interest in view of the fact that RNA synthesis is shut down[379] and polyribosomes break down to monosomes[592] during mitosis. It will be interesting to determine if this synthesis is sensitive to puromycin or actinomycin or if it possibly resembles the DNA-dependent synthesis described by Wang for the residual protein fraction.

Response to Hormones and Other Agents

An acceleration of nonhistone protein synthesis has been noted in response to estrogen in the rat uterus (but not rat liver);[686] to isoproterenol in rat salivary glands,[663] to phenobarbitol in rat liver;[576] to ME virus in Ehrlich ascites cells;[318] and to coritsol in rat liver.[614] In the latter there was a marked increase in a nonhistone protein of 41,000 molecular weight.[614] The function of this protein is yet to be determined.

Nonhistone Proteins and Specificity in Gene Regulation

To summarize, the following observations fuse gracefully into a body of evidence which is at least consistent with the possibility that within the nonhistones there are some which supply the specificity for gene regulation in eukaryotes. (1) Studies of the lac and lambda repressors show that some acidic proteins have the capability of binding tightly and specifically to DNA regions possessing a certain sequence of bases and as a result inhibit the attachment or movement of RNA polymerase. (2) A small fraction of the nonhistone proteins are capable of binding to DNA with a degree of specificity that is sufficient to distinguish between rat and fish DNA.[374] (3) Electrophoretic patterns of some of the nonhistones show species and organ specificity.[540] (4) Nonhistones can interact with histones to inhibit their effectiveness in repressing template activity. (5) Reassociation experiments suggest the nonhistones supply specificity needed to allow the reconstitution of chromatin with organ-specific template properties.[267,521,729] (6) The nonhistone proteins are metabolically active, are synthesized throughout the S period, and respond to hormone administration.

It is likely that the nonhistone proteins that are of the greatest interest in relation to specific gene regulation are individually present in minute concentrations. It will require the judicious use of DNA cellulose or poly-

acrylamide chromatography,[3,109] radioiodine labeling,[374] nitrocellulose membrane filters,[557–560] somatic cell genetics, and other techniques to study these elusive proteins.

NUCLEAR RNA

Rapidly Labeled, Heterodisperse, Intranuclear RNA

The original concept of bacterial messenger RNA as detailed by Jacob and Monod[341] required only a smooth flow from DNA to messenger RNA to ribosomal translation. The first suggestion that things might not be that simple in eukaryotes came from biochemical and autoradiographic studies of Harris and Watts.[287,288,290,736] They concluded that most of the rapidly labeled RNA was broken down within the nucleus and that the later appearance of label in the cytoplasm did not prove a transfer of RNA from the nucleus to the cytoplasm, since RNA could have been synthesized there from the breakdown products of nuclear RNA. Although the suggestion of cytoplasmic synthesis of RNA has subsequently found no ringing confirmation, the proposal that there are RNA molecules with a rapid turnover that are specific to the nucleus has been verified. This has been termed heterodisperse nuclear RNA or HnRNA, and has a number of interesting characteristics.

Properties of HnRNA

1. *Heterodisperse DNA-like RNA.* When mammalian cells are pulse labeled with RNA precursors and the RNA is immediately isolated and centrifuged in sucrose, it is possible to distinguish two types of rapidly labeled nuclear RNA. One is a 45*S* GC-rich precursor to ribosomal RNA, and the second is a highly heterogeneous population of RNA molecules ranging from 20 to 100*S*, and having a DNA-like base composition.[20,71,173,321,593,613,636,734,775] By comparison, the *S* values of cytoplasmic messenger RNA are in a much lower range of 6–16*S*.[173]

2. *Tight binding to MAK columns.* HnRNA may also be separated from other RNA on the basis of its behavior on methylated albumin Kieselguhr columns. If pulse-labeled RNA is placed on a MAK column and eluted with salt, some RNA remains tightly adherent and can be removed only with sodium dodecyl sulfate or 1 M ammonia.[39,219,387,573] This RNA has a high molecular weight, a DNA-like base composition, and a poorly ordered secondary structure. Although generally considered to be HnRNA, one investigator has suggested it could be a precursor to 16*S* ribosomal RNA.[57]

3. *Rapid turnover.* Calculations based on the turnover and relative amounts of cytoplasmic messenger RNA and ribosomal RNA suggest that an intranuclear precursor to cytoplasmic messenger RNA should be synthesized no faster than about one-sixth the rate of synthesis of preribosomal RNA.[173] In reality, about three times as much radioactivity in pulse-labeled cells is found in the HnRNA than in the pre-rRNA.[173] This observation, plus the numerous studies of the rate of loss of label from HnRNA into the acid-soluble pool, indicate that HnRNA has a rapid rate of turnover.[20,173,288,613,637,734]

4. *Restriction to the nucleus.* The proposal by Harris that much of the intranuclear RNA was both created and rapidly destroyed without even entering the cytoplasm was confirmed by Shearer and McCarthy[612] and others.[117,118,200,257] This was accomplished by competition hybridization experiments which showed that RNA sequences in the cytoplasm were also represented in the nucleus, but many sequences in the nucleus were not represented in the cytoplasm. Nuclear RNA was complementary to five times as much of the genome as cytoplasmic RNA.[612]

5. *First appearance with multicellular organisms.* HnRNA does not occur in all eukaryotes. It appears to be absent in nucleated unicellular organisms.[545] This suggests that it came upon the evolutionary scene with the development of the genetic complexity required for multicellular organisms, rather than appearing when nuclei were first developed.

6. *Chromosome associated.* HnRNA is not associated with the nucleolus.[528] In this respect it may represent what cytologists have referred to as "chromosomal RNA".[173,215]

7. *HnRNA shows greater sequence similarity among tissues of a given animal than cytoplasmic mRNA.* Again, studies utilizing competition hybridization suggest that in a given organism there is greater similarity between the HnRNA sequences from different tissues than between cytoplasmic mRNA sequences of different tissues. In fact, there is greater similarity between the HnRNA sequences of, for example, calf thymus and calf liver, than between the HnRNA of calf thymus and the cytoplasmic mRNA of calf thymus.[673] One possible explanation for this is that many of the genes that are transcribed for HnRNA may be "on" in all tissues, while the genes for cytoplasmic mRNA are different in different tissues.

8. *Between different genera there is a greater sequence diversity in HnRNA than in cytoplasmic RNA.* In studies utilizing the temperature of dissociation of hybrids between mouse L-cell nuclear RNA and DNA of other rodents, Shearer and McCarthy[613] observed that there was greater sequence diversity in the nuclear than in the cytoplasmic RNA. They suggested that this might be the result of a relatively recent reduplication of the DNA sequences coding for nuclear RNA. This could be analogous to the markedly different sequences within the satellite DNA of closely related species.[717] An alternative possibility is that the HnRNA may be transcribed in large part from junk DNA and as such it may be free to shoulder a greater mutational load than essential DNA sequences.

9. *A greater portion of the HnRNA is released to the cytoplasm in rapidly dividing compared to nondividing cells.* Following hepatectomy, some of the intranuclear RNA sequences begin to appear in the cytoplasm.[117] In normal rat liver a large number of nuclear RNA species are never transferred to the cytoplasm, while in hepatomas nearly all of the nuclear RNA species were detected in the cytoplasm.[200] These studies suggest that the selective transport of HnRNA to the cytoplasm may play a role in gene regulation.

10. *Some HnRNA transport is responsive to hormonal changes.* When rabbit uterine cells are exposed to estradiol, some of the nuclear restricted RNA sequences are released to the cytoplasm.[119]

11. *HnRNA and cytoplasmic mRNA contain transcripts from both repetitive and unique sequences.* Studies of the kinetics of hybridization of both HnRNA and cytoplasmic messenger RNA indicate that although they are composed predominately of unique sequences hybridizing at very high *Cot* values, both contain a small amount of RNA which hybridized very rapidly at low *Cot* values.[535a] Although both contain repeated sequences, the most highly reiterated ones occur in the HnRNA fraction.[174,175]

12. *HnRNA is not inhibited by cordycepin.* Cordycepin or 3′-deoxyadenosine is a nucleotide analogue. It can be added to a growing RNA molecule by the usual 5′,3′-phosphodiester bond. However, another nucleotide cannot be added because of a hydrogen atom instead of a hydroxyl at the 3′ position. This compound inhibits synthesis of messenger, ribosomal, and mitochondrial RNA but has no effect on HnRNA. It has been suggested that this may mean that HnRNA and cytoplasmic mRNA are distinct entities.[529,530]

13. *Hybridization to total DNA.* Utilizing high concentrations of RNA and prolonged incubation times, Scherrer *et al.*[594] have shown that HnRNA hybridizes to 5–10% of the homologues DNA in HeLa cells, while polyribosomal messenger RNA hybridizes to 0.5–1%. Thus the cytoplasmic messenger RNA is homologous to ten times less DNA than HnRNA.

14. *HnRNA and cytoplasmic mRNA probably share some sequences.* This problem is rather difficult to answer unambiguously because even a small number of broken nuclei will result in the contamination of cytoplasmic with nuclear RNA; because hybridization reactions are not all that precise; and because cytoplasmic messenger RNA must have come from the nucleus even if it is not a product of HnRNA. Within the scope of these limitations, there is some hybridization data which suggest that high molecular weight HnRNA and lower molecular weight cytoplasmic mRNA share some sequences.[594,638]

15. *Both HnRNA and mRNA contain transcripts of SV40 DNA.* When cells are transformed by the oncogenic virus SV40, the viral DNA is integrated into the host genome, and transcripts of that DNA appear in the cytoplasmic RNA. Lindberg and Darnell[414] found that nuclear RNA of much higher molecular weight than the cytoplasmic virus-specific RNA, also contained virus RNA sequences. The ability to follow a specific marker like this suggested that the cytoplasmic mRNA is produced by the cleavage of the larger HnRNA.

16. *Polyadenylic acid in both HnRNA and mRNA.* Edmonds and Carmela[212a] found that about 0.5% of the nuclear RNA of Ehrlich ascites cells consisted of a stretch of polynucleotides containing predominately (90%) adenosine monophosphate. Further studies of this system have shown that the polyA-rich section is about 200 nucleotides long and is found in both HnRNA and in cytoplasmic mRNA. It constitutes an even greater percentage of the total in mRNA.[176,213,399,407] It is covalently bound to the RNA and is ribonuclease resistant. On the basis of these findings, Edmonds *et al.*[213] have postulated that each HnRNA molecule transcribed contains within it one poly-A sequence adjacent to a potential mRNA sequence. The HnRNA is broken down within the nucleus, presumably until it reaches the nuclease-resistant poly-A segment which in combination with protein binding[653] protects the mRNA, which is then passed to the cytoplasm. It is interesting that studies of hemoglobin messenger RNA indicate that even after the mRNA reaches the cytoplasm it is still much larger than it should be.[253]

17. *Balbiani rings produce HnRNA.* Is the heterogeneity of HnRNA due to the fact that it includes transcripts of many different genes or sets of

genes, or is it due to the fact that RNA polymerase moves along producing one long RNA molecule which gets broken at different places, thus resulting in a heterogeneous collection of sizes? The extremely long RNA molecules that were directly observed to be synthesized by lampbrush RNA (Fig. 11) are compatible with the latter hypothesis. In another approach to this problem, Daneholt and Edstrom[169,215] isolated the RNA synthesized by single Balbiani rings of *Chironomus tentants* and showed that it contained high molecular weight, rapidly labeled RNA. This has been confirmed by Pelling.[527] This finding indicates that a very restricted portion of the genome is capable of producing HnRNA, suggesting that its heterogeneity is due more to the release of variable lengths of RNA rather than to the transcription of many different sequences.

Relationship Between HnRNA and mRNA

The most interesting aspect of HnRNA concerns its relationship to messenger RNA. The similarities and differences between these two RNA species are summarized in Table II. The two major proposals are that

TABLE II. Features of HnRNA and mRNA[a]

	HnRNA	Messenger RNA
Differences		
Location	Nucleus	Cytoplasm
Turnover	Rapid	Less rapid
S Value	20–80	4–20
Molecular weight	$1–15 \times 10^6$	$0.1–1 \times 10^6$
Cordycepin	Not inhibited	Inhibited
Hybridization with DNA	Hybridizes to 5–10% of total DNA	Hybridizes to 0.5 to 1% of total DNA
Similarities		
Base composition	DNA-like	DNA-like
Base sequences	Some shared sequences	
SV40-specific RNA in transformed cells	Present	Present
Poly A regions	Present	Present
Reiterated sequences	Some present	Some present

[a] See text for references.

(1) HnRNA and cytoplasmic mRNA are completely independent entities, or (2) giant HnRNA contains within it mRNA sequences which are released after much of the nuclear RNA is degraded. Most of the items in Table II are compatible with either hypothesis. The features which tend to favor the precursor-product relationship are the observations that (1) a specific gene product (SV40-specific RNA) is found in both types of RNA, (2) restricted segments of the genome such as Balbiani rings synthesize HnRNA, and (3) direct visualization of lampbrush loop transcription shows (Fig. 11) that RNA polymerase sweeps the entire loop, forming giant RNA molecules rather than two (HnRNA + mRNA) classes of RNA.

CHROMOSOMAL RNA

A Histone-RNA Complex

If histones lack the diversity to be capable of acting as specific genetic repressors, how is such specificity attained? The two major proposals have been either (1) the nonhistone proteins are responsible, or (2) a unique species of RNA may function as an adaptor molecule having one portion combine with histones and another portion recognize specific DNA sequences and thus guide a given histone to the proper gene. The latter would be a negative gene control mechanism that would allow a nonspecific histone to become a specific repressor. Alternately, the RNA could recognize a specific gene sequence, cause strand separation, override the repressive effect of histones, and thus act as a positive control element.[238]

When pea bud nucleohistone was centrifuged in 2.09 M cesium chloride, Huang and Bonner[327] noted the presence of a histone-RNA complex. The RNA of this complex was rich in dihydrouridylic and adenylic acid and poor in guanylic and cytidylic acid. They termed it chromosomal RNA. The only other RNA that contains dihydrouridylic acid is transfer RNA. Although transfer RNA is also rich in adenylic and poor in cytidylic acid, it contained significantly more guanylic acid than chromosomal RNA and the two were felt to be distinct. The chromosomal RNA could be removed from the histone by strong acid, thus explaining its absence in previous histone preparations. It was resistant to ribonuclease while complexed to histone, but sensitive after its release. It had a chain length of about 40 nucleotides. RNA with similar properties was found in rat liver[35] and ascites cells,[168] calf thymus,[616] and chicken embryos.[328] Some investigators using slightly different methods were unable to find it.[158,186]

Additional features that were consistent with the proposal that chromosomal RNA may play a role in gene regulation included the observation that it (1) possessed a very heterogeneous base sequence;[45] (2) caused organ-specific RNA to be synthesized from chromatin that was reconstituted from histones, DNA, and chromosomal RNA while such specificity was lacking in chromatin reconstituted from histone alone;[33,167,328] (3) appeared to be related to the HnRNA synthesized immediately after hepatectomy;[443] (4) hybridized to 5% of DNA while ribosomal and transfer RNA hybridized to only 0.24 and 0.054%;[45] (5) hybridized to repetitious DNA sequences[623] that were proposed by Britten and Davidson[65] to contain some regulator sequences; and (6) hybridized to both native and single-stranded DNA while messengerlike DNA hybridized to only single-stranded DNA.[32] On the basis of the latter it was suggested that chromosomal RNA could interact in some unique manner with native DNA and that this characteristic might be related to its high dihydropyrimidine content.[82]

Chromosomal RNA and Transfer RNA

This imposing edifice of biochemical evidence has recently been challenged by the suggestion that chromosomal RNA thay be a breakdown product of transfer RNA.[305] For example, in the technique used by Bonner and co-workers, the protein-RNA complex that floats on top of 4 M CsCl is treated with pronase to release the chromosomal RNA,[45,616] which is subsequently purified by DEAE-Sephadex chromatography. However, Heyden and Zachau[305] reported that if the pronase step was omitted, a protein-transfer RNA complex was obtained. Treatment of this with tritiated $NaBN_4$ introduced amounts of radioactivity into the tRNA similar to those introduced into chromosomal RNA. Their studies suggested that ribonuclease contaminants in the pronase resulted in the degradation of transfer RNA to chromosomal RNA and that the latter then hybridized to DNA to a greater extent than undegraded tRNA. These observations are capable of explaining some aspects of chromosomal RNA, especially its high content of dihydrouridylic acid. It is also possible that some of the theories relating to the evolution of life which suggest that transfer RNA at one time may have been able to act as an adaptor molecule by direct interaction with DNA, might have some bearing on the ability of chromosomal RNA to bind to native DNA. To explain the chromatin reconstitution experiments it is necessary to assume that small amounts of nonhistone protein present along with the RNA were accounting for the specificity observed, or that the hybridization reactions at the temperatures used were too nonspecific.[186]

RNA POLYMERASE, SIGMA, RHO, AND PSI

Introduction

The concept of the operon resulted in emphasis on the repressor as the chief mechanism of gene control, and within about 6 years this idea was proven by the isolation of specific lambda and lac repressors. However, as the decade of the 1960's came to a close, studies on RNA polymerase demonstrated a new mechanism of gene control which proved to possess remarkable versatility. This explosive chapter began with the demonstration by Burgess *et al.*[82] that when bacterial RNA polymerase is fractionated on a phosphocellulose column, it is possible to separate the molecule into a fraction which binds to the column (PC enzyme or core enzyme) and a fraction which does not bind and is found in the initial runoff peak. It was observed that when T4 DNA was used as a template, neither the core enzyme nor the nonbinding fraction possessed enzyme activity, but when they were combined, RNA polymerase activity was restored. Acrylamide gel electrophoresis demonstrated a component in the runoff peak that was termed sigma factor, σ. It had a molecular weight of 95,000.[82] Studies of the molecular weight and electrophoretic properties of the core enzyme indicated the following subunit composition: $\alpha_2\beta\beta'\omega_2$ with molecular weights of α, 39,000; β and β', 155,000; and ω, 9,000.[81] The early suggestion that the sigma factor acted as a specific initiator was soon confirmed.

Sigma Factor and Site-Specific Initiation of RNA Synthesis

By studying its effect on the incorporation of label into the first nucleotide compared to subsequent nucleotides, Travers and Burgess[702] demonstrated that sigma factor was required for chain initiation but played no role in subsequent chain elongation. After initiation sigma factor was released for reuse (Fig. 14), thus functioning as a catalyst. Early evidence that it acts as a specific initiator was as follows. It was shown that RNA polymerase nonspecifically transcribes both strands of phage fd DNA, but when sigma factor was added it restored asymmetrical or single-strand transcription and the RNA synthesized changed from a heterogeneous molecular weight to specific RNA species.[672] In addition, core enzyme transcribed T4 DNA randomly and with low efficiency. When sigma factor was added, it stimulated transcription of a specific set of pre-early T4 genes.[26] These observations, demonstrating that sigma factor acts as a specific initiator

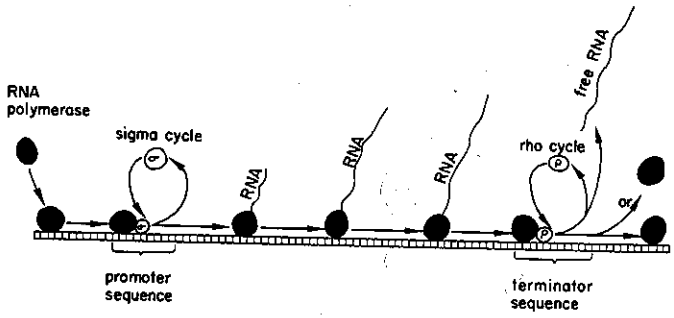

Fig. 14. The transcription cycle. RNA polymerase becomes attached to and detached to from the DNA. In the presence of the sigma factor, however, it may specifically fix to the promoter site. Once transcription is initiated, the sigma factor is released. RNA polymerase continues to transcribe DNA with RNA chain elongation. Through the interaction of rho factor, terminator sequences are recognized and the RNA chain is released. Rho is also released and RNA polymerase may either be freed or continue down the DNA molecule.

guiding the core enzyme to certain promoters, have been amply confirmed in many subsequent studies. In this respect it causes positive control and as such the mechanism of its action is of considerable interest.

Sigma Factor and Localized Strand Separation as a Mechanism of Positive Control

Since the core enzyme can readily bind to DNA, the sigma factor does not appear to be required in the formation of an RNA polymerase-DNA complex. However, these nonspecific complexes readily dissociate and sigma factor appears to function by stabilizing the complex at specific promoter sites.[310] There are several observations which suggest that sigma factor may function in part by melting or causing strand separation of a segment of DNA. (1) The initiation of RNA synthesis is much more rapid at 37° than at 20°.[721] (2) This temperature-sensitive step requires sigma factor.[310] (3) Sigma is not required for transcription of single-stranded DNA or DNA with a number of nicks.[82,712] Although the β' subunit of the core polymerase appears to be the subunit responsible for primary binding of the enzyme to DNA,[784] the presence of sigma factor is necessary for this to be site specific. It may act by binding tightly to a sequence of nucleotides on one of the two strands, thus locking this portion of DNA into an open configuration.[310]

Control by Alteration of the Core Enzyme

Although changing from one sigma factor to another was the most obvious mechanism to provide for a change in transcription from one set of genes to another, it soon became apparent that mechanisms involving changes in the subunits of the core enzyme itself were also being utilized by different systems. These included sporulation in *B. subtilis*, and the transcription of early and late genes in T7 and T4.

Sporulation of *Bacillus subtilis*

As *B. subtilis* cells pass from a vegetative stage into a sporulation stage, some enzyme activities disappear and some new ones appear. The RNA polymerase of the vegetative stage is able to transcribe phage ϕe DNA, but early in sporulation this capability is lost.[416] Furthermore, a class of mutants was found that resulted in a failure of *B. subtilis* to sporulate.[643] The RNA polymerase in these mutants failed to read ϕe DNA and was also rifampicin resistant. These observations suggested that during sporulation there was a change in the RNA polymerase which resulted in a change in its specificity of transcription. This was confirmed by the demonstration that the β subunit of the sporulating enzyme had decreased from a molecular weight of 155,000 to 110,000 daltons.[417] This change was probably brought about by proteolytic cleavage of the β subunit of the vegetative core enzyme (Fig. 15).

Bacteriophage T7, Early and Late Genes

When some of the bacteriophages enter *E. coli* cells, one set of early phage genes is immediately transcribed. In time a switching event occurs

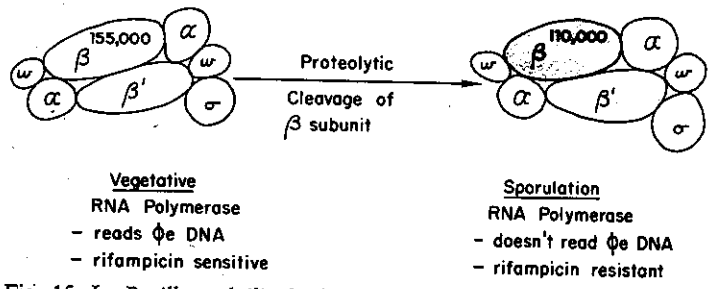

Fig. 15. In *Bacillus subtilis* the RNA polymerase molecule is altered by cleavage of the β subunit during transition from the vegetative to the sporulating cell (see text).

and a second set of late genes becomes expressed. In T7 phage, late gene synthesis is under the control of a product of a gene transcribed with the early genes (gene 1). Although it was initially felt that the product of gene 1 was a new sigma factor,[675] it was later shown that gene 1 was in fact coding for a new phage polymerase that was distinct from the host polymerase.[112]

T4 Phage: Pre-early, Early, and Late Genes

The situation is somewhat more complicated in T4 phage infection. In this case there are three stages—pre-early, early, and late. During the pre-early stage, a few phage genes are transcribed by the host core polymerase and sigma factor. A short time later, alpha subunits are modified by adenylation, and new phage ω subunits and sigma factor are added which appear to play a role in the specific transcription of early genes. Still later, a new phage β' subunit and still another phage sigma factor are added. By this time only the β subunit of the host remains and the late genes are transcribed.[166,270,697–700] This, then, is an example in which both the sigma factor and several different subunits of the core polymerase are involved in a series of modifications which result in the transcription of successive sets of different RNA molecules.

The Rifampicins

A series of antibiotics, rifamycins, rifampicin, and the closely related streptovaricin, specifically inhibit DNA-dependent RNA synthesis. They differ from actinomycin in that instead of interacting with DNA, and thus also inhibiting DNA replication, they interact with RNA polymerase and specifically inhibit RNA synthesis.[291] They are of some clinical importance since they inhibit viral as well as bacterial growth. Specifically, rifampicin blocks the addition of the first ribonucleotide at the initiation of RNA synthesis[694] and binds to be β subunit of the core polymerase,[784] which is the subunit that is altered in *B. subtilis* sporulation, rendering the polymerase rifampicin resistant. During normal transcription, once the initiation complex has been formed, it is resistant to rifampicin.[27]

Rho Factor and Chain Termination

The isolation and characterization of sigma factor required the tools of phosphocellulose chromatography and the effect of fractions on incorporation of label into the first as opposed to subsequent ribonucleotides. Utilizing these same tools, Roberts[571] was able to isolate a factor which

had no effect on chain initiation but had a marked effect on chain elongation. This factor termed rho (ϱ), for release, was found to be responsible for specific chain termination. In the absence of rho, RNA molecules released from λ DNA show a heterogeneous size distribution. In the presence of rho, two size classes of RNA, 12*S* and 7*S*, corresponding to the *N* and *X* genes that are located to the left and to the right of the C_I immunity region of λ DNA, are synthesized. Like sigma, rho is cycled and reutilized[172] (Fig. 14). Not all specific termination required rho factor, and the exact mechanism by which rho functions is unknown. It has been variously suggested that it binds to the RNA,[556] RNA polymerase,[172] and DNA.[172] It may serve to merely break the RNA chain without dissociating the RNA polymerase from the DNA. The latter may take place at a second type of termination signal.[111] The fact that its presence helps to limit T4 transcription to early genes raises the interesting specter of yet another type of control, antitermination factors.

Antichain Terminators

One proposed mechanism of controlling the transition from pre-early to early gene transcription in T4 bacteriophage was the synthesis of a new sigma factor.[698] An alternative possibility suggested by Schmidt *et al.*[600] is that the reason the transcription of the early genes follows so closely on the heels of the pre-early genes is that the rho-mediated termination of pre-early gene transcription has been prevented by a rho inhibitor. A similar type of mechanism was proposed by Roberts[571,572] to account for the stimulation of transcription by the product of the *N* gene of λ phage. Namely, it was suggested that the product of the *N* gene was acting as an antiterminator factor, thus stimulating the transcription of genes distal to *N*.

Psi Factor, ppGpp, and the Transcription of Ribosomal Genes

Even though ribosomal RNA genes constitute only a fraction of the genome of *E. coli*, their gene product constitutes up to 40% of the RNA of a rapidly growing culture.[703] However, when *E. coli* DNA is transcribed *in vitro* using purified RNA polymerase with sigma factor, no ribosomal RNA is synthesized. Since the *in vivo* synthesis of ribosomal RNA is sensitive to rifampicin inhibition, alterations in the core enzyme are probably not involved. These observations led Travers *et al.*[703] to suspect that a specific cofactor was involved in the transcription of ribosomal RNA genes. Utilizing

the stimulation of rRNA synthesis as an assay, they reported such a factor in *E. coli* extracts. This factor was termed psi.

Inactivation of Psi by ppGpp

When normal (stringent) *E. coli* are starved for amino acids there is an inhibition of ribosomal and transfer RNA synthesis but little effect on mRNA synthesis. Associated with this is an increase in the level of a nucleotide guanosine tetraphosphate, ppGpp.[98,99] This nucleotide appeared to bind to psi and inactivate it.[97] When mutant relaxed (RC) cells are grown in starvation conditions, the ppGpp does not accumulate and there is no inhibition of psi-mediated ribosomal RNA synthesis. Like the proposed anti-rho factors, this seemed to be another example of a situation in which a factor (ppGpp) inhibiting a stimulator (psi) played an important role in gene regulation (inhibition of RNA synthesis).

Psi as a Nonspecific Stimulator of Transcription

More recent studies of psi action confirmed its responsiveness to ppGpp but the specific effect on ribosomal RNA synthesis has not been reproducible.[701] Although it may act as a nonspecific stimulator of transcription, the ultimate role of the factor remains to be elucidated.

CAP, Cyclic AMP, and Multiple Gene Control

When bacteria are grown in glucose or other similar carbon sources, catabolites accumulate and there is a general repression of a number of inducible enzymes which are involved at different sites in the pathway for utilization of these compounds. This phenomenon, known as catabolite repression, appears to be due to a concomitant suppression in cyclic AMP synthesis, or loss of cyclic AMP by diffusion through the cell membrane.[431] Catabolite repression has been most extensively studied in relation to the lactose operon of *E. coli*. Zubay and colleagues[788] have found that both cyclic AMP and a protein factor termed catabolite activator protein (CAP, or CGA-catabolite gene activator) are necessary for activation of the lactose operon. *In vitro* studies indicate that CAP binds to DNA at promoter sites, and that this binding is markedly stimulated by the association of CAP with cyclic AMP.[561] Studies of a complete *in vitro* system of lac gene regulation[185] suggest that the lac promoter may possess two sites, one for RNA polymerase binding and one for CAP binding, and the two act synergistically to initiate transcription of the lac operon. It is of interest that the binding of

CAP-cAMP may not be specific for lac promoter DNA. It binds equally well to such diverse species as herring sperm DNA. The apparent specificity of action may arise from the possibility that only catabolite-sensitive promoters require CAP binding to function.[561]

There are several aspects of this system that deserve emphasis. (1) CAP represents a class of proteins which bind to DNA and require an association with cyclic AMP to do so. The possible relevance of this to hormone action and gene regulation in eukaryotes[196] is obvious. (2) CAP-cAMP is capable of activating a whole set of different genes. Such a mechanism would be of significant value higher on the evolutionary ladder for controlling whole sets of genes in different organs. (3) The binding of CAP-cAMP to DNA is nonspecific. This should be borne in mind in studies of DNA binding properties of nonhistone proteins in higher organisms. It cannot be assumed that proteins which nonspecifically bind to DNA of heterologous species are not involved in specific gene regulation.

Four Mechanisms of Positive Control and Their Relation to Gene Regulation in Eukaryotes

Negative control of transcription, involving the binding of a repressor protein to DNA to prevent RNA polymerase from binding or traveling, is easy to visualize. However, positive control, or stimulation of transcription which does not involve release of repression, is more difficult to conceive. From the above discussion of transcription control in prokaryotes it is possible to visualize four mechanisms by which this may occur.[111]

1. Sigma and sigmalike factors which primarily interact with RNA polymerase may help to melt a short but specific segment of DNA to initiate transcription.

2. The synthesis at a later stage in development of a new RNA polymerase which may be able to interact with promoters to which a previous RNA polymerase was insensitive. Early *versus* late gene transcription in T7 may be accomplished in this manner.

3. Inhibition of rho, or rholike termination factors. Keeping RNA polymerase functioning once it starts can be just as effective as stimulating it in the first place.

4. DNA binding proteins which interact primarily with DNA but function by either stimulating RNA polymerase or by melting a short segment of DNA. CAP, which has been shown to bind to DNA rather than to RNA polymerase, may function in this manner.

One might ask what all these prokaryote factors have to do with eukaryotes where gene activation is brought about simply by counteracting the repressive effect of histones. The answer is, possibly a great deal. It is not unlikely that gene activation in eukaryotes may require at least two steps: (1) the removal of histone repressors, and (2) the activation of transcription at specific promoter sites by one of the above mechanisms.

Mitochondrial RNA Polymerase—A Single Polypeptide

The studies of Chamberlin *et al.*[112] showed that the RNA polymerase of phage T7 was a single polypeptide of 100,000 molecular weight. Kuntzel and Schafer[386] have uncovered an even simpler example. They find that the RNA polymerase of the mitochondria of *Neurospora* is a single polypeptide of only 64,000 molecular weight. These monomeric enzymes indicate that multiple subunits are not always a necessity. On simple genomes, initiation, chain elongation, and termination can be accomplished with a single protein. Like the *E. coli* polymerase, the mitochondrial one is sensitive to rifampicin.

RNA Polymerases in Eukaryotes

The studies of RNA polymerase in prokaryotes have shown the diverse ways in which transcription can be controlled by alterations in the core subunits and in the sigma, rho, and psi factors. Although the exquisite beauty of some of these studies is difficult to reproduce in eukaryotes because of the lack of adequate genetic markers, a number of observations suggest that similar control may be possible in higher organisms because of the presence of three different RNA polymerases.

Earlier studies utilizing the effect of variations in concentration of magnesium, manganese, and ionic strength had suggested that there were two forms of RNA polymerase, one localized to the nucleolus and the other extranucleolar but still within the nucleus.[441,744] Utilizing DEAE cellulose chromatography, Roeder and Rutter[574,575] have isolated three forms of RNA polymerase termed I, II, and III, from sea urchin gastrula and rat liver. Others have found similar enzymes in fungi[320] and other organisms. Their characteristics are listed in Table III. RNA polymerase III is a minor fraction, which like *E. coli* polymerase, is sensitive to rifampicin. It may be a vestigial remnant of the prokaryotic enzyme or possibly a contaminating mitochondrial polymerase. Type I is localized in the nucleoli, binds only to ribosomal DNA, and is inhibited by cyclohexamide. Type II is found in the nucleus at extranucleolar sites and is sensitive to α-amanitin. Studies of

TABLE III. Eukaryote RNA Polymerases

Type[320,574,575]	I	II	III
Location[574,575]	Nucleolar	Extranucleolar	Extranucleolar
Inhibitors	Cyclohexamide[320]	α-Amanitin[320,413,574]	Rifampicin[320]
Mn^{++} 1–2 mM[574]	++	+++	++
Mg^{++} 2–8 mM[574]	++	±	+
Mn^{++}/Mg^{++} [574]			
sea urchin	1	10	2.5
mammalian	2	5	
rDNA binding[320]	++	0	
Ionic strength for[574,575] maximum activity	0.04 M	0.1 M	0.2 M
Comments[320]			Possibly a vestigal prokaryotic enzyme or a mitochondrial enzyme

agents which stimulate transcription suggest that sigmalike factors also exist for the eukaryotic polymerases.[165,285,693] The possible utilization of variations in these polymerases and their factors in differentiation are just beginning to be studied.

DNA HETEROGENEITY

The DNA of *E. coli* consists of about 4000 genes, most of which are present as single copies. For a time it was felt that the much larger amount of DNA present in higher organisms represented a simple extrapolation of this to result in a relatively homogeneous mixture of many more genes. However, it is becoming clear that this is only partially true and that the DNA of higher organisms represents a markedly heterogeneous collection of different families of DNA sequences. One of the early clues to this heterogeneity came with the discovery that if DNA is centrifuged to equilibrium in cesium chloride, so that molecules separate according to their mean base composition, in some species one or more satellite bands could be distinguished from the main band DNA.[368] Other types of heterogeneity based on timing of replication, differences in base composition, and degrees of repetitiveness have since been described.

Satellite DNA

In 1961 Kit[368] noted that when DNA from a number of different animals was centrifuged to equilibrium in cesium chloride, satellite peaks of varying buoyant density and size were observed. The rather prominent satellite that constitutes 10% of the total DNA of the mouse has been a particularly popular subject of study. When this satellite was isolated by preparative ultracentrifugation, and denatured to single strandedness by boiling, it was observed to renature very rapidly.[735] This phenomenon occurs because it is composed of a large number of similar or repetitious sequences.[735] Thus the task of a strand finding a complementary one to hybridize with is simple and takes place very quickly. Because of this, the mouse satellite, and the satellites of many other species, are also referred to as highly repetitious or rapidly renaturing DNA. The term rapidly renaturing is more descriptive and noncommittal since it does not make any prejudgments as to whether the repeated sequences are identical or merely similar. On the basis of renaturation data it has been estimated that mouse satellite is composed of a basic unit of 300–400 nucleotides[227] repeated some 10^6 times.[735] However, actual sequencing studies suggest a basic repeat consisting of 8–13 bases.[646] This overestimation of basic sequence length by renaturation data is probably the result of some mismatching of bases, which in turn is the result of the accumulations of numerous mutations in different copies of the basic sequence.[646,647,677]

Two features are necessary to allow a portion of the genome to occur as a distinct satellite. (1) It must have a mean base composition that is distinct from that of the main band. Thus satellite DNA which is enriched in guanine and cytosine will show a higher buoyant density, and satellite enriched in adenine and thymidine will show a lower buoyant density compared to the main band. (2) The sequences must be clustered. If they were interspersed between larger blocks of normal DNA they would never separate out as a distinct band. Since the DNA bands as a Poisson distribution, the greater the degree of repetitiousness, the smaller the deviation in base composition and the sharper the peak.

Asymmetrical Distribution of Bases in the Half DNA Helices

The satellite DNA can be separated into single strands (half DNA helices) by denaturation. If this is done with alkali (pH 12) the strands do not renature and their individual properties can be studied during centrifugation. If the base composition of the two half-helices were the same,

only a single peak would be seen. However, with mouse satellite two distinct peaks are actually observed,[226,227] indicating that the bases are nonrandomly distributed between the 5′-3′ and 3′-5′ strands. That this is also true for guinea pig α-satellite[228] can be seen from the analysis of its base composition which shows 3′-GGGATT-5′.[646] It is easy to understand that when the basic repeat is only 6 nucleotides long, as in the α-setellite of the guinea pig, or 8–13 bases in the mouse, by chance the two half-helices would be asymmetrical.

Different Species, Different Satellites

Studies of related species of rodents have shown that each species has its own distinct type of satellite. The buoyant densities vary such that the satellites range all the way from much heavier to much lighter than the main band with all gradations in between.[16,303,717] They also differ in base sequence. Hybridization studies show that the base sequence in the satellite of one species is distinct from that of even a closely related species.[227] This presents an interesting problem in evolution and speciation and suggests that satellites are lost and reevolved with some facility; otherwise a given species would have remnant satellites from all its predecessors.

Isolation of Hidden Satellites

If examination of many different species shows satellites ranging from heavier to lighter than main band DNA, it is apparent that some species will have satellites that are hidden in the main band because they have the same mean base composition as main band DNA, yet still possess the characteristic of repetitious clustered sequences. How are these detected and studied? There are several ways. (1) The rapidly renaturing DNA can be isolated from the bulk of the DNA by hydroxyapatite column chromatography. When this DNA is centrifuged, hidden satellites may be seen.[388] (2) Small fractions of main band DNA may be isolated by preparative ultracentrifugation. When these are recentrifuged, satellites are enhanced.[40] (3) The DNA may be centrifuged in cesium sulfate to which either Ag^+ or Hg^{++} has been added. This preferentially binds to certain bases, making some satellites significantly lighter or heavier than main band DNA.[161–163,777] (4) Finally, the DNA may be denatured, renatured for a short time, and then centrifuged. Since denatured DNA has a higher buoyant density than native DNA, and since repetitious satellite DNA renatures much more rapidly than the bulk of main band DNA, this allows the separation of hidden satellites.[143,161–163]

Human Satellites*

Studies by Corneo have shown that when native human DNA is centrifuged in cesium chloride, a small satellite, constituting less than 1% of the total DNA, with a buoyant density of 1.687 g/cm^3, is present[159,160] (the mean buoyant density of the main band of most mammals is 1.700 to 1.701 g/cm^3). When Ag^+ or Hg^{++} — $CsSO_4$ centrifugation was utilized, a second satellite (II) with a density of 1.693 g/cm^3 and constituting about 2% of the total was found.[163] More recently, with changes in the ratio of Ag^+ to DNA, yet a third satellite has been observed.[352] Centrifugation in Hg^{++} — $CsSO_4$ was noted to produce a homogeneous main band shoulder constituting about 15% of the total DNA. These components were isolated and used to prepare complementary RNA for *in situ* hybridization experiments. Complementary RNA of satellite I hybridized to all chromosomes with no specificity. Satellite II showed preferential hybridization to the centromeric regions of A_1, C_9, and E_{16}.[350a] The homogeneous main band component also hybridized nonspecifically to all chromosomes. There was no evidence for preferential hybridization of repetitious main band DNA to the Q or G banding areas (see below).

In Ag^+ — $CsSO_4$ centrifugation studies of human DNA, Saunders *et al.*[19,590] also found a satellite that was distinct from Corneo's I and II and had a buoyant density of 1.703 g/cm^3. They termed it satellite III and showed that it hybridized specifically to the centromeric heterochromatin of C_9. Whether this is the same as the third satellite mentioned above remains to be determined. Saunders *et al.*[590] also isolated by thermal elution on hydroxyapatite a repetitious fraction which showed preferential hybridization to the centromeric region of A_1.

Time of Synthesis of Mouse Satellite DNA

Because of the correlation between satellite DNA and constitutive heterochromatin, the time of synthesis of satellite during the S period has been of considerable interest. Several different techniques have been used. In studies by Smith[630] in which DNA synthesis was initiated in a confluent sheet of mouse kidney cells by infection with polyoma virus, the first DNA to be replicated was satellite DNA. However, when mouse L-cells were synchronized, labeled at different times in the S period, and the specific activity of the isolated satellite and main band determined,[692] the satellite

* Not to be confused with the cytological term satellite, referring to the secondary constrictions on the short arms of the acrocentric chromosomes.

seemed to be replicating very late in S. When the DNA was analyzed by preparative ultracentrifugation of pulse-labeled and control DNA rather than by isolating the satellite, this very late replication was not observed[141] although most of the satellite replicated in the last half of the S period.[229] In a study by Bostock and Prescott,[49] mouse L-cells were first pulse labeled, then "synchronized" by collecting cells during mitosis at different times after the label. When pulse-labeled DNA was centrifuged with DNA from unsynchronized cells, it was apparent that the satellite was labeled in the last half of the S period but completed its replication before the end of S. Thus under conditions of normal growth, mouse satellite DNA replicates in about the third quarter of the S period. The observation of Smith[630] that under certain circumstances the satellite can be made to replicate out of phase with the rest of the DNA remains to be explained. However, it is not difficult to believe that such a peculiar type of DNA may do peculiar things.

GC-Rich Satellites, Ribosomal DNA, and Gene Amplification

Utilizing RNA–DNA hybridization it has been shown that some of the GC-rich satellites that are heavier than the main band are enriched in ribosomal DNA.[40,73,410] In most tissues the amount of satellite DNA is constant, but in many amphibians and insects,[248] it has been shown that during prophase of oogenesis there is a massive amplification of nucleolar DNA and with it ribosomal DNA.[40,73,247,410] This results in an increase in the amount of heavy satellite in prophase oocytes compared to spermatocytes or somatic cells. Biochemical studies have shown that the 16*S* and 23*S* ribosomal DNA genes are adjacent to each other and sets are separated by spacer DNA.[40] Both the existence of gene amplification and the presence of spacers are clearly demonstrated by the electron micrographs of Miller (Figs. 9 and 10). In some cases the spacer DNA may actually have a higher GC content than the ribosomal DNA.[40] Not all heavy satellites contain ribosomal DNA. In the land crab[624] and red-backed salamander,[426] ribosomal RNA hybridized well with main band but poorly with heavy satellite DNA.

The possibility that gene amplification may occur was first raised by studies of DNA puffs in *Rhynchosciara* by Pavan[60,524] and later by Keyl.[363] Initially this idea was looked upon as heresy to the doctrine of DNA constancy, but since the studies on ribosomal DNA it has become well established. In fact, there is now the possible danger of going too far in the

opposite extreme by suggesting that structural genes coding for enzymes and other proteins may all undergo gene amplification during some stage of development. Some evidence for it has been found in the rather special condition of mouse myelomas,[384] but when specifically looked for in rat hepatomas it was not found.[611] The general constancy of DNA content in different organs makes it seem unlikely that gene amplification is extensively used for other than a few specific instances. The relatively unusual nature of ribosomal gene amplification was emphasized by the proposal that this may represent the presence of independent, nonchromosomal, self-replicating rDNA satellites restricted to the germ line.[720] The interesting possibility that the reverse transcriptase may play a role in gene amplification remains to be investigated.

Highly, Moderately, and Nonrepetitious DNA

A significant advance in our understanding about the composition of the eukaryotic genome came with the demonstration by Britten and Kohne[66] that DNA is composed of three major classes: highly, moderately, and nonrepetitious. This observation was based on studies of the rate of renaturation of DNA of higher organisms. DNA is sheared into short pieces of approximately 500 nucleotides in length, heat denatured, and then allowed to renature. The extent of renaturation is followed by the decrease in optical

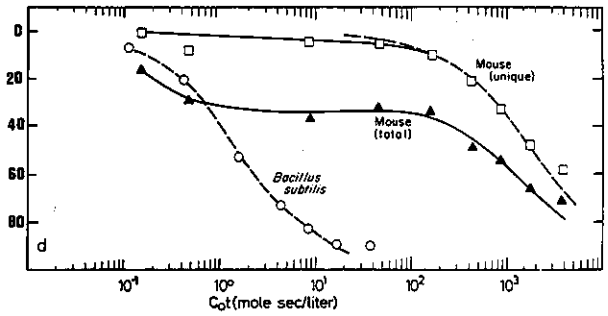

Fig. 16. *Cot* curves. Ordinate: Percent DNA renatured, Abscissa: Concentration of DNA at time zero in moles of DNA phosphorus × time in seconds. *Bacillus subtilis* DNA with a relatively simple genome renatures rapidly. Mouse DNA with a large number of unique sequences renatures a thousand times more slowly than *B. subtilis* DNA. Total mouse DNA containing both highly repetitious and unique DNA has a biphasic curve. The repetitious DNA renatures rapidly, the unique slowly. (From Laird,[388] by permission.)

density or by periodically separating the renatured strands by hydroxyapatite column chromatography. The rate of renaturation is expressed as *Cot*, in which *Co* is the initial concentration of DNA expressed in moles of DNA phosphorus or moles of nucleotides per liter, and *t* is time in seconds. It is expressed on a logarithmic scale ranging from 10^{-4} to 10^{4}. DNA renaturing at low *Cot* values (10^{-4} to 10^{-1}) is composed of highly repetitious sequences, DNA renaturing at intermediate *Cot* values is moderately repetitious (10^{0} to 10^{2}), and DNA renaturing at high *Cot* values is minimally or nonrepetitious (Fig. 16). The content of these classes varies considerably. In general, highly repetitious DNA constitutes 3–30%, nonrepetitious DNA 30–80%, and intermediate the remainder. Minimally or nonrepetitious DNA is usually the predominant type, but in some species such as *Amphiuma*, which contain huge amounts of DNA per cell, repetitious DNA predominates.[66]

Not all of the highly repetitious DNA is satellite DNA. A significant portion is dispersed throughout less repetitious sequences in main band DNA.[66]

Four Types of Rapidly Renaturing DNA

For an adequate appreciation of the relationship between repetitious sequences and heterochromatin it is important to point out that there are several categories of rapidly renaturing DNA.

Satellite DNA

As discussed above, these represent clustered repetitious sequences which because they have a distinct base composition can be separated by centrifugation from the rest of the DNA.

Hidden Satellite DNA

Clustered repetitious sequences which just happen to have the same average base composition as main band DNA are satellites in every sense of the word except they do not form a distinct band by centrifugation. They can be studied by one of the techniques described above for isolation of hidden satellites.

Highly Repetitious, Nonclustered Sequences

Species both with and without satellite DNA have significant amounts of rapidly renaturing DNA which is interspersed throughout the genome.[66]

Such DNA, even if it has a distinct base composition, could not be separated by centrifugation. It has been suggested that some of these sequences may play a role as regulator genes.[65] If this is true, they would of necessity have to be dispersed throughout the genome and would be unavailable for accumulation into either satellites or into constitutive heterochromatin.

Foldback DNA

On some occasions, regardless of how low the *Cot* values, or how dilute the DNA, there is a certain amount of almost instantaneous though somewhat imperfect, renaturation. This suggests that some sequences do not have to find each other in solution because they are already attached, that is, a single strand may fold upon itself and renature with some similar but reversed repeats. Britten and Smith[68] have termed this foldback DNA. There is also a certain degree of decrease in O.D. upon dropping the temperature from denaturing conditions (100°C) to renaturing conditions (60°) purely as a result of random coil formation.[194]

Recent reviews have covered many other aspects of repetitious and satellite DNA.[48,66,67] The relationship between repetitious DNA and heterochromatin and the possible functions of satellite DNA are discussed later.

Transcription of the Different Classes of Repetitious DNA

The question of whether all or only some of the different classes of repetitious DNA are transcribed is central to a number of theories concerning both gene regulation and the role of repetitious DNA in heterochromatin. For example, if satellite DNA is luxuriantly transcribed, this would be difficult to fit with its relationship to heterochromatin; if structural genes exist as single copies, then cytoplasmic messenger RNA should hybridize predominately to nonrepetitious DNA; and if moderately repetitious DNA plays a role in gene regulation at the posttranscriptional level, then some of the cytoplasmic messenger RNA should hybridize to moderately repetitious DNA. What is the evidence on these points?

Satellite DNA

The sequence studies of the α-satellite of the guinea pig[646] indicate that it is extremely unlikely that a protein would ever be synthesized from this DNA. Of the six possible reading frames, two give either nonsense or chain

terminating codons, and a protein of such monotonous repetition of two amino acids would seem useless. Although the sequence of mouse satellite has not been completed, the same objections would hold. If mouse satellite does not produce a protein, does it produce RNA? In the RNA from rat liver, Flamm *et al.*[227] found essentially no molecules that were complementary to satellite DNA. However, when rapidly labeled nuclear RNA was examined, Harel *et al.*[286] found that some hybridized to the light dA-rich strand of mouse satellite, although the counts were quite low. If this is not just some type of artifactual nonspecific cross-reaction, these results would suggest that (1) if satellite DNA is transcribed, the RNA is rapidly broken down and probably serves no functional purpose; (2) even though the satellite DNA travels with the heterochromatin fraction, some of it may be transcribed, and (3) it suggests that the cell is not able to completely shut off transcription of what surely must be described as junk DNA.

Moderately Repetitious DNA

The studies of Britten and Kohne[66] indicate that with the concentrations and incubation times usually employed, the RNA–DNA hybridization technique examines only the highly and moderately repetitious RNA sequences. The fact that these reactions go at all indicates that there is a certain amount of repetitious DNA that is transcribed into the nuclear and cytoplasmic DNA. The role of this DNA in the regulation of gene expression remains to be clarified.

Some of the moderately repetitious sequences consist of ribosomal and transfer DNA, and possibly histone genes.[361]

Nonrepetitious DNA

To examine the RNA that hybridizes to nonrepetitious DNA requires special techniques. These include the use of extremely high concentrations of RNA with high RNA–DNA ratios,[74,178,594] or the removal of more repetitious DNA sequences by hydroxyapatite chromatography,[74] or preincubation with repetitious RNA to cover up repetitious DNA sequences,[175] or the use of excess DNA and removal of RNA–DNA hybrids by hydroxyapatite chromatography.[361,434]

Using a combination of such techniques Brown and Church[74] studied transcription from nonrepetitive DNA of the mouse. They found that 10% of unique DNA was complementary to *unlabeled* brain RNA while less than 2% was complementary to liver, kidney, or spleen DNA. This suggests that 5 times as many unique sequences are active in brain cells compared

to other organs. Since about two-thirds of the genome consists of unique sequences in the mouse, this also suggests that at least 8% of the genome in mammals contains functional genes. Similar results were obtained by Hahn and Laird.[281] In a study by Gelderman *et al.*[256] 12% of single-copy DNA, or 8% of the genome was complementary to *rapidly labeled* RNA from neonatal and fetal mice. In an examination of the complexity of RNA synthesized by *Xenopus* oocytes, Davidson and Hough[178,179] found that 1.2% of the nonrepetitive DNA, or about 2.5% of the genome was complementary to the oocyte RNA. Of particular significance was the fact that this represented a diversity of approximately 40,000 genes of 500 nucleotides in length. Since this RNA was derived primarily from synthesis by the lampbrush chromosomes, and since there are in the order of 5000 loops per haploid set*,[94] this suggests the possibility that more than one type of RNA molecule is synthesized by each lampbrush loop.

Gelderman *et al.*[256] suggested they were underestimating the degree of hybridization in their study and felt that up to 25% of the genome was being transcribed in fetal and neonatal mice. It should be emphasized that they were using rapidly labeled RNA that was derived from the whole mouse. This would thus include the transcribed sequences from all organs, and since rapidly labeled RNA was used it would also include many RNA sequences that would be rapidly broken down and never transcribed. Nevertheless, this figure is of considerable interest for several reasons. (1) If only 10 to 20% of the genome contains useful genes and the rest is junk (see below), this would be further evidence that the cell has not learned to turn off the transcription of all of its useless DNA. Or to put it differently, a gene may lose its translational usefulness before it loses its transcriptional abilities. (2) It suggests that during embryogenesis a significant number of genes may be active that are inactive in adult animals, and if only 10–20% of the genome contains functional genes, then almost all of them must be in use during embryogenesis.

Early and Late Replicating DNA

When mammalian cells are synchronized in tissue culture and pulse labeled at different times during the S period, it has been observed that early replicating DNA is relatively GC-rich and late replicating DNA is relatively

* Although this value is for *Triturus cristatus* which has 67 pg per diploid complement[214] compared to 6 pg for *Xenopus*,[182] the loops are smaller in *Xenopus*[177] and the number in the two species are comparable.

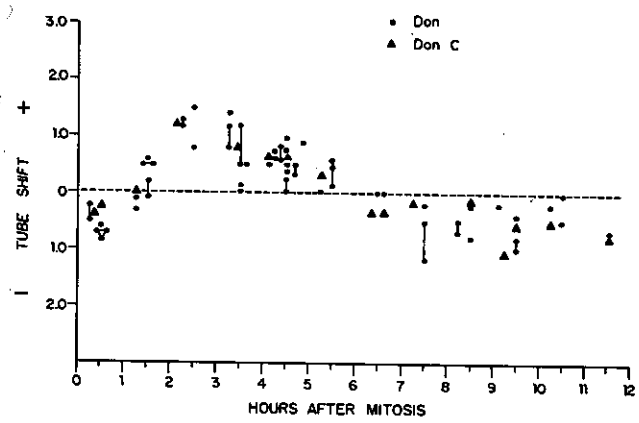

Fig. 17. Base composition and time of DNA replication. A + tube shift indicates relative GC-richness, a – represents relative AT-richness. The data are based on preparative ultracentrifugation of Chinese hamster cell ^{3}H-DNA (from 30 to 60 min pulse labeling of synchronized cells) with ^{14}C-DNA (from unsynchronized cells). The very earliest DNA to replicate is relatively AT-rich, the early replicating euchromatin is GC-rich, and the late replicating heterochromatin is AT-rich.[134]

AT-rich.[49,50,50a,134,229,692] This has been demonstrated by utilizing several different methods of determining base composition, including preparative ultracentrifugation in cesium chloride,[49,50,50a,134,692] DNA Tm,[143] and direct chemical analysis.[692] There are two major alternatives to explain this observation. (1) There may be something inherent in the base composition of replicons that predisposes GC-rich ones to replicate early and AT-rich ones to replicate late,[50,50a] or (2) the DNA of euchromatin may be relatively GC-rich and the DNA of genetically inactive heterochromatin may be relatively AT-rich.[134] The observation[134] that in synchronized Chinese hamster cells the very earliest replicating DNA actually tends to be relatively AT-rich (Fig. 17) suggests that timing *per se* is not the major factor. Further evidence that AT richness may be a characteristic of heterochromatic DNA comes from the observation that on the basis of ultracentrifugation the main band DNA of highly purified mouse heterochromatin has a significantly decreased buoyant density (more AT-rich).[439]

Although the tendency for late replicating DNA to be relatively AT-rich has now been demonstrated in human, mouse, Chinese hamster, and rabbit cells, there is some evidence that there may be some exceptions. For example, in the quail there is a prominent GC-rich heavy shoulder

component which is enriched in the heterochromatin fraction[142] and in the quail much of the heterochromatin is restricted to the late replicating microchromosomes.[140] It is therefore likely that at least in this species the late replicating DNA would actually be relatively GC-rich.

Heavy Shoulder DNA

When a homogeneous species of DNA is examined by analytical ultracentrifugation the resulting peak is a perfect Gaussian distribution. However, in a number of species there is a non-Gaussian distribution to the GC-rich heavy side of the main band. This has been termed heavy shoulder DNA[133] (Fig. 18). The reproducibility with which it is observed in some species, its absence in other species, the fact that it presents as a distinctly bimodal curve when the heavy third of the meain band is isolated and recentrifuged, the observation of bimodality in the DNA Tm curve of DNA from the heavy one-third of the main band, and its localization to specific subnuclear sites in some organisms,[133,142,143] all indicate that it is a real component and not an artifact of centrifugation. Studies of isolated heterochromatin and euchromatin in the chicken and quail show that heavy shoulder DNA is enriched in the heterochromatin fraction of the Japanese quail, but not in the chicken. The fact that the microchromosomes are heterochromatic in the quail[140] but not in the chicken suggests that heavy shoulder DNA may be localized to the microchromosomes. Thus if the microchromosomes are heterochromatic, heavy shoulder DNA comes down with the heterochromatic fraction; if the microchromosomes are not heterochromatic it does not.[142] The presence of a distinct family of DNA molecules in the tiny chromosomes raises the interesting question: Did the microchromosomes arise from some type of hybridization between two different species early in the evolution of birds and reptiles? The presence of heavy shoulder DNA in the Indian Muntjac[182] which has only large chromosomes, suggests it is not always associated with microchromosomes. The significance of this type of DNA heterogeneity is unknown.

Replicative Heterogeneity

When highly synchronized Chinese hamster cells are pulse labeled with ^{3}H-thymidine at short intervals throughout the S period, and this DNA is centrifuged with ^{14}C-labeled DNA from unsynchronized cells, it can be seen that the pulse-labeled DNA frequently shows one or more subcomponents (Fig. 19), while the control DNA shows a smooth Gaussian curve.[134]

This suggests that within the main band there are several subfamilies composed of DNA sequences with a similar base composition and replicating at similar times. It is possible that these may represent groups of related sequences that have arisen through extensive gene duplication. A suggestion of a similar type of main band heterogeneity was noted by thermal denatu-

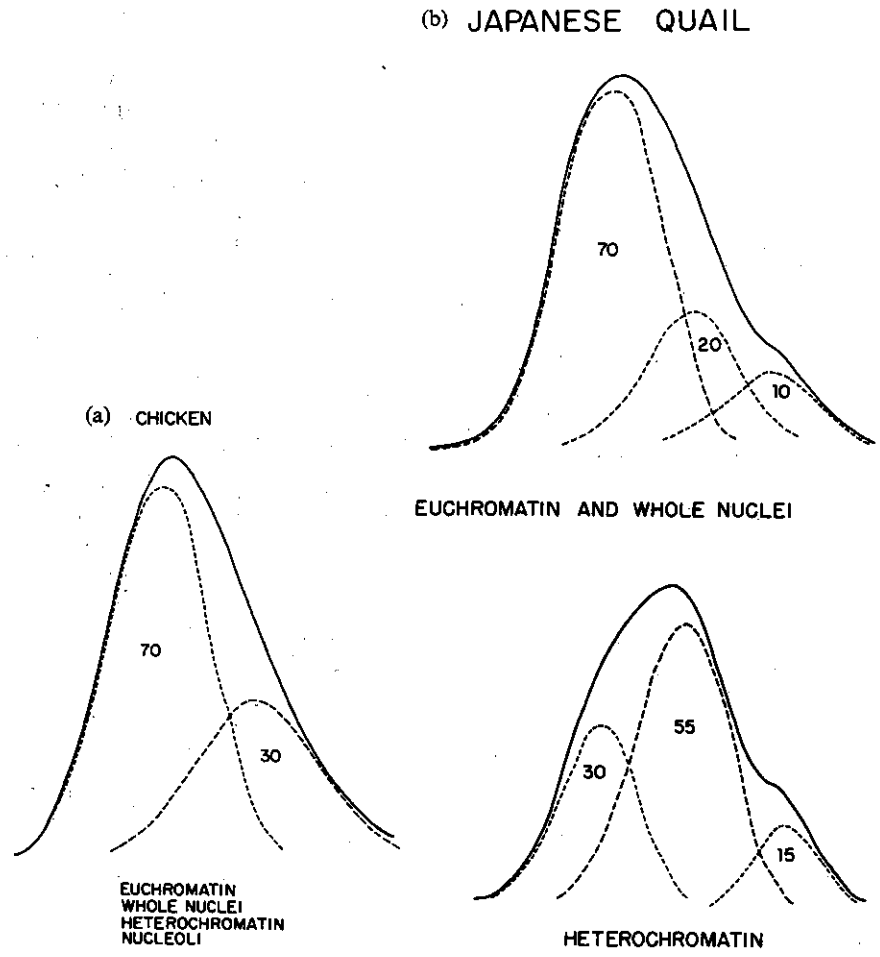

Fig. 18. (a) Heavy shoulder DNA in the chicken. The main band DNA has a non-Gaussian distribution with a heavy shoulder component constituting 30% of the total DNA. It is not enriched in the DNA from the heterochromatin fraction.[134] (b) Heavy shoulder DNA in the Japanese quail. There are three components in DNA from whole nuclei: main band = 70%, heavy shoulder = 20%, and satellite = 10%. In DNA from the heterochromatin fraction there is a marked enrichment of the heavy shoulder DNA to 55%.[142]

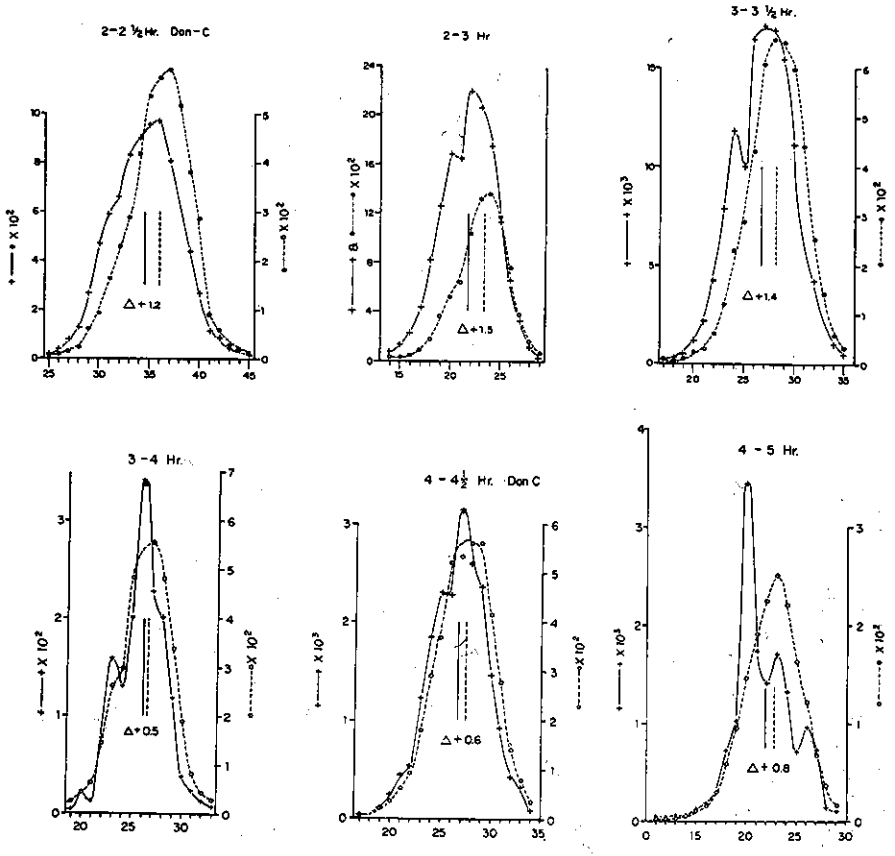

Fig. 19. Replicative heterogeneity of DNA. DNA from synchronized Chinese hamster cells labeled for 30–60 min with ^{3}H-thymidine (solid line) is centrifuged with DNA from unsynchronized cells labeled with ^{14}C-thymidine (dotted line). It can be seen that the pulse-labeled DNA frequently shows one or more subcomponents, suggesting there are families of DNA with similar base compositions which replicate at similar times in the S period.[134]

ration studies and by recentrifugation of mammalian DNA subfractionated by preparative ultracentrifugation.[449] The subcomponents have the same composition in regard to repetitious DNA as the unfractionated DNA.

A somewhat different type of heterogeneity was noted following the stimulation of DNA synthesis in lymphocytes. Different antigens stimulated the synthesis of DNA with a distinct base composition,[645] raising the interesting possibility that such stimulation may have called forth specific sequences to be preferentially replicated.

Summary

From the above it can be seen that the DNA of higher organisms is far from homogeneous. Various techniques are capable of demonstrating a remarkable degree of heterogeneity. This encompasses different degrees of repetition of similar sequences, a different base composition of early and late replicating DNA, the presence of several subfamilies of sequences with a similar base composition that replicate at similar times, satellite DNA's of different base composition and sequence, and nonsatellite heavy shoulder components. Even greater microheterogeneity may be detectable by RNA-DNA hybridization of the type used by Kedes and Birnstiel[361] to detect the presence of multiple histone genes in the GC-rich portion of DNA.

The recent development of *in situ* hybridization and a number of staining techniques which distinguish between different portions of the chromosome are either directly detecting some of this heterogeneity by interacting with the DNA, or indirectly detecting it by interacting with the non-histone proteins it is associated with.[132,143] The continued development of such techniques will put this heterogeneity of DNA to good use in cytogenetic identification.

DNA REPLICATION AND THE NUCLEAR MEMBRANE

Introduction

In 1963 Jacob *et al.*[340] proposed that in bacteria, DNA replication was controlled and initiated at a site where the DNA was attached to the cell membrane. The unit of DNA replication was termed a replicon. In the subsequent years many studies have lent support to this concept.[225,251,282,342,396,619,633,635,704] Although the Kornberg DNA polymerase long held sway as *the* polymerase for normal DNA replication, studies of mutant cells[191] which were defective in this enzyme now suggest that it is primarily a repair enzyme. A contender for the role of true polymerase is DNA polymerase II, isolated from polymerase I negative (Pol A_1-) mutants. This enzyme is intimately associated with the bacterial cell membrane and has a number of distinctive characteristics.[378,380,500]

In view of the apparent importance of membranes in regulating bacterial DNA replication, Comings and Kakefuda[139] investigated the possibility that DNA replication in eukaryotes might also be initiated at membranes, namely the nuclear membrane. They synchronized human amnion cells to the beginning of the S period with excess thumidine and amethopterin, then labeled

cells for 10 and 20 min with ^{3}H-thymidine. Control, unsynchronized cells were also labeled. Electron microscope autoradiography of the synchronized cells labeled for 10 min showed that in most of the nuclei the label was restricted to the region around the nuclear membrane and nucleolus. In some cells, the label was diffuse throughout the nucleus. In the synchronized cells labeled for 20 min the restriction of label to the membrane was less striking, and in the unsynchronized cells most of the nuclei showed grains scattered throughout the nuclei, although in some there was a ring of grains at the nuclear membrane. These results were interpreted as suggesting that in some or all replicons, DNA synthesis was initiated at the nuclear membrane, but that the replication point soon left the site of membrane attachment and traveled along the DNA to other parts of the nucleus. What is the present status of the relation between DNA replication and the nuclear membrane in eukaryotes?

Other Autoradiographic Studies

The above findings suggested that there should be two times during the cell cycle when replication is predominately associated with the nuclear membrane, very early in S when replication is just getting started, and late in S when the membrane-associated heterochromatin is replicating. To investigate the possibility that whenever grains were seen at the nuclear membrane it was due solely to the replication of heterochromatin, we repeated these studies in *Microtus agresti* cells. This species was chosen because the majority of its heterochromatin is localized to the giant sex chromosomes which form two large heterochromatic masses in the cell nucleus.[756] If membrane-associated grains are due entirely to heterochromatin replication, then such grains should be found only where these masses attach to the membrane. However, as shown in Fig. 20, from a culture of cells synchronized with amethopterin, the grains were all around the nuclear membrane. Interestingly, in this experiment a significant number (30%) of cells in the unsynchronized control culture also showed a perinuclear distribution of grains. Among other things, this could mean that the addition of fresh labeled media to the unsynchronized cultures stimulated a number of cells resting in G_1 to pass into S, or it could have some relevance to the biochemical studies (see below) which suggest that the replication point may remain associated with the membrane. Others have made similar observations of a perinuclear ring of grains after pulse labeling in synchronized *Microtus*[485] and CMP cells,[358] in HeLa cells in which DNA polymerase was first temporarily blocked with caffeine,[385] and in unsynchronized dog

Fig. 20. *Microtus agrestis* unsynchronized tissue culture cells labeled with ^{3}H-thymidine. (a). 10 min, × 7800. (b). 10 min, × 7800. (c). Pulse-labeled for 4 hr with 2.5 μCi/ml, then chased with cold thymidine for 36 hr, × 7700. (d). 60 min, × 6800. In (a) the label is entirely around the nuclear periphery, not just at the regions of heterochromatin, while in (b) presumably a late-replicating cell, the label is confined to the areas of heterochromatin. The clear distinction between these two indicates that when nuclear membrane labeling is seen it is not because the cell is in late S. The diffuse label of the pulse chase (c) indicates the membrane labeling is not due to an excess concentration of chromatin at the periphery; (d) is a typical example of heavy membrane labeling.

kidney[770] and HeLa[484,472a] cells. In some cases peripheral labeling has not been observed.[221]

In a study of Chinese hamster cells synchronized by both mitotic selection and FUdR, and pulse labeled for 10 min, Williams and Ockey[749] were unable to find a nuclear membrane-associated pattern of grains in mitotically synchronized cells exposed to amethopterin or FUdR for 11 hr, or in mitotically synchronized cells labeled 2.5 hr after mitosis. They subsequently observed that the cells exposed to the inhibitors were still undergoing DNA replication and were thus not stopped at the beginning of S.[485] After long exposures to these inhibitors the nuclear pattern of grains was observed and attributed to the breakdown of DNA occurring with thymineless death.[485] However, in our own studies with Chinese hamster cells we found that some DNA replication actually begins almost immediately after mitosis,[134] and when the cells are labeled at this time the peripheral localization of grains is clearly seen in some cells without the use of any blocking agents.[153]

In studies of ^{3}H-thymidine labeled lymphocytes stimulated with phytohemagglutinin, Milner[462a,b] noted, that when a considerable amount of condensed heterochromatin was still present, the label preferentially occurred at the junction between the condensed chromatin and the euchromatin. When transformation was complete and there was little condensed chromatin left, the label preferentially occurred at the nuclear membrane. We have noticed similar tendency for the label to occur at the interface between condensed and dispersed chromatin in unsynchronized Chinese hamster cells.[153] The exact significance of this is unclear but it certainly makes it unlikely that the replication point is always associated with the nuclear membrane.

Other Cytological Techniques

A completely different approach to this problem has been utilized by Freeman *et al.*[236] They utilized fluorescent-tagged antibody to single-stranded DNA and demonstrated that during the earlier part of the S period, fluorescence was detected around the nuclear membrane of human fibroblasts stimulated to undergo DNA synthesis by subculturing. During the latter part of the S period the label was more diffusely distributed throughout the nucleus.

A DNA-Protein-Lipid Complex in Somatic Cells

When chromatin is sheared for increasing periods of time, increasing amounts of DNA are solubilized. Jackson *et al.*[339] noted that despite extensive shearing of calf thymus, chromatin, about 1% of the DNA remained in an insoluble form. Analysis of this fraction showed it contained a significant amount of lipid. By thin-layer chromatography the major phosphatides were shown to be phosphatidyl choline and phosphatidyl ethanoline. Analysis of the nonhistone protein in the complex showed it was rich in asparagine and glutamine, low in net charge, and was hydrophobic. These properties are compatible with its insolubility and lipid binding capability.

When attention has been focused on the nuclear membrane rather than the chromatin, it has been observed that analysis of the inner nuclear membrane fraction shows it to contain DNA.[783]

When mammalian cells are gently lysed with detergent and centrifuged on sucrose gradients, rapidly sedimenting material which appears to be DNA of a very high molecular weight is observed. On the basis of a series of experiments utilizing varying doses of X-ray to break DNA, and ^{14}C-acetate to label lipids, Ormerod and Lehman[503] concluded that this high molecular weight complex was due to the attachment of DNA to the nuclear membrane at numerous sites. In cesium chloride, the complex banded at 1.40 g/cm^3 compared to 1.70 g/cm^3 for DNA. DNA was released from the complex following its breakage with X-ray. From a mathematical model it was estimated that the attachment points were spaced at intervals of 2×10^9 daltons, or every 1000 μ of DNA. Although this is considerably longer than the average replicon sizes of 7–100 μ,[92,332] this technique measures only detergent-stable attachment points, and these may be fewer than the total number of attachment points.

A DNA-Protein-Lipid Complex in Meiotic Cells

In studies of DNA replication in microsporocytes of the lily, Hecht and Stern[293] noted the appearance, during the premeiotic S phage and during zygotene, of a complex of DNA with protein. This complex was detected on the basis of the buoyant density of DNA centrifuged in cesium sulfate. The complex was stable in solutions of high ionic strength, but could be dissociated with sodium dodecyl sulfate. The complex also contained phospholipid, DNA polymerase, and could synthesize DNA from triphosphate precursors. The DNA associated with the complex was recently replicated DNA. The M band technique of Trembly *et al.*[704] whereby phospholipid membrane material bands at a characteristic density in the presence of Mg^{++} and sarkosyl, was also effective in isolating this complex. No such complex was found during pachynema when the DNA synthesis that occurs represents repair-replication. These findings are consistent with

the association of replicating DN with the nuclear membrane during both the premeiotic S phase and zygotene. The significance of the zygotene DNA replication is unknown.

Replicating DNA and the Nuclear Membrane—Biochemical Studies

It has frequently been observed that in the usual phenol[37,239] or isoamyl–alcohol–chloroform[239,404] techniques for the extraction of DNA, nonreplicating DNA was easily released into the aqueous phase, while recently replicated nascent DNA was difficult to release and remained associated with the protein in the interface between the water and the phenol or chloroform. In studies of this property, Friedman and Mueller[239] found that it required 60–120 min post-replication before the DNA could be released to the aqueous phase. Appropriate pulse and chase experiments suggested it was the site of replication rather than the site of initiation that was attached to some cellular component. Because of the hydrophobic characteristics of this complex they suggested it contained lipopolysaccharides.

In additional studies of the portion of chromatin that remains insoluble after shearing, Yoshikawa-Fukada and Ebert[776] demonstrated that it contains a polymerase and is capable of immediately incorporating nucleotide triphosphates into DNA while cytoplasmic polymerase showed a lag time of 5–8 min. This lag could be abolished if the cytoplasmic polymerase was preincubated with heat-denatured DNA. The cytoplasmic polymerase required added DNA template while the membrane polymerase did not. If the insoluble fraction was allowed to replicate for 30 min, then centrifuged, DNA synthesizing activity was present in the supernatant, suggesting that some DNA polymerase molecules had been released as free enzyme. These observations suggested that in the insoluble membrane fraction, DNA and DNA polymerase exist in an active complex. The results of using BUdR incorporation indicated that the DNA replication taking place in the insoluble fraction was true semiconservative replication rather than terminal repair replication. It was also observed that the DNA extracted from the insoluble fraction hybridized to the RNA of Rous sarcoma virus to a greater extent than DNA of the soluble chromatin. This was of interest in view of the fact that one property of this tumorogenic virus is its ability to stimulate DNA replication, and that many viruses appear to associate with the nuclear membrane.[470,520] This observation that the membrane-associated DNA may be a distinct type of DNA is consistent with other studies which suggest that the initial portion of the replicon may be AT-rich[134,683] and may be

composed of a piece of repetitious DNA.[141] This would also be consistent with a distinct initial portion of the replicon (replicator)[340] being associated with the membrane prior to the onset of DNA synthesis.

When the nuclear membranes of regenerating rat liver cells that had been pulse labeled with ^{3}H-thymidine were isolated by sucrose gradient centrifugation, it was found that the DNA with the highest specific activity was found to be associated with the inner nuclear membrane.[468] Although this fraction contained only 6% of the total DNA, its specific activity was five times that of the bulk DNA in the pellet. Pulse chase experiments showed that the label could be chased from this membrane-associated fraction.

In a related experiment, differing in that HeLa cells were used and the membrane-bound DNA was isolated by the Mg^{++}-sarkosyl technique, Hanaoka and Yamada[283] also found that in pulse chase experiments label could be observed to move away from the membrane-bound DNA. A similar result was observed by O'Brien *et al.*[484] They utilized HeLa cells that had been labeled for only 1 min. The isolated nuclei were sonicated and a nuclear membrane fraction, containing only 6% of the DNA, was isolated by differential centrifugation. The ratio of the specific activity of the DNA of this fraction compared to the rest of the DNA was 4.80. After only a 5-min chase it dropped to 0.60 and plateaued at 0.24 by 30 min. Electron microscopy of the cells pulsed for 1 min showed label at the nuclear membrane. When pulsed for 1 hr the label was distributed throughout the nucleus.

This biochemical data suggests that the replication point may always remain at the nuclear membrane. However, the autoradiography studies show that when unsynchronized cells are pulse labeled for 10 min many cells show label throughout the nucleus suggesting some replication forks are not attached to the nuclear membrane. These differences might be explained by suggesting that the replication fork quickly moves away from the membrane and thus, after a 10-min pulse, some label would be seen in the center of the cell. To clarify this point, we have pulse labeled unsynchronized Chinese hamster cells for 0.5, 1, 2, 4, 10, and 20 min. There was no evidence, by electron microscope autoradiography, for a greater amount of membrane label in the shorter pulses.[153] Even in the cells pulse labeled for 30 sec, a significant number of cells showed label throughout the nucleus suggesting that many replication forks must be independent of the nuclear membrane.

Conclusions

There are three major alternatives in the interpretation of this data: (1) DNA replication in some or all replicons may be initiated at the nuclear

membrane. (2) The replication fork may remain at the nuclear membrane. (3) The nuclear membrane may have nothing to do with DNA replication and the patterns observed may be due merely to the association of heterochromatic DNA with the nuclear membrane. There is evidence both for and against each of these alternatives, and the data at present is insufficient to allow an unambigious choice as to which is correct. Further studies are clearly needed.

One-Way vs. Shuttle Replication

There are two possible ways a replicon can be synthesized. For example, during the first cell cycle, replication would proceed from point *A* to *B*. During the second cell cycle it could once again proceed from point *A* to *B*. This might be called "one-way" replication. Alternatively, during the second cell cycle it could proceed from point *B* back to point *A*. This might be called "shuttle" replication. The appropriate autoradiographic experiment, with two different isotopes, or the use of ^{3}H-thymidine and ^{14}C-BUdR, might distinguish between these alternatives.

Spacing on the Nuclear Membrane and DNA Replication

It was suggested by Comings[129] that if the replicator portions of replicons are attached to the nuclear membrane, the spatial arrangement of these attachment sites might play a role in controlling several aspects of DNA replication. This could have relevance to the simultaneous replication of homologous portions of homologous chromosomes, to the replication of heterochromatin, and to various facets of X chromosome inactivation. A similar idea for bacteria was expressed by Marvin[437] who suggested a relationship between the rate of cell growth, DNA replication, and the surface area of membrane. This site-territory concept suggests that the initiation of DNA replication requires the creation of a new attachment site for the daughter strand, and if the membrane is overcrowded such sites are limited and a new round of DNA replication cannot occur. Several observations in eukaryotes offer some support for this concept. One is the finding by Alfert and Das[6] that the rate of DNA synthesis in tetraploid snapdragon seedlings was closely related to the increase in nuclear surface area. The second is the observation by Harris[289] that when chicken erythroblast nuclei are stimulated to undergo DNA replication by hydridization with HeLa cells, they show significant increase in nuclear size before either

DNA or RNA synthesis takes place. Also, some observations concerning the changing replication patterns of chromosome segments following deletions or translocations could be explained on this basis.[34]

The observation that the number of nuclear pores, which probably represent the site of attachment of the chromatin to the nuclear membrane, increases during the S period[440] is also consistent with the need for new attachment sites during DNA replication. In general, however, the concept that the nuclear membrane plays other than a passive role in regulating DNA replication has a good way to go before attaining more than theoretical validity.

HOW MUCH DNA IS JUNK?

Why should the disturbing possibility that some of the DNA of our genome is relatively useless junk even be considered? There are several reasons: (1) Some organisms have an unreasonable excess of DNA, clearly more than they require. (2) Reasonable estimates of the number of genes necessary to run a eukaryote seem significantly less than the amount of DNA available. (3) The mutational load would be too great to allow survival if all the DNA most eukaryotes carry was composed of essential genes. (4) Some junk DNA, such as mouse satellite, clearly exists.

Surplus DNA

The haploid DNA content of a number of species spanning the plant and animal kingdoms is given in Table IV. It can be seen that for a while there is a reasonable degree of increase in DNA content with increase in complexity. Thus *E. coli* has 80 times as much DNA as phage lambda, and man has 800 times as much DNA per haploid complement as *E. coli*. Being a little chauvinistic toward our own species, we like to think that man is surely one of the most complicated species on earth and thus needs just about the maximum number of genes. However, the lowly liverwort has 18 times as much DNA as we, and the slimy, dull salamander known as *Amphiuma* has 26 times our complement of DNA. To further add to the insult, the unicellular *Euglena* has almost as much DNA as man. Apparently these species have endoploided or polyploided themselves into acquiring more DNA than they need. Since organisms like *Amphiuma* show no more genetic polymorphism than man,[138] it seems likely that most of this excess DNA is not used. It is interesting that within certain orders of eukaryotes, the more advanced species have the least amount of DNA.[309,655] This could

TABLE IV. DNA Content per Haploid Genome[467,492,569,710]

	Organism	DNA in picograms
Viruses	OX 174	0.0000026
	Lambda	0.000050
	T2	0.000208
Bacteria	*Mycoplasma*	0.00084
	E. coli	0.004
Fungi	Yeast	0.022
Protozoa	*Plasmodium berghei*	0.06
	Astasia longa	1.5
Invertebrates		0.06 to 1.5
Chordates	*Amphioxus*	0.6
Fish		0.06 to 2.5
Reptiles		1.5 to 2.5
Mammals	Man	3.2
Amphibians	Toad	3.7
	Frog	7.0
	Necturus	25.0
	Amphiuma	84.0
Plants	*Aquilegia*	0.6
	Euglena gracilis	3.0
	Lillium longiflorum	53.0
	Tradescantia	58.0

either mean that species must shed some DNA to advance in complexity or more likely, excessive polyploidization squeezes an organism into an evolutionary dead end.

Minimum Number of Genes

A haploid content of 3.0 pg is equivalent to approximately 3.0×10^9 base pairs. This would constitute 3 million genes 1000 base pairs long. Since the hemoglobin genes are half this size, on the average this figure should be enough to include most structural genes plus their adjacent regulator sequences. Five thousand should be a liberal estimate of the maximum number of genes devoted to enzymes and structural proteins. If we once again are liberal and suggest that 20 times this many genes are needed for morphogenesis, and an equal number are devoted to repetitious genes such as those for ribosomal, transfer, and 5*S* RNA, and for histone genes and any others that may be present in repetitious sequences, this leaves us with

a rough estimate that 200,000 genes should be adequate to make and run one human. This is $20 \times 10^5/3.0 \times 10^6$, or about 7% of the genome devoted to essential functions. Since this may seem low, are there other ways to approach this problem?

Maximum Genetic Load

Studies of a number of organisms indicate that on the average each structural gene sustains a deleterious mutation in 10^5 generations. This suggests that the presence of 10^5 gene loci would produce one deleterious mutation per generation,[365,491] an unbearable load. Taking into consideration this frequency of spontaneous mutations and the maximal mutational load, Muller[473] estimated the number of functioning gene loci in mammals to be approximately 3×10^4, or about 1% of the genome, and Lyon[420] estimated there might be only 10^4 essential genes. When Mueller divided this number into the total number of nucleotides present, he concluded that each gene was more than 100,000 nucleotides long. Although this is far too long for the average structural gene, it is not unreasonable for a piece of HnRNA. However, rather than assigning this length to each gene, it is more likely that following gene duplication there is a subsequent degeneration rather than either specialization to new functions or maintenance of old functions, and most of this excess DNA probably becomes irrelevant compared to the basic set of functional genes.[491a,499]

It is conceivable that this number of 3×10^4 might be increased somewhat by allowing that many traits are polygenic and the presence of a deleterious mutation in, for example, one of 100 genes that help form a nose, would not be at all that serious. Nevertheless, the principle that the mutational load places an absolute upper limit on the total number of genes is valid. Even if the estimate of 3×10^4 is off by a factor of 10, this would still account for only 10% of the genome.

Do the Bands on Polytene Chromosomes Represent Single Genes?

Studies of the number of different mutations that can occur at a given band locus in *Drosophila* suggest that each band represents only a single gene function.[31,401,610] Since there are less than 5000 bands in the *Drosophila* salivary chromosome map,[63] this would suggest a rather small number of useful genes. Assuming a gene length of 1000 nucleotides, this would imply that $5 \times 10^6/10^8$ or 5% of the *Drosophila* genome is composed of

essential genes. The DNA content (per haploid strand) of the bands ranges from 5×10^3 to 5×10^5 base pairs.[578] Thus the smaller bands would be largely composed of essential genetic material, while the larger bands should have a great deal of "silent" DNA.[401] This agrees with recombination data on the vermilion alleles which show them to be restricted to a short interval about 0.10 map units from the left edge of 10A1-2 band but 0.5 units from the right edge.[274,401] Similarly, the length of the white locus is approximately 0.025 map units,[356] while the band it occurs in is about 0.3 map units in length.[401] This may not be true of all bands, however.[401]

Studies of recombination intervals compared to band size show that the amount of recombination is proportional to the amount of DNA in a band rather than the distance between bands. This is inherently reasonable and indicates that the silent DNA is participating in the recombination processes. This is incompatible with the master-slave hypothesis which requires that recombination take place primarily between master genes.[93,690] It is also unlikely that the silent DNA is repetitious since there is too little repetitious DNA in *Drosophila*[389] compared to the amount of excess DNA. On the basis of present data it seems most likely that the silent DNA of the bands is junk DNA and may in part be coding for HnRNA.

Use of Junk DNA

These considerations suggest that up to 20% of the genome is actively used and the remaining 80+% is junk. But being junk doesn't mean it is entirely useless. Common sense suggests that anything that is completely useless would be discarded. There are several possible functions of junk DNA. (1) Despite being unused, junk DNA may be difficult to get rid of because it is interspersed between useful genes. Large spaces between genes may be a contributing factor to the observation that most recombination in eukaryotes is inter- rather than intragenic.[690] Furthermore, if recombination tended to be sloppy, with some mutational errors occurring in the process, it would be an obvious advantage to have it occur in intergenic junk. (2) Frameshift mutations would be restricted to single genes or parts of genes if there was a lot of unused spacer between each cistron.[491] (3) Small deletions occurring during translocations, during DNA replication, or as a result of various types of radiation would be less detrimental if a lot of unused buffer DNA was around. (4) Satellite DNA which tends to be localized to centromeric heterochromatin could play an important role by merely being dispensible during the process of Robertsonian transformation.[439]

Other Implications of 80+% Junk DNA

Relation to HnRNA

The observation that up to 25% of the genome of fetal mice is transcribed into rapidly labeled RNA, despite the fact that probably less than half this much of the genome serves a useful function, indicates that much of the junk DNA must be transcribed. It is thus not too surprising that much of this is rapidly broken down within the nucleus. There are several possible reasons why it is transcribed: (1) it may serve some unknown, obscure purpose; (2) it may play a role in gene regulation;[593] or (3) the promoters which allow its transcription may remain sufficiently intact to allow RNA transcription long after the structural genes have become degenerate.

Relation to Heterochromatic DNA

It has frequently been suggested that the DNA of genetically inactive heterochromatin represents the degenerate and useless DNA of the genome. However, heterochromatin rarely constitutes more than 20% of the genome. This suggests there are two categories of junk DNA, (1) DNA of constitutive heterochromatin which is neither transcribed nor translated, and (2) nonheterochromatic junk DNA which is probably transcribed, but not translated. This distinction adds one more dimension to the mystery of heterochromatic DNA. Why is it singled out to be nontranscribable when being nontranslatable seems adequate for most of the junk DNA? Perhaps there is clustered junk (heterochromatic DNA) and nonclustered junk, just like there is clustered repetitious DNA (satellite DNA) and nonclustered, repetitious DNA.

Relation to the Percent of the Genome that Is Repressed

Another interesting facet of this subject is the observation that stable RNA of the brain, when hybridized to saturation of the nonrepetitive sequences, anneals to approximately 10% of the genome.[74,281] Since this is stable RNA, presumably it does not include most of the HnRNA sequences that are rapidly broken down in the nucleus, but does include messenger RNA's. Since this is in the same range as the proposed proportion of the genome devoted to essential genes, perhaps a much higher percentage of the useful genome may be turned on than has previously been suspected.

Relation to the Hybridization of Specific mRNA's

As discussed previously, specific histone mRNA appears to hybridize to moderately repetitious DNA despite the evidence in histone V for allelic polymorphism at a single locus. Another disturbing feature of specific mRNA hybridization is the observation that globin mRNA hybridizes to some 0.5% of the genome or about 30,000 sequences* despite the excellent genetic evidence that it is coded for at only a few loci.[750] This may in part be owing to the presence of repetitious regulator genes along with the mRNA. Another factor may be the existence of a significant number of sequences which are similar to histone or globin genes, but which have long, since joined the junk pile of degenerate, nontranslated DNA. Thus, despite the fact that only a few genes actively synthesize stable histone or globin messenger RNA, that RNA may be able to anneal to many additional DNA sequences.

Base Composition of Useful and Junk DNA

Utilizing the amino acid sequence of a number of known mammalian proteins, and assuming that the frequency of bases in the third position of the codon is random, it is possible to calculate the base composition of that portion of the genome which is used to produce these proteins. These results give GC contents of between 44 and 48%, yet the GC content of the DNA as a whole is only 40%. This suggests that the junk DNA may be relatively AT-rich with a GC content close to 40%, while the actively functioning DNA responsible for producing useful proteins may be relatively GC-rich. If the further assumption is made that the nonrepetitious main band DNA in constitutive heterochromatin is relatively enriched in junk DNA and deficient in useful DNA, then the AT-richness of late-replicating heterochromatic DNA and GC-richness of early-replicating, euchromatic DNA is understandable.

If these assumptions are correct, and junk DNA is more AT-rich than essential DNA, a legitimate question to ask is, why degenerate DNA tends to become more AT-rich than essential DNA. The answer to this is unknown but some speculations are possible. For example, deamination of methylcytosine will cause it to be changed to thymidine.[278,591] It is possible that methylation of many of the cytosine bases might be an important characteristic of essential, transcribed, and translated DNA. Any transition from methylcytosine to thymidine in an essential part of the genome would

* Recent studies indicate globin mRNA hybridizes to only a few genes.[408]

be selected against. However, such a transition in duplicated, unessential, junk DNA would not be selected against and could result in the gradual shift of the base composition of junk DNA to AT-richness. This implication that the DNA of essential genes may be more highly methylated than in nonessential DNA is, of course, still speculative.

Junk DNA and Units of Recombination

For some unknown reason the number of nucleotide pairs in a map unit* is closely related to the amount of DNA in the genome.[542] The larger the map unit the lower the efficiency of recombination between two genes. If the significantly greater size of the map unit in eukaryotes were due entirely to the presence of nuclear proteins surrounding the DNA, then there should be two general size classes of map units—one for organisms with naked DNA and one for organisms with covered DNA. This is clearly not the case. For example, Fig. 21 illustrates the size of a map unit in nucleotides plotted against the size of the genome in nucleotides. It can be seen that there is almost a linear relationship, with no significant deviations from the curve as the organisms pass from prokaryotes (phage T4 and *E. coli*) to eukaryotes. If chromosomal proteins are not responsible for the decreased efficiency of recombination of higher organisms, then what is? It could be suggested that the larger the genome the less the chance for two homologous genes to find and pair with each other. However, the precise pairing brought about by the synaptonemal complex[147,148] in all eukaryotes makes this explanation unlikely. On the assumption that increased amounts of DNA in the genome means increased amounts of junk DNA between functional genes, is there any way this could result in a decreased amount of recombination between the genes? One possibility is that the amount of mutational degeneracy in the DNA between genes may be sufficiently great that there is significantly less base sequence homology between homologous chromosomes in this junk than between homologous functional genes.[136] Assuming that recombination is initiated by heteroduplexes formed by the annealing of half-helices of each parental DNA molecule,[313,742] such sequence dissimilarity could provide a mechanism by which an increasing distance between genes might not be accompanied by a proportional increase in the amount of recombination between them. Alternately, if certain sequences are necessary in one or more steps of the pairing and recombination process[156,313] and these became degenerate, recombination would be inhibited.

* A measure of recombination between two genes. 1 Map Unit = 1% recombination.

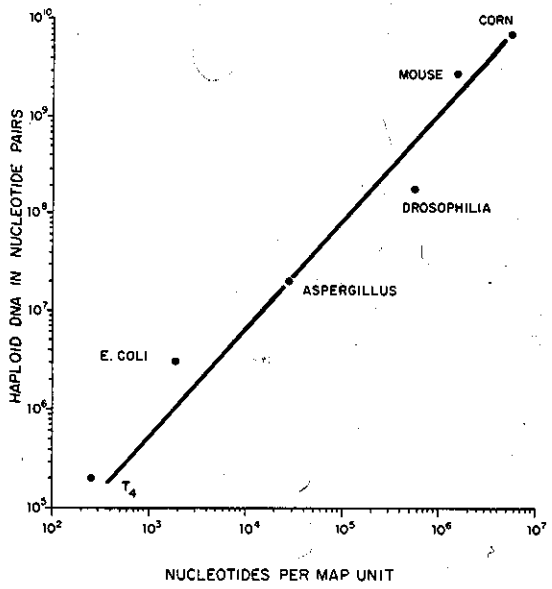

Fig. 21. The direct relationship between the haploid genome size (ordinate, in nucleotide pairs) and size of a map unit (abscissa in nucleotides). (Data modified from Pontecorvo.[542])

JUNK DNA *VS.* THE MASTER-SLAVE HYPOTHESIS

On the basis of morphological studies of lampbrush chromosomes of the oocytes of newts, Callan and Lloyd[94] proposed that each loop was composed of a series of repeated copies of a single gene. This proposal had a number of implications and corollaries. (1) It implied that each chromomere was the equivalent of a single genetic function, with a series of repeated genes being fed out at the proximal end of the loop and coiled back into the chromomere at the distal end. (2) To alleviate the problem of mutational diversity among the repeated genes it was proposed that the set consisted of a single master gene and a series of slave genes. Through a process of rectification, the slave genes were periodically aligned with the master gene and any mutations they had accumulated were corrected to match the master copy.[93,743] It was originally proposed that the synaptonemal complex played a role in this process but since mutations and recombination also occur in somatic cells it was necessary to propose that rectification could also occur outside of the meiotic system.[690] (3) Because of the amount of excess DNA involved in some chromomeres, the degree

of repetitiousness of each structural gene must be in the range of 100 to 10,000 times. This implies the existence of very little in the way of unique sequences in eukaryotic genomes. (4) Most meiotic recombination takes place in the master genes.

In order to provide this interesting proposal the privilege of serious consideration it must (1) explain one or more observations that cannot otherwise be explained, and (2) it must be consistent with the present body of genetic and biochemical knowledge about eukaryotes.

Advantages of the Master-Slave Hypothesis

The hypothesis can offer its services toward providing a potential explanation for a number of observations. These include the following.

1. It could explain the excessive amount of DNA present in many eukaryotes.

2. It could provide a mechanism by which the repetitious ribosomal, transfer, and 5*S* RNA sequences could maintain similar sequences.

3. In eukaryotes more recombination occurs between genes than within genes, while in prokaryotes, which have no slave sequences, most recombination is within genes.[690] (Even though one of the conditions of the hypothesis is that most recombination takes place between master genes, the sheer bulk of the slave genes would still shift the balance to intragenic recombination.)

4. There is a lower rate of deleterious mutation per nucleotide in eukaryotes than prokaryotes. This would be expected if there were a large number of correctable slave genes between the master genes.[690]

5. In eukaryotes little damage is incurred by translocations or by the recombination process, while similar events in prokaryotes are frequently deleterious.[690] This would also be understandable if these events took place in slave sequences.

6. Evidence from *Drosophila* suggests a correlation between a single gene function and a single salivary band. The presence of many slave sequences would explain the marked disparity between the amount of DNA per band and the DNA needed for a single structural gene.[690]

7. Analysis of the RNA produced by a single Balbani ring in Diptera shows it to have a partially restricted degree of diversity, suggesting it is not composed of a large number of different genes.[169]

8. Thomas *et al.*[691] have shown that after short treatment with nucleases it is possible to generate circles in eukaryotic DNA. They have interpreted this as strong evidence for tandem repetition of sequences.

Evidence Against the Master-Slave Hypothesis

1. One of the strongest and most devastating pieces of evidence against the master-slave hypothesis comes from studies of the renaturation kinetics of eukaryotic DNA which show the presence of large amounts (up to 80%) of sequences which are present as unique sequences.[66,388,667] The master-slave hypothesis implies a diametrically opposite conclusion, that all essential genes are present in multiple copies. The RNA-DNA hybridization data show that it is the class of unique sequences that hybridizes to the cytoplasmic messenger RNA sequences.

2. All of the above observations that have been proposed as evidence for a number of slave genes being interposed between master genes, apply equally well to the proposal that there is a large amount of junk DNA between essential genes. The junk DNA, although originally derived from the essential genes by duplication, is free to undergo a sufficient number of mutational changes so that it behaves as unique sequences and is thus consistent with the data on the kinetics of DNA renaturation. And yet, the similar origin of the essential and junk DNA could provide enough relatedness to allow cyclization of the DNA and explain the tendency for Balbiani rings to synthesize RNA with a relatively limited degree of heterogeneity.

3. The master-slave hypothesis proposes a marked decrease in recombination between slave genes. However, studies of LeFevre[401] and Rudkin[578] show that recombination is proportional to the amount of DNA in the bands rather than to the number of bands *per se*. There is no necessity to ask for a restriction of recombination in junk DNA (although some may occur on the basis of its heterogeneity).

4. Studies of the synaptonemal complex[147,148,151,155] show it to be a rather simple-minded proteinaceous structure which probably serves primarily to pull together homologous chromosomes that have already been partially paired by other mechanisms.[151,155,156] It is unlikely that it has the capability to perform the complicated maneuvering required by the master-slave hypothesis. Rectification occurring in somatic cells is conceptually even more difficult to visualize despite some brave attempts to explain it.[690]

5. In Hemoglobin Constant Spring[463] and Hemoglobin Tak,[230] polypeptides containing 31 and 10 amino acid residues have been added on to the end of the α- and β-chains respectively. This has most likely occurred as a result of a mutation in a stop signal, with the result that DNA distal to the-structural genes was read. These polypeptides bore no resemblance to known globin chains. This is consistent with the presence of junk DNA following the α and β genes. However, if slave genes existed it would be necessary to conclude there is a significant amount of junk spacer between them.

6. Finally, the master-slave hypothesis is difficult to reconcile with the evidence that a large piece of HnRNA is the precursor to a much smaller mRNA, and that most of the HnRNA is degraded. The master-slave hypothesis would suggest that a long stretch of DNA, such as that seen in the lampbrush loop, was synthesizing repeated copies of mRNA, all of which were being used.

Perhaps the only thing junk DNA can't explain that a master slave mechanism can, is the question of how repetitious sequences such as those coding for transfer, ribosomal, and 5*S* RNA can be kept similar. This may not be a problem for ribosomal DNA since there is some evidence for heterogeneity in these sequences.[471] One possibility is that these sequences may be so vital that even one mutant gene out of several hundred might be lethal and thus allow selection to act despite much gene duplication. Other possibilities have been considered by Edelman and Gally.[211]

In summary, although the master-slave hypothesis can offer a potential explanation for a number of biological observations, these observations can also be explained as well or better by the presence of junk DNA, and the hypothesis is inconsistent with biochemical studies of DNA renaturation which suggest that the major portion of most eukaryotic DNA exists as single copies.

HETEROCHROMATIN

Introduction

The difficulties involved in formulating a totally unambiguous definition of heterochromatin have been recognized ever since the term first came upon the cytogenetic scene.[603] The earliest use of the word can be traced to the turn of the century when some sex chromosomes were called heterochromosomes because of their tendency to undergo heteropycnosis during meiosis.[752] The autosomes were called euchromosomes. It began to take on its present connotation as a result of a series of studies by Heitz between 1928 and 1934.[298–301]

Using both plants and animals, he showed that the chromatin of specific parts of certain chromosomes remained condensed during interphase. These parts, which he termed heterochromatin, retained the heavily staining properties characteristic of the metaphase chromosomes. An important aspect

of his studies was the statement that this characteristic persisted *throughout interphase*. This simple aspect alone helps to distinguish the heterochromatin of specific chromosomes from the nonspecific condensation that the chromatin of all chromosomes can undergo in the G_1 period of some cells. Early work of Bridges[62] had shown that the chromatin of the Y chromosome of *Drosophila* appeared to be relatively inert. In 1932, Muller and Painter[474] noted that the X chromosome contained a segment that was comparable to the Y, in that it was heterochromatic and genetically inert. This provided genetic evidence that in addition to the pycnosis described by Heitz, heterochromatin was also genetically inactive. The final major characteristic of heterochromatin had to await the development of radioisotopes. In the late 1950's Lima-de-Faria[408] showed that the DNA of the heterochromatic sex chromosomes of grasshoppers replicated out of phase from the DNA of the rest of the chromosomes. Appropriate experiments indicated that this DNA replicated late in the S period compared to early replicating euchromatin.

On these fundamentals, a basic definition can be formulated. Heterochromatin represents specific portions of specific chromosomes which (1) remain heteropycnotic throughout interphase, (2) are genetically inactive, and (3) undergo DNA replication out of phase with remaining euchromatic parts of the chromosomes. Although not perfect, this forms a reasonably firm foundation upon which to base an elaboration of many additional aspects of heterochromatin.

Condensed Chromatin

The use of heteropycnosis and heterochromatin as interchangeable terms has resulted in much of the confusion surrounding the definition of heterochromatin. This problem is partially avoided when the original criterion of Heitz is adhered to, namely that heterochromatin be restricted to that which remains heteropycnotic throughout the cell cycle. For example, a number of studies of "heterochromatin"[238] have utilized mature lymphocytes or nucleated red blood cells of birds.[181] However, these represent special cells which are relatively dormant and up to 80% or more of their chromatin is heteropycnotic. On the basis of late replication patterns and heterochromatin staining, this is clearly far in excess of the amount of true heterochromatin present. When these cells are stimulated to move out of the G_0 state and into G_1, S and G_2, by exposure to phytohemagglutinin or by hybridization with HeLa cells,[289] most of the heteropycnosis disappears. In the metabolically active cell most of the condensed chromatin represents

true, late-replicating, genetically inactive heterochromatin. This is not to say that some of the characteristics that have been described for condensed chromatin of lymphocytes, such as poor RNA synthesis and decreased amounts of nonhistone protein[238] are not valid for heterochromatin also. However, when other aspects of true heterochromatin, such as its content of unique types of DNA (see below) are looked for, they are difficult to detect because of the large amounts of euchromatin present in condensed chromatin from these sources.

Facultative vs. Constitutive Heterochromatin

One of the most useful subclassifications is that of facultative *versus* constitutive heterochromatin[76] (Table V). Constitutive heterochromatin occurs on homologous portions of both homologous chromosomes. As such it forms a permanent structural characteristic of a given chromosome pair. This characteristic plus its genetic inertness make it a logical site to investigate as a possible region of accumulation of special types of DNA. The heterochromatic blocks on *Drosophila*[284,301] and many plant chromo-

TABLE V. Facultative vs. Constitutive Heterochromatin

	Facultative	Constitutive
Occurrence on homologous chromosomes	Occurs on only one of two homologues	Occurs at homologous sites on both homologous chromosomes
Asynchronous DNA replication	+	+
Genetic inactivity	+	+
Heteropycnotic	+	+
Enriched in satellite DNA	No	Frequently
Enriched in AT-rich DNA	No	Frequently
Distinctive fluorescence after quinacrine staining	Usually not	Frequently
C-Band staining	Negative	Especially centromeric heterochromatin
Reversibility	Reversible	α-Irreversible β-May be reversible
Subclassification	Facultative and semifacultative (occurring on hemizygous chromosomes)	α and β (*Drosophila*) Centromeric and intercalary (mammals)

somes;[170] on the long arms of the X and Y of *Microtus agrestis*;[756] and the centromeric regions of many organisms, are typical examples of constitutive heterochromatin.

Facultative heterochromatin exists on only one of a pair of homologous chromosomes. One of the best-studied examples of this type is the genetically inactivated, single X chromosome of human XX females. This heteropycnotic chromosome forms the sex chromatin body or Barr body of female interphase cells (see Lyon hypothesis, below). Since the inactivation of one of the X chromosomes is a random event, the DNA of facultative heterochromatin is the same as that of the other nonheterochromatic chromosome. This is of importance since it demonstrates that the failure to synthesize RNA, the late DNA replication, the synchronous firing of the replicons, and the heteropycnosis, at least in this instance, cannot be attributed to the presence of distinct types of DNA such as repetitious DNA. In general, facultative heterochromatin does not show distinct fluorescence following quinacrine staining, and is not stained by specific techniques which distinguish at least some of the constitutive heterochromatin.

Timing of Heterochromatin Replication

Since the initial observation that heterochromatin of the grasshopper tended to replicate out of phase with euchromatin,[408] many additional organisms have been studied. These provide substantiation for the general rule that heterochromatin replicates later in the S period than the remaining portion of the chromosomes.[409] Within this large group of species showing late replication there are a number of situations when a distinct block of heterochromatin is seen to replicate in the third rather than the fourth quarter of the S period,[49,75,132] and in *Microtus agrestis* where the two types of heterochromatin can be easily distinguished, the facultative heterochromatin replicates earlier than constitutive heterochromatin.[599] When the replication of heterochromatin is timed throughout the S period, it is apparent that the delay in onset of replication is frequently greater than the delay in cessation of replication, and the intensity of replication is significantly greater for heterochromatin than for euchromatin.[130] These observations suggest that perhaps the most basic characteristic of heterochromatin replication is the tendency for its individual replicons to fire off in unison, rather than to fire off throughout the S period.[130] This may be correlated with the fact that the attachment sites for heterochromatin are all clustered onto a small area on the nuclear membrane. The mechanism responsible for the late replication is unknown.

Failure of Heterochromatin Replication

Late replication of heterochromatin indicates that whatever is responsible for the genetic inactivation also affects such basic elements as the timing of DNA synthesis. Even more striking evidence of this is provided by the observation that heterochromatin undergoes little or no replication in the polytene chromosomes of Diptera. This was first clearly demonstrated by the spectrophotometric and autoradiographic studies of Rudkin.[577,579] It can also be appreciated by the comparison of the amount of heterochromatin in somatic cell chromosomes compared to the salivary gland chromosomes. For example, as shown in Fig. 22, the pericentromeric heterochromatin of *Drosophila* somatic cell chromosomes constitutes approximately one-third of the total length of the chromosomes. These same rather massive blocks of heterochromatin are virtually missing from the salivary gland chromosomes. This might be looked upon as late replication taken to an extreme, or as the selective tissue-specific disposal of heterochromatin. It also presents some conceptual difficulties in regard to visualizing what is taking place at the junction of unreplicated heterochromatic DNA with the polytenized euchromatic DNA. Are multiple replication forks left in a state of frozen progression, or is there an absence of continuity by covalent bonding of the euchromatic and heterochromatic DNA?

The observation that heterochromatin is underreplicated during endomitosis in the testis sheath nuclei of the mealy bug[415,481,483] indicates that replicative failure is not unique to the polytene chromosomes of Diptera, and that it can also occur in facultative heterochromatin.

The Sex Chromatin Body and the Lyon Hypothesis

In association with some studies on the morphology of cat neurons in response to various stimuli, Barr in 1949[24] noted that female cats possessed a heteropycnotic mass of chromatin which was absent in males. This has been called the Barr or sex chromatin body. Although it was initially thought to have been derived from parts of both X chromosomes, Ohno demonstrated it arose from a condensed single X chromosome.[493,496] On the basis of this type of cytological evidence and studies of X-linked coat color genes in mice, Lyon[421] proposed that in mammals (1) one of the two X chromosomes is genetically inactivated, (2) this inactivation occurs early in embryogenesis, (3) the inactivation is random, affecting in a given cell either the maternal or paternal X, and (4) once the inactivation has occurred it remains inactivated in all subsequent generations of that cell. The numerous sub-

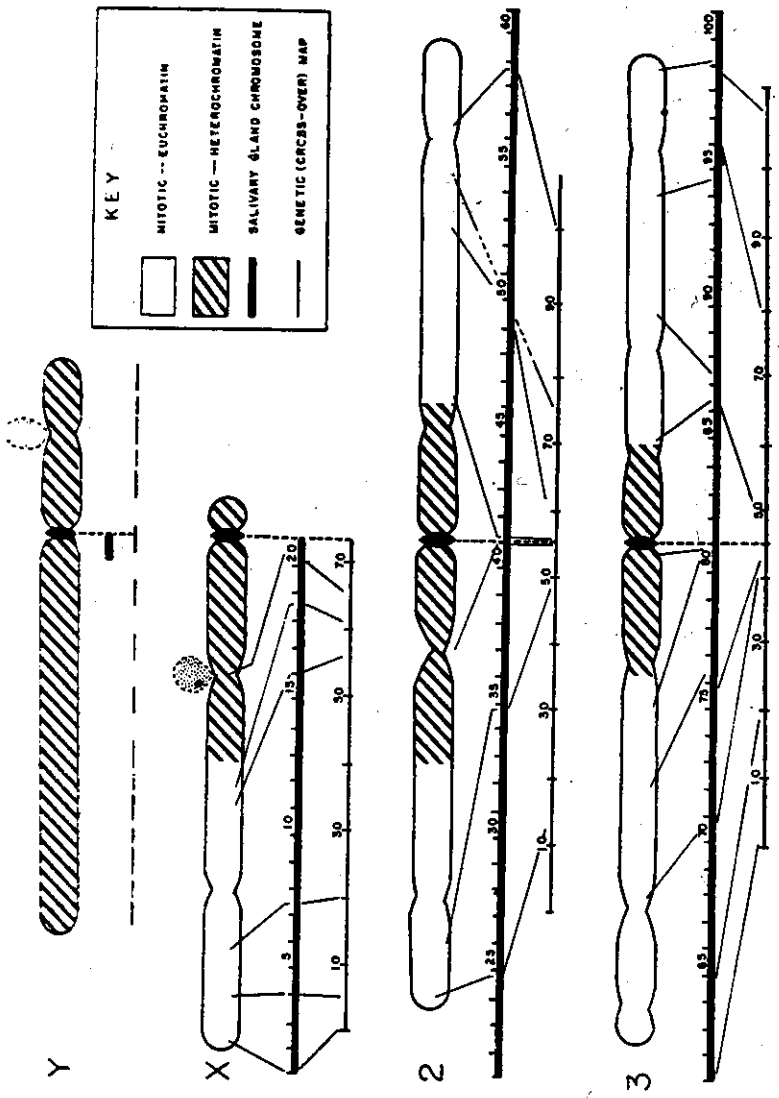

Fig. 22. Comparison of the amount of heterochromatin and euchromatin in *Drosophila* mitotic chromosomes (upper diagram for each chromosome) with the salivary gland chromosome map (middle heavy line), and genetic map (thin lower line). This illustrates how the large amount of heterochromatin (hatched) in the mitotic chromosomes is almost absent in salivary gland chromosomes, and markedly underrepresented in the genetic map, indicating relatively little crossing-over in heterochromatin. The salivary map is reduced to 1/200 in comparison to the mitotic map. (From Hannah,[284] by permission.)

sequent studies which have led to a verification of this hypothesis are reviewed by Lyon.[422] This inactive X chromosome forms the classical example of facultative heterochromatin. In man abnormal X chromosomes are preferentially inactivated, and when more than two X's are present (with a normal number of autosomes), all X's in excess of 1 are inactivated.[279] The mechanism by which this type of random but permanent inactivation is brought about remains as one of the intriguing unanswered problems of gene regulation in eukaryotes. Some of the possible explanations have recently been reviewed.[423]

Studies of glucose-6-phosphate dehydrogenase levels in early embryos suggest that the X chromosome inactivation is a positive act of genetic repression rather than a failure of activation of one of the X's.[220]

Genetic Inactivation Precedes Chromatin Condensation

One interesting aspect of the sex chromatin body is that it is not always present in all XX cells. For example, in rapidly growing human fibroblasts it is seen in only 30–50% of cells, while in confluent sheets of cells it is present in almost 100%.[128,687,688] The idea has frequently been expressed that the inactivity of heterochromatin is due to the condensation of the chromatin, and that if the inactive X is decondensed then it has become genetically reactivated. Several lines of evidence suggest this is incorrect and that the condensation is secondary to the inactivation.

One piece of such evidence comes from human fibroblasts in culture. If cells are taken from a person who is heterozygous for the electrophoretically distinguishable A and B variants of the X-linked glucose-6-phosphate dehydrogenase, it is possible to clone these and obtain a line in which the B allele is inactivated in all the cells. During the log phase of growth over 50% of these cells show no sex chromatin body. Despite this, only the single A allele is seen upon electrophoresis of the cell lysate, indicating that in the presence of decondensation the X still remains inactivated.[125] Similar evidence has been obtained from the study of the total amount of G-6-PD present in XY, XX, and XXXY human cells. These showed the same average enzyme content despite the presence of a significant number with decondensed X's.[689] A further example has been provided by the autoradiographic studies of Sieger *et al.*[620] on cultured *Microtus agrestis* cells. Although the constitutive heterochromatin on the sex chromosomes of this species is clearly seen in brain, liver, and kidney cells, it is relatively decondensed and poorly seen in tissue culture cells. However, when these cells are labeled with ^{3}H-uridine and autoradiographed, blocks of unlabeled

chromatin, representing genetically inactivated but uncondensed heterochromatin are seen. This is also consistent with the observation that during the maturation of chicken erythrocytes, the restriction of RNA synthesis occurs before the chromatin is fully condensed.[95]

A situation in which condensation is clearly secondary to genetic inactivation is seen when lampbrush chromosomes are exposed to actinomycin[338] or α-amanitin.[432] After transcription is inhibited by these agents, the loops contract into the chromomeres.

These types of experiments indicate that genetic inactivation comes first, and the condensation of chromatin is probably a secondary phenomenon.

α- *and* β-*Heterochromatin*

In a 1934 description of heterochromatin in the salivary gland chromosomes of *Drosophila*, Heitz[301] proposed a distinction between densely compacted chromatin at the site of confluence of the centromeres (chromocenter) which he called α-heterochromatin, and less densely compacted heterochromatin that was located in the chromosome arms, away from the centromere. The latter was more vesicular in character and was termed β-heterochromatin (Fig. 23). It is the β-heterochromatin which is reduced to only a few bands in the giant salivary chromosomes.[250] Recent electron microscopy studies of Sorsa[644] show more clearly the densely compacted α and more vesicular β-heterochromatin (Fig. 24). That this distinction is not merely the idle product of morphological nitpicking has been shown by *in situ* hybridization studies which show that the repetitious satellite sequences tend to be especially well localized in the α-heterochromatin.[250,302,549]

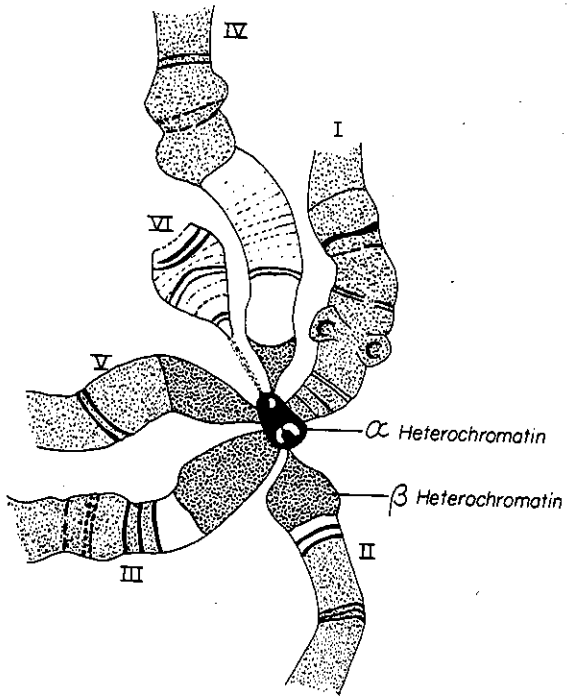

Fig. 23. Heitz's original differentiation of α- and β-heterochromatin of *Drosophila*. (Redrawn from Heitz.[301])

Fig. 24. Electron microscopy of the chromocenter region of *Drosophila* salivary gland chromosomes showing the vesicular nature of the β-heterochromatin and more condensed nature of the α-heterochromatin. (From Sorsa,[644] by permission.)

On the basis of morphology it has been assumed that α-heterochromatin is also localized to the telomeres.[30] This has been confirmed by the *in situ* hybridization studies of Hennig *et al.*[302] They were also able to demonstrate that some of the bands in the chromosome arms contained α-heterochromatin enriched in repetitious satellite sequences.

Positive and Negative Heteropycnosis

The concept of positive and negative heteropycnosis was introduced by White[738a,739] to describe the behavior of the X chromosome during the spermatogonial cycle in *short*-horned grasshoppers (Acrididae) and crickets (Gryllidae). Although this phenomenon might seem to merely add more confusion to the subject, it does serve to illustrate further some of the multifaceted aspects of heterochromatin. During the *early* spermatogonial divisions of these species, the X chromosome remains incompletely condensed. For example, during metaphase, at the height of condensation of the autosomes, the X has the appearance of an early prophase chromosome. However, in *later* spermatogonial divisions this negative heteropycnosis disappears, and still later during the first meiotic division the X becomes positively heteropycnotic and shows the type of prophase condensation typical of heterochromatic chromosomes.

A negatively heteropycnotic chromosome condenses more slowly or to a lesser extent during prophase and undergoes decondensation more rapidly at anaphase. A positively heterochromatic chromosome is already well condensed by prophase and remains so as the others are undergoing decondensation at anaphase.

In the females of these species, the two X chromosomes show neither positive nor negative heteropycnosis during oogenesis. Furthermore, negative heteropycnosis does not occur in constitutive heterochromatin and it is not universally seen among grasshoppers. The X chromosomes of the *long*-horned grasshoppers (Tettigoniidae)[739] show positive but not negative heteropycnosis.

This then provides the interesting situation of a chromosome which is not permanently heteropycnotic. When the X is in the female it is euchromatic, when it is in the male it is negatively heterochromatic in early spermatogonia, euchromatic in late spermatogonia, becomes positively heterochromatic in spermatocytes and spermatids, and if involved in fertilization it reverts to euchromatic status in the XX female zygote.

Semifacultative Heterochromatin

If we must feel compelled to have everything fit nicely into some type of classification, then the single X chromosome of the male short-horned grasshoppers and crickets seems to be left in a semantic form of purgatory. It does not represent constitutive heterochromatin because it is not a permanent structural aspect of the chromosome. It does not represent facultative heterochromatin since as a hemizygous chromosome it is not presented

with the capability of inactivating either of two homologues in the original sense of the term.[76] Furthermore, the heterochromatinization of this X chromosome does not seem to play any role in providing for a balanced gene set or dosage compensation. It is as though the X is needed in spermatogonia and probably in early embryogenesis, but not at other times and is thus inactivated. It comes closer to being facultative heterochromatin than constitutive. Perhaps, if for no other reason than to call attention to this distinctive situation, it might be called semifacultative heterochromatin.

It is probably not restricted to these insects. Chromosomes with these same characteristics occur in mammals. For example, in most mammals, during spermatogenesis, the X and the Y are heterochromatic, while during oogenesis the two X chromosomes are both euchromatic.[494,495] Assuming that the Y is essentially not a homologue of the X, then this X heterochromatin is not facultative, not constitutive, but semifacultative.

The Y-Chromosome and Constitutive Heterochromatin

By this rather circuitous route we come to another interesting thought, that the heterochromatin on the Y chromosome of man and some other mammals may also be somewhat distinctive. Offhand it would seem to be an example of constitutive heterochromatin, despite its hemizygous state, since it seems to be permanently heterochromatic. However, one characteristic makes it stand out from all the other constitutive heterochromatin. This is its appearance during pachynema of meiosis. At that time it is intimately associated with the X chromosome to form the sex vesicle. In this situation, both show a highly distinctive type of chromatin condensation characterized by a rather loosely compacted but very regular condensation with no association with intranuclear ribosomelike granules (Fig. 25). This is quite distinct from the highly compacted nature of the constitutive heterochromatin (Fig. 25). This distinctive coiling of the X and Y is also apparent in whole-mount preparations.[155] Since in this situation its partner, the X, is semifacultative, perhaps the Y is also. Again, this has been brought up, not for the sake of pedantic semanticism, but to point out some of the distinctive aspects of the heterochromatin on the Y chromosome.

Cold, Colcemid, and Heterochromatin

When plant cells are exposed to cold, the metaphase chromosomes show areas which are reduced in diameter and stainability.[170,171,255] Similar effects have also been seen with Colcemid.[209,402,631,678,679] These regions

Fig. 25. Electron microscopy of a diplotene cell from mouse testis. Nu, nucleoli; S.V., heterochromatic X and Y chromatin in the sex vesicle; H, centromeric constitutive heterochromatin. The chromatin of the sex chromosomes has a pattern of condensation which is strickingly distinct from that of euchromatin and constitutive heterochromatin.

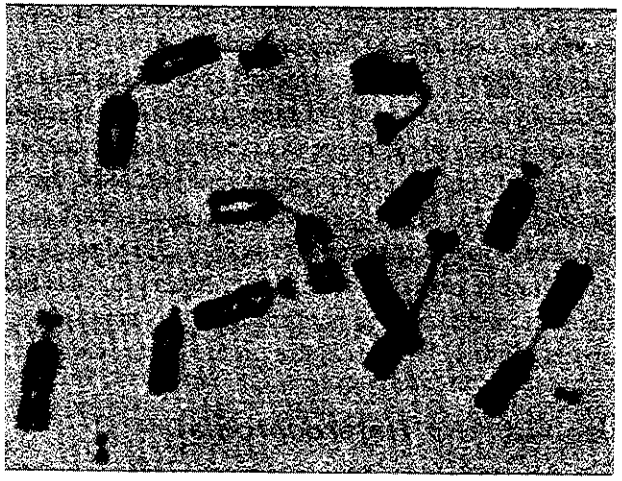

Fig. 26. Chromosomes of the Indian Muntjac treated with Colcemid. The centromeric heterochromatin is undercondensed compared to the other regions, producing extended necklike regions.[132]

correspond to areas of constitutive heterochromatin. Darlington and La-Cour[170,171] termed this "differential reactivity" and suggested it was caused by undercharging of the heterochromatin owing to nucleic acid starvation. However, on the basis of measurements of chromosomes with and without exposure to cold, Wilson and Boothroyd[46,753] demonstrated that this effect was due to the undercontraction of the heterochromatic segments. This proposal was substantially verified by autoradiographic and microspectrophotometric studies which showed that differential replication of DNA played no role in the differential reactivity.[47,767,768] Similar differential contraction with Colcemid can be seen in animal cells.[132,589,618,631] This can be particularly well seen in the large chromosomes of the Indian Muntjac[132] (Fig. 26). Since chromosomes which have different amounts of heterochromatin will undergo different degrees of contraction after exposure to Colcemid compounds, a series of similar chromosomes, such as the C group in man, might end up being arranged differently, depending upon the length of time they were exposed to mitotic inhibitors.

A further implication of differential reactivity to cold and Colcemid is that the pattern of chromatin folding in heterochromatin seems to be permanently set. Thus, it both fails to decondense during interphase and fails to hypercondense during metaphase. One possible explanation for this phenomenon is discussed later.

Unusual Types of Heterochromatin

Early Replicating, Reversible, Constitutive

Tanaka[681] has described some interesting characteristics of heterochromatin in an orchid *Spiranthes sinesis*. DNA replication studies suggest that it replicates early in the S period while the euchromatin replicates later.[680] The heteropycnotic chromatin also undergoes decondensation during DNA replication while this does not occur with other heterochromatin.[127,376] A further interesting aspect was the observation that subspecies occurring in the colder, Northern part of Japan showed a decrease in the number of chromosomes containing the large blocks of heterochromatin. It was suggested that deheterochromatinization was playing a role in the activation of some genes, resulting in an increase in plant size and invasion into a new habitat. Perhaps the most disturbing feature of this situation was the rather large amount of heterochromatin, constituting over 70% of the genome. This suggests the possibility that this may be more like condensed chromatin even though it was occurring on specific parts of the chromosome

(perhaps the condensed chromatin of lymphocytes and other cells occurs on specific parts of chromosomes). An additional plant, *Pellia nessiana*, has also been described with early replicating heterochromatin.[682]

Late Replication Without Heteropycnosis

This in itself is not such an unusual situation since some euchromatin probably replicates just as late as heterochromatin. However, White and Webb[741] described an interesting situation in marabine grasshoppers (*Moraba virgo*) which were permanently heterozygous for various structural rearrangements. Late replication was occurring in these segments without heteropycnosis. Assuming this was a form of genetic inactivation at the chromosomal level, it was not constitutive since it occurred on only one of two homologues, and it was not facultative since it always occurred on the same homologue. It was suggested that mutations had occurred in these segments that led to genetic inactivation with late replication but heteropycnosis had not yet developed.

Facultative, Negative Heteropycnosis

In the short-horned grasshoppers males are XO. In Marabine grasshoppers the X is fused to an autosome, giving rise in the male to a neo-XY system with the X being an X-autosome fusion chromosome and the "Y" being the unattached autosome. The X part of the X-autosome shows negative heteropycnosis in early spermatogonia. However, when tetraploidy occurs, and there are now two X-autosome chromosomes, the X portion of only one of them shows negative heteropycnosis.[740] This has obvious similarities to the positive facultative heterochromatin in XX mammals.

Subtypes of Constitutive Heterochromatin

Comparative results of the response to the different staining techniques provide evidence for various subtypes of constitutive heterochromatin (Table VI). For example, Vosa[713] showed that in various plants some heterochromatin showed differential contraction to cold starvation while some did not, and in both of these classes some heterochromatin showed increased staining with fluorochromes while others showed decreased staining. Similarly, Ganner and Evans[252] could identify four different types of late replicating bands in relation to whether they stained positively or negatively to the Q- and G-banding techniques. What role different types of repetitious DNA or other factors play in producing these variations remains to be determined.

TABLE VI. Different Types of Constitutive Heterochromatin Based on the Differential Staining Techniques

A. Data of Vosa on Plant Chromosomes[713]

	Q-staining	Cold "starvation" effect
Trillium, Vica	+	+
Allium carinatum	+	−
Tulbaghia	−	+
Allium cepa, Scilla sibirica	−	−

B. Data of Ganner and Evans on Human Chromosomes[252]

	Q-staining	G-staining	Late DNA replication
Most G bands	+	+	+
2° constrictions of 1 and 16, centromeric regions of 7 and 22	−	+	+
long arm of Y	+	±	+
2° constriction of 9	−	−	+

Position Effects

One of the most intriguing aspects of heterochromatin is its ability to cause suppression of genetic activity of euchromatin that is placed next to it by a translocation. This concept that the activity of a gene may be dependent upon its relationship to neighboring genes is known as position effect.[670] Many aspects of this phenomenon are reviewed elsewhere.[405] The following is a brief account of some of its more blatant aspects in relation to the light that it casts on the physiology of heterochromatin.

Variegation in *Drosophila*

The white locus and its surrounding genes on the X chromosome of *Drosophila melanogaster* provide an excellent example of what Lewis[405] has described as V or variegation-type position effect. This refers to the variable phenotype expressed by the genes that are translocated to the region of heterochromatin. As shown in Fig. 27, the effect of the heterochromatin can spread along a considerable expanse of the euchromatin. Thus in N^{264-52}, an expanse of five genes from rst (roughest) to bi (Bifid) all show variega-

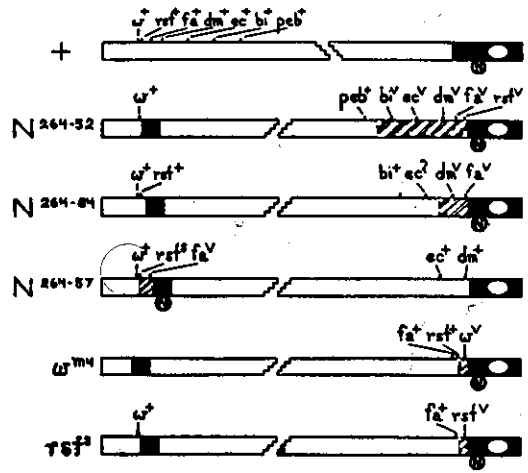

Fig. 27. V-type variegation in *Drosophila*. Examples of X-chromosome inversions associated with variegation of genes with reference to the normal chromosome at the top of the figure. Black, heterochromatin; shaded, extent of spreading of variegation; N, nucleolar region; V, variegated; S, stable; +, nonvariegated or wild type. (From Lewis,[405] by permission.)

tion. This figure also shows that different segments of heterochromatin vary in their ability to cause variegation. Thus, while in N^{264-52} there is extensive induction of variegation by the nucleolus-containing segment of heterochromatin, the translocated heterochromatin has caused no variegation of the white eye (w^+) gene. In N^{264-57} the nucleolus has now been translocated and has caused variegation of facet(fa) while the untranslocated segment has caused no variegation.

Variegation Within a Single Cell

The variegation phenotype is seen when the wild-type genes are on the rearranged chromosome and the mutant genes are on the homologous normal chromosome (example, $R(w^+)/w$). A logical basis for variegation would be a mixture of some cells in which the gene was completely inactivated and some cells in which it was completely normal. However, in studies of bristles, in which the degree of genetic activity of certain genes could be assayed in each cell, evidence has been obtained for more than an all-or-none effect.[405] Thus, whatever it is that causes the inactivation, it appears to have the capability of only partially repressing gene activity.

Effect of Additional Heterochromatin

Changing the total amount of heterochromatin in the genome by adding or subtracting X or Y chromosomes has a marked and seemingly paradoxical effect on V-type variegation. Thus the removal of the Y chromosome can cause an increase in the amount of inactivation of the normal but rearranged genes,[602] and the addition of X or Y chromosomes results in a decrease in the inactivation of the normal rearranged genes.[272,273] Brosseau[72] has suggested that the effect of the Y chromosome is the result of two position effect suppressor genes, one on the short and one on the long arm, rather than the effect of the heterochromatin *per se*.

"Reverse" Variegation

The variegation discussed above represents the repressive effect of heterochromatin acting on genes which are normally distant from it. There are some cases in which the converse is true—genes which function normally while in close proximity to heterochromatin may show variegation when removed to the distant sites.[405]

Skipped Genes

Further adding to the conceptual difficulties of position effects is the occasional observation that some genes closer to the heterochromatin may be unaffected while more distant ones are variegated. For example, in the case of the cubitus interruptus gene, some rearrangements were found in which the position effect spread through the bent gene without affecting it.[405] This could be interpreted as indicating that (1) whatever factors are elaborated by the heterochromatin, they do not act as completely nonspecific repressors, or (2) some genes, or their regulators, are able to overcome a mild dose of the repressing factor from the heterochromatin.

Variegation in Mammalian Cells

Variegation resulting from the positioning of autosomal genes next to facultative heterochromatin on the X chromosome has been extensively studied by Russel[580–582] and Cattanach.[105–108] This system also shows a spreading effect with decreasing repression as the successive genes are further removed from the heterochromatin. There is also evidence for the existence of a few centers of inactivation on the X chromosome.[107,582]

Implication of V-type Position Effects

The various facets of the V-type position effect suggest that the heterochromatin may elaborate some substance which spreads locally along the continuity of the chromosome. It is easiest to visualize this as being some type of nonhistone protein which has capabilities of gene repression and may show some limited degree of specificity. This presumptive nonhistone protein could be synthesized in the cytoplasm and preferentially accumulated by the heterochromatin, or it might be synthesized in the heterochromatin, perhaps utilizing a system of DNA-dependent protein synthesis suggested by Wang (see above) for residual proteins. It would be of interest to see if this type of protein synthesis preferentially occurs in the residual fraction of heterochromatin. Other alternatives are possible. For example, Cattanach and Isaacson[107] have suggested that some of the properties of the X chromosome inactivation center have features in common with the controlling system described by McClintock[447] in maize. By contrast, in the coccids, despite fragmentation of the paternal set of chromosomes, all fragments regardless of size become heterochromatic,[77] ruling out the presence of only a few inactivation centers.

S-Type Position Effects

Not all position effects are due to the translocation of genes to the proximity of heterochromatin. There is a class which Lewis[405] described as stable or S-type position effect which does not depend upon the presence of a chromosomal rearrangement. These include those cases in which, for example, mutant genes *a* and *b* show a different effect when they are in the *cis*-position $ab/++$ than when they are in the *trans*-position $a+/+b$ (exclusive of the phenomenon of complementation in pseudoalleles). The linked genes bithorax and bithoraxoid[406] exhibit this effect. One possible explanation of this phenomenon is that the two genes each control a step in a sequential series of reactions such that they would act more effectively when on the same rather than different chromosomes. This is more easily acceptable if the enzymes involved are synthesized within the nucleus.

The Bar Mutation

A further classical example of position effect is found in the tandem duplication known as Bar (B), and the product of unequal crossing-over called double bar (*BB*).[64,670] Females with Bar in the *trans*-configuration,

B/B, have larger eyes than those with bar in the *cis*-configuration $BB/+$. Since eye size decreases with increasing doses of *B*, this indicates that two adjacent *B*'s are more effective in producing this effect than two *B*'s in the *trans*-position.

Ectopic Pairing

A number of observations suggest that in addition to the large blocks of heterochromatin located around the centromeres in *Drosophila*, there are probably numerous smaller segments of heterochromatin out in the arms. These observations include the fact that there are numerous sites that show a significant increase in break frequency after exposure to X-rays, a characteristic of typical heterochromatin. In addition, a frequent morphological feature of squash preparations of salivary chromosomes is the presence of adhesions between bands on different chromosomes.[625] This ectopic pairing is not a random phenomenon. It occurs at specific mapable sites. For example, Fig. 28 shows the probable location of regions of intercalary heterochromatin[284] based on the localization of sites of ectopic pairing and easy breakability. Some of these correspond to repeats which are areas where duplications have become fixed during evolution.[603]

What is the basis behind such ectopic pairing? There is good evidence that the presence of repetitious DNA plays a significant role. For example, *in situ* hybridization studies have shown that RNA synthesized from *Drosophila* satellite sequences hybridize to centromeric, telomeric, and intercalarysites of α-heterochromatin—all of which are involved in extracurricular, nonhomologous pairing.[302,549]

There are alternative explanations such as the presence of a stretch of similar (but not repetitious) sequences scattered at various sites in the arms of different chromosomes. These could find and pair with each other through the same mechanism that brings about precise somatic pairing in the Diptera.

A possible related phenomenon is the tendency for the arms of mammalian chromosomes containing constitutive heterochromatin to stick together. This is seen in the X chromosomes of *Microtus agrestis*,[534,598,400] and other rodents[598] and in the long arms of the Y chromosome in man. There is good evidence that the constitutive heterochromatin in *Microtus agrestis* contains repetitious DNA.[17,143] Thus, unusual pairing properties in some instances are probably due to the presence of highly repetitious DNA. In other cases they may be the result of a very low order of repetition resulting from simple duplications.

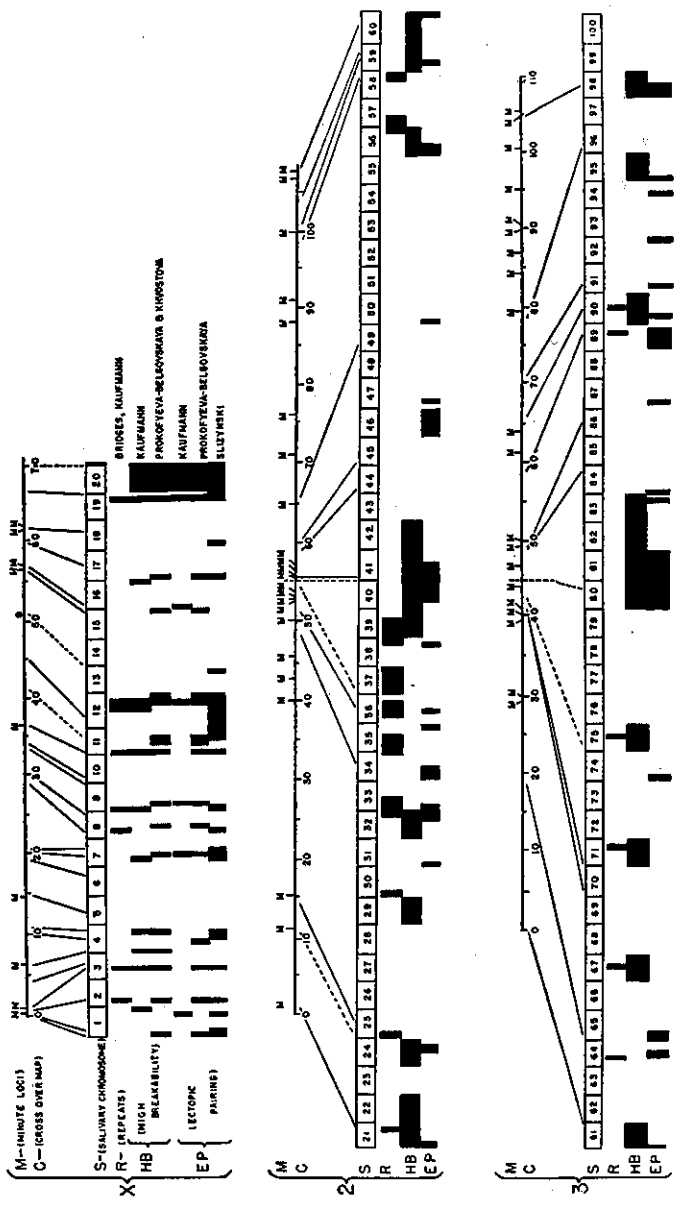

Fig. 28. Localization of intercalary heterochromatin in the X, 2nd, and 3rd chromosomes of *Drosophila* on the basis of ectopic pairing and high breakability. These regions are also compared to sites of repeats (R), minute loci (M), and relationship between genetic map distance (C) and salivary gland chromosomes (S). (From Hannah,[284] by permission.)

Recombination in Heterochromatin

There is an interesting paradox concerning the relationship between recombination and heterochromatin. It has long been appreciated that there is a significant decrease in the amount of genetic recombination that takes place between homologous chromosomes in regions of heterochromatin. By contrast, there is an increase in the amount of sister strand crossing-over in regions of heterochromatin.[184] These observations might be explained in the following manner. If genetically inactive, heterochromatic DNA shows a greater amount of mutational divergence than essential genes, there would be a greater likelihood for poor sequence homology to occur between homologous chromosomes and thus decreased recombination. This would not be present in the heterochromatin of sister chromosomes—these regions would have identical sequences and their closer proximity by virtue of greater condensation during interphase would increase the chance of recombination.[135] In this regard it would be of interest to determine if the heterochromatic DNA of related species (exclusive of satellite DNA) shows greater sequence diversity than the euchromatic DNA. Alternative explanations are possible and it could just as easily be stated that the repetitious DNA in heterochromatin should increase the chance of heteroduplex formation and thus increase recombination.

Variation in Amount of Heterochromatin During Development

Is the amount of constitutive heterochromatin constant throughout development or does it change? This question is of fundamental importance, since it could be suggested that genes in some of the constitutive heterochromatin of adult animals might be active and useful during embryogenesis. Studies of *Microtus agrestis* suggest that large blocks of constitutive heterochromatin on the X and Y chromosomes are present throughout all stages of embryogenesis.[400] This suggests that this DNA is relatively useless during development, a conclusion which is consistent with the demonstration of increased amounts of repetitious DNA in the heterochromatin of this species.[17]

In contrast to this, however, are studies of late-replicating DNA in developing frog embryos. Stambrook and Flickinger[654] observed a change in the pattern of late-replicating DNA during the period of cell determination. Further studies of this system by Remington and Flickinger[554] showed a progressive increase in the proportion of late-replicating DNA as develop-

ment proceeded from gastrulation. These studies suggest that some types of constitutive heterochromatin might be capable of some genetic activity[438] early in embryogenesis while other types are permanently heterochromatic. It is tempting to suggest that the former may possess DNA that is similar to euchromatic DNA in its degree of repetitiousness, while the later is more profusely enriched in repetitious DNA.

Euchromatinization

Can the genetic inactivation of heterochromatin be reversed? The answer to this reflects the variety of the types of heterochromatin. At one extreme lies the nonspecifically condensed chromatin, which is not true heterochromatin but has some of its characteristics, such as heteropycnosis, lack of RNA synthesis, and decreased content of nonhistone proteins.[238] This is readily reversible merely by exposing the cells to phytohemagglutinin in the case of lymphocytes, and by fusion with active cells in the case of nucleated red cells.[289]

Next is facultative and semifacultative heterochromatin. In the male short-horned grasshoppers for example, the single X is densely heteropycnotic in spermatids.[738a,739] However, if the X-bearing sperm fertilizes an egg, the result is an XX female in which both X's are euchromatic. Obviously, at some stage in this process this X must have passed from heterochromatin to euchromatin.

Similarly, in the XX mammalian females, the embryonic primordial germ cells contain chromatin bodies[754a] while in the oogonia both X's are euchromatic,[493,494] suggesting activation of the heteropycnotic X early in the formation of the ovary. Finally, in the male mealy bug some tissues show a developmental euchromatinization of the heterochromatic set of chromosomes.[77,78,482] These are examples of physiological euchromatinization. Genetic activation of heterochromatin has also been induced by chemicals such as polyethylene sulfonate.[238,455]

A more difficult question to answer is: Can constitutive heterochromatin be reversed? This is important in regard to the concept of whether it always contains degenerate genes, or whether in some cases the genes may only be inactivated.

The deheterochromatinization described by Tanaka[680] for orchids is a possible example, but as mentioned before, this is an atypical type of constitutive heterochromatin. Wolf[755] has described experiments with a fly, *Phryne cincta*, which when grown at 22–26°C has a small X chromosome composed entirely of vesicular β-heterochromatin. However, when they are

grown at 2°C so that the developmental processes are retarded without affecting growth, the flies are twice the size of those grown at higher temperatures, and in salivary gland preparations the X is now a fully extended euchromatic chromosome with many bands, suggesting that the inhibition of the β-heterochromatic DNA replication has been removed and many potential but repressed bands expressed. α-Heterochromatin at the centromere and on the Y showed no change under the environmental extremes. This situation is reminiscent of the finding in the orchids. Those growing in the colder environment were larger and showed deheterochromatinization of some chromosomes.[680]

I am aware of no reports of the reversal of α-heterochromatin.

It is tempting to draw a parallel between the propensity to euchromatinization and the type of DNA that the different types of heterochromatin contain. When the DNA is the same as euchromatin, condensation is readily reversible (non specific condensation, facultative heterochromatin). When the DNA is degenerate or composed of highly repetitious sequences such as satellite DNA, it is totally irreversible (α-heterochromatin). When the DNA is unaltered or only moderately altered, and has the same average content of repetitious DNA as euchromatin, it may under some circumstances be euchromatinized.

DNA OF HETEROCHROMATIN

The demonstration that much of the satellite DNA in various organisms (see below) is localized to constitutive heterochromatin has infused new interest into the problems surrounding the nature of this intriguing form of chromatin. At the same time it has raised a number of fundamental questions. These include: (1) Is all of the DNA of constitutive heterochromatin highly or moderately repetitious? (2) Is all highly repetitious DNA localized to the heterochromatin? (3) What are some other types of DNA that tend to accumulate in heterochromatin?

Satellite DNA and Heterochromatin

The first clear indication that the DNA of constitutive heterochromatin might be different from that of euchromatin came with studies which showed an enrichment of mouse satellite DNA in the nucleolar[595] and heterochromatic[772] fractions of nuclei, and from *in situ* hybridization studies[350,512] which showed a marked enrichment of satellite sequences in the pericentromeric heterochromatin of mouse chromosomes. It has subsequently been shown

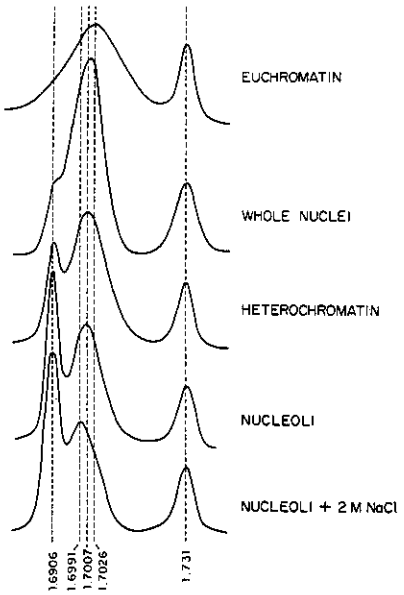

Fig. 29. Analytical ultracentrifugation of DNA from nuclear subfractions of the mouse. DNA from whole nuclei shows a light satellite band (1.6906 g/cm³) constituting 10% of the total and a main band component with a buoyant density of 1.701. The peak at 1.731 g/cm³ is *M. lysodeikticus* marker DNA. As the heterochromatin is increasingly purified (nucleolar preparations), the satellite DNA increases to a maximum of 49% and the buoyant density of the main band shifts to 1.6991 (higher AT content). About 50% of the heterochromatic DNA is main band DNA which is not enriched in repetitious sequences.[439]

that there is an enrichment of satellite DNA in the heterochromatin and nucleolar* fractions of the mouse;[143,439] guinea pig;[439,777] calf;[773] chicken;[143] Japanese quail;[143] *Microtus agrestis*;[143] human;[596] Chinese hamster;[143] kangaroo rat;[446] and green monkey;[430] and *in situ* hybridization studies have shown a localization of satellite or repetitious DNA to the centromeric and other heterochromatic regions in *Drosophila*;[302,351,549] *Rhyncoschiaria*;[210] *Microtus agrestis*;[17] salamanders;[426] and man.[18]

Typical variations in the amount of satellite DNA in various nuclear subfractions are shown in Fig. 29. These show the buoyant density and relative concentration of mouse satellite and main band DNA in euchromatin, whole nuclei, heterochromatin, nucleoli, and nucleoli treated with DNase and 2 M NaCl to further remove adventitious chromatin. The maximal enrichment of mouse satellite that we have been able to obtain in the most highly purified of heterochromatin preparations was less than 50%.[143,439] The fact that the main band DNA from these increasingly purified heterochromatin fractions (Fig. 29) showed an increasing shift to AT-richness, typical of late replicating DNA, indicated that the main band

* Heterochromatin is intimately associated with the nucleolus and nuclear membrane. The rapidly sedimenting fraction of sonicated nuclei contains both heterochromatin and nucleoli. The greatest purification of heterochromatin comes from using highly purified nucleolar preparations.

DNA in these purified samples of heterochromatin was not simply from contaminating euchromatin but was truly nonsatellite heterochromatic DNA. Despite the fact that this was not satellite DNA, it might be suggested that it is still highly repetitious. Denaturation-renaturation studies, however, suggest it is no more repetitious than the rest of the main band DNA.[141,143]

Thus, the answer to the question of whether there is an enrichment of satellite DNA in constitutive heterochromatin is clearly affirmative.

Heavy Shoulder DNA

Comings and Mattoccia[142] showed that there was a significant enrichment of heavy shoulder DNA in the heterochromatin fraction of Japanese quail, and that this was probably due to the localization of this type of DNA to the heterochromatic microchromosomes. Some enrichment of heavy shoulder DNA was also found in several mammals, but not in the chicken, in which the microchromosomes are not heterochromatic. Renaturation studies of heavy shoulder DNA indicate it is no more repetitious than main band DNA.[133] These observations have several implications. (1) They indicate that there are some distinct species of DNA molecules, other than satellite DNA, which are enriched in heterochromatin. (2) Since heavy shoulder DNA is not unusually repetitious, this is a further example of nonrepetitious heterochromatic DNA. (3) One might suggest that all constitutive heterochromatin was the result of the presence of a specific type of repetitious or degenerative DNA which predestined that segment of the chromosome to be genetically inactive. However, if the suggestion that heavy shoulder DNA is localized to microchromosomes in birds is correct,[142] then it would imply that here is a distinct type of DNA that can be heterochromatic in some species (Japanese quail) but not in others (chicken). (4) Since this heavy shoulder DNA is GC-rich, and presumably late replicating in the quail, it suggests that not all late-replicating main band DNA is AT-rich.

AT-Rich DNA and Heterochromatin

As discussed earlier, in a number of organisms early replicating DNA tends to be GC-rich and late replicating DNA tends to be AT-rich. This is most likely a reflection of the relative GC-richness of euchromatic DNA, and the relative AT-richness of nonsatellite, main band heterochromatic DNA.[134,439] What is the basis behind this difference? One possible explanation may be related to DNA methylation. In mammalian DNA, most

methyl groups are attached to cytosine. If the amino group on 5-methylcytosine is enzymically removed, this changes the base to thymidine.[278,591] When late replicating DNA is methylated with ^{14}C-methyl-methionine and this centrifuged with ^{3}H-labeled DNA, there is a shift of the Carbon-14 curve away from the AT-rich DNA. No such shift is seen in early replicating DNA or DNA from unsynchronized cells. This suggests that the AT-rich late-replicating DNA is undermethylated.[137] Thus it is possible to suggest that mutations (transitions) from C to T (GC to AT) are constantly occurring by the deamination of methylcytosine; they are selected against if they occur in genetically useful euchromatin, but would not be selected against if they occurred in heterochromatin. This could provide a mechanism for the slight shift of heterochromatic DNA to AT-richness, and explain the tendency for this DNA to be undermethylated.[137] The relative content of junk and useful DNA in heterochromatin compared to euchromatin may also be pertinent to this problem (see p. 318).

Is All Heterochromatic DNA Highly Repetitious?

Although studies of the mouse are extremely informative because of its high content of satellite DNA, this very characteristic may be somewhat misleading as far as drawing conclusions about the relationship between repetitious DNA and heterochromatin is concerned. For example, at the opposite extreme it would be informative to study an organism which contains a large amount of heterochromatin but has little satellite DNA. The Chinese hamster is just such an organism. Heterochromatin staining shows from 15 to 20% of the genome devoted to constitutive heterochromatin,[143] but satellite DNA could only be demonstrated by centrifuging denatured and renatured DNA from highly purified nucleoli.[143] This indicated that it possesses less than 0.5% satellite DNA. Studies of the renaturation kinetics of early and late replicating DNA from this species showed that early replicating euchromatic DNA and late replicating heterochromatic DNA had the same relative amount of repetitious DNA.[141] This would seem logical since if one eliminates the clustered repetitious sequences (satellite DNA) only nonclustered repetitious sequences are left and it is reasonable to find that these are not sequestered into any particular intranuclear location (but see below).

Thus there are a number of indications that not all of the DNA of constitutive heterochromatin is highly repetitious. (1) Renaturation studies of early- and late-replicating Chinese hamster DNA show they have approximately the same average content of repetitious DNA.[141] (2) Renaturation

of isolated euchromatic and heterochromatic DNA of the Chinese hamster show the same average content of repetitious DNA.[143] (3) Heavy shoulder DNA enriched in the heterochromatin of the Japanese quail is not unusually repetitious.[142] (4) Centrifugation of mouse nucleolar DNA after denaturation and renaturation indicates that the AT-rich main band component is not unusually repetitious,[143] and renaturation studies of the late-labeled AT-rich DNA of Chinese hamster suggest it is not unusually repetitious.[134] (5) It has been suggested that heterochromatinization is the first step in changing two ancestral homologous autosomes into Z and W chromosomes in the differentiation of sex chromosomes in snakes.[552] Such a mechanism would require that at least for a time the heterochromatic DNA of the W would be similar to that of the Z. The same applies to the early developmental stages of heterochromatic chromosomes in other systems.[741] Some arguments in favor of the proposal that some noncentromeric heterochromatin may be enriched in repetitious sequences are presented later.

Is All Repetitious DNA Localized to Heterochromatin?

The answer to this is clearly no. (1) Again, the studies of the renaturation kinetics of early replicating euchromatic Chinese hamster DNA indicates it contains just as many repetitious sequences as late replicating DNA,[141] and similar studies of isolated euchromatic DNA have shown it to have the same renaturation kinetics as isolated heterochromatic DNA.[143] (2) In the *in situ* hybridization studies that have utilized RNA synthesized from rapidly renaturing main band DNA, there are significant numbers of grains over euchromatic portions of the chromosomes.[17,302,351,549] (3) If the theories which suggest that some of the repetitious DNA is utilized as control genes[65,258] are correct, this type of repetitious DNA would be precluded from being sequestered into heterochromatin.

Thus, despite the fact that satellite DNA is usually and perhaps always localized to constitutive heterochromatin, the observation that other types of nonrepetitious DNA also go to make up the DNA of heterochromatin, and that not all repetitious DNA is localized to the heterochromatin fraction, indicates that the terms repetitious DNA and constitutive heterochromatin should not be thought of as interchangeable.

Heterochromatin and Repetitious DNA in Drosophila

Since heterochromatin is underreplicated in *Drosophila* salivary gland chromosomes, studies of the content of repetitious DNA in these cells

compared to nonpolytene nuclei should provide further information on the relationship between heterochromatin and repetitious DNA. In *Drosophila hydei*, Dickson *et al.*[194] found that DNA from whole larvae contained 20% repetitious sequences while the repetitious fraction was decreased to 5% in salivary gland chromosomes. Gall *et al.*[250] found that in *Drosophila virilis* and *D. melanogaster*, satellites constitute 41% and 8% of the DNA of diploid nuclei but are present in only tiny amounts in DNA from salivary gland chromosomes. In *D. virilis*, RNA complementary to the DNA of the largest satellite hybridized at about the same extent to both mitotic and polytene chromosomes, and in the chromocenter of the salivary gland chromosomes it hybridized primarily to the α-heterochromatin. In *D. melanogaster*, RNA complementary to total DNA hybridized to both the α- and β-heterochromatin. These and other observations indicate that the α-heterochromatin is underreplicated in the polytene chromosomes and that both the α- and β-heterochromatin contain repetitious DNA. Some repetitious sequences were also found in intercalary heterochromatin in the chromosome arms.[250,302]

An intriguing observation was the fact that the chromocenter contained repetitious sequences from both the satellites and from the main band DNA.[250] This indicated that even though repetitious DNA in the main band is not free to band as a distinct satellite it is free to cluster in heterochromatin.

Heterochromatin and Moderately Repetitious DNA

The question of whether some heterochromatin is the repository of significant amounts of moderately repetitious DNA is presently a difficult one to answer. Studies of *Microtus agrestis* by subnuclear fractionation suggest that the heterochromatin may be enriched in DNA that renatures at an intermediate rate,[143] and the *in situ* hybridization studies of Arrighi *et al.*[17] were compatible with this. However, on the basis of centrifugation of partially renatured DNA, no similar evidence for this was found for a number of other species.[143] It has been suggested that some of the Q- and G-banding (see below) in humans[674,778] might be due to the presence of moderately repetitious DNA. This would require clustering of the moderately repetitious seguences. Although there is some biochemical evidence against this,[68] it is not all that clear-cut.[647] In the human chromosomes these bands constitute approximately half the genome while there is only about 15–20% repetitious DNA.[163] The renaturation studies of late replicating[141] and heterochromatic DNA[143] of the Chinese hamster also speak against

an excessive enrichment of moderately repetitious DNA in heterochromatin. On the other hand, the findings in *Drosophila* indicate there is no real conceptual difficulty in having the major portion of the repetitious DNA of the genome localized to heterochromatin, and even suggest that the repetitious sequences in the main band can be clustered in chromocenters.[250] Still another aspect of this question is the suggestion by Southern[647] that some or all of the moderately repetitious DNA may not be a distinct class of sequences, in which case they certainly couldn't be separated into distinct chromosomal regions.

This subject obviously requires further study.

Function of Satellite DNA

Ever since satellite DNA was discovered, there has been great interest in the question of what function it serves. Although a number of possible roles have been suggested, such as involvement in chromosome folding, many of these can be ruled out by the observation that satellite DNA is localized to the centromeric heterochromatin in most species. In addition, the base sequence studies of the α-satellite of the guinea pig indicate that satellites are unlikely to produce useful gene products. Whatever function it may serve, it is likely that it is served by the DNA itself rather than any posttranscriptional product. Walker[718] has suggested that by virtue of its repetitious nature it may play a role in chromosome pairing. It would seem to me, however, that any substance that gave rise to nonspecific pairing of all chromosomes would severely hinder rather than help the cell to achieve specific homologous pairing. Besides, the existence of precise somatic pairing in the arms of *Drosophila* salivary chromosomes, well away from the centromeric heterochromatin, indicates that the cell has the means at its disposal to bring about precise pairing in the absence of both heterochromatin and the synaptonemal complex.[155,156] The observations of Hennig *et al.*[302] strongly support the idea that satellite DNA plays no role in bringing about somatic pairing in *Drosophila*. They demonstrated that the satellites of different species of *Drosophila* showed marked differences in their nucleotide sequences, in agreement with the findings in mammals. Despite this, there is remarkable accuracy in somatic pairing in hybrids formed from the different species.

It is also unlikely that satellite DNA plays any role in the attachment of microtubules to the centromere since some chromosomes, such as the Y in the mouse, have no satellite DNA.[350,512] Although the fact that mouse satellite is retained in heteroploid cultures of L-cells suggested that it played

a vital role,[429] the observation by Maio[430] that one of the satellites of the green monkey may be lost in some cell lines, suggests that they may not be vital to immediate metabolic needs.

One phenomenon in which satellite DNA may play a role is that of affinity. Studies by Michie[452,453] showed that during meiosis the parental chromosomes segregate nonrandomly with those of a given species tending to pass to the same daughter cell. Their ability to do this may be related to the presence of a species-specific satellite at the centromeres,[48] and this phenomenon might serve to help keep species isolated.

It has also been suggested that since chromosomes probably lose some DNA in the process of Robertsonian or centromeric fusion, it would be advantageous for the cell to place expendable DNA at the centromeres.[439] The presence of junk DNA at this site would prevent the loss of essential genes during chromosomal evolution.

Finally, there is the interesting observation that the satellites of even closely related species seem to come with distinct base sequences and in varying amounts.

Thus, of all the possible functions of satellie DNA, those which stand out the most seem to potentially play a role in speciation, whether it be to allow the development of new species by chromosomal rearrangements, or the maintenance of distinct species by allowing common chromosomes to segregate together. This is compatible with a role of satellite DNA, not in the immediate metabolic needs of the cell, but in the evolution and maintenance of new species.

Centromeric and Intercalary Heterochromatin

From the evidence presented above, there seem to be several different types of DNA which may tend to accumulate in constitutive heterochromatin. These include (1) highly repetitious satellite DNA, (2) nonrepetitious, AT-rich main band DNA, and (3) nonrepetitious heavy shoulder DNA. The satellite DNA tends to be localized to centromeric regions but on the basis of DNA replication studies, the effect of cold on heterochromatin, and fluorescent staining, it is apparent there is also a great deal of noncentromeric heterochromatin. This, plus other observations cited above and in the section of differential staining (below), suggest there are at least two types of constitutive heterochromatin. Although objections and exceptions can be raised to almost any classification, perhaps it might be easier to refer to these as centromeric *versus* intercalary heterochromatin.[132,143] Their respective features are listed in Table VII. Figure 30 illustrates their possible

TABLE VII. Centromeric *vs.* Intercalary Heterochromatin

	Centromeric	Intercalary
Location	Primarily at centromeres but can occur elsewhere	Primarily in chromosome arms but can occur at the centromere
Satellite DNA	Present	Not a major component
AT-Rich DNA	Not a major component	Present
Heavy shoulder DNA	Not a major component	May be present
C-Band staining	+	+ or −
Q-Band staining	Increased or decreased	Increased or decreased
Late DNA replication	+	+
Heteropycnotic	+	+
Genetically inactive	+	+
Reversibility	Irreversible	May be reversible

modes of origin. This begins with a set of active genes separated by repetitious spacer genes(r) (the junk DNA is not shown). It is suggested that the first event giving rise to intercalary heterochromatin is the genetic inactivation of a segment of the genome including repetitious and nonrepetitious sequences. Once inactivated, this segment would be susceptible to degenerative base changes that would eventually render it permanently inactive. Included in these changes would be GC to AT shifts, possibly through the mechanism of deamination of methylcytosine (see above).

The end result would be a block of intercalary heterochromatin* that was genetically inactive and slightly AT-rich, but which had the same average composition of repetitious sequences as the euchromatic DNA. Alternatively, a portion of some of the repetitious but nonclustered sequences might undergo rapid expansion to give rise to repetitious but clustered sequences or satellite DNA. This origin from already repetitious sequences would be consistent with the observation that some satellite DNA sequences are homologous to sequences that are scattered throughout the main band DNA.[210,227] The satellite would eventually be translocated to centromeric positions, or may have arisen there in the first place, to form the bulk of the DNA of centromeric heterochromatin.

* This classification does not apply to *Drosophila* where α and β and intercalary heterochromatin all contain an excess of repetitious DNA.

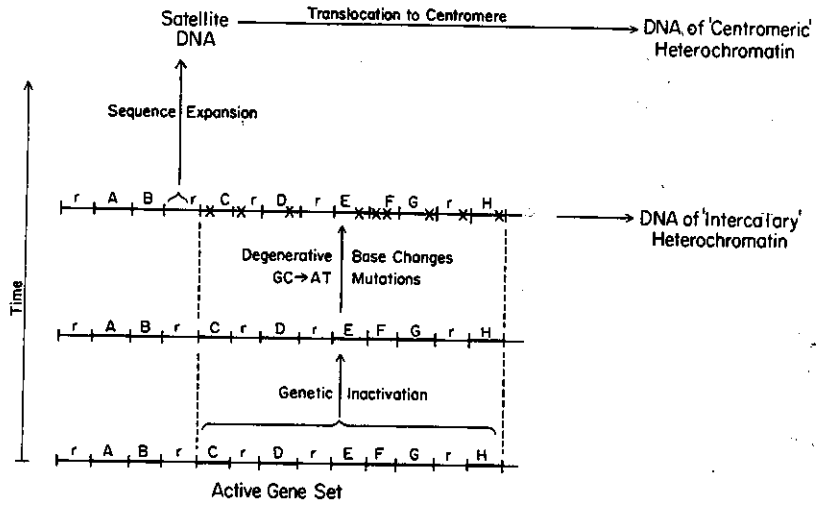

Fig. 30. A possible origin of two types of heterochromatic DNA. It is suggested that originally an active set of genes *A–H* was separated by interstitial repetitious sequences (bottom line). If a set of these genes became genetically inactivated (middle line), they would be susceptible to degenerative base changes. If these changes were more often GC to AT rather than AT to GC (possibly by deamination of 5-methylcytosine), the end result would be heterochromatic DNA that has the same average composition of repetitious sequences as euchromatin DNA but would tend to be slightly AT-rich, and would represent the DNA of intercalary type heterochromatin. Some of the already repetitious sequences may undergo sequence expansion to form satellite DNA, and constitute the DNA of centromeric type heterochromatin. Since it originally arose from repetitious sequences, there would be some cross hybridization between satellite and main band DNA.

Perhaps the greatest value in emphasizing the existence of at least two types of constitutive heterochromatin lies in the aid it may render in attempting to interpret the many new staining reactions that have surfaced in recent years.

Conclusion

Heterochromatin is somewhat like human society—it is a complex subject and simple slogans are inadequate to characterize it. For every apparent rule it is possible to cite an exception. The only solution is to become acquainted with its many and varied facets. The unsolved mysteries it presents remain the source of its fascination.

DIFFERENTIAL DETECTION OF CHROMOSOME SEGMENTS

One of the most exciting advances in cytogenetics in recent years has been the development of techniques to differentiate between varying segments of the chromosome. Some methods for making this differentiation have been around for a long time; they include enhancing heterochromatic segments of plant chromosomes by treatment with cold[170,171,255] and some largely forgotten staining techniques for centromeric and telomeric heterochromatin.[403] However, during the 1960's the most extensively utilized technique was the rather laborious one of autoradiography. The recent proliferation of new methods began with the development by Caspersson and his colleagues of quinacrine fluorescence[101] and the development by Pardue and Gall[511,512] and Jones[350] of *in situ* hybridization and the coincident finding that some of the areas that were detected by annealing to rapidly renaturing DNA could also be detected by Giemsa staining alone. The following is a brief summary of the techniques that allow differential detection of chromosome segments and comments on what they may mean.

Autoradiography

The present state of the art of autoradiography of chromosomes has been covered in recent reviews.[262,457] In regard to human chromosomes, it aids in the distinction between chromosomes 4 and 5 and 13–18 but is of no consistent value for distinguishing between chromosomes 19 and 20, is of questionable use for 21 and 22, and does not allow much identification in the C group (6–12). This technique, however, is of considerable value in that what it does is precisely known (i.e., it identifies chromosome segments that are undergoing DNA replication) while the precise mechanism of many of the other techniques is not known. It is clear that in human cells there are many noncentromeric areas of late replication which are not detected by *in situ* hybridization or by the C-banding technique.

Autoradiographic Detection of Differences in Base Composition

Since tritiated thymidine is incorporated into regions containing AT base pairs, and tritiated guanidine or cytosine into GC regions (after removal of RNA by RNase), autoradiography with these compounds, followed by quantitative grain counting, provides a means of detecting AT and GC-

rich chromosomal segments. This technique has been utilized by Hook and Hatcher[319] in the study of human chromosomes and has suggested that late replicating segments of chromosomes 17, and F and G groups were enriched in GC, and regions in the B group, the Y and 2 were enriched in AT. The minimal variation in base ratio for the Y was inadequate to account for its bright fluorescence.

Cold Treatment

The effect of cold treatment on detecting areas of heterochromatin in plant chromosomes has been discussed above. Its mode of action is not known, but it presumably has something to do with the factors responsible for permanent *versus* nonpermanent chromosome condensation.[156]

Fluorescence with Quinacrine Compounds

Fluorescence microscopy of chromosomes stained with quinacrine mustard or quinacrine dihydrochloride shows a striking degree of differential staining.[101,102] This is of particular value in human cytogenetics[100,104] and mouse genetics since it allows the individual identification of all the chromosomes (Fig. 31). By international convention this has been referred to as the Q-banding technique.[231]

The possible biochemical significance of this differential staining is discussed later.

Ethidium Bromide Fluorescence

Staining of chromosomes with ethidium bromide results in a pattern of fluorescence which tends to be the converse of that obtained with the quinacrine compounds.[103]

Fluorescent Antibodies to Single-Stranded DNA

The ability to specifically detect single-stranded DNA while giving no fluorescence with double-stranded DNA (or vice versa) would provide a mechanism to clarify many of the questions concerning the relationship between base composition or repetitiousness of DNA and Giemsa or quinacrine staining. With denaturation of the DNA, followed by different periods of renaturation, it would be possible to stain chromosome regions that vary in their content of repetitious DNA, and thus determine if renaturation of

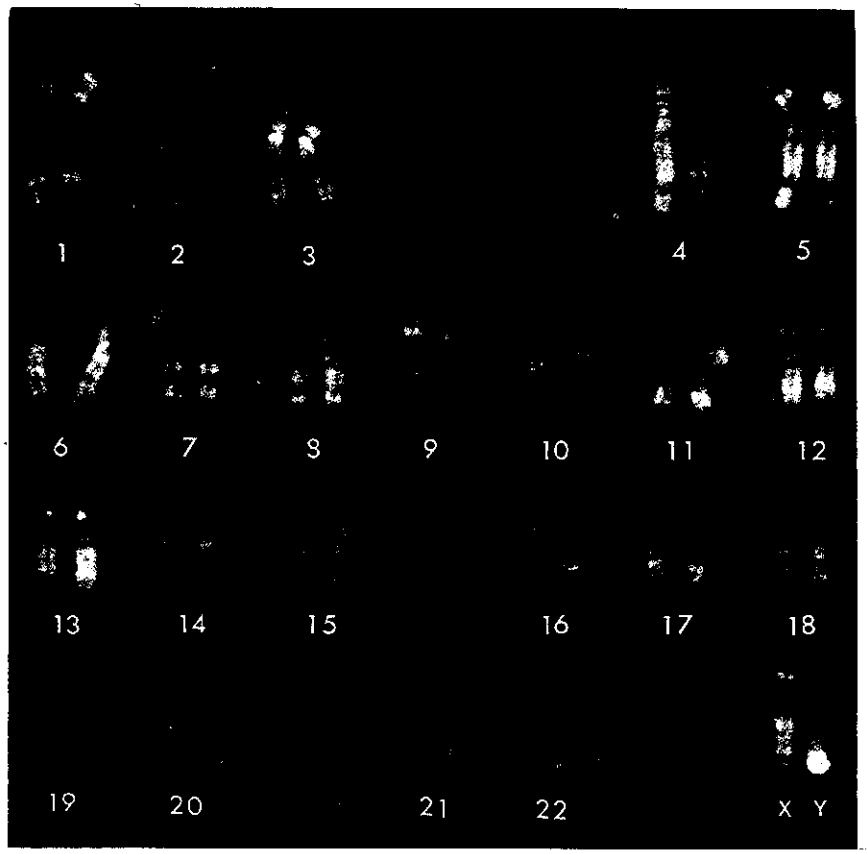

Fig. 31. Fluorescence pattern of human chromosomes. (Courtesy of Drs. Linaud and Irene Uchida.)

nonrepetitive DNA takes place more rapidly *in situ* than *in vitro*. By denaturing the DNA at various temperatures (AT-rich DNA denatures at lower temperatures than GC-rich DNA) it would be possible to detect variations in base composition. With some of these ends in mind, Freeman *et al.*[236,237] have utilized antibodies to guanidine that are specific for single-stranded DNA. Chromosomes with native DNA do not stain; those with denatured DNA stain brightly. Under specific conditions they were able to obtain some differential staining. However, even this seemingly straightforward procedure is fraught with many problems. For example, simple thermal denaturation did not give the expected differential staining; fixation must be brief and carefully controlled; denaturation by extremes of pH takes longer than expected for pure DNA; and since the chromosomes are not

composed of naked DNA, the question of the effect of proteins on allowing access of the antibody to the DNA remains a complicating factor.

In situ Hybridization

The technique of hybridizing nucleic acids directly to the DNA of cytological preparations was independently developed in three different laboratories by Pardue and Gall,[511] Jones,[350] and Buongiorno-Nardelli and Amaldi.[79] Although more laborious than the staining procedures, the beauty of this technique is that there is precise knowledge of what is taking place. Thus it is possible to localize in cells and chromosomes sites where DNA is homologous to ribosomal,[79,249,511,513] transfer,[660] and 4*S* and 5*S*[12,754] RNA, and satellite and highly repetitious DNA.[17,350,351,512] Using refined techniques it is possible that the specific annealing sites of some nonrepetitious RNA's for structural genes might be determined in polytene chromosomes but at the present state of the art, the renaturation kinetics seem to be against attaining such localization using the usual somatic metaphase chromosomes.

Early Stains for Centromeric Heterochromatin

In 1946 Levan[403] described a technique for staining the centromeric and telomeric heterochromatin of plant chromosomes. Young root tips were prefixed for 4 hr in 0.005 M mercuric nitrate, then fixed in Navashin solution (chromic acid–glacial acetic acid–formalin). The root tips were subsequently cut longitudinally and stained in crystal violet. The results (Fig. 32) were very similar to those obtained with some of the recent Giemsa techniques. This staining reaction suggested to Levan that there were two types of constitutive heterochromatin, one which occurred primarily at the centromeres and telomeres and stained intensely and another which occurred away from these regions and did not stain. He reviewed much of the older literature in which previous authors had also obtained some differential staining of centromeric heterochromatin. The important conditions seemed to be the use of heavy metal fixatives and treatment with acid or alkali.

C-Banding Techniques

Pardue and Gall[512] noted that following the treatments necessary for *in situ* hybridization, the centromeric heterochromatin was preferentially stained with Giemsa. Acting on this observation Arrighi and Hsu[18] developed a stain that was specific for this type of constitutive heterochromatin.

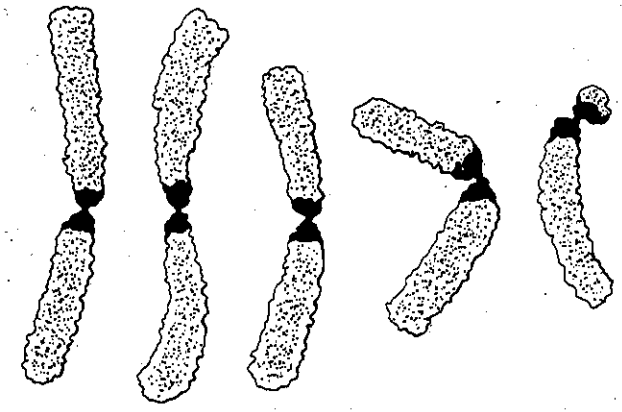

Fig. 32. Staining of centromeric heterochromatin in *Allium carinatum*. (From Levan,[403] by permission.)

The technique involved denaturation of the DNA with sodium hydroxide, followed by a period of renaturation in 2X SSC (SSC = 0.15 N NaCl, 0.015 N Na citrate) with subsequent staining in Giemsa. In human chromosomes (Fig. 33) this resulted in preferential staining of pericentric heterochromatin and especially heavy staining of segments on A_1, C_9, E_{16} and the Y chromosome.[18,113,243] These particular regions on the autosomes correspond to sites of secondary constrictions and are also sites of late DNA replication.[597] This heterochromatin is sometimes found at noncentromeric positions as indicated by blocks on the long arms of the human Y, intercalary regions on Chinese hamster, fruit bat,[323] and horse[143] chromosomes and extensive staining of the long arms of the X and Y chromosomes of *Microtus agrestis*.[17]

Relation to Satellite and Other Repetitious DNA

The precise correspondence between the localization of satellite and highly repetitious DNA and sites of C-banding[231] in the mouse[350,512] and *Microtus agrestis*,[17] and the apparent necessity for denaturation and renaturation to obtain a positive stain, suggest that repetitious DNA has something to do with the reaction. It could be as simple as the proposal that the Giemsa preferentially reacts with double-stranded (renatured) DNA. However, one observation suggests some caution in this interpretation. There is a large amount of Giemsa-positive heterochromatin in the Chinese hamster (15–20%) but very little (less than 0.5%) satellite DNA.[143] This could mean (1) very little satellite DNA is required to give a positive reaction; (2) other

Fig. 33. Staining of centromeric heterochromatin in man. (From Arrighi and Hsu,[18] by permission.)

factors, such as the presence of certain types of nonhistone protein in the constitutive heterochromatin, are also important; or (3) some of the centromeric heterochromatin may be composed of tandem repeats which give a positive reaction despite relatively little overall repetitiousness.[116]

G-Banding Techniques

Soon after the development of the technique for staining centromeric heterochromatin, it became apparent that by utilizing a number of modifications it was also possible to obtain differential staining in the arms of the chromosomes[199,519,601,674,778] (Figs. 34 and 35). These modifications are so numerous, and variable, and essentially empiric, that it is difficult to determine the important factors in the reaction. Although a denaturation

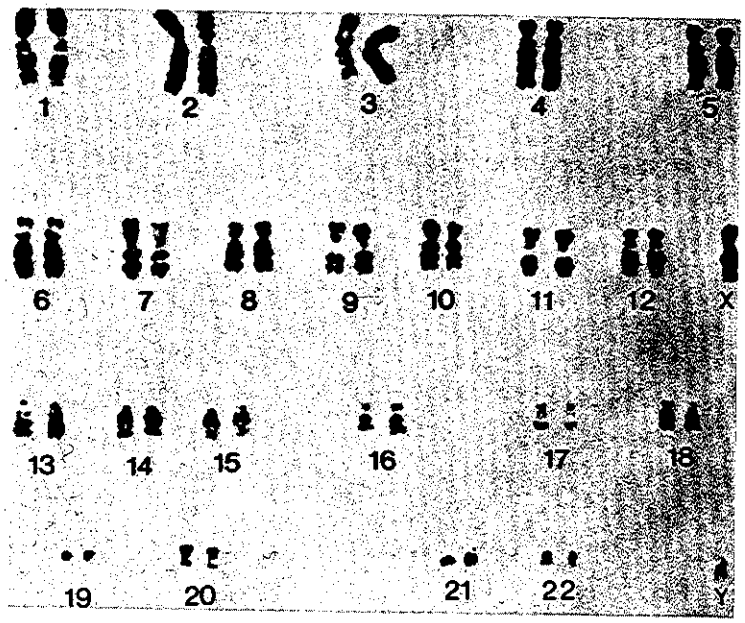

Fig. 34. Human chromosomes stained with one of the G-banding techniques. (From Summer *et al.*,[674] by permission.)

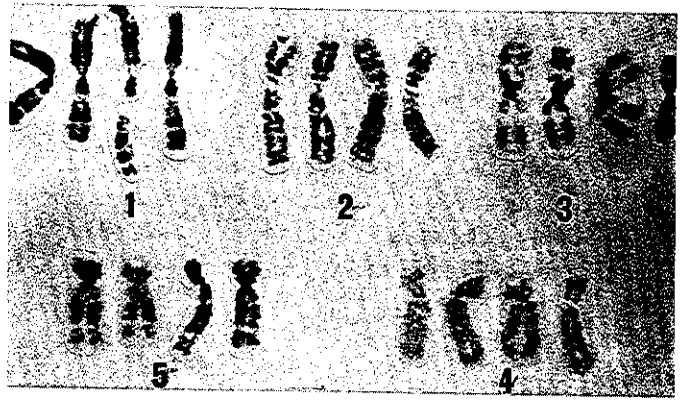

Fig. 35. Human chromosomes 1–5 stained with another variation of a G-banding technique. (From Drets and Shaw,[199] by permission.)

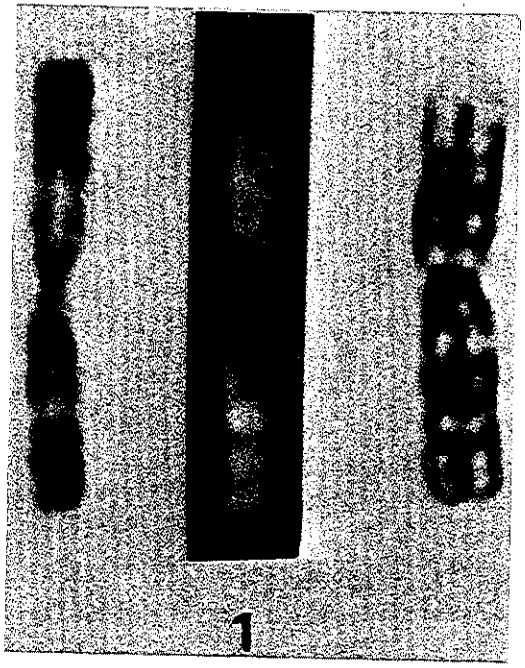

Fig. 36. G-band staining as produced by pretreatment of chromosomes with pronase (right) of chromosome 1. On left is reverse staining (see Fig. 38) and in center Q-banding. (From Dutrillaux and Lejeune,[208] by permission.)

step is a feature of many,[199,601] this may be left out[519,674] and is either not necessary or may be accomplished by the fixation itself. A heating step at 60–65°C for 1–72 hr[674,601] is frequently employed but this too is not essential and exposure to a pH of 9 alone may suffice.[519] Of particular significance is the observation that simple treatment of the chromosomes with pronase[208] or trypsin[606a] followed by Giemsa staining can produce the same result (Fig. 36). These modifications all produce the same patterns and have been termed the G-banding techniques.[231] With the exception of the centromeric heterochromatin on A_1, E_{16} and the Y, they precisely mimic the results of Q-banding.[231]

R-Banding

To add further intrigue to the situation, Dutrillaux and Lejeune[207] found that by increasing the temperature of the heat treatment to 87–89°C

for 10–12 min, a staining pattern was obtained that was the exact reverse of the G-bands, with the exception of the centromeric heterochromatin of C_9 (Fig. 37). This has been termed the R-band technique.[231] Even though these chromosomes were stained with Giemsa, it was necessary to observe them with phase contrast microscopy to fully appreciate the pattern. This suggests the possibility that something may have been leached out of the normal G-band regions and primarily the rest of the chromosomes are being visualized by this technique. Zakharov *et al.*[780] treated human chromosomes

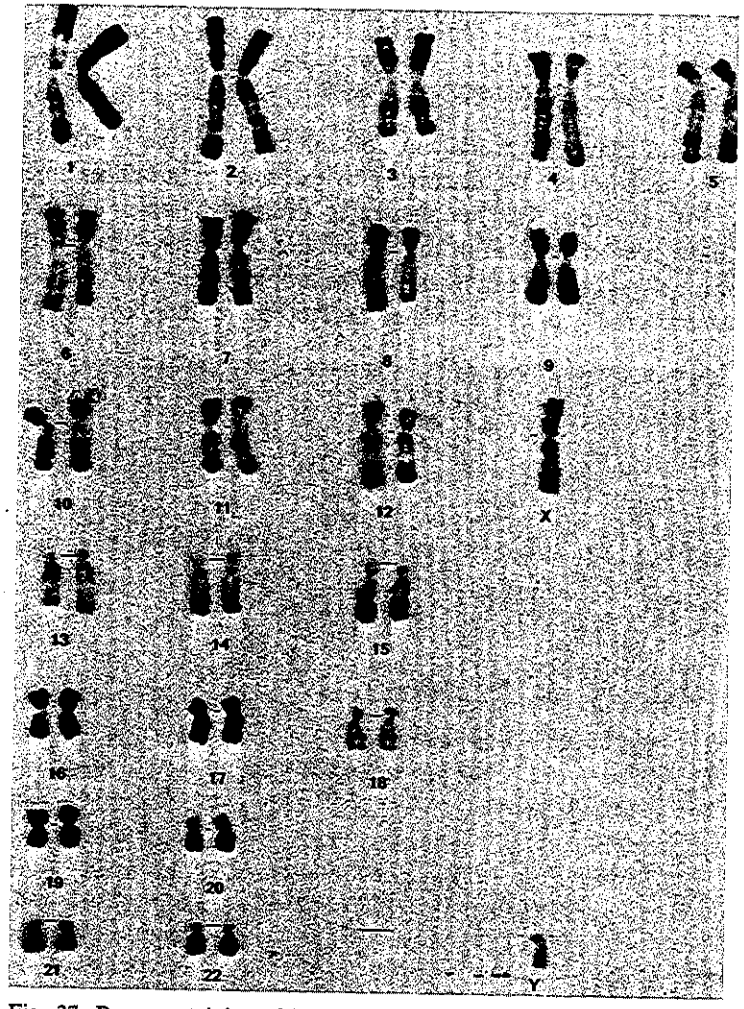

Fig. 37. Reverse staining of human chromosomes. (From Dutrillaux and Lejeune,[207] by permission.)

with β-mercaptoethanol (or BUdR) and noted that there was a despiralization of the late replicating regions. Since it is predominantly the late replicating segments in the arms of the chromosomes which stain by the G-banding techniques,[252] this despiralization gives a pattern that is very similar to that obtained by the R-banding technique described above. Patau[514] observed that in a few cells from lymphocyte cultures of patients with Bloom's syndrome (autosomal recessive dwarfism with skin changes and chromosome breakage)[41] the same despiralization patterns were seen to occur spontaneously. Similar phenomena have been reported in Chinese hamster cells following prolonged treatment with Colcemid.[668,779]

Staining of Chromosome Coils

Heat has played a prominent role in many of the G-banding and R-banding techniques. When carried to extremes it produces interesting results. For example, Fig. 38 illustrates Chinese hamster chromosomes which have been treated as in the C-banding technique,[18] except that the denaturation was carried out by the dry heat of an oven (100°C for 30 min). The pattern most closely resembles that of the coiling configuration of

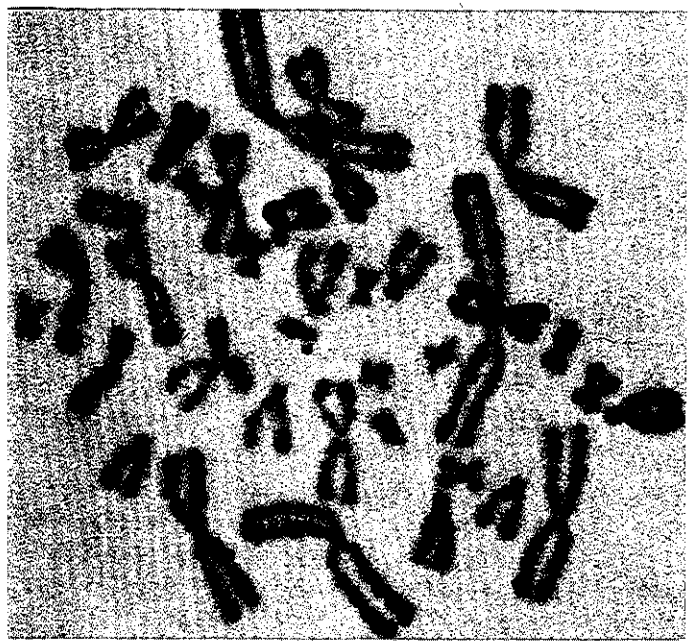

Fig. 38. Chinese hamster chromosomes treated as in the C-banding technique except that denaturation was with dry heat at 100°C for 15 min.

chromosomes[498] and suggests that the most dense part of the chromosome at the peaks of the coils is the part that is staining most intensely.

The Biochemical Significance of the Banding Patterns

As an approximate generalization, at least in human chromosomes, it can be said that the fluorescent or Q-banding = G-banding = late replication patterns and that the R-banding is the complement of these. The major exception to this is that the centric heterochromatin is also late replicating but it usually (but not always) stains poorly by the Q and G techniques. These observations suggest that the different techniques are responding in different ways to the same basic chromosomal characteristics and that these characteristics are (1) euchromatin, (2) centromeric heterochromatin, and (3) late-replicating, noncentromeric or intercalary[132] heterochromatin.

Thus the question of the biochemical basis behind these staining reactions may be partially equated with the biochemical differences between euchromatin and heterochromatin. On the basis of *in situ* hybridization and other studies there is little question that at least part of the staining of the centromeric heterochromatin is related to its content of highly repetitious DNA, and this has been discussed above. The following then is concerned primarily with the question of the biochemical nature of the noncentromeric Q, G, and R banding. There are several possibilities.

GC Content

An early suggestion was that the fluorescent banding patterns were due to variations in the guanine content (GC content) of different areas of the chromosome.[102] However, there are a number of reasons to suggest that although the quinacrine mustard may be binding to guanine,[1a] changes in GC composition are probably of minor importance in producing the banding.

1. Although quinacrine mustard contains an alkylating group that is thought to react with the N7 atom of guanine,[101] quinacrine dihydrochloride which lacks the alkylating group works just as well and gives the same basic patterns as do many other widely varying techniques.

2. In studies of the Indian Muntjac, Comings[132] observed the presence of a satellite DNA that was hidden within the main band and thus had the same base composition as the rest of the DNA. C-Band staining suggested it was localized to the centromere regions. These centromere regions stained very poorly by fluorescence, even though the DNA did not have a distinct base composition. This suggests that the fluorescence reaction was due to some property other than the base composition of the DNA.

3. Quantitation of the fluorescence of the human Y chromosome shows an increase of up to 2.2 times the average intensity of the other chromosomes. There is thus a greater variation in fluorescence than there is in base composition since the mean GC content of mammalian DNA is 40% and even the heavy satellites show only up to 53% GC content.[16]

4. Greensher *et al.*[276] have reported the fluorescence of the inactive X chromosome (Barr body) in Wharton-jelly cells of the umbilical cord of human females, and the occasional fluorescence of the Barr body in other cells. Since this is facultative heterochromatin, and its DNA is thus identical to that of the other nonstaining X, the DNA itself must play no role in the fluorescence of the Barr body.

5. If the quinacrine staining of any of the chromosomes were to undergo changes during differentiation or following translocation to new sites, this would also suggest that factors other than the composition of the DNA (which does not change) are involved in the fluorescence. Fraccaro *et al.*[232,789] have reported preliminary evidence from a study of a Y/autosome translocation in *Drosophila* that the intensity of fluorescence of the heterochromatin may decrease when translocated to another chromosome.

6. There is good correlation between Q and G banding and late DNA replication,[252] and studies in many organisms have shown that the late-replicating DNA is AT- rather than GC-rich (see above).

Variations in Chromosome Condensation

It might be suggested that the various treatments inherent in the G-band techniques result in an uncoiling of euchromatic regions, and Giemsa then merely stains the more condensed heterochromatic bands.* If this plays any role, it is more likely to be a secondary effect since the same patterns are seen by Q-banding and this does not involve any treatments likely to induce differential uncoiling. Furthermore, some of the poorest staining areas are the densely coiled regions of centromeric heterochromatin.

RNA

There is no change in the fluorescent pattern of chromosomes treated extensively with RNase,[132,216] indicating that the association of different types or amounts of RNA to the chromosomes does not contribute to the banding patterns.

* We have recently obtained biochemical and ultrastructural evidence which supports this possibility.

Histones

There is also no change in the fluorescent pattern following complete removal of all the histones by treatment with 0.2 N HCl.[132] It is not surprising that histones play no role in view of the fact that there is no difference in the amounts or types of histone present in heterochromatin compared to euchromatin (see above).

Repetitious DNA

The question of whether repetitious DNA is playing a role in the generation of banding patterns is difficult to answer unequivocally at the present time. However, there are a number of observations which suggest it does not. This has been discussed in the section on DNA of heterochromatin and the evidence for an intercalary type of heterochromatin that is not unusually enriched in repetitious DNA was reviewed.

Nonhistone Proteins

Largely on the basis of indirect evidence, a case can be made for the possibility that the primary factor responsible for the banding patterns is the presence of different DNA–nonhistone protein complexes in euchromatin compared to heterochromatin.

1. There is a considerable body of evidence suggesting there is a difference in the amount of nonhistone protein in heterochromatin compared to euchromatin (see above).

2. The reproduction of the G-banding pattern by treatment of chromosomes with pronase or trypsin can best be explained by the proposal that the enzymes are selectively removing certain types of proteins.

3. The uncoiling of the G-bands by treatment with β-mercaptoethanol might be the result of the breakdown of S-S bonds in mitotic proteins. In this regard it is of interest that one of the principal differences between proteins of interphase and metaphase chromatin was the presence of more S-S bonding in the latter.[585] These observations are consistent with DNA-protein interaction playing an important role in the staining reactions, and for reasons stated above it is unlikely that the histones are involved.

Although these considerations are compatible with the interaction of DNA and nonhistone proteins in the Q-, G-, and R-band staining reactions, *in situ* hybridization of moderately repetitious DNA to human chromo-

somes, the nonhistone proteins of euchromatin and heterochromatin, and the chromosomal components removed by the various techniques, must be studied further.

ORDER IN THE INTERPHASE NUCLEUS—REVISITED

In 1968 some reasons for the proposal that chromatin is nonrandomly arranged in the interphase nucleus were presented.[129] It was suggested that interphase chromosomes were attached to the nuclear membrane at multiple sites and that this allowed them to occupy stable rather than changing positions in the nucleus, allowing the chromatin to be reproducibly folded into mitotic chromosomes. The following is a brief updating of some recent evidence that adds further support to this proposal.

Nonrandom Arrangement of Chromosomes on the Spindle Apparatus

The spindle fibers form and attach to the chromosomes before the nuclear membrane has completely disintegrated at mitosis.[129] This potentially provides a mechanism by which any order that may be present in the nucleus could be maintained through mitosis. Some evidence that chromosomes may be nonrandomly arranged on the mitotic spindle was provided by Costello.[164] In a study of a turbellarian he found a distinctly ordered arrangement of chromosomes during metaphase of the first cleavage division. A reasonable mechanism is that the arrangement of chromosomes in the spermatozoa was in precisely the same linear order as the chromosomes in the egg. This in turn could have resulted from the end-to-end association of chromosomes or the ordered intranuclear arrangement via attachment to the nuclear membrane, or both. In the rat kangaroo, Heneen[301a] has shown that the chromosomes with heavily labeled centromeric regions seem to have a nonrandom orientation on the spindle. In a similar vein, I have noted that among 18 mitoses of the rat kangaroo, photographed from tissue culture cells that were not exposed to Colcemid, four showed a remarkably similar arrangement of chromosomes. Two such mitoses are shown in Fig. 39. These had to be turned or inverted in order to see this similarity. The observations have not been subjected to the cold realities of statistical evaluation, and in other studies where a nonrandom arrangement between nonhomologous chromosomes was looked for, it was not found.[584] Nonetheless, this rather striking similarity of arrangement of chromosomes in different

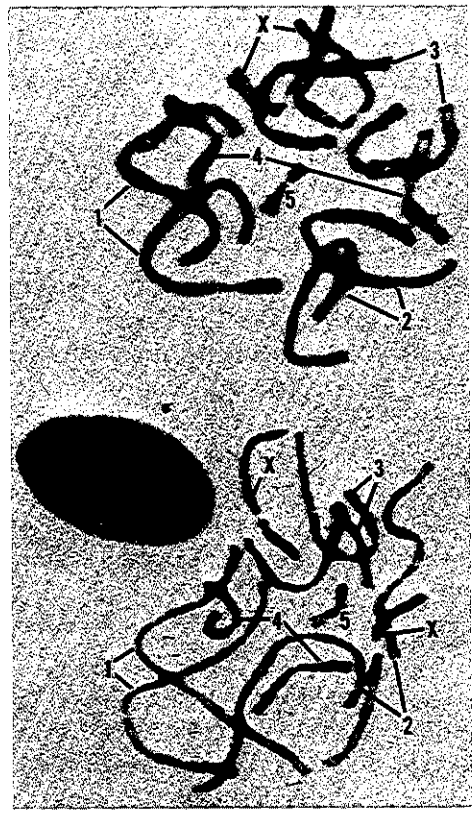

Fig. 39. Metaphase chromosomes from the rat kangaroo, *Potorous tridactylus*, not exposed to Colcemid. In most of the preparations the single chromosome 5 (hypodiploid line) was at the center and homologous chromosomes tended to lie close to each other. In several, such as these two, the different sets of homologues occupied similar positions in respect to one another.

mitoses and the observations of Costello suggest that, in addition to being spatially fixed in the nucleus, some chromosomes *might* have a nonrandom relationship to each other.

End-to-End Association of Chromosomes

Wagenaar[714,715] has reported the end-to-end association of chromosomes in several different plants. Such an arrangement would help maintain

chromosomes in a specific relationship to each other, and the fact that homologous chromosomes were adjacent to each other in the interphase cells suggested a mechanism by which they could easily find and pair with each other during meiosis.

Multipolar Mitoses

In a study of *Microtus agrestis* cells, Pera and Schwartzacher[531–533] noted diploid tripolar mitoses with one daughter nucleus that had a diploid DNA content and a diploid chromosome number and two nuclei with haploid DNA contents and haploid chromosome numbers. They also observed tripolar mitoses in tetraploid cells that resulted in haploid, triploid, and tetraploid nuclei. This capability of segregating out haploid sets suggests that the chromosomes are not just randomly piled into the nucleus. This was further indicated by the observation that the position of the chromocenters of binucleated cells immediately derived from the division of mononucleated cells tended to exactly mirror each other.[531]

Hypotonic Treated Cells

Further evidence for the fixed position of chromatin in the interphase nucleus was provided by studies by Brasch *et al.*[58] of chicken erythrocyte nuclei treated with hypotonic solutions. Photographs of the nuclei with Nomarski optics showed a three-dimensional topology of the nuclei. After treatment with water, the nuclear chromatin was completely dispersed. When the cells were resuspended in saline, a topology that was identical to the pretreated cells returned (Fig. 40). Electron microscopy of thin sections of the water-treated nuclei showed the chromatin attached to the nuclear membrane and streaming from there into the center of the nucleus (Fig. 41).

Whole-Mount Electron Microscopy

Further evidence for the association of chromatin with the nuclear membrane was provided by whole-mount electron microscopy studies of Comings and Okada.[144–146] They observed that the chromatin of metaphase chromosomes frequently showed many residual attachments to the nuclear membrane. In some cases the chromatin of sister chromatids was seen to share attachment sites on the same fragment of nuclear membrane (Fig. 42), suggesting they were attached there together in the interphase nucleus. Whole-mount electron microscopy of interphase cells showed the tendency

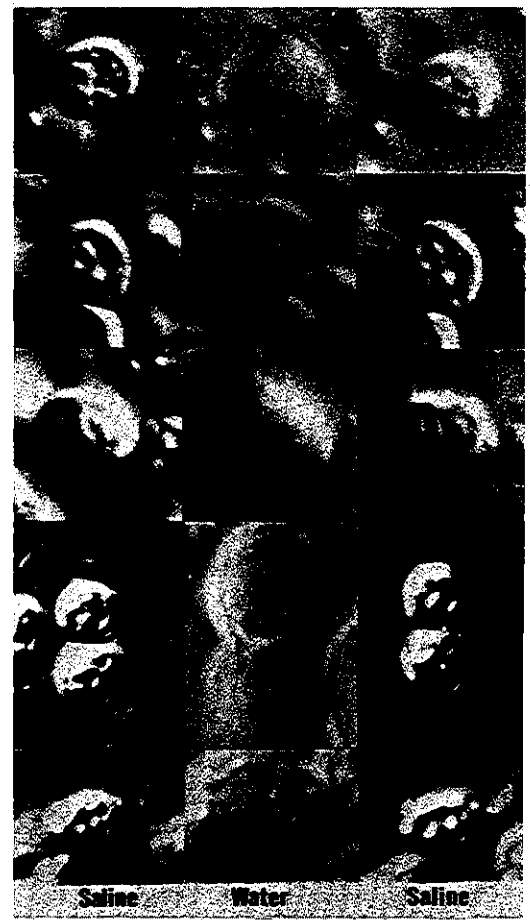

Fig. 40. Photomicrographs of six individual chicken erythrocyte nuclei as viewed by Nomarski interference-contrast optics. Each horizontal series of three micrographs represents the same nucleus, first in saline, then in water, then returned to saline. Despite the disruption of the nucleus by hypotonic swelling, the nucleus returns to the same topography when returned to saline. (From Brasch *et al.*[58] by permission.)

for chromatin fibers to converge and attach to the pores of the nuclear membrane (Fig. 43). This pattern of chromatin converging to attachment sites could be seen to persist in metaphase chromosomes.[145] This could provide a mechanism by which the arrangement of chromatin would be set by attachment to the nuclear membrane during interphase and this arrange-

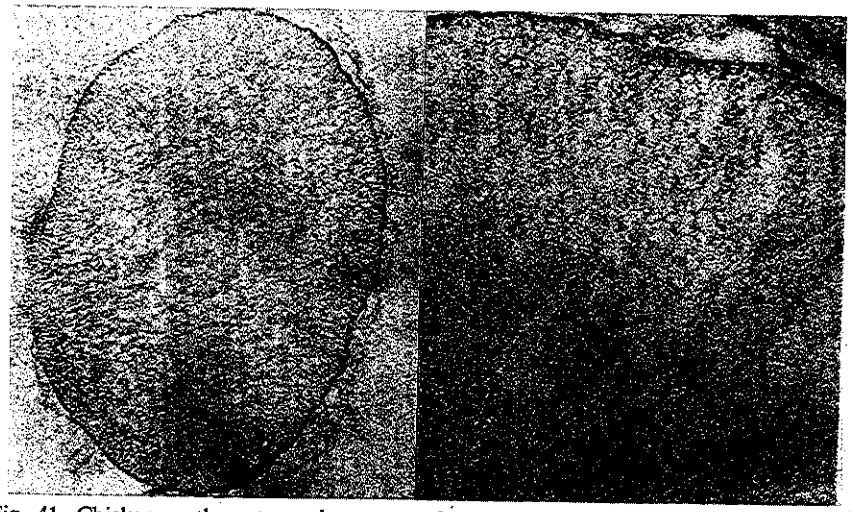

Fig. 41. Chicken erythrocyte nucleus exposed to water and fixed. Left: Chromatin is attached all around the inner surface of the nuclear membrane from which it centripetally extends into the center of the nucleus. × 30,000. Right: At a higher magnification. × 72,000. (From Brasch *et al.*[58] by permission.)

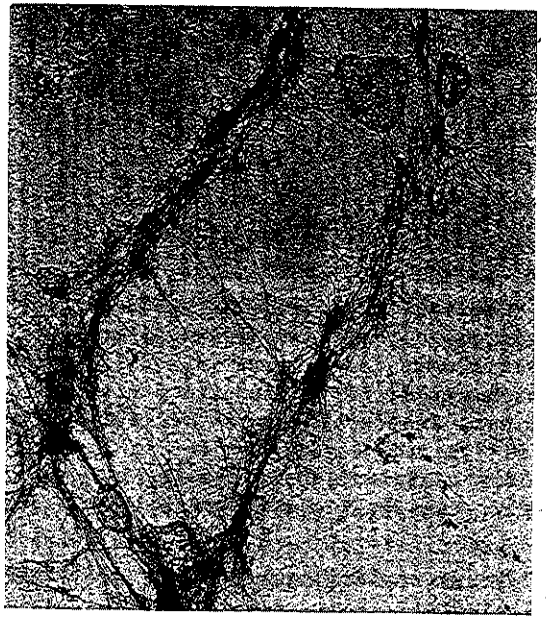

Fig. 42. Whole-mount electron microscopy of Chinese hamster metaphase chromosomes showing the chromatin fibers from both sister chromatids sharing attachment sites on remnants of the nuclear membrane. × 6600.[144]

Fig. 43. Whole-mount electron microscopy of an interphase cell from human testis. Chromatin fibers can be seen to cluster around the annuli of the nuclear membrane. × 20,000.[144]

ment could be maintained relatively unaltered in metaphase chromosomes. At prophase, the chromatin contracts onto the undersurface of the nuclear membrane, leaving the center of the nucleus relatively free of chromatin.[146]

Spatial Arrangement of Chromatin

Once the maternal or paternal mammalian X chromosome is inactivated, it remains inactivated in all subsequent divisions of that cell. The mechanism behind this epigenetic type of inheritance is not well understood. It is possible that once the pattern of chromosome condensation has been set it may remain fixed through the mechanism of these converging patterns of chromatin association. For example, one might conjecture that in heterochromatin, adjacent chromatin is associated in a small number of neighboring attachment sites, while in euchromatin it is associated with many different attachment sites. This concept is illustrated in Fig. 44. This proposal is complicated somewhat by the possibility that the attachment sites may

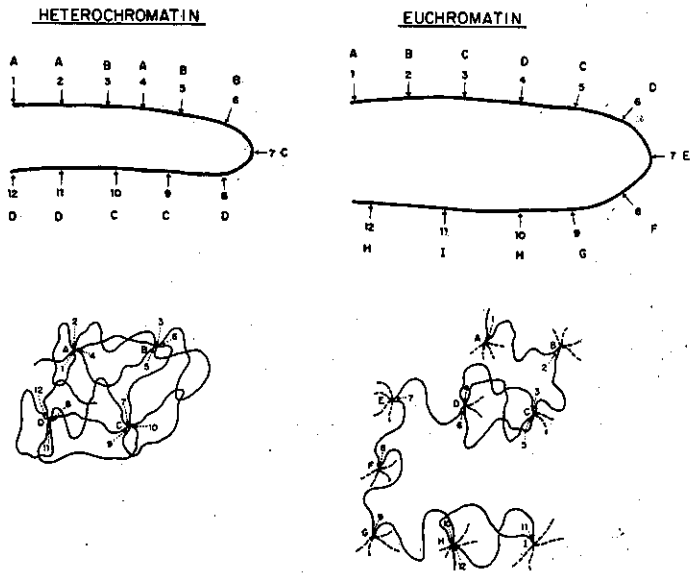

Fig. 44. A hypothetical mechanism by which some of the properties of heterochromatin could be related to the spatial arrangement of the chromatin fibers. In the upper figure is an extended heterochromatic and euchromatic chromatin fiber in which 12 successive sites of convergence are numbered. It is suggested that in heterochromatin adjacent sites may attach to the same annulae (A–D), while in euchromatin they attach to different annulae (A–H).

not be permanent, and other explanations for heritable chromosome condensation are possible (see p. 390).

THE ARRANGEMENT OF CHROMATIN INTO CHROMOSOMES

One of the most interesting and popular controversies about chromosomes revolves around the question of whether they are single-stranded (uninemic) or multistranded (polynemic). A significant body of data which has a bearing on this question has accumulated and arriving at a reasonable answer to this question requires that it all be consulted and weighed. The following is a brief tour of the battleground. An (S), an (M) or an (N) after each heading indicates this reviewer's opinion as to whether the evidence under discussion favors single-strandedness, multistrandedness, or neither.

General Morphological Aspects of Chromosome Structure (N)

The basic question about chromosome structure is: How is the DNA arranged in the chromatid? The logical approach to this question is to examine the chromosomes by electron microscopy. Unfortunately, thin-section electron microscopy provides very little information other than details about the structure of the kinetochore, the synaptonemal complex,[472] and the fact that chromosomes of meiotic cells possess a protein corelike structure (the prospective lateral element of the synaptonemal complex) while such structures are absent in mitotic chromosomes. The chromosomes themselves show only a homogeneous compacted mass of chromatin fibers.[23,477,543] Considerably more details are apparent in whole-mount preparations. This technique involves allowing mitotic cells to spread out on a surface of distilled water. They are then picked up on grids, fixed, and either air dried or dried in a critical point apparatus.[13] This procedure has been utilized extensively.[1,144,145,147–150,201–208,206,757–762] These preparations show primarily a netlike arrangement of chromatin fibers (Fig. 45). In an examination of several thousand preparations from eight species of mammals and birds we found no evidence for any subdivision other than that of the chromatin fiber itself.[150] Counting across the width of the chromatid shows from 25 to 100 fibers, depending upon the degree of chromosome dispersion.

Longitudinal Fibers

There are three general ways in which the chromatin fibers can be arranged. These are (1) repeatedly folded such that the chromatin fiber builds up to the thickness of the chromatid but essentially progresses uniformly from one telomere to the other[149,152,202] (Fig. 46); (2) longitudinal fibers which pass from telomere to telomere two or more times;[204] and (3) a combination of both. Frequently, partially dispersed or stretched chromosomes give the appearance of having multiple longitudinal fibers. Such an arrangement is unlikely, however, for the following reasons. (1) The appearance of longitudinal fibers can be easily produced by compaction or stretching of the chromosome.[152] In well-spread preparations there is no evidence for such fibers (Fig. 45). (2) Recombination data and studies of polytene salivary gland chromosome indicate the presence of a single linkage map which correlates well with the linear arrangement of chromosome bands. Any model which suggests the presence of longitudinal fibers violates this basic fact, including models which suggest that the chromatin fiber passes from telomere to centromere, turns around at the centro-

mere, and returns to the telomere. Any complaints that salivary gland chromosomes are atypical are unconvincing since they merely represent the polytenization of metaphase chromosomes and there is no reason to suppose the metaphase chromosomes of the Diptera are unique. (3) The fluorescent microscopy of chromosomes (Fig. 31) provides similar evidence against longitudinal fibers. Since the fluorescent bands extend completely across the chromatid, and since they are highly reproducible, it would be quite unlikely that they would be produced by multiple distinct and separate sections of many longitudinal fibers rather than by a single contiguous region of a folded chromatin fiber. (4) Multiple longitudinal fibers are incompatible with the existence of reciprocal translocations.[152]

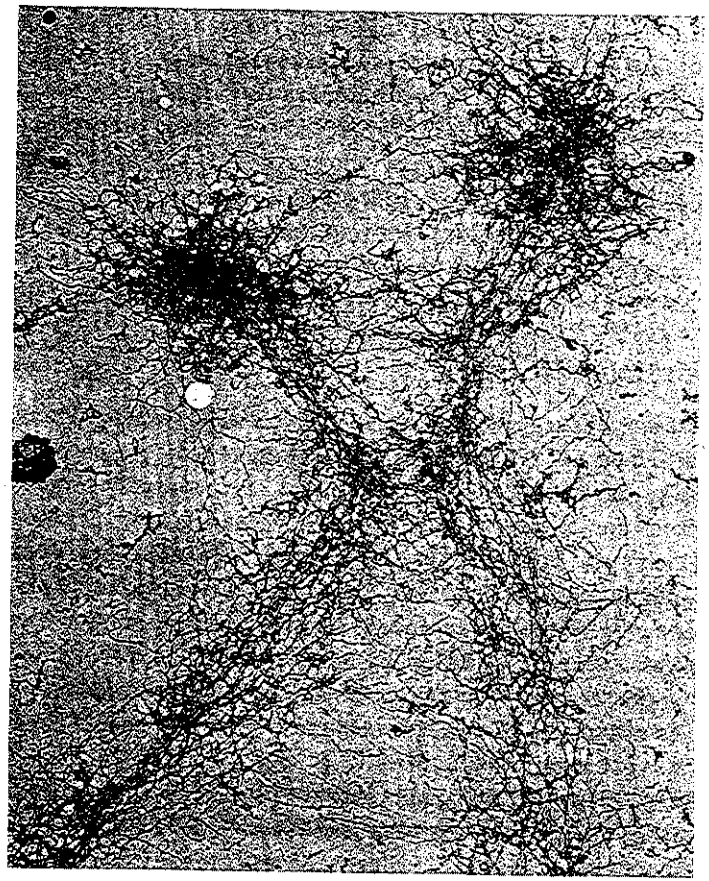

Fig. 45. A well-dispersed whole-mount preparation of a human submetacentric chromosome. × 19,300.[150]

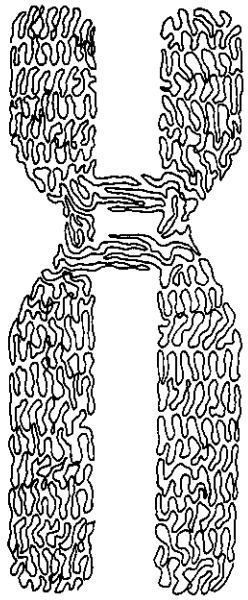

Fig. 46. A single-stranded model of chromosome structure. This suggests that a single DNP fiber beginning at one telomere folds upon itself to build up the width of the chromatid and eventually progresses to the opposite telomere without lengthy longitudinal fibers, with no central core and no half- or quarter-chromatids. The centromere region in this metacentric chromosome is depicted as the result of the fusion of two telocentric chromosomes with retention of their individual centromere regions. The fibers at the point of chromatid association briefly interdigitate.

Chromosome Cores

It has frequently been proposed that metaphase chromosomes have central cores composed of protein (or longitudinal chromatin fibers). Recently this banner has once again been raised with the suggestion that cores can only be seen if chromosomes are isolated in hexylene glycol.[669,769] This embellishment seems unlikely for the following reasons.

1. In our experience the appearance of cores is an artifact of extensive handling or stretching of chromosomes. In well-spread preparations only a netlike arrangement of chromatin fibers is seen.[1,149,150,201,202] If the chromosomes are only partially dispersed, the central chromatin fibers tend to remain together. When these central fibers are stretched they adhere to each other like wet spaghetti and give the illusion of cores. This is produced especially well by forcing a chromosome through a needle.[769] It is unlikely that cores are only seen when chromosomes are prepared in hexylene glycol. We have seen no cores in well-dispersed chromosomes isolated with or without hexylene glycol, and apparent "cores" in chromosomes isolated with or without glycol.[153] It is unlikely that the water-spread technique destroys protein structure since proteinaceous elements such as the axial filament of meiotic chromosomes[147,151] and the delicate spindle fibers of mitotic chromosomes[83,153] are readily seen by this technique.

2. Cores are not seen in thin sections of mitotic chromosomes but are readily seen in meiotic chromosomes where the presence of axial filaments[472]

has long been known. These axial filaments form the lateral element of the synaptonemal complex which is then discarded before the chromosomes enter first meiotic metaphase.[155]

3. In a teleological vein, there is no need for cores in somatic metaphase chromosomes. They only add unnecessary complexity to what can be a relatively simple system.

In regard to the question of strandedness, it is unlikely that purely morphological studies will provide convincing evidence one way or another,[152] except in the case of lampbrush chromosomes which seem clearly to have loops composed of single DNA helices[459] (Fig. 11).

Half-Chromatids (N)

By light microscopy the chromatid frequently appears to consist of two strands coiled around each other.[269,433,479,705,706] These have been termed half-chromatids. Their appearance is enhanced by treatment of chromosomes with trypsin,[706,762] and they do not represent fixation artifacts since they are seen in living specimens.[22] This appearance of doubleness has occasionally been seen by whole-mount electron microscopy.[762] However, in an extensive study of whole-mount chromosome preparations there was no evidence that they represented a true subdivision of the chromatid.[150] It has been suggested[150] that half-chromatids are an illusion of doubleness that is uniquely seen in coiled structures, and their existence is valid as an observation, but the conclusion that they are proof of double strandedness is not. Since a coiled chromosome, whether it is uninemic or polynemic, could produce this appearance of half-chromatids, they provide no clear evidence on the question of strandedness.

Half-Chromatid Exchanges (S)

When cells are irradiated during prophase of mitosis, aberrations are frequently seen at anaphase which have the appearance of involving only a portion of the chromatid in an exchange. These have been termed half-chromatid or subchromatic exchanges. They have frequently been used as evidence for the existence of half-chromatids and thus double strandedness. However, if they truly represent half-chromatids, at anaphase of the next mitosis (A_2) they should show up as half-chromatid lesions on both sister chromatids.[131] If they represent incompletely resolved complete chromatid breaks, then at A_2 they will present as chromosome breaks (a break in both sister chromatids). When this type of data has been collected it has been found that the lesions at A_2 are chromosome in type,[364,505] occasionally chromatid in type,[294] but double half-chromatid lesions have not been found. The morphological aspects of half-chromatid lesions are consistent with either single or double-stranded chromosomes,[131] and these data on A_2 lesions favor single strandedness.

Mutations (S)

The rather formidable body of knowledge of classical Mendelian genetics indicates that in diploid organisms recessive mutations must be present in double dose to the expressed. These and other data are most readily understood on the basis of a paternal and maternal chromosome, each consisting of a single DNA helix. If each chromosome were composed of more than one helix (multistranded) it would be necessary to find some mechanism of having a mutation spread from one strand to another. To do this requires complex and improbable models making extensive use of undiscovered enzymes.

Recombination (S)

A similar problem is encountered with the mechanism of recombination. If each chromosome is multistranded, elaborate mechanisms are required to have all strands break and recombine at precisely the same point. These problems are not encountered with single-stranded chromosomes.

Chromosome Replication (S)

The Watson–Crick model predicts that DNA replicates semiconservatively. This prediction was verified by analytical ultracentrifugation studies.[451] Thus, if DNA is replicated in the presence of labeled precursors, two daughter molecules are produced, each containing one unlabeled strand and one labeled strand. Since on this basis, precise predictions can be made concerning the segregation of label in single *versus* multistranded chromosomes, autoradiography has been a popular tool for the study of this question, and a number of different observations have been made.

Semiconservative Segregation of Label (S)

In 1957 Taylor *et al.*[684] showed that if plant chromosomes were labeled with ^{3}H-thymidine at S_1, then placed in cold media during S_2, at M_2 one sister chromatid was labeled while the other was unlabeled. This is precisely the result expected with a single-stranded chromosome.

Sister Strand Crossovers (S)

Taylor[682a] noticed that occasionally the label on one M_2 chromatid was translocated to the unlabeled sister chromatid. In the plant system it was possible to determine whether this occurred during the first or the second cell cycle. The ratio of the frequency of the two events indicated that polarized strands were being exchanged. This polarization is best interpreted as the 3′ to 5′ and the 5′ to 3′ strands of a single DNA helix.

Endoreduplicated Chromosomes (S)

Occasionally a cell will pass from S_1 to S_2 without going through mitosis. When mitosis does occur, diplochromosomes are seen with four sister chromatids. If two mitoses in a row are skipped, quadruplochromosomes will result. Autoradiography of these chromosomes shows that in diplochromosomes the labeled strand is in chromatids 1 and 4, and in quadruplochromosomes it is in chromatids 2 and 7.[304,605,719] This is the expectation on the basis of semiconservative replication of a single-stranded chromosome in which the daughter strands were consistently placed to the outside of the parental ones. This system also allows calculation of the ratio of exchanges occurring in the first and second cell cycle and the results confirm those of Taylor.[304]

Replication in Dicentric Chromosomes (S)

When breakage and reunion occur to form dicentric chromosomes, precise predictions can be made as to the results expected with single *vs.* multistranded chromosomes. The results indicate single strandedness.[61]

Isolabeling (N)

An apparent exception to the semiconservative segregation of label in chromosomes is occasionally seen. This consists of the occurrence of label at the same place on both sister chromatids. This has been termed isolabeling and has been used as evidence that chromosomes are multistranded.[184,525,526] However, isolabeling can be equally well explained on the basis of single or double sister strand exchanges occurring in a multifolded single-stranded chromosome.[135]

Delayed Isolabeling (N)

The occurrence of isolabeling as late as the fourth or fifth mitosis after labeling has been reported.[184] Although this has also been claimed to indicate

multistrandedness (up to 64 strands would be required), it could just as well be the result of the sister strand exchanges occurring in the immediately preceding cell cycle.[135]

When all of the autoradiography data are considered, it is strongly in favor of single-stranded chromosomes.

X-Ray Studies and the Cell Cycle (N)

When chromosomes are irradiated early in G_1, prior to DNA replication, at the next mitosis chromosome aberrations are seen (a break in both sister chromatids). When they are irradiated during the S period, after DNA replication has begun, at the next mitosis chromatid aberrations are seen (a break in only one of the sister chromatids).[222,324,766] In some studies it has been noted that the transition from chromosome to chromatid aberrations occurs in late G_1 rather than in early S.[222,766] On the basis of this and studies with various chemical agents, it has been suggested that this indicates chromosomes must be multistranded and that the strands separate, to allow chromatid aberrations in G_1.[763–765,193] However, there are alternative explanations. For example, the same results would be obtained if the replicators (initial portion of the replicons) were to replicate before the S period gets into full swing, or if the attachment of DNA polymerase to DNA prior to replication were to bring about some strand separation. If these regions were particularly radiosensitive, chromatid aberrations could be produced in single-stranded chromosomes in the late G_1. This interpretation is favored by the observation that exposing cells to ^{3}H-uridine during G_1 results in chromatid type aberrations, while exposure to ^{60}Co results in chromosome aberrations.[412] The ^{60}Co would hit the native DNA helix, while the ^{3}H-uridine would preferentially hit DNA undergoing strand separation in the process of transcription. At their present state, irradiation studies do not provide clear-cut evidence on the question of strandedness. The results can be interpreted either way, according to one's prejudices.

X-Ray Studies and DNA Content (S)

There is a second type of evidence from X-ray studies which presents results whose interpretation is a little more clear cut. Sparrow and Evans[648] demonstrated a direct relationship between the DNA content of an organism and its sensitivity to radiation. This is understandable on the basis of the additional DNA being added on longitudinally to a single-stranded chromosome. The more DNA the more DNA to break. However, the opposite

result would be expected if the additional DNA was added on as extra strands. Then the more DNA, the more strands, and the less the likelihood of breakage completely across the chromosome.

DNA Renaturation (S)

The time it takes to attain one-half renaturation of DNA is directly proportional to the complexity of the genome in relation to the number of different sequences it contains.[66] This value can thus serve as a measurement of the amount of unique DNA present in a given genome. Utilizing data of this type, Laird[388] and Straus[667] have shown that after correction for the highly repetitious DNA, this amount is equal to the haploid content of the genome. This means that these sequences are present as single copies, rather than double copies as predicted by a double-stranded model of chromosome structure, or in multiple copies as predicted by a multistranded model or by the master-slave hypothesis.

DNA Content and Chromosome Number (N)

There have been some reports in plants of a geometric increase in DNA content (1:2:4) in related species without a change in chromosome number. This has been interpreted as evidence for polynemy. There are, however, more exceptions to this than there are good examples of it.[544] The non-geometric increases are best explained by addition of interstitial DNA at various places. This can be visually appreciated by the meiotic pairing between *Allium cepa* and *Allium fistulosum*. *A. cepa* has 27% more DNA than *A. fistulosum* and at meiosis of hybrids, the *A. cepa* chromosomes loop out at numerous places.[553] Thus, this type of data on DNA content and chromosome number does not make a convincing case for polynemy. At the same time, since there could be interstitial additions in a polynemic chromosome, the frequent absence of a geometric series doesn't prove uninemy either.

Microspectrophotometry of Drosophila *Ganglion Cells (M)*

Gay *et al.*[254] have reported that during the course of development from first to third instar in larvae of *Drosophila*, there is an increase in the DNA content from 4C to 8C without a change in the chromosome number. The chromosomes themselves also appear larger. This provides good evidence

that these mitotic chromosomes are polynemic. They may represent chromosomes that are in the early stages of polytenic development.

Studies of Lampbrush Chromosomes (S)

Studies of the kinetics of chromosome breakage after treatment of lampbrush chromosomes with DNase led Gall[245] to conclude that these chromosomes were single stranded. Recent studies by Heddle and Bodycote[295,296] have shown the difficulties involved in interpretation of this type of data. In support of it, however, are the electron microscopy studies of Miller[459] which show that the lampbrush chromosomes contain only a single DNA helix. Thus the evidence for single strandedness in lampbrush chromosomes of newts, which contain excessive amounts of DNA and should be excellent contenders for having multistranded chromosomes, provides strong evidence for uninemy.

Meiosis in Chlamydomonas reinhardi *(N)*

Studies by Sueoka *et al.*[114,671] of meiosis in *Chlamydomonas reinhardi* (an alga) have shown that after gametes fuse, there is one round of DNA replication followed by three divisions to produce eight zoospores. There are several possible interpretations of this observation. Wolff[763] has interpreted it to mean that 1*n* gametes fuse to produce 2*n* zygotes and zygospores, that DNA replication results in 4*n*, and three divisions result in $\frac{1}{2}n$ zoospores each containing a half-chromatid. Sueoka *et al.*[671] have diagrammed the process as 2*n* gametes to 4*n* zygotes and zygospores, DNA replication to 8*n*, and three divisions to 1*n*, then a DNA replication to 2*n*. This implies diploid vegetative cells. A third interpretation is that prior to nuclear fusion the gametes undergo DNA replication as they do in many eukaryotes.[621,622] Although difficult, cytogenetic studies might allow an unambiguous distinction between these alternatives.

Mitotic Segregation in Sea Urchins (N)

In studies of sea urchin eggs treated with mercaptoethanol, Mazia and Bibring[444] were able to prevent cleavage division of cells at mitosis, but division of the centrioles continued. When the block was removed at the time for the normal second cleavage division, a tetrapolar mitosis occurred with each daughter cell seemingly containing a normal diploid number of chromosomes. Since DNA synthesis was also inhibited by the mercapto-

ethanol, it was initially suggested that a four-way split of the chromosomes was occurring with a separation into half-chromatids. This would imply that each chromosome contains two DNA helices.[314] However, subsequent microspectrophotometric studies indicated that the four daughter nuclei contained approximately a 4*n* amount of DNA and the DNA content of each was not the same, thus making a separation into half-chromatids unlikely.[445]

Llaveiella *Chromosomes (M)*

Hughes-Schrader[333] reported that subunits of the chromosomes of the coccid *Llaveiella taenechina* completely disjoined at first meiotic anaphase and then later reassociated in parallel pairs. This certainly suggests the presence of multistranded chromosomes if the half-chromatids can separate completely. However, the skeptic, upon looking at the pictures, might counter with the complaint that rather than being separated they are only a clear example of half-chromatids, and an actual photograph rather than an interpretative drawing would be more convincing. It is of some interest in regard to the question of light microscopic observations of chromosome separation that the achromatic gaps in metaphase chromosomes which appear to be devoid of chromatin material by light microscopy have been shown to contain continuous chromatin fibers by whole-mount electron microscopy.[70]

DNA Ultracentrifugation (S)

A novel approach to the problem of chromosome strandedness has been to determine the molecular weight of unsheared DNA and determine the number of different molecular weight classes of DNA. Thus if a primitive eukaryote had 6 chromosomes and 6 different molecular weight classes of DNA, and when added up they accounted for the total molecular weight of the DNA of the entire genome, this would indicate 1 DNA molecule per chromosome. Preliminary studies along this line suggest just that.[359]

Conclusion—A Single-Stranded Model of Chromosome Structure

In regard to the question of chromosome strandedness, there is some evidence which does not clearly contribute one way or the other, some which favors single-stranded chromosomes, and some which favors multistranded

chromosomes. However, the evidence which lies in the mainstream of genetic relevance concerning mutation, recombination, replication, chromosome breakage, and DNA renaturation all favor single-stranded chromosomes. The evidence that speaks for multistranded chromosomes tends to cluster at the periphery of relevance and includes mitotic chromosomes which are probably at early stages of polyteny in *Drosophila*.

The basic genetic dictum of the one chromosome—one gene map, and the multifaceted evidence for single strandedness suggests that eukaryotic chromosomes are composed of a single DNA helix which is surrounded by histone and nonhistone proteins and supercoiled to form a chromatin fiber, which folds upon itself to make up the width of the chromatid[149,150,152,202] and eventually progresses from one telomere to the other. This is illustrated in Fig. 46. This chromosome has no longitudinal fibers and no central core. Electron microscopic studies suggest that metacentric chromosomes are formed by the fusion of two telocentric chromosomes,[149] and the kinetochore is a disc-shaped structure occurring at the centromere region, to which the spindle fibers attach.[154,349] It is likely that the chromatin fibers of the metaphase chromosome converge to form sites which attach to the nuclear membrane[145] and that this arrangement is responsible for the reproducible patterns of chromosome condensation that are so clearly seen by quinacrine fluorescence microscopy. This model has the advantage of stark simplicity and is consistent with the bulk of available genetic, biochemical, and morphological data.

GENE REGULATION

The literature on gene regulation is far too vast to be even partially reviewed here. However, within the scope of the present subject there are some pertinent points to be made concerning transcriptional control and the relevance of prokaryote systems to higher organisms.

Positive Control

Although prokaryotes classically possess a negative system of gene control, there are some situations in which genes appear to be under positive control. For example, the *N* and *Q* genes of phage lambda seem to produce products which act as positive controlling elements, and in the arabinose operon of *E. coli*, deletions of the *C* gene (which synthesizes the repressor-activator protein) result in the shutting off of the operon—providing clear evidence for a positive control mechanism. Evidence to date suggests that

the *C* gene produces a repressor which turns off the operon by binding to the operator. However, in the presence of arabinose the repressor is changed to an activator which binds to an initiator sequence and activates the operon.[745] The mechanisms by which positive control might be brought about have been discussed previously.

Negative Control

The lactose operon of *E. coli*[341] and numerous other loci in prokaryotes are under negative control. That is, a regulator gene (*i*) synthesizes a repressor protein which specifically binds to the promoter region of the operator (Fig. 47) and prevents the attachment or function of RNA polymerase. This system is well known and requires no rehashing here. However, there are a number of mutants of the lactose operon which may provide important insights into potential genetic mechanisms in eukaryotes. A compilation of these mutants is presented in Table VIII, and the implications of some of them are discussed below.

i^{-d}: A Model for Dominant Mutations in Man

The complaint is often heard that outside of the hemoglobinopathies there are as yet no models to explain the mechanism of action of dominant mutations. There is, however, an intriguing mutant of the *i* gene of the lactose operon that provides a clear precedent for one mechanism. The complete repressor is a tetramer composed of four identical monomers.[265] In the presence of the i^{-d} mutation, defective monomers are made which

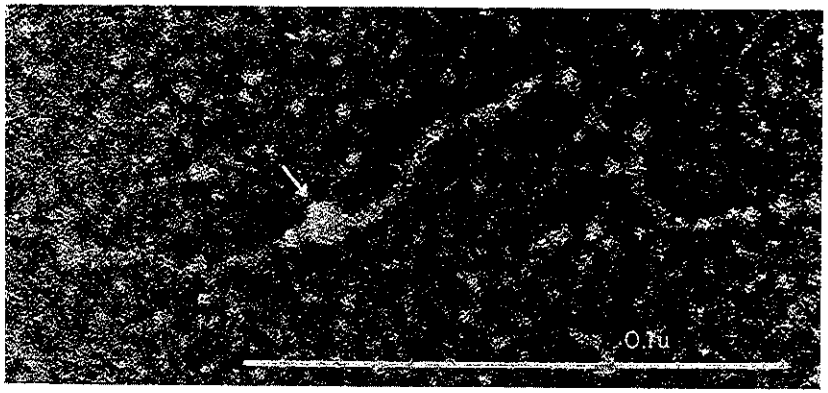

Fig. 47. Lambda repressor bound to the operator site (as measured from the ends that are not shown) of lambda DNA. (Courtesy of Dr. Jack Griffith.)

TABLE VIII. Mutants of the Lactose Operon

Mutant	Mechanism and phenotype	Genetics	Reference
Repressor mutants			
i^-	Repressor gene absent or nonfunctioning → constitutive synthesis of enzymes.	Recessive	341
i^s	Mutant repressor protein is unable to bind to inducer but does bind to operator → noninducibility of enzyme synthesis.	Transdominant	52, 751
i^{TL}	Thermolabile repressor. Growth at increased temperature results in derepression.	Recessive	586
i^{TSS}	Temperature-sensitive synthesis of repressor. Growth at increased temperatures results in decreased synthesis of repressor and derepression.	Recessive	586
i^Q	Probable mutation of the *i* promoter—tenfold increase in repressor synthesis.	Dominant	475
$i^{super\ Q}$	Similar to i^Q but 50× increase in repressor synthesis.	Dominant	265
i^{-d}	Defective repressor → monomer which can combine with i^+ monomers and render entire repressor molecule defective → failure of repressor to bind to operator → constitutive enzyme synthesis.	Transdominant (recessive to i^Q and $i^{super\ Q}$)	265
Operator mutants			
O^c	Operator unresponsive to repressor → constitutive synthesis of enzymes.	cis dominant	341
Promotor mutants			
p^-	Decreased affinity of promoter for RNA polymerase → decreased synthesis of enzymes.	cis dominant	335, 343
Structure gene mutants that can masquerade as regulator mutants			
z^-	Defective or deleted β-galactosidase gene. Since galactose must be acted on by this enzyme to make inducer, the *y* and *a* genes of z^- cells cannot be induced by galactose.	Recessive	265
y^-	Defective or deleted permease. *z* and *a* genes cannot be induced by lactose since it cannot enter the cell without permease.	Recessive	265
o^o	Originally thought to be operator negative, mutants are actually extreme polar mutants with nonsense mutants in structural genes close to the operator.	Recessive	29

combine with normal i^+ monomers and affect the entire molecule so that it will not bind to the operator. Here then is a potential mechanism by which a disorder that is inherited in a dominant fashion could be the result of a complete enzyme deficiency, despite the fact that the mutation is present in heterozygous dosage.

Structural Mutants that may Masquerade as Regulator Mutants

Human genetics is rife with mutations that were originally thought to be regulator gene mutations but subsequently have been shown to be structural gene mutations. These difficulties can be appreciated by an examination of some of the lactose operon mutants which have required the precision of genetic mapping that is possible in prokaryotes to demonstrate they too are structural rather than regulator gene mutants. These are the z^-, y^-, and o^o mutants (see Table VIII). An interesting recent example of the problems in making this distinction are illustrated by the testicular feminization mutation in the mouse. A number of its characteristics suggest that it could be a regulator gene mutation[195,489,497] but studies of the cytosol of kidney cells suggest it could also be a mutation of the structural gene for a testosterone binding protein,[696] which may also function as a regulatory protein.

The Promoter

The promoter is the region of the operon at which initiation of transcription is controlled. This initiation includes the recognition and binding of RNA polymerase to DNA, and the opening of the duplex.[456] It is an important site and concept since it serves as the focal point of many different factors. These include (a) different RNA polymerases and their cofactors; (b) DNA binding proteins (such as CAP) and their modification by cyclic AMP; (c) mechanisms of producing localized opening up of the DNA helix in preparation for transcription (and the implications this has for positive control); and (d) the effect of base substitutions on the effectiveness of the promoter.

The existence of the promoter allows one to bring to a swift halt chicken or egg type of questions about gene regulation such as: "If the *i* gene controls the transcription of the lac operon, what gene controls transcription of the *i* gene, and what controls that, etc.?" The answer is, that the base composition of the promoter region of the *i* gene sets its rate at a specific low level of constant transcription. This is indicated by the presence of i^Q and i^{superQ}

mutants. These are most likely point mutations which reset the *i* promoter at a different rate of transcription.

The lac Repressor

The concept of negative gene control as proposed by Jacob and Monod[341] was proven by the isolation of the repressor molecules. They were shown to be acidic proteins and to specifically bind to the appropriate operators.[264,546,547,557–560] The lac repressor or product of the *i* gene of the lactose operon is a tetramer with a molecular weight of 150,000 composed of 4 identical monomers of 38,000 molecular weight.[265] Its binding to the lac operator is remarkably tight, with a dissociation constant of 10^{-13}.[558] It has been estimated that there are on the average only 10 copies of the lac repressor per cell.[265] It binds to native but not single-stranded DNA; the binding is greatest at low ionic strength,[557–560] and it preferentially binds to AT-containing regions.[411]

Other Sequence-Specific DNA Binding Proteins

Other examples of proteins that bind to specific regions of the DNA molecule continue to be reported and will obviously mark an important chapter in understanding the use and control of different genomes. At present these include the lac, lambda,[546,547] 434 phage,[538] galactose,[462] arginine,[708] histidine,[634] and coliphage[538] repressors; some specific antibodies, basic proteins, glucosylating and methylating enzymes[771]; and some RNA polymerases and their cofactors (see above). Other proteins which bind to DNA and which may or may not select specific sequences include DNA polymerase, the ω protein of *E. coli* which may produce the swivel for DNA replication,[730] the product of gene 32 of bacteriophage T4 which is essential for DNA replication and recombination,[4] numerous excision and repair enzymes,[357] and nonhistone[374] and cytoplasmic proteins[587] in eukaryotes. Some of the repressors, such as those of the lambda, histidine, and arginine systems are able to repress several different operators, and the CAP-cyclic AMP system of *E. coli* is able to activate several different promoters (see above). These characteristics would obviously be of some use in higher organisms. In the histidine system in *Salmonella*, several operons are sensitive to a single repressor and the hutC gene which codes for this repressor is in one of those operons.[634] A repressor such as this, which can repress its own synthesis, has properties that would be helpful in higher organisms.

Nucleosteric Chromosome Pairing and Folding Proteins

Some of the properties of the lac repressor could be very useful for performing some chromosomal housekeeping functions in higher organisms. For example, meiotic pairing and recombination in higher organisms appears to take place in three steps: (1) premeiotic alignment or pairing of homologous chromosomes, (2) tight synapsis of homologous chromosomes mediated by the synaptonemal complex, and (3) formation of heteroduplex DNA molecules with ensuing recombination.[148,155] The mechanism by which homologous chromosomes become aligned prior to the formation of the synaptonemal complex, or the mechanism of precise somatic pairing in *Drosophila*, has remained an enigma. Comings and Riggs[156] have suggested that a class of "nucleosteric" proteins may exist with the following characteristics: (1) They are able to bind to specific DNA sequences. (2) After DNA binding they undergo allosteric changes which (3) allow them to bind to each other. This is not difficult to conceive since repressor and other proteins are already known to individually possess such properties. Such proteins would be ideally suited to initiate homologous pairing. The only two proteins in the nucleus that would bind to each other would be the two that are bound to a specific sequence on the two homologous chromosomes. Such proteins could also bind to a moderately repetitious sequence localized only in constitutive heterochromatin and keep it condensed throughout the cell cycle without affecting euchromatic DNA. Finally, nucleosteric proteins could provide a mechanism for chromosome condensation at mitosis, and could allow the site-specific inactivation of histone repression.[156] Although purely speculative at present, techniques are available to test for such useful proteins.

Models of Genetic Regulation in Eukaryotes

Fiction writers say that there are only a few basic plots and all the world's stories merely consist of the insertion of different characters and different places. The following models are somewhat analogous although the characters and places seem fairly well set and the problem is to find the right plot. Any proposal for gene regulation in higher organisms should contain the following ingredients: (1) structural genes which are controlled by (2) adjacent operator or promoter sequences, and (3) a mechanism to control the operators and promoters both singly and in groups. When completed, the edifice should provide a role for repetitious and unique DNA sequences, account for the existence of large HnRNA molecules which are

degraded in the nucleus, and smaller molecules which escape into the cytoplasm, and still smaller RNA sequences which actually code for the structural proteins, and accommodate the histone and nonhistone proteins along with the cyclic AMP effects. The most successful author will be the one whose plot contains the correct interaction of these characters.

The Britten-Davidson Model

One of the earliest entries to accommodate the repetitious DNA sequences was the proposal by Britten and Davidson.[65] Without getting into their special terminology, the essence of this model was that attached to each structural gene were a series of operators rather than only one. This meant that a series of conditions, rather than only one condition would be necessary to activate a given gene, and it also meant that several different structural genes could be coordinately controlled if they each had an operator in common. To aid in the coordinate control of different structural genes it was further suggested that whole sets of regulator molecules (acting on the operators) could be produced by a single stimulus. Although they proposed that the regulators were RNA molecules (thus attempting to account for HnRNA and chromosomal RNA) the system could also work with proteins as regulator molecules.

The Georgiev Model

Georgiev[258] has proposed a similar model which seems to provide a more convincing role for HnRNA. Here again a series of operator sites were attached to each structural gene, allowing one or more conditions for gene activation, and allowing several genes to be coordinately controlled by a single regulator molecule. In this model the entire operon was transcribed to give rise to HnRNA and the operon RNA was then degraded in the nucleus, allowing the structural mRNA to pass to the cytoplasm.

An mRNA Ticker-Tape Model

If Georgiev had also suggested that some of the operon RNA sequences were allowed into the cytoplasm, a proposal similar to the mRNA ticker-tape model would have resulted. For example, Sussman.[676] has suggested that each messenger RNA molecule may contain a series of repetitious sequences prior to the beginning of the structural message, and that each time the mRNA is transcribed one of these sequences is chopped off. It was also suggested that the repetitious sequences might bind to some types

of ribosomes but not to others; that is, there might be specific mRNA-ribosome interactions. The length of the stretch of repetitious sequences could control the number of times the mRNA was used and thus the level of enzyme synthesis, and the ribosomal specificity might allow favored classes of mRNA to attach to ribosomes and be carried to the cytoplasm. This model is consistent with the length of hemoglobin mRNA being greater than necessary for the structural message, with the ability of mRNA to hybridize to a larger portion of the genome than expected, and with the apparent necessity to have a nucleolus before mRNA can be transported from the nucleus.[289] A suggestion containing some of the properties of these two models was proposed by Scherrer and Marcaud[593] and termed "cascade regulation." Here also, repetitious regulatory sequences were transcribed into HnRNA, providing degradable sequences for regulation of the RNA.

Each of these models assumes the presence of repetitious sequences interspersed between unique DNA sequences, and the latter two assume the presence of repetitious RNA sequences in HnRNA or cytoplasmic mRNA. As discussed in this review, there is some evidence for these conditions. The intriguing observation that the euchromatic DNA of *Drosophila* contains very little repetitious DNA[194,250] suggests that a considerable amount of genome usage in higher organisms can take place without a lot of repetitious regulatory sequences. Studies of whether HnRNA in *Drosophila* also shows an absence of repetitious HnRNA would be of interest. None of these models has taken into account the possible existence of large amounts of junk DNA. This would seem to provide a better explanation for the huge disparity between the size of HnRNA and mRNA than would the proposal that the degraded part of the HnRNA is composed of repetitious regulatory sequences.

Cyclic AMP and Hormone Action

An exciting discovery in the effect of hormones on target tissues has been the emergence of the seemingly ubiquitous "second messenger," cyclic AMP. The general mechanism appears to consist of the association of hormones with specific receptors on the cell surface of target organs → intracellular activation of adenyl cyclase → generation of cyclic AMP from ATP → activation of protein kinases → phosphorylation and alteration of activity of various enzymes[266,334] and other proteins such as ribosomal proteins[722] and histones.[369,393] It is apparent that by a single stimulus it is possible to change the activity of a number of different proteins and either alter enzyme activity or alter the genetic activity of the chromatin.

Target-Tissue Receptors and Hormone Action

Hormones may have both nonspecific and specific effects on their target tissues. The cyclic AMP-protein kinase system appears to accomplish relatively nonspecific gene activation. How can more specific activation be brought about? There is increasing evidence from a number of systems that there are specific receptor proteins in the cytoplasm of target cells that bind the hormone and that this complex is transported to the cell nucleus where it associated with the chromatin,[344,435,661,695] probably binding to non-histone proteins. The chromatin of the target tissues appears to contain specific acceptor sites for the hormone-receptor complexes.[661] The exact result of this mechanism in terms of whether specific genes are activated and if so which ones and how many, remains to be elucidated. However, this potentially provides an example of a relatively simple mechanism by which a small molecular weight effector can potentially regulate coordinated sets of genes in different tissues.

Sex as a Regulator Gene

The experiments of Jost[353–355] demonstrated that the female phenotype automatically develops in the absence of the male gonad and that the male phenotype is induced by testosterone. In view of this simple on/off system, Ohno[489,497] suggested that maleness and femaleness may represent the induced and noninduced state of a single regulatory gene.

CONCLUSION

Many of the differences between prokaryotes and eukaryotes could be directly attributed to increases in the genome size. Thus gene duplication may lead to the development of new genes, but it is more likely to lead to the accumulation of junk DNA and functional or marginally functional repetitious DNA. Genomes of increasing size are more easily handled and distributed in small units or chromosomes. If membranes are important in the mechanism of DNA replication, there would be an obligatory need for nuclear membranes. It is reasonable to suggest that most of the unique features of eukaryotes such as chromosomes, nuclei, mitosis, meiosis, the synaptonemal complex, junk and repetitious DNA, HnRNA, and heterochromatin may be the natural outgrowth of the expansion of the genome size. In a similar vein, it should not be too surprising to find that those processes which are independent of genome size, such as transcription,

translation, processing of ribosomal RNA, interlinking of hormonal controls by cyclic AMP, DNA replication, and most of all, positive and negative gene controls, may have remained relatively unaltered during the transition from the first and second to the third billion years of life on earth.

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