CHROMATIN, AND GENE REGULATION IN EUKARYOTIC CELLS AT THE TRANSCRIPTIONAL LEVEL

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INTRODUCTION

Considerable progress has been made in our understanding of the molecular mechanisms for the regulation of bacterial gene expression. The extensive reviews[1,2] as well as some of the current literature in this area[3-5] clearly illustrate that the model proposed by Jacob and Monod in 1961 provided the much needed paradigm to initiate and catalyze almost a decade of extensive experimental work in studying the mechanism of gene regulation in prokaryotic cells. Through a massive trust in public interest and support, and by the judicial use of a limited number of biochemical, biophysical, and genetic techniques, we now understand details about the genes of these cells that would probably have been regarded as unattainable a few years ago.

What about eukaryotic cells? Is it too early to propose how gene regulation takes place in these cells? A number of models have been proposed in recent years. With the ever-increasing interest in the structure and function of the eukaryotic cell chromosome – particularly the chromosome in the interphase cell which we call "chromatin" – there is a clear need to examine these models, to reassemble ideas, explore, test, and possibly modify our conceptions in an attempt to arrive at some sort of paradigm for gene regulation in higher organisms. The underlying difficulty in arriving at such a basic paradigm is clearly a combination of both the complexity of the problem and the lack of detailed experimental data.

Despite suggestions that the basic mechanisms for gene regulation in eukaryotes may not be as complicated as we fear, when we compare the amount of DNA present in the eukaryotic cells with that of the prokaryote[6] it is apparent that gene expression in higher organisms is a multistage phenomenon. From the complexity of rRNA synthesis[7] and mRNA synthesis,[8-12] to the complexity of the cell's own control of the differentiation process[61] involving little understood gene regulation mechanisms in cell and tissue communication,[13] it is clear that the mere production of an RNA molecule from the template DNA is but the start of a long series of steps before the final function of the gene is attained. This complexity has in the past led us to what are to a large extent artificial, sequential stages in the gene expression process. These stages include the "turning on of

the gene," its transcription into RNA, the little understood "processing" of this RNA in the nucleus and its transport into the cytoplasm, and its metabolism and translation into protein in the cytoplasm followed by the storage, secretion, metabolism, or decay of this protein. The study of many of these stages of gene expression has evolved into complex branches of biochemistry. These stages contain multiple regulatory elements both within themselves and with respect to other stages in the gene expression process. In arriving at a paradigm for gene regulation in eukaryotic cells we should not lose sight of the overall complex process. It must be remembered that all stages in this process are interconnected, and it is possible that there are further connections between different stages that we are unaware of at present. Thus, any model that crosses into the boundaries of numerous stages in the gene expression phenomenon is likely to kill or severely wound a few "sacred cows" in the process. The value of models should be weighed in terms of their ability to explain the many diverse phenomena in the regulation of gene expression in eukaryotes, rather than in terms of their ability to enlarge upon and fortify one small aspect of the whole process of events.

There are presently available a number of excellent up-to-date articles that review the structure and metabolism of chromosomes[14,15] and the four major components of the chromosome: DNA,[16,17] histones,[18-20] nonhistone proteins,[20,21] and RNA.[22] Although we will not attempt to enlarge upon the content of these reviews, it may be valuable at this stage to look at some of the major features of these components.

CHROMATIN DNA

Satellite DNA

The chromosome of the average mammalian cell contains about 9×10^9 base pairs. Fortunately, nature has not presented us with a situation where we have a vast number of sequences within this DNA all distinctly different from each other. Instead we find that there are distinct groups of sequences within the genome of the cell which "stand out," in that they differ considerably from the rest of the DNA of the genome. As we shall see later, their presence has played a fundamental role in proposed models for gene regulation in eukaryotes.

Perhaps the first experimental procedure capable of "picking out" a discrete DNA fraction was CsCl equilibrium density gradient centrifugation, introduced by Meselson, Stahl, and Vinograd[23] in 1957 and used by Kit[24] and Szybalski[25] to isolate a light density component of mouse DNA. Within a year, numerous components of higher or lower density to that of "main band" DNA had been observed in a diverse range of animal species.[27] These fractions became known as "satellite DNA." This field has been extensively reviewed by Walker.[17]

Both analysis of isolated chromatin fractionated into heterochromatin and euchromatin,[27] and in vitro hybridization of labeled satellite DNA (or its RNA) to metaphase chromosomes partially denatured on a microscope slide[28] indicate that most of the satellite DNA is localized in heterochromatin and near the centromere in metaphase chromosomes. Schildkraut and Maio[29] showed that the nucleolus is also considerably enriched with satellite DNA.

The functional significance of satellite DNA has remained problematic since its initial discovery. It could well be one of the key cellular components which would tie together questions about the structure of the chromosome and the regulation of its genes. However, a few of its unique properties make it difficult to assign a function to it at present. With the exception of cistrons coding for rRNA[30] and histone mRNA[31] it has not been possible to detect RNA's synthesized in vivo complementary to satellite DNA.[32] Thus it seems that satellite DNA plays no part in coding for RNA or proteins. As we shall see later when we look at reassociation rates of eukaryotic DNA, satellite DNA is characterized by its ability to reassociate rapidly,[33] and on the basis of the reassociation data it was proposed that satellite DNA is made up of repeated sequences. Several hundred of these repeated sequences may be joined together. Indeed, the sequence can be very simple; a repeating dAT polymer is found in the crab *Cancer borealis*[34] (Figure 1), and more complex sequences also occur.[17] The amount of satellite DNA can vary considerably among different species. The highest reported proportion of satellite DNA was found in the Mongolian horse *Equus przewalskii*, 31% of the total cellular DNA.[35] Can we assign a function for this large amount of DNA? One observation that considerably restricts possible roles we may assign

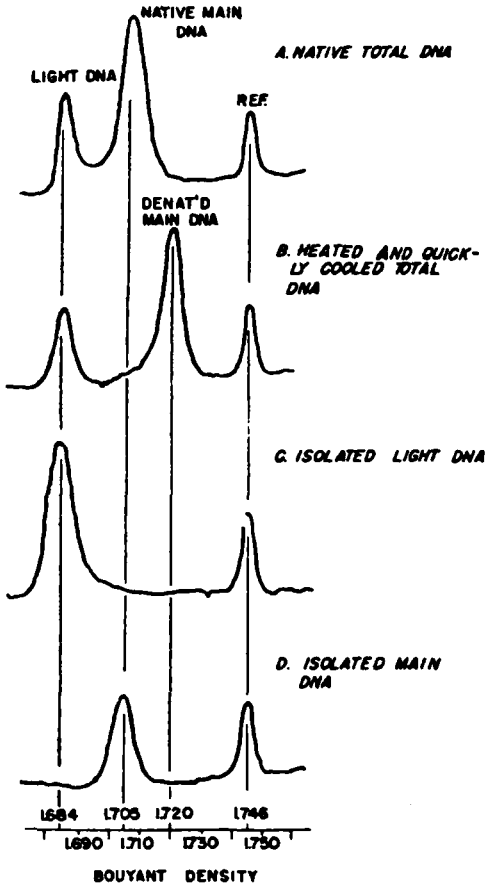

FIGURE 1. Isolation of light (satellite) DNA component from *C. borealis*. N^{15} *Pseudomonas aeruginosa* DNA (ρ = 1.746) is a density reference. A. The original DNA from *C. borealis*. B. The same material, heated and quickly cooled. C. An isolated light DNA component from 0.7 *M* eluate fraction of a chromatogram of heated and quickly cooled DNA. D. An isolated main DNA component prepared by subjecting the original DNA (without heat treatment) to chromatography and by taking an earlier fraction. (Reproduced from Sueoka, N. and Cheng, T., *Proc. Natl. Acad. Sci. U.S.A.*, 48, 1851, 1961. With permission.)

to satellite DNA is that even among very closely related species there can be large differences in the type and content of satellite DNA. This was well illustrated by Hennig, Hennig, and Stein[36] when they showed that the three species of *Drosophila, hydei, neohydei,* and *pseudoneohydei,* have three satellites of different buoyant densities. These species are so closely related that they can interbreed yet there are distinct differences in their satellite DNA's.

Spacer DNA

There is some evidence that satellite DNA could have evolved from the "spacer DNA" between the 18 and 28S ribosomal RNA cistrons.[37,38] It appears that in many species during maturation of the oocytes the ribosomal cistrons along with the spacer regions are amplified.[39,40] At least in the case of *Xenopus,* it appears that, in contrast to the ribosomal cistrons themselves, the spacer DNA regions have little homology between each other for two related species, *Xenopus loevis* and *Xenopus mulleri.*[41] Such DNA regions could thus undergo, during evolution, base substitutions without affecting the ribosomal cistrons. Walker[17,42] suggests that a number of events are involved in the appearance of satellite DNA in a species. He suggests that spacer DNA – and ribosomal spacer DNA appears to be the only candidate available at present – undergoes in evolution a long period of slow multiplication during which some base substitutions can occur, giving rise to new sequences. These sequences were probably originally situated near the centromere. A tandem arrangement of spacer DNA-derived sequences evolved. Suddenly, by chance or otherwise, this whole structure underwent a rapid multiplication. This is suggested as conferring selective advantage to the chromosome itself. There is some evidence that the amount of heterochromatin in the region of the centromere does alter its properties at meiosis.[43-45] Walker then suggested that this satellite DNA was translocated to other parts of the chromosome and to other chromosomes to give the distribution we see today for each species. He proposes that there are also cases where some or all of the satellite DNA was lost when by chance its presence was no longer advantageous to the cell.

Britten and Kohne,[46] on the other hand, speculate that satellite DNA arises as a sudden "soltatory" multiplication of a selected sequence at some stage in evolution. This highly repetitive DNA sequence undergoes, with time, random base substitutions. As the nucleotide sequences diverge they become part of the "intermediate DNA" fraction (we shall discuss this fraction in more detail later) and thus assume a regulatory role, thereby conferring a selective advantage to the cell. Thus, the range of satellite DNA's found in organisms today would represent different stages of this process.

In any event, it would appear from the data

available at present that the satellite DNA fraction of the chromosome is not intimately involved in regulation of specific gene expression. Rather, it seems that the "main band DNA" is the fraction that codes for and contains the receptor elements for the gene regulatory processes within the cell.

DNA Hybridization

Can we subfractionate this main band DNA? Here we must introduce the property of nucleic acid denaturation and renaturation. When DNA is heated above a certain temperature it separates into single complementary strands. This process in known as "melting." The temperature at which 50% of the DNA is melted is known as the Tm. When cooled below this Tm the complementary strands recombine according to a second order reaction.[47,48] The rate of the strand renaturation reaction will depend upon the concentration of DNA in the solution and the time the strands have had to renature. The product of the initial DNA concentration (moles of nucleotides per liter) and the time (in seconds) of the hybridization reaction has been given a special term, the "cot" value. For any given denatured DNA sequence its renaturation will depend upon its cot value. Thus, sequences that are highly repeated (i.e., sequences that occur over and over again) in sheared eukaryotic DNA will renature rapidly, while those that do not occur often, termed "unique sequences," will renature slowly. Other factors of course affect the rates of strand renaturation but this simplified account will suffice for our purpose.

It is possible to separate renatured double stranded DNA from single stranded denatured DNA on a hydroxyapatite column.[49,50] In this way we can examine the repetitive and unique regions of eukaryotic DNA. When this was first done by McCarthy (1967) and Britten and Kohne (1968) it quickly became apparent that there were three distinct renaturing DNA fractions in higher organisms (Figure 2).[46,51] The first is a rapidly renaturing fraction composed of satellite DNA, other highly repetitious sequences (these presumably happen to have the same base composition as main band DNA), and "foldback DNA" – an array of sequences ordered such that a single strand of DNA can fold back upon itself.[52]

Intermediate DNA

The second DNA fraction is the so-called "intermediate DNA."[46,53] This fraction renatures more slowly than satellite-like DNA. It possesses the distinguishing feature that it will hybridize with in vivo-synthesized RNA. In fact, until very

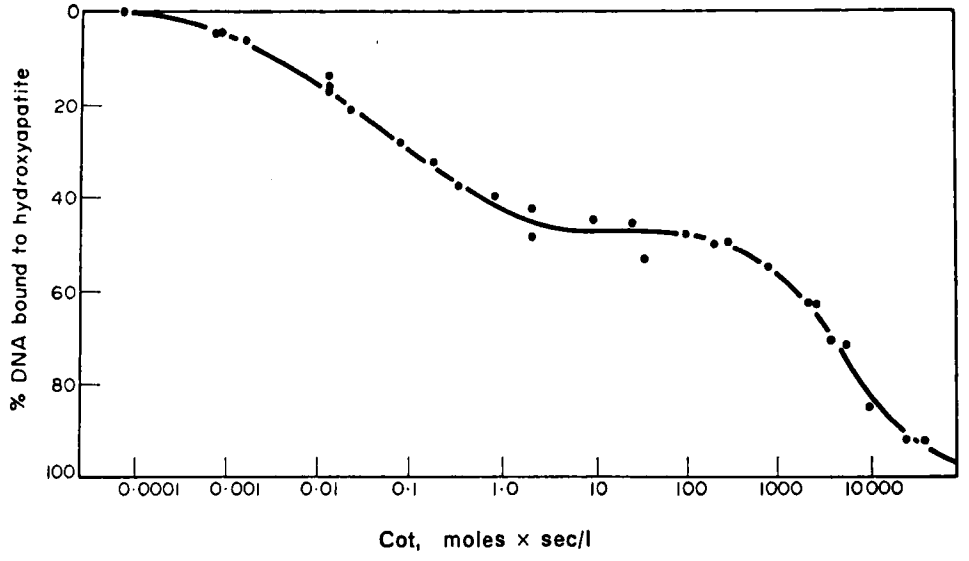

FIGURE 2. The reassociation of calf DNA. This DNA was sheared into pieces approximately 400 nucleotides long, denatured, and annealed at 60°C in 0.12 *M* phosphate buffer, and the proportion of reassociated DNA was assayed by hydroxyapatite fractionation. (Reproduced from Britten, R. J. and Davidson, E. H., *Q. Rev. Biol.*, 46, 111, 1971. With permission.)

recently almost all in vivo-synthesized RNA's from higher organisms that were experimentally hybridized (or competitively hybridized) to DNA originated from this fraction of the cellular DNA. This is simply because the concentrations of RNA and DNA used and the period of time over which the hybridization was carried out were not sufficient to pick up hybrids between RNA species from the third DNA fraction, the "unique DNA fraction."

In calf, for example, the intermediate DNA fraction represents about 37% of the nuclear DNA. Britten et al.[52,54] calculate that there are about 66,000 copies of closely related sequences, each about 17,000 nucleotide pairs long, in this fraction. Also in marked contrast to the satellite DNA, the intermediate DNA appears to be spread throughout the genome. This observation comes from both in vitro hybridization studies[55-57] and direct examination of sheared fragments of DNA.[52,58,59] In *Xenopus*, Davidson et al. found that 50% of the DNA had closely interspersed repetitive and nonrepetitive sequences. The average length of repetitive sequences was estimated to be 300 ± 100 nucleotides, while the adjacent nonrepetitive sequences were estimated to be 800 ± 200 nucleotides long.[54] It should be remembered, however, that renatured intermediate DNA is characterized by a very broad range of thermal stability.[46,53] While the intermediate DNA fraction may contain many copies of a sequence, it is quite likely that there are small differences within the multiple repeats of this sequence. We shall return to this broad range of terminal stability later when we look at models that suggest a function for this DNA.

What can we say about the RNA transcribed from "intermediate DNA"? It appears that the type of RNA transcribed from intermediate DNA changes during tissue development;[60,61] distinct differences can be found when various differentiated cells are examined.[62-64] A very characteristic property of this RNA is that it is largely confined to the cell nucleus where it turns over very rapidly.[65-67] However, some "intermediate transcribed RNA" does appear in the cytoplasm as polysomal RNA.[62,68] There appears to be little evidence at present that a major portion of the intermediate DNA fraction codes for polypeptides. Rather, it has been suggested that the RNA itself is the major active endproduct of these sequences.[61] A regulatory role has been assigned to the intermediate DNA sequences. We shall examine this suggestion at length later.

Unique DNA

The third and final DNA fraction of the chromosome is the so-called "unique DNA" fraction. In this fraction the sequences are nonrepetitious. In vivo-synthesized RNA hybridizes with this fraction; however, its detection requires special procedures due to the low occurrence of each unique sequence within the total DNA preparation. These procedures include removal of repetitious DNA by hydroxyapatite chromatography[69,72] using high RNA concentrations for long time periods[70] or hybridization in DNA excess.[71] For the eukaryotic cells examined to date, it appears that in vivo-synthesized unique RNA hybridizes with about 2 or 3% of the total DNA.[69] Brain tissue seems to be unusual in that its unique RNA sequences hybridize with higher amounts of total DNA, about 9% of the total DNA.[73] Because of their diverse and unique nature it is believed that the "unique" fraction of DNA contains sequences that code for the cell's structural genes. Polyribosomal RNA hybridizes with unique DNA sequences,[74] as do duck[74] and mouse[75] globin mRNA's. However, this need not always be the case; the sea urchin 9S histone mRNA gene seems to be multiple and partially clustered as it bands on the heavy side of the DNA peak in a CsCl gradient.[31] Little is known about the chromosomal distribution, species, and tissue variations of DNA unique sequences. Although a gene regulatory role for the RNA transcribed from unique DNA remains largely unexplored, one possibility is that some of this RNA codes for the nonhistone proteins of chromatin.

These three DNA fractions, the "rapidly renaturing," the "intermediate," and the "unique sequences," while extensively studied, are not the only way chromosomal DNA has been subfractioned. Thomas et al.[59] have found that specific regions of eukaryotic DNA, after shearing and limited exonuclease treatment, followed by an annealing treatment, can form circular structures (Figure 3). These ring structures suggest tandem repetitious sequences in the genome. Their distribution is not restricted to specific regions of a CsCl density gradient.[59] They are not, for example, specifically localized in satellite or ribosomal DNA sequences. More recently, Bick et al.[76] suggested that the regions of DNA called

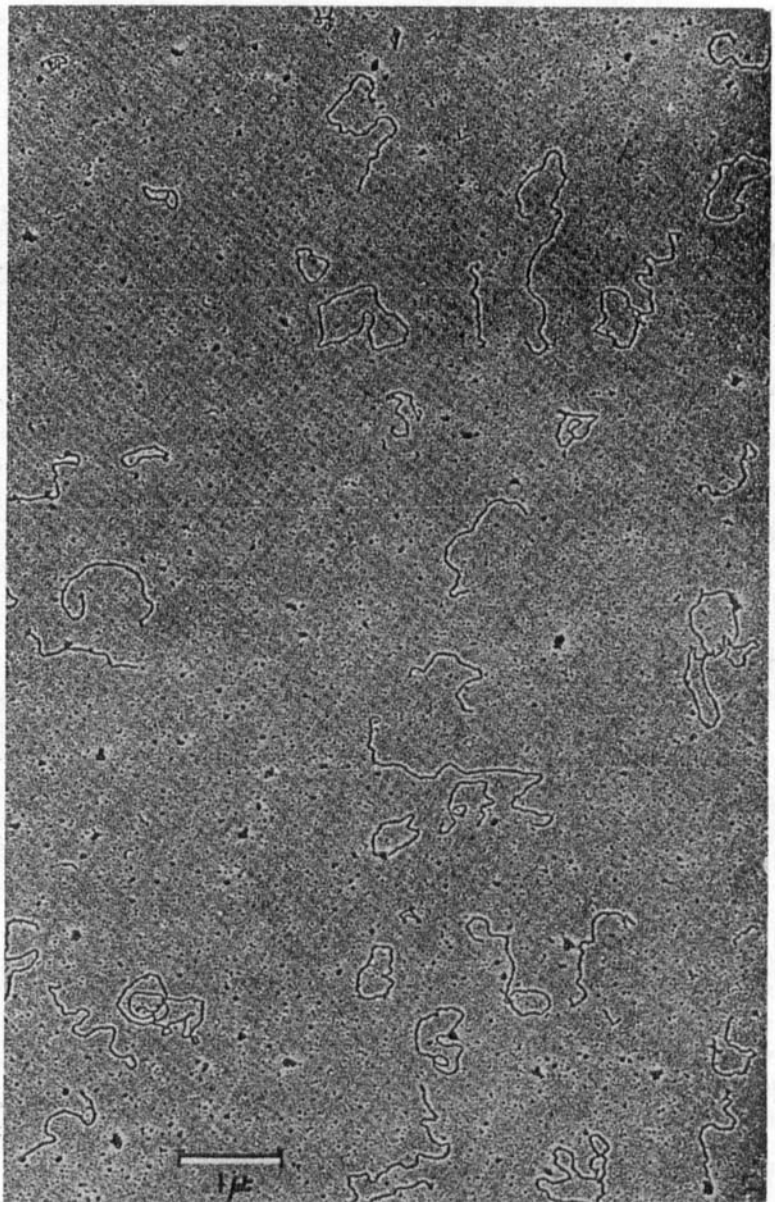

FIGURE 3. An electronmicrograph of *Necturus* DNA fragments showing circular structures formed after exonuclease treatment, followed by annealing for 2 hr at 60°C in 2 x SSC. (Reproduced from Thomas, C. A., Jr., Hamkalo, B. A., Misra, D. N., and Lee, C. S., *J. Mol. Biol.*, 51, 621, 1970. With permission.)

"g-regions," capable of forming the rings, contain tandem repeated sequences. The average length of a g-region in *Drosophila* is about 5 μm. It appears that, at least for this organism, as much as 50% of the chromosomal DNA resides in g-regions. There appears to be no nonrepetitious DNA between the repeated sequences within a g-region. Bick et al. suggest that there may be one g-region for each chromomere of the chromosome.

There is clearly some difficulty in correlating the observations of Bick et al. with that of Davidson et al.,[54] since they are unable to observe such a large fraction of the total DNA-containing repetitive sequences not closely interspersed with nonrepetitive sequences.

Because of its unique physical and chemical properties the nucleolus can be readily separated from the rest of the genome.[7] For this reason it

has been possible to isolate nucleolar DNA free of extranucleolar DNA. However, it now appears that even for such a specific DNA fraction further heterogeneity seems to exist. Amalric et al.[77] have recently shown the presence of two DNA components, a single strand and a double strand DNA fraction. Crick[78] has proposed that "gene recognition sites" for gene regulation at the transcriptional level in eukaryotes should contain such single stranded DNA regions. We shall return to this idea later when we look at specific gene regulatory models.

The subfractions of DNA presented so far have been largely "man made," in the sense that the cell does not in itself fractionate the DNA into these separate entities, at least in the physical sense. Indeed, we must stress the importance of clearly distinguishing between such terms that mean a physical classification and those that imply a function. There are few functional terms for eukaryotic DNA. One exception to this is in the case of the DNA of the ciliated protozoa *Stylonychia*. During the development of a "macronucleus" from a "micronucleus" in this organism the DNA undergoes a series of complex alterations, the final result being the loss of 93% of the original DNA in the micronucleus.[79] The remaining 7% is amplified approximately 600-fold in the form of small pieces, most of which are about 0.75 μm long. When we come to look at the requirements for a functional gene in eukaryotes we will see that *Stylonychia* sp. must come very near to the absolute minimum. It has been suggested (and is very conceivable) that the 0.75-μm fragments are individual genes. Interestingly, each "gene" has an AT-rich region at one end of the 0.75-μm fragments. A similar AT-rich region has been suggested for the prokaryote *lac* gene of *E. coli*.[4] *B. subtilis* RNA polymerase preferentially binds to one end of the fragments.

Since some strains of *Stylonychia* can survive for many cell generations with only these fragments of DNA, it would appear that 93% of the cellular DNA is not essential for many of the cellular functions in this organism.[79] These organisms without the "extra" 93% of normal DNA do die eventually; however, it remains to be seen what the role of the macronuclear DNA is. Could it have a gene regulatory role, perhaps long-term gene regulation controlling gene expression for the future cell generations? In any event, we must take the precaution of remembering that here we are discussing a simple unicellular organism. It remains to be seen if gene regulation in such creatures is a stepping stone from *E. coli* to mammals, or only a side track.

THE HISTONES

An extensive coverage of histone chemistry, their isolation, characterization, and metabolism is given in a number of recent reviews.[18-20] We shall briefly mention only a few pertinent facts

The histones are a group of basic proteins of relatively low molecular weight (10,000 to 25,000), of apparently limited heterogeneity, found in chromosomes of all eukaryotic cells (Figure 4). For most organisms five distinct subclasses can be seen. These subclasses have two nomenclature systems: F1, F2a2, F2b, F3, and F2a1 of Johns and Butler[80] and I, IIa, IIb, III, and IV of Rasmussen, Murry, and Luck.[81] We shall adopt the former nomenclature in this review. Other subclasses of histones have been found in highly specialized cells.[82-84] In many spermatozoa, histones are replaced by low molecular weight (~ 6,000) arginine-rich proteins known as protamines.[85] The five subclasses of histones appear to be quite distinct from each other both in terms of structure and function. The following is a summary of their most apparent properties.

F1 Histones

This fraction is often called the "lysine-rich histone fraction," and is characterized by a high lysine and alanine content (for calf thymus chromatin, 28 and 24 mol %, respectively[86]). The F1 histones tend to have a higher molecular weight than that of other histone subclasses (21,000 for calf thymus histone F1[87]). This fraction possesses a useful property: it can be selectively removed from chromatin with low concentrations of ionic dissociating agents such as sodium chloride (0.6 *M*),[88] or with 0.5 *M* sodium phosphate, and 1 *M* urea (pH 5.5).[89] Removal of the F1 histones from chromatin brings about a distinct alteration in its structure,[14,18,19] and makes chromatin soluble at any ionic strength.

Smart and Bonner[91] suggest that F1 histones are deposited on the DNA or on the outside of a "sheath" of DNA and the other histone fractions such that they are exposed to the environment.

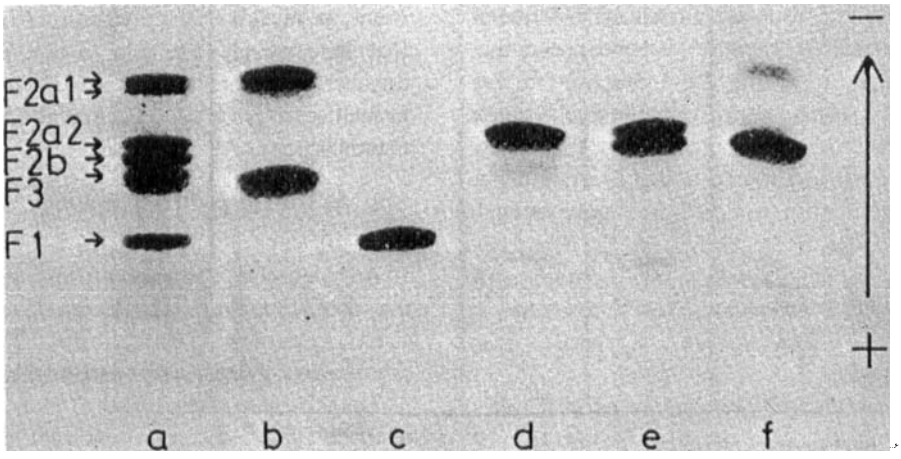

FIGURE 4. Polyacrylamide gel electrophoresis of the products of calf histones. (a) Total histones, (b) F2a1 and F3, (c) F1, (d) F2a2, (e) F2a2 and F2b, (f) F2b. (Reproduced from Van Der Westhuyzen, D. R. and Von Holt, C., *FEBS Lett.*, 14, 333, 1971. With permission.)

This may be why they are very susceptible to proteolytic degradation[92] and reaction with formaldehyde[93] in chromatin preparation. From a study of the melting profiles of chromatin with and without F1 histone fraction, Ohlenbusch et al.[88] concluded that F1 histones are not arranged consecutively over long stretches of DNA. Proton magnetic resonance studies[94,95] suggest that the calf thymus F1 histone remains complexed to chromatin via the lysine residues at the N and C terminal ends. Bradbury et al.[94] suggest that at physiological ionic strengths the central portion of the molecule is free to interact with other F1 histones or possibly other proteins in chromatin.

In contrast to other histone fractions the F1 fraction displays some degree of heterogeneity and tissue specificity.[97,98] The tissue specificity is probably a result of differential phosphorylation[99] rather than the presence of different amino acid sequences. *Drosophila* F1 histone appears to have a higher molecular weight than that of mammals and birds.[100] Structural studies with purified rabbit thymus F1 histone subfractions indicate an asymmetric distribution of basic and acidic amino acids. The C terminal is highly basic and invariant in structure, while the N terminal amino acids are less basic and vary from one subfraction to another.[101,102] There may be a very specific interaction in the binding of the C terminal ends of F1 histones to DNA, one so specific that no amino acid changes in this part of the molecule can be tolerated without drastically altering the binding of the molecules to the DNA.

F2a2 and F2b Histones

These are collectively also known as the "slightly lysine-rich histones," and contain less lysine and alanine but more arginine than F1 histones. Except for differential phosphorylation they appear to be a homogeneous group of proteins.[19,104] Low concentrations of sodium deoxycholate have recently been used to selectively dissociate these two fractions from chromatin.[105] The selectivity is, however, not as good as sodium chloride for F1 histones, because some of the other histones are also removed. Again, melting profiles of this partially dehistonized chromatin suggest the absence of long stretches of F2a2 and F2b histones bound in a tandem manner to DNA.[105] However, the limits of the resolution of this method are unclear for such a complex structure as chromatin. The N terminal region of calf thymus F2b histone is basic, being especially rich in lysine.[105] The C terminal is also basic, being poor in lysine but rich in arginine. The middle of the molecule is nonbasic. Proton magnetic resonance studies suggest the N terminal region of calf thymus histone F2b binds to DNA while the C terminal region remains free.[103] One should remember, however, that these studies may not simulate the situation in vivo where the histone/DNA complex is only a part of a complex structure containing many other components.

F3 Histones

These "arginine-rich histones" are characterized

by a high content of glutamic acid and arginine and a low lysine content. They are also unique in that the F3 fraction is the only histone fraction that contains sulfhydryl groups. The presence of cysteine in F3 histone preparations may account for the early heterogeneity seen in this fraction.[107] The F3 histones have the ability to form dimers and polymers by the formation of intermolecular disulfides.[20,108,109] This oxidation of cysteine also appears to have a biological role. In metaphase chromosomes the F3 histone of HeLa cells is polymerized or complexed with other proteins.[109] In interphase chromatin the F3 histone contains mainly cysteine and is almost completely in the monomeric state. Highly purified chicken erythrocyte F3 histone shows little heterogeneity except for a partial acylation of one lysine residue.[110] Calf thymus and pea embryo F3 histones appear to be quite similar.[111] DeLange et al.[112] found distinct sequence differences between calf thymus F3 and F2a1 histones.

F2a1 Histones

A second type of "arginine-rich histone," these are characterized by a high arginine and glycine content. The fraction appears to be a single component although tissue-specific degrees of phosphorylation and acylation of lysyl residues have been well demonstrated.[113-115] The F2a1 histone is perhaps the best illustration of the extremely conservative nature of histones during evolution. Only two relatively minor amino acid differences are found in pea and calf F2a1 histones. This conservation suggests a very specific function for the entire F2a1 molecule, perhaps binding to DNA other protein fractions or RNA. There is some evidence for a clustered arrangement of F3 and F2a1 histones in chromatin.[116] F2a1 histone seems to be involved in maintaining the supercoiled structure of DNA in chromatin.[103,201]

The Function of Histones in Vivo

Stedman and Stedman[117] first proposed that the function of the histones was "to act as gene suppressors." This suggestion required remarkable foresight on their behalf at that time, since the mechanism of gene suppression was not known then. It was not until 12 years later that Huang and Bonner presented the first experimental data to support this hypothesis.[118] They showed that histones reduced the template activity of the pea embryo chromatin when assayed with pea embryo RNA polymerase. Removal of the histones with salt solution led to a drastic increase in the template activity of the dehistonized chromatin. This was later shown to be a general phenomenon for all eukaryotic cells. This restricted template activity of chromatin was seen with eukaryotic and prokaryotic exogenous polymerases, or with the endogenous polymerase present in chromatin preparations.[18] It is still not clear to what extent each histone fraction is responsible for restricting the template activity of chromatin in vivo. Georgiev[18] has reviewed this question in some detail. He points out that removal of F1 histone from chromatin does not lead to an increase in the hybridizability of the RNA product to DNA when the endogenous RNA polymerase present in chromatin is used. This is in contrast to the situation wherein exogenous RNA polymerase is added to the chromatin. In this situation, removal of F1 histone leads to longer RNA products and a greater hybridizability of the RNA to the DNA.[18,119] He suggests that the F1 histones are on or near the initiation sites for the polymerase molecule in vivo. Presumably the F1 histones are also bound to other regions of the DNA to account for chromatin's increased template activity when assayed with exogenous polymerase.

Spelsberg and Hnilica[120] in a stepwise manner removed F1, F1 and F2b, then all the histones from rat thymus chromatin and concluded that the arginine-rich F2a1 and F3 histones were the two fractions most responsible for the histone restriction of chromatin template activity. Smart and Bonner,[91] on the other hand, by using two different techniques to extract histones in different stepwise orders concluded that the template activity of the pea bud chromatin was a function of the amount of histone removed rather than the type.

It is apparent that even for such relatively uncomplex chromatin components as the histones we are a long way from assigning with certainty a specific role for them in the gene regulatory process. Perhaps they have a structural role which indirectly gives rise to a regulatory role.

There are indications that F1 histones are preferentially bound to repeated DNA sequences in vivo.[18,123] The alteration in structure seen when F1 histones are removed from DNA particles[18,121,123] or chromatin fib-

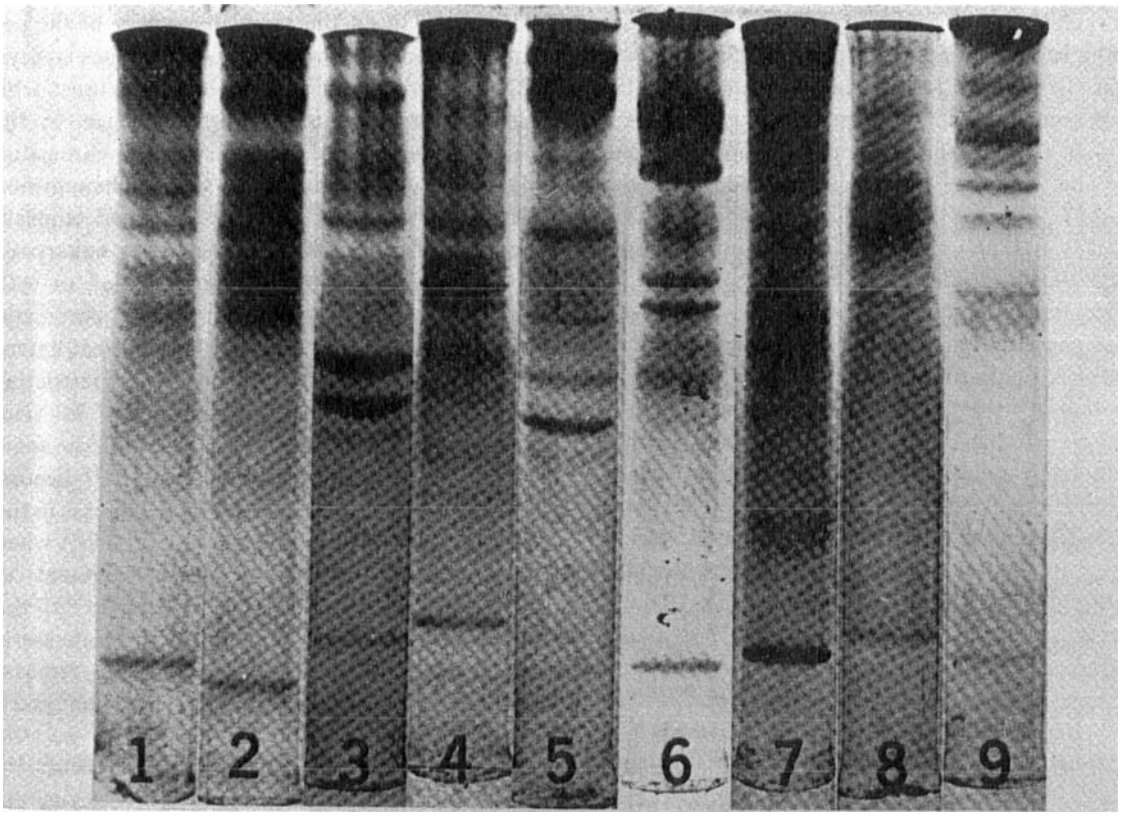

FIGURE 5. Electrophoretic comparison of the nonhistone chromosomal proteins obtained from various tissues. Samples of approximately 200 μg were run in 10% anionic polyacrylamide gel systems at 4 mA/gel column for 1 hr. 1. Mouse liver. 2. Mouse ascites. 3. Ehrlich ascites. 4. Mouse sarcoma. 5. Transplanted mouse sarcoma. 6. Chicken erythrocytes. 7. Rat liver. 8. Old rat liver. 9. Calf liver. (Reproduced from Kostraba, N. C. and Wang, T. Y., *Int. J. Biochem.*, 1, 327, 1970. With permission.)

rils[18,122,123] may be an unfolding of a compact chromatin structure linked together via F1 histones situated strategically at repeated sequences along the DNA. Crick[78] has suggested the presence of "globular control DNA" and "fibrous coding DNA" regions in the eukaryotic chromosome. While we shall look at this model in more detail later, we will mention the possibility that such homogeneous chromatin components as histones may be all that is required to segregate the genome into these two elements.

Sutton[124] more recently goes further: for monotene chromosomes he proposes that single copy genes are interspersed in a matrix of repetitious DNA and histone complexes. Because the charge on the DNA is neutralized by the histones the chromosome will be capable of folding into a tight "crystalline" structure. Many single copy genes will be entrapped in this structure and repressed, being unavailable to the RNA polymerase. We shall return to this model again later. At this stage we should keep in mind the fact that while there are a limited number of histone components in chromatin it is very conceivable that they are intimately involved in the gene regulatory process in that they may be the tools by which the regulation is performed.

CHROMATIN NONHISTONE PROTEINS (NHP)

In marked contrast to the well-characterized histones of limited apparent heterogeneity is the NHP fraction, still poorly characterized and of considerable apparent heterogeneity (Figure 5). Because of the diversity of the methods used to prepare this chromatin fraction, the NHP's represent different proteins to different investigators. In this review, by NHP we mean all proteins, except the histones, present in chromatin

that has been isolated by use of the more classical procedures.[121,125-127] A number of extensive reviews of methods used to isolate this fraction are available.[14,15,20,21] The nuclear phosphoprotein fraction described by Langan[128] is probably an element of the total NHP fraction. The "nuclear acidic proteins"[129,130] presumably contain the NHP fraction as a component. Less clear is the extent to which the nuclear cysteine-containing proteins modified with *N*-ethylmalamide and extracted with urea[131,132] represent the NHP fraction. Because of the complexity of the NHP fraction a direct comparison of proteins isolated by different techniques is difficult to interpret.

One of the earliest studies on the isolation and fractionation of NHP's was carried out by Patel and Wang.[133] With rat liver nuclei washed free of nucleoplasmic "soluble proteins" the chromatin was solubilized with 1 *M* NaCl. Dilution of the salt concentration down to 0.14 *M* leads to a DNA-histone precipitate. Many of the NHP's are not precipitated and can be collected by centrifugation. Starch gel electrophoresis and later polyacrylamide gel electrophoresis[134] showed a heterogeneous group of proteins in this NHP preparation.

Marushige et al.[135] used a different approach: after removing the histones from chromatin with HCl, the NHP was dissociated from the DNA with 1% sodium dodecyl sulfate (SDS). The DNA was removed by high speed centrifugation, leaving the NHP in the supernatant. Polyacrylamide gel electrophoresis[136] of such an NHP preparation displays a limited number of major protein components (often less than 15). Shelton and Allfrey[137] used phenol to extract "nuclear acidic proteins" from acid washed nuclei. A wide spectrum of proteins was seen in this extract with polyacrylamide gel electrophoresis. MacGillivray et al.[138] used urea to dissociate the chromatin components and chromatography on hydroxyapatite to obtain a pure NHP preparation.

The above four procedures should help to illustrate the diversity of the methods used to isolate the chromatin NHP's. Therefore, it is not surprising that different "protein profiles" are obtained with each method. The way the nuclei are isolated and purified can alter the final profile.[138] Acid extraction of nuclei extracts more than histones,[139,140] probably a number of NHP's as well Chromatin is associated with protease activity.[141] In many cases it is not clear to what extent the nucleolar proteins are contributing to the NHP fraction.[129,131,142] In a number of cases it is clear that not all NHP's are isolated; they either remain bound to the DNA,[143] to columns,[138] or aggregate on the surface of acrylamide gels.[134]

In spite of these difficulties, however, a number of interesting and general phenomena are associated with NHP's, almost irrespectively of their means of isolation. They are often called chromatin acidic proteins because of their high content of glutamic and aspartic acids and relatively lower basic amino acid content. The total fraction displays a wide spectrum of molecular weights (5,000 to 100,000).[134,136,137] At physiological pH values they have a marked tendency to aggregate and precipitate out of solution,[127,131] and they readily form insoluble precipitates with histones.[127] A number of well-defined enzymatic activities can be found in the NHP fraction, including RNA polymerase,[144] DNA polymerase,[145] and DNase activities,[146] polynucleotide ligase,[147] histone methylase[148] and deacetylase,[149] and NHP kinase activities,[150] as well as more diverse enzymes such as glutamate dehydrogenase, lactate dehydrogenase, ATPase, and malate dehydrogenase.[127] From the presence of these enzymes alone, it is not surprising that the NHP profile on acrylamide gels displays a heterogeneous profile. Indeed, the major bands of stained proteins seen with high resolution one-[136] or two-dimensional[132] SDS polyacrylamide gel electrophoresis may well be these proteins as well as "structural proteins" of the chromosome itself.

Gene-specific Regulation by Nonhistone Proteins

The major factors that led to increased interest in the NHP's stem not from their enzymatic activities, but from the fact that they appear to display some of the properties a gene-specific regulatory component should have. Much of the experimental data are in agreement with the concept that underneath the pattern of major NHP bands seen as described above is a "background" of an extremely heterogeneous mixture of proteins that play a major role in deciding which regions of the DNA will be transcribed into proteins and which will be repressed. Space allows us to only briefly review the support for this idea. One complication is the fact that the NHP fraction is not a group of specific regulatory proteins and

nothing else. The enzymes and possibly "chromatin structural proteins" in the NHP fraction probably contribute significantly to "patterns" seen on polyacrylamide gels. For this reason, claims that such "patterns" are species or time specific,[132,134,139] that the content in chromosomes of differing RNA synthesizing ability is different,[154] or, conversely, that such patterns are not species or time specific[136,138] should be taken with the understanding that we may be looking at some enzyme components in chromatin or some type of chromatin "structural proteins." For example, the NHP polyacrylamide gel electrophoresis patterns of rat liver nucleolar and extranucleolar chromatin are quite similar.[174] It may be that the gene regulatory proteins are too diverse and in too low a concentration to be detected. Certainly in prokaryotes this appears to be the case. Only a few *lac* repressor molecules are present in the wild type *E. coli* cell.[1-4] For eukaryotes in the few cases that have been examined, the number of nuclear or chromosomal receptor sites available per cell to bind gene-specific effector molecules often appears to be less than a few thousand.[151-153] Even the tissue-specific selective synthesis of some NHP fractions after cell stimulation by hormones[129,137,155,156] must be taken with caution, for such changes may reflect nothing more than a change in the amount of chromosomal enzyme or a structural protein in chromatin, having little to do with the regulation of transcription of a specific gene or group of genes.

The detection of a small (0.01%) group of proteins in the NHP fraction that can bind in a species-specific manner to DNA[157,158] is probably a more convincing piece of evidence for proteins with a gene-specific regulatory role – though these could be simply factors analogous to prokaryotic RNA polymerase σ factor.[159] Perhaps the two most convincing types of evidence for a gene-specific regulatory role in the NHP fraction are

1. The ability of the NHP's to modulate the template activity of "reconstructed" chromatin.
2. Their tissue-specific response to hormones in "target cells" followed by alterations in the template activity of chromatin.

Let us look at each of these properties briefly.

Reconstructed Chromatin and Nonhistone Proteins

Competitive hybridization studies[160] have shown that chromatin as it is isolated from the cell has a limited number of DNA regions available to prokaryotic RNA polymerase, and that the RNA transcribed from these regions is identical to the RNA transcribed in vivo. We shall overlook for the moment the facts that the type of polymerase used can alter the amount of RNA transcribed from each site,[161] and that the assay conditions allow us to only look at highly repeated and intermediate RNA fractions.[162] With these limitations in mind we can say that the isolated chromatin behaves exactly as it did in the cell, at least with respect to its template activity. There appears, therefore, to be no "sliding" of chromosomal proteins along the DNA backbone during its isolation. In spite of the complexity of chromatin, it was found that it was possible to dissociate most of the chromatin components in a solution of 5 *M* urea, 2 *M* sodium chloride and recombine them to give a native-like material by dialyzing away first the salt, then the urea.[163-166] The reconstructed chromatin displayed the same appearance under the electronmicroscope[189] and the same template properties as chromatin in vivo.

In all such chromatin reconstruction experiments, it became quite clear that the histones were necessary for the quantitative restriction of DNA in chromatin, and that they played no role in the specificity of the restriction. Using "artificially" constructed chromatins it was found, for example, that the specificity of RNA synthesized in vitro could be changed from a pattern characteristic of rat liver to that of rat thymus and vice versa by exchanging the NHP fractions (together with some chromatin RNA components).[166] In many of these types of experiments it appears that there is always a small content of RNA in the NHP fraction. In some cases it is not clear if the sequence-specific interactions in the reconstruction process are due to the NHP's themselves[165,166] or to the RNA in this fraction.[163,164] This point does not appear to be adequately resolved at present. There is some indirect evidence that the NHP's themselves possess all the needed specificity to bring about the correct template activity to reconstructed chromatin.[157,158]

There is nothing unique in the NHP's ability to alter the histone restriction of the DNA's template

activity per se; for example, bovine serum albumin can do this.[167] What is unique is that the template restriction is controlled in a sequence- (and tissue-) specific manner. We have seen that at least some NHP's can recognize particular DNA sequences. Kamiyama and Wang[168] showed that an NHP fraction from rat liver chromatin containing no RNA components (the RNA and other NHP's were not eluted from the DEAE cellulose column with the conditions used[133,169]) was able to increase the template activity of rat liver chromatin ("activated chromatin") above that of the control chromatin containing presumably its normal complement of NHP's. RNA transcribed from the activated chromatin appears to have additional sequences not transcribed in the control chromatin.[168] It is not immediately clear why a homologous NHP fraction should expose new DNA sequences on chromatin containing these NHP's. Some type of nonspecific polyionic effect, like that of polystyrene sulfonate[170] or polyethylene sulfonate,[171] could be taking place. However, further work in this series[172] showed that the additional sequences transcribed from rat liver chromatin activated with rat liver NHP's differed from the additional sequences transcribed from rat liver chromatin activated with Walker 256 carcinosarcoma NHP's. This suggests that the NHP's are capable of differentially augmenting the transcription of RNA in vivo from chromatin. The NHP fraction used in these studies contained 1.5 to 2.0% RNA. Kostraba and Wang[172] went on to show that this RNA fraction inhibits the template activity of chromatin. However, chromatin has been shown to contain a number of RNA components,[169,203,222,223,227,229] some of which inhibit, others that increase the template activity of chromatin. It is not clear which components are present in their NHP fraction. The suggestion that NHP's activate the template activity of chromatin is in agreement with the many observations that there are greater amounts of NHP's in tissues that are, or become, active in RNA transcription.[126,130,154,155,173,175]

Shea and Kleinsmith[176] have demonstrated that phosphorylated rat liver NHP's can stimulate RNA synthesis from rat DNA using rat liver RNA polymerase. This stimulation was not observed with salmon, calf, or *E. coli* DNA. Partial removal of phosphorus from the NHP fraction with alkaline phosphatase abolishes this ability to stimulate RNA synthesis in this system. Unfortunately, the source or purity of the alkaline phosphatase was not given. Hydrolysis of the nucleic acid component present in this fraction[178] remains a distant possibility as the active component, since at least some batches of enzyme contain RNase activity.[177]

Nonhistone Proteins Involved in Hormonal Receptors

The tissue-specific response to hormones in "target cells" is often followed by complex changes in the metabolism of these cells;[179,181-184,190] in many cases alterations in the rate and type of RNA transcribed from chromatin in vivo occur.[185-188] Alterations in chromatin specificity are expected in many of these situations. There is some evidence that the NHP's are intimately involved in some of these processes. Space allows us only to illustrate one well-characterized system.

The progesterone-induced synthesis of a specific protein, avidin, in estrogen-prestimulated chick oviducts is a good example of a tissue-specific involvement of the chromatin NHP's in gene regulation induced by a hormone. This system has been recently reviewed,[179,184,190,191] so we shall mention a few relevant points. points.

O'Malley et al.[192] demonstrated that with diethylstilbestrol-pretreated chick oviduct tissue it was possible to isolate a radioactive progesterone-protein receptor complex from the cytoplasm of the cells. Exposure of the tissue to labeled progesterone of 0°C resulted in the appearance of the labeled complex almost exclusively in the cytosol. When the tissue was then warmed to 37°C, the presence of a labeled hormone-protein complex in the nucleus became apparent. Both the nuclear and cytosol receptor complexes have been characterized on sucrose gradients[193] and by their elution from agarose columns.[194]

Both complexes sediment at about 4S in 5 to 20% sucrose gradients containing 0.3 *M* KCl.[193] The steroid binding to the cytosol receptor is specific for progesterone and other progestational compounds. In general, no binding is seen with aldosterone, cortisol, estrogens, or androgens;[190,193,195] testosterone, however, appears to exhibit some binding.[194] There is an absolute requirement for oviduct cytosol to obtain nuclear binding. Incubation of oviduct nuclei with tritiated progesterone does not yield an extractable nuclear

progesterone-receptor complex unless oviduct cytosol is present.[196] Lung or liver nuclei are unable to take up tritiated progesterone as a 4S protein complex from oviduct cytosol.[196] This and the fact that with progesterone stimulation of oviduct tissue there is a progressive decrease in the cytosol receptor content as the nuclear 4S progesterone-protein complex increases[196] strongly suggest a two step mechanism involving first the initial binding of the hormone to a protein in the cytoplasm. This complex then moves into the nucleus and binds to a second receptor there.

The nuclear receptor has been shown to be present in chromatin of chick oviduct tissue.[197] Much less binding of the progesterone-receptor complex to spleen or erythrocyte chromatin was seen. O'Malley et al.[198] demonstrated that selective removal of the histones from chick oviduct and their replacement with histones from other tissues in reconstructed "hybrid" chromatins did not alter the binding of the cytosol progesterone-receptor complex to oviduct chromatin. If both the histones and 90% of the NHP's were dissociated from oviduct DNA, and these were replaced in "hybrid" reconstructed chromatin with histones and NHP's from other tissues, these workers found that the resultant "hybrids" could only bind a little of the cytosol progesterone-receptor complex. It thus appeared that the chromatin acceptor for the cytosol progesterone-receptor complex was contained in the NHP fraction.

It is interesting to note that chromatin RNA components are not involved in the specific binding of the cytosol steroid-receptor complex to chromatin, since treatment of dissociated chromatin with RNase before reconstruction does not appear to affect the chromatin acceptor ability. This, of course, only tells us about the ability of chromatin to "accept" a cytoplasmic "signal." Clearly, much remains to be done to see how this "signal" bound to the NHP fraction brings about the production of avidin mRNA in these cells.[185]

While we have discussed the progesterone stimulation of the avidin system in some small depth, we should point out that for a number of other hormones a similar type of picture is beginning to emerge. Well-characterized cytosol and nuclear receptors have been found for estrogens, androgens, cortisol, aldosterone, dexamethosone, possibly 25-hydroxycholecalciferol, and ecdysone.[179, 199,200] In many cases it appears that the NHP fraction of chromatin is the primary site to which these hormones bind in the nucleus. For this reason it is highly probable that at least a distinct fraction of the NHP's is intimately involved in the regulation of eukaryotic gene transcription. We should also take care not to restrict our thinking of chromatin responding as such to only the above "classical" hormones. It is quite possible that similar systems will be found not only for other hormones but also for metabolites within the body, within tissues, and within the cell itself.

CHROMATIN RNA COMPONENTS

Despite the fact that almost all chromatin preparations contain a distinct RNA fraction,[125-127,202] this RNA has, with the possible exception of a protein-bound "chromosomal RNA" component,[203] remained largely unstudied. While the content of RNA in chromatin preparations is small in comparison with that of DNA, histones, and NHP's, we should not on this basis dismiss it as being incapable of having any gene regulatory functions in the cell. Indeed, from what we have seen of the NHP fraction it appears that any gene-specific regulatory role this fraction has may also be represented by a component that itself is only a small part of the total mass of the chromosome.

There are additional difficulties in isolating chromatin RNA components from chromatin that are not seen with the histones or NHP's. There is almost a 500-fold excess of DNA over RNA in chromatin. In many cases it is difficult to separate the two nucleic acids completely.[169] The presence of nucleolar RNA components[204] is often an unexplored source of chromatin RNA. Metaphase chromosomes are known to bind ribosomes.[205] In any nonsynchronized population of cells there will be a few cells at this part of the cell cycle. It is possible that these metaphase chromosomes become part of the chromatin preparation together with the attached ribosomes. Contaminating nuclear membranes have been suggested as being a major source of chromatin RNA.[206] Even nuclear ribonucleoprotein particles could lead to some RNA contribution to the total chromatin RNA fraction,[207] particularly since a distinct RNase activity has been shown to be associated with chromatin.[208]

However, when all of these difficulties are taken into account, chromatin preparations made by a number of diverse procedures, many of which

eliminate the above difficulties, yield a reproducible RNA content in the final chromatin preparation.

Let us now briefly look at the properties of the chromatin RNA fraction.

Chromosomal RNA

In 1965, Bonner and Huang,[203] in their studies of the pea bud histones that had been dissociated from chromatin by salt rather than acid, observed that such histones did not bind in a 2.09 *M* CsCl isopycnic gradient characteristic of that of free protein. They attributed this to a protein-bound RNA complex which they suggested attached to histone aggregates by ionic and hydrogen bonds. The protein portion of the complex had an amino acid composition similar to that of an NHP.[203] The base composition of the RNA was unusual in that it contained a very high content of dihydrouridylic acid (27.5%).[203] Since then this RNA fraction has been isolated from a number of tissues,[22,163,164,209-214] and methods used for its isolation have been improved. A more accurate estimation of its size (about 3.2S) and base composition (about 7 to 10% dihydropyrimidine) has been obtained for some tissues.[215,216] These workers assigned a special name to this RNA, "chromosomal RNA" (cRNA). They demonstrated that cRNA had some unique properties. It hybridized to DNA to a much larger extent than any other well-characterized RNA.[210,213,216] Rat cRNA hybridizes to about 4 to 9% of rat DNA. Pea bud cRNA hybridizes to the intermediate DNA fraction.[210] At least ascites cRNA has been shown to contain some methylated bases, though these have not been characterized.[213] There are indications that the cRNA fraction contains a very heterogenous population of molecules.[211] Tissue-specific differences in rat cRNA can be demonstrated[209] as well as an increase (above normal) in the amount of cRNA in regenerating rat liver chromatin.[217]

Much of the interest in cRNA centers around the observations by Huang and Huang[164] and Bekhor et al.[163] that a chromatin RNA fraction (their experiments do not point specifically to cRNA) is necessary to bring about a tissue-specific reconstruction of chromatin that has had its protein components dissociated from the DNA with salt and urea. We have seen when we briefly looked at the chromatin NHP's that in such chromatin reconstruction experiments the NHP's were necessary to ensure that the correct regions of DNA were exposed to RNA polymerase, while the remainder of the DNA was unavailable. There are indications that it is not the NHP's per se that bring about this sequence-specific reconstruction of chromatin but the chromatin RNA contained in the NHP fraction. Huang and Huang[164] and Bekhor et al.[163] observed that if the dissociated chromatin components were treated with RNase or $ZnNO_3$ under conditions suitable to hydrolyze RNA, the chromatin protein components were unable to reattach to the DNA in a tissue-specific manner. While the histones and NHP's reattached to the DNA, the regions of DNA available to RNA polymerase were not the same as those available originally. For this reason it was suggested that cRNA has a fundamental role in specific gene expression in eukaryotic cells. This work does imply an important role for the chromatin RNA fraction; however, we should bear in mind that there are a number of distinct RNA fractions present in chromatin preparations most of which are not protein bound.[169,222,223,227-229] It is not clear at present if all or some of these components are involved in the above sequence-specific reconstruction of chromatin.

Unfortunately, other workers have experienced some difficulties in the isolation[218,219] and characterization of cRNA.[220-222] An explanation for at least some of these difficulties has been given by Holmes et al.[216] The mode of the sequence-specific interaction of cRNA with DNA is unclear. Salt solutions like CsCl should not dissociate cRNA hybridized to DNA, nor should cRNA be able to hybridize back to DNA as a DNA/RNA hybrid under the chromatin renaturation conditions used.[224] Possibly the presence of the high content of dihydropyrimidine, a triple stranded hybrid,[225] or the action of some of the NHP's that "melt" segments of the DNA like "32 protein" from bacteriophage T4[226] are responsible for ensuring a sequence-specific interaction with the DNA.

Arnold and Young[223] isolated a cRNA-like RNA from rat liver. It had many of the properties described above except that dihydropyrimidines were not present. Getz and Saunders[334] found that human leukocyte cRNA can hybridize with only about 0.4% of human DNA.

Other Chromatin RNA Components

The structure and function of other chromatin

RNA components is even less well understood.

A wide range of nonprotein-bound RNA components has been isolated from chromatin in a wide variety of eukaryotic cells. Utilizing a hot-phenol extraction procedure, Prestayko and Busch[227] could detect at least 14 RNA fractions in Novikoff hepatoma chromatin. Twelve distinct RNA fractions could be obtained from KB, L, Don-c, and rat liver hepatoma cells grown in tissue culture.[169] Kanehisa et al.[228-230] have isolated four distinct RNA fractions from chicken liver chromatin. These RNA's inhibit the template activity of DNA using *E. coli* RNA polymerase – an effect common to many other cellular RNA's. However, unlike other RNA species tested when homologous tissue chromatin is used as template, two of the fractions considerably stimulate the template activity of chromatin. These two RNA's show an unusual affinity for histones,[230] presumably removing them from the DNA and in this way increasing the template activity of chromatin. Szeszak and Pihl[266] found that only 1% of the total RNA in rat liver chromatin was sensitive to RNase H, an enzyme which specifically degrades the RNA moiety of DNA/RNA hybrids, and that 0.1% of the DNA of rat liver chromatin is present in the form of RNA/DNA hybrids. They tend to overlook the fact that this RNA could have a regulatory role in gene transcription. There are other reports of eukaryotic DNA with RNA hybridized to DNA;[231,232] again, they have not been well characterized.

Almost all the chromatin RNA fractions isolated to date are of unknown heterogeneity since the separations were obtained only in terms of polynucleotide chain lengths. Almost nothing is known about their metabolism. Clearly this is one area of chromatin research that needs to be more deeply explored so that we may understand the structure and function of the eukaryotic cell chromosome.

Lastly, before we look at some suggestions as to how the above chromosomal elements interact with each other, we must stress the fact that chromatin as it exists in the cell is a very active material in the metabolic sense. Not only are some of its components turning over rapidly in terms of synthesis and degradation, but they are often actively modified (through phosphorylation, methylation, acetylation). Also, it is possible that there are a number of protein or RNA components that reversibly associate with chromatin in the cell. Because of their feeble interaction they may be lost in the final purified chromatin preparations. For this reason it may be difficult to build models for a gene regulatory system in eukaryotes, simply because we do not yet know all the major regulatory components.

GENETIC ORGANIZATION OF THE EUKARYOTE CHROMOSOME

There is one further problem that considerably limits the detail we can instill into models for a gene regulatory system: at present the genetic organization of the eukaryotic cell chromosome remains unknown to us. The reader is directed to an interesting review of some suggestions on this topic by Thomas.[234] To date it would appear that much of the experimental data is in favor of the "unineme chromatid."[234] In this view it is suggested that a single double helix of DNA runs along the complete length of the chromatid. In this way the chromatid replicates, segregates, and mutates, as if its axis were a single DNA double helix.

This correlation between the gross organization of the chromosome and its source of genetic information appears to go one step further. Belling,[235] after developing a cytological technique to look at the detailed structure of pachytene chromosomes of *Lilium*, showed that they contained numerous pairs (2,000/cell) of "spherules," which he called "chromomeres." On the basis of their number, varying sizes, and fixed linear order in any specific chromosome he suggested a one to one correspondence between genes and chromomeres. Some years later, Painter[236] called attention to the giant sized chromosomes of *Drosophila* larvae salivary glands, which undergo endomitosis. The repeated duplication of chromatids of these chromosomes during endomitosis is accompanied by a tight somatic pairing throughout the length of the chromatids. The chromomere regions of all chromosomes associate together as distinct "bands." They each have a distinct size and location within the chromosome. Bridges[237] was among the first to note a one to one correspondence between genes and bands, of which there were at least 5,000 in *Drosophila melanogaster*. A reasonable correlation between a genetic linkage map of this organism and the corresponding cytological preparation of its chromosomes was seen.[237] The loss of a piece

of the chromosome as in "notch" mutants[237] or the duplication of a piece of the chromosome as in "bar" mutants[237] could be clearly seen as the absence or extra addition of bands in cytological preparations of salivary gland chromosomes.

Genes and Chromomeres

Can we then indirectly "see" genes on the chromosomes? The elegant work of Judd et al.,[333] for example, who could assign 121 lethal and gross morphological point mutations to 16 complementation groups, which corresponded to 16 chromosome bands in the *giant* to *white* region of the X chromosome of *D. melanogaster*, suggests that each chromomere does operate as a single functional unit. However, there is also evidence to suggest that the chromomeres (or bands) contain a number of genes that show intraallelic recombination and *cis/trans* effects.[238] Depending on what experimental data one accepts, it appears that the estimated number of functional genes per chromomere ranges from about 1[234,239] to about 20 or 30.[240,241] The reader is directed to an explanation by O'Brien[240] of possible errors in the lower estimates and a structure proposed by Bonner and Wu[241] which accounts for much of the physical chemistry and molecular biology of *Drosophila* DNA. If we for the moment accept a value of 30 as the number of functional genes per chromomere, we arrive at a value of some (30 x 5,140 =) 154,200 as the approximate total number of functional genes in *D. melanogaster*. To some people this number may appear to be too large or too small. When we consider that *E. coli* has about 4,000 genes[6] and yet it can carry out a very diverse number of molecular and biological operations, 1.5 x 10^5 may appear too large. Yet when we look at the complexity of *Drosophila* or, extrapolating this point further, when we see just how identical two monozygotic twins are, we may feel we are unable to propose how such a limited number of genes can organize a species to such fine detail.

Permutations and Combinations

The theoretical biologists, of course, will point out that there is no need for astronomically high numbers of functional genes to store and generate the necessary data to build a complex organism. If we assume for the moment that each of our 154,200 genes can exist only in 2 states (expressed or repressed) then each *Drosophila* cell would be capable of existing in $2^{154,200}$ or $10^{46,418}$ different states of gene activity. Even if we allow for a few hundred (say 256 [2^8] for *Drosophila*) cell types and allow each differentiated cell to "modulate" its state by turning on and off a certain number of its genes (say 100 genes), then we see that the 2^{108} or 10^{32} states of gene activity compose but a small subset of the enormous potential for variation in gene activity. Doubtless, the success of the eukaryotic cell in bringing about this refined regulation of gene activity has been built around the organization of its functioning parts, the genes.

Redundancy Minimizes Errors

Goodwin[242] was among the first to suggest the necessity for built-in functional redundancy in a system for gene regulation, to ensure that errors do not occur in the decision of which genes will be expressed and which will not. Kauffman[243,244] has built gene regulation networks, where genes are looked upon as binary switches (i.e., capable of being on or off, 0 or 1). In these switching networks the output of some genes serves as the input of others. A Boolean function for each gene specifies for each integer of time how it will behave based on the current set of values of its input. A gene state "net" consists of a list of the present value (1 or 0) of each of the set N genes. This present value determines through the net structure the subsequent state of the net. Such a net has a finite number of states (2^N). Kauffman demonstrated that if we assume two inputs per gene,then, even with a randomly arranged state net initially, as time proceeds the switching network quickly becomes trapped in a cycle of states no matter what the initial set of gene net values were. Using more realistic models of the in vivo situation, he demonstrated, using computer simulations, that if N = 15 genes, almost half of the time was spent among 0.1 to 0.6% of the 32,768 states. Such state cycles correspond to cell types. If we perturb such a system by changing the value of a single gene, the system may return to its state cycle or flow into another state cycle. This flow between state cycles, Kauffman suggests, models differentiation. It appears that in a switching network with two random inputs per gene, one state cycle can only move into a limited number of other state cycles. Further, the transition between the two states is usually favored for one state to the other and not vice versa.

Thus we see that an extremely ordered dynamic behavior can be observed in these randomly constructed gene nets with two inputs per gene. No such homeostatic tendency is seen with one or N inputs per gene. In some cases three inputs per gene can give rise to state cycles, i.e., simulated cell types.

Since these models were built with a random arrangement of two inputs per gene, it should be clear that networks evolving by random mutation will tend to be homeostatic. It is thus quite possible that the eukaryotic cell, by utilizing a number of such simple gene interactions of high molecular specificity, has built into the system homeostatic mechanisms that are derived from the system as a whole and may not be immediately apparent to us.

MODELS OF GENE REGULATION

From our lack of knowledge of the chromosome structure, models for gene regulation tend to deal with the rather specific "mechanics" of individual gene regulation. Only when we have some knowledge of this can we hope to understand the ways whole systems interconnect and thus build themselves into stable homeostatic mechanisms.

A Model in Which Histones Play an Important Role

The model proposed by Tsanev and Sendov,[245,246] while not the first extensive consideration to how such gene regulation is attained in eukaryotes, does differ considerably from the other models. For this reason we shall consider it first.

This model is unique in that in it the histones do not play the part of relatively nonspecific proteins that inhibit the template activity of chromatin under the direction of the other chromatin components. Instead they are assigned to the specific gene recognition process. It is suggested that the operon is composed of three regions (Figure 6): recognition, repression, and transcription sites. The recognition site is coded by a specific arrangement of histones; these specify the initiation site of the operon. They point out that, with 60 histone molecules each occupying only 2 or 3 turns of the DNA,[247] for 5 different types of histones there would be 5^{60} or 10^{41} possible histone arrangements. The one or more repression sites are regions on the DNA – presumably beside the recognition sites – where the reversible attachment of allosteric repressors can occur. The transcription site is where the different mRNA's are transcribed. The arrangement of histones at the repression and transcription sites need play no part in the mechanism of action of the model, while those at the recognition sites have a dual role. They block transcription of the operon, also, because their unique arrangements make the recognition site recognizable to a specific NHP. Each NHP would therefore recognize its own arrangement of histones as its own recognition site. Having done this the histone-NHP complex at the recognition site *deblocks* the operon; that is, if there is no repressor(s) at the repressor sites the operon can be transcribed into RNA. Should there be a repressor at the repressor site, the operon still cannot be transcribed unless some factor (E) is able to inactivate the repressor – a system analogous to the *lac* repressor in *E. coli*.[1,2] It is suggested in the model that the histones once bound to the DNA never leave it. Further, during replication of the DNA, the fixed arrangement of histones on each old strand will determine a complementary histone arrangement on each new strand (Figure 6). An NHP in the process of deblocking the operon can bind almost irreversibly with the DNA, so even if it is no longer synthesized that operon will remain deblocked. Only after two rounds of mitotic diversions with no further synthesis of this NHP will such an operon become blocked again. This model is clearly different from that which we are familiar with in prokaryotes, in that here there are two distinct processes involved in regulation of gene transcription. There is the blocking/deblocking control and repression/derepression control. Figure 7 illustrates a simple type of interaction between two operons of the type described by Tsanev and Sendov.

Let us suppose operon A is deblocked by an NHP (NHP_2 in Figure 7) from some other source initially; for example, in the case of the initial stages of development of the egg, they suggest that the NHP may be translated from stored mRNA in the egg cytoplasm. It can be seen that operon A will deblock (with NHP_1) and repress (with RP_1) operon B. In the presence of a factor E_1 (which inactivates RP_1) operon B becomes active and in fact keeps operon A active (with NHP_2). Should the source of E_1 be removed, then RP_1 will again

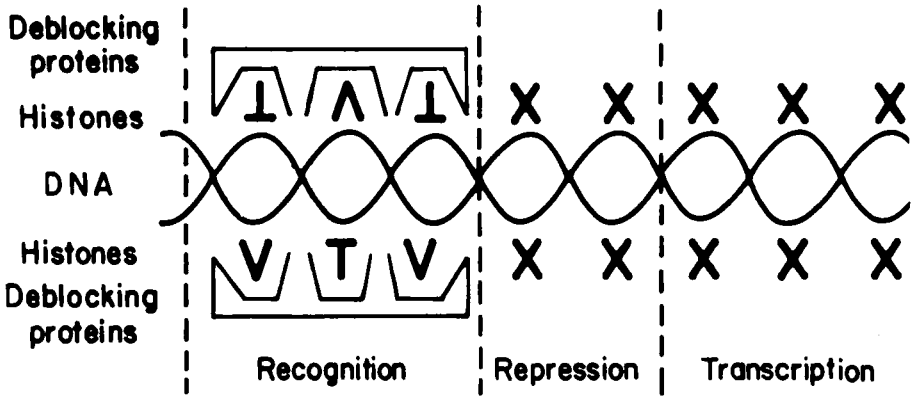

FIGURE 6. A schematic representation of the chromatin elements involved in genetic control in eukaryotic cells. V and T signify different histones in specific arrangements forming the recognition sites of the chromatin; X indicates other histones also bound to DNA, but which play no role in the control mechanisms. (Reproduced from Tsanev, R. and Sendov, B., *J. Theor. Biol.*, 30, 337, 1971. With permission.)

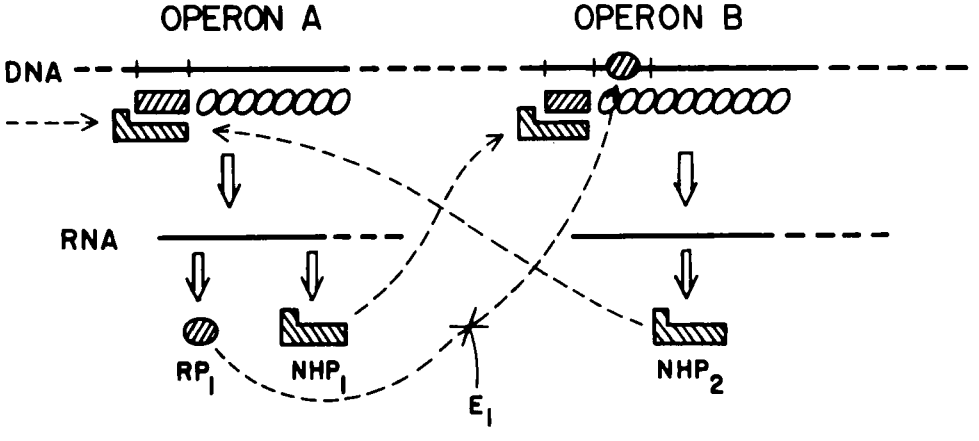

FIGURE 7. A scheme for the interaction of two operons, according to the model of Tsanev and Sendov.

repress operon B, closing it down. Here we have a case for reversible cell transformations brought about by some factor in the cell's environment.

We can also have situations where there will be an irreversible interaction between two genes (Figure 8). Operon A again deblocks and represses operon B. In the presence of a factor E_1 that inactivates RP_1, operon B becomes active. Operon B is responsible for the production of repressor RP_2, which will turn off operon A. In the presence of E_1 even without the supply of NHP_1 from the now turned off operon A, we see that operon B can remain active up to the first or second mitotic division, since the NHP deblocking proteins bind irreversibly to the recognition sites. Removal of E_1 will not return operon A to its initial state since RP_2 will continue to repress operon A. This may be the type of operon interaction in the process of embryonic induction.

Tsanev and Sendov then built a mathematical model of cells with genetic nets of these types (utilizing, for example, the elimination of repressors, by blocking of genes after mitotic divisions). In extending this system to explain the mechanism of cell differentiation they conclude that cell differentiation is determined by deblocking genetic units, while the different functional states within the differentiated cells are determined by the repression/derepression process. The model illustrates the requirements for mitotic divisions to

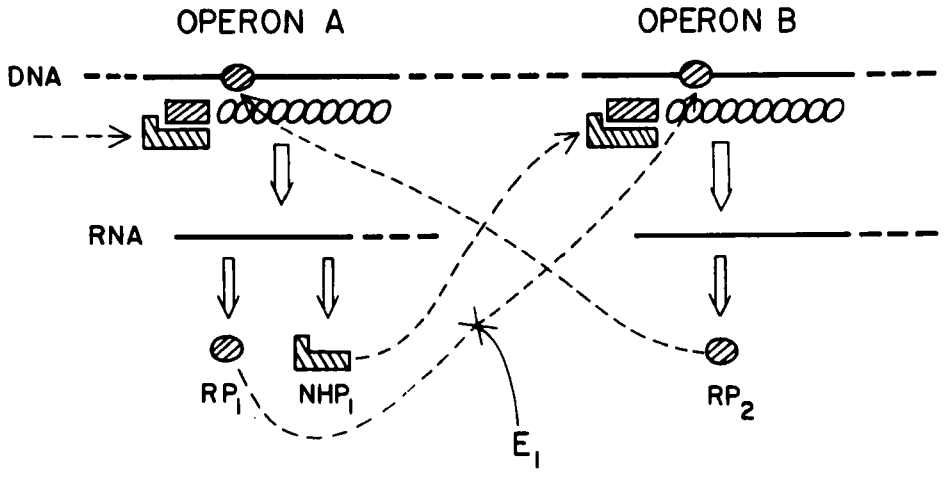

FIGURE 8. A scheme for the interaction of two operons, according to the model of Tsanev and Sendov.

obtain cytodifferentiation and carcinogenesis. Of interest also is the fact that the mathematical treatment shows the association constants of the repressors to the DNA to be a very sensitive parameter in regulating changes for the pattern of gene expression in the whole system. Even slight changes in the structures of these proteins, not even large enough to eliminate their functions as repressors, can lead to drastic changes in the overall gene expression of the cell.

Tsanev and Sendov put forward a number of points (many of which are circumstantial) to support their hypothesis. Histones do bind tightly to DNA and do appear to be well conserved throughout the cell cycle and even cell generations.[248] They appear to be synthesized almost synchronously with DNA.[249] A mechanism has been proposed by Lewin[250] whereby histone pairs in the wide grove of DNA can, by rupture of weak hydrophobic linkages to the DNA, turn on their ionic linkage away from the wide grove. In this way it would be possible for one DNA strand to be transcribed while the complementary strand remains masked by the other histone partner. Possibly the NHP's in Tsanev and Sendov's model bring about such a rotation of the histones.

The role of the NHP's as deblocking groups seems quite plausible. The restoration of histone-inhibited RNA synthesis from DNA template by NHP's is well known.[251] There is also evidence for NHP's firmly bound to the DNA,[252] which would presumably represent the NHP's bound at the recognition sites. Tsanev and Sendov point out that not all histones may be involved in this operon-specific recognition property of the DNA histone complex (almost certainly not the F1 histones, which, as we have already seen, are probably concerned in a nonspecific way with the compactness of the chromatin).[14,18,90] From this model we can see why the continued presence of a factor that brings about a switched differentiated state may not be further required (as it is in Figure 8, for example). There are a number of situations like this in vivo.[253] Perhaps the model's most useful prediction is that rearrangement of gene expression in the process of cell differentiation will commonly occur with DNA replication and mitotic division of the cells. It is a commonly observed fact that most forms of cell differentiation are in fact preceded by DNA replication and mitosis.[254,256] In fact, in some cases it can be shown that direct inhibition of DNA synthesis prevents differentiation.[257] Cases of the positive appearance of tissue-specific proteins (not previously present in the undifferentiated cell) are known to occur after DNA replication.[258]

This model has a number of attractive features in the mathematical treatment of how genes may interact. However, in terms of the known biochemistry of chromatin it tends to be unsatisfactory. Apart from the fact that there is no evidence for such a specific arrangement of histones in DNA it is difficult to see how a complementary histone arrangement would form on each new DNA strand during DNA replication. Extensive replacement of histones with protamines takes place in the sper-

matozoa of some animals.[85,259] A complex process requiring, among other things, additional gene recognition sites to ensure that histones from the operon-specific recognition site are not removed would appear necessary in these cases. It is difficult to see how nuclear transplantation experiments of the type described by Gurdon[260] could work with this model. Here nuclei from a differentiated tissue were transplanted into enucleated eggs and were able to give rise to normal adult organisms. When we have a situation wherein a component is irreversibly bound to the DNA, as in the case of the deblocking NHP's, it is difficult to incorporate into the model a process whereby the chromosome can "step back in time" in its ordered process of gene deblocking. Reversible attachments of operon regulator functions to the chromosome are less difficult to handle in these cases.

We have seen that all of the chromatin components can be dissociated with high salt and urea, and that these components can be recombined into chromatin with the same electromicroscopic appearance and template properties as chromatin in vivo.[163-166,189] Tsanev and Sendov suggest that "chromosomal RNA" (cRNA) may represent RNA transcribed from the recognition sites of operons, and that in the chromatin recombination process cRNA, by hybridizing to the DNA, ensures that the NHP's attach themselves to the DNA in the correct manner. This prevents the initiation sites from being blocked by the histones. However, it is difficult to believe that the RNA transcribed from chromatin in vivo with recognition sites deblocked by a histone/NHP complex should be the same as RNA transcribed from chromatin with recognition sites deblocked by RNA.

One possible test for this model would be to look at the content of NHP's firmly bound to DNA[252] in the egg or early embryo of an organism and compare this with those in an adult. One might expect more deblocked operons and thus more firmly bound NHP's in the latter case.

All Somatic Cells Are Unique

The use of the DNA replication phase as a biological clock in models for gene regulatory controls in cell differentiation has been extensive, perhaps the most recent ingenious hypothesis put forward by Wassermann[261] illustrates a case where it has been used to its fullest extent. Wassermann (extending a hypothesis put forward by Sperry for neurons[262]) suggests that all somatic cells that are not members of a clone are individually, differentiated. Such cells synthesize "cell unique proteins" which could number 40,000 distinct types per mammalian cell. No two cells have the same complement of cell unique proteins. Moreover, the type of cell unique proteins any cell contains is under control of "process algorithms" operating on gene sequences in a hypothetical chromosome model. These cell unique proteins are suggested to be the components that give cells the ability to sort themselves into the highly specified assemblies of organs and structures. Clearly an enormous number of diverse proteins would be required. Wassermann attempts to provide a mechanism to assure the genetic determination of these cell unique proteins in the following way: he utilizes the observation by Wu[263] that at high relative humidities X-ray diffraction patterns of DNA fibers suggest that the secondary structure of DNA in these fibers consists of partially intercalated double helices (Figure 9). Such a four stranded model of DNA would still retain all the biological implications of the Watson-Crick double helix. For semiconservative replication each of the two duplexes come apart, and nucleotide pairs are inserted onto each duplex and polymerized. No unwinding of the DNA double helices is involved in this process. Wassermann postulates that much of DNA which codes for proteins that are not cell unique proteins (e.g., enzymes, hemoglobin, etc.) is present on a large continuous circular DNA of a Wu-type DNA double duplex. This large circular DNA is folded longitudinally a number of times along the length of the chromosome (Figure 10). It is also looped out into "immobile side loops" at regular intervals. A number of "mobile side loops" – themselves Wu-type DNA double duplexes – are catenated to each immobile side loop (Figure 10). Wassermann in his monograph describes how each mobile side loop may be propelled unidirectionally past a fixed complex on the nuclear membrane. The essence of this model is that each mobile side loop rotates only once after each DNA replication. During this replication RNA fragments are transcribed from one DNA duplex of the mobile side loops; these are later assembled to form mRNA's for the cell unique proteins. The types of RNA fragments synthesized from each mobile side loop are determined by a complex "process algorithm" explained in detail in the monograph. As mentioned earlier the model is concerned with gener-

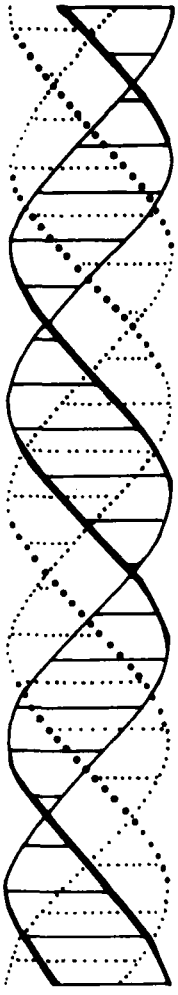

FIGURE 9. Schematic representations, one by the solid lines and the other by the dotted lines, of the two partially intercalated double helices. The pitch of the helices is 67.2 A, and the separation between the base-pairs on the same double helix is 6.4 A. (Reproduced from Wu, T. T., *Proc. Natl. Acad. Sci. U.S.A.*, 63, 400, 1969. With permission.)

ating large numbers of diverse cell unique proteins in a gene controlled and ordered manner. Such a system could also conceivably be involved in antibody production. However, it does not suggest how regulation of "regular" cell enzymes and proteins is controlled. The presence of cell unique proteins must still be regarded as hypothetical until further experimental evidence illustrates their presence. Further, there appears to be little experimental evidence for a DNA double duplex in eukaryotic chromosomes. All physical chemistry studies are in agreement with a single DNA duplex.[264,265] The electron microscopic studies cited by Wassermann are difficult to interpret; the 250 Å "unit fiber" described by Ris and Fubai[267] could conceivably contain fold-back loops of DNA rather than a double duplex of the type described above.[122] With the exception of the one reference cited[268] there appears to be little evidence for RNA transcribed from DNA being assembled piecewise into mRNA; in fact, the evidence is strongly in favor of the reverse process, namely, disassembly of transcribed RNA into smaller pieces of mRNA.[274,277,281,289]

Turnover of Nuclear RNA

This discussion of the breakdown of newly synthesized RNA in eukaryotes brings us to a topic that has been used extensively in two well-known models for gene regulation in higher organisms, namely the turnover of nuclear RNA. It was first observed by Harris[66] and Scherrer et al.[269] and subsequently confirmed by other workers[67,269] that the newly transcribed RNA from DNA undergoes a partial, rapid degradation within the nucleus. The newly transcribed nuclear RNA has a high molecular weight, is of a heterogenous nature in terms of size, and is usually known as DNA-like RNA or heterogenous nuclear RNA (HnRNA). While it is not clear just how large HnRNA is,[270-272] it is generally agreed that the molecules contain more nucleotide sequences than is required for translation of an average protein. Competitive hybridization studies clearly demonstrated that almost all cytoplasmic mRNA sequences are contained in HnRNA but that only a part of the sequences in HnRNA were found in cytoplasmic mRNA.[65,268,273,274] It should be noted that many of these early experiments were done under conditions that would only give information about RNA transcribed from repetitive DNA sequences. More recently it has been demonstrated that unique mRNA sequences are contained in HnRNA.[11,275] When the transcription, "nuclear processing," and transfer out into the cytoplasm of one unique operon transcript (that from the rRNA genes) became clear,[7,276] it seemed logical to suggest that just as the 45S precursor rRNA was cleaved in several steps to give rise to 28S and 18S rRNA's so also cytoplasmic mRNA arose by selective cleavage of HnRNA molecules.

Scherrer and Marcaud,[277] utilizing the duck

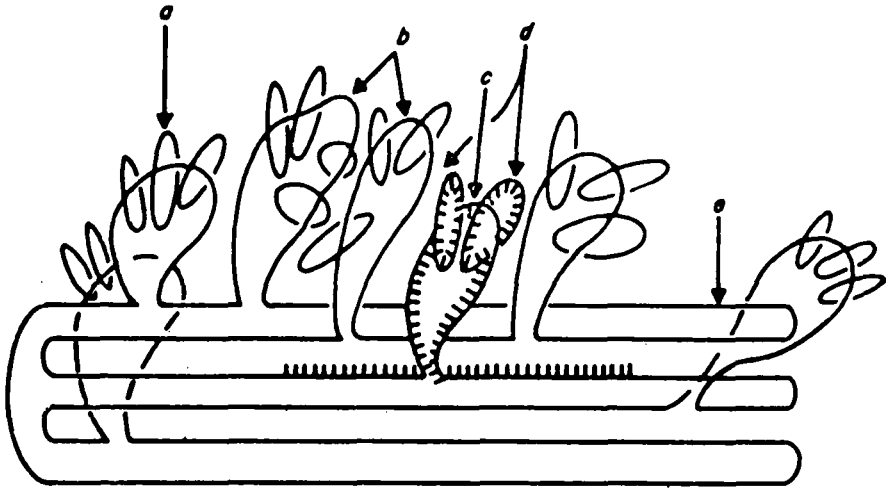

FIGURE 10. Schematic representation of some of the structures of a eukaryotic chromosome which are postulated in the model of Wassermann. Only some of the axial strands (e) of the principal circular DNA are shown. A few typical fixed side loops (b) and a few typical mobile side loops (a) (catenated with the fixed side loops) are drawn in. The striated structures indicate parts that become exchanged completely during a reciprocal crossover (e.g., a typical fixed side loop [c] and mobile side loops [d] are exchanged as complete units). Reprinted from Reference 261, p. 191, by courtesy of Marcel Dekker, Inc.

erythroblast system – a system in which more than 60% of the protein synthesized in the cell is hemoglobin – have done an extensive examination of the steps involved in the transcription, "processing," transportation, and translation of the hemoglobin mRNA. The RNA synthesized in the nuclei of these cells (which we shall call HnRNA) has a heterogenous high molecular weight. It sediments between 30S to 90S on a 5 to 20% sucrose gradient. There is but a very small contribution of 45S and 35S precursor rRNA species in this HnRNA fraction in erythroblasts. One can thus easily follow the events in hemoglobin mRNA synthesis and "processing" in this system. No nascent globin mRNA could be seen in the nuclei of these cells. All RNA rapidly synthesized existed as HnRNA, which on pulse labeling followed by actinomycin D treatment gave rise to species of lower molecular weights. It was concluded that a large fraction of HnRNA decays with a half-life of 15 min. There was a small but definite fraction of this HnRNA that moved out into the cytoplasm. A third fraction of this HnRNA decayed slowly in the nucleus. Hybridization experiments indicated that a surprisingly large fraction (4 to 7%) of the genome in these cells was activated for RNA synthesis. Some difficulty was experienced in getting polysomal RNA of sufficiently high specific activity to conclusively show that HnRNA contained homologies to functional mRNA. However, cold polysomal RNA did not compete for more than about 10% of the sites on DNA homologous for HnRNA. Using these data and those of others with similar observations in other systems,[65-67,278] Scherrer and Marcaud put forward their "cascade regulation theory" for gene regulation in eukaryotes.[277]

Cascade Regulation Theory

In this model (Figure 11), a large polycistronic HnRNA molecule is transcribed from DNA, possibly only one type of HnRNA molecule from each active chromosomal chromomere. This HnRNA species will contain a number of mRNA species, coding for up to an estimated 30 cistrons. Before passing out into the cytoplasm the HnRNA passes through one or more "regulation thresholds" during which only selected gene transcripts from the HnRNA actually reach the stage where they can be translated in the cytoplasm. The rest of the HnRNA, containing at least in part other gene transcripts, is broken down in the nucleus. Sections of the HnRNA may also be stored rather than being immediately expressed. Scherrer and Marcaud suggest that there are "recognition molecules" which in a highly specific manner recognize specific mRNA sequences, or secondary structure on one hand and regulatory effectors on

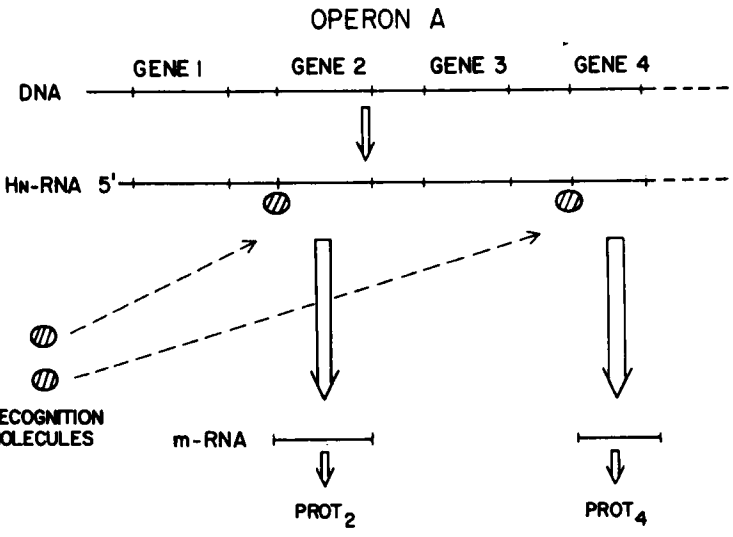

FIGURE 11. A scheme for the expression of one operon, according to the model of Scherrer and Marcaud.

the other. These recognition molecules would thus be, at least, partially tissue specific. These workers did not suggest the arrangement of mRNA sequences on the HnRNA, the scheme shown in Figure 11 clearly being but one possibility.

This model has a number of attractive features. It explains why HnRNA is considerably larger than the mRNA we might expect to code for an average size polypeptide. Giant RNA transcripts from the BR 2 region of *Chironomus tentans* have been well identified;[271] this region is rich in newly synthesized RNA coding for one or a few of the salivary gland polypeptides.[279] The model accounts for the rapid turnover of HnRNA[65-67,274] as well as the low content of mRNA sequences in HnRNA.[277] HnRNA would not, therefore, appear to be RNA from a number of identical cistrons all functioning and coding for the same protein, as suggested by Callan.[280] The model would suggest a reason for HnRNA containing ribonucleoprotein (RNP) particles,[281] namely to "protect" and transport the selected gene transcripts out into the cell cytoplasm. At the time Scherrer and Marcaud suggested that the RNP particles may themselves select the appropriate mRNA sequences; this seems unlikely now, in view of the very limited protein heterogeneity in RNP particles.[281-283]

There is one point often brought up in models of this nature, that of "infinite regression." In this case, if we have recognition molecules controlling gene expression, then we must have genes that

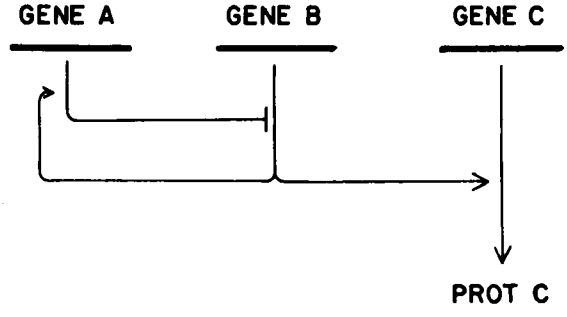

FIGURE 12. A "closed system" regulation of gene C by genes A and B.

control the recognition molecules, and so on. Paul[284] has pointed out that we can always get around this problem by using both positive and negative controls. Figure 12 illustrates a very simple example of how such a principle can work. Gene C is responsible for the final gene product protein C. Expression of C requires the presence of B gene product which is also required for A gene product. However, A gene product inhibits the expression of gene B. In this way a closed system is set up. Expression of C could be modulated by effectors that alter the actions of A or B gene products. Scherrer and Marcaud[277] did not specifically suggest negative control elements in their model though it is easy to see how they could be inserted.

There are a few serious difficulties with this model, however. While a unique endoribonuclease

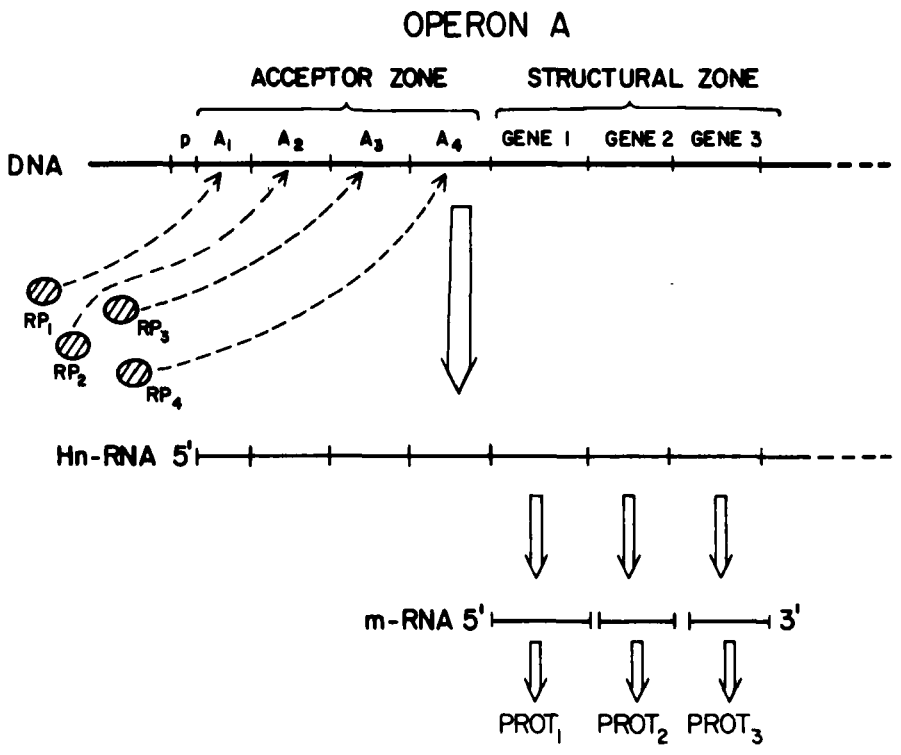

FIGURE 13. A scheme for the expression of one operon, according to the model of Georgiev.

activity has been shown to be associated with RNP particles[285] and a cytoplasmic endonuclease activity specific for HnRNA has been identified,[286] there is little evidence for regulatory proteins that can specifically select particular mRNA sequences from HnRNA. Such proteins would have to be reasonably tissue specific as well as in some cases being capable of having their activity modulated by regulatory effectors. It has been suggested that some effectors act at this level;[287] however, the weight of evidence seems to support the idea that effector modulated control of mRNA formation for specific genes takes place on the chromosomes, with the NHP's taking at least a part of the regulatory role. The model also tends to be incomplete in that it does not explain how the production of HnRNA is regulated, or in what way the cell decides which operon (possibly chromomere) is to be transcribed into HnRNA.

Structural and Acceptor Zones of the Operon

Georgiev[288] put forward a model that answers some of these difficulties. This was subsequently enlarged upon by Georgiev et al.[289] In this model (Figure 13), Georgiev suggests that each operon contains two functional "zones." A "structural zone" contains cistrons for cellular structural proteins, enzymes, and regulatory proteins, and is located at the latter part of the operon. The "acceptor zone" is in many ways analogous to the operator region in the *lac* operon,[3] and is located after the promoter region (a region where the RNA polymerase binds to the DNA) but before the structural zone. This acceptor zone is, Georgiev suggests, very large in higher organisms, almost comparable to the structural zone in size. It contains a number of different sites (A_1, A_2, A_3, and A_4 in Figure 13) which are capable of interacting with different regulatory proteins. It is possible and probable that different operons have a number of acceptor sites in common. Thus one regulatory protein may influence the transcription of a number of operons. Such multiple acceptor sites may correspond to a fraction of repetitive DNA base sequences. Other possibilities such as terminations and sequences to be recognized by specific endoribonucleases may also be present in this zone and exist as repetitive DNA sequences. Georgiev et al[289] recently pointed out that the 3' end of HnRNA also contains some repetitive base

sequences. These may move out into the cytoplasm with mRNA, and could act as terminators of transcription or translation.

It is suggested in this model that the RNA polymerase transcribes the complete operon, first the acceptor zone sequences, then the structural zone sequences. This transcription is regulated by the presence or absence of the regulatory proteins (RP_1 to RP_4 in Figure 13) at their acceptor sites in the acceptor zone. Georgiev points to two possibilities as a role for the regulatory proteins. They could repress the operon by binding to DNA analogous to the *lac* repressor 3 or they could derepress the operon by removing the inhibitory action of the histones in RNA synthesis. It is not specified in the model if the simultaneous presence of all regulatory proteins at the acceptor zone is required for transcription. Because of the rapid synthesis of HnRNA presumably this is so in many cases. On the other hand, a stepwise passage of the RNA polymerase along the acceptor zone, each step awaiting the presence of a regulatory protein (or its effector), could give rise to a useful gene regulatory system, particularly if the structural zone contained regulatory genes.

Each newly transcribed HnRNA molecule will thus contain not only the cytoplasmic mRNA sequences from the operon but also sequences transcribed from the acceptor zone. Georgiev suggests that the RNA transcribed from the acceptor zone sequences serves no further purpose, being rapidly degraded in the nucleus. Some specific cleavage of the structural zone sequences also occurs, yielding mRNA species which pass out into the cell's cytoplasm. In contrast to Scherrer and Marcaud's model[277] the breakdown of the HnRNA is not gene specific. It occurs for all genes in the same manner. One way this could happen is via unique exo- and endoribonucleases that recognize a specific sequence or sequence conformation common among all mRNA sequences.

It appears that much of this HnRNA degradation takes place either during or after RNP particle formation.[281]

This model has a considerable amount of experimental data in its favor. It accounts for many of the properties of HnRNA. The high molecular weight, rapid turnover, and low occurrence of mRNA sequences in this RNA fraction are in agreement with the model. A progressive decrease in the size of HnRNA can be seen with time after its synthesis.[277,289] High molecular weight HnRNA contains a nucleotide triphosphorylated at the 5′ end.[289] In lower molecular weight HnRNA species only nucleotides monophosphorylated at the 5′ end are found. This clearly agrees with transcription of one large HnRNA followed by endonuclease activity. Georgiev et al.[289] demonstrated that polysomal mRNA only slightly inhibits hybridization of high molecular weight HnRNA to DNA, and that the 3′ end sequences of HnRNA strongly compete with such mRNA species for sites on the DNA. This implies that mRNA sequences are in fact located towards the distal end of transcribed operons. It is suggested in this model that at least some of the acceptor zone DNA sequences may be identical or similar and occur in a number of operons. Such sequences should thus be present in the repetitive DNA fraction. There are in fact indications that this is the case. Ryskov et al.[290] found that the hybridizability of 5′ end sequences of HnRNA was 3 to 4 times greater than that of whole HnRNA. Mantieva and Arion[291] found that the hybridizability of HnRNA synthesized in cells after UV preirradiation was increased. Such UV irradiation leads to incompletely synthesized HnRNA molecules lacking sequences at their 3′ ends.

All the experimental evidence taken together lends considerable support to Georgiev's model. There are a few problems with the model, however. It seems unlikely to us that the cell should simply degrade and discard all the HnRNA transcribed from the acceptor zone. Apart from the evolutionary burden of maintaining a supply of enzymes to remove this HnRNA fraction, it would appear feasible that the cell could put this large transcript from the operon to further use. We shall discuss this point in more detail later. Greenberg and Perry[292] suggest, after studying the thermal stability of HnRNA/DNA and mRNA/DNA hybrids performed in formamide solutions at DNA excess, that there is less similarity among the unique sequences of DNA coding for HnRNA than there is among the unique sequences coding for mRNA. Furthermore, the distribution of repetitive DNA sequences transcribed into HnRNA and mRNA is not quite as different as this model might expect. Dina et al.[293] find that *Xenopus* embryo mRNA contains at least 13% of its sequences that are not transcribed from unique DNA sequences. Vorob'ev and Volfson[294] observed in the early developmental stages of sea urchin embryos a transfer of RNA's synthesized

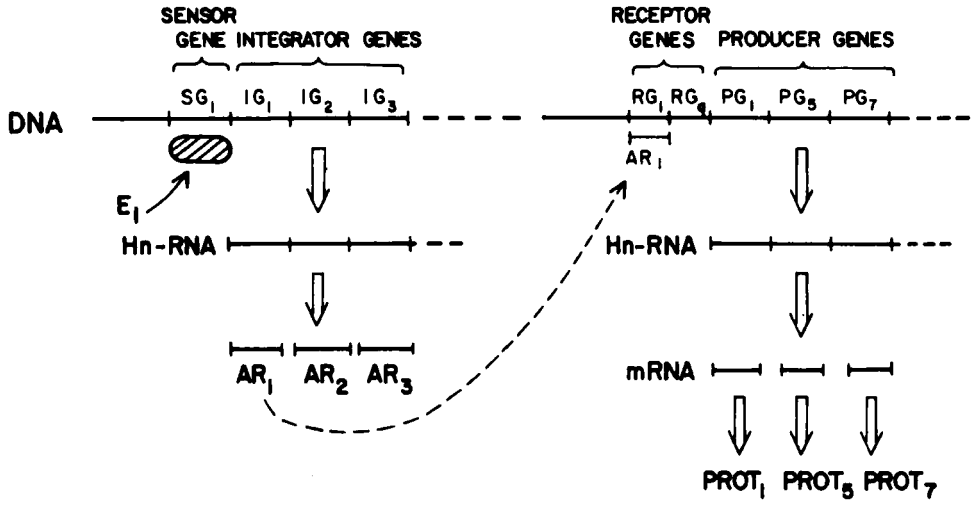

FIGURE 14. A scheme for the regulation of an operon, according to the model of Britten and Davidson.

from repetitious DNA to the cell cytoplasm. Much of this repetitious cytoplasmic RNA was unable to compete with nuclear HnRNA sequences. There may even be complications in the breakdown of the structural zone into mRNA species. Ostertag et al.[295] have recently found five polysomal RNA species in mouse leukemic cells with globin template activity. These species were of three different size classes (8S, 12S, and 16S, approximately).

In this model by Georgiev the mode of synthesis and regulation of the regulatory proteins has remained largely undefined.

Producer Genes

A rather different type of model which also accounts for many of the properties of HnRNA has been put forward by Britten and Davidson.[296]

In this model (Figure 14), genes that code for structural proteins or enzymes will be termed "producer genes." One or more producer genes are contiguous to one or more "receptor genes." The sequence-specific interaction of an "activator RNA" molecule with its receptor gene causes transcription of the contiguous producer genes. For example, in Figure 14 the attachment of activator RNA 1 (AR_1) to receptor gene 1 (RG_1) leads to transcription of producer genes 1, 5, and 7 (PG_1, PG_5, PG_7). The activator RNA's are transcribed from "integrator genes." One or more integrator genes are contiguous to a "sensor gene." This is a sequence serving as a binding site for agents that can induce changes in the patterns of gene transcription in the genome. It is suggested that in general these agents mediate their effect via a specific protein which binds to the sensor gene. Thus in Figure 14 effector E_1 binds to the protein attached to sensor gene 1 (SG_1); this leads to transcription of the contiguous integrator genes IG_1, IG_2, and IG_3 which give rise to activator RNA's AR_1, AR_2, and AR_3. In this way E_1 leads to the eventual production of proteins $PROT_1$, $PROT_5$, and $PROT_7$. An essential feature of this model is that large "batteries" of producer genes can be turned on in a simple way. There may be many RG_1 receptor genes spread throughout the genome. All these receptor genes will be capable of turning on their contiguous producer genes when sensor gene SG_1 is activated. We see in Figure 14 that E_1 also leads to production of activator RNA's AR_2 and AR_3 which (not shown in Figure 14) lead to activation of other producer genes. The presence of effector E_1 is not the only way producer genes PG_1, PG_5, and PG_7 could be turned on. In this example (Figure 14) we see that they are also contiguous to receptor gene RG_9. Thus an effector that interacts with a sensor gene contiguous to an integrator gene IG_9 will also turn on these genes. The reader is directed to the original model[296] and further reports[297-299] which explore in some detail how this model allows complex interactions in gene regulation. The authors point to the possibility that the activator RNA's may in fact take the form of proteins translated from these RNA species. In this model the protein component of a sensor gene is also the product of a producer gene. Because of

this it is possible to have sequential patterns of gene activity, a characteristic feature of cell differentiation and development.

Because of their proposed multiple occurrence Britten and Davidson suggest that integrator and receptor genes belong to the repetitive DNA sequence. An elegant manner in which these genes can give rise to evolutionary novelty is explained by the above authors.[299]

As with the previous two models there is considerable experimental support for this model. It accounts for the presence of repetitive DNA sequences in eukaryotic cell genomes, although a function for highly repeated sequences and satellite DNA sequences would still remain unexplained. We have seen that there is considerable evidence for the sensor gene type binding proteins in the NHP fraction. The demonstration of activator RNA type components is less clear, although, as we have seen, some RNA components in chromatin could fulfill this role. Sivlop and Bonner[210] have shown that "chromosomal RNA" hybridizes largely with repetitive and intermediate DNA base sequences.

Congote and Trachewsky[300] find that soon after injection of aldosterone to adrenalectomized rats there is a specific increase in the nuclear RNA's transcribed from repetitive DNA sequences in the rat kidney cortex cells. No such large increase was seen with RNA transcribed from unique DNA sequences. Vorob'ev and Konstantinova[301] observed that cortisone preferentially activates RNA transcription from repetitive DNA sequences in rat liver cells compared to the less repetitive and unique sequences. Such effects could be interpreted as alterations in the nuclear "profiles" of activator RNA's, an event preceding large changes in the types of producer genes to be expressed.

There are problems associated with this model also. Mature *Xenopus laevis* oocyte repetitive DNA sequences transcribed into RNA display no homology with such sequences found in late blastula stages of development.[302] One would expect some overlap of active producer genes at these two stages of development. This model suggests two classes of HnRNA's, one containing RNA transcribed from repetitive DNA sequences, the other transcribed from largely unique DNA sequences. The experimental data are not in agreement with this point. All HnRNA sequences appear to contain sequences transcribed from repetitive as well as unique DNA sequences.[303,331]

The model is also lacking in detail. For example, it is not clear how the activator RNA's turn on producer genes, or if one or all activator RNA's must be present at receptor genes before the contiguous producer genes can be transcribed.

One problem that cannot easily be explained by models that utilize the repetitive sequences in DNA as a key component in their structure is that there are cases where species have little such functional DNA yet differentiate and function like other eukaryotes. The euchromatin of *Drosophila melanogaster* contains little repetitious DNA,[329,330] yet the organism has little difficulty in regulating its gene expression. We have already mentioned the more extreme case of the protozoa *Stylonychia*, which can survive for many cell generations with only unique DNA base sequences.[79]

Conformational Changes as a Means of Regulation

There are other models dealing with some aspects of control of gene transcription in eukaryotes which, unfortunately, space will allow us to only briefly mention. Sutton[124] points out the possible importance of chromatin packing in the nucleus as a rather broad form of gene regulation. This was also indicated earlier by Batchan et al.[317] Sutton points out that some nucleotide sequences in double stranded DNA will lead to local differences in the conformation of the double helix;[304] further, these differences could be amplified through association of histones with the DNA. Sutton suggests that similar DNA sequences in chromatin will tend to arrange themselves in "three-dimensional crystals" within the nucleus. These packing forces will keep large segments of the DNA unavailable to RNA polymerase and thus suppress these genes. Should some of the repeated sequence families evolve, the chromatin packing forces would weaken, thereby exposing initially new nongenetic DNA to a natural selection process.

Fibrous and Globular DNA

Crick[78] suggests that chromosomal DNA falls into two classes. One class, "fibrous DNA," contains the cell's structural genes and is present in the interband regions of *Diptera* polytene chromosomes (or between chromomeres in other cases where such giant polytene chromosomes are not

found). The second class of DNA, "globular DNA," present in chromosomal band regions of *Diptera* (or chromomeres in other cases) contains the recognition sites for the control of the structural gene transcription. Within these globular regions are twisted hairpin loops of double stranded DNA. A region of such DNA loops, because of the geometry involved, will split into two single stranded DNA chains. Crick suggests that at these regions gene regulatory proteins attach to the genome. Thus, in this hypothesis the RNA polymerase attaches at the globular DNA regions (bands) and transcription extends out into the fibrous DNA regions (interbands) where the cell's structural genes are transcribed.

However, as pointed out by Paul,[284] it appears that much of the experimental data suggest that transcription occurs in the bands, rather than in the interbands.

Destabilizer Molecules

Paul has recently proposed a model[305] in which the interband region contains the RNA polymerase binding site, a site for regulatory proteins, and a site for a "destabilizer molecule." The destabilizer molecule brings about local relaxation of DNA supercoiling in the interband region, allowing the polymerase to attach to the DNA, to continue this destabilizing process, and to transcribe RNA in the adjacent chromosome band. Much of the DNA from the chromosome band region (for the polytene chromosomes, in chromomeres in other cases) will be transcribed as a large HnRNA molecule. Paul suggests that only a fraction of this RNA will contain useful mRNA sequences. The remaining "nonsense RNA" will be degraded in the nucleus in a way not unlike that suggested by Georgiev.[288,289]

A COMPREHENSIVE MODEL

Having briefly reviewed these recent models for regulating of gene transcription in eukaryotes we see that all have some experimental evidence for their support. Is control of gene transcription in eukaryotes this simple? Could it be that there exist other explanations for our experimental observations? Our own feeling is that there probably do exist within the cell higher orders of gene regulation, possibly involving complex theoretical regulatory phenomena[306] which regulate alterations in gene expression in such complex processes as cell differentiation and tissue development. However, an elementary subset of this process probably does involve regulatory processes of the type we have reviewed. We would further like to introduce a new model for such "elementary" gene regulatory processes.[233] The basis of this model is partially derived from a synthesis of the models already described.

We suggest that there are two major elements controlling gene transcription in eukaryotic cells: an RNA element turning genes on and a protein element turning them off. In this model we suggest that the RNA in the "noninformative" part of HnRNA as described by Georgiev[288,289] gives rise to activator RNA's which behave in many respects like the activator RNA's described by Britten and Davidson.[296] In this model we assign a role for the NHP's, repetitive DNA sequences, and HnRNA, and speculate in some detail about the process involved in turning a gene on.

Before discussing the model we would first like to introduce the following functional definitions.

Operon – This is a term usually applied to a part of the genome of a prokaryote. In using the term "operon" here we shall restrict our meaning to a part of the eukaryotic cell DNA that is transcribed by the RNA polymerase beginning at one region, which we shall call the promoter region (P), and continuing through an undefined number of genes until some undefined terminator signal is reached. No further analogy with the prokaryotic operon is implied. An inactive operon is influenced by a protein layer. It is suggested that this layer can be divided into two main functional regions, a tightly bound (TB) protein zone and a loosely bound (LB) protein zone. When the TB protein zone is present alongside the DNA, the RNA polymerase cannot transcribe the operon. For transcription of the operon, the TB protein zone must first be removed. The remaining protein layer on the operon, the LB protein zone, does not impede the passage of the polymerase along the DNA strand. It either falls away when the TB protein zone is removed, is pushed away by the polymerase, or is otherwise removed in an as yet unknown manner. There may even be regions of the repressed operon which never have their LB protein zone covered with protein.[307] We would like to emphasize that the proposed TB and LB protein zones need not correlate directly with the known ease by which certain proteins can be dissociated from DNA by increase in ionic

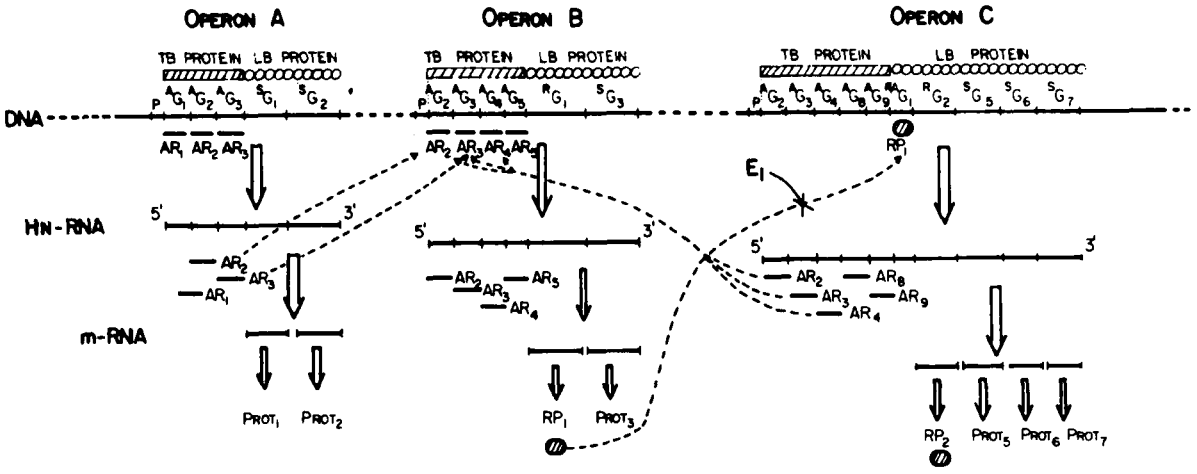

FIGURE 15. A scheme for the interaction and regulation of three operons, A, B, and C.

strength, changes in pH, or some other simple environmental change.[86,105] Rather, we mean to distinguish between one region of the protein layer that must somehow be removed by the cell in order to make transcription of the operon possible and another region of the protein layer that in itself does not impede the transcription process.

We propose that the HnRNA transcribed from each operon is transcribed as one continuous polycistronic message. In Figure 15 three such operons, A, B, and C, are illustrated. In this model we will assume all genes are read from left to right. Each operon contains one of the following genes or any combination of two or more.

Structural genes ($^{S}G_n$) – These contain the base sequences on the DNA that carry information for the synthesis of structural proteins or enzymes used by the cell. They do not contain information for proteins that have a regulatory role at the transcriptional level, although other levels of control within the cell are of course possible. All $^{S}G_n$ genes are coated by the loosely bound protein zone in the repressed state of the operon. The subscript "n" is used here to distinguish between different structural genes.

Activator genes ($^{A}G_n$) – We suggest that these genes are contained largely in the repetitive sequences of the DNA of the cell genome. These genes generally would be located immediately after the promoter region of the operon. The HnRNA transcribed from each operon would thus have located on its 5' end RNA sequences complementary to these genes. In the process of breakdown of the giant operon HnRNA into monocistronic units of $^{R}G_n$ and $^{S}G_n$ gene products, we propose that short sequences of RNA complementary to the repetitive sequences of DNA on the $^{A}G_n$ genes would be formed. The $^{A}G_n$ genes are coated by the tightly bound protein zone when the genome is switched off. The subscript "n" is used to distinguish different activator genes.

Activator RNA (AR_n) – These are the fragments of RNA released after the breakdown of HnRNA from that part of the operon that contained the $^{A}G_n$ type genes, and will thus contain sequences complementary to repetitive sequences in DNA. The concentration of each activator RNA will vary throughout the life of the cell in an extremely complex way, since its concentration at any one time will depend on a balance between breakdown due to nuclease activity and supply from different operons, each producing different amounts of activator RNA's. An essential proposed feature of the activator RNA molecules is that they can displace those parts of the tightly bound protein zone of the operon to which they bind. Thus in operon A (Figure 15), when AR_1, AR_2, and AR_3 are present simultaneously, all of the tightly bound protein zone proteins are removed from this operon; the RNA polymerase is thus free to transcribe the complete operon. Should there later be an absence of one or all the activator RNA's AR_{1-3} some or all of the tightly bound protein zone proteins would bind back to the DNA of operon A, turning it off.

Repressor genes ($^{R}G_n$) – These regions of the DNA carry information for synthesis of the

repressor proteins (RP_n). The subscript "n" is used to distinguish different repressor genes and their proteins. In the absence of effector (E), these proteins will bind reversibly to a region of the DNA – the repressor protein acceptor gene ($^{RA}G_n$). In doing so they remove the tightly bound protein zone of the operon that they bind to; however, the polymerase cannot transcribe the whole operon since the repressor protein firmly bound to the DNA prevents its passage along the operon to the structural genes. In some cases these proteins can have their binding to the $^{RA}G_n$ type gene inhibited by the presence of effector molecules (E_1) (for example, RP_1 in Figure 15). Under these conditions, before the tightly bound protein zone can rebind, the polymerase can transcribe the operon at least once. Activator RNA molecules (AR_n) formed from the transcribed RNA will then bind back to the $^{A}G_n$ genes, keeping the operon on, by displacing any of the tightly bound protein zone that had formed on the operon. We also suggest that there are cases in which the repressor proteins are not acted upon by any effector molecules but always display a strong affinity for an $^{RA}G_n$ type gene. In doing so the further production of RNA along this operon is prevented for as long as this regulator protein is maintained within the nucleus. All $^{R}G_n$ genes are coated by the loosely bound protein zone when the operon is repressed.

Repressor protein acceptor genes ($^{RA}G_n$) – These regions of DNA contain sequences that are recognized by the repressor proteins. The recognition is highly specific; for example, the $^{R}G_1$ gene repressor protein binds to only an $^{RA}G_1$ type repressor protein acceptor gene. In this model no essential function is assigned to the RNA transcribed from $^{RA}G_n$ type genes. Again, all $^{RA}G_n$ genes are coated by the loosely bound protein zone when the operon is switched off.

The Model

An essential feature of our model is that it has a series of interlocking elements. We assume that at any one time in the cell there are always some active genes. It is via complex interactions of these genes that the decision of which further genes will be turned on or off is made.

It is proposed that in the cell the passage of RNA polymerase along the operon requires the presence of RNA sequences (AR_n) complementary to the base sequence of the DNA for those $^{A}G_n$ genes that are covered by the tightly bound protein zone. We will not explain how the presence of the AR_n at the $^{A}G_n$ genes facilitates passage of the RNA polymerase along the operon, but it is possible that this is intimately involved with the removal of F1 histones, since, as we have seen, these appear to be bound to repeated DNA sequences and may be near the initiation sites for the RNA polymerase.[18,119]

Operon A (Figure 15) represents one of the simplest organizations of an operon: the polymerase passes through the $^{A}G_n$ genes, then transcribes the structural genes $^{S}G_1$ and $^{S}G_2$. A high molecular weight DNA-like RNA (HnRNA) molecule is formed in this process. From the proposed organization of the activator genes and structural genes we suggest therefore that the 5′ terminal end of the transcribed HnRNA will contain a number of repeated RNA sequences. In the process of the polycistronic HnRNA breakdown into individual cistrons of structural genes and these being sent out into the cytoplasm,[269,274,277,281,289,303] we suggest that by means of specific nucleases present in the nucleus, activator RNA molecules are formed from the repetitive sequences of HnRNA that were transcribed from the $^{A}G_n$ genes. We have seen that HnRNA breakdown appears to begin from the 5′ terminal end,[289,303] and that repeated RNA sequences are preferentially located at this end of HnRNA.[288-291] This fragmentation of the HnRNA 5′ part would require the presence of nuclease within the nucleus capable of breaking the HnRNA in a specific manner. This does not seem unreasonable if we remember that such nucleases are almost certainly required to release the mRNA sequences.[303] For such a mechanism it is probable that along the HnRNA there are linker base sequences between different $^{A}G_n$ genes, possibly giving a unique conformation to this region of the HnRNA[308] (double stranded regions in HnRNA are known,[327] as well as interesting "hairpin loops"[335]) or in some other way causing a preferential hydrolysis by the ribonuclease at these points. Cases of sequence-specific breakdown of viral[309] and ribosomal RNA's[7] are well known. Indeed, specific nucleases for HnRNA have been isolated.[285,286] While this model suggests a function for some of the repetitive sequences in DNA, it does not exclude the possibility that other repetitive sequences that have other functions are present. Such functions could include linker

sequences between AR_n or mRNA sequences of a defined conformation such that the nuclear nuclease activity can recognize the correct locations to hydrolyze the HnRNA molecule, or sequences to bring about the correct assembly of the RNP particles.[281] Other transcribed repeated sequences probably have as yet unknown functions; for example, it is not clear at present what the function of repeated sequences in mRNA is.[293] The AR_n genes should contain repeated DNA sequences from the "intermediate" repetitive DNA fraction rather than from the highly repetitive or satellite DNA fractions, since, as we have seen, these remain untranscribed.[32]

From the arrangement of the ^AG_n gene sequences, we might expect a tandem arrangement and distribution throughout much of the cellular DNA of some repetitive DNA sequences. We have seen that this has been suggested by Thomas.[59,76] The identity of the activator RNA's is perhaps the most serious problem with this model. A number of low molecular weight RNA components have been isolated from nuclei that could have this property; one of these, "chromosomal RNA," described by Bonner and others,[210-214,216] is one possible candidate. It has been shown to be essential for a sequence-specific interaction of chromosomal proteins with DNA.[163,164] The chromosomal RNA appears to hybridize with a large fraction of the total cellular DNA;[22,209] further, the hybridization is preferentially to the repetitive sequences of DNA.[210]

The unique base composition of "chromosomal RNA" would require that the AR_n molecules be modified before binding to the ^AG_n genes.

Other RNA components which have been isolated from chromatin[169,206,223,227,231,232,310-312] could serve such a role. The chromatin-associated low molecular weight RNA components described by Kanehisa et al.,[228-230] which increase the template activity of chromatin, are clearly possible candidates. Also, the suggestion that the association of activator RNA's with chromatin is a reversible phenomenon should be remembered. Such activator RNA's may not be found associated with isolated chromatin; the majority may be contained in the nucleoplasm. We have seen that the labeling kinetics work of Scherrer and Marcaud,[277] in fact, demonstrated the presence of a fraction of HnRNA that decayed slowly in the nucleus. We would also like to point out that many of the techniques used to fractionate chromatin[22,127,128,135,138,143,160,163-165] will not yield an RNA species that is hybridized to DNA. Our hypothetical activator RNA, because it is suggested as interacting with the DNA in a sequence-specific manner, may be hybridized to DNA and may in many cases remain hidden in the DNA fraction of chromatin isolated in the above procedures. Szeszak and Pihl[266] demonstrated by treating rat liver chromatin with RNase A and RNase H that as much as 0.1% of the DNA may be present in the form of DNA-RNA hybrids in vivo.

Operon A, illustrated in Figure 15, once activated by the presence of activator RNA's AR_{1-3}, will thus continuously produce proteins 1 and 2, assuming that here there are no other posttranscription controls. This is the kind of operon we might expect in a cell that has genes that are used in some fundamental biochemical pathway of the cell where continuous production of mRNA is required. An ever-increasing level of activator RNA's will of course be prevented by the presence of nuclear nuclease activity.[285,286,313,314] However, it is suggested here that at least some of the activator RNA's are present on the DNA long enough that the polymerase can produce more HnRNA, and their further production is facilitated. At least in the case of avian nucleated red blood cells it appears that the degraded HnRNA remains bound to the chromosome for a period of time before moving through the nucleus.[315] Daneholt and Svedhem also present some evidence for this in *Chironomus tentans* salivary glands.[316]

An essential feature of any model accounting for regulation of gene transcription in eukaryotic cells is that different batteries of genes must be able to interact with each other.[296,297] We suggest that an operon may interact with other operons in one or two ways: it can induce the production of HnRNA in another operon via the production of activator RNA's, or inhibit this formation of HnRNA by the production of repressor proteins. Let us consider each in turn.

Operon B of Figure 15 could be activated by the combined effect of operon A, which provides activator RNA's AR_2 and AR_3, and some other operons (not shown) which provide AR_4 and AR_5. It could also be activated by an operon that provides AR_5 and operon C, when C itself is active, since it contains activator gene sequences for AR_{2-4}. As we will explain later, C is normally only active in this diagram in the presence of an

inducer, E_1. It must be remembered that activation of an operon (for example, operon B) by activators AR_{2-4} and AR_5 is not a single isolated event within the cell. The polymerase will have to wait until there is a sufficient build-up in the concentrations of AR_{2-4} and AR_5 within the nucleus such that sufficient activator RNA's are available to simultaneously bind to the $^{A}G_{2-4}$ and $^{A}G_5$ genes. The polymerase at this operon will then be able to transcribe its HnRNA, which in turn gives rise to further species of AR_{2-4} and AR_5. These of course could bind back to the $^{A}G_{2-4}$ and $^{A}G_5$ genes (facilitating their further production), diffuse to other operons, or be broken down by nuclease activity. A further complexity is that an operon containing one or more multiple identical activator genes will require not only a representative activator RNA for each $^{A}G_n$ gene it contains before transcription but will have to wait until a high concentration of the multiple $^{A}G_n$ gene activator builds up in the nucleus. Once this happens it tends to maintain itself and also turn on other operons containing a high content of that $^{A}G_n$ gene.

Clearly, a negative control element is required to repress the expression of certain genes once they have served their purpose for a period in the lifetime of a cell. This is the function we propose the repressor proteins serve.

Operon B (Figure 15) differs from A in that it contains a regulator gene ($^{R}G_1$). Thus repressor protein RP_1 is formed when operon B is activated by AR_{2-4} and AR_5. Operon C could be activated by the combined action of operon B, which gives rise to activator RNA's AR_{2-4}, and some other operons (not shown) which provide AR_8 and AR_9 from among their $^{A}G_n$ genes. However, since in this case operon B is producing RP_1, this repressor protein will by binding to the $^{RA}G_1$ gene prevent the repressor gene $^{R}G_2$ and structural genes $^{S}G_{5-7}$ of operon C from being transcribed. This could be an essential process for the cell if it wants to have operon B and other operons containing $^{A}G_8$ and $^{A}G_9$ genes on all the time but C turned off. If there was no $^{RA}G_1$ gene present in C the activator RNA's AR_{2-4}, AR_8, and AR_9 would have turned it on.

The degree of binding of RP_n molecules to $^{RA}G_n$ genes could vary considerably, some binding so strongly as to permanently turn off operons. For other RP_n molecules this may be quite reversible. At least some nonhistone proteins turn over rapidly.[20,21] In such cases a way to turn on operon C without the preexistence of activator RNA's would be to shut off RP_1 synthesis (for example, via a reduction in AR_5 concentration in the nucleus). After the RP_1 concentration decreases there will be insufficient RP_1 to bind to $^{RA}G_1$. The polymerase will transcribe the operon at least once before the reformation of a tightly bound protein zone on operon C, giving rise to AR_{2-4}, AR_8, and AR_9, which in turn feed back and keep the operon switched on.

There is another way the cell could turn on operons without the preexistence of activator RNA's to bind to its $^{A}G_n$ genes. As seen in the case of operon C, RP_1 could be a nonhistone protein that has its conformation altered by some effector E_1 such that it can no longer bind to the $^{RA}G_1$ gene, or binds in a manner that does not inhibit the action of the RNA polymerase. For the same reason as explained above, the absence of the inhibition effect of RP_1 on the RNA polymerase at the $^{RA}G_1$ gene will allow operon C to be switched on.

As in the bacterial system[1-4] removal of E_1 will allow RP_1 to again inhibit the action of the RNA polymerase at the $^{RA}G_1$ and cause the operon to revert to a switched off state. We cannot estimate what proportion of the nonhistone proteins exist as effector-sensitive and effector-nonsensitive types.

This model can explain how a large series of gene transcription alterations occurs, as for example when tissues are stimulated by hormones.[175, 176,178,180,184-186,199,200] Let us call the state of gene expression before the cells were stimulated state A; that after stimulation, state B. Operons D and E (Figure 16) could be the type of key linking operons between many operons in state A soon to be turned off and operons in state B to be turned on. We call these types of operons an "activator RNA amplifier operon combination" for reasons that will become clear below.

Let us say operons of major importance in state A require a high concentration of activator RNA's AR_4 and AR_5 while operons in state B require a high concentration of activator RNA's AR_8 and AR_9. Among the operons expressed in state A is operon D (Figure 16). This operon is activated by AR_2, AR_4, AR_5, and AR_7, and will give rise to the repressor protein RP_3 which will bind to the DNA sequence of the $^{RA}G_3$ gene in operon E. The tightly bound protein zone will be removed

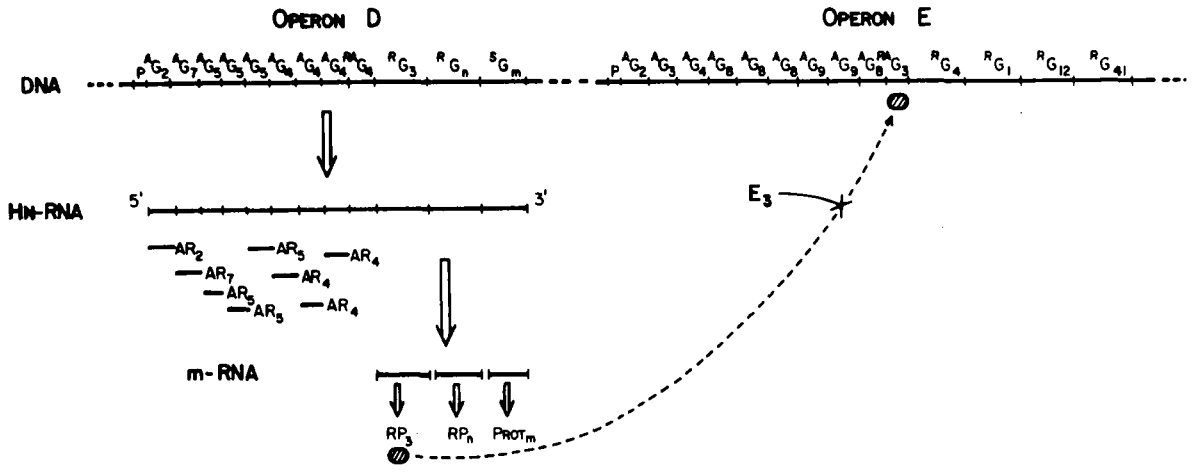

FIGURE 16. A scheme for an "activator RNA amplifier operon combination."

from the $^{A}G_{n}$ genes of operon E; however, because the RP_3 protein remains bound to the DNA the operon is not transcribed into RNA. The RP_3 protein bound to the $^{RA}G_3$ sequence prevents the RNA polymerase from transcribing the operons. It is suggested that an effector (E_3) capable of causing transformation of cell state A to state B can, by binding either directly to RP_3 or via some other intermediary protein (as for example in the case of steroid cytoplasmic and nuclear hormone receptors[190-194,196-200]), inhibit or alter the binding of RP_3 to the $^{RA}G_3$ gene. The RNA polymerase can then transcribe operon E, giving rise to new activator RNA's, AR_8 and AR_9. Also in the case of this example, a new repressor protein RP_4 is formed. The RP_4 will in this case bind to the $^{RA}G_4$ on operon D, closing it down. The newly formed activator RNA's per se will be capable of starting up other operons within the cell. Recent work by Congote and Trachewsky[300] has shown that in the case of at least one effector, aldosterone, acting on rat kidney cortex tissue, an early effect of hormone treatment was a specific increase in nuclear RNA capable of hybridizing with repetitive DNA sequences. The new operons switched on could be like operons A, B, or C, but it is possible that AR_8 and AR_9 could bring about expression of many other operons that the cell at this time does not wish to express. Therefore, operons D and E would contain a number of $^{R}G_n$ type genes to ensure that such operons are not expressed. For example, the $^{R}G_1$ gene on operon E of Figure 16 would prevent the expression of operon C of Figure 15. We would thus expect the appearance of new nonhistone proteins in the nuclei of cells involved in such an effector-dependent change from state A to state B.

We propose that the repressor proteins are contained in the NHP fraction. We have already reviewed the suggestion that they have gene-specific regulatory roles. To quickly summarize: the NHP is a heterogeneous population of proteins probably containing many diverse proteins in low amounts. The amounts of NHP present in isolated chromatin vary markedly from one cell type to another.[14,15,20,21,126] In general there appears to be more NHP in template active chromatin than in template inactive chromatin.[126,129,130] This we would expect from our model since, as already explained, the further production of many new activator RNA species in template active chromatin necessitates the specific repression of other operons that would otherwise be turned on as well. At least some NHP's interact specifically with DNA.[157,158,165,166] In contrast to histones that turn over at a low rate,[19,20] at least some NHP's turn over rapidly in the cell.[21] Also, unlike many other proteins of the cell, their synthesis is maintained at mitosis.[318] Our model clearly requires that proteins bind specifically to DNA, and that some at least be tissue specific. A rapid turnover of these proteins is in agreement with a highly flexible differential expression and repression of some operons. The NHP composition appears to be altered during the process of differential gene expression, i.e., when cells undergo a state A to state B type transition. We have seen that a wide range of effectors has been suggested as being involved in these processes.[190,192-194,196-200] Clearly, these changes

in NHP compositions are in line with the effector-dependent changes of NHP we would expect from our model.

We have suggested that the binding of the repressor proteins RP_n to their $^{RA}G_n$ genes brings about removal of the tightly bound protein zone of each operon. Sufficient data are not yet available to suggest what components are involved in this process or how it takes place. At least one study has indicated removal of the tightly bound F3 arginine-rich histone by a specific NHP after stimulation of uterine tissue with estradiol.[319] We have seen that there are numerous reports of NHP's increasing the template activity of chromatin[21,168,172,176] as well as reports of increased RNA transcribed from chromatin that had an increase in its content of NHP's.[154,155,166,173,175] It might thus appear contradictory that we suggest a repressor protein role for the nonhistone proteins. However, careful analysis of the model reveals no major contradiction with the experimental results reported. Increased RNA transcribed from chromatin could arise when a few NHP's have their binding to the DNA inhibited by one or more effectors, as in the case of operon E (Figure 16) already discussed. As pointed out, formation of many new activator RNA's also requires formation of new repressor proteins to turn off unwanted operons that would otherwise be activated by the new profile of activator RNA's. The increase in template activity of chromatin seen in vitro when NHP is added to chromatin preparations[168,172,176] could be due to exposure of the DNA previously hidden by the tightly bound protein zone but now available to the prokaryote polymerase for transcription. This observed increase in template activity of DNA appears to lie in the repetitive sequences of the DNA.[172] Such increases in template activity seen in vitro have been suggested as bringing about an increase in the length of the transcribed RNA rather than as exposing new initiation sites on the DNA.[168] We interpret this to mean that once the RP_n has bound to the $^{RA}G_n$ gene further RP_n molecules only increase the removal of the TB protein zone within this operon and not at the many other repressed operons within the cell. There is also the possibility that much of the increase in NHP content in active chromatin[154,155,166,173,175] is largely due to an increase in the content of one or more enzyme activities[144-147] associated with the NHP fraction.

Georgiev,[303] in discussing the observations of Thomas et al.[59,76] of tandem repetitions of repetitive sequences in eukaryotic genomes, suggests the possibility that operons may contain a number of identical structural genes each separated by identical acceptor sites (repetitive DNA sequences to which regulatory proteins may bind). Callan's well-known master and slave genes hypothesis,[280] in which he suggests the presence of multiple gene copies in eukaryotic cells, as well as Whitehouse's[320] suggested mechanism for slave rectification, clearly fits into such a scheme. Bonner and Wu[241] proposed a similar gene arrangement for each chromomere of the *Drosophila* genome, except that the structural genes between the repetitive DNA segments are each different structural genes. We can incorporate these ideas in our model along with many observations made by Tomkins et al. about inducible enzyme systems.[287]

Consider the case of $Prot_5$ transcribed from $^{S}G_5$ (Figure 17). A basal level of $Prot_5$ is maintained with AR_{1-3}. However, also produced is a repressor protein RP_5 which in the absence of inducer E_5 binds to the $^{RA}G_5$ genes located between other $^{S}G_5$ genes. In this situation, therefore, only one $^{S}G_5$ gene is transcribed (note this is only a simple example; there may be many $^{S}G_n$ genes in the HnRNA). The attachment of RP_5 to $^{RA}G_5$ genes is inhibited by the "inducer" E_5. The RNA polymerase will then be able to transcribe several copies of $^{S}G_5$, giving rise to more $^{S}G_5$ mRNA and thus an induced level of $Prot_5$. Removal of E_5 will clearly return the operon to its original state, reducing eventually the levels of $Prot_5$. There is as yet insufficient data available to propose how the premature termination (or alternatively, extended transcription) of the HnRNA is effected. Possibly RP_5 or other proteins bring this about.

A posttranscriptional regulatory system has been suggested by Tomkins et al.[287,321] to account for many of the phenomena seen with inducible enzymes. While posttranscriptional control is very possible, we would like to point out that many of the effects used as evidence for it could also be reinterpreted in terms of the above model. In this model we see that enzyme-specific mRNA accumulates in the presence of inducer even when protein synthesis is inhibited. We see

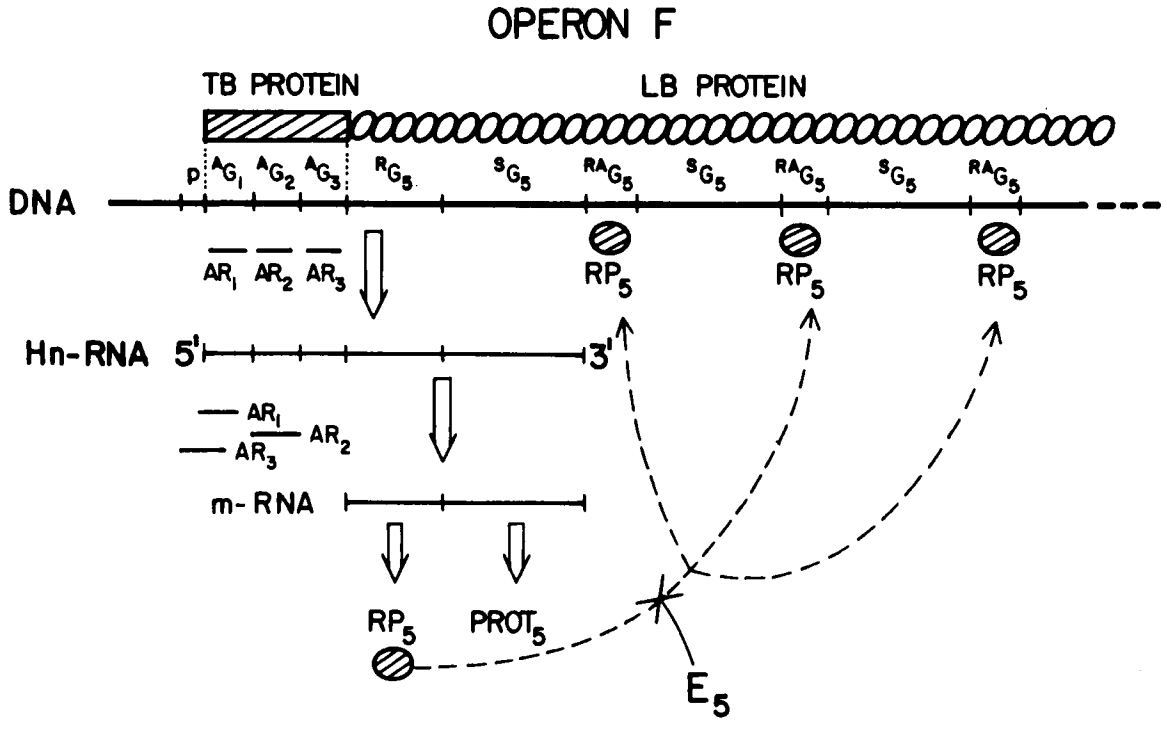

FIGURE 17. A scheme for an operon containing an inducible enzyme.

that the constant presence of the inducer is required to maintain the induced state, and that RNA synthesis is required for induction. These are all properties of the inducible enzyme tyrosine aminotransferase (TAT) induced in HTC cells by dexamethasone.[287,321] The well-known "super-induction" effect[287] seen when actinomycin D is added to HTC cells with induced levels of TAT could be accounted for by the ability of actinomycin D to increase the rate of peptide chain elongation.[322,323] Butcher et al.[328] point out a number of other explanations for this effect. This scheme accounts for the migration of a steroid-receptor complex to the nucleus[324] and its possible combination with a site on the chromosome[325,326] if we assume the action of the steroid is similar to the other well-characterized systems.[190,192,196,198-200]

The operations presented above are in many cases too simple to account for the sophisticated control of gene expression that appears to take place in eukaryotic cells. We feel, however, that similar gene arrangements and interactions, containing multiple $^{A}G_n$, $^{R}G_n$, $^{RA}G_n$, and $^{S}G_n$ genes in such operons, would be in many ways sufficient to explain many of the known experimental facts of the eukaryotic chromosome, its components, and their functions.

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