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EUKARYOTIC GENE REGULATION

Volume II

Gerald M. Kolodny

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Eukaryotic Gene Regulation

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Editor

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FOREWORD

The control of gene expression in eukaryotic cells is of fundamental importance in understanding development, regeneration, aging, genetic diseases, and cancer.

Our ability to treat many human diseases is limited by our lack of knowledge as to why one cell type expresses one gene pattern and another cell type expresses another. For example, adult epithelial cells divide, whereas adult neurons do not. If the genes for cell division could be turned on in neurons, one might be able to repair spinal cord injuries by regenerating new functional neurons. Kidney transplantation might give way to kiney regeneration. Diabetes following pancreatitis might be cured by regenerating new islets cells.

The regulation of gene expression is also an important component of the mechanism of normal human development. Anomalies in development involve these regulatory pathways and attempts to understand, prevent and repair these anomalies would be strengthened by an understanding of how gene control is achieved.

The cause of cancer and its many manifestations is at present unknown. Since many of its manifestations, including its control of cell division, appear to represent abnormal patterns of gene expression, studies of the regulation of gene expression will provide important insights in the understanding and treatment of cancer.

This volume attempts to present some of the recent work on regulation of gene expression in eukaryotic cells. It is, however, purposely limited to an examination of nuclear transcriptional events. Gene regulation at the post nuclear and translational level is not covered and the reader is referred to other excellent reviews on this subject now available.

There is an attempt in the following chapters to review those components of the nucleus which may have gene regulatory functions and to explore how each of them may be contributing to gene regulation. These components include chromatin, RNA, RNA polymerases, histones, nonhistone proteins and hormones. We have not included salts, lipids, and polysaccharides simply because they are probably too limited in their structural and chemical complexity to offer more than the most gross specificity.

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Chapter 1

EUKARYOTIC DNA-DEPENDENT RNA POLYMERASES: AN EVALUATION OF THEIR ROLE IN THE REGULATION OF GENE EXPRESSION

Trevor J. C. Beebee and Peter H. W. Butterworth

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I. CONTROL OF GENE EXPRESSION AT THE LEVEL OF THE RNA POLYMERASE

Any temptation to believe that the regulation of gene expression in nucleated cells might lie primarily at the level of transcription has its origin in the precedents established for prokaryotic systems. Simple DNA sequence (where sequences are represented in the chromosome once or only a few times), multiple structural genes often existing within one transcription unit, very rapid turnover of messenger RNA (mRNA), and concurrent transcription and translation limit the scope for regulation to occur at any point other than at the level of transcription in prokaryotes. On the other hand, eukaryotes contain amounts of DNA grossly in excess of that required to code for cellular proteins, and the DNA is extremely complex (in terms of the multiplicity of sequence repeats); a large proportion of the primary gene transcripts, constructed within the nucleus, never reaches the cytoplasm; there is a spatial separation of transcriptional from translational events which requires the transport of information from the nucleus to the site of protein synthesis. Thus, the potential for regulating the expression of specific sections of the chromosome can be dictated by any number or all of these steps in this highly organized process. With the current state of our knowledge and the limited but rapidly developing technology, one must exercise real caution in ascribing primary regulatory significance to any one of these phenomena.

This chapter concerns one stage in the overall process of expression of genetic information in eukaryotic cells: the role of DNA-dependent RNA polymerases in the controlled expression of specific (classes of) genes. The real question is whether any RNA polymerase exerts a direct regulatory influence in any system. To invoke this enzyme as being directly involved in control processes, variable initiation specificity, elongation rate, or termination efficiency must be defined in terms of some structural modification of the enzyme itself.

A. Prokaryotic Precedents

In bacterial systems, not all potential control mechanisms satisfy these strict terms of reference. In general terms, two categories of regulatory mechanism are prevalent: positive and negative control, both of which operate at the level of transcription (reviewed by Lewin[1]). Both mechanisms require the binding of a control element to DNA in the proximity of the RNA polymerase binding site: a positive control element interacts with DNA at a site adjacent to and promotes the recognition of this site by the polymerase (for example, the cyclic-AMP binding protein which facilitates the binding of the polymerase to the lac promoter[2]); a negative control element binds to the DNA at a site which prevents the movement of the RNA polymerase from the promoter into the region containing the structural genes, e.g., the binding of repressor to the operator of the lac operon.[3] Neither of these control proteins interacts directly with the RNA polymerase, and therefore, they do not satisfy the criteria for control operating directly on the enzyme.

However, there are at least three other possible control mechanisms which, under certain conditions, may be operative in bacterial cells. There is ample evidence that different RNA polymerases bind to and transcribe from different promoters; that is, they have different template specificities. Following infection by the phage T_7, the host *Escherichia coli* RNA polymerase transcribes a limited portion of the phage genome (the "early" genes); one of the early gene products is an RNA polymerase which recognizes the promoters for the "late" phage genes.[4] A more subtle situation is displayed in the modification of the *Bacillus subtilis* RNA polymerase following infection by phage SP01;[5] expression of viral genes results in the binding of virus-coded proteins to the host polymerase which promote the transcription of specific sections of the phage genome. Perhaps the most challenging mechanism of control which has been proposed for prokaryotes is that of Travers[6] which suggests a role for auxiliary factors which associate with the RNA polymerase mediating the recognition of specific classes of promoters, e.g., those for ribosomal RNA coding sequences. While positive and negative control elements are involved primarily in the "fine" regulation of expression of small groups of genes under coordinate control, these alternative mechanisms dictate a "coarse" form of control, where gross changes in cellular function are to be expressed.

Although this brief survey of prokaryotic control systems begs the criticism of superficiality, it serves to place in perspective different systems of control which may be operative at the level of transcription of genetic information in bacteria.

B. The Eukaryotic Problem

We are faced with a much more complex problem when attempting to rationalize the types of mechanism regulating gene expression in nucleated cells. From a single fertilized egg, the complete organism develops through embryogenesis into a complex array of different cell phenotypes. As phenotype changes during this process, the patterns of gene expression must be changed. Ultimately, the adult phenotype is maintained through subsequent generations. It is conceivable that the "fine" contol of cellular processes may still be achieved by mechanisms similar to those operating in bacterial cells through the transient association of regulatory proteins with the chromosome. However, during replication, when associated proteins must be released from the chromosome, the opportunity arises to replace or maintain a complex array of regulatory factors which direct the expression of phenotype. This group of proteins are normally referred to as "nonhistone" proteins, whereas the histones (which form the protein core of the nucleosomal particle[7]) have a predominantly structural role in the organization and packaging of the chromosome.

If discussion is restricted to the differentiated eukaryotic cell, it is known that, in general, 50 to 80% of the genome is made up of sequences represented only once (or a few times) in the chromosome: the unique or nonrepetitive sequences. The remainder of the genome (excluding satellite DNA) is composed of "moderately repetitive" sequences (see Lewin[8] for review). While there are some notable exceptions (such as the coding sequences for histones), most structural genes exist as single copies in the haploid genome. Current models of the organization of DNA sequence suggest the interspersion of unique with repetitive regions and invoke a regulatory function for the latter.[9] However, the *modus operandi* for these putative regulatory sequences is still far from clear: it is conceivable that they constitute multiple recognition sites for the binding of RNA polymerase and/or regulatory factors (of the "positive" or "negative" control type) or sequences which, when transcribed, constitute processing enzyme cleavage points. The fact that only a small proportion of the genome (up to 10%) may be transcribed at any time confers a major role on proteins which restrict the access

of the RNA polymerase to the chromosome. Almost invariably, the initial RNA transcripts are considerably longer than the sequences which ultimately appear in the cytoplasm, the latter representing sequences complementary to only about 2% of the genome.[9] The generation of mature RNA by progressive cleavage of the primary gene transcript is, of course, a potential regulatory step. In the case of stable RNA species (rRNA and tRNA), the structural relationships and the cleavage steps required to generate mature RNAs are comparatively well characterized (extensively reviewed by Perry[10]), and there is some experimental evidence to suggest that control is exercised during these processes.

However, the precursor-product relationship between rapidly synthesized, high molecular weight, nuclear (HnRNA) and mRNA has been a contentious issue for more than 10 years[11] and has only recently received unequivocal experimental support. A high proportion of HnRNA is turned over within the nucleus. From a detailed comparison of total nuclear HnRNA and messenger populations of single cell types, HnRNA has been shown to be as much as five- to tenfold more complex than mRNA and contains repeated sequences. The evidence suggests that repetitive and nonrepetitive sequences are interspersed in the region of the HnRNA molecule 5′ to the mRNA component (assuming that message is located at the 3′ termini of these molecules).[12] During the maturation process, these 5′ sequences may have a role in the (controlled) selection of RNA to be transported from the nucleus to the cytoplasm; some of the repetitive sequences may form regions of secondary structure which may be recognized by processing enzymes[13] or which link remote portions of the HnRNA molecule[14] so that the sequence which appears in the cytoplasm is made up of noncontiguous sections of the primary gene transcript.[15] The opportunity now exists to examine in detail the relationship between the structure of the macromolecular precursor of a specific mRNA and subsequent processing events: Bastos and Aviv[16] have identified a precursor for the globin mRNA in both mouse Friend cells and mouse spleen some seven times larger (27S, approximately 5000 nucleotides) than the mature 10S message (approximately 750 nucleotides) together with a partially processed molecule (15S, approximately 1600 nucleotides). The 27S species has a halflife of only 5 min, but it is still uncertain whether this molecule represents the primary gene transcript or some partially processed intermediate. It should also be pointed out that even the mRNA is oversized with respect to the absolute coding requirement for globin (430 nucleotides): this excess in length is a common feature of those mRNAs which have been purified.[8] These phenomena, as well as further posttranscriptional events such as poly-A addition,[17] "capping" of mRNA precursors[18] and translational controls[19] all have regulatory potential and will be discussed in detail elsewhere in this volume. Our purpose in summarizing the complexity of the eukaryotic genome and the proposed structure and processing of primary gene transcripts is to draw attention to the fact that transcriptional controls are only part of a much more complex picture of regulation. It also serves to illustrate the problems inherent in any attempt to diagnose a direct role for the RNA polymerase per se in regulating the expression of specific coding sequences.

Transcriptional controls operate primarily at the level of the initiation of RNA synthesis. Considerable work is possible (and will be discussed later) on the initiation of the synthesis of rRNA and tRNA because detailed information is available on the precise structure of the primary gene transcript and the DNA sequences which code for them. From the foregoing discussion, it is apparent that, for mRNA, often (if not invariably) the structural gene may be separated from the RNA chain initiation site by a sequence many times longer than the message itself. The information necessary to enable us to study the factors which regulate the expression of a specific structural gene at the level of initiation of RNA synthesis will be contained in the DNA sequence

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5′ to the initiation site for the precursor molecule: this remains a considerable challenge for the future. In the meantime, using suitable hybridization techniques, it is possible to identify the transcription (in vivo or in vitro) of coding sequences for stable RNA species or mRNA. However, the conclusion that a sequence is transcribed does not necessarily mean that it will be processed to a biologically active form. One of the simplest examples of this is in the resting peripheral lymphocyte where the 45S precursor of 18S and 28S rRNA is synthesized but, in the maturation process, the 18S sequence is degraded.[20]

For all these reasons, studies on the regulation of RNA synthesis in eukaryotes at the level of the RNA polymerase remains a somewhat phenomenological art!

II. THE STATUS OF THE DNA-DEPENDENT RNA POLYMERASE IN NUCLEATED CELLS

The extent to which our understanding of the process of RNA synthesis in prokaryotes has developed over the last few years has been dramatic. This has been due largely to the ease with which the RNA polymerase may be isolated and purified from bacteria in large amounts. A heteromultimeric enzyme is involved in the expression of all types of RNA in the exponentially growing bacterial cell. The classical description of this multimer presents it as being made up of at least four types of subunit (B′, β, σ, and α_2) having a molecular weight of about 500,000 daltons. Even in the case of the well-characterized *Escherichia coli* enzyme, it has been argued that what is isolated and finally purified is the "most stable assembly" of polypeptide chains: efforts to achieve a homogeneous enzyme by classical protein purification techniques may eliminate certain less stable interactions which may be crucial in the selective expression of different classes of coding sequence.[21]

Although techniques were available 20 years ago for the study of transcription in vitro in animal cell nuclei, the general observation was that the RNA polymerase activity was (to all intents and purposes) exclusively associated with the chromosome as a transcription complex: the so-called "aggregate" enzyme.[22] This conclusion was probably conditioned by the types of experimental system used which was usually derived from rat liver! Had the problem been approached using rapidly dividing eukaryotic cells, it is likely that the whole development of this area of investigation might have taken on a rather different complexion. However, using the "aggregate" nuclear enzyme system, evidence appeared in 1966 which suggested that a multiplicity of polymerase activities was present in eukaryotic cells.[23] With the advent of techniques which "solubilized" the aggregate enzyme activity, the concept of multiplicity was confirmed, and three classes of enzyme were identified according to (1) their sequential elution from DEAE-Sephadex® (types I, II, and III)[24] and (2) their differential sensitivity to amanatoxins (class A [or I] were insensitive to α-amanitin concentrations up to $10^{-3}M$; class B [or II] were inhibited by concentrations in the range 10^{-9} to $10^{-8}M$, and class C [or III] were inhibited by intermediate concentrations in the range 10^{-5} to 10^{-4} M).[25] Neither of these two classifications is without ambiguity: we shall adopt the former and, as far as possible, relate published work to this system of nomenclature.

Several reviews of the multiplicity, structure, and function of these eukaryotic enzymes have been published recently.[26-29] In the brief synopsis of this vast area of experimental effort which follows, we examine the principles underlying the progress which has been made to construct a general picture of the machinery of RNA synthesis in nucleated cells. Conceptually, this area has been dominated by the notion that polymerase activity exists primarily as a nucleoprotein complex. This may be the case in cells with a low mitotic index (such as liver and thymus, probably the two most heavily

studied systems). Whether the same situation pertains in rapidly dividing cells whose metabolic state resembles more closely that of bacteria remains an open question.

A. Isolation and Purification of Eukaryotic RNA Polymerases

The common approach to the isolation of RNA polymerases is to resolve nuclei from whole cells or tissues. While giving rise to a formidable initial protein purification, this technique may select for polymerase which is tightly bound to the chromosome and, thus, may eliminate a large proportion of other polymerase pools (see below). In general terms, there are two techniques which are used to solubilize polymerase activity from nuclei: (1) by sonication in high-salt concentrations (0.3 *M* ammonium sulfate) or (2) by incubating nuclei in media containing Mg^{++} and low-salt concentrations.[30] The wide variation in detailed application of these techniques has been summarized elsewhere.[26] The precise mechanism by which these procedures gives rise to "soluble" polymerase activity is somewhat obscure. Treatment with high salt probably releases large amounts of protein from the chromatin, and sonication will then fragment the DNA.[31] The absolute dependence of the low-salt extraction procedure on the presence of Mg^{++} implies that chromosome fragmentation by nucleases is probably the primary phenomenon. Whether either of these procedures actually dissociates transcription complexes is not clear. A certain amount of evidence is accumulating (our own unpublished observations) to suggest that this is not the case: even after extensive purification of activities released from chromatin by either of these two techniques, the enzymes appear to remain bound to very short DNA fragments. The implications of this will be discussed below.

The initial resolution of multiple RNA polymerases is best accomplished by salt gradient elution from DEAE-Sephadex®, coupled with an assessment of α-amanitin sensitivity. In certain instances, it has been shown that the sequence of elution of different classes of enzymes is not invariant, and, in some lower eucaryotes, there is a decreased sensitivity to α-amanitin. This subject has been reviewed elsewhere.[28,29] Experience has shown that extensive purification procedures are required to yield homogeneous enzyme preparations.

B. Localization of RNA Polymerase Activities in Eukaryotic Cells

By carrying out simple cell fractionation techniques and making use of differential sensitivity to α-amanitin, the intracellular distribution of polymerase species has been studied. In approaching this problem, it must be recognized that an enzyme which is not associated with nuclear chromatin may still be contained within the nucleus or may be free to diffuse between the nucleus and the cytosol. Such a pool of polymerase activity may be diagnosed, even in the presence of enzyme bound to and transcribing chromatin: that which is "bound" to the chromosome (actively elongating RNA chains in vitro) can be blocked by high concentrations of actinomycin D, while that which is "free" or soluble will continue to be able to transcribe the synthetic template poly-d[A-T];[32] for reasons which are described in detail elsewhere[29] it is desirable to titrate the mixed system with actinomycin D to attain total suppression of the "bound" RNA polymerase. Although this technique will define the existence of a pool of "free" enzyme within a cell fraction, it is difficult to quantitate the relative size of this pool by this procedure alone as the transcriptional efficiency by polymerases of poly-d[A-T] varies between enzyme species.

Virtually regardless of the cell type, nucleoli contain only form I RNA polymerase.[33] The question arises as to whether this is the only location of this enzyme, and, on this point, information is scarce. The answer is probably conditioned by (1) the type of cell under scrutiny (having either a high or a low mitotic index) and (2) the technique used to isolate organelles. In our experience, nuclei which have been prepared by ho-

mogenization of rat liver in isotonic sucrose contain no pool of "free" polymerase I;[34] however, if homogenization in hypertonic sucrose is used, nuclei are found to contain "free" polymerase I,[34,35] and the data suggest that nuclei may be leaky with respect to the pool of "free" enzyme. It is unlikely that the pool of "free" enzyme is very large in rat liver as Seifart et al.[36] failed to identify significant quantities of type I polymerase in their analyses of a postnuclear supernatant, whereas another species of polymerase (III) was present. On the other hand, in the extranuclear fraction of HeLa cells,[34] mouse plasmacytoma,[37] and phytohemaglutinin- (PHA) stimulated periperal lymphocytes (unpublished data),[185] a considerable "free" pool of polymerase I was apparent. The comparison between these rapidly dividing cells and hepatic tissue may indicate a difference in the status of any polymerase depending on the growth state of the cells.

The situation with respect to type I polymerase serves to emphasize several important criteria which need to be considered if an overall view of RNA metabolism is to be appreciated. Firstly, any species of enzyme need not be (and, in fact, *is* not) restricted to a single locus in the cell: the cycle of reactions involving chain initiation and termination requires that enzyme be both bound to the chromosome and free at some time and, in the latter state, may equilibrate with an extrachromosomal pool; secondly, in restricting one's attention to a single locus, a part of the overall process may be overlooked; thirdly, by extracting the enzymes from whole cells, one obtains a complex of enzymes derived from more than one pool, and it is questionable at the moment whether it is possible to identify components of these pools in the mixture.

What has been said here with respect to polymerase I may equally well apply to both polymerases II and III. Both of these species exist as nucleoprotein complexes in the nucleoplasm. Using the poly-[dA-T]/actinomycin procedure, "free" pools of these enzymes have been recognized in rat liver nuclei prepared in hypertonic sucrose.[34] That there may be a considerable pool of "free" polymerase III is indicated by the common finding of this enzyme in the postnuclear supernatant of rat liver,[36] mouse myeloma,[37] and many other cell types.[28]

There is enough evidence available to suggest that the classical definition of intracellular distribution of these enzymes (I, nucleolar; II, nucleoplasmic; and III, nucleoplasmic and cytoplasmic) is too restrictive and should be extended to account for two pools of any one polymerase (at least). It is not yet practical to define the sizes of the pools of any single polymerase species, but it may be anticipated that at appropriate time during the cell cycle, the balance between that which is involved in the synthesis of RNA ("bound" enzyme) and that which constitutes the "free" pool may change. This situation and other factors regulating the "level" of polymerases in eukaryotic cells will be dealt with in depth in the section below concerned with control mechanisms.

C. Structure and Microheterogeneity and Eukaryotic RNA Polymerases

The analysis of the subunit structure of the nuclear RNA polymerase activities has confirmed that higher organisms contain three structurally distinct enzyme species (mitochondrial and chloroplast RNA polymerases will be omitted for this review). In general terms, all these enzymes bear a marked structural relationship to the well defined *E. coli* holoenzyme in being heteromultimers having molecular weights in the region of 500,000 daltons; each contains two large subunits (in excess of 100,000 daltons), which appear to be characteristic for each class of enzyme, and a number of smaller subunits (less than 100,000 daltons). Each class of enzyme is probably genetically distinct although there is immunological evidence that there may be some sharing of antigenic determinants: in yeast,[38] three small polypeptide chains appear to be common to types I and II polymerase, and there is evidence of a similar phenomenon in calf thymus[39] and in mouse plasmacytoma.[40] This whole subject is reviewed extensively

Table 1
THE SUBUNIT MOLECULAR WEIGHTS OF CLASS I, II, AND III RNA POLYMERASES FROM MURINE PLASMACYTOMA MOPC 315

Class I				Class II					Class III			
Common subunits		I_A	I_B	Common subunits		II_O	II_A	II_B	Common subunits		III_A	III_B
				(o)		240						
				(a)			205					
(a)	195											
				(b)				170				
									(a)	155		
				(c)	140							
									(b)	138		
(b)	117											
									(c)	89		
									(d)	70		
(c)		60	—									
									(e)	53		
(d)	52											
									(f_1)	49		
				(d)	41				(f_2)	41		
									(g)		32	33
(e)	29			(e)	29				(h)	29		
				(f)	27							
				(g)	22							
(f)	19			(h)	19				(i)	19		
				(i)	16							

Note: The molecular weights ($\times 10^{-3}$) of the subunits of each class of mouse myeloma MOPC 315 RNA polymerase is presented: those for classes I and II are derived from Sklar et al.[47] and for class III from Sklar et al.[40] Different RNA species within any one class of polymerase contain a number of "common" subunits, and microheterogeneity is defined in terms of the presence, absence, or variation in the molecular weight of one subunit only.

elsewhere.[21,27,28] Our review will concentrate on current concepts of microheterogeneity within polymerase classes. The subunit structures of mouse myeloma polymerases is summarized in Table 1.

1. Class I RNA Polymerase

Two species of form I RNA polymerase (I_A and I_B) have been described in a wide variety of systems.[29] The problem here is to distinguish between the possibilities that the two species are both physiological forms of the enzyme or that one may be derived from the other as an experimental artifact. The multiplicity of form I polymerases has been diagnosed chromatographically on DEAE-Sephadex® and phosphocellulose. In our experience, their order of elution is reversed on phosphocellulose, and, in constructing a common nomenclature between laboratories, we have taken this into account.[29] The two forms differ in their subunit composition. When extracted by the high-salt, sonication procedure from rat liver nucleoli, the I_A enzyme is composed of six subunits of molecular weight 195K, 128K, 60K, 44K, 26K, and 19K;[41] form I_B is identical, save that the 60K subunit is missing. Similar data arise from studies on enzymes purified from calf thymus[42] and the mouse plasmacytoma.[40] There is some question as to whether both species are physiological forms of the enzyme: from a detailed

analysis of the high-salt, sonication procedure,[34] it was shown that the conversion of I_A to I_B occurs, the extent of interconversion depending on the precise extraction conditions. When rat liver nucleoli were extracted by the low-salt incubation method, quantitative extraction was achieved which comprised only form I_A (although there was an apparent reduction in the molecular weight of the largest subunit from 195K to 175K, presumably by limited proteolysis[41]). In nucleoli, most (if not all) of the form I enzyme is in the form of a transcription complex; form I polymerase isolated from the cytoplasm of HeLa cells[34] and an extranuclear fraction of *Xenopus* ovaries[34] has chromatographic properties similar to I_B polymerase, although a subunit analysis of this enzyme has not been carried out to confirm true I_B structure as defined above. These findings have led us to conclude that the complete enzyme (I_A) is the transcribing form and I_B may constitute the unbound pool of this class of enzyme. Similar conclusions have been reached by Matsui et al.[43] More evidence is required before we can be certain that intracellular pools of this (or other) species of polymerase differ structurally, but the present evidence of experimental interconversion indicates that caution must be exercised before coming to this conclusion. In general, it has been difficult to demonstrate differences in the catalytic properties between the purified I_A and I_B enzymes.

2. Class II RNA Polymerase

Multiple species have been identified in form II enzymes isolated from a variety of eukaryotic cells. The difference between them lies in the molecular weight of the largest subunit: the form II enzymes from mouse plasmacytoma all contain subunits of molecular weight 140K, 41K, 30K, 25K, 20K, and 16K and a large subunit of 240K, 205K, or 170K (forms II_O, II_A, and II_B, respectively).[37] Biochemically, these forms are indistinguishable, and it cannot be discounted that the differences may arise as proteolytic artifacts.[44,45] Therefore, in mammalian systems, the physiological significance of form II heterogeneity remains unresolved. A potentially interesting situation has been shown to exist in certain plants. Two forms of type II RNA polymerase have been identified in germinating soybean which again differ only in the molecular weight of the largest subunit: in ungerminated embryos, the enzyme (II_A) is in a soluble form and has a 200K subunit; as germination proceeds, the enzyme becomes bound to the chromatin template, and, following extraction, the largest subunit is found to be reduced to 170K.[46]

3. Class III RNA Polymerase

While heterogeneity in type III enzymes has been demonstrated in a number of systems, the structural differences between enzymes of this class seem to be subtle. In the only well-characterized instance, the mouse plasmytoma (MOPC 315), a reduction in the molecular weight of one subunit (from 33K to 32K) appears to be the only recognizable distinction between forms III_B and III_A.[40] Again, one must be cautious concerning the physiological significance of this microheterogeneity. It may be relevant to point out that in initial cell fractionation experiments on these cells, Schwartz et al.[37] showed that forms III_A and III_B were concentrated in the nucleus and the cytoplasm respectively.

D. The Function of Each Class of Eukaryotic RNA Polymerase

The involvement of each class of RNA polymerase in the expression of different classes of genes has been implied from studies of the intracellular localization of the enzymes and from following the effects of α-amanitin on the synthesis of RNA species in vivo and in vitro. It is not our intention to duplicate the extensive reviews which already exist, but attention will be drawn to a number of pertinent pieces of recent work.

As nucleoli contain only form I RNA polymerase, a role in the expression of ribosomal RNA coding sequences was ascribed to this activity. Studies on the α-amanitin sensitivity of RNA synthesis by form I enzyme in isolated nuclei and nucleoli provided evidence of this.[48-50] Recently, the hybridization characteristics[51] and fingerprint analyses[52] of in vitro transcripts by the endogenous nucleolar form I enzyme both confirm the expression of rRNA coding sequences by this enzyme.

It is generally accepted that form II transcribes HnRNA (the putative precursor of mRNA) in its nucleoplasmic locus, and extensive studies both in vivo and in vitro using α-amanitin support this view.[28] Much of this evidence is circumstantial, but direct confirmation has emerged from a number of different approaches to the problem. Mutants have been produced in Chinese hamster ovary cells[53] and a BHK cell line[54] which contain an α-amanitin-insensitive form II RNA polymerase. Whereas the expression of polyoma virus genes in infected mouse 3T3 cells is inhibited by α-amanitin in the growth medium, somatic cell hybrids between these infected cells and the α-amanitin-resistant BHK cell mutant results in the α-amanitin-insensitive expression of polyoma functions.[54] This implies the direct involvement of form II polymerase in the transcription of viral messenger RNAs. Furthermore, SV40 can be isolated from infected mammalian cells in a form which contains polymerase II as a transcription complex.[55] An alternative approach has been to raise antibodies to form II polymerase of *Drosophila melanogaster*[56] and to show that these antibodies bind in the chromosomal interband regions and puffs which are known to be transcriptionally active in the larval salivary gland, adding further weight to the conclusion that form II is involved in mRNA synthesis.

Having established that polymerase III was inhibited by high concentrations of α-amanitin, 5S and pre-4S genes were shown to be transcribed by this enzyme in mammalian nuclei[50] and chromatin.[57] Furthermore, this enzyme has been implicated in the expression of viral 5.5S and cellular 5S and pre-4S RNA in adenovirus-2 infected HeLa cells[58] and 5.5S and 5S RNA synthesis late in productive infection of KB cells by adenovirus.[59]

E. Selective Transcription by Eukaryotic RNA Polymerases In Vitro

Functions for each class of RNA polymerase have been established, but the problem of reproducing selective expression of specific coding sequences in vitro has been intractable. There are a number of obvious reasons for this. The vast complexity of the eukaryotic genome has tended to restrict attention to those sequences which are heavily reiterated (particularly those for the stable RNAs, tRNA and rRNA). Even in these cases, attempts to reproduce in vitro the in vivo function of the different RNA polymerases has been complicated by our inability to isolate intact DNA duplex, devoid of single-stranded breaks ("nicks") which constitute pseudopromoters for the polymerases. Small bacteriophage DNAs provided the answer to these problems in studies of bacterial polymerase specificity, but animal viral DNAs have not proved to be the solution in the eukaryotic case. The reader is referred to Chambon's review article[28] where these difficulties are discussed in depth. We shall concentrate on recent experiments concerning the transcription of rRNA and 5S RNA coding sequences in vitro using purified RNA polymerases I and III respectively.

The work of Van Keulen et al.[60] and Van Keulen and Retel[61] on the transcription of rRNA coding sequences by the purified form I enzyme from yeast deserves careful scrutiny. Throughout their experiments, they use an enzyme prepared from whole cells of exponentially growing *Saccharomyces carlsbergensis*. The following facts are established:

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