EUKARYOTIC RNA POLYMERASES

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I. INTRODUCTORY BACKGROUND

The reading unit for genetic information is a complex enzymatic machinery named "DNA-dependent RNA polymerase" (EC 2.7.7.6). In bacteria, a unique enzyme, flanked with a variety of satellite proteins, performs the synthesis of both informational and structural RNAs. A decisive breakthrough in the understanding of the prokaryotic transcription system was made in 1969 with the discovery of the initiation factor sigma and of the termination factor rho. At the same period of time, the study of the eukaryotic transcription system was initiated by the successful solubilization and resolution of the mammalian enzymes by Roeder and Rutter.[1] After them, there were many investigations to demonstrate the multiplicity of DNA-dependent RNA polymerases in different eukaryotic cells. Now, after more than a decade of research, the existence in eukaryotes of three forms of nuclear RNA polymerases is well established on the basis of structural and, more recently, functional criteria. RNA polymerase A (or I) synthesizes ribosomal RNA precursors in the nucleolus, RNA polymerase B (or II) synthesizes mRNA precursors, and RNA polymerase C (or III) makes essentially tRNAs and 5S RNA.

The differences between the prokaryotic and eukaryotic RNA polymerases do not reside in the overall mechanism of the synthetic reaction. DNA is transcribed into RNA using ribonucleoside triphosphates as substrates. RNA synthesis requires a divalent cation, usually magnesium, and shows an absolute requirement for DNA or a synthetic nucleic acid as template. Direction of synthesis is 5′ to 3′ and the base sequence of the synthesized RNA is complementary to the DNA template strand. The particularity of the eukaryotic transcription system resides first in the exceptional complexity of the enzymatic machinery. Each of the three forms of nuclear RNA polymerases has more polypeptide components than the unique bacterial enzyme. This molecular complexity, in relation with that of the eukaryotic genome, probably reflects the variety of regulatory controls that are exerted on these enzymes through interactions with chromatin components and specificity factors. The second major difference between bacterial and eukaryotic RNA polymerases is the complete deficiency of the purified eukaryotic enzymes to carry the accurate transcription of an isolated gene. Besides the fact that this situation delayed the analysis of gene expression in eukaryotes, it also suggested the possibility of new regulatory mechanisms involved in gene activation.

In the past years the study of nuclear RNA polymerases at the structural and functional level has gone through successive phases of development. The finding of multiple RNA polymerase activities,[1] supported by their differential sensitivity to the fungal toxin α-amanitin,[2] has encouraged a general search for this family of enzymes in a large number of different organisms, from lower eukaryotes, to plants, insects, crustacea, amphibians, and mammals. In all cases where they have been sought with appropriate techniques, three main forms of RNA polymerases have been found. By 1972, it was clear that the eukaryotic

transcription system was really tripartite. Confirmation of this conclusion had to come from the structural analysis of the purified enzymes. Therefore, during a second phase of research, nuclear RNA polymerases were purified, again from all sorts of organisms, with variable success. Lower eukaryotes being more amenable to biochemical studies, yeast RNA polymerases have been the most extensively investigated, although RNA polymerases of other organisms, including *Acanthamaeba*, wheat germ, *Drosophila*, *Xenopus laevis*, mouse, and chicken, and calf, have also been characterized in some detail. RNA polymerases purified from a single cell type are large molecules with molecular weights between 400,000 and 700,000, have complex subunit structures, and can be differentiated on the basis of their polypeptide content.

At the functional level, after repeated unsuccessful attempts to transcribe properly isolated viral or cellular genes using the purified enzymes, the selectivity of in vitro transcription has been examined in cellular extracts supplemented or not with the appropriate RNA polymerase. The discovery of soluble cell-free systems capable of accurate transcription of exogenous DNA opened a third phase of research: the identification of DNA sequences, promoters, terminators and regulatory signals, which direct the accuracy and efficiency of RNA chain initiation and termination. It also allowed the fractionation of the *factors* required for specific transcription by each of the three forms of RNA polymerase. Factor isolation was complicated by the fact that multiple components intervene in gene expression in addition to RNA polymerases.

This review focuses on the structure, genetics, regulation, and properties of the nuclear RNA polymerases and their specific factors and for the most part neglects the description of the genetic signals in the DNA which are involved in gene expression. Pioneer work on isolation, characterization, and function of multiple forms of eukaryotic DNA-dependent RNA polymerases has been treated in three major reviews.[3-5] A comprehensive review focusing mainly on nuclear RNA polymerases has been published recently,[6] as well as a specialized review on yeast RNA polymerases.[7] A mini-review on the molecular structure of nuclear RNA polymerases is available.[8]

II. PURIFICATION OF RNA POLYMERASES

As mentioned previously, an enormous amount of time and effort has been invested in the purification and characterization of eukaryotic nuclear RNA polymerases from an increasing number of organisms (see Figure 1 for references). This situation has not been very favorable for in-depth analysis of RNA polymerases because of unavoidable redundancy. Starting from different tissues also precluded the use of standard purification procedures.

A. General Approach to Large-Scale Purification

From lower eukaryotes to man, RNA polymerases present the same overall physical properties which can be exploited for purification and resolution of the three forms of enzymes. Most purification procedures were adapted from past experience with *Escherichia coli* RNA polymerase. The most helpful parameters were the large size of the proteins, their net acidic charge, and their affinity for binding nucleic acids or other anionic polymers. Because of their acidic charge, the RNA polymerases bind to DEAE cellulose or DEAE Sephadex, but they also bind strongly to phosphocellulose, heparin Sepharose, or CM Sephadex, like the bulk of basic proteins, probably because of their general affinity for nucleic acids. This dual affinity for both cationic and anionic exchangers gives a very powerful means of purification. However, this may also lead to a specific enrichment into a family of DNA binding proteins or enzymes which could interfere with enzyme activity. A sizing step is, most of the time, introduced to get rid of small-molecular-weight proteins at the end of the purification. The sizing procedure is generally a sedimentation in glycerol

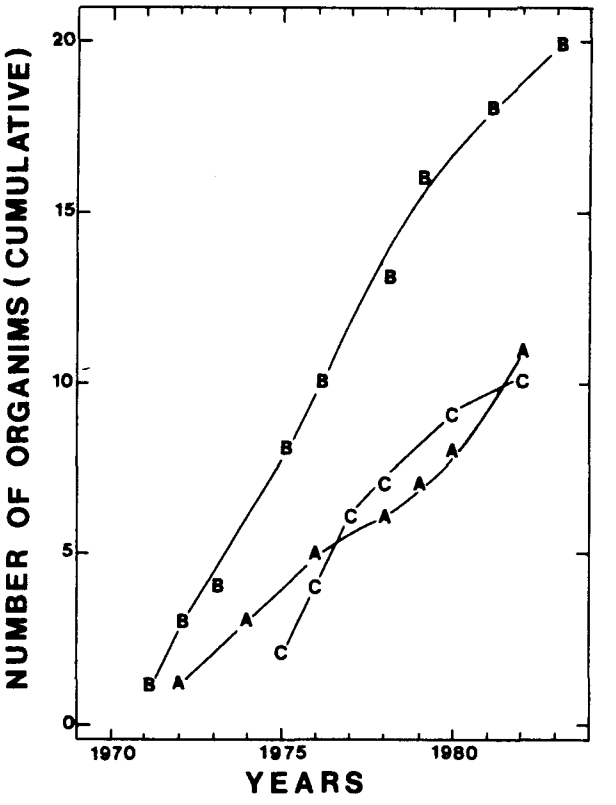

FIGURE 1. Organisms from which RNA polymerases A, B, or C were purified to near homogeneity. RNA polymerase A was purified from calf thymus,[9] mouse plasmacytoma,[10] *Saccharomyces cerevisiae*,[11] rat liver,[12] soybean,[13] *Acanthamoeba castellanii*,[14] *Aspergillus nidulans*,[15] cauliflower inflorescence,[16] *Drosophila*,[17] *Schizosaccharomyces pombe*, and *Candida tropicalis*.[18] RNA polymerase B was purified from rat liver,[19,20] calf thymus,[20,21] *Saccharomyces cerevisiae*,[22] human Hela and KB cells,[23] mouse plasmacytoma,[24] *Drosophila*,[25] wheat germ,[26] parsley,[27] hen liver and oviduct,[28] cauliflower,[29,30] *Physarum polycephalum*,[31] rye and maize,[32] pea,[33] *Agaricus bisporus*,[34] *Acanthamoeba castellanii*,[35] *Aspergillus nidulans*,[36] *Chiromus thummi*,[37] mouse ascites cells,[38] and *Xenopus laevis*.[39] RNA polymerase C was purified from mouse plasmacytoma,[40,41] *Saccharomyces cerevisiae*,[42] *Bombyx mori*,[43] wheat germ,[44] KB cells,[45] *Acanthamoeba castellanii*,[46] *Drosophila*,[47] and *Podospora comata*.[48] The figure shows the cumulative number of enzyme sources as a function of time. Enzyme B was always the most extensively investigated, while enzyme C after a lag has attracted much interest.

or sucrose gradients which keeps the enzyme concentrated and protects it from denaturation. Gel filtration has also occasionally been used.

There has been little evolution of the methodology in recent years. It concerns mostly the early steps of purification, i.e., the solubilization of enzyme activity and the elimination of nucleic acids from the crude extract. Various procedures were used to dissociate RNA polymerase from the chromatin, the most efficient and popular, originally described by Roeder and Rutter,[1] is sonication in high-ionic-strength media (see the review article of Jacob[49]). Direct measurements with radioactively labeled amanitin confirmed the efficiency of this method to quantitatively release enzyme B from animal tissues.[50] However, low salt

extraction procedures are increasingly being used for solubilization of RNA polymerases A, B, and C, mostly from lower eukaryotes,[14,15,31,35,36,46,51] but also from plants,[26,30,33,44] *Xenopus laevis*,[39] and calf thymus.[52] Actually, the first eukaryotic RNA polymerase to be extensively purified (the B enzyme) was solubilized from yeast in a low-salt buffer by Frederick et al.[53] in 1969. Surprisingly, however, there is little information on the effect of varying the salt concentration, in the extraction buffer, on the extent of solubilization of RNA polymerases.

Another improvement in the methodology concerns the removal of nucleic acids by precipitation with polyethylene imine (Polymin P). First used by Zillig et al.,[54] this procedure was adapted to plant[26] and mammalian enzyme B.[52] Polyethylene imine (PEI) fractionation now appears to be of general value for purification of the three forms of RNA polymerases from all eukaryotes.[14,15,31,32,34,35,39,46,48,51,52,55] One advantage of the method is that at low-salt RNA polymerases coprecipitate with nucleic acids. The enzymes are thus rapidly separated away from proteases and can be eluted sequentially by buffers of increasing ionic strength. Alternatively, the pellet can be stored frozen. Some progress has been mentioned in the use of DEAE Sepharose CL-6B, which has a higher capacity than other DEAE-substituted ion exchangers.[31,55] Heparin-agarose is also increasingly used.[14,31,44-46,48,55] However, this material is not well characterized and, in our hands, showed more variability than phosphocellulose in the salt concentration required for elution of the yeast enzymes. On occasions, enzyme activity is lost after elution, possibly through binding of the enzyme to coeluted solubilized heparin. Hydrophobic chromatography on amino butyl-Sepharose has been used with some success with plant enzymes B,[56] and C.[44] The unusual behavior of RNA polymerase C on DEAE-substituted matrixes[1,58] could well be related to its special aptitude for engaging nonionic interactions.[57] In the future, affinity chromatography with monoclonal antibodies could provide a new and powerful means to purify RNA polymerases. Dissociation of the enzyme-antibody complex might be feasible under conditions which maintain its physical integrity and activity.[59]

Demonstration of enzyme purity can be best achieved by analytical electrophoresis on polyacrylamide gels under nondenaturing conditions. The polypeptides present at the level of the protein band should correspond to the polypeptide pattern of the enzyme preparation observed prior to electrophoresis. There is the perplexing problem that the enzymes may lose some weakly bound components upon electrophoresis. Those are not necessarily contaminants and could play a crucial role in regulation of transcription. Partial dissociation of RNA polymerase is likely to be a reversible process. Often, both the complete and dissociated forms of enzyme can be visualized on the gel.[10,42,60] It is important that the enzyme activity be localized at the level of the protein band after electrophoresis. This was shown only in few cases.[6] Data on the overall purification based on determination of enzyme activity at the various steps of purification are not very informative. Activity at the earlier stages is likely to be affected by contaminant enzymes, DNA binding proteins, or stimulatory substances. Adding a known amount of pure enzyme to crude fractions provides a means to correct the activity data.

B. Microscale Purification

Methods of purification from small amounts of cells were developed to investigate regulation of enzyme levels or enzyme modifications during physiological transitions. First, the three enzymes can be separated from a crude extract on DEAE Sephadex, and their activity quantitated.[1] However, activity measurements may be influenced by the presence of varying interfering components (proteases, nucleases, phosphatases, and stimulatory factors) which themselves can be affected by the physiological transitions under study. More recently, small-scale purification procedures have been developed for all three forms of yeast RNA polymerases.[18,51,60] ^{32}P or ^{35}S-labeled RNA polymerases A or B can be rapidly obtained in homogeneous form, starting from 2 g of yeast cells.[60-63] Immunoprecipitation

of RNA polymerase from partially purified fractions has been used to obtain yeast enzyme A[62] as well as RNA polymerase B from yeast,[63] growing plant tissues,[64,65] *Drosophila*,[25] and CHO cells.[66] The enzymes are pure enough for their polypeptide content to be visualized by SDS-gel electrophoresis. Because the three RNA polymerases are immunologically related (see below) it is necessary to use polyclonal or monoclonal antibodies to unrelated subunits or to separate the three enzymes by a preliminary purification step.

III. THREE FORMS OR CLASSES OF RNA POLYMERASES?

Three main forms of enzymes were originally separated by chromatography on DEAE Sephadex, and classified on simple criteria like chromatographic properties, salt optimum for activity, divalent cation requirement, template preference, and more specifically by their differential α-amanitin sensitivity.[1-5,67] There was a general agreement that these criteria, which were the only ones available in the early period of eukaryotic RNA polymerases, were not stringent enough. However, despite their obvious weaknesses, they proved, in general, adequate to properly differentiate the three forms of RNA polymerases originating from a particular cell type. They were somewhat less reliable when trying to correlate enzyme forms of different organisms.

Increasing resolution in the chromatographic analysis of RNA polymerases disclosed the existence of minor chromatographic forms which could be related, on the basis of α-amanitin sensitivity, to one of the three major forms. Each major form could also be further resolved into subspecies by additional ion exchange chromatography or gel electrophoresis under nondenaturing conditions.[4,5] These observations and the finding of multiple forms of the largest subunit in mammalian RNA polymerase B led to the proposal of the existence of three distinct classes of RNA polymerases, each constituted of distinct subspecies.[3,68,69] This concept of enzyme classes suggested that intraclass enzymes could be endowed with distinct specificity or be under different regulatory control.[24,35,70-74] (A Gram-positive bacterium, like *Bacillus subtilis*, accommodates five different forms of holoenzyme, each containing a different type of sigma subunit which directs RNA polymerase to different classes of promoters.) Now, after almost a decade, how does this notion of enzyme classes stand? As discussed below, one will see that enzyme alterations during purification appear to be one of the major causes for RNA polymerase heterogeneity. Two kinds of gross structural alterations were shown to occur: proteolysis of subunits and dissociation of some polypeptides.

1. Proteolysis of Subunits

The finding of multiple forms of the largest subunit of form B enzymes was the first demonstration at the structural level of enzyme B heterogeneity.[3] RNA polymerase B purified from calf thymus,[21] mouse,[24] yeast,[74] or *Physarum*[31] can be resolved into different species, by electrophoresis under nondenaturing conditions, which differ essentially by the molecular weight of their largest subunit in the range of 240,000 to 180,000. The number of enzyme B subforms differing by their largest subunit vary depending on the organism and cell type. Hence, hen liver,[28] mouse ascite cells,[38] *Drosophila* larvae,[37] ungerminating plant embryos, plant actively proliferating tissues,[33] or fungi,[36] give one main form of enzyme B; yeast,[75] calf thymus,[21] and amoeba[35] enzyme B often appears as two main species called B_I and B_{II} (or II_A and II_B); enzyme B purified from rat liver,[19] hen oviduct,[28] and mouse plasmacytoma,[24,40] can be resolved into three clearly defined species called B_0, B_I, and B_{II} (or II_0, II_A, and II_B); at the extreme, a multiplicity of forms, each varying slightly in the size of the largest subunit, were isolated from *Physarum polycephalum* by Smith and Braun.[31] The number of enzyme B subforms also varies with changes in purification procedures. Improvements in speed of purification and care in prevention of protease activity yield enzymes with a largest subunit of increased size.[37,75-77b] The nature of enzyme B species varies also

with the physiological state of the cells, or the cell type. Greenleaf et al.[76] found a subunit of 215,000 daltons in a *Drosophila* cell line instead of a 174,000-dalton component in the larvae or in embryos. Similarly, RNA polymerase B of *Physarum* has a subunit of 215,000 daltons when isolated from microplasmodia in early exponential growth, whereas in late exponential phase a different enzyme form is found, with a subunit of 170,000.[77a] During the germinating process, the plant enzyme B is progressively altered, its largest subunit being modified in size from 215,000 daltons in ungerminated embryo to 180,000 daltons in the enzyme purified from growing axes.[65,72,73]

Several observations strongly suggest that this subunit heterogeneity is a proteolytic artifact occurring during purification:

1. Yeast,[75] *Drosophila* embryo extracts,[76] and proliferating plant extracts,[73,77b] contain a protease activity capable of cleaving the 220,000-dalton subunit to the 180,000-dalton subunit. Plant embryo tissues do not have this protease activity.
2. Immunoprecipitation of enzyme B from crude or partially purified extracts of yeast,[63] plant,[65] *Drosophila*,[76] and mammalian cells[66] yields an enzyme highly enriched in the large subform of the largest subunit. This was particularly striking in yeast where the 185-kDa subunit was totally replaced by the 220-kDa polypeptide[63] and also in growing plant tissues[65,77b] where was found predominantly a 220 kDa polypeptide previously present only in enzyme B from ungerminated plant embryos.
3. One- or two-dimensional peptide mapping showed that the various subunit subforms are structurally related in primary sequence in yeast,[78] plant,[73,79,80] and mammalian enzyme B.[81] Moreover, the different subunit subforms are strongly immunologically related.[37,81-84] Although these structural and immunological studies cannot be used to assess the absolute degree of homology between these polypeptide subforms, they strongly suggest that they derive from the same polypeptide by proteolysis.
4. A mutation in the gene for the largest subunit of yeast enzyme B alters both the peptide pattern of the large native subunit of 220 kDa and that of 180 kDa.[78] This conclusively demonstrates that, at least in yeast, these two polypeptides are the product of the same gene. A similar conclusion can be reached with mammalian cells. The two subforms of RNA polymerase B, B_I, and B_{II}, from a myoblast α-amanitin-resistant cell line, exhibit the same partial resistance to the drug.[85] This indicates that the mutant allele codes for a subunit common to both enzyme subforms. This subunit being recently identified as the largest subunit (see below), it follows that the largest subunit of the polymerase B subforms, although varying in size, are the product of the same gene.

The present observations can be interpreted in simple terms: there appears to be a general susceptibility of the largest subunit of RNA polymerase B from all organisms to proteolytic attack; this proteolysis occurs predominantly in vitro, and at selected sites to give defined subspecies of the large subunit (in mammalian enzyme B, $B_{240} \rightarrow B_{215} \rightarrow B_{180}$). The primary cleavage step is probably very rapid, and in some cases, could make the native unproteolyzed subunit remain elusive.[76,77b] At this stage, the simplest conclusion is that this molecular heterogeneity of RNA polymerase B is unlikely to have a physiological significance for transcription regulation. Furthermore, in practice, whenever a single form of RNA polymerase B is isolated with a largest subunit under 200 kDa,[30,33,34,36,38,73,79] it can be suspected that this RNA polymerase preparation is proteolyzed.

Proteolysis of the largest subunit is not restricted to enzyme B. There are several reports on the occurrence of a similar phenomenon with RNA polymerase A from plant,[16,29,64] *Artemia salina* larvae,[86] and from rat liver.[87] Proteolytic cleavage, as with enzyme B, generates a smaller discrete subunit of about 170 kDa, which was found by peptide mapping to be structurally related to the larger component (190 kDa).[16] In *Artemia salina*, the ap-

pearance of a new chromatographic form of RNA polymerase A was detected in extracts from developing larvae but not in embryos. The enzyme conversion could be mimicked in vitro by a protease which is induced in the larvae, and could be prevented by addition of soybean trypsin inhibitor to the larvae extracts.[86] Generally, however, proteolysis of RNA polymerase A does not seem to be a major problem, in contrast to enzyme B. Indeed, immunoprecipitation of enzyme A from yeast crude extracts yields an enzyme whose molecular structure and subunit size are identical to that of the enzyme obtained by a large-scale purification procedure.[62,63] There is no indication as yet on a possible proteolysis of the large subunits of RNA polymerase C.

2. Enzyme Variants Lacking Polypeptides

Dissociation of polypeptides during purification without concomitant loss of general polymerase activity can be another cause for enzyme heterogeneity. Dissociation of enzyme components has been reported for all three forms of RNA polymerases from the yeast *Saccharomyces cerevisiae*. The first instance reported was the dissociation of two polypeptides from enzyme A during phophocellulose chromatography or electrophoresis, to give an enzyme variant called A*.[60] The A → A* transition was also observed with RNA polymerase A from other genetically distant yeast species.[18] The same phenomenon occurs with yeast RNA polymerase B which gives rise, upon DEAE-Sephadex chromatography or polyacrylamide gel electrophoresis (PAGE), to an enzyme variant called B*, devoid of two polypeptide components.[75] In both cases, this partial enzyme dissociation could be favored by a mild urea treatment,[78,88] and the dissociated polypeptides could be recovered. This argued in favor of a true dissociation reaction rather than a preexisting heterogeneity revealed by chromatography or electrophoresis. Yeast RNA polymerase C similarly loses three components upon electrophoresis on polyacrylamide gel under native conditions.[42] In all these cases, the complete enzyme and its simplified form were separated by chromatography and/or gel electrophoresis.

Heterogeneity of RNA polymerase A due to the loss of dissociable polypeptides has been reported in other systems. The mouse myeloma enzyme was the first to be resolved in two species by gel electrophoresis,[10] one of which lacked a 60-kDa component. Calf thymus[89] and rat liver[12] enzyme A can also be obtained in a simplified form lacking a similar 50 to 65 kDa polypeptide by chromatography. Disparities in the subunit composition of RNA polymerase A purified by different procedures from yeast,[90] *Acanthamoeba*,[14] and *Drosophila*[17] have also been noted. Although in all these cases one could not exclude a preexisting heterogeneity, it remains likely that dissociable polypeptides can reversibly interact with the basic catalytic unit of enzyme A as demonstrated in the yeast system.

Loss of polypeptides was seldom reported for RNA polymerase B, with the above-mentioned exception of the yeast enzyme. During electrophoresis, several components dissociate from *Acanthamoeba* enzyme B,[35] but there is no report on the activity of this simplified enzyme. Actin (~45,000 kDa) was detected in several highly purified preparations of RNA polymerase B from *Physarum polycephalum*,[91] *Acanthamoeba*,[35] or animal cells.[38] There is no clear evidence that actin interacts with the enzyme molecule. If it does, the complex can be easily dissociated by phosphocellulose chromatography or gel electrophoresis[91] or sedimentation.[38] RNA polymerase C from *Podospora comata* tends to lose a polypeptide of 87 kDa upon phosphocellulose chromatography or centrifugation in a glycerol gradient.[48] The enzyme fraction depleted in that polypeptide has altered chromatographic properties. A polypeptide of similar size (80 kDa) dissociates from yeast,[42] and *Acanthamoeba*[46] enzyme C upon electrophoresis but not from the mammalian[41] or *Xenopus* enzyme.[92]

3. Microheterogeneity

Gross structural alterations, like proteolysis and loss of subunits, which are easily detected by one-dimensional electrophoresis in the presence of SDS, do not account for all reported

cases of enzyme chromatographic or electrophoretic heterogeneity.[4,5,43,92] Increased sophistication in analytical techniques has also disclosed minute variations in the molecular size or isoelectric point of enzyme components which do not detectably change the enzyme electrophoretic or chromatographic properties.[18,32,41,73,93] The biological significance of these small variations is uncertain. In view of the complexity of the enzyme molecular structure, the scarcity of information on subunit function (see below) and the many possibilities of structural modifications during purification, the analysis in great detail of subunit heterogeneity would, at the present time, rather confuse the issue than bring useful information.

To conclude this survey on RNA polymerase heterogeneity, it appears that, in many cases, enzyme subforms can be generated by gross structural alterations occurring during purification (proteolysis, loss of subunits), while other reported cases of chromatographic or electrophoretic heterogeneity could not be attributed yet to a detectable structural change and remain unexplained. As discussed by Lewis and Burgess,[6] enzyme chromatographic behavior in partially purified fractions could be influenced by multiple interactions of RNA polymerases with a variety of contaminants. Microheterogeneity of purified enzyme preparations, on the other hand, could originate from discrete structural alterations during purification (dephosphorylation, minor proteolytic degradation, etc.) and does not necessarily reflect the in vivo situation.

From these considerations, it appears that the experimental basis which originally suggested the existence of enzyme classes has not received additional support in recent years. When juxtaposed to the concept of *promoter classes*, which has been well established on structural and functional grounds for class B and class C promoters, the notion of classes of RNA polymerases similarly implies a difference in specificity (or regulatory state) of enzymes within a given class. Until it is demonstrated that distinct subspecies of RNA polymerases have different function or are differently regulated, it would seem more appropriate to return to the original concept of *three forms of nuclear RNA polymerases* in a given cell type, a concept which fits better to the emerging idea that enzyme specificity is determined primarily by general or promoter-specific transcription factors rather than by changes in the basic catalytic unit. The term of class A, B, or C RNA polymerase could be reserved to refer to the corresponding form of enzyme in different eukaryotic organisms.

IV. MOLECULAR PROPERTIES AND POLYPEPTIDE COMPOSITION

A. Molecular Complexity of RNA Polymerases

The large size of the nuclear RNA polymerases was the first indication that they were multimeric enzymes. Molecular weights of 500,000 to 650,000 have been deduced for each form of enzyme from their sedimentation behavior,[4,6] and their migration in nondenaturing polyacrylamide gels of graded porosity.[22,94] RNA polymerase B (or C) and *Escherichia coli* RNA polymerase can be cosedimented in a glycerol gradient and assayed independently by virtue of their differential sensitivities to α-amanitin and rifampicin.[24,43] The eukaryotic enzymes sedimented faster than the bacterial holoenzyme (480,000 Da). Analysis of purified eukaryotic RNA polymerases progressively revealed their enormous structural complexity.[3-5] Indeed, the three forms of RNA polymerases, A, B, and C, are each multisubunit enzymes composed of two large distinct polypeptides and a collection of several smaller polypeptides.[4,5]

More recent results have confirmed this complex structural organization, and have shown that the subunit structure was even more complex than originally reported. The main reason for increasing complexity with time was essentially a better inventory of the polypeptide content of purified enzyme preparations using high-resolution PAGE in one or two dimensions, and also the realization by the investigators that the presence of small components or of heterogeneous protein bands may prove to have some implication in enzyme activity, or

regulation of transcription. The same trend had been observed earlier after the initial separation of eukaryotic RNA polymerases into three major chromatographic forms by Roeder and Rutter.[1] Duplication of their experiment using different cell extracts, with increasing resolution has disclosed the existence of additional minor chromatographic forms which were carefully indexed.

The first investigators who have succeeded in purifying sufficient amounts of nuclear RNA polymerases from vertebrates or[10,24,28,41,94] plant,[26] insect,[25] or yeast cells,[42,75,60] spent many efforts to convince themselves that all the polypeptides present in their RNA polymerase preparations were part of the enzyme. Several approaches were followed to demonstrate the association of a given polypeptide with the enzyme molecule: it should cochromatograph and cosediment with enzyme activity and comigrate with the enzyme activity by electrophoresis in polyacrylamide gel under nondenaturing conditions. Gel electrophoresis is certainly the best technique to separate the enzyme in homogeneous form, based both on the size and charge of the protein. However, identification of the small polypeptides by subsequent electrophoresis under denaturing conditions was often hampered by the limited amount of enzyme which could be applied to the native gel. In subsequent works, much less care was taken to rigorously prove the association of the multiple polypeptide components with the enzyme molecules — this conclusion being often derived by analogy with previously characterized enzymes.

B. Enzyme Subunit: A Changing Concept

The term "subunit" was originally loosely defined on simple operational criteria; any polypeptide which was shown to be associated with the enzyme molecule using a variety of purification techniques could be qualified as a subunit of RNA polymerase. In addition, its molar ratio, by reference to one of the large subunits, had to assume an integral value (of one, or admittedly two for small polypeptides). The sum of the molecular weights of all the putative subunits had to be consistent with the estimated molecular weight of the native enzyme (see Roeder[5] for discussion of the subunit status). Unfortunately, even in cases where such information was only partly available or which contradicted the above rules, the term "subunit" has been largely adopted to refer to all the polypeptides identified in purified RNA polymerase preparations.

In recent years, new information has accumulated on the molecular structure of eukaryotic RNA polymerases and progressively, the possible role and importance of certain components has emerged.

1. The *universality* of certain polymerase components is strongly suggested by the comparison of the molecular structure of RNA polymerases from an increasing number of different eukaryotic organisms.
2. One important finding is the discovery of a *core of common subunits* shared by the three forms of enzymes. Additional polypeptides are also shared by RNA polymerases A and C (see the section on common subunits). These polypeptides are likely to be part of the fundamental enzyme structure.
3. Immunological studies of RNA polymerases with antibodies directed to individual enzyme components have disclosed *immunologically related polypeptides* in the same form of RNA polymerase from different eukaryotes or in the three enzyme forms in the same cell type. Hence, antigenic determinants are remarkably conserved in the pair of large polypeptides in different organisms (see below).
4. Isolation of *enzyme variants* lacking certain polypeptides has in certain cases contributed to approach the minimal structure required to carry the basic steps of transcription.
5. *Probes for subunit function*, specific inhibitors, or subunit-directed antibodies have been used in attempts to identify essential subunits. An interesting finding was the identification of a polypeptide interacting with an α-amanitin derivative.

6. Although the genetic approach is still very limited, *mutants with altered RNA polymerases* have been isolated. In two occasions the mutated subunit could be identified.

These new developments call for a reformulation of the definition of enzyme subunit based on more stringent, structural, functional, and genetic criteria. Tentatively, a polypeptide component could be accepted as an enzyme subunit if, in addition to being structurally associated to the active enzyme molecule, it satisfies at least one of the following criteria:

1. It is structurally altered in mutants of RNA polymerase
2. It is the target of a specific inhibitor of RNA polymerase
3. It belongs to the pool of common subunits
4. It is structurally related to an homologous polypeptide universally present in the same class of RNA polymerase from different eukaryotic organisms
5. It gives a lethal phenotype upon gene disruption or deletion in cases of single copy genes

Dissociable polypeptide components which are dispensable for RNA polymerase activity with nonspecific templates in vitro will not be considered as subunits until they are shown in the future to play a role in specific transcription. Polypeptides arising by proteolysis of larger subunits (in vitro or in vivo) are not considered as distinct components until they are shown to have a special role in transcription in vivo.

Ultimately, the definition of the polymerase subunit structure should rest on demonstration of the requirement of each enzyme component for the structural or the functional integrity of RNA polymerase. The obtention of mutants for each structural gene will be required for demonstrating the need of each constituent in vivo.

In Table 1, the above criteria were applied to yeast RNA polymerases which are to date the best-characterized enzymes.

Two different nomenclature systems have been used to name the polypeptide chains associated to the RNA polymerases. The simplest one is the alphabetical or numerical order starting from the largest component. This system has the disadvantage of being difficult to adapt to changes in polypeptide composition which arise from deletion of irrelevant components (contaminants or proteolyzed polypeptides) or insertions of new components identified by using higher-resolution separation techniques or improvement of enzyme purification procedures. Furthermore, it does not facilitate the direct comparison of the structure of a given class of enzyme from different sources. We prefer a second system, which adapts easily to modifications and facilitates comparisons of enzyme structures. Polypeptide components are identified by a letter (or a number) indicating the RNA polymerase from which it derives, and a subscript, corresponding to its molecular weight $\times 10^{-3}$. Common subunits can be easily identified by two or three letters, like ABC_{27} or AC_{40} in yeast RNA polymerases, or by the one-letter system, when the enzyme origin of the subunit has to be clearly specified.

C. Polypeptide Content of RNA Polymerases A, B, and C from Different Organisms

1. RNA Polymerase A

Table 2 summarizes the polypeptide content of highly purified preparations of form A RNA polymerase from lower eukaryotes, plants, insects, amphibians, and mammals. The best source of RNA polymerase A is the yeast *Saccharomyces* for which rapid large-scale and microscale purification techniques have been described.[7] With a remarkable consistency, class A RNA polymerases show a pair of large polypeptides of about 190 and 130 kDa. The number of the small associated polypeptides varies widely, from a total of four in some plant and mammalian enzyme A preparations to 10 or more in lower eukaryotes. This difference could be attributed to several causes. Early reports on the molecular structure did

Table 1
APPROACH TO THE SUBUNIT STRUCTURE OF RNA POLYMERASES FROM *SACCHAROMYCES CEREVISIAE*

RNA polymerase form		
A	**B**	**C**
190[aij]	220[bcjp] (185)[d]	*160*[e,f]
135[am]	150[bc]	*128*[f]
49[ef]		*82*[f]
43[fgj]		53[fg]
40[a,i,hk]	44.5[bl]	*40*[h]
		37[f]
34.5[ej]	32[e]	34[n]
		31[n]
27[ai]	*27*[i]	*27*[i]
23[aij]	*23*[hij]	23[ij]
19[hj]	16[e]	*19*[hj]
14.5[i]	*14.5*[ih]	*14.5*[i]
14[n]	*12.6*[b]	11[n]
12.2[n]		
10[o]	10[o]	10[o]

Note: In italics are the polypeptides which have qualified as subunit following the criteria listed in the text.

[a] Bear antigenic determinants present in RNA polymerase A from genetically distant yeast species.
[b] Bear antigenic determinants present in enzyme B from different eukaryotic organisms.
[c] Gene essential for growth (simple copy gene in haploid cells).
[d] Proteolytic product of the B_{220} subunit.
[e] Absent in simplified form of enzyme (A* or B*). Dispensable with nonspecific templates.
[f] Absent in certain enzyme preparations and dispensable with nonspecific templates.
[g] Molar ratio <1.
[h] Polypeptide common to RNA polymerases A and C.
[i] Polypeptide common to RNA polymerases A, B, and C.
[j] Phosphorylated in vivo and in vitro.
[k] Homologous polypeptide identified in genetically distant yeast species.
[l] A polypeptide of that size is present in the same form of polymerase from all sorts of eukaryotic organisms.
[m] Monoclonal antibody inhibits enzyme activity.
[n] No specific comment.
[o] Poorly characterized.
[p] Structurally modified in mutant RNA polymerase B.

Table 2
POLYPEPTIDE COMPOSITION OF RNA POLYMERASE A (OR I) FROM VARIOUS EUKARYOTIC CELLS

Saccharomyces cerevisiae[7]	*Acanthamoeba castellanii*[14,93]	Cauliflower inflorescence[16]	Wheat germ[6]	*Drosophila melanogaster*[17]	*Xenopus laevis*[39]	Mouse plasmacytoma[10,40]	Calf thymus[89,94,96]
190	185	190	200	195	195	195	197
		(170)					
135	133	125	125	125	125	125	126
49	41.5		50	48	55	61	51
43							
40	39—37	38	38	41	41	52	44
34.5	35			38			
27	22.5	25	20	25	29	29	25
23	17.5	22	17.8	18.5	23	19	16.5
19	15.5	17.5	17		19		
14.5	13.3				18		
14	<10						
12.2							
<10							

Note: Polypeptides are identified by their molecular weight $\times 10^{-3}$

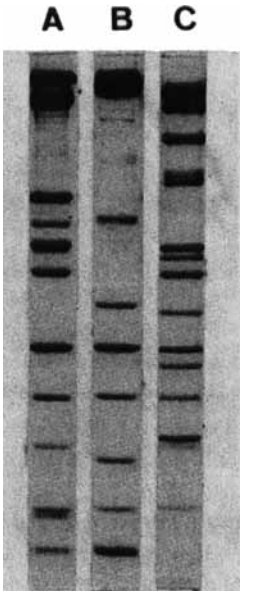

FIGURE 2. RNA polymerases A, B, and C from *S. cerevisiae* analyzed by electrophoresis on a polyacrylamide gel under denaturing conditions with SDS. The molecular weight of the polypeptides are given in Table 1. Enzyme B is form B_{II} (with B_{185} polypeptide). (Figure courtesy of J. Huet.)

not use appropriate techniques to resolve all the components; low-molecular-weight polypeptides were not systematically taken into account or, being faintly stained, could have remained undetected; finally, some components loosely associated to the enzyme could be more easily lost in certain cases, depending on the purification procedure or the cell type.

The actual association of all these polypeptides into a multimeric enzyme has been well demonstrated in the case of the yeast,[60] and mammalian enzyme,[4,10] by their cosedimentation with enzyme activity and their comigration in nondenaturing polyacrylamide gel. Additionally, subunit-specific antibodies have been used to identify the polypeptides that are an integral part of the yeast enzyme A structure. When ^{35}S-labeled enzyme A was immunoprecipitated with antibodies to its largest subunit, A_{190}, all but one of the polypeptides, $A_{34.5}$, were coprecipitated at the same molar ratio as in the purified enzyme.[60]

A careful comparison of the polypeptide content of RNA polymerase A from different yeast species has shown the existence of a natural variation in its molecular structure.[18] Several criteria (size, charge, dissociation upon electrophoresis, and in vivo phosphorylation) were used to establish a correspondence between homologous polypeptide components of the various enzymes. Whereas enzyme A from different *Saccharomyces* species showed the same polypeptide pattern, the enzyme from *Schizosaccharomyces pombe* or *Candida tropicalis* lacked components homologous either to A_{49}, A_{43}, $A_{34.5}$, A_{14}, or $A_{12.2}$. Based on these correlations, the minimal subunit composition of enzyme A from the yeast *Saccharomyces* was tentatively defined as A_{190}, A_{135}, A_{40}, A_{27}, A_{23}, A_{19}, and $A_{14.5}$. Remarkably, this minimal structure of seven polypeptides is made of the two large polypeptides plus the subunits common to enzymes A and C. Incidentally, this study showed that similarity of molecular weight is not a sufficient criterion to identify cognate subunits in enzymes of the same class. For instance, in *S. pombe*, the polypeptide equivalent to A_{23} and $A_{14.5}$ (which both belong to the core of common subunits in *S. cerevisiae*) did not correspond to the polypeptides of

similar molecular weight but to smaller components based on phosphorylation and isoelectric point.[18]

2. RNA Polymerase B

Of the three forms of nuclear RNA polymerases, the B enzyme has been the most widely studied at the structural level in a large variety of organisms, in lower eukaryotes, *S. cerevisiae*,[75] *Physarum*,[77a] *Aspergillus*,[36] the soil amoeba *Acanthamoeba*,[35] in higher plants,[30,32,33,65,72,73,79,97] insects,[25,37] *Xenopus laevis*,[39] and mammals[21,24,39,52,96] (see Figure 1 and Table 3).

RNA polymerase B has the largest of all the polypeptides associated with the three forms of enzyme, in a molecular weight range of 220,000. As discussed previously, this subunit is highly susceptible to proteolysis. In early works, and even in more recent ones, it was completely missing in some enzyme B preparations which contained, instead, one or several polypeptides of reduced size (under 200,000 Da).[22,25,30,33,34,36,52] The best source of unproteolyzed enzyme B is probably seed embryo tissues like wheat germ,[32,97] or ungerminated soybean embryonic axes,[32,65,73] which are in a quiescent state and have a low protease content.

The second largest subunit also has a very conserved molecular weight in all organisms (around 140 to 150 kDa). In contrast to subunit B_{220} it generally gives an homogeneous band on SDS-PAGE and therefore should be preferred as a reference to calibrate the molar ratio of the other components. The small polypeptides always have a molecular weight less than 45,000 and their number varies from six in most vertebrate enzyme preparations to eight or nine in plants and lower eukaryotes. This variation again seems to depend more on the exhaustive compilation of the small components than to reflect a fundamental difference.[52,98] Interspecies comparison of enzyme B structure is in support of the subunit status of all these components. Insect RNA polymerase B from *Drosophila* and *Chironomus* are very similar in the size and number of components.[37] The small components of plant enzyme B from soybean, wheat, cauliflower, rye, and maize have only minor differences in size and show highly similar two-dimensional gel electrophoretic patterns.[32] This indicates a conservation of not only molecular weight, but also of isoelectric point of enzyme subunits. More remarkably, the mouse and *Xenopus* enzyme B (form B_I or B_{IIA}) are indistinguishable in the number and size of their associated polypeptides.[39] SDS-PAGE does not necessarily give accurate molecular weight values, but these comparisons are valid since, in these studies, the protein samples were analyzed simultaneously under the same conditions. Finding similar polypeptide patterns in enzyme B from different eukaryotes is not a decisive proof, however, for their being *bona fide* enzyme subunits. If the same purification protocol is used to isolate the enzymes to be compared, the preparations could carry similar contaminants.

The $B_{44.5}$ polypeptide appears to have its counterpart (based on size) in enzyme B from all organisms. In yeast this polypeptide is sensitive to proteolytic degradation.[75] In *Acanthamoeba*[35,93] and wheat germ enzyme B,[32,97] the protein of that size comes also as a doublet of bands which are very similar in charge.[93,97] That this protein is part of RNA polymerase B is further supported by the strong cross-reactions observed between yeast and wheat germ RNA polymerase B using antibodies specifically directed to yeast $B_{44.5}$ polypeptide.[99] Similarly, antibodies to yeast $B_{12.6}$ also strongly recognized the wheat germ enzyme and weakly the one from *Drosophila*. The immunological relationship of class B RNA polymerase from different organisms will be described in more detail in a later section.

3. RNA Polymerase C

RNA polymerase C has been purified from a variety of cell types: from lower eukaryotes, *S. cerevisiae*,[42,90,100,101] *Acanthamoeba castellanii*,[46] *Podospora comata*,[48] wheat germ,[55] insects, *Drosophila hydei*,[47] *Bombyx mori*,[43] *Xenopus laevis*,[39,40] mammals, mouse plas-

Table 3
POLYPEPTIDE COMPOSITION OF RNA POLYMERASE B (OR II) FROM VARIOUS EUKARYOTIC CELLS

Saccharomyces cerevisiae[75]	*Acanthamoeba castellanii*[35,93]	*Physarum polycephalum*[77]	Soybean axes[65,73]	Wheat germ[6]	*Drosophila melanogaster*[37]	*Xenopus laevis*[39]	Mouse plasmacytoma[24,39]	Calf thymus[52,98]
220	193	215	215	220	215	220	240	240
(185)	(178)	(170)	(180)		(175)		(205)	(214)
							(170)	(180)
150	152	135	138	140	140	142	140	140
44.5	40—38.5	38	42	42—40	32	40	41	34
32	22.5	26	27	27—25	25	29	30	25
27	18	23	22	21—20	20	23	25	20.5
23	15.5	17	19	20	18	20	22	18
16	13.3	14	17.6	17.8	17.5	19	20	17.5
14.5	12.5	13	17	17	15	16	16	16.5
12.6	12	9	16.2	16.3	<15			
10	<10		16.1	16				
			14	14				

Table 4
POLYPEPTIDE COMPOSITION OF RNA POLYMERASE C (OR III) FROM VARIOUS EUKARYOTIC CELLS

Saccharomyces cerevisiae[7,42]	*Acanthamoeba castellanii*[46,93]	*Podospora comata*[48]	Wheat germ[55]	*Bombyx mori*[43]	*Drosophila hydei*[47]	*Xenopus laevis*[39]	Mouse plasmacytoma[41]	KB cells[45]
160	169	174	150	155	154	155	155	155
128	138	129	130	136	135	138	138	138
82	82	87	94	67	62	94	89	86
53	52	50	55	62	58	68	70	63
40	39—37	39	38	49	38	41	53	43
37	34	23	30	39	32	39	49	34
34	30	21	28	36	31	33	41	32
31								
27	28.5	19	25	31	27.2	29	33—32	27
23	22.5	17	24.5	28	26.5	24	29	22
19	17.5	16.5	20.5	18	21.5	23	19	
14.5	15.5	13.5	20		17.5	19		
11	13.3	11	19.5					
	<10	10	17.8					
			17					

macytoma,[41] and KB cells.[45] The polypeptide content of RNA polymerase C is distinctly different from that of enzyme A or B from the same organism. This was well established in the case of mouse,[41] amphibian,[39,40] plant,[55] yeast,[42] and amoeba,[46] where the three forms of enzymes have been purified and their polypeptide content analyzed in the same slab gel in the presence of SDS. The existence of common subunits in the three forms of enzymes or in two of them has much simplified the identification of *bona fide* subunits (see the section on common subunits). Putting aside the two large components and all the common subunits, there remains a group of several polypeptides whose subunit status is less convincing.

An indirect way to reach a "consensus" molecular structure for an enzyme as complex as RNA polymerase C is to compare the enzyme isolated from different cell types (Table 3). From lower eukaryotes to human cells, the overall structure of RNA polymerase C is strikingly conserved. Like RNA polymerase A and B, it is characterized by two large subunits of molecular weight around 160 and 130 kDa and a complex collection of smaller components with a molecular weight range between 10 and 90 kDa. Roeder and collaborators have purified to homogeneity RNA polymerase C from human, mouse, *X. laevis*, and insect cells. Comparing their molecular structure on the same gel slab under denaturing conditions, they found a striking homology in the number and size of their polypeptide components.[39,43,45] The size range of most polypeptides in all eukaryotic cells (including higher plants) appears to be fairly conserved. One characteristic component of intermediate size, around 80 to 90 kDa is found in all cases except in insects.[43,47] The lack of this component has been initially correlated with the anomalous resistance to α-amanitin of the insect enzyme C compared to form C enzyme from other animal sources.[43] This hypothesis now appears unlikely since the yeast enzyme C is also resistant to the toxin, while having a component of that size (C_{82}). No immunological studies have been conducted yet to identify homologous subunits in class C enzymes from different sources. This approach would probably contribute to establish a fundamental molecular structure based on the conservation of important antigenic determinants. Only in a few cases were the polypeptide components shown to meet the elementary criteria of subunits as discussed by Roeder.[5,41] Some polypeptides were not found consistently when the enzyme was purified by alternative procedures, as in the case of wheat germ[44,55] or yeast enzymes.[42,102,103] One problem concerns the low-molecular-weight subunits which may be only partially resolved, with incidence on molar ratios and number of associated components. For this reason, with improvements of analytical techniques, the number of putative subunits of enzyme C has risen steadily, reaching values of 14 to 17 components.[46,55] RNA polymerase of class C therefore appears to be the most complex of all three forms of RNA polymerases. Whether this complexity is real and reflects particularities of function and regulation of this enzyme, this sort of question, as for enzymes A and B, would require the assignment of a function to the various polymerase components.

There were reported observations of enzyme C variants which lost some components upon chromatography[48] or gel electrophoresis.[42,46] Another type of structural heterogeneity is the replacement of one low-molecular-weight subunit by a polypeptide of slightly different size: component C_{32} is replaced by C_{33} in two chromatographically different forms of the mouse enzyme.[41] These observations have not yet been exploited to compare the specificity of these enzyme variants in reconstituted transcription systems.

D. Structural Relationship of the Multiple Components of Each Enzyme Form

Are the polypeptide components of a given enzyme distinct gene products? This question arose in view of the molecular complexity of nuclear RNA polymerases, which altogether comprise about 30 components (see Table 2). Could some of the smaller polypeptides be generated by partial proteolysis of larger components, as was already demonstrated for the largest subunit of the B enzyme? The available biochemical and immunological evidence do not support this view. Rabbit antibodies to each individual polypeptide component of

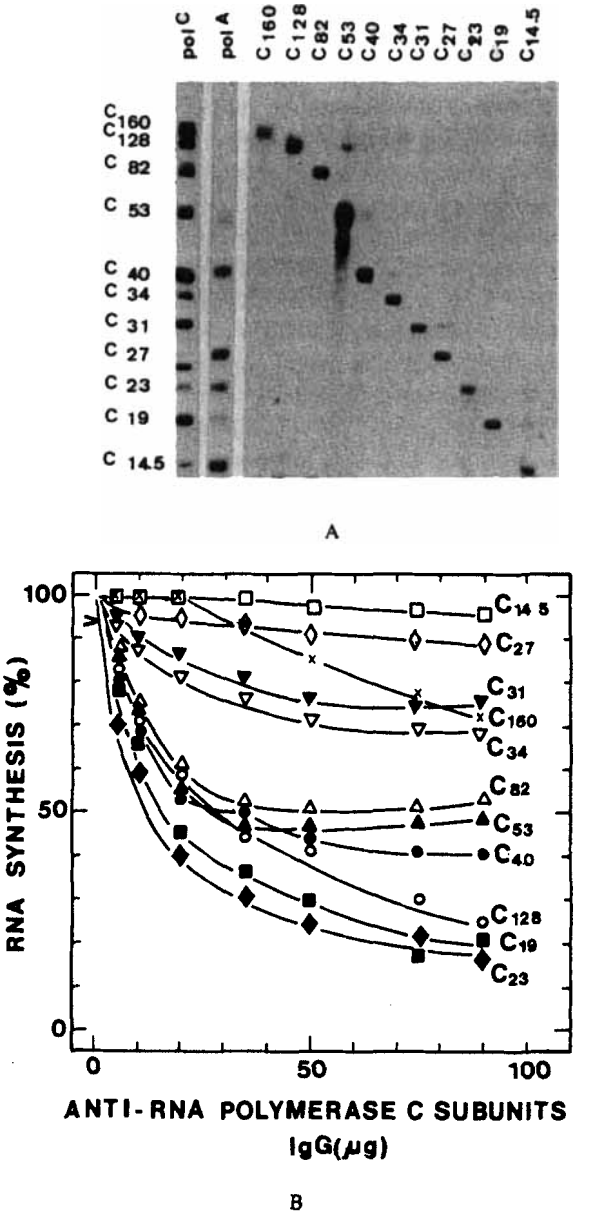

FIGURE 3. (A) Specificity and inhibitory properties of antibodies to each polypeptide component of yeast RNA polymerase C. (A) The subunits of enzyme C were separated by electrophoresis with SDS and probed with different antibodies. (Antibodies are identified by the name of the enzyme or polypeptide chain used as antigen: pol C, C_{160}, etc.) (B) The effect of the same antibodies on enzyme C activity was determined with poly d(A-T) as template.

yeast RNA polymerases A, B, or C reacted specifically by blot-immunodetection with the corresponding subunit[104a,104b] (see also Figure 3). Weak side reactions of antibodies to unrelated subunits, which were occasionally detected on a nearest-neighbor polypeptide, could simply reflect a trace contamination of the injected antigen. This comprehensive survey of the immunological relatedness of the yeast RNA polymerase components did not disclose any precursor-product relationship except, of course, in the case of the $B_{220} \rightarrow B_{185}$ conversion. The results were confirmed for the animal and plant enzyme B; in the case of the

large components, antibodies to B_{220} subunit from *Drosophila* or wheat germ enzyme B reacted specifically with B_{220} and not with B_{140} from the same enzyme.[83,112a] (However, a slight cross-reaction with B_{220} was found in the reverse situation with anti-B_{140}.[83,112a]) In these experiments, performed with poly-specific antibodies, mostly the immunodominant sites of the protein were explored, specifically those which persisted after electrophoresis of enzyme subunits in the presence of SDS. Experiments with monoclonal antibodies directed at a unique epitope, though not appropriate to give a general description of antigenic structures, are also interesting as they could possibly disclose conserved sites. A collection of monoclonal antibodies directed at several subunits of yeast enzyme A (A_{190}, A_{135}, A_{49}, A_{43}, and $A_{14.5}$) was obtained after immunization of mice with the native enzyme.[105] All of these antibodies reacted specifically by blot-immunodetection with one single subunit confirming the results obtained with poly-specific antibodies. Similarly, monoclonal antibodies to the largest subunit of mammalian enzymes A[106] or B[82,84] did not bind significantly to other subunits. A monoclonal antibody to the second-largest subunit of RNA polymerase A of the silkworm also seemed highly specific.[106] In contrast, the first described monoclonal antibody directed to a eukaryotic RNA polymerase, by Krämer et al.,[107] cross-reacted with the two large subunits of insect RNA polymerase B. The antibody bound more tightly to the largest component,[37] suggesting a region of incomplete homology.[363] Taken together, the above results support the idea that the two large subunits of A, B, and C enzymes are distinct gene products and are unrelated to the smaller components.

Peptide mapping of the two large subunits of yeast,[78] plant,[73,79,80] and mammalian enzyme B[38,81] led to a similar conclusion. Analysis of the tryptic peptide maps of the iodinated large subunits of wheat, parsley, and maize enzyme B showed that they are not structurally related to each other[80] (one does not consider here the proteolytic byproduct of the largest subunit). Comparing the two-dimensional tryptic map of each subunit of RNA polymerase B from maize,[108] or from Ehrlich ascites tumor cells,[38] clearly confirmed that they were distinct proteins not structurally related. Dahmus,[81] however, noted some similarities in the tryptic or thermolysin peptide patterns of the two large subunits of calf thymus enzyme B, but only when the subunits were iodinated before dissociation of the native enzyme. In most of the preceding experiments, the subunits had been first isolated prior to iodination. Regions of limited homology could explain the cross-reactions mentioned above. At any rate, it remains clear that the two large subunits of enzyme B are the products of separate genes. Possible regions of homology will be best analyzed at the level of the genes rather than with the proteins (see the gene cloning section).

From the above immunological and biochemical results, it seems reasonable to conclude that the different polypeptide components of a given enzyme are distinct proteins and the product of different genes. This gives the impressively large total of 22 to 25 *distinct* polypeptides in the three isoforms of RNA polymerases.

V. STRUCTURAL AND PHYLOGENETIC RELATIONSHIPS OF THE THREE FORMS OF RNA POLYMERASES

A. Common Subunits

The presence of common subunits in the three forms of nuclear RNA polymerases was originally suggested by the finding of polypeptide chains having identical molecular weights in enzymes A and B from calf thymus[3,94] and yeast,[11] and extended to RNA polymerase A, B, and C in the mouse system.[5,40] Early immunological studies also suggested some common antigenic determinants in A and B enzymes.[109,110]

The existence of common subunits is now well established, especially in the yeast enzymatic system which has been the most extensively examined (see Sentenac and Hall[7] for a detailed discussion). In yeast, the common subunits are AC_{40}, ABC_{27}, ABC_{23}, AC_{19}, and $ABC_{14.5}$. This conclusion was based on a variety of criteria (see Table 1):

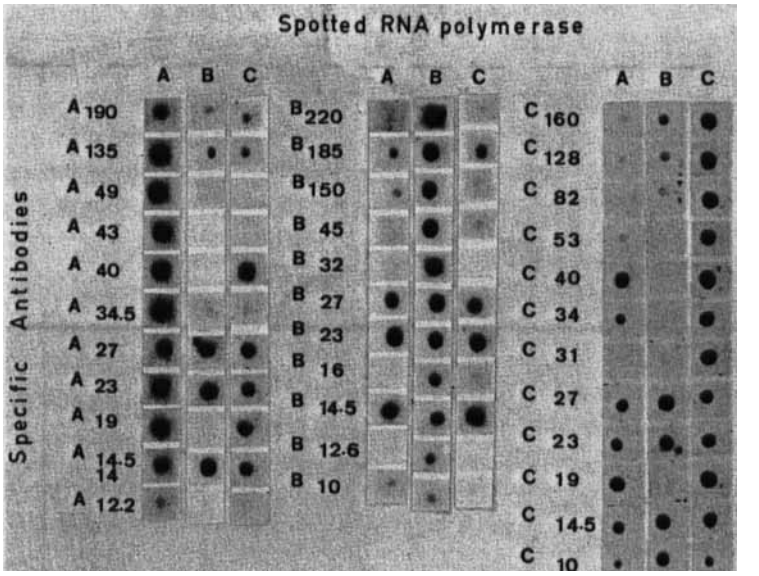

FIGURE 4. Immunological relatedness of the three forms of yeast RNA polymerases investigated with antibodies to the isolated subunits of each enzyme form. Purified RNA polymerase A, B, or C was spotted on nitrocellulose filters and challenged with the different antibodies. Bound antibodies were revealed by ^{125}I-labeled protein A. Antibodies are identified by the name of the subunit used as antigen. The cross-reaction of anti-C_{34} with enzyme A is due to contamination with anti-C_{40}. (Figure courtesy of J. Huet.)

1. Common subunits have identical molecular weights and identical isoelectric points as determined by two-dimensional mapping of the dissociated enzymes.[61,102,103,111] The charge difference between AB_{23} and C_{23}, observed by electrophoresis in urea, possibly arose from a different degree of phosphorylation.[103]
2. The fingerprint pattern of ^{35}S-labeled polypeptides are indistinguishable in the case of AB_{27}, AB_{23}, and $AB_{14.5}$.[61]
3. Furthermore, ABC_{23} as well as AC_{19} are phosphorylated in vivo.[51,62]
4. The common polypeptides were also identified after transfer to a membrane using antibodies to the native enzymes A or B,[104a] or directed against the individual components of enzymes A, B,[104b] or C,[364] or, in the case of subunit $ABC_{14.5}$, using a monoclonal antibody directed to $A_{14.5}$.[105]

On the basis of these antibody data, it was concluded that yeast enzymes A, B, and C share three polypeptides (ABC_{27}, ABC_{23}, and $ABC_{14.5}$) while enzymes A and C have two additional polypeptides in common (AC_{40} and AC_{19}). There are no subunits in common between enzymes A and B only. Figure 4 summarizes the immunological relatedness of the yeast enzymes.

Similar observations were reported by Paule and collaborators which analyzed the structure of the three forms of RNA polymerase of *A. castellanii* by two-dimensional gel electrophoresis; RNA polymerases A, B, and C appear to share three subunits ($ABC_{22.5}$, $ABC_{15.5}$, and $ABC_{13.3}$), and RNA polymerases A and C share three additional components ($AC_{17.5}$, AC_{37}, and AC_{39}).[93] The last two components of 37 and 39 kDa sometimes migrate as a single band on polyacrylamide gel and have the same charge.[14,46] Therefore, they could be structurally related, the AC_{37} component possibly deriving from AC_{39} by proteolysis.

In plants, two-dimensional gel analysis of RNA polymerases A and B from cauliflower

inflorescence suggested that these two enzymes share three small polypeptides.[16] These three subunits from enzyme A reacted with antibodies raised against cauliflower enzyme B. Enzyme C also contains three polypeptides of similar size as analyzed by one-dimensional SDS-PAGE. Wheat germ enzymes A, B, and C also have three polypeptides of identical size (20, 17.8, and 17 kDa).[55] If no additional subunits, shared by enzymes A and C only, were originally detected in cauliflower,[16] wheat germ enzymes A and C have one 38,000-dalton component which was reminiscent of subunit AC_{40} of the yeast system. These results have been reexamined and extended in a recent immunological study of plant RNA polymerases with antibodies raised against several subunits of enzymes B, by Guilfoyle et al.[112a] The general conclusions were similar to those reached previously with the yeast enzyme system, with a few interesting differences. Based on size and antigenicity, the three forms of cauliflower RNA polymerases were found to share a core of three subunits, ABC_{25}, ABC_{19}, and $ABC_{17.5}$. In addition, enzymes A and C also share one subunit, AC_{39} (instead of two in yeast). Also at variance with the yeast system, wheat germ enzymes B and C appear to share one polypeptide, BC_{25}, and the B_{40} subunit is antigenically related to AC_{38}.

Originally, only two putative common subunits have been detected in mammalian enzymes (25 and 16.5 kDa in calf thymus enzymes A and B,[94] or 29 and 19 kDa in mouse RNA polymerases A, B, and C[40]). In addition, one polypeptide of 52 kDa was found in both enzymes A and C and one polypeptide of 41 kDa in B and C enzymes from mouse cells.[40] Later, Dahmus showed that the tryptic peptide map of the iodinated 25-kDa subunits of calf thymus RNA polymerases A and B were identical.[96] A recent report on *X. laevis* RNA polymerases A, B, and C clearly demonstrated the existence of three components of identical size in the three forms of enzymes (29, 23, and 19 kDa).[39] Antibodies prepared against the amphibian enzyme C recognized the common 29-kDa polypeptide. This work also confirmed the existence of one additional polypeptide of identical size (41 kDa) in enzymes A and C whereas no polypeptides were detected, possibly common to enzymes A and B or B and C only.

Some general considerations or comments can be made at the present time concerning common subunits in nuclear RNA polymerases:

1. The presence of common polypeptides appears to be a general feature of RNA polymerases from all eukaryotic organisms. Their universality make them good candidates as *bona fide* subunits of RNA polymerases.
2. A core of three subunits, belonging to the pool of small-molecular-weight components, is shared by the three enzymes. This applies to lower eukaryotes and plant and amphibian RNA polymerases. That only two common components were detected in mammalian RNA polymerases, rather than being regarded as an exception, could be simply due to the difficulty of visualizing the small-molecular-weight components. Remarkably, the largest component of this core of common subunits is extremely basic (pI = 9)[16,61,93] and is always present with a stoichiometry of two (based on Coomassie blue staining). The other two common subunits are acidic in charge (pI $\sim$ 4.5) and with a stoichiometry of one.
3. A and C RNA polymerases share additional components which are not present in enzyme B. The number of these polypeptides specific to A and C enzymes varies with the organisms; there are two in *S. cerevisiae*, possibly three in *A. castellanii* (with the possibility discussed above that two of these could be structurally related), and one in mammalian cells and possibly also in plants. This variation may reflect the looser association of common regulatory components or again a difficulty in analyzing small components. Interestingly, the common subunit to A and C enzymes in the 40- to 50-kDa range seems universally conserved in all organisms.
4. Reports on possible common subunits specific to RNA polymerases A and B or B and

C only in plants or mammalian cells, though interesting, are still preliminary. Clearly, more definitive studies are required to confirm these observations.

5. There is very limited information on the function of the common polypeptides. One could suppose that the core of three common subunits shared by all three enzymes plays an essential part in the function of the fundamental enzymes. Subunits shared by enzymes A and C could possibly be involved in the coordinated regulation of these two enzymes which are responsible for synthesis of stable RNA species.
6. The presence of common polypeptides in three multimeric enzymes which otherwise are made of different polypeptides has interesting structural and evolutionary implications which are discussed in the following section.
7. Finally, it will be interesting to investigate the organization of the genes coding for the common subunits, whether or not there are multiple genes under different regulatory control, and how the balanced and coordinated synthesis of the common and unique components of each RNA polymerase form is achieved.

B. The Large Subunits of the Three Enzymes

In each form of RNA polymerase, the two large polypeptides represent between 50 to 70% of the total enzyme mass. They constitute the structural core of the enzymes with the common subunits. They are likely also to constitute the functional core together with the common subunits and to participate in the basic steps of RNA synthesis. Their large size, on the other hand, makes them the preferential target for interactions with a variety of specificity or regulatory factors or chromatin components as the enzymes transcribe different classes of genes and are located in different parts of the nucleus. These considerations suggest that two sorts of divergent constraints were exerted on these large polypeptides. One was to *conserve* in the cognate subunits some domains essential for the interaction with the common subunits, and for the enzyme activity (like the template, substrate, or RNA product binding sites). The other was to *evolve* some protein domains particular to the specific function, location, and regulation of each enzyme. Several observations are in favor of this dual evolutionary trend.

The first observation is the general conservation of an α-amanitin binding site in both form B and C RNA polymerases. Exceptionally, in yeast, it is form A enzyme which retains some α-amanitin sensitivity,[60] not enzyme C.[7] In the animal enzyme B it is the largest subunit which is the likely target of the toxic peptide (see below). The cognate subunit in C enzyme has probably conserved a region of limited homology.

Work with polyspecific antibodies directed at each of the large subunits of yeast enzymes A or B using a sensitive spot-immunodetection technique revealed a small but discrete cross-reaction between the three yeast enzymes (see Figure 4).[99] The cross-reacting polypeptides were identified by blot-immunodetection after separation of the enzymes subunits by SDS-PAGE;[104b] the largest subunit of A and B enzymes (B_{220} and A_{190}) were found slightly immunologically related, as well as A_{135} and B_{150}. Therefore, there is a size conservation in cognate subunits. These observations were confirmed in higher cell systems. A monoclonal antibody to the second largest subunit of enzyme A of the silkworm showed some cross-reactivity with polymerase B and C.[59] A monoclonal antibody to A_{190} subunit from hepatoma cells RNA polymerase A was inhibitory to both enzyme A and C.[106] Subunit B_{140} and A_{125} of the plant enzymes share some related antigenic sites.[112a] Although encouraging, these immunological data should be considered with some caution as only weak cross-sections were observed. There was no attempt to compare the tryptic peptide map of the large subunits of the three enzymes. Their map complexity would preclude a meaningful comparison (inasmuch as no similarity in tryptic peptide maps were detected in the homologous large subunits from different plant species).[80] Again, more information will be derived in the near future from gene studies.

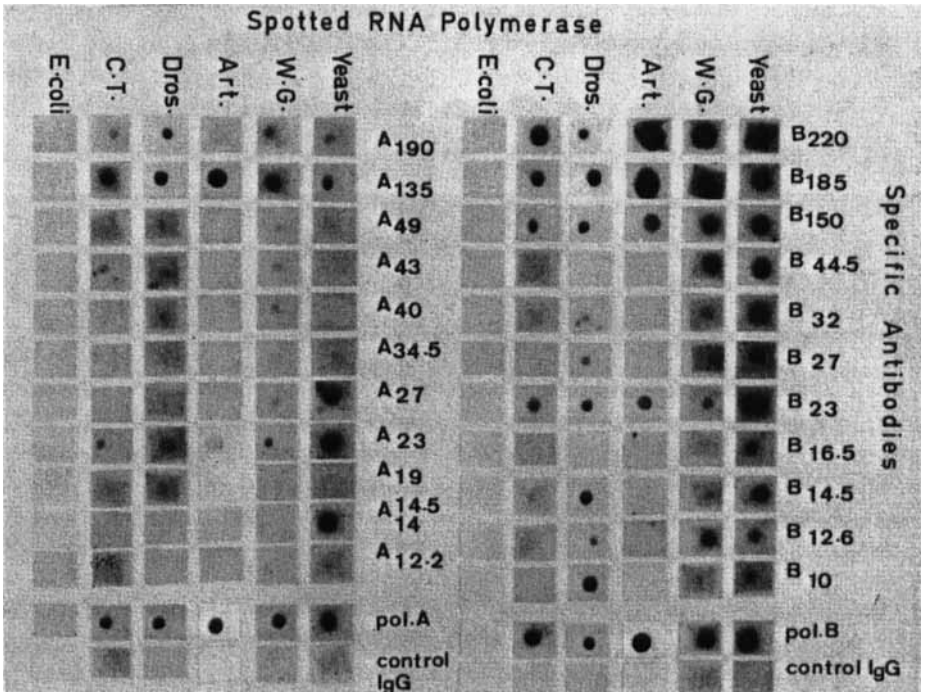

FIGURE 5. Immunological relationship of class B RNA polymerases as visualized by spot-immunodetection. Samples of purified RNA polymerases B from various eukaryotic sources (wheat germ, *Artemia salina*, *Drosophila*, calf thymus) were spotted and analyzed with specific antibodies to yeast RNA polymerases A or B subunits as indicated in Figure 3. Note the cross-reactions with the large subunits and the common subunit AB_{23}. *E. coli* RNA polymerase was also used and showed no cross-reaction with any antibody.

C. Phylogenetics Studies

1. Conservation of Immunological Determinants

Studies with polyspecific antibodies raised against native RNA polymerase B from different sources have in general revealed a cross-reactivity of class B enzymes.[37,83,104a,110] The conserved immunological determinants were found, by blot-immunodetection, to reside mostly in the two large subunits,[37,83,104a] with the largest one being more conserved.

Studies with antibodies raised against isolated subunits confirmed and extended these observations. RNA polymerase A from genetically distant yeast species was probed with polyspecific antibodies to individual subunits of *S. cerevisiae* enzyme A.[18] There was a marked conservation of immunologically related sites in the two large subunits as well as in AC_{40} (common to enzymes A and C). On the other hand, only weak cross-reactions were detected with the small subunits. Huet et al.[99] explored the immunological relationship of form B RNA polymerase from yeast, plant, insect, crustacea, and mammal, using a collection of antibodies to each of the components of the yeast enzymes B or A (Figure 5). There was a general cross-reaction of all B enzymes with antibodies to the large subunits of yeast enzyme B (B_{220} and B_{150}). Remarkably, a small cross-reaction was also observed with antibodies to the large components of yeast enzyme A (A_{190} and A_{135}). All of the B enzymes also shared a few immunological determinants with ABC_{23}, one of the common subunits. In addition to these general cross-reactions, other specific antibodies reacted with wheat germ or *Drosophila* enzyme B (see Figure 5). From these results, it appears that all the subunits did not evolve at the same pace. A stronger degree of structural and functional constraint must be exerted on the two large subunits as well as on some of the common subunits (AC_{40} and ABC_{23}).

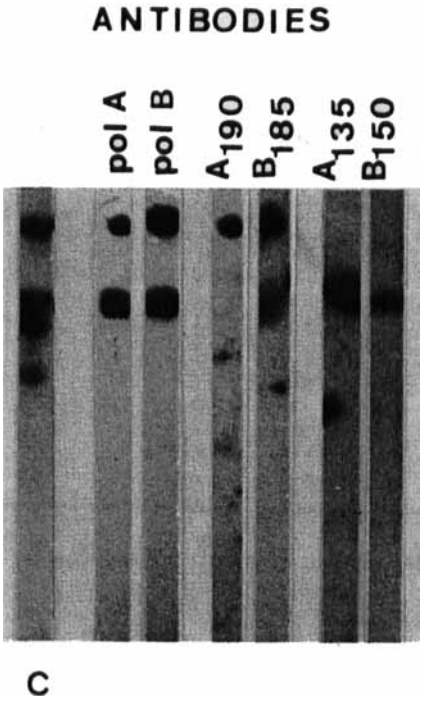

FIGURE 6. Antibodies to the large subunits of yeast RNA polymerases A and B recognize the large components of the unique archaebacterial RNA polymerase (from *Halobacterium halobium*) blotted on nitrocellulose. In lanes marked C, the polyacrylamide gel was stained with Coomassie blue after blotting. Only the large subunits are seen.

Weeks et al.[83] identified by immunoblotting the subunits of enzyme B from different organisms which cross-reacted with antibodies to each of the large subunits from wheat germ or *Drosophila* enzyme B. They found a clear correspondence between the largest subunit of yeast, wheat germ, *Drosophila*, and calf thymus enzyme B. Similarly, the second largest subunit of all these organisms was immunologically related. Clearly, there is a size conservation for the cognate subunits.

Studies with monoclonal antibodies confirmed these observations. A monoclonal antibody directed at the largest subunit of *Drosophila* enzyme B recognized the corresponding subunit from calf thymus enzyme B (but had no effect on enzyme activity).[107] Conversely, a monospecific antibody to the largest subunit of calf thymus enzyme B bound to *Drosophila*, plant, and yeast enzyme B.[84] (Incidentally, it is surprising that one could generate in mouse an antibody against a determinant which appears to be highly conserved in mammals.) Several monoclonal antibodies directed at the largest subunit or at two small components of *Podospora comata* enzyme B recognized the B enzyme from yeast, wheat germ, and calf thymus.[112b]

Using the same approach and a collection of antibodies directed at yeast RNA polymerase subunits, Huet et al.[113] discovered a striking immunological relationship between yeast RNA polymerases A and B and the unique form of RNA polymerase from six different archaebacteria. Cross-reactions were again localized in the large subunits and occasionally in some common subunits (no significant cross-reactions were found with RNA polymerases from eubacteria). The same large subunit of the archaebacterial enzymes was recognized by antibodies to either B_{220} and A_{190} or to the couple B_{150} and A_{135} (see Figure 6). This strongly

Table 5
α-AMANITIN SENSITIVITY OF RNA POLYMERASE B FROM DIFFERENT ORIGINS

Cell species	α-Amanitin concentration for 50% inhibition (μg/mℓ)
Mammalian cells	0.01
Xenopus laevis	0.05
Drosophila melanogaster	0.03
Bombyx mori	0.01—0.05
Zea mais	0.1
Wheat germ	0.3
Acanthamoeba castellanii	0.1
Saccharomyces cerevisiae	1
Agaricus bisporus	6.5
Podospora anserina	50
Aspergillus nidulans	>400

suggests that the three nuclear RNA polymerases must be derived from a *common ancestral multimeric protein after triplication of the large subunit genes.* The separation of the three forms of nuclear RNA polymerases may have occurred after the branching of the eukaryotes from ancestral archaebacteria.[115]

2. Conservation of Important Structural Domains

One should first recall here the general sensitivity of the largest subunit of class B RNA polymerase to proteolysis. This subunit appears to have conserved some protease-sensitive sites at one end of the protein, which leads to the loss of a ~40-kDa fragment after proteolytic action. It is this protein domain which contains a cluster of sites phosphorylated in vivo in yeast, plant, and animal cells[62-65,98] (see the Regulation section). Since the proteolyzed enzyme is active in vitro, this domain is not essential for enzyme activity and is more likely to be endowed with some regulatory or specificity function. Actually, Christmann and Dahmus[82] isolated a monoclonal antibody to the largest subunit of calf thymus enzyme which does not bind form B_{II} enzyme (with B_{180} instead of B_{215}) and therefore is probably directed at the domain removed by proteolysis. This antibody does not interfere with enzyme activity in vitro but inhibits specific transcription in a reconstituted system.[115]

Conservation of essential structural or functional domains in eukaryotic RNA polymerases is also attested by the general, though variable, sensitivity of class B enzymes to α-amanitin (Table 5), and by the possibility of obtaining hybrid RNA polymerase in vivo. Riva et al.[18] isolated a meiotic segregant from a diploid hybrid cell derived by conjugation of two genetically distant yeast species and demonstrated the presence in that segregant of a hybrid RNA polymerase A with five subunits coming from one parent (*S. douglassii*) and two from the *S. cerevisiae* parent. The homologous subunits were identified by a slight difference in molecular weight (see Figure 7). The cell transformation experiments of Ingles and Shales also imply that an hybrid RNA polymerase B was formed when the gene for the largest subunit of the human enzyme was transferred into a mutant hamster cell.[116]

Another common feature of nuclear RNA polymerases that they share with other DNA-dependent RNA polymerases is their association with zinc atoms. No information is available as to which subunit is involved in zinc binding. For a detailed review on this particular point, see Lewis and Burgess.[6]

These results suggest a striking conservation of some structural domains in the large polypeptides of RNA polymerases A and B from different eukaryotes. Very little is known on enzyme C but it is tempting to extrapolate this conclusion to all three forms of enzymes.

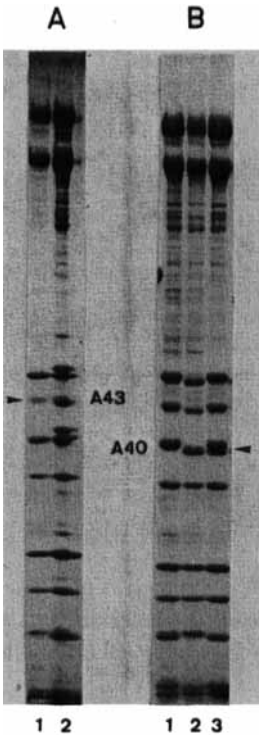

FIGURE 7. Polypeptide pattern of RNA polymerase A from *S. cerevisiae*, *S. douglasii*, an interspecific hybrid, and two segregants. (A) Subunits of enzyme A were separated by high-resolution electrophoresis on a slab gel with a gradient of polyacrylamide; (Lane 1) a segregant with a doublet band for A_{43}; (Lane 2) the interspecific hybrid, with double bands for A_{49}, A_{43}, A_{40}, $A_{34.5}$, A_{19}, and A_{14}. (B) (Lane 1) *S. cerevisiae* enzyme A; (Lane 2) *S. douglasii* enzyme A; (Lane 3) a segregant with a doublet band for A_{40}. See text for further details. (Figure courtesy of M. Riva.)

Also, some of the common subunits appear strongly conserved, which supports the view that they are part of the fundamental enzyme. The three forms of enzymes probably derived from an ancestral multimeric protein, but the large cognate subunits then diverged to meet various functional and regulatory requirements for the proper transcription of different classes of genes. This working hypothesis will hopefully be further explored with RNA polymerase structural genes.

VI. FUNCTIONAL ROLE OF INDIVIDUAL POLYPEPTIDE CHAINS

A. Partial Enzyme Dissociation

1. RNA Polymerase A

As mentioned earlier, some components, not as closely associated with the enzyme as other polypeptides, can spontaneously dissociate upon electrophoresis,[10,60,75] chromatography,[12,17,60,89,117] or sedimentation.[118] The missing polypeptide is usually the largest of the pool of small components (in the range of 50 to 60 kDa, depending on the organism).[10,12,17,75,89] The lack of this polypeptide does not affect enzyme activity on nonselective templates.[12,89] The role of this component could now be reinvestigated with RNA polymerase-dependent reconstituted transcription systems as it could be involved in the selection of correct promoter site on rDNA.

The variant of yeast enzyme A, called A*, is depleted of two polypeptides A_{49} and $A_{34.5}$ and has severely impaired enzymatic properties.[60] The A* enzyme becomes practically inactive on native DNA at ionic strength above 0.1, whereas at low salt concentration it transcribes poly|d(A-T)| as efficiently as the complete enzyme.[120] An enzyme A deprived of A_{49} and A_{43}, but retaining $A_{34.5}$, is also less active than the complete enzyme.[90] Therefore, it could be that the critical polypeptide is A_{49}. It is clear that although the loss of these subunits markedly alters the properties of the enzyme, they are not essential for the polymerization reaction. A good correlation was found between the activity of yeast enzyme A and its content in polypeptides A_{43} and A_{23}.[118] Also, the presence of A_{23} in all three enzymes argues for its essentiality to enzyme activity. Treatment of yeast RNA polymerase A at acid pH (below pH 5) induces the irreversible enzyme inactivation as well as the sequential loss of A_{43} and A_{23} followed by A_{43} and $A_{34.5}$.[119] In all experiments, however, where subunit losses were correlated with enzyme inactivation, a reconstruction experiment should be performed to substantiate that the subunit loss is a cause rather than a result of enzyme inactivation.

2. RNA Polymerase B

No drastic impairment in the activity of yeast RNA polymerase B was brought about by the removal of B_{32} and $B_{16.5}$.[75] The deficient enzyme, B*, is only half as active as the complete enzyme, with poly (rC) as template, and has a lower affinity for denatured DNA.[78] These observations are not sufficient to ascribe or deny a function to these polypeptides which clearly are not mandatory for nonspecific RNA synthesis in vitro. No selective transcription system is yet available in yeast to transcribe class B genes. Therefore, the possible role of these components in specific transcription could not be examined.

3. RNA Polymerase C

Partial dissociation of the C_{87} subunit from *Podospora comata* enzyme is accompanied by a loss in enzyme specific activity on a nonselective template, which suggests a role for that subunit in RNA synthesis.[48] Reconstruction experiments where the deficient enzyme is supplemented with C_{87} would confirm the essentiality of that universal subunit.

B. The α-Amanitin Binding Subunit

Subunit markers have been sought to identify the basic catalytic unit of RNA polymerases. The most promising was α-amanitin, a toxic cyclic octapeptide of the amatoxin family produced by the poisonous *Amanita* mushrooms. This toxin is a potent inhibitor of mammalian RNA polymerase B. There is a large variability in the sensitivity of form B RNA polymerase from different origins, of about three orders of magnitude (Table 3). At the limit, some class B enzymes like the one from *Aspergillus nidulans* are fully resistant to the toxin.[36] Amatoxins bind to the mammalian enzyme B with a high affinity, forming a 1:1 stoichiometric complex with an equilibrium association constant (KA) of about 10^8 to 10^{10} M^{-1} (see Reference 121a for a comprehensive review). The toxin inhibits RNA chain elongation, and the pyrophosphate exchange reaction,[4] but allows the formation of the first phosphodiester bond.[121b] It possibly interferes with the translocation step. Subforms of RNA polymerase B with the large subunit proteolyzed are similarly inhibited,[73,75] or bind similarly a ^{3}H-labeled amanitin derivative.[28,94] Brodner and Wieland[122] took advantage of the high binding constant of the tritiated amanitin-polymerase B complex to purify it by various chromatographic and gel filtration steps. Interestingly, when purified in this way, calf thymus enzyme B appeared less sensitive to proteolysis.[122] It contained a considerable proportion of B_{240} subunit which was barely present in the enzyme preparation obtained by the standard procedure, without α-amanitin. A water-soluble carbodiimide reagent was successfully used to cross-link the labeled peptide to the enzyme. When analyzed on denaturing gels, the

radioactivity was found exclusively at the level of the second largest subunit, B_{140}. This result implied that this subunit represented the true amatoxin receptor of the RNA polymerase B. This was therefore consistent with this subunit having a direct role in transcription. A small weakness of this approach was the possibility of a cross-linking between subunits of RNA polymerases during the reaction and therefore a misinterpretation of the protein banding pattern. On the other hand, in a preliminary report, Bateman and Nicholson[123] have indications that α-amanitin can be covalently attached to the two large subunits of wheat germ RNA polymerase B. The identification of the α-amanitin receptor is further questioned by the isolation of *Drosophila* mutants resistant to α-amanitin (see the Genetics section). It was recently found that mutations affecting α-amanitin sensitivity are located in the gene coding for the largest subunit B_{220}.[124] Pertaining to the same question is a former observation by Guialis et al.,[66] who were studying the regulation of RNA polymerase B in $ama^S \times ama^R$ hybrid cells grown in the presence of α-amanitin. Concomitant to the accumulation of the α-amanitin-resistant form of RNA polymerase, they observed a more rapid degradation of subunit B_{214} than of B_{140} and an increase of the rate of synthesis of B_{214} but not of B_{140}.

The discrepancy could have various explanations. In the cross-linking experiment, α-amanitin could be attached to a neighboring subunit instead of its true receptor. Indeed, in view of their size, the two large subunits are likely to interact, or the toxin binding site could overlap on the two large subunits (this is rather unlikely since all the α-amanitin resistant mutants fall in the same complementation group). Alternatively, mutations to α-amanitin resistance could indirectly modify the receptor site on another subunit through conformational changes (actually, mutations directly located on the α-amanitin binding site could inactivate the polymerase if it is an essential part of the active site), or the α-amanitin binding subunit could be of a different size in mammalian cells and *Drosophila* (this, however, is very unlikely as the largest subunit of insect and mammal enzyme B are immunologically related; see the Phylogenetics section). It remains that the genetic evidence is in favor of the largest subunit being the α-amanitin target, but the question remains open. The fact that all the mutations characterized so far in different organisms are in the gene for the largest subunit of enzyme B, whether α-amanitin was used as a selection agent or not, also underscores the universal importance of that subunit (see the Genetics section).

C. Antibodies as Functional Probes

Specific antibodies directed at each individual component of yeast RNA polymerases A and B were used to probe the activity of the three forms of enzymes.[103,104] Inhibition of nonselective transcription by these subunit specific polyclonal antibodies underscores the participation of the target subunit in the active enzyme structure but does not necessarily mean that the subunit itself is required for RNA synthesis. As shown in Figure 3, not all antibodies to the various components of yeast RNA polymerase C are inhibitory, although all were found by spot immunodetection to bind the native enzyme. The fact that anti-C_{82} and anti-C_{53} antibodies are inhibitory indicates that these polypeptides are part of the enzyme structure (see Table 1) despite their occasional absence from some enzyme preparations. Based on DNA protection experiments, the largest subunit of enzymes A and B as well as the common subunit ABC_{23} appear to be involved in DNA binding.[104] Huet et al.[105] obtained a monoclonal antibody, directed at yeast subunit A_{135}, which was much less inhibitory when yeast enzyme A was engaged in a binary complex with DNA. Similarly, Carroll and Stollar[125] isolated a monoclonal antibody to the largest subunit of calf thymus RNA polymerase B which blocks the formation of enzyme-DNA complexes. Whether the antibodies directly blocked the DNA binding site in these experiments or changed the enzyme conformation is not known. Therefore, the direct involvement of these particular subunits in DNA binding is not firmly established. Rose et al.[106] described a monoclonal antibody reacting with the largest subunit A_{190} of the rat form A enzyme. Their antibody preparation partially inhibited

RNA elongation by RNA polymerase A in isolated nucleoli or by the purified enzyme in vitro on calf thymus DNA. The antibody behaved as a competitive inhibitor with respect to UTP concentration. In view of the tight binding of antibodies to their epitope it is puzzling that the Km for UTP progressively increased in the presence of increasing antibody concentrations. The results deserve to be reexamined with purified antibodies. The monoclonal antibodies to several subunits of *Podospora* RNA polymerase B which inhibit the enzyme in a nonspecific assay could also prove useful to identify functional components of the enzyme.[112b]

Noninhibitory monoclonal or polyclonal antibodies directed at specific subunits can also be potentially useful to identify subunits involved in the interaction with transcription factors. A monoclonal antibody directed at the largest subunit, B_{240} (or B_{214}), from calf thymus RNA polymerase B does not inhibit nonselective transcription by purified RNA polymerase B but inhibits factor-dependent specific transcription of the adenovirus-2 major late gene as well as the conalbumin and ovalbumin genes in a Hela cell extract.[115] Interestingly, this monoclonal antibody does not bind B_{180} (which is a proteolyzed derivative of B_{240}, like B_{214}). Since specific transcription is fully inhibited when the extract was supplemented with partially purified enzyme which mainly contained B_{214} and B_{180} polypeptides, it is likely that the enzyme subform with B_{180} component does not participate in specific transcription. One possible explanation of these results is that the bound antibody molecule interferes with the interaction of a transcriptional factor with RNA polymerase, possibly with B_{240} itself, and that the factor interaction site has been lost, through partial proteolysis, in the B_{180} polypeptide. These encouraging results obtained with monoclonal antibodies and the generalization of this technology will probably encourage the increasing use of these specific probes to investigate subunit structure and function.

D. Other Enzyme Ligands

Some attempts have been made to demonstrate directly the interaction of isolated subunits of RNA polymerases with nucleic acids. Huet et al.[126] subjected yeast RNA polymerase A to SDS-PAGE containing ^{32}P-labeled $(rA)_n$. After extensive washing of the gel in a SDS-free renaturation buffer, several subunits (A_{190}, A_{49}, $A_{34.5}$, and A_{27}) were found to retain the polynucleotide, thereby giving dark bands on the autoradiogram.

Similarly, Gundelfinger and Stein[127] and Gundelfinger,[128] found that after dissociation with SDS and transfer to nitrocellulose, the two large subunits of *Drosophila* enzymes A, B, and C can bind tightly to DNA. In all these experiments, however, nucleic acid binding could occur nonspecifically to clusters of basic amino acids on partially renatured subunits. UV cross-linking of ternary complexes under more physiological conditions identified again the two large subunits of the polymerases as DNA-binding subunits, whereas nascent RNA was bound mainly to the largest subunit.[128] This binding pattern is consistent with the immunological data and is also consistent with the findings on the prokaryotic enzyme.

Affinity labeling of the polymerases active site with nucleotide photoaffinity probes has not yet been explored although it was successfully used with *E. coli* RNA polymerase.[129,130] Pyridoxal 5′-phosphate has been used to probe a possible phosphate binding site in rat liver and yeast RNA polymerases. Yeast RNA polymerase A is inhibited by this compound through formation of a Schiff base at a few lysyl residues.[131] The subunits that react with the aldehyde are predominantly A_{190}, A_{49}, $A_{34.5}$, and, to a lesser extent, A_{135}.[132] DNA prevents binding of the reagent to the four polypeptides, whereas nucleosides triphosphate decrease its binding to A_{190}. These results suggest that A_{190} contains the nucleotide binding site, with a lysine residue involved in enzyme activity, and also participates in DNA binding, perhaps in conjunction with A_{135}, A_{49}, and $A_{34.5}$.

It has been postulated that Cibacron blue F3GA binds to the nucleotide fold in enzymes which interact with nucleotides and polynucleotides. The dye binds reversibly to yeast RNA

polymerase A in a 1:1 stoichiometry and inhibits its activity.[117] However, the inhibition is noncompetitive with respect to ATP or DNA, in contrast to the results obtained with *Azotobacter vinelandii* RNA polymerase.[133] The subunit interacting with the dye has not been determined.

Reagents for sulfhydryl groups in yeast RNA polymerase A inactivate the enzyme.[134] Statistically, 2 mol of *p*-chloromercuribenzoate per mole of enzyme suffice to totally inhibit transcription, suggesting that at least one cysteine residue is involved in the polymerization reaction. The most reactive residues were located with ^{14}C-NEM in subunits A_{190}, A_{135}, and A_{40}. However, it is difficult to correlate this subunit labeling with the effect on enzyme activity.

E. Enzyme-Associated Enzymatic Activities

The molecular complexity of nuclear RNA polymerases suggests the possible involvement of aggregated multienzyme systems acting on regulation of DNA transcription and/or processing of the RNA product. Such an hypothesis was explored on two occasions. Rose et al.[135] reported that purified preparations of rat hepatoma RNA polymerase A contain an endogenous protein kinase activity capable of phosphorylating several components of the enzyme. They noted that two polypeptides of the polymerase A preparation were nearly identical in size to the two subunits (42 and 24.6 kDa) of nuclear, cyclic nucleotide-independent protein kinase II from the same cells. Based on these observations and on coarse immunological data, the authors concluded the physical association of a protein kinase with RNA polymerase A. Other authors also proposed that maize RNA polymerase B may also contain protein kinase as an integral component.[136] Dahmus confirmed the presence of casein kinase II in standard preparations of calf thymus RNA polymerase A.[96] However, the tryptic peptide map of the two subunits of casein kinase II were clearly different from the map obtained for the subunits of similar size in RNA polymerase A.[96] Similarly, calf thymus protein kinase I also failed to cross-react with RNA polymerase B subunit of similar size and gave a different peptide map. Clearly, calf thymus RNA polymerases A or B do not contain casein kinase I or II polypeptides. Although the kinase activity copurifies with RNA polymerases, this contamination can be totally removed by chromatography[96] or by sedimentation as in the case of the yeast enzymes A[51] or Morris hepatoma enzyme B.[137]

A ribonuclease H activity, which degrades RNA-DNA hybrids, copurifies with yeast RNA polymerase A through various fractionation procedures including polyacrylamide gel electrophoresis under native conditions.[138] Two distinct RNase H species could be renatured after electrophoresis of RNA polymerase A in an SDS-polyacrylamide gel containing a labeled hybrid substrate.[139] They co-migrated with subunits A_{49} and A_{40}. The partial proteolytic peptide pattern of subunit A_{49} corresponded to that obtained from an RNase H activity of 49 kDa isolated from yeast chromatin. The dissociation of A_{49} from enzyme A was accompanied by the loss of RNase H activity.[139] The role of a ribonuclease H activity associated with RNA polymerase A remains obscure. They could be unrelated proteins that happen to have a strong affinity for the polymerase. Alternatively, they could be involved in transcription termination or in the removal of transient RNA-DNA hybrids required for initiation of replication.[88] There are some indications that RNA polymerase A could play a role in DNA replication by making RNA primers.[140] Note that RNase H was not found in enzyme A from other eukaryotes.[17]

To conclude this topic, there is yet no clear evidence that enzymatic activities other than the polymerizing activity are integral parts of one form or another of RNA polymerase. The kinase hypothesis was ruled out. The presence of RNase H in yeast RNA polymerase A resides partly in a component, A_{49}, which can dissociate easily from RNA polymerase and which is dispensable for polymerizing activity. This later example illustrates the difficulty of reaching a conclusion on the physiological significance of some components associated to purified RNA polymerases.

VII. GENETICS OF EUKARYOTIC RNA POLYMERASES

In contrast to bacterial RNA polymerases whose structural genes for the major subunits (β, β′, α, σ) have been identified and extensively studied at the different levels of organization, expression, and regulation (reviewed by Yura and Ishihama[141]), the genetics of eukaryotic RNA polymerases is still in its infancy. Slow progress in this area has several causes. Few eukaryotic organisms lend themselves to genetic studies. The best inhibitor of RNA polymerase B, α-amanitin, does not act in vivo in all type of cells. The presence of three forms of RNA polymerases also discourages a brute force approach based on enzyme assays. Nevertheless, a considerable amount of work has been invested in the isolation of mutants of mammalian cells, *Drosophila*, and yeast with altered RNA polymerase B. Interesting results were obtained on the importance of the largest subunit of enzyme B, on the role of enzyme B in the expression of viral genes and the regulation of enzyme synthesis in mammalian cells; analysis of α-amanitin-resistant *Drosophila* mutants has led to the isolation and identification of the altered structural gene; cloning of all the structural genes for RNA polymerases is being successfully undertaken in yeast.

A. Mutants of Mammalian Cells Resistant to α-Amanitin

Chan et al.[142] have been the first to use α-amanitin as a selective tool to isolate mutants resistant to the toxin from a CHO cell line. In most cases, the resistance phenotype was due to the appearance of a new form of enzyme B highly resistant to the drug. Ama-1 mutant enzyme B required 800-fold higher concentrations of α-amanitin for inhibition compared to the wild-type enzyme.[143,144] Similar mutations were described afterwards in rat myoblast,[85] human fibroblasts,[145] BHK-hamster cells,[146] mouse myeloma,[147] and lymphoblastoma cell lines,[148] as well as in nonmammalian organisms, insect cells,[149] *Drosophila*,[150] and the nematode *Caenorhabditis elegans*.[151]

The altered enzyme B from the mutants was indistinguishable from wild-type polymerase with respect to chromatographic or catalytic properties,[85,152] or antigenicity,[85] although it may present in some occasions,[143,153] but not generally,[85,152,153] an increased thermosensitivity. The altered enzymes showed primarily a reduced affinity for the inhibitor.[144,147,148,152] The equilibrium dissociation constant (Kd) of the complex polymerase B-inhibitor (a labeled derivative of α-amanitin) increased from $3.8\ 10^{-11}$ *M* with the control enzyme to 3.10^{-10} *M* with RNA polymerase from the Ama-6 mutant.[144] The α-amanitin sensitivity of different mutant cell lines varied largely and was well correlated with the variable degree of α-amanitin sensitivity of their enzyme B.[144,152] These results suggest that these various mutations brought about a modification in the amino acid sequence of one enzyme subunit and that a series of different alterations was possible and tolerated by the polymerase since the growth rate of the cell was not significantly affected. There is the possibility that the mutations did not change the toxin binding site but modified its conformation at a distance, as a direct change in the toxin binding site could give rise to a dominant lethal mutation. Alternatively, to explain this variety of mutations it could be that the toxin binding site is not essential for activity, the drug blocking RNA chain elongation by steric hindrance or by opposing enzyme conformation changes. Note, however, that all mutant enzymes retained a partially functional α-amanitin binding site since they were inactivated at sufficiently high drug concentrations. This is reminiscent of the large natural variation in the α-amanitin sensitivity of enzyme B from different eukaryotes (see Table 3). At any rate, the different mutations could not complement each other: in hybrid cells coming from parent cells of different α-amanitin sensitivity, no wild-type RNA polymerase was generated. The mutations are therefore likely to affect the same subunit, possibly the polypeptide target of α-amanitin, as discussed earlier.

Ama-1 mutation and other similar mutations are co-dominant. Most cultured mammalian cell lines resistant to α-amanitin contained two forms of RNA polymerase B, Ama^R and

Ama^S, and were therefore functionally diploid at the locus coding for the altered subunit.[85,144,146-148,154,155] The variable ratio between these two forms could reflect a gene dosage effect in aneuploid cell lines.[152,154,155] On the other hand, mutants of CHO cells have only one functional allele.[144,155,156]

At the functional level, α-amanitin-resistant mutants helped demonstrate the participation of host cell RNA polymerase B (at least of its altered subunit) in the expression of viral genes from polyoma virus,[146] herpes simplex virus,[157] and vaccinia virus.[158]

B. Study of Thermosensitive Mutants

The analysis of α-amanitin-resistant polymerase was somewhat disappointing since the enzyme appeared functionally unaltered in vitro. Furthermore, the modified subunit could not be identified. These considerations led Ingles and colleagues to search for conditional-lethal thermosensitive (Ts) mutations affecting RNA polymerase B in mammalian cells. In a collection of 168 α-amanitin-resistant mutants obtained after nitrosoguanidine treatment of CHO cells, 5% were found to be thermosensitive.[153] Genetic evidence indicated that the Ts mutation affected RNA polymerase B and more specifically, the polypeptide conferring α-amanitin resistance. Indeed, when grown at nonpermissive temperature, the cell hybrids Ts Ama^R × Ts^+ Ama^S lost their α-amanitin-resistant phenotype. Similarly, reversion of the Ts phenotype was often accompanied by a change in α-amanitin sensitivity.[153]

Ts AF_3 cells are a Ts mutant of BHK cells isolated by Meiss and Basilico[159] in 1972. This mutant arrests at the nonpermissive temperature in the G_1 phase of the cell cycle. Several results suggested a defect either in the synthesis, the assembly, or the stability of RNA polymerase B.[160] The Ts mutation is recessive, affects a locus functionally hemizygous,[161] and can be complemented in interspecific cell hybrids by human chromosome 3.[162] Although RNA polymerase B from the mutant is probably not thermosensitive,[163] genetic evidence indicate that Ts and Ama^R mutations are in the same complementation group. An elegant demonstration of this was afforded by transforming a Ts Ama^S mutant with DNA originating from Ts^+ Ama^R cells. Transformed cells selected for the Ts^+ phenotype became simultaneously α-amanitin resistant.[116] The gene coding for α-amanitin resistance was co-transferred with the Ts^+ locus. Unless several polymerase B genes were genetically very closely linked, the gene for a single subunit responsible for both phenotypes was probably transferred. Human DNA can complement the Ts mutation in the Syrian hamster cells,[116] as previously observed by Ming et al.[162] In that case, a hybrid functional RNA polymerase should result.

It was somewhat unexpected that the only Ts mutation in RNA polymerase obtained independently of all α-amanitin selection steps happened to map in the same gene, conferring α-amanitin resistance. All this considerable amount of work to isolate and characterize mutants affected on RNA polymerase B illustrates the difficulty of carrying genetic studies with mammalian cells despite the advantage of a powerful selective tool.

C. *Drosophila* Mutants

The search for mutants in organisms more amenable to genetic and biochemical analyses has resulted in the isolation of *Drosophila* flies resistant to α-amanitin.[150] The mutant Ama C_4 has an altered RNA polymerase B,[150] with a much reduced affinity for α-amanitin.[164] Except for a small difference in Km for nucleotides, the general catalytic properties of the enzyme are unchanged, like in the case of mammalian mutants. The locus Ama C_4, conferring the α-amanitin resistance phenotype, was genetically mapped on chromosome X, to position 35.7, and localized to the polytene chromosome band interval 10 C_2 to 10 D_4.[150] This locus is an allele of a lethal-mutable locus, L_5, mapped previously in the same region.[150,165,166] A known allele of L_5, Ubl (Ultrabithorax-like) causes abnormalities of development.[166,167] Furthermore, individuals homozygous for a Ts mutation in the Ubl locus exhibit a discrete

temperature-sensitive period extending from gastrulation to pupation, whereas adult flies are viable if placed at nonpermissive temperature.[167] The results suggest the existence of critical events in developmental pathways requiring either a minimum concentration of active enzyme or the proper transcription of certain genes.[166,167]

The cloning of the locus Ama C_4 (or $RpII^{C4}$) is a splendid demonstration of the power of genetics and cytogenetics in *D. melanogaster*. Searles et al.[169] took advantage of the insertion of a transposable P element into the L_5 locus, causing a lethal mutation, to clone a genomic DNA fragment containing P element and flanking non-P sequences from the L_5 (or RpII) locus. Non-P sequences hybridized on the polytene chromosome at the band location of $RpII^{C4}$. In the mutant used for making the gene bank, the transposon was probably inserted outside the structural part of the RNA polymerase B gene since precise excision of P element was not required to restore function of the RpII locus. The localization of the gene, undefined at that stage, was clarified by analysis of Poly(A)$^+$ RNA transcripts.[170] Among other RNAs hybridizing to subclones of the RpII region, a 7-Kb RNA was a good candidate as a transcript from a large subunit gene. The mammalian genome contains a related sequence detected by hybridization under conditions of reduced stringency. Remarkably, the mammalian sequences recognized by hybridization were the same which, when transferred into α-amanitin-sensitive (Ama^S) Ts AF_8 cells conferred the Ts^+ and the Ama^R resistance phenotypes.[170] This confirmed the conclusion that the 7-Kb RNA coded for a polymerase subunit which, considering its size, was suspected to be B_{240} rather than B_{140}. Greenleaf, by cloning a part of the structural gene into an expression vector, recently demonstrated that the hybrid fusion protein was specifically recognized by antibodies directed at the largest subunit and not by anti-B_{140}.[124] As discussed previously, this result suggests that this subunit was involved in α-amanitin binding.

This remarkable series of investigations on *Drosophila* mutants which led to the isolation of the first RNA polymerase structural gene demonstrates the interest of the genetic approach but also draws its limits. Its great interest was to set the way to studies on the role of one essential constituent of RNA polymerase B, the regulation of its biosynthesis, and to the role in general of enzyme B in the development of a multicellular organism. Its limits are those which founded its strategy since the type of mutants analyzed were so far restricted to the gene(s) involved in α-amanitin sensitivity.

D. Yeast Mutants

The isolation of yeast mutants with altered RNA polymerase would be of great help to correlate loss of a function in vivo and in vitro with a structural defect as the yeast enzymes are well characterized. Unfortunately, yeast cells are not permeable to α-amanitin and there is no straightforward means of selecting for RNA polymerase mutants. Thermosensitive mutants have been described that may be altered in components essential for RNA synthesis.[171-173] Thonart et al. isolated three independent thermosensitive mutants defective in the synthesis of all RNA species at nonpermissive temperature (37°C). It was suggested that the mutations were in genes encoding the three common subunits.[173] However, this is unlikely as analysis of RNA polymerases A and B never revealed increased thermosensitivity of the purified enzymes.[171,364]

One mutant (rpoB 1), deficient in RNA polymerase B activity in vitro, was isolated from a collection of mutagenized cells altered in RNA production[174] using a polymerase assay technique which allowed one to differentiate the three RNA polymerase activities in crude yeast extracts.[175] The enzyme B from the rpoB 1 mutant was defective in RNA chain initiation and elongation reactions.[78] Enzyme-DNA binding was comparatively much less affected. The B_{220} subunit in the rpoB 1 mutant had an altered subunit map, indicating that the polymerase lesion resided in the largest subunit. The structural alteration of B_{220} also brought about the spontaneous dissociation of B_{32} and $B_{16.5}$.[78] This was the first characterization at

the subunit level of an eukaryotic RNA polymerase mutation. It established the essential functional and structural role of the largest polypeptide of enzyme B. Unfortunately, the rpoB 1 mutant had no detectable in vivo phenotype and it remains the only yeast strain affected in one specific RNA polymerase.

As an alternative to mutant analysis, Riva et al.[18] took advantage of a natural variation in the molecular size of seven subunits of RNA polymerase A from two different yeast species, *S. cerevisiae* and *S. douglassii*, to investigate the expression and organization of the corresponding genes. The diploid hybrid, derived by crossing the two species, contained all the subunits characteristic of the two parents. Therefore, all the corresponding alleles were expressed. Tetrad analysis suggested that the genes for several subunits are located on different chromosomes. Analysis of disomic strains (with one haploid genome plus one chromosome coming from the two parents) showed that two out of the seven subunits, A_{43} and A_{40} (common to enzymes A and C), are each placed by themselves on a different chromosome (see Figure 7).

E. Cloning the Structural Genes of Yeast RNA Polymerases

The availability of a unique collection of antibodies directed at RNA polymerase subunits, together with the powerful development of recombinant DNA techniques in *Saccharomyces* has encouraged the complex enterprise of cloning the genes for all the subunits of the three forms of enzymes. Young and Davis developed an expression vector, λgt 11, that promotes the synthesis of hybrid proteins and permits the screening of large libraries of recombinant DNA.[176] Screening a genomic bank of sheared yeast DNA, inserted into this vector, with antibodies to yeast RNA polymerase B led to the isolation of several recombinant phages which were likely to harbor part of some structural genes for RNA polymerase.[177] Two clones were specifically identified with antibodies to the large subunits, B_{220} or B_{150}, which were characterized previously.[104b] Quantitative Southern experiments showed that the genes for these two subunits exist in simple copy in the haploid yeast.

This successful experiment demonstrated the feasibility of cloning the whole genetic system for yeast nuclear RNA polymerases. In the laboratory, we have undertaken the cloning and identification of this family of genes. Two groups of genes were of special interest: the genes for the large subunits, and those coding for the common polypeptides — altogether, 11 genes. Using specific antibodies to these important subunits, one could isolate a collection of recombinant phages representing all of these genes[365] (see Figure 8). The gene coding for subunit AC_{40}, a subunit common to A and C enzymes, is present in a single copy, like the genes for the large subunits of enzyme B.[366] Besides these important groups of genes, other genes unique to each RNA polymerase may also prove to be essential for polymerase function and were also cloned similarly. A total of 23 different genes are presently available based on the immunodetection assay. Examples of gene cloning are given in Figure 8. Other identification criteria are needed to firmly establish that they correspond to RNA polymerase genes. Preliminary evidence is encouraging: in protein blots from different bacterial clones, a protein of the correct size was identified in three cases, corresponding to subunits A_{43}, A_{40}, and $A_{34.5}$. In other cases, as expected, either fusion proteins or protein fragments of a smaller size than the corresponding subunit were found.

The isolation of genes for yeast RNA polymerases will open a new field of investigation at the structural and functional levels. The question of the evolution of RNA polymerases will be more easily addressed at the gene level than with the proteins. Hence, the conservation of some important domains in the large subunits of the three enzymes, which was suggested by immunological analysis (and by their probable interaction with common subunits) will be investigated more precisely. Random in vitro mutagenesis of the genes and the substitution of the altered gene for the genomic wild-type copy is feasible with the yeast cell. Conditional lethal mutants will hopefully be isolated in this way and will help in defining the function

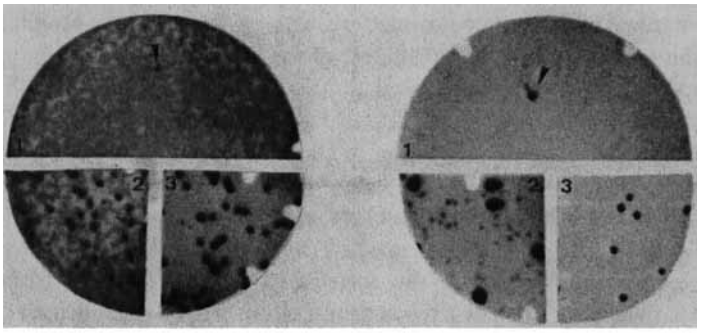

FIGURE 8. Cloning the structural genes for yeast RNA polymerases using specific antibodies. A bank of sheared yeast DNA inserted into the λgt11 vector[177] (provided by R. Young) was screened with antibodies to each RNA polymerases subunit. The two examples shown are with anti-C_{128} and anti-C_{53}. (1) About 100,000 recombinant phage plaques were probed after blotting onto a nitrocellulose filter (ϕ 8 cm); (2 and 3) positive phages were purified until all phage plaques bound the specific antibodies. (Figure courtesy of M. Riva.)

of the altered subunit both in vitro (by analyzing the functionally altered enzyme) and in vivo. Disposing of the yeast genes, one can envision the extension of RNA polymerase gene cloning to other eukaryotic cells. Since there is an extensive immunological relationship between the large homologous subunits of different eukaryotic form B enzymes (see preceding section), one can expect that a residual sequence conservation of the genes will permit their isolation using the yeast probes. Recently, Ingles, Greenleaf, and their collaborators, using a *Drosophila* probe coding for part of the largest subunit of polymerase B, isolated a yeast DNA sequence coding probably for the homologous polypeptide in yeast.[363]

VIII. REGULATION

How are nuclear RNA polymerases regulated? This most important question has multiple facets depending on whether one considers RNA polymerase biosynthesis or activity. Is the synthesis of the three forms of enzyme distinctly regulated or subjected to coordinated regulation? Is there a balanced synthesis of the various polypeptide chains belonging to the same enzyme form? How is the problem of the common polypeptides solved? These are important questions concerning the regulation of enzyme levels. Despite a considerable amount of work, there is limited sound data in this area. Concerning the regulation of RNA polymerase activity, the question is why are RNA polymerases so large if not to engage multiple reversible interactions with regulatory components? In addition, are there covalent modifications of the RNA polymerase functional core which modulate enzyme activity or achieve drastic alterations of enzyme properties? Again, only partial information is available.

A. Regulation of Enzyme Levels

1. Changes in Activity Levels

The existence of three forms of RNA polymerase with distinct functions made attractive a model of control of the different RNA classes by differential regulation of each enzyme level. In the absence of appropriate quantitative techniques to isolate and quantify the various forms of nuclear RNA polymerase, most studies concerned with regulation of enzyme levels have relied on the determination of polymerase activity. To the coarse determination of endogenous nuclear enzyme activity and the assay of solubilized enzymes in nuclear extracts succeeded the separation of the various enzyme forms on DEAE Sephadex. As discussed

previously, a number of factors could interfere with enzyme activity. Inhibitors acting on the template, the enzyme itself, the substrates, or the RNA product can copurify with RNA polymerases and themselves be influenced by the physiological transitions being studied. For instance, studies of enzyme levels during larval development of *Artemia salina* were complicated by the simultaneous induction of proteases which inactivated forms A and C RNA polymerases in vitro.[178] Shields and Tata[179] observed that the stability and the recovery of rat liver forms A and B enzymes, upon chromatography, were strongly influenced by the growth status of the cells. An inhibitor masked two forms of enzymes in mycelial cells of *Histoplasma capsulatum*, but not in the corresponding yeast cells.[180] Isolation of RNA polymerases from nuclei or nucleolar preparations might also be complicated by the variable and selective losses of some enzyme forms.[181,182] Hence, most studies have focused on class A and B enzymes because of their inability to recover enzyme C from nuclei extracts. The search for interfering substances would be endless, but it can be compensated to some extent in mixing experiments where the two different cell types being compared are mixed prior to enzyme extraction and chromatography. This was done on a few occasions.[178,183,184] These comments indicate that some caution should be exercised in interpreting in terms of enzyme content the following works where activity measurements of partially purified enzyme preparations were compared. One should see Roeder[5] for a discussion of early work on RNA polymerase regulation.

a. Animal Cells

Increased RNA polymerase activity was found in more rapidly growing mammalian cells. During the transition from the resting to the growing state of fibroblasts, enzyme A activity level increased whereas enzyme B remained unchanged.[185] The increase in form A enzyme was about proportional to tumor cell growth rates.[186] Levels of both form A and C enzymes were markedly and preferentially increased during liver regeneration[71,187] or in rapidly proliferating mouse tissues.[188]

Various hormonal or drug treatments bring about variable changes in RNA polymerase levels, either a preferential stimulation of A enzyme with little or no variation in the B form,[189-193] or a specific stimulation of form B, in response, e.g., to vitamin D,[194] or a stimulation of B and C enzymes only,[195] or of all three forms of enzymes,[196] or no change at all in enzyme levels (see Roeder[5] for additional references and comments). After inhibition of protein synthesis by cycloheximide there is a gradual leakage of enzyme A from the nucleolus,[71,182] but no marked decrease in recovery of intracellular enzymes A and B. The half-life of these two enzymes appears to be quite long,[182,197] more than 24 hr for enzyme B, possibly a little less for enzyme A. This is in contrast to an earlier conclusion that enzyme A had a rapid turnover time.[198]

The level of several forms of RNA polymerases remained unchanged in logarithmically growing Hela cells or in synchronized population of cells undergoing mitosis or in middle-S phase cells.[199]

During oogenesis, in *Xenopus laevis*, the three forms of RNA polymerases increase dramatically in the same proportion, whereas during early development, form A and B enzymes are selectively increased.[183] No drastic change in levels of the three forms of RNA polymerases were detected during larval development of *Artemia salina*.[178] In contrast, upon starvation of the larvae, there was a selective decrease of form A and C enzymes.

Interpreted in terms of polymerase content, all these results would rather suggest that in animal cells the regulation of the three forms of RNA polymerases is not tightly coordinated and that distinct regulatory controls operate. Some coordinated regulation of A and C enzymes which are involved in the synthesis of stable RNA species, rRNA, and tRNA, remains a likely possibility, however, which should be explored with more direct approaches.

b. Plant Cells

Regulation of plant RNA polymerases has been investigated during germination of soybean embryonic axes and axis growth, and after auxin treatment.[64,65,200] The amount of RNA polymerases A and B (calculated on a dry weight basis) remained unchanged during germination whereas endogenous polymerase activities in isolated nuclei increased dramatically.[65] Similarly, the amount and proportion of form A and B RNA polymerases remained constant during illumination of etiolated pea seedlings.[201] Mechanisms other than modulation of enzyme concentration must regulate RNA synthesis. In contrast, upon auxin treatment of mature soybean hypocotyl, RNA polymerase A and B levels increased three- to six- and twofold, respectively (on a tissue DNA basis).[64,200] Incorporation of $|^{35}S|$ methionine into immunoprecipitated RNA polymerases A or B subunits suggested a *de novo* synthesis of RNA polymerases. There was no preferential synthesis of any subunit suggesting a coordinated regulation of synthesis of enzyme multicomponents.[64]

c. Lower Eukaryotes

Lower eukaryotes which can be grown under defined conditions and are subjected to developmental transitions provide simple experimental models for the study of transcription control at the polymerase level. Young and Whiteley[184] found that the transition of the dimorphic fungus *Mucor rouxii* from yeast-like to mycelial growth was accompanied by a specific change in the relative amounts of polymerases A and B, while enzyme C was unchanged. Detke and Paule[202] noted a selective increase in form B enzyme during encystment of the soil amoeba *Acanthamoeba castellanii*, with no change in enzyme A. The RNA polymerase spectrum in *Dictyostelium discoideum* was reported to be different in the vegetative amoeba and at the culmination stage during morphogenetic development,[203] in contrast to an earlier report.[204] In this organism, however, the identification of all the different chromatographic forms could not be clearly established. Induction of the incompatibility response in the fungus *Podospora comata* did not result in gross alteration of RNA polymerases level.[205]

Sebastian et al.[206] investigated the effect of growth rate on RNA polymerase content of the yeast *Saccharomyces cerevisiae*. Fast-growing cells contained proportionally more RNA polymerase A than slow-growing cells, whereas the activity of enzyme B was almost independent of growth rate. This result was confirmed by direct quantitation of A and B enzyme by selective immunoprecipitation.[7] As the rate of rRNA and tRNA accumulation increases with the growth rate, synthesis of new RNA polymerase A (and probably C) molecules may be required for the production of stable RNA species. On the other hand, neither amino acid or glucose starvation nor cycloheximide treatment affected the chromatographic peak pattern of the solubilized enzymes in yeast.[207]

Independent regulation of RNA polymerases A and B was observed during the cell cycle of *S. cerevisiae*.[208] In contrast, in a comparable study, equal activities of both enzymes A and B were detected throughout the synchronous mitotic cycle of *Physarum*,[209] in agreement with findings in animal cells.[199] In these experiments, to the difficulty of properly estimating RNA polymerase activity was added the problem of collecting enough cells at different stages of the cell cycle.

Taken together, these studies on regulation of enzyme levels in lower eukaryotes suggest an independent regulation of form A and B RNA polymerases. As all three RNA polymerases can be radioactivity labeled and selectively immunoprecipitated in yeast, regulation of A, B, and C enzyme levels would be interesting to investigate in this organism.

2. Regulation of RNA Polymerase B Level

The high affinity of animal enzyme B for amanitin[210] made possible the precise determination of RNA polymerase B molecules in crude tissue extracts by simply measuring the

binding of labeled amatoxin.[3] Cochet-Meilhac et al.[211] found the highest numbers of RNA polymerase B molecules in the most metabolically active cells. Less active cells like uterine cells of ovariectomized rats contained five to ten times less RNA polymerase molecules per haploid genome. The effect of steroid hormones on enzyme B level was investigated by the same binding assay.[212,213] Steroid hormones have dramatic effects on the transcriptional activities of their target tissues. Upon administration of estradiol to immature or castrated rats, there was first no change in the amount of enzyme B for about 12 hr, then there was a progressive rise (+40% at 24 hr) in the number of enzyme B molecules on a cell DNA basis. Remarkably, there was a tight correlation between the increase in uterine enzyme B activity and α-amanitin binding capacity.[212] Similar experiments were conducted in chickens. The administration of estrogen to roosters was accompanied by a twofold increase in the number of RNA polymerase B molecules per nucleus in the liver, which also closely reflected the increased enzyme activity of the extracts.[213] During withdrawal from estrogen the amanitin-binding capacity gradually declined to the unstimulated level. This decline could be related to the following observations.

Isolation of mammalian cell line mutants containing two populations of enzyme B, sensitive (wild-type) and resistant (mutant) to α-amanitin, disclosed a very interesting regulatory phenomenon. When grown in the presence of α-amanitin, the cells compensate in a generation time the inactivation of the sensitive enzyme species by a corresponding increase of the resistant ones.[214-216] The effect is reversible; 2 days after withdrawal of the inhibitor, the ratio of the two enzyme species returns to its original level. Therefore, the cells have a way to maintain constant enzyme B activity. These variations in enzyme B are not accompanied by a change in enzyme A activity. Binding studies with labeled α-amanitin showed clearly the rapid drop in the number of sensitive enzyme molecules (or at least of the binding subunit) induced by the inhibitor.[215,216] Degradation of the large subunits of enzyme B was demonstrated by quantitative immunoprecipitation.[66] The largest subunit, B_{214}, disappeared faster than B_{140}. This observation correlates well with the recent finding that it is the largest subunit which is altered in *Drosophila* α-amanitin-resistant mutants (see the Genetics section). *De novo* synthesis of the resistant form of enzyme B in the presence of α-amanitin was also confirmed, by immunoprecipitation, for subunits B_{214}, B_{25}, and $B_{20.5}$.[66] The rate of synthesis of several other subunits, including B_{140}, did not change. This study suggests the existence of a "fail-safe system" which disposes of inactive, but potentially DNA-binding, RNA polymerase B molecules, and keeps the number of active enzyme B constant. It also suggests the coordinated synthesis of at least some subunits of RNA polymerase B. As Somers et al. suggested,[214] RNA polymerase B synthesis in mammalian cells may be regulated autogenously.

B. Free and Bound Enzymes

As shown amply in the preceding section, regulation of transcription is not simply dependent on RNA polymerase concentration. Various other mechanisms probably intervene to modulate enzyme activity or selectivity. To have a closer view at actually transcribing enzyme molecules, several authors have tried to estimate the fraction of enzyme tightly bound to the chromatin. Several operational approaches were used to define the free and bound enzyme populations. The "free" enzyme readily solubilized during homogenization of tissues and nuclei in isotonic media;[3,181,217] the so-called "engaged" enzyme required sonication in high ionic strength media to be solubilized.[3] Treatment of the chromatin with DNase is another way to release template-bound enzyme.[218-222] The concept of free and template-bound pools was also supported by a functional assay where endogenous RNA polymerase engaged in transcription is inactivated by a high dose of actinomycin-D to allow the free enzyme to be measured independently with the addition of a synthetic template not sensitive to the inhibitor.[223] Also using actinomycin-D, but in this case to prevent the release of enzyme A from the chromatin, Matsui et al. could also define two pools of free and

bound enzyme A.[71] More quantitative methodologies like immunoprecipitation or α-amanitin binding could not be applied as they would not distinguish between nontranscribing (free) and actively transcribing enzyme pools.

Like all operational definitions these are not without weaknesses. Sonication tends to alter RNA polymerases;[217,219] although salt has a biphasic effect on RNA polymerase release, other interpretations rather than free and chromatin-bound pools are possible;[224] the ingenious functional assay of Yu,[223] using actinomycin-D, presents some technical problems as discussed by Kellas et al.[219] and Okai,[225] and may result in conflicting reports.[225,226] These general comments should be kept in mind while interpreting the experimental results summarized below.

Free RNA polymerase may represent a normal phase of the RNA polymerase cycle which includes its release after termination (though it is still not known if enzyme C is released after one round of transcription of the small tRNA and 5S RNA genes). Free and bound enzymes would therefore be functionally equivalent. Matsui et al.,[71] however, found that free enzyme A from rat liver was structurally different from the bound enzyme. The transcriptionally active enzyme is form A_{II}, which contains a 62-kDa subunit, while the free enzyme contains A_{II} and another form, A_I, which lacks that subunit.[71] As discussed previously, the physiological significance of enzyme forms lacking subunits is uncertain inasmuch as the high salt-sonication treatment was found to change the relative proportion of these two forms of enzyme A.[219] However, conflicting results were reported.[227] The soluble "free" enzyme B found in plant quiescent tissues is form B_I, with the B_{220} subunit. A new form, B_{II}, was found in actively growing tissues, with a smaller subunit, B_{170}. Form B_I was originally thought to be a precursor or storage form of enzyme.[72] As discussed in a previous section, this apparent structural change was in fact a proteolytic artifact. Therefore, there are no indications that free and bound enzymes might not be functionally equivalent.

In rat liver cells, free nuclear RNA polymerase activity is a large fraction (40 to 50%) of the total RNA polymerase population.[217,223,228] There are great variations in the relative distribution between the free and the engaged forms of enzymes within each RNA polymerase forms: if most enzyme A appears engaged (80%), only about 50% of enzyme B and 30% of form C are engaged.[187,228,229] The fact that there is a large pool of free RNA polymerases B and C suggests that the main control of transcription by these enzymes is at the level of their rate of initiation. On the other hand, the rate of rRNA synthesis might be more directly dependent on enzyme A level.

There is no shortage of reports on the effect of a number of physiological transitions on free and bound forms of RNA polymerases. In general, an increase in bound enzyme A was found upon stimulation of the cell growth rate,[186,187] or after various hormonal[192,212,230,231] or drug treatments.[189] The pool of free enzyme A is increased simultaneously,[187,192,231] though not always.[189] This could suggest the existence of an equilibrium between the free and bound fractions. On the other hand, upon prolonged exposure of lymphocytes to phytohemagglutinin, while there was no further increase in bound enzyme A, the free pool of enzyme A kept rising drastically as if there was no direct control of the size of the free pool by nuclear function.[231] These two conclusions, however, are not mutually exclusive. At variance with the behavior of enzyme A, bound and free enzyme B often remained constant, or increased slightly, or only after much delay.[186,187,189,212,230,231] In the case of enzyme B, the control of the selectivity of transcription is expected to be more determinant than large alterations in enzyme pools. The distribution of enzyme C in free and bound pools was investigated during liver regeneration.[187] Both pools were increased similarly. As there is already an excess of free enzyme in control liver cells, this could suggest that the intranuclear enzyme concentration is one of the parameters which regulate the rate of tRNA or 5S RNA synthesis. The toxic and carcinogenic aflatoxin B_I which inhibits RNA synthesis markedly increased the pools of free enzymes A and C while, concurrently, it decreased the bound pool of these

two enzymes, suggesting again an equilibrium between these two pools.[229] There is no information yet as to the factors which determine this putative equilibrium between the different pools of RNA polymerases. These could be the template availability, the presence of transcriptional factors required for gene recognition, or other subtle modifications of RNA polymerases themselves.

C. Phosphorylation of Enzyme Subunits

The possibility of a regulation of eukaryotic RNA polymerases via phosphorylation has been the subject of considerable investigations at several levels: (1) to demonstrate an effect of phosphorylation on enzyme activity in vitro; (2) the phosphorylation of enzyme subunits in vivo and in vitro; and (3) a selective change in the phosphorylation pattern during development or physiological transitions.

At the structural level, there is good evidence that form B RNA polymerase is phosphorylated in vivo in yeast,[51,62,63,232] plant,[64] and animal cells.[98] Phosphorylation of enzymes A[51,62,63,232] and C[51] in vivo was only demonstrated in yeast. Attempts to detect the in vivo phosphorylation of enzyme A in plant or animal cells have not been successful.[64,98]

Five subunits of yeast enzyme A are phosphorylated in vivo: A_{190} (the largest), A_{43}, $A_{34.5}$, A_{23} (shared by the three enzyme forms), and A_{19} (shared by A and C enzymes). Some subunits are phosphorylated at multiple sites. The average distribution of phosphate per molecule was A_{190} (6), A_{43} (4), $A_{34.5}$ (2), A_{23} (1 to 2), and A_{19} (1 to 2), corresponding to an average of 15 ± 3 phosphate groups per enzyme.[63] Phosphoserine and phosphothreonine were identified.[51,62] Yeast RNA polymerase C is phosphorylated on subunit C_{23} (which belongs to the core of common subunits) and C_{19} (shared by A and C) with an average of 0.4 to 2 mol of phosphate per mole of enzyme. These stoichiometry values are compatible with the idea that most enzyme molecules are phosphorylated. There was some uncertainty as to the number of RNA polymerase B subunits phosphorylated in vivo. In yeast, immunoprecipitation and two-dimensional gel analysis established the phosphorylation of B_{220}[62,63] (the largest subunit) and B_{23}.[51,62,63,232] Therefore, the common polypeptide, ABC_{23}, was phosphorylated in the three enzymes. Interestingly, the proteolytic byproduct of B_{220}, B_{180}, was not phosphorylated.[51,62] There are indications that $B_{44.5}$ could also be phosphorylated.[62,63] These general observations were confirmed in plant and animal enzyme B. The largest subunit is phosphorylated, but not its proteolyzed form.[64,98] In Hela cells, both B_{240} and B_{214} are phosphorylated but not B_{180}, although both B_{214} and B_{180} are derived from B_{220} by proteolysis. As discussed previously, there must exist in B_{220} a protein domain with multiple phosphorylated sites which is highly susceptible to proteolysis and which contains a strong antigenic site.[82] The plant enzyme is also possibly phosphorylated on B_{42} (homologous to yeast $B_{44.5}$?) and the mammalian enzyme on $B_{20.5}$.

RNA polymerases A, B, and C from yeast are phosphorylated in vitro by a yeast protein kinase[51,232] on the same subunits phosphorylated in vivo, plus some additional ones (A_{49}, B_{32}, C_{53}). The extent of in vitro phosphorylation was relatively low, about 0.2 to 2 mol phosphorus per mole of enzyme.[63] This probably reflected the fact that the enzymes were fully phosphorylated in vivo under the growth conditions used. The sites of phosphorylation of enzyme A subunits in vivo and in vitro were found to be related as the patterns of ^{32}P-labeled peptides were strikingly similar.[63] This is in favor of the involvement of protein kinase in vivo. Actually, the phosphorylation of yeast enzymes A and B is, for the most part, a post-translational process since it can occur in the absence of protein synthesis.[62,63] Mammalian RNA polymerases A and B are phosphorylated in vitro by homologous protein kinases[98,233-235] on several subunits, including the largest, in both cases.

Whether phosphorylative modification of RNA polymerase is constitutive or modulates enzyme activity in vivo remains an open question. There was no change in the phosphorylation pattern of plant RNA polymerase B subunits after auxin treatment which induces

cell proliferation.[64] There is a preliminary report on the possible modulation of the degree of phosphorylation of the B_{214} subunit upon stimulation of confluent glioma cell cultures by isoproterenol.[236] Protein kinases have been reported to stimulate purified mammalian RNA polymerase A and B activity using nonspecific transcription systems.[233,234,237,238] This is somewhat surprising in view of the low degree of phosphorylation achieved in vitro, and the fact that enzyme B with the proteolyzed and unphosphorylated B_{180} subunit is normally active in nonspecific assays. A tight correlation between subunit phosphorylation and enzyme activity remains to be established using nonspecific as well as specific transcription systems. No post-translational modifications of nuclear RNA polymerases other than phosphorylation were clearly demonstrated. Preliminary observations on modifications by cyclic GMP or by ADP-ribosylation have not been further substantiated.[239,240]

D. Regulation by the Substrates

Recent kinetics studies with purified enzymes have suggested that the RNA chain elongation reaction could be more complex than previously thought. Chain elongation in vitro on a supercoiled or linear template is discontinuous, elongating RNA polymerase B molecules pausing at certain sequences along the DNA.[241-244] Transcription of poly d(A-T) proceeded as if the wheat germ enzyme B was a nonprocessive enzyme.[245] The non-Michaelis-Menten kinetic behavior observed for plant germ RNA polymerases A and B may suggest that they are allosterically regulated by ribonucleoside triphosphates.[246-248] If RNA polymerase B contains more than one nucleotide binding site as suggested,[246-248] this could contribute to explain the pausing and nonprocessivity of RNA polymerase at low nucleotide concentration. These preliminary observations certainly warrant a detailed study of the interaction of ribonucleoside triphosphates and other possible small effectors with the RNA polymerase molecule.

IX. TRANSCRIPTION SPECIFICITY

A. Studies with Purified Enzymes

Early studies on the template specificity of various eukaryotic RNA polymerases showed a marked preference, if not a requirement, for unpaired DNA or related DNA defects such as nicks and single-stranded gaps.[5,53,249] A minimal level of template activity was obtained with native double-stranded DNA. Detailed studies on DNA binding and transcription initiation by wheat germ and calf thymus RNA polymerase B have disclosed its rapid and specific binding to single-strand nicks to form long-lived transcriptionally competent complexes. The interaction of enzyme B with nonpromoter sites (reviewed by Lewis and Burgess[6]) is similar in several respects to the complexes formed between *E. coli* core enzyme and DNA.[250]

This global lack of specificity, as well as the development of transcription extracts, had the general result to reduce the interest for specificity studies with purified enzymes. The lack of similar extracts for accurately transcribing class B genes in plants and yeast and the availability of well-characterized enzyme B from these two sources has nevertheless encouraged attempts to demonstrate some sequence selectivity with these purified enzymes. The problem of the selectivity of chain initiation was examined by Lescure et al. in the presence of selected dinucleotides as primer. (We use the term of *selectivity* to refer to nonrandom initiation on selected DNA sequences and reserve the term of *specificity* for accurate initiation at physiological sites.) In a dinucleotide-primed system, yeast enzyme B was able to selectively direct a very efficient synthesis of a trinucleotide,[251] and to initiate selectively on a supercoiled template that contained the yeast alcohol dehydrogenase I gene.[241] Priming by a dinucleotide was not an essential requirement, but the pattern of RNA transcripts was strongly affected by the ribonucleoside triphosphate concentration.[252] The presence of

a few short discrete transcripts made at low substrate concentration indicated precise initiation and termination (or pausing). An initiation site within the yeast DNA insert was precisely located in a region where the DNA sequence had several features reminiscent of eukaryotic promoter sites.[252] However, the physiological significance of this initiation site is still unknown. Coarse mapping of in vitro initiation sites confirmed a similar selectivity of initiation in different systems with the yeast[253] but also the soybean enzyme B.[244] In each case supercoiled templates containing cloned homologous genes were used at a low enzyme to DNA molar ratio.

More surprisingly, pursuing the same approach, Lescure demonstrated a striking selective initiation by yeast enzyme B within the (putative) TATA box of the yeast iso-1-cytochrome *c* gene promoter region, in the proper orientation.[254] Sequences in the 5′ vicinity of the TATA box were found to be required for efficient transcription. Tsuda and Suzuki[255] showed by S_1 mapping that purified RNA polymerase B from *Bombyx mori*, wheat germ, and mouse ascites tumor cells each initiated at the same site (at about nucleotide position +25 of the fibroin gene) on the coding strand.

These observations contrast with the general assumption that purified enzyme B initiates transcription at random. Clearly, under appropriate conditions, the eukaryotic enzyme B is capable of selective initiation. Supercoiling of the DNA is essential. The formation of hairpin loops or a B to Z transition of the DNA helix could be an important feature for regulating enzyme-DNA interaction.[252,256-258] Keeping the substrate concentration low also favors selectivity, but different class B enzymes are not equally sensitive to this parameter.[255] Although the in vitro initiation sites mapped in these studies did not precisely coincide with the major in vivo starts, it is likely that the selectivity exhibited by purified enzyme B has some relevance to its physiological function.

Comparatively, there is little information on RNA polymerases A and C template specificity. Transcription of cloned yeast ribosomal DNA by yeast RNA polymerase A is strand-specific but nonaccurate.[259] The in vitro initiation region was located upstream of the in vivo site,[259] in regions enriched in A-T sequences.[260] Purified RNA polymerase C transcribes randomly homologous templates containing class C genes.[45,261-263] Discrete RNA transcripts from restriction endonuclease-digested DNA templates containing a yeast 5S RNA structural gene were found to be initiated from the ends of the DNA and terminate at selected sites.[262] Cozzarelli et al.,[263] using a sensitive hybridization technique, demonstrated that purified enzyme C from *Xenopus laevis* terminates transcription specifically and efficiently at the consensus sequence at the end of the 5S RNA gene. The termination signal is a simple cluster of at least four thymine residues on the noncoding strand.[264]

B. Transcription Factors

1. Transcription Systems

The finding of crude in vitro transcription systems carrying the accurate transcription of exogenous DNA templates has been a major advance in a field which was at a standstill. It allowed the identification of promoter and regulatory DNA sequences controlling gene expression and initiated the search for general transcription factors as well as specific regulatory components. It is not the topic of this review to describe in detail this important development of research. There are excellent reviews which describe the chronological development of transcription systems and their properties in relation with the in vivo situation.[265,266] This author will mention only the general situation.

Soluble cell-free systems are available for the transcription of class A, B, and C genes.[267-271] Initiation is remarkably specific and accurate and can even mimic qualitatively, in some cases, the relative efficiency of different initiation sites. Cell-free extracts from cultured transformed cells, supplemented or not with RNA polymerase B, were successfully used to transcribe a great variety of protein coding genes: viral genes like adenovirus 2 early

and late genes,[268,269,272-274] SV40[275-277] and polyoma[278] early genes, herpes simplex virus, thymidine kinase, and other early genes,[279,280] retrovirus DNA,[281] hepatitis B core and surface antigen,[282,283] as well as cellular genes like chicken conalbumin and ovalbumin,[284] mammalian α- and β-globin genes,[285-287] fibroin,[288] chick α_2(I) collagene[289] and apolipoprotein II,[290] histone genes,[291,292] protamin,[283] human corticotropin β-lipotropin,[294] *Drosophila* heat shock protein and actin,[295] and others. Simplified systems with endogenous enzyme B are being increasingly used to map class B promoter sites. Most genes were transcribed in heterologous systems, using extracts from cultured human cells (KB or Hela) which showed a high degree of permissivity. Exogenous RNA polymerase B could also come from different vertebrate organisms, albeit not from yeast, wheat germ, or *Drosophila*.[268,284] Regulated transcription might require homologous systems. Homologous extracts have been used successfully from insect[296] and chicken cells.[297]

Class C genes were transcribed by endogenous enzyme C in a variety of crude homologous or heterologous cell-free systems from human,[267] insect,[298] murine or amphibian cell lines,[261] *X. laevis* germinal vesicles,[299,306] mature oocytes,[300] *Bombyx mori* posterior silk gland and ovaries,[301] and from the yeast *Saccharomyces*.[302-304] The genes transcribed included viral genes Ad2 (VA) RNA I and VA RNA II,[261,267] Epstein-Barr virus DNA,[305] a large collection of tRNA genes,[298,299,301-303,306] 5S RNA genes,[261,298-300,304] and the Alu family.[307,308] Correct initiation and termination were demonstrated.[265,266]

In vitro transcription of the class A ribosomal genes has lagged several years as it appeared to require the appropriate homologous extract. These were obtained from mouse ascites cells,[270] mouse, human, and insect cultured cell lines,[310-312] and *X. laevis*.[313] Species specificity of rDNA transcription in vitro has been reported in a number of cases.[270,310,312,314] One should note that the term "species" was generally used inappropriately. When a human cell extract does not transcribe mouse rDNA or vice versa,[270,310,314] one compares two different mammalian orders, not two species within the same order. In fact, *X. laevis* rDNA is transcribed accurately in *X. borealis* oocytes.[313] The cell specificity may not be as stringent as previously thought.[310,313]

Crude cell extracts could lend themselves to studies on the regulation of gene expression. Tjian and collaborators have first shown the involvement of large T antigen in repression of SV40 early transcription and autoregulation in vitro.[275] The large T antigen, a multifunctional phosphoprotein (96 kDa), binds specifically at three closely spaced sites on SV 40 DNA in the early promoter proximal region and this binding results in inhibition of initiation of transcription.[275,315] A mutation in binding site II which overlaps the cap sites prevents autoregulation by purified T antigen in vitro.[316] Soon after infection of host cells by SV40 there is activation of rRNA synthesis in response to large T antigen. Purified T antigen was indeed found to stimulate the rate of rRNA synthesis in a whole-cell extract prepared from uninfected cells.[317] The activity of the factors involved in rRNA synthesis has been shown to correlate with the cell growth rate.[270] The stimulation of transcription of yeast $tRNA^{Tyr}$ gene in a yeast cell-free extract by tyrosyl-tRNA synthetase (TyrRS) suggested a role for TyrRS in the regulation of $tRNA^{Tyr}$ synthesis;[318] studies with complex transcription mixtures, however, are complicated by the presence of unrelated components which makes the interpretation of results as simple as the effect of the ionic conditions or DNA concentration difficult[271] on the preferential utilization of specific class B promoters.[319] The mode of action of regulatory components will be best investigated in purified transcription system.

The development of these cell-free systems has been essential to delineate the sequences in the DNA which control transcription like the TATA box, the enhancer elements, the intragenic control region of class C genes (see References 266,320-322 for an overview). They also contributed to establish the gene specificity of each RNA polymerase form. Also, they reactivated the search for transcriptional factors which had met with limited success when specific transcription assays were not available.

2. Stimulatory Factors

A basic 37-kDa protein (P_{37}) which stimulates nonspecific transcription by RNA polymerase A and B has been purified from yeast cells.[323] A complex of P_{37} and RNA polymerase B in a 1:1 molar ratio was isolated by sedimentation through a glycerol gradient and electrophoresis.[323] The P_{37} protein bound preferentially to RNA polymerase B_I (the nonproteolyzed enzyme form). The complex had a dissociation constant of 5.10^{-8} *M*. The common subunit ABC_{23} appeared involved in the enzyme-factor interaction,[324] which suggested a general role for this protein in transcription, possibly at the step of chain elongation.[325]

A protein with similar general properties was purified from Ehrlich ascites tumor cells by Natori and collaborators. This protein (40.5 kDa), termed "S-II",[326] interacted weakly with homologous purified RNA polymerase B (the proteolyzed subform B_{II} was used) and seemed to remain associated to the transcription complex during chain elongation.[327] S-II protein can occur in phosphorylated form.[328] There is good evidence that S-II protein or immunologically related protein(s) is one of the general transcription factors of class B. Antibodies raised against murine S-II protein inhibited accurate transcription from adenovirus-2 major late promoter in a human cell extract.[329] Apparently, the Mn^{2+}-dependent stimulatory activity of the factor is not necessary for accurate transcription, but it makes a convenient assay for purifying homologous proteins from other sources. Immunofluorescence studies showed the presence of immunologically related proteins in the nucleoplasm of various eukaryotic cells.[330]

3. Multiple Transcription Factors

The term "factor" refers here to protein components distinct from the subunits of RNA polymerases which are required as essential components of the transcription complexes at the various steps of RNA synthesis, chain initiation, elongation, or termination.

Fractionation of transcription extracts for isolating class B factors has been attempted from human cell lines. Matsui et al.[331] demonstrated the requirement for multiple components distinct from RNA polymerase B for accurate transcription from the Ad_2 major late promoter. The transcription system was reconstituted with RNA polymerase B and four fractions obtained by a succession of salt-step elutions from different columns and designated TFIIA, TFIIB, TFIIC, and TFIID. An additional component, TFIIE, was identified.[332] These authors also postulated the existence of gene-specific factors in addition to these general transcription factors. TFIIC was later found to be identical with poly (ADP-ribose) polymerase.[333] This enzyme suppresses random initiation by binding to nicks in the DNA template and does not seem to be required in more purified systems. From its chromatographic behavior, TFIIB appears similar to the stimulatory factor described in yeast and mouse ascites cells. A number of other groups have repeated these observations using a similar fractionation approach.[334-335] Davison et al.,[336] using a prebinding assay where two different templates compete sequentially for a transcription factor, demonstrated the stable binding of a Hela cell factor to DNA fragments containing the TATA box region of the conalbumin gene or the Ad2 major late promoter. Deletions of the TATA box sequence affected the formation of this preinitiation complex which occurred as a primary step of transcription in the absence of RNA polymerase B. The interactions engaged by the other components are unknown. Recently, Bunick et al.[337a] have reported that ATP hydrolysis was required for specific transcription. The particular transcription factor that requires ATP (or dATP) hydrolysis is involved in the early step of transcription.[337a] It could correspond to a DNA-dependent ATPase which copurifies with TFIIE factor.[337b]

As the run-off assay used in these experiments eliminated the need for proper termination, the presence of a putative class B termination factor was not investigated. RNA-dependent nucleoside triphosphate phosphohydrolases were described in yeast[338] and rat liver.[339] They do not seem to act as termination factors like *E. coli* rho protein.

Dynan and Tjian described the first promoter-specific transcription factor of class B. Fractionating a Hela cell extract, they partially purified a factor, termed "Sp_1," that was required for transcription from SV40 early and late promoters but not from Ad_2 major late, human β-globin, and retrovirus LTR promoters.[340] The same authors further showed by DNase footprinting that SP1 factor activity copurified with a specific DNA binding component interacting with sequences in the 21-bp repeat upstream from the SV40 early promoter.[341]

Fractionation of class C factors, following the identification of internal control (promoter) regions within the class C genes,[321] has given the first clue as to how gene recognition could be achieved in eukaryotic cells. First, a protein factor TFIIIA (38.6 kDa) required for 5S RNA gene transcription, which alone can recognize the intragenic control region was purified by Engelke et al.[342] from *Xenopus* oocytes. The factor being purified to homogeneity was well characterized and its physical interaction with the 5S DNA gene was investigated in detail.[343-347] The *Xenopus* factor makes specific contacts on the noncoding strand of the 5S gene promoter[344] and slightly unwinds the DNA.[346] Zinc is required for binding and activation of transcription.[348] The factor also interacts stoichiometrically with 5S RNA, which suggests a mechanism of autoregulation of synthesis.[349-351] However, TFIIIA is not sufficient for directing 5S DNA transcription, and it does not act on tRNA genes. Other factors are involved.

Roeder and colleagues partially purified two factors (termed "TFIIIB" and "TFIIIC"), from human[352] and amphibian cells,[353] which were required for transcription of tRNA and VA RNA genes, and also, when supplemented with TFIIIA, of 5S RNA genes. Lassar et al.[354] conducted a systematic exploration of gene-factor interactions with the indirect approach of template exclusion by preincubation steps involving different factors and template combinations. Human factor TFIIIC appeared as a pivotal component for recognition of class C genes. By itself, it formed a stable complex with VA_1 RNA gene, with a $tRNA^{Met}$ gene, in conjunction with TFIIIB, and finally, it stabilized the otherwise weak interaction of TFIIIA with the 5S RNA gene.

The requirement for two general class C factors distinct from RNA polymerase C was also found in *Drosophila*[355] and yeast[303] cells with various tRNA genes. A yeast factor, termed τ, which sedimented as a high molecular weight protein (~300 kDa), was shown in template sequential competition experiments to form a very stable complex with yeast tRNA genes in the absence of divalent cations, RNA polymerase C, or any other transcriptional component.[356] That the yeast τ factor could by itself form a stable complex with some tRNA genes, at variance with human TFIIIC, could simply result from differences in DNA sequences or in the distance between the A and B blocks, although other explanations can be envisioned. The τ factor very probably corresponds to the footprinting component first identified by Klemenz et al.[357] in a partially purified yeast extract which interacted strongly and specifically with the B block region of VA RNA 1 and tRNA genes. DNA binding was correlated with transcription factor activity.[358,359] An equivalent DNA footprinting factor activity was similarly obtained from Hela cells.[360] Whether these fractions contained a unique component is not completely clear. In any case, the pattern of factor(s)-DNA interaction, strong and rapid with the B block in the posterior region, then weaker with the A block, suggested that the protein(s) binding to these two separated regions interact, possibly folding the template during this process.[359] Based on DNA mutagenesis studies, Hall et al. proposed a model where the A and B blocks would interact within the transcription complex.[361] Study of the formation and structure of class C transcription complexes using purified components will shed more light on these fascinating protein-DNA interactions.

These preliminary studies on eukaryotic transcription factors underscore the central role of factor-DNA interactions in promoter recognition and gene activation. Once formed the factor(s)-DNA complex would in turn be recognized by RNA polymerase through protein-protein contacts, at least in part.[362] This type of regulation could explain the molecular complexity of RNA polymerases engaged in multiple protein-protein interactions.

X. FUTURE PROSPECTS

Two fields of interest are likely to be strongly attractive in the near future. Analysis of the structure and function of RNA polymerases and their subunits will be investigated at the level of the structural genes rather than the proteins. The cloning of a *Drosophila* and of the yeast RNA polymerase genes is very stimulating, as it allows the study of the genetic organization, evolution, and regulation of this family of genes. In vitro mutagenesis of these genes will provide the tools for in-depth analyses of the role of each enzyme form and their constituents. Isolation of mutants will be best developed in yeast cells, which are well amenable to genetic studies.

Study on gene expression in vitro will aim at reconstituting specific transcription systems with purified components. The factor hunt will hopefully be accelerated and lead to a better description of the mechanisms of recognition of specific regulatory sequences in the DNA. How RNA polymerases interact with factors and promoter sites and how transcription is regulated in the eukaryotic cell are questions that soon will be addressed at the molecular level.

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