the intramolecular water bridge between the 2′-OH and 3′-phosphate proposed elsewhere (Bolton & Kearns, 1978, 1979) which might be expected to hinder the local motion at the 2′ position.

There is now a fair amount of evidence for the presence of conformational fluctuations of the backbone, phosphate and ribose of polynucleotides on the time scale of nanoseconds. Thus, the concept of the "rigid nucleotide" should be limited to a description of the equilibrium conformation of nucleic acids and not to the conformational rigidity of polynucleotides.

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Escherichia coli Ribonucleic Acid Polymerase Binding to the Deoxyribonucleic Acid of the Echinoid *Paracentrotus lividus*: Properties of the Complexes and Distribution of Stable Binding Sites†

Ernesto Di Mauro,* Paola Ballario, and Francesco Pedone

ABSTRACT: We describe the properties of the complexes that form between *Escherichia coli* RNA polymerase and *Paracentrotus lividus* DNA: dissociation kinetics, temperature dependence of the complex formation, resistance to heparin, and range of RNA polymerase–DNA weight/weight ratios that give rise to the stable binding events. The amount and distribution of the sites that form stable binding [class A sites as defined by Hinkle & Chamberlin [Hinkle, D., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157]] with *E. coli* RNA polymerase were determined by the analysis of the dissociation of complexes formed by the enzyme on DNA fragments of various length. The *P. lividus* appears to form 3.1×10^5 stable ($t_{1/2} \geq 15$ min) complexes per haploid genome; the great majority of these complexes shows a short-range distribution (1000–2000 base pairs). The observed attributes of the stable binding sites of *P. lividus* DNA for *E. coli* RNA polymerase (amount, distribution, and quantitative ability to start in vitro RNA chains) point to the conclusion that *E. coli* and sea urchin DNA are nearly indistiguishable by the criteria adopted. The behavior of the sea urchin stable binding sites for the *E. coli* enzyme is not consistent with the expected behavior of the in vivo promoters.

It has been shown that *Escherichia coli* RNA polymerase can form stable complexes with DNA at sites [class A sites as defined by Hinkle & Chamberlin (1972)] which correspond to genetically identified promoters; the evidence obtained is limited to the interaction of procaryotic RNA polymerase with bacteriophagic, genetically well-defined genomes and with a few bacterial promoters [for a review, see Chamberlin (1976)]. We (Pedone et al., 1978) have described the distribution of class A sites on the relatively more complex genome of *E. coli*, on the assumption that such an analysis provides a rapid and detailed description of the procaryotic genome under consideration; the validity of such an assumption is based (a) on the observed correspondence between known bacterial and bacteriophagic promoter sites and stable binding sites (Chamberlin, 1976), (b) on the similarities of the properties of the stable complexes we (Pedone et al., 1978) have described in the *E. coli* system with the properties of complexes formed on genetically identified promoters, and (c) on the correspondence between the number and distribution of stable binding sites and the available knowledge of genetic complexity and organization of the *E. coli* genome.

In the present communication we describe the analysis of a much more complex genome (the echinoid *Paracentrotus lividus*) carried out with the same methodological approach that we have used for the *E. coli* genome (Pedone et al., 1978) and discuss the validity of the use of this bacterial enzyme as a tool in the analysis of eucaryotic genomes and in the study of their organization.

Material and Methods

Materials, the preparation of the DNA fragments, and the nitrocellulose filter assay conditions for the binding of RNA polymerase to DNA are as described in Pedone et al. (1978). Purification of *E. coli* RNA polymerase was also performed

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as described in Pedone et al. (1978); the enzyme obtained was more than 90% homogeneous, as judged by gel electrophoresis in polyacrylamide gel, contained approximately (±10%) equimolar amounts of σ, and had a specific activity of 600 units/mg [defined according to Burgess & Travers (1971)].

P. lividus [^3H]*DNA Labeling and Extraction.* The procedures described by Di Mauro et al. (1979) were used. The specific activity of the DNA was 90000 counts/(min μg). The recovered DNA had a molecular weight of 20000 base pairs, as judged by EM length analysis, and contained no more than 2.2 single-stranded nicks/molecule, as judged by alkaline sucrose gradient sedimentation (Studier, 1965).

Results

Characterization of Class A Sites. (1) Sea Urchin DNA Retention on Filters. P. lividus DNA binds to *E. coli* RNA polymerase, and the resulting complexes can be retained on nitrocellulose filters when present either as a high molecular weight species (20000 base pairs) or as a low molecular weight fragment of subgenic size (300 base pairs) such as can be obtained by sonication. The affinity of *E. coli* RNA polymerase has been described for various templates (Hinkle & Chamberlin, 1972; Pettijohn & Kamiya, 1967; Chamberlin, 1970) and is explained by the non-promoter-specific, general affinity of the enzyme for polynucleotide sequences (Chamberlin, 1976).

In our system the efficiency of retention ranges from 60 to 90% of the DNA input depending on the conditions of filtration. Under the chosen conditions (filtration speed = 1 mL in 15 s; Pedone et al., 1978) a lower efficiency (60%) and a lower background (<0.5% of the input DNA) are obtained. It should be noted that the retention as a function of the RNA polymerase/DNA ratio (w/w) is identical with the retention of the *E. coli* DNA [see Figure 1 in Pedone et al. (1978)] at the same RNA polymerase/DNA ratios, showing that in the retention on filters [mostly due to binding to class B sites (Hinkle & Chamberlin, 1972); see below] there is no major preferential binding effect to any of the two templates. Retention efficiency on the filters of the input DNA did not vary as a function of the size of the DNA molecules used; we therefore conclude that no preferential loss of a certain size class of molecules takes place.

(2) Sensitivity of RNA Polymerase to Heparin. The sensitivity to heparin, higher for free or loosely bound RNA polymerase than for RNA polymerase bound to class A sites (Pedone et al., 1978; Zillig et al., 1970; Schafer et al., 1973; Giacomoni et al., 1977), allows one to discriminate between the complexes formed on the two types of sites.

Heparin can be considered in excess for the reaction with the non tightly bound RNA polymerase (for the reported amount of enzyme) at a concentration higher than 1 μg/mL (titration not shown). Figure 1 shows the dissociation kinetics of *P. lividus* DNA–RNA polymerase complexes in the presence of various amounts of heparin. Two classes of complexes with different stabilities are observed; the first has a half-life of ≤15 s, and the second is characterized by a complex behavior. Figure 1 shows how the half-life of the stable complexes was calculated by extrapolating to zero heparin concentration. A half-life of 15 min is obtained. The half-life of the stable binary complexes formed by *E. coli* RNA polymerase with *P. lividus* DNA is lower than that obtained for the complexes formed with the homologous DNA (38 min) (Pedone et al., 1978); the lower stability is probably related to the heterology of the system. In addition to these complexes, other more stable binary complexes exist (see Figure 1, the experimental points above the extrapolation line) with an

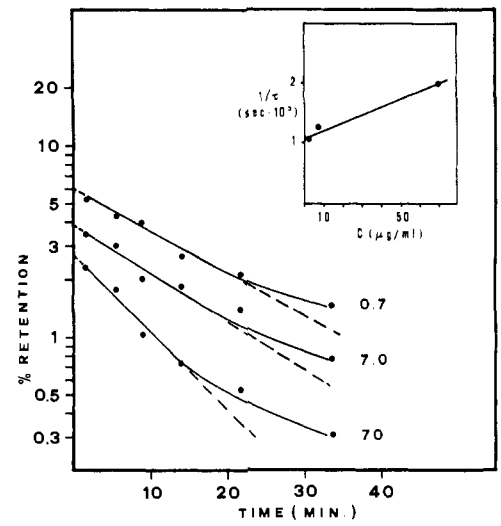

FIGURE 1: Dissociation kinetics of *E. coli* RNA polymerase–^{3}H-labeled *P. lividus* DNA complexes in the presence of heparin. For each dissociation curve, 1 μg of ^{3}H-labeled *P. lividus* DNA (300 base pair fragments) and 25 μg of *E. coli* RNA polymerase were incubated for 10 min at 37 °C in 3 mL of binding buffer; heparin was added at the concentrations in micrograms per milliliter reported in figure, and the dissociation was followed during the indicated time by filtration of 0.3-mL samples diluted to 1 mL with binding buffer. The background (0.5%) of the input DNA due to nonspecific retention to the filter is substracted. Insert: Plot of rate constant $1/\tau$ against heparin concentration. Data are taken from the dissociation kinetics reported in Figure 1.

estimated half-life of 5 h. As in the case of stable complexes formed by *E. coli* RNA polymerase with *E. coli* DNA (Pedone et al., 1978), we refer to class A sites as the sites which form complexes with a half-life of 15 min or higher.

(3) Temperature Dependence. The formation of stable complexes is temperature dependent (data not shown). The increase of heparin resistance is constant starting from 0 to ~25 °C and is very similar to what was observed in the homologous *E. coli* system (Pedone et al., 1978). Fast dissociating binary complexes (half-life < 15 s) form readily at 0 °C. An appreciable although low amount of stable complexes (~15% of the total) forms even at 0 °C. The same picture observed for the homologous *E. coli* RNA polymerase–*E. coli* DNA system (Pedone et al., 1978) seems thus to hold also for this heterologous system.

Amount and Distribution of Class A Sites. The distribution of the DNA sites able to form complexes with *E. coli* RNA polymerase which are resistant to heparin can be studied by analyzing the dissociation kinetics of complexes formed by excess RNA polymerase on DNA fragments of different length. Figure 2 shows three examples of these dissociations carried out on fragments of 300, 400, and 600 base pairs. It can be observed (Figure 3), as already reported for the homologous *E. coli* system (Pedone et al., 1978), that for the short-sized fragments the level of resistance does increase linearly with the increase of the fragment length.

Figure 3 shows that the increase of resistance will not be linear for longer fragments; the figure reports the fraction of DNA fragments that contains a nucleotide sequence able to form stable binding with *E. coli* RNA polymerase as a function of the fragment length.

It can be observed that almost all of the DNA is retained by 2000 base pairs (by 5000 base pairs the retention is complete); that is, there are no binding sites for *E. coli* RNA polymerase that are spaced more widely than this value. From these data also the total amount of sites per haploid genome can be calculated; given a genome complexity of 6.5×10^8 base

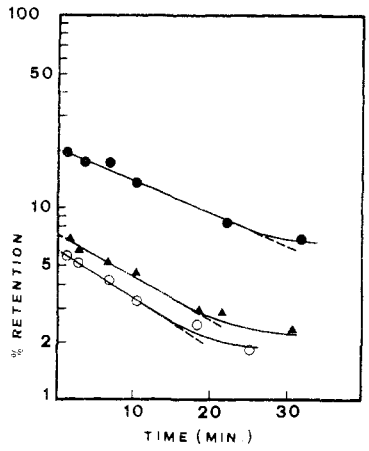

FIGURE 2: Determination of the dissociation kinetics of *E. coli* RNA polymerase–^{3}H-labeled *P. lividus* DNA complexes for different molecular weight DNA fragments. 1 μg of ^{3}H-labeled *P. lividus* DNA, 300 (○), 400 (▲), and 600 (●) base pairs, and 25 μg of *E. coli* RNA polymerase were incubated at 37 °C for 10 min in 3 mL of binding buffer, and dissociation kinetics, in the presence of 10 μg/mL heparin, were followed as described.

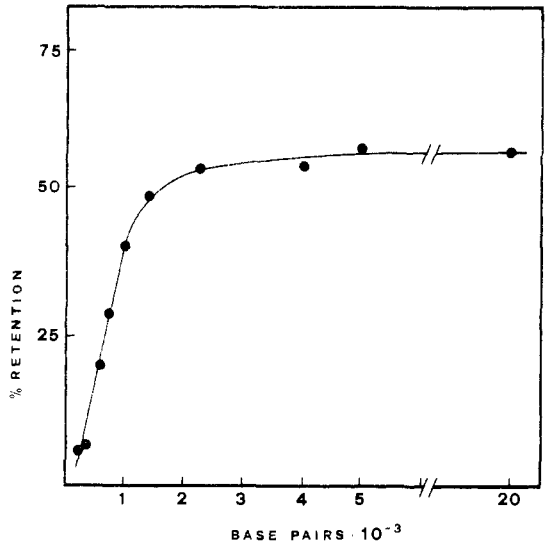

FIGURE 3: Fraction of *P. lividus* DNA fragments bearing a class A binding site for *E. coli* RNA polymerase as a function of fragment length. Data are derived from dissociation kinetics similar to the ones described in Figure 2.

pairs (Venderly & Vendrely, 1949), an average presence of 1 site every 2090 base pairs, we obtain a total of 3.1×10^5 sites/genome. The average presence of sites has been calculated as follows. The curve reported in Figure 3 has been resolved into five linear components, each referring to the following fragment-length intervals: 0–1000, 1000–1500, 1500–2300, 2300–5000, and 5000–20 000 base pairs. For each interval the increment of retention has been calculated and corrected for the retention efficiency. The number of class A sites for each interval has been calculated by

$$N = (6.5 \times 10^8)\Delta r/(100n)$$

N = number of class A sites of the fragment, n = base pairs of the fragment length interval (upper value), and Δr = retention increment. The number of the class A sites for the whole genome is given by the sum of the values obtained for the five intervals.

The same distribution of class A sites for *E. coli* RNA polymerase can be observed by making use of a different approach; an RNA polymerase/DNA ratio (w/w) can be

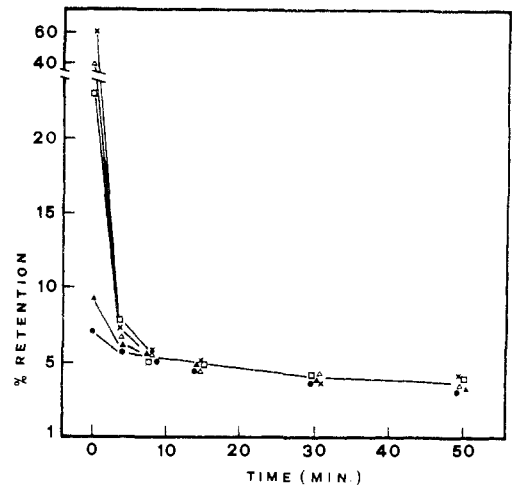

FIGURE 4: Dissociation kinetics of complexes formed at various *E. coli* RNA polymerase–*P. lividus* DNA ratios. 1 μg of ^{3}H-labeled *P. lividus* DNA (300 base pairs) was incubated for 10 min at 37 °C with the quantities of enzyme reported below in 3 mL of binding buffer; heparin (final concentration 10 μg/mL) was then added, and the dissociation kinetics were followed as already reported. RNA polymerase: 1 (●), 2 (▲), 4 (□), 10 (△), and 40 (×) μg. The maximal percent retention before heparin addition was for each of the indicated RNA polymerase/DNA ratios: 7, 9.5, 23, 40, and 60. Data are not corrected for the retention efficiency of the assay.

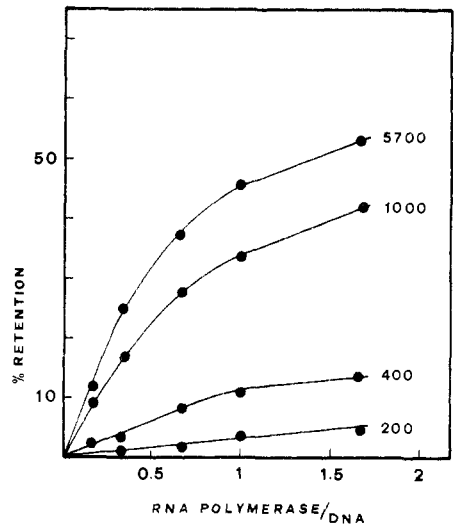

FIGURE 5: Retention of ^{3}H-labeled *P. lividus* DNA as a function of the *E. coli* RNA polymerase/*P. lividus* DNA ratio (w/w) for DNA fragments differing in length. 0.1 μg of ^{3}H-labeled DNA was incubated at 37 °C for 10 min in 0.3 mL of binding buffer in the presence of the amounts of RNA polymerase indicated on the abscissa. Samples were diluted to 1 mL with binding buffer and filtered as described (Pedone et al., 1978). The base pair values, for each saturation curve, are indicated in the figure.

identified experimentally that gives rise only to stable binding. This method relies on the assumption that the *E. coli* RNA polymerase has a sufficiently higher affinity for class A sites to be found, at a limiting enzyme concentration, preferentially bound to them. This condition, already verified in other systems [see the review article by Chamberlin (1976)], can be satisfied also for *P. lividus* DNA; Figure 4 shows in fact that at the lower w/w RNA polymerase/DNA ratio reported (ratio = 1) only complexes were formed that dissociate with a kinetic characteristic of the class A complexes (cf. dissociation in Figure 2). An increase of the RNA polymerase/DNA ratio only slightly increases the amount of stable complexes formed and results mostly in the formation of increasingly higher amounts of rapidly dissociating complexes.

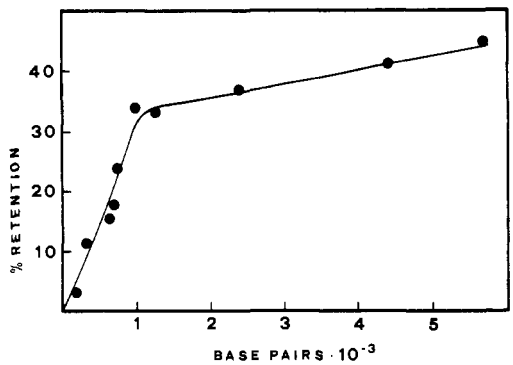

FIGURE 6: Fraction of *P. lividus* DNA fragments bearing a class A binding site for *E. coli* RNA polymerase as a function of fragment length. Each point indicates the percentage of retention obtained for the DNA fragment whose length is indicated on the abscissa at an *E. coli* RNA polymerase–*P. lividus* DNA ratio (w/w) of 1.

Figure 5 shows the retention on filters of various *P. lividus* DNA populations differing in length by increasing amounts of RNA polymerase.

If the RNA polymerase/DNA ratios reach high values (not shown), the maximal retention value of 60% will be reached also for low molecular weight DNA fragments. The values of percent retention that are observed at the relevant RNA polymerase/DNA ratio of 1 for the four DNA fragment populations described in Figure 5 and for the six other populations are reported in Figure 6. It can be observed how the distribution of stable binding sites obtained with this technique corresponds to the distribution obtained through the analysis of the levels of resistance to heparin as a function of the DNA fragment length (cf. Figures 3 and 6).

Discussion

The description of the number and of the distribution of stable binding sites for *E. coli* RNA polymerase might be considered a useful tool in the analysis of eucaryotic genomes and in the study of their organization. The results obtained in this study emphasize that caution should be taken in utilizing a procaryotic enzyme on a eucaryotic DNA.

Data on the nature of eucaryotic promoters are very scarce, and the way they function is still only conjectural, although one may think that the basic mechanism identified in procaryotes should not have been lost during evolution, at least for the requirement of the local limited melting of the double-stranded structure and of the formation of an "open" "rapid starting" complex. In this sense the sites that on eucaryotic DNA are able to form a temperature-dependent stable complex may correspond to the sites that are in vivo committed to start RNA synthesis. There is so far no way to obtain a direct evaluation of the degree of correspondence between stable binding sites and genetic promoters in eucaryotic genomes. In order to examine the degree of such correspondence and therefore to assess the descriptive relevance of the *E. coli* RNA polymerase binding analysis we have performed, we will bring into consideration the following indirect arguments.

Commitment to Start an RNA Chain. *E. coli* RNA polymerase forms stable complexes with *P. lividus* DNA at sites from which it can start RNA synthesis, that is, with sites that behave as in vitro promoters; we (Di Mauro et al., 1977) have shown that *E. coli* RNA polymerase can start RNA synthesis at 32 500 sites/*P. lividus* genome under restrictive ionic conditions [100 mM $(NH_4)_2SO_4$]. In the present evaluation we calculate that in low salt binding buffer (50 mM NaCl) *E. coli* RNA polymerase forms 3.1×10^5 stable complexes/*P. lividus* genome; as the amount of stable complexes that forms in 100 mM $(NH_4)_2SO_4$ is 3 times lower than the corresponding amount in 50 mM NaCl (data not shown), it can be stated that only about one-third of the molecules that form stable complexes can start the synthesis of an RNA molecule. The direct correspondence of the enzyme molecules that form stable complexes with the ones that start the synthesis of an RNA chain will be detailed elsewhere.

The ability to start an RNA chain in vitro (that in the heterologous system we describe here is missing in two-thirds of the sites that form stable complexes) may be considered a necessary requirement for the identification of a promoter region but is by no means a sufficient one.

Amount of Binding Sites. How many promoters does one expect to find along the *P. lividus* genome? Omitting from the consideration the rRNA and tRNA species, one can assume the number of promoters to be equal to the number of expressed nuclear RNAs and cycloplasmic mRNAs. It has been shown (Galau et al., 1974) that *Strongilocentrotus purpuratus* gastrulas express 14 000 different mRNA sequences. The number of nuclear RNAs expressed at the same stage is 2 to 3 times higher. This evaluation can be deduced from the observation [Hugh, Smith, and Davidson, as quoted in Galau et al. (1974); Davidson, 1976] that the *S. purpuratus* gastrular nuclear RNA has a complexity 10 times higher than that of the polysomal mRNA of the same developmental stage and considering that the apparent mean length in nucleotides is 8×10^3 nucleotides for nuclear RNA and 1.8×10^3 nucleotides for mRNA (Davidson, 1976). We can therefore estimate that the number of total transcription events in gastrulas is $\sim 5 \times 10^4$. These figures derive from the analysis of a particular developmental stage and are therefore a lower limit estimate of the total transcriptive events that take place in the sea urchin organism. The data discussed have been obtained in the sea urchin *S. purpuratus*; the genus used in the present study is *P. lividus*. Both the amount of DNA per haploid genome and the relative amounts of the different repetitive classes are very similar (C. T. Baldari and F. Amaldi, personal communication). However, taking into consideration the likelihood that a large fraction of the genetic potential of an organism is expressed in all of its cells [Bishop et al., 1974; Izquierdo and Bishop, as quoted in Levy & McCarthy (1975); Levy & McCarthy, 1975; Axel et al., 1976], the reported value of 5×10^4 transcriptive events should be a reasonable estimate. Our present observation that *E. coli* RNA polymerase forms more than 3×10^5 stable complexes per *P. lividus* genome indicates therefore a large degree of nonspecificity in the binding process.

Distribution of Binding Sites. The pattern reported in Figure 3 indicates that the retention increase is directly proportional to the increase of the fragment length until ~1000 base pairs; this is taken as an indication that 1000 base pairs is the minimal distance between two class A sites. Most of the sites have a spacing that goes from ~1000 to ~2000 base pairs [the base pair value at which a plateau of 60% of the DNA input (see Sea Urchin DNA Retention on Filters) is reached]. From these data it seems clear that no sizable fraction of widely spaced class A binding sites for *E. coli* RNA polymerase exists on *P. lividus* DNA. The only basis of comparison on which one can test the observed distribution of the in vitro stable binding sites on the *P. lividus* genome comes from the data on the sizes of mRNAs and hnRNAs in sea urchins. The apparent size of nuclear RNA molecules has a mode value of ~8000–9000 nucleotides as measured in denaturing velocity sedimentation gradients or formamide–agarose–acrylamide gels (Kung, 1974; DuBroff & Nemer,

1975). Aqueous sedimentation analysis has given values in the range 50–100 S (Brandhorst & Humphreys, 1972; Sconzo et al., 1974). RNA is present under a broad range of sizes, the number average of which is at 2000 nucleotides (Kung, 1974; Nemer, 1975; Nemer et al., 1974, 1975); some very large cytoplasmic RNAs have also been reported (Kung, 1974; Brandhorst & Humphreys, 1972; Sconzo et al., 1974; Giudice et al., 1972, 1974; Rinaldi et al., 1974). It seems therefore that the distribution of class A in vitro binding sites observed does not satisfy the minimal spacing required by the size of the reported in vivo transcriptions.

In summary, we observe that *E. coli* and *P. lividus* DNA have a very similar behavior as far as the *E. coli* RNA polymerase binding is concerned. The number, the distribution, and the quantitative ability to start RNA chains that characterize the stable binding sites on *P. lividus* DNA for the heterologous RNA polymerase are not consistent with the expected corresponding attributes of the nucleotide sequences endowed in vivo of direct positive operative character. The reported finding that *E. coli* RNA polymerase transcribes chromatin but not naked DNA in a manner which reflects the in vivo specific gene expression (Axel et al., 1973; Biessmann et al., 1978; Harris et al., 1976; Smith & Huang, 1976; Stein et al., 1975; Gilmour & Paul, 1973; Swetley & Watanabe, 1974) may indicate that chromosomal proteins are involved in the restriction of DNA sequences available for transcription.

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