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Transcription of nucleosomal DNA in SV40 minichromosomes by eukaryotic and prokaryotic RNA polymerases

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ABSTRACT

SV 40 minichromosomes can be transcribed by prokaryotic and eukaryotic RNA polymerases. Size analysis of transcripts indicated that DNA in nucleosomes was accessible for transcription by both enzymes. Sedimentation of the transcription complex showed that minichromosomes which were being transcribed had a full complement of nucleosomes. Strand selection by E.coli RNA polymerase was reduced by the presence of nucleosomes. No region of SV 40 DNA was preferentially transcribed on minichromosomes by either enzyme.

INTRODUCTION

Permissive cells infected with papovaviruses contain viral DNA complexed with histones in chromatin-like structures, sometimes called viral "minichromosomes" (1-3). This protein-associated form appears to be the normal state of papovavirus DNA, since it is found throughout the infective cycle as well as inside the virions (3-6). Obviously then, studies on replication and transcription of these DNAs must consider the possibility that minichromosomes, rather than free DNA, are the actual templates. In view of the close similarity between minichromosomes and cell chromatin structure (2), such studies should be of interest for the more general problem of transcription in eukaryotic cells.

We have recently shown that BK-virus minichromosomes can act as transcription templates in an in vitro system using E.coli RNA polymerase (7). In the present study, SV40 minichromosomes extracted from virions were transcribed with eukaryotic RNA polymerase B and with E.coli RNA polymerase. Transcript

analysis indicated that DNA in nucleosomes is accessible for transcription by both the prokaryotic and the eukaryotic enzyme. Sedimentation of the in vitro transcription complex showed that minichromosomes transcribed in our experiments had a full complement of nucleosomes. Strand selection by E.coli RNA polymerase was reduced by the presence of nucleosomes. No region of SV40 DNA was preferentially transcribed on the minichromosomes by either enzyme.

MATERIALS AND METHODS

Enzymes. E.coli RNA polymerase was purified from MRE 600 strain ($RNase^-$) as already described (8). Calf thymus RNA polymerase B was purified by a modification of the method of Kedinger et al. (9). Fresh calf thymus was homogenized at 4° in Tris-HCl (pH 7.2)-0.25 M sucrose-25 mM KCl-5 mM $MgCl_2$-1.5 mM $CaCl_2$-0.15 mg/ml phenylmethyl sulfonylfluoride and centrifuged for 15 min at 5,000 rpm. The supernatant and floating lipids were carefully decanted and the crude nuclear pellet (150 g) was suspended in 500 ml of sonication buffer (20 mM Tris-HCl (pH 7.9)-3 mM $MnCl_2$-1 mM DTT-0.1 mM EDTA-30% glycerol-0.15 mg/ml PMSF) on ice and lysed in 0.3 M NH_4 sulfate. The viscous extract was sonicated and then centrifuged in a Spinco 30 rotor at 29,000 rpm and 2° for 2 hrs. Two volumes of DEAE buffer (50 mM Tris-HCl (pH 7.9)-3 mM $MnCl_2$-1 mM DTT-0.1 mM EDTA-30% glycerol) were added to the supernatant. Total RNA polymerase activity was batch-adsorbed on 600 ml (packed volume) of DEAE cellulose (Whatman DE 32) precycled as described (9) and equilibrated against 50 mM NH_4 sulfate in DEAE buffer. The suspension was stirred for 45 min and filtered on filter paper. The adsorbent was washed with 500 ml of the above buffer and transferred to a glass column, from which total RNA polymerase activity was eluted with DEAE buffer containing 0.5 M NH_4 sulfate. After precipitation with NH_4 sulfate (60% of saturation) and desalting on a Sephadex G-25 column, protein-containing fractions were pooled, clarified by centrifugation at 30,000 rpm for 45 min and adsorbed on a DEAE-Sephadex A-25 column

previously equilibrated with DEAE buffer. RNA polymerase A activity was eluted with DEAE buffer containing 0.15 M NH_4 sulfate, whereas B activity was eluted with the same buffer containing 0.3 M NH_4 sulfate. After ammonium sulfate precipitation of fractions containing B activity, the precipitate was dissolved in a minimal volume of 50 mM Tris-HCl (pH 7.9)-0.1 mM DTT-15% glycerol and centrifuged through a sucrose gradient as described (10). Fractions of the activity peak were precipitated with ammonium sulfate as above, the sediment was dissolved in 50 mM Tris-HCl (pH 7.9)-0.1 mM EDTA-1 mM DTT-50% glycerol and stored at -85°. Enzyme preparations contained no appreciable DNase activity, as measured by conversion of superhelical polyoma virus DNA to nicked circular form in the conditions of transcription assay.

Restriction endonuclease Hin c III was obtained from Miles. Pancreatic and T1 ribonucleases were purchased from Merck.

Cells and virus. Confluent CV-1 cells grown in H21 medium supplemented with 5% fetal bovine serum and 1% Tryptose (Difco) were infected with plaque purified SV40 strain VA54-45 at a moi of 0.05 pfu/cell. After 10 days, cells and medium were collected and centrifuged for 15 hrs at 13,000 rpm in a Beckman JA-14 rotor at 3°. The pellet was sonicated, frozen and thawed three times and treated with 0.01% trypsin and 1% Na deoxycholate at 37° for 30 min. Virus was then purified by differential centrifugation and finally banded in a CsCl gradient. Tritium-labeled virions were obtained by addition of 1 µCi of [^{3}H]-thymidine (CEA, sp. radioact. 25 Ci/mmol) per ml of culture medium 7 days after infection.

Purification of SV40 nucleoprotein complex. Minichromosomes were extracted from purified SV40 virions by mild alkaline treatment as already described (6, 7). The lysed virions were then centrifuged through 5 to 20% sucrose gradients in 10 mM Tris-HCl (pH 7.5)-1 mM EDTA-0.1 M NaCl-0.25% Triton X-100. Centrifugation was at 45,000 rpm at 4° for 75 min in a Spinco SW 50.1 rotor. Gradient fractions were assayed for template activity with E.coli RNA polymerase as described (7). Fractions corresponding to peak activity (60 S) were used for subsequent

experiments.

Sedimentation analysis of transcription complex. Purified [^{3}H]-thymidine labeled SV40 minichromosomes or SV40 superhelical DNA were transcribed with E.coli RNA polymerase in the presence of [α-^{32}P]-UTP (sp. radioact. 45 Ci/mmol). Reaction conditions were as described (7). Final reaction volumes were 100 µl. Reaction kinetics were followed, to compare relative template efficiencies of DNA and minichromosomes. After 20 min at 37°, 5 µl of 0.1 M ATP were added (to lower final background) and the reactions were immediately loaded on 15 to 30% gradients in 10 mM Tris-HCl (pH 7.5)-4 mM EDTA-0.15 M NaCl-0.25% Triton X-100, previously cooled to 4°. Centrifugation was for 60 min at 50,000 rpm and 4° in a Spinco SW60 rotor. In control samples, the templates (minichromosomes or DNA) were mixed with all the reaction components except RNA polymerase just before centrifugation. Gradient fractions were precipitated in 5% TCA-1% Na pyrophosphate and filtered on GF/C glass fiber filters, which were then washed with the same TCA solution and finally with ethanol.

Other methods. DNA purification from virions, RNA synthesis and purification, sedimentation through sucrose gradients in formamide and self-hybridation of RNA have been described previously (7, 11, 12).

RESULTS

Template efficiency. Minichromosomes can be transcribed by either eukaryotic or prokaryotic RNA polymerase, although they are less efficient templates then deproteinized SV40 superhelical DNA. When purified SV40 minichromosomes (taken from the 60 S peak fractions of a preparative sucrose gradient, see Materials and Methods) or an equivalent amount of SV40 DNA form I were added to an in vitro transcription system the results shown in Fig. 1 were obtained. It can be seen that with both RNA polymerases, transcription of minichromosomes amounted to 25-30% of that of "naked" SV40 superhelical DNA (after 30 min of synthesis). Thus, the presence of nucleosomes on the viral DNA does not completely inhibit transcription,

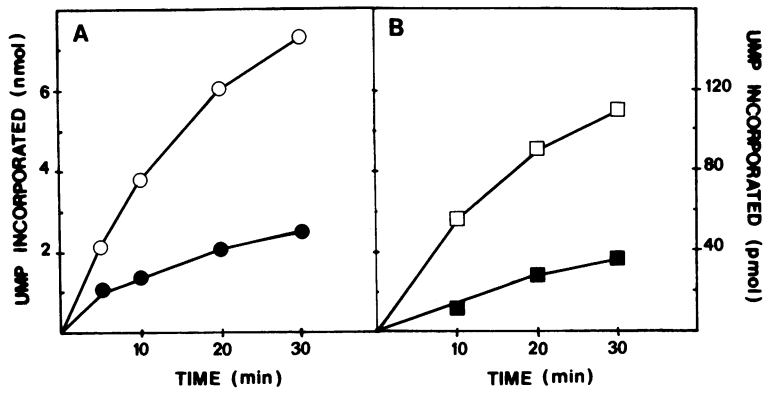

Fig. 1. UMP incorporation kinetics. Reactions (400 µg of superhelical SV40 DNA (open symbols) or an equivalent amount of SV40 nucleohistone complex (solid symbols). Incorporation was started by addition of 0.3 units of calf thymus RNA polymerase B (A) or 5.7 units of E. coli RNA polymerase (B).

and this is true for both the eukaryotic and the prokaryotic enzyme. The fact that the extent of inhibition is the same for the two polymerases suggests that it is due to the template structure and that the mechanism of inhibition is the same for both enzymes.

Nucleosomal DNA can be transcribed by eukaryotic and prokaryotic RNA polymerases. RNAs synthesized in the above experiment (30 min) were analyzed by sedimentation through denaturing sucrose gradients in formamide (12). The results of such an analysis are shown in Fig. 2. It can be seen that the size of RNA made on minichromosomes by either polymerase was smaller than the corresponding RNA made on deproteinized superhelical DNA. Reduction in average size was approximately from 1,500 to 1,000 nucleotides with E.coli RNA polymerase and from about 1,200 to 900 nucleotides with calf thymus RNA polymerase B. The average length of internucleosomal DNA in SV40 minichromosomes, on the other hand, has been evaluated as 40 to 60 base pairs (1, 3, 13). The simplest interpretation of these results is that, although RNA chain elongation is partially inhibited by

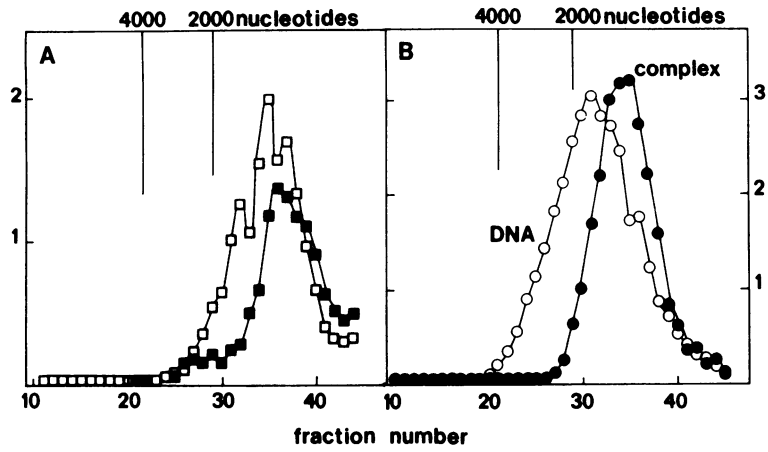

Fig. 2. Purified ^{32}P-labeled RNAs synthesized on superhelical SV40 DNA (open circles) or on SV40 minichromosomes (solid circles) by eukaryotic (A) or prokaryotic (B) RNA polymerase were sedimented through denaturing sucrose gradients in formamide as described (12).

the presence of nucleosomes, both eukaryotic and prokaryotic RNA polymerases can transcribe portions of minichromosomal DNA that include a few nucleosomes.

Minichromosome structure is not substantially altered by transcription. Size analysis of RNA transcripts (Fig. 2), although strongly suggestive of the availability of nucleosomal DNA as a transcription template, says nothing about possible mechanisms through which this transcription may occur. One possibility could be that nucleosomes are somehow disrupted during RNA chain elongation, either by advancing RNA polymerase or simply by incubation conditions. Another more trivial explanation of our results could be that a fraction of our minichromosome preparation is in fact nucleosome-depleted, and that transcription occurs selectively on that fraction of the population. Considering the relative template efficiency of deproteinized superhelical DNA and of minichromosomes (Fig. 1), this putative nucleosome-depleted fraction should constitute approximatley 25-30% of the whole population. Alternatively, our minichromosome preparation

could in fact be homogeneous, but contain only 70 to 75% of the full complement in nucleosomes, leaving nucleosome-free regions available for transcription.(Both these latter possibilities were made unlikely by direct visualization of minichromosome preparations in the electron microscope, as described (7) and by the fact that only the 60 S peak fractions were used for transcription). Finally, one could think that eukaryotic and prokaryotic RNA polymerases are in fact able to transcribe DNA in nucleosomes without substantially alter the nucleosomal structure, or reforming it after transcription.

In order to test the validity of some of these hypothesis, we analyzed the state of template minichromosomes after transcription. To this end,[^{3}H-thymidine]-labeled SV40 minichromosomes were transcribed with E. coli RNA polymerase into ^{32}P-labeled RNA. At the end of synthesis, the reaction mixture was loaded onto a neutral sucrose gradient and analysed by sedimentation. As a control, a similar amount of tritium-labeled minichromosomes were mixed just before sedimentation with the same reaction components as the incubated sample, but excluding RNA polymerase. The two samples were sedimented in parallel and gradient fractions were assayed for TCA-precipitable radioactivity (Fig.3). The untranscribed sample gives the reference position of minichromosomes which have not undergone incubation or possible disruptive interactions during the transcription process. These minichromosomes sedimented as 60 S material (Fig. 3a), that is, they re-sedimented with the same velocity as the peak from which they were originally isolated (see Materials and Methods). This indicates that only intact SV40 minichromosomes were present at the beginning of transcription. After transcription (Fig. 3b), no tritium-labeled material sedimenting slower than the untranscribed sample was detected, indicating that no major disruptions of the minichromosome structure had occured. A shoulder on the heavy side of the tritium peak was consistently found, due most likely to the presence of RNA polymerase and nascent RNA chains on minichromosomes that were being transcribed. In fact, the profile of synthesized ^{32}P-RNA in the same gradient (Fig. 3b) showed a peak centered on this shoulder. The majority of RNA molecules however, were detached from the complex, and sedimented free in a region

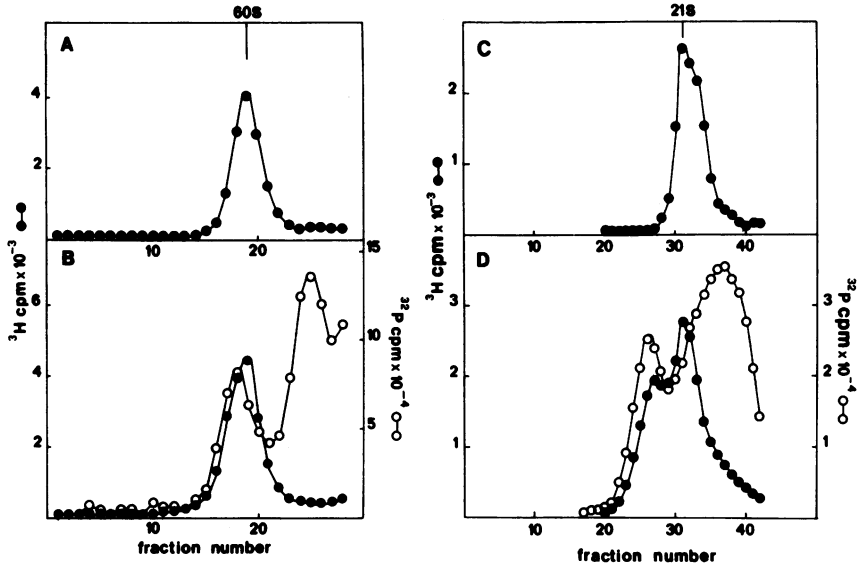

Fig. 3. Sedimentation through non denaturing sucrose gradients of transcription reaction mixtures containing ^{3}H-labeled templates (solid circles) and ^{32}P-labeled incorporated UMP (open circles).
(A): SV40 minichromosomes, no polymerase added. (B) SV40 minichromosomes, E. coli RNA polymerase added. (C) SV40 DNA, no polymerase. (D) SV40 DNA, E. coli RNA polymerase added.

of the gradient where no template was present. Possible reasons for RNA detachment from the transcription complex are discussed below. Whatever the causes of this chain release, it is nonetheless clear that in this experiment nascent RNA chains were only found associated with minichromosomes that had a full complement of nucleosomes.

That RNA chains on the complex were precursors to those found in the major, slow sedimenting RNA peak was confirmed by a similar experiment in which tritium-labeled "naked" SV40 superhelical DNA was used as the template (Fig. 3c and d). Again, part of the DNA label was displaced to the heavy side of the gradient, indicating association of the template with RNA polymerase and nascent RNA. (The displacement in this case was more consistent than with minichromosomes, because the relative increase in mass

due to association was larger). As in Fig. 2b, RNA distribution was bimodal (Fig. 3d), with a peak superposed to the "complexed" DNA and a broader, slower peak. These latter RNA chains sedimented more slowly than any form of viral DNA present in the reaction, and therefore must have been released from the fast sedimenting transcription complex. The cause of this release was most likely the lack of adequate RNA protection in the non-denaturing gradients although premature chain termination cannot be excluded. Gradients composition was chosen to preserve the integrity of minicromosomes.

Strand selection is reduced by the presence of nucleosomes. Superhelical SV40 DNA is transcribed asymmetricaly by E. coli RNA polymerase, the majority of transcripts being complementary to the early (E) strand (14,15). The relevance of this observation to SV40 transcription in infected cells was made doubtful by the finding that DNAs of two other members of the papovavirus group (polyoma virus and BKV), closely related to SV40, are transcribed symmetrically under the same conditions (16,12). However, the ability of E. coli RNA polymerase to preferentially recognize promoters for one of the two strands on SV40 DNA allows one to ask whether the constraints imposed on viral DNA by nucleosomes result in an alteration of this selectivity. In view of the close similarity between viral minichromosomes and cellular chromatin, the question is related to the more general problem of signal recognition on eukaryotic DNA in the cell nucleus.

In the experiment described in Fig. 4, SV40 minichromosomes or an equivalent amount of deproteinized superhelical SV40 DNA were transcribed in vitro with E. coli or calf thymus B RNA polymerases. Transcripts synthesized in 30 min at 37° were purified and assayed for self-complementarity. It can be seen that with E. coli RNA polymerase, the fraction of RNA which became RNase resistant after self-hybridization was over 30% in the case of minichromosomes, as compared to 15% when "naked" DNA was transcribed. A marked reduction in strand selectivity seems therefore to be imposed by nucleosomes on transcription with the prokaryotic enzyme.

When eukaryotic RNA polymerase B was used to transcribe superhelical SV40 DNA, a large fraction of the RNA produced was

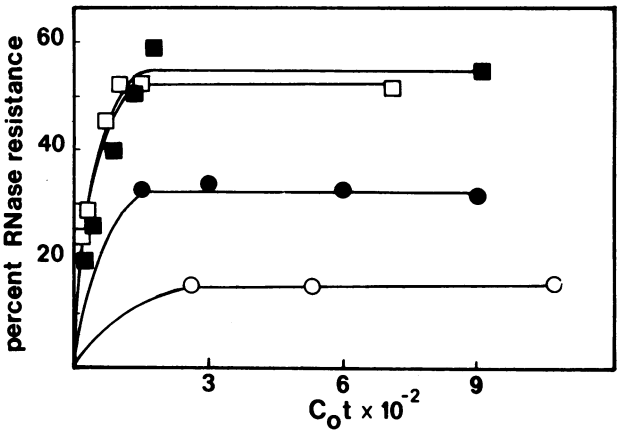

Fig. 4. Self-complementarity of in vitro RNAs made on minichromosomes (solid symbols) or on deproteinized SV40 DNA (open symbols) by E. coli (circles) or calf thymus - B (squares) RNA polymerase. Points represent the percent of input radioactivity that became RNase resistant at the indicated $C_o t$ values. $C_o t$ units are moles of nucleotides x l^{-1}x s.

self-complementary (Fig. 4), in agreement with previously reported results (17). The poor strand-selectivity exhibited by the eukaryotic enzyme was not improved when minichromosomes were used as templated instead of naked DNA, since the same level of self-complementarity was found in the transcripts.

DISCUSSION

Our understanding of transcription in eukaryotic cells is severely limited by the complexity of the cellular template. On one hand, the enormous amount of information contained in eukaryotic DNA makes it very difficult to identify and locate with any precision nuclear transcripts of the cell genome. On the other hand, the organization of eukaryotic chromatin, with DNA packed in closely spaced nucleosomes, allows no obvious visualization of processes like sequence recognition, chain elongation and termination. The first of these difficulties can be partially circumvented by the use of viral "chromatins" as model transcription templates. Papovaviruses are particularly interesting in this respect, since their

small DNA is associated to cell histones to give nucleosomes practically undistinguishable from those of cellular chromatin. Since practically all of the viral DNA is found in these nucleoprotein complexes at all stages of the viral cycle (3-6), it is very suggestive to think that actual transcription of papovavirus DNA in the infected cell occurs on such templates rather than on "naked" DNA. These viral chromatins, called by some authors "minichromosomes" (1-3), were in fact isolated from infected cells and studied as templates for endogenously catalyzed transcription (18-21). In those studies, however, only a small fraction (less than one per cent) of the minichromosomes were transcriptionally active, making the precise nature of the template somewhat uncertain. Presence of cellular contaminants was also a problem (Chambon, personal communication).

The same minichromosomes are present inside the papovavirions, and large quantities of them can be isolated intact and free of cellular contaminants by mild alkaline treatment (6,7). We think that minichromosomes isolated in this way are very convenient templates for model studies of viral and cellular chromatin transcription by exogenous RNA polymerases.

One crucial question that can be asked to such a system is whether advancing RNA polymerase can transcribe DNA in nucleosomes. The results presented here show that transcripts produced by prokaryotic or eukaryotic-B RNA polymerases on SV40 minichromosomes cover DNA lenghts that must include several nucleosomes. The possibility that synthesis of such transcripts was achieved on nucleosome-depleted minichromosomes could be excluded (at least in the case of E. coli RNA polymerase) by sedimentation analysis of the nucleoprotein template before and after transcription. The simplest interpretation of these results is that RNA polymerases can actually read and transcribe DNA coiled around nucleosomes, although with a lower efficiency compared to naked DNA. More sophisticated interpretations cannot be excluded. One could imagine that RNA polymerase "pushes ahead" nucleosomes while elongating RNA chains. Our reactions being carried out with limiting enzyme concentrations (see Fig. 3b), this putative nucleosome displacement would seldomly be counteracted by a polymerase advancing in the opposite direction. Constraints imposed on DNA structure could

be released by redistribution of nucleosomes on the circular DNA. This hypothesis, however, implies a relatively fast sliding of nucleosomes along the DNA, a mechanism that has not yet been demonstrated at the salt concentrations used here (22, 23). Another possible mechanism compatible with our results could be a transient disruption or alteration of nucleosomal structure, which would be reconstituted after transcription. In this case, however, additional assumptions would have to be made to explain fast reconstitution of nucleosomes in a medium (that of transcription assay) which should not favor it (24). Whatever the mechanism involved, it follows from our results that eukaryotic and prokaryotic RNA polymerases can, without any additional factor, transcribe portions of DNA that contain nucleosomes. Similar conclusions were drawn from experiments on endogenous transcription of viral chromatin isolated from infected cells (21, 25), although in those cases integrity of the nucleoprotein template could not be directly demonstrated. Our results are also in agreement with hybridization studies of histone-protected sequences in cellular chromatin (26-28) and with work on transcription of histone-covered T7 DNA by Williamson and Felsenfeld (29). Somewhat different conclusions, although not totally contradictory with ours, were obtained by Cremisi et al. (30) when studying exogenous transcription of SV40 minichromosomes isolated from infected cells.

If it is not necessary to peel off nucleosomes in order to transcribe the DNA, then it would be interesting to know whether the same promoters are recognized in chromatin as in naked DNA. Again, SV40 minichromosomes are very useful tools to investigate such a question. Naked SV40 DNA is transcribed asymmetrically by E. coli RNA polymerase, the promoters recognized being mainly for the E strand (14). The data presented here show that this strand selectivity is partially obscured by the presence of nucleosomes on the DNA. A similar finding was reported by Williamson and Felsenfeld studying transcirption of histone-covered T7 DNA (29). Although we cannot offer a quantitative interpretation of our results, this seems consistent with the idea that nucleosomes mask DNA sequences normally recognized by the prokaryotic enzyme. In fact, comparison of the reduction in RNA synthesis (70%) to the reduction in average RNA size (25 to 30%) proves that, besides elongation,

initiation must also be reduced by the presence of nucleosomes.

Limitations imposed on initiation, on the other hand, do not result in new transcription specificity of SV40 minichromosomes. This was shown directly by blot-hybridization of transcripts made on DNA or on minichromosomes to Hin c III fragments of SV40 DNA. With both prokaryotic and eukaryotic-B RNA polymerases, hybridization patterns were all undistinguishable, and indicated that all regions of viral DNA were uniformly transcribed (our unpublished observations). This suggests that factors other than the chromatin template and RNA polymerase are needed for transcription specificity in the in vitro system. One could say, on the other hand, that recent spectacular findings on gene discontinuity and post-transcriptional splicing make it even unnecessary to postulate site-specific initiation and termination of RNA chains in eukaryotic cells, the precise base sequence of mRNA being constructed by splicing of what could very well be an imprecisely initiated and terminated primary transcript.

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