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Escherichia coli RNA polymerase *in vitro* mimics simian virus 40 *in vivo* transcription when the template is viral nucleoprotein

(simian virus 40 minichromosome/transcriptional control/transcriptional initiation/simian virus 40 late promoters)

EDWARD B. JAKOBOVITS*, SENTOB SARAGOSTI†, MOSHE YANIV†, AND YOSEF ALONI*

*Department of Genetics, The Weizmann Institute of Science, Rehovot, Israel; and †Department of Molecular Biology, Pasteur Institute, Paris, France

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ABSTRACT We have used a low-salt detergent-free extraction procedure on cells infected with simian virus 40 to obtain viral nucleoprotein late after infection. Addition of *Escherichia coli* RNA polymerase and ribonucleotide triphosphates to the viral minichromosomes permitted transcription of RNA from viral templates. This synthesis was initiated predominantly within a fragment of DNA spanning 0.67 to 0.76 map unit on the genome. The synthesis from this region proceeded primarily along the "late" strand in a clockwise direction. These results were in contrast to the synthesis obtained with naked viral DNA in which initiation occurred on other regions of the genome and from which transcription proceeded counterclockwise along the early strand. These findings indicate that the nucleoprotein template or factors tightly associated with it may be responsible for site(s) and strand selection in transcription of simian virus 40.

A major problem in the understanding of transcription in eukaryotes is whether the structure of the template or factors associated with it is responsible for the temporal regulation of this process. Results of extensive transcriptional studies of purified chromatin *in vitro* with both endogenous and exogenous DNA-dependent RNA polymerases have proved to be exceptionally difficult to analyze because of the great complexity of these templates and the difficulties of obtaining native-like structures *in vitro* (1–3).

To overcome these problems, it has been possible to use the DNA tumor viruses as model systems for eukaryotic transcription (4–6). These viruses offer the advantages that (*i*) they display a well-defined transcriptional program throughout their life cycle (7) which is carried out by host cell enzymes (8, 9) and (*ii*) they are small and it is easy to obtain large quantities of their DNA either pure or within a chromatin-like structure.

Studies using the naked viral DNA from simian virus 40 (SV40) and polyoma as templates for purified DNA-dependent RNA polymerases have demonstrated that the DNA itself can provide recognition sites for RNA polymerase binding and initiation (10–15). However, these sites did not fall within the natural *in vivo* transcriptional initiation regions (6, 16, 17). Recent success in achieving correct initiation on naked DNA has been obtained by transcription of adenovirus type 2 (Ad2) DNA with purified RNA polymerases II (18, 19) and III (20–24). The correct initiation was completely dependent on the addition to the assay mix of either soluble cytoplasmic or whole cell extracts obtained from uninfected cells.

Various methods have been developed for the extraction of viral chromatin from infected cells late after infection with SV40 and polyoma viruses (25–31). These extracted complexes have been shown to have many structural features similar to cellular chromatin (32–36). It was therefore of interest to investigate these molecules in *in vitro* transcriptional systems to determine whether they possess the necessary factors required for the initiation of transcription at natural sites, using, as a first approach, exogenous *Escherichia coli* RNA polymerase.

We demonstrate here that the initiation specificity of the bacterial RNA polymerase is highly altered by the presence of histones or other factors on the template and that initiation of transcription in the region of the major "late promoters" of SV40 (16, 17) occurs efficiently on the viral nucleoprotein with this enzyme but to only a minor extent, if at all, on the naked viral DNA. Furthermore, the RNA synthesized on the viral nucleoprotein is primarily a copy of the late strand, in contrast to the early strand transcripts obtained from the naked DNA. These results suggest that the structure of the template or factors tightly associated with it determine, at least in part, the transcriptional specificity *in vivo*.

MATERIALS AND METHODS

Cells and Virus. Growth of BSC-1 cells, infection with SV40, and the low-salt detergent-free extraction of SV40 nucleoprotein have been described (31).

Assay for RNA Polymerase Activity. The standard RNA polymerase assay mixture (final volume, 100 μl) contained: ATP, GTP, and CTP at 1 mM each; 1 mM dithiothreitol; 10 mM $MgCl_2$; 125 mM NaCl; 2 μg of α-amanitin per ml; 10 μCi of [α-^{32}P]UTP (2000–3000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham); 5 units of *E. coli* RNA polymerase prepared according to Burgess and Jendrisak (37); 50 μl of nuclear extract containing 50 mM Tris·HCl at pH 7.9; 1 mM $MgCl_2$; 5 mM 2-mercaptoethanol; approximately 1 μg of SV40 DNA. Assays were incubated at 37°C for the required time.

Purification of Labeled RNA. Assays were stopped by the addition of 10 μg of tRNA, 20 μg of Pronase (self-digested for 60 min at 37°C), EDTA to 10 mM, and $NaDodSO_4$ to 0.5%. Incubation was continued at 20°C for 30 min followed by extraction with phenol/chloroform/isoamyl alcohol, 50:49:1 (vol/vol). The deproteinized mixture was passed through a 5-ml syringe packed with Sephadex G-25 (fine) by centrifugation for 5 min at 3000 × *g* to remove unincorporated nucleotides. The RNA in the void volume was treated with DNase, extracted with phenol as above, and precipitated with ethanol (9).

Preparation of Restriction Fragments and RNA·DNA Hybridization. SV40 DNA fragments were prepared and electrophoresed on 1.4% agarose gels as described (38). Preparation of single-stranded SV40 DNA fragments by electrophoresis on 2.0% agarose gels was carried out as described (39). Fragments were transferred to nitrocellulose paper (Schleicher & Schuell BA85) as described by Southern (40). RNA·DNA

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Abbreviations: SV40, simian virus 40; m.u., map unit (1 m.u. is the equivalent of 0.01 of the viral DNA length); Ad2, adenovirus type 2; NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M Na citrate, pH 7).

hybridization was carried out in 4X standard saline citrate [0.15 M NaCl/0.015 M Na citrate, pH 7 (NaCl/Cit)] at 68°C for 36 hr. The RNA was heated at 100°C for 2 min before hybridization. Filters were incubated at 20°C for 30 min in 2X NaCl/Cit containing pancreatic RNase at 1 μg/ml followed by washing at 50°C for 30 min in 2X NaCl/Cit, dried, and exposed to Kodak X-R5 film at −70°C.

RESULTS

Determination of Initiating Site. We have recently shown that SV40 DNA–protein complexes can be extracted from infected cells late after infection by using a low-salt detergent-free procedure (31). The nuclear extract obtained with such a procedure contains a population of active endogenous viral transcriptional complexes capable of synthesizing RNA from the viral template (41). This activity could be completely abolished by the addition of a low concentration (2 μg/ml) of α-amanitin, indicating that cellular RNA polymerase II was responsible for this synthesis (42).

In the subsequent experiments using exogenously added *E. coli* RNA polymerase, we incubated the nuclear extract obtained from infected cells late after infection in an assay mixture for transcription containing this concentration of α-amanitin. The results in Fig. 1 show that the addition of *E. coli* RNA polymerase initiated incorporation of [^{32}P]UMP into RNA which proceeded actively for at least 1 hr.

In order to determine the initiation site(s) and direction of this synthesis, we allowed transcription to continue for various lengths of time after addition of *E. coli* RNA polymerase and ribonucleotides to the nuclear extract. The labeled RNA was then extracted, purified, and hybridized to DNA restriction fragments produced by cleavage of SV40 DNA with *Eco*RI, *Hpa* I, and *Bgl* I restriction endonucleases (see diagram, Fig. 2*B*). If there existed a single initiation site on the DNA (or a small defined number), then in the shortest pulse time there should be enrichment of radioactivity appearing in a fragment of DNA containing this site. With increasing labeling times, more distal fragments should hybridize with the radioactive RNA as the enzyme progresses along the DNA. On the other hand, if there were no specific initiation site, then the labeled RNA should hybridize to all the fragments even with the shortest pulse times.

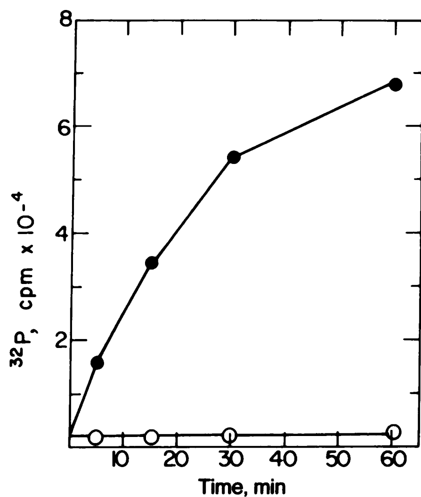

FIG. 1. Time course of [^{32}P]UMP incorporation. Nuclear extract prepared from infected cells at 48 hr after infection was assayed for RNA synthesis in the absence (○) or presence (●) of exogenous *E. coli* RNA polymerase. Aliquots (0.01 ml) were removed at the indicated times, spotted onto Whatman no. 3 filters, and precipitated with trichloroacetic acid as described (31).

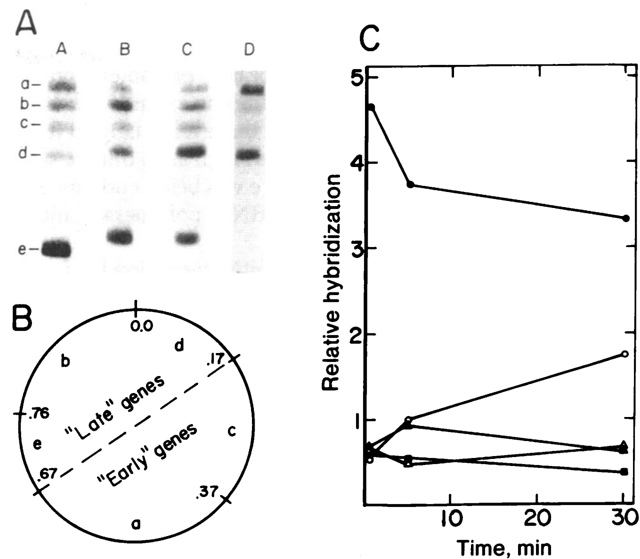

FIG. 2. Initiation sites of RNA synthesis. (*A*) *E. coli* RNA polymerase was added to an assay mixture for RNA synthesis containing infected cell nuclear extract prepared at 48 hr after infection and incubated for various times. The ^{32}P-labeled RNAs were purified and hybridized to Southern blots of SV40 DNA fragments. Shown are the patterns of hybridization with RNA synthesized on nucleoprotein for 0.5 min (lane A), 5 min (lane B), and 30 min (lane C) and on naked DNA for 0.5 min (lane D). (*B*) Map positions of SV40 DNA fragments generated by cleavage of form I SV40 DNA with *Hpa* I [which cuts at 0.17, 0.37, and 0.76 map unit (m.u.)], *Eco*RI (which cuts at 0.0 m.u.), and *Bgl* I (which cuts at 0.67 m.u.). (*C*) The autoradiograms shown in *A* were scanned with a spectrophotometer, and the peaks corresponding to the bands of hybridization were quantitated as a percentage of total hybridization per strip and divided by the expected intensity based on the size of each fragment. Fragments: ■, a; ▲, b; △, c; ○, d; ●, e.

We found that RNA synthesis for a very short time (0.5 min) by *E. coli* RNA polymerase on naked SV40 DNA led to the appearance of labeled transcripts essentially from two regions of the genome, as detected by hybridization of the purified RNA to restriction fragments a and d (Fig. 2*A*, lane D). In some preparations the RNA hybridized only to one or the other of these fragments. This is in agreement with previous studies which demonstrated the presence of two major promoters for the *E. coli* enzyme at 0.17 and 0.43 m.u. on the SV40 DNA (10, 12, 13, 15).

In contrast, when transcription was carried out on the infected cell nuclear extract (with the same enzyme) for 0.5 min, a major fraction of the RNA hybridized to fragment e which spans 0.67–0.76 m.u. on the viral genome (Fig. 2*A*, lane A). Scanning of the autoradiograms in Fig. 2*A* showed that at least 40% of the RNA synthesized at very short times came from fragment e which comprises only 9% of the genome. Thus, this short fragment is at least 4 times more efficient in permitting initiation than the rest of the genome at all the times tested (Fig. 2*C*). Increasing the labeling time to 5 min (Fig. 2*A*, lane B) and 30 min (Fig. 2*A*, lane C) led to the appearance of increased radioactivity associated with fragment b (0.76–0.0 m.u.) and to some extent also with fragment d (0.0–0.17 m.u.) in addition to the radioactivity associated with fragment e. The region of DNA in fragment b is immediately adjacent to that of fragment e when moving in a clockwise direction (that is along the late strand) which is in turn followed by the region of fragment d. However, in some preparations of RNA incubated for extended times, both fragment e and fragment a or fragment a sequences alone were found to be enriched together with fragment e sequences which were always present in high concentrations. This

suggested elongation of the RNA from fragment e into fragment b followed in some preparations by partial elongation of the RNA into fragment d or exposure of the "promoters" on the early strand of fragments a and d during the extended *in vitro* incubation. It is interesting to note that the level of labeled RNA complementary to fragment e remained high at all times tested, suggesting that the region of fragment e was being continuously transcribed by reinitiating *E. coli* RNA polymerase molecules.

These results indicate that a major initiating region for *E. coli* RNA polymerase exists on SV40 nucleoprotein and that, in contrast to the initiation sites on naked DNA, it is found within a region of the viral DNA located between 0.67 and 0.76 m.u. Furthermore, the above results suggest that the transcription on the viral nucleoprotein complex initiated in the region 0.67–0.76 m.u. proceeded in a clockwise direction.

Strand Selection on the Template. In order to confirm that transcription occurred from the late strand, we incubated *E. coli* RNA polymerase for short times with a nuclear extract obtained from infected cells late after infection. The labeled RNA was purified and hybridized to the separated strands of SV40 DNA restriction fragments produced by digestion of the viral DNA with *Hpa* I and *Bgl* I (Fig. 3*B*).

The results of this experiment, in agreement with those shown in Fig. 2, demonstrated that a major fraction of the RNA synthesized for 5 min (Fig. 3*A*, lane A) or 30 min (Fig. 3*A*, lane B) hybridized to fragment d which spans 0.67 to 0.76 m.u. (identical to fragment e of the previous ensemble of fragments). In addition, a substantial amount of this RNA was complementary to the late strand. The level of hybridization to fragments other than fragment d indicated that additional regions of the viral DNA were transcribed. However, of this additional RNA only fragment a (spanning 0.76 to 0.17 m.u., the equivalent of fragments b and d of the previous ensemble) showed a high proportion of RNA hybridizable to the late strand compared to its complementary early strand at both 5 and 30 min (Fig. 3*A*, lanes A and B). In contrast, transcription of naked SV40 DNA with *E. coli* RNA polymerase for 0.5 min (Fig. 3*A*, lane C) led to the appearance of labeled RNA complementary in large part to the early strands of fragments a, b, and c. Essentially no fragment d sequences of either strand were detected. Thus, the increased transcription from the additional regions of DNA seen at longer incubation times with the viral nucleoprotein seemed to come both from elongation along the late strand from RNA initiated within the region 0.67–0.76 m.u. and from early strand transcripts, presumably initiated at the "promoter" sites available on the naked DNA.

These results support the conclusion that the very high selectivity for initiation on the early strand of SV40 displayed by *E. coli* RNA polymerase on naked DNA is reversed for specificity of initiation on the late strand of the viral chromatin within the region 0.67–0.76 m.u.

Template for Transcription. The nuclear extract obtained by leaching infected cell nuclei in a low salt detergent-free buffer contains various viral specific structures, including replicating intermediates, viral minichromosomes, previrion structures, and fully encapsidated virus (31). In order to determine which of these structures was acting as the template for the exogenous *E. coli* RNA polymerase, we infected cells with SV40, labeled the DNA between 32 and 48 hr after infection, and extracted the viral structures from the nuclei at 48 hr after infection. The labeled nuclear extract was layered onto a sucrose gradient and centrifuged (Fig. 4*A*). Two peaks of labeled viral deoxyribonucleoprotein structures were obtained—a 250S peak containing intact SV40 virions and previrion structures, and a 75S peak of replicating intermediates and viral minichromosomes (31). The material in each peak was pooled and aliquots were assayed for incorporation of [^{32}P]UMP with *E. coli* RNA polymerase. As shown in Fig. 4*B*, the material in both peaks acted as template for transcription, but the material from the 75S peak was somewhat more efficiently transcribed than that from the 250S peak. Analysis of the RNA

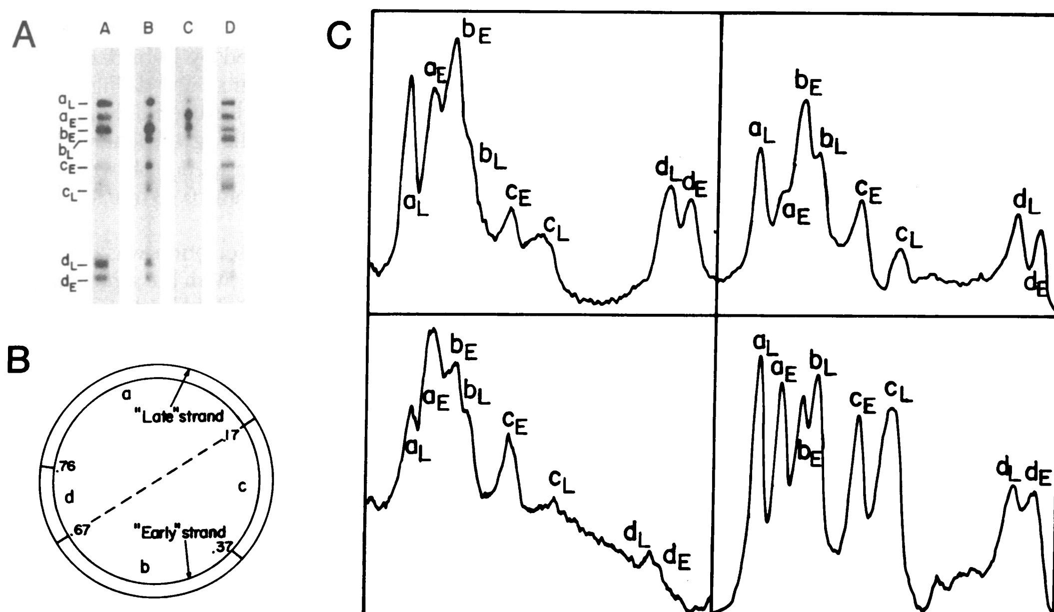

FIG. 3. Strand selection by *E. coli* RNA polymerase. *E. coli* RNA polymerase was added to an assay mixture for RNA synthesis containing infected cell nuclear extract prepared at 48 hr after infection and incubated for various times. The labeled RNAs were purified and hybridized to Southern blots of separated strands of SV40 DNA fragments (39). (*A*) Patterns of hybridization with RNA synthesized on nucleoprotein for 5 min (lane A) and 30 min (lane B). Lane C shows the pattern of hybridization with RNA synthesized on naked DNA. For lane D, the hybridization was carried out with ^{32}P-labeled nick-translated SV40 DNA (43); this is a control experiment indicating the position of the separated strands of the fragments in which the intensity of each band is directly related to its size. (*B*) Diagram indicating the map positions of SV40 DNA fragments generated by cleavage of form I SV40 DNA with *Hpa* I and *Bgl* I. (*C*) Spectrophotometer scans of the autoradiograms displayed in *A*: upper left, lane A; upper right, lane B; lower left, lane C; lower right, lane D.

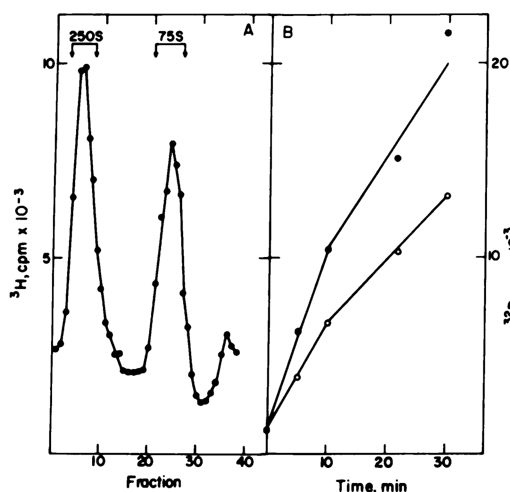

FIG. 4. Determination of the nucleoprotein template for transcription. (*A*) SV40-infected cells were labeled with [*methyl*-^{3}H]-thymidine as described (31) between 32 and 48 hr after infection. The labeled viral nucleoproteins were extracted (3) and loaded onto a 5–30% sucrose gradient in 50 mM Tris·HCl, pH 7.9/1 mM $MgCl_2$/5 mM 2-mercaptoethanol/0.1 M NaCl. Centrifugation was for 120 min at 32,000 rpm at 4°C in the SW 41 rotor. Fractions (0.3 ml) were collected from the bottom of the tube and aliquots (0.05 ml) were assayed for radioactivity in Triton-based scintillation fluid. The two labeled peaks of viral nucleoprotein were pooled as shown. (*B*) An equal amount of radioactivity was assayed for RNA synthesis with *E. coli* RNA polymerase. At the indicated times, aliquots (0.01 ml) were spotted onto Whatman no. 3 filters and trichloroacetic acid precipitated as described (31). ●, From 75S peak; ○, from 250S peak.

synthesized on each peak showed similar patterns of hybridization with the restriction fragments as described above for whole nuclear extracts (results not shown).

It has been reported (44–46) that *E. coli* RNA polymerase is capable of carrying out RNA-dependent RNA transcription by utilizing the endogenous RNA normally present in nuclear extracts. In order to ascertain whether the transcription products obtained in our assay system were derived from DNA or RNA templates, we incubated a SV40 nuclear extract for a short time (5 min) with *E. coli* RNA polymerase in the absence and in the presence of actinomycin D in a concentration that completely inhibits DNA-dependent RNA transcription (44–46). The addition of the drug greatly reduced the viral specific transcription (Fig. 5). Furthermore, the pattern of hybridized RNA synthesized in the presence of actinomycin D closely resembled the characteristic pattern obtained by hybridizing nuclear late RNA to these SV40 DNA fragments (16, 47) with no enrichment of labeled RNA complementary to fragment e. These results demonstrate that there was some RNA-dependent RNA synthesis occurring but the enrichment of RNA complementary to the 0.67–0.76 m.u. fragment was wholly a DNA-dependent process.

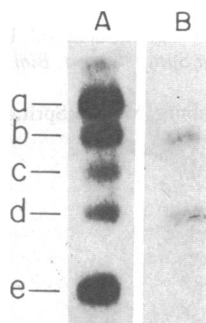

FIG. 5. Effect of actinomycin D on RNA synthesis. *E. coli* RNA polymerase was added to two equal assay mixtures for RNA synthesis containing infected cell nuclear extract prepared at 48 hr after infection and incubated for 5 min in the absence (*A*) or presence (*B*) of actinomycin D at 20 μg/ml. The labeled RNAs were purified and hybridized to Southern blots as in Fig. 2.

DISCUSSION

The results presented here demonstrate that the initiation specificity of *E. coli* RNA polymerase is dramatically altered when transcription is carried out on SV40 nucleoprotein rather than on naked viral DNA. This is true both for the selection of the initiation site(s) and for the strand that is transcribed. Thus, we have found that, when labeled RNA is isolated very rapidly after initiation of transcription on the viral nucleoprotein and is hybridized to viral DNA fragments, a region of DNA close to the *in vivo* viral late promoters is preferentially transcribed. This RNA is transcribed mainly from the "late" strand. This is in contrast to the transcription initiated on promoters present at 0.17 and 0.43 m.u. on the naked DNA and from which transcription proceeds along the "early" strand (10, 12, 13). The addition of α-amanitin to inhibit the endogenous RNA polymerase activity on the minichromosome did not alter the initiation specificity of the added *E. coli* polymerase; transcription of the viral chromatin in the absence of α-amanitin with a large excess of the *E. coli* enzyme gave a similar pattern of synthesis. The mechanism of strand selection on the viral nucleoprotein presumably reflects the fact that the template was extracted late in infection. In this respect, it would be of interest to see whether the early template possesses a different set of transcriptional characteristics.

It has been reported that transcription of Ad2 DNA by RNA polymerase II (18, 19) and by RNA polymerase III (20–24) can be modified by the addition of soluble factors obtained from either the cell cytoplasm or whole cell extracts. However, no indication was given as to how these soluble factors operated. Thus, it is not clear whether the transcription was modified by interaction of these factors with the transcribing enzymes themselves or with the DNA template.

As reported here the site and strand selection mechanisms operating on SV40 nucleoprotein seem to be regulated by the nucleoprotein structure or controlled by tightly associated factors. This is supported by the observation that the specific initiation region available on the chromatin of SV40 is present also on material purified through a sucrose gradient. This reduces the likelihood that the soluble factors released together with the nucleoprotein during extraction are necessary during transcription with *E. coli* RNA polymerase for the activation of this region. However, it is possible that such factors could remain associated with the template during centrifugation in sucrose gradients.

It has been reported that roughly 20% of SV40 minichromosomes contain an "exposed" region near the origin of replication. This exposed region has been identified by its sensitivity to DNase I (48, 49), staphylococcal nuclease (50), an endogenous endonuclease (48, 51), and various site-specific restriction endonucleases (52, 53). In addition, it has been reported that a fraction of SV40 minichromosomes display, precisely within this region, a stretch of DNA not contained within a typical nucleosome structure (49, 54). Recent studies have indicated that transcription occurs on the SV40 minichromosome (41, 55), and that the late *in vivo* promoters map in a region spanning 0.67 to 0.76 m.u. (16, 17). The results presented here demonstrate that *E. coli* RNA polymerase also initiates on SV40 nucleoprotein within this region *in vitro*. The presence of an appreciable percentage of SV40 minichromosomes with exposed DNA in this part of the genome (49, 54) may suggest that *in vivo* transcription initiates within this region on such "gapped" molecules. The presence within this region of an active promoter determined by the primary sequence of the DNA would make available for the RNA polymerase enzyme

the necessary recognition signals for initiation. Furthermore, it is possible that such a mechanism for the selection of transcriptional initiation sites may occur also in eukaryotic chromatin because it has been shown that actively transcribed regions are preferentially sensitive to various nucleases (56–60) and display nucleosome-deficient stretches of DNA in the electron microscope (61–64).

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