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Accurate Transcription of Simian Virus 40 Chromatin in a HeLa Cell Extract

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During simian virus 40 viral maturation, a series of modifications occur which alter the composition of viral nucleoprotein complexes. As a consequence, the chromatin that is extracted from extracellular simian virus 40 virions exhibits properties that are similar to those of transcriptionally active eucaryotic chromatin. The influence of this chromatin structure on specific RNA initiation by RNA polymerase II was examined by using the in vitro HeLa cell extract of Manley et al. (Proc. Natl. Acad. Sci. U.S.A. 77:3855–3859, 1980). The 5′ ends of RNA transcripts were positioned by the "run-off" assay, in which transcripts extend from the initiation site to termination sites created by restriction cleavage and by S1 nuclease analysis, using DNA probes labeled at their 5′ termini. Two major early RNA transcripts, which originated at map positions 5,240 ± 10 and 5,145 ± 10, and two major late RNA transcripts, which originated at map positions 325 ± 10 and 185 ± 10, were identified. Transcripts were initiated with comparable relative efficiencies at the same 5′ site when either purified DNA or chromatin was used as the template. Our results suggest that extracellular simian virus 40 virion chromatin modifications do not regulate simian virus 40 promoter selection but function to increase the accessibility of RNA promoter sequences to RNA polymerase II and allow efficient elongation of the RNA chain after the initiation event.

The DNA of eucaryotic cells is associated with octomers of four histone proteins which condense the DNA into the basic repeat unit of chromatin, the nucleosome (29). The nucleosome structure effectively blocks the access of enzymes to DNA and therefore inhibits RNA chain initiation and elongation (7, 9, 46). Recent studies have shown that changes in chromatin structure occur as genes are activated for transcription; this suggests that chromatin structure is one determinant of regulation of transcription in eucaryotic cells (6, 8, 10, 12, 16, 25, 32, 33, 37, 44, 47, 48). Analyses of several genetic loci have demonstrated that during periods of transcriptional activity, chromatin DNA becomes hypersensitive to DNase I, apparently due to modifications in the chromatin structure, including histone acetylation, decrease in H_1 stoichiometry, and interaction of nonhistone proteins with chromatin.

Two in vitro RNA transcription systems which accurately transcribe purified DNA templates have been developed recently. Weil et al. (43) used a combination of purified RNA polymerase II and a crude HeLa cell cytoplasmic extract to provide specific transcription of DNA templates, whereas Manley et al. (26) promoted specific transcription by adding DNA templates to a concentrated HeLa whole-cell extract. Using a variety of DNA templates, several investigators have demonstrated that both of these soluble systems recognize specific DNA regulatory sequences (e.g., the Goldberg-Hogness sequence TATA) and that RNA chain initiation starts at sites identical to those that occur in vivo (2, 18, 20, 21, 27, 38, 41, 42).

We have previously reported that the 110S chromatin complex isolated from extracellular simian virus 40 (SV40) virions is an efficient template for RNA transcription by *Escherichia coli* RNA polymerase (4, 5). We had not characterized the suitability of this complex as a template for the homologous eucaryotic RNA polymerase II or determined if the chromatin structure altered the ability of RNA polymerase II to recognize promoter sequences and initiate specific RNA synthesis. In this paper, we describe the transcription of extracellular virion chromatin by RNA polymerase II in the HeLa extract of Manley et al. (26).

MATERIALS AND METHODS

Viruses and cells. SV40 strain 776 was originally obtained from K. Takemoto, National Institute of

Allergy and Infectious Diseases. The virus was grown in BSC-1 cells.

Infection and purification of SV40 virions. Confluent monolayers of BSC-1 cells maintained in Eagle minimal essential medium supplemented with 0.03% glutamine, 10% fetal calf serum, streptomycin, penicillin, ampicillin, and mycostatin were infected with SV40 at a multiplicity of infection of 10 PFU/cell. After 2 h of adsorption at 37°C, Eagle minimal essential medium supplemented with 2% fetal calf serum was added. When cell lysis was complete (approximately 9 days), the infected cells and supernatant were collected, and the virus was purified as previously described (5).

Preparation of chromatin complexes and conditions for in vitro RNA synthesis. Preparation of SV40 chromatin complexes was carried out by ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA)–dithiothreitol dissociation of SV40 virions as described previously (4, 5), except that the NaCl concentration was decreased to 100 mM. HeLa cell extracts were prepared by the method of Manley et al. (26), using cells that were harvested at a cell concentration of 5×10^5 to 6×10^5 cells per ml instead of 8×10^5 cells per ml as originally described. The concentration of template DNA required for specific transcription was decreased when the cells were harvested at the lower cell density. Each standard transcription reaction mixture (60 μl) contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 8 mM Tris (pH 8.6), 50 mM KCl, 16 mM NaCl, 6.25 mM $MgCl_2$, 0.8 mM EGTA, 0.05 mM EDTA, 1.5 mM dithiothreitol, 0.16 mM phenylmethylsulfonyl fluoride, 0.008% Triton X-100, 8.5% glycerol, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.04 mM UTP, 20 μCi of [α-^{32}P]UTP (500 Ci/mmol), and 0.5 to 2.5 μg of DNA as chromatin or purified DNA. Transcription assay mixtures were incubated for 60 min at 30°C. DNase I (RNase-free; Worthington Diagnostics) was then added to a final concentration of 15 μg/ml, and the reaction mixture was incubated for 5 min at 30°C. Sodium dodecyl sulfate (SDS) was then added to a final concentration of 0.2%, and this was followed by the addition of 25 μg of proteinase K per ml and incubation at 30°C for 15 min. The RNA was then purified as described by Lavialle et al. (24).

Analysis of RNA transcripts by glyoxal gel electrophoresis. SV40 DNA fragments which were used as size markers were generated by restriction endonuclease cleavage of superhelical SV40 DNA with (i) *Bam*HI, (ii) *Hin*FI, or (iii) *Bam*HI and *Hpa*II. An equimolar mixture of the three digests was labeled at the 5′ termini with phage T4 polynucleotide kinase and [γ-^{32}P]ATP as described by Maxam and Gilbert (28). Purified RNA samples and DNA markers were denatured by glyoxal as described by McMaster and Carmichael (30). Denatured samples were electrophoresed on a 1.5% agarose gel (10 mM phosphate buffer, pH 7.0) at 150 V (50 mA) for 2.5 h. The gel was fixed with 1% streptomycin for 30 min at room temperature, dried, and exposed to X-ray film.

S1 nuclease analysis of RNA transcripts. SV40 DNA fragments were labeled at their 5′ termini as described above. RNA transcripts and the labeled DNA probe were suspended in 20 μl of a solution containing 0.4 M NaCl, 0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 1.25 mM EDTA, and 80% formamide and heat denatured by incubation at 85°C for 5 min. The samples were then transferred immediately to a 50°C water bath and incubated for at least 3 h. Hybridization reactions were stopped by adding 9 volumes of a solution containing 0.25 M NaCl, 0.03 M sodium acetate (pH 4.6), and 1 mM $ZnSO_4$ (preequilibrated to 4°C). S1 nuclease was added to a final concentration of 5,000 U/ml, and the reaction sample was incubated for 45 min at 30°C. Protected DNA fragments were then purified and analyzed by glyoxal gel electrophoresis.

Analysis of the superhelical structure of chromatin DNA. A 2.5-μg sample of SV40 chromatin or purified SV40 F_I DNA was incubated with the soluble HeLa cell extract as described above for transcription reactions. After 0, 0.5, 5, 15, 30, and 60 min of incubation, portions were removed, and the reaction was stopped by adding an equal volume of 20 mM EDTA–0.4% SDS. Samples were then treated with proteinase K (50 μg/ml) for 1 h at 37°C. The DNA was purified and analyzed by gel electrophoresis on a 1.5% agarose gel at 50 V for 16 to 20 h with a 40 mM Tris-acetate (pH 7.2)–20 mM sodium acetate–1 mM sodium EDTA buffer. The gel was stained with ethidium bromide and photographed under a UV light source. To distinguish between nicked circular and covalently closed circular SV40 DNA molecules, DNA samples were electrophoresed in the presence of 6 μg of ethidium bromide per ml.

Isolation of RNA by using Southern blots. The separated strands of two SV40 DNA fragments generated by cleavage with *Bam*HI and *Hpa*II restriction endonucleases were electrophoresed and transferred to nitrocellulose paper by the method of Southern (36), as previously described (3). Hybridization of [α-^{32}P]UTP-labeled RNAs was carried out in a buffer containing 50% formamide, 2 mM EDTA, 0.75 M NaCl, 0.5% SDS, and 200 μg of tRNA per ml in 100 mM Tris (pH 7.4) for 16 h at 37°C. Hybridization strips were washed with 10 successive rinse solutions (5 ml each) containing 0.2% SDS and 2 mM EDTA in 10 mM Tris (pH 8.0). Nitrocellulose strips were dried and exposed to X-ray film. Individual bands were excised, and the RNA was eluted by incubation at 85°C for 5 min in hybridization wash buffer.

RESULTS

Stability of SV40 chromatin in HeLa cell extract. HeLa cell extracts prepared by the method of Manley et al. (26) contain topoisomerase. This enzyme removes superhelical turns from covalently closed DNA molecules by introducing a single-strand nick in the DNA and resealing the nick after the torsional tension of the double helix is relieved, allowing the release of one superhelical turn each time that the cycle is repeated. By virtue of the torsional constraints that nucleosomes exert on the DNA duplex, chromatin structure prevents the loss of superhelical turns (17, 29). Therefore, an analysis of DNA structure after incubation with the extract provides an assay for the stability of the SV40 chromatin complex. Control superhelical SV40 DNA (F_I) and SV40 chromatin were added to complete transcription assay reaction mixtures. Samples were removed at different times, depro-

teinized, and analyzed by agarose gel electrophoresis. The action of topoisomerase on F_I SV40 DNA was apparent after 0.5 min of incubation since most of the superhelical DNA had been converted either to intermediates with a lower superhelix density or to relaxed covalently closed DNA molecules and nicked circular DNA (SV40 DNA F_{II}) molecules (Fig. 1, lanes A and B). The latter two forms of SV40 DNA comigrated in this gel system. Further incubation of purified DNA resulted in complete conversion of F_I DNA to relaxed covalently closed DNA molecules and nicked circular molecules (Fig. 1, lanes C through F). In contrast, incubation of chromatin DNA under identical conditions revealed that the superhelical DNA was stable in the presence of the enzyme (Fig. 1, lanes G through L). After 0.5 and 5 min of incubation, no significant change in superhelical DNA structure was detected in the chromatin samples. After 10, 30, and 60 min of incubation, there was a partial loss of the F_I superhelical DNA band, with a concomitant increase in the amount of F_{II} or relaxed covalently closed circular DNA. To differentiate between F_{II} and relaxed covalently closed circular DNA molecules, duplicate DNA samples were electrophoresed in an agarose gel containing 0.6 mg of ethidium bromide per ml. This analysis demonstrated that in addition to DNA molecules with an unaltered superhelix density, the only DNA species present in the chromatin samples was DNA F_{II} (data not shown).

The stability of SV40 chromatin during incubation with HeLa cell extracts was also analyzed by velocity sedimentation and isopycnic banding in CsCl. [^{3}H]thymidine-labeled SV40 virions were dissociated and incubated with the soluble HeLa cell extract as described above. After incubation, the sample was compared with control chromatin on isokinetic 10 to 30% sucrose gradients and CsCl density gradients. The results of these experiments demonstrate that chromatin structure was preserved since the sedimentation coefficient (110S) and buoyant density (1.40 g/cm^3) were not altered after incubation with the extract (data not shown). The fact that no free DNA was detected in either of these analyses also demonstrates that the F_{II} circular DNA molecules noted above were in a chromatin structure before deproteinization of the DNA for gel analysis.

Transcription of SV40 chromatin in soluble HeLa cell extracts. SV40 virions were dissociat-

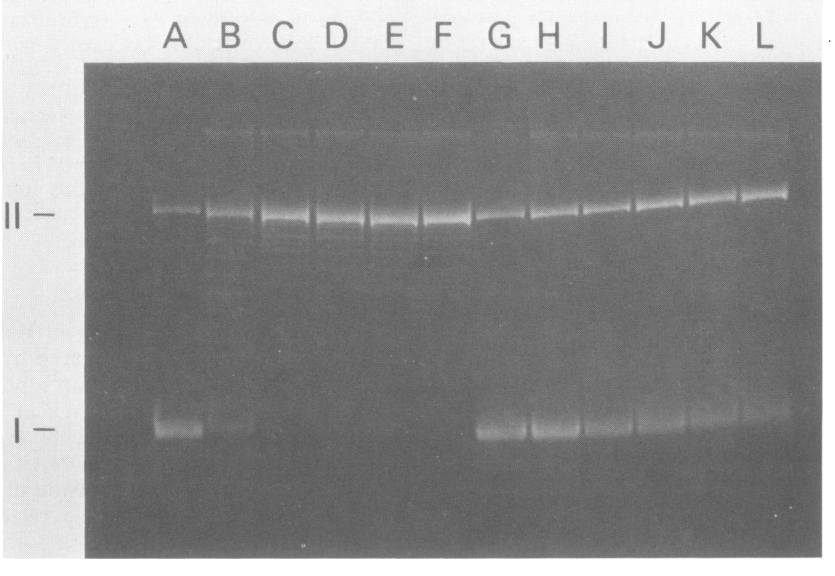

FIG. 1. Superhelical structure of chromatin DNA after incubation with HeLa cell extract. A 2.5-μg sample of SV40 chromatin or SV40 F_I DNA was incubated in the standard transcription mixture, which included all four nucleotide triphosphates. After 0, 0.5, 5, 15, 30, and 60 min of incubation, portions were removed, and the reaction was stopped by adding an equal volume of 20 mm EDTA–0.4% SDS. DNA samples were then treated with proteinase K (50 μg/ml) for 1 h at 37°C, purified, and analyzed by gel electrophoresis on a 1.5% agarose gel at 50 V for 16 to 20 h with a 40 mM Tris-acetate (pH 7.2)–20 mM sodium acetate–1 mM EDTA (sodium salt) buffer. The gel was stained with ethidium bromide and photographed under a UV light source. Lanes A through F, zero time and 0.5-, 5-, 15-, 30-, and 60-min purified DNA incubation samples, respectively; lanes G through L, zero time, and 0.5-, 5-, 15-, 30-, and 60-min chromatin DNA incubation samples, respectively. I and II indicate the migration positions of SV40 superhelical (F_I) and nicked circular (F_{II}) DNAs, respectively. Relaxed covalently closed circular DNA migrated at the same position as F_{II} DNA.

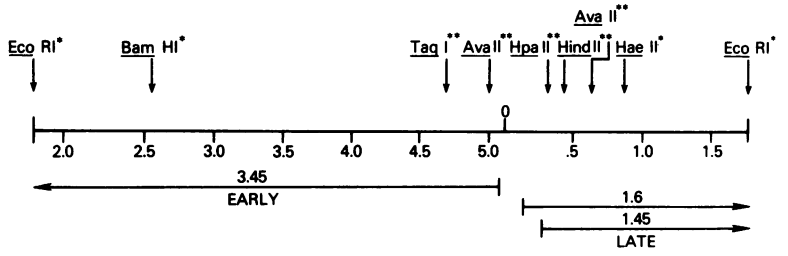

FIG. 2. Transcriptional map of SV40 DNA. The diagram represents an SV40 DNA template which has been digested with endonuclease *Eco*RI (Fig. 3). The orientations and lengths of RNA transcripts from early and late SV40 promoters are shown below the map. The restriction endonuclease sites used for transcription "run-off" assays (single asterisks) (Fig. 3 and 4) and S1 nuclease analysis (double asterisks) (Fig. 6) are indicated above the map. The map coordinates and RNA transcript lengths are indicated (in kilobases).

ed with EGTA and dithiothreitol as described previously (4, 5), and the chromatin DNA was restricted with endonuclease *Eco*RI (map position 1,783) (Fig. 2). The restricted chromatin template was then added to the transcription reaction mixture at different concentrations ranging from 10 to 50 μg/ml. After incubation at 30°C for 1 h, the RNA transcripts were purified and analyzed by glyoxal gel electrophoresis (Fig. 3). The major RNA bands detected in this analysis were transcripts of approximately 3,450, 1,600, and 1,460 nucleotides. Assuming that transcription terminates at the endonuclease restriction site, the 3,450-nucleotide transcript is consistent with an RNA molecule originating near the early SV40 promoter and extending to the *Eco*RI restriction site. Similarly, the 1,600- and 1,450-nucleotide transcripts would originate from late SV40 promoters. The radioactive band migrating at 1,800 nucleotides was not a SV40 transcript, but apparently was generated by radiolabeling of the endogenous RNA present in the extract (26). The lengths of all RNA transcripts have been corrected by 5.6% to compensate for the different migration rates of RNA and DNA in glyoxal gels.

The surprising aspect of this experiment was that accurate initiation of transcription on SV40 chromatin occurred over a wide range of DNA concentrations. This is in direct contrast to the strict DNA concentration requirement for accurate initiation of transcription on naked DNA, as noted by Manley et al. (26) in the original description of the soluble transcription system. We observed a similar DNA concentration dependence in our analysis of transcription of purified DNA templates. Specific transcription from SV40 early and late promoters occurred only over a very narrow range (20 to 30 μg/ml) when the template was purified DNA. Increasing purified DNA concentrations to 40 to 50 μg/ml resulted in a decrease in specific transcription and a concomitant increase in nonspecific transcription (data not shown). Transcription of both DNA and chromatin at concentrations less than 10 μg/ml reduced transcription below the level of detection.

We have previously shown that extracellular SV40 virion chromatin is an efficient template

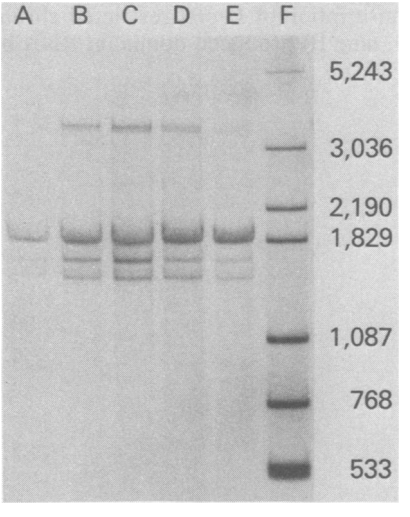

FIG. 3. Transcription of SV40 chromatin in HeLa cell extracts. SV40 virions were dissociated by treatment with 5 mM EGTA–3 mM dithiothreitol–100 mM NaCl in 50 mM Tris (pH 8.7) for 30 min at 32°C. After dissociation, $MgCl_2$ was added to a concentration of 6 mM, and the chromatin template was restricted by incubation at 32°C for 1.5 h in the presence of 5 to 10 U of *Eco*RI per μg of DNA. [α-^{32}]UTP-labeled RNAs were synthesized in standard reaction mixtures containing 10 μg (lane A), 20 μg (lane B), 30 μg (lane C), 40 μg (lane D), or 50 μg (lane E) of the *Eco*RI-restricted chromatin template per ml. Purified RNA transcripts were purified and denatured with glyoxal as described by McMaster and Carmichael (30). Denatured samples were electrophoresed in a 1.5% agarose gel in 10 mM phosphate buffer (pH 7.0) at 150 V (50 mA) for 2.5 h. Gels were fixed with 1% streptomycin (30 min, 25°C), dried, and exposed to X-ray film. Lane F contained a ^{32}P-end-labeled DNA marker. The lengths of all RNA transcripts have been corrected by 5.6% to compensate for the different migration rates of RNA and DNA molecules in glyoxal gels.

for *E. coli* RNA polymerase (4, 5). Similar results were observed in a comparison of the transcriptional efficiencies of chromatin and SV40 DNA in the extract of Manley et al. (26). A quantitative analysis of densitometric scans of [α-^{32}P]UTP-labeled transcript autoradiograms and hybridization data indicated that at optimum DNA concentrations, the chromatin template was approximately 75% as efficient as SV40 DNA (data not shown).

Termination of transcription occurs at restriction endonuclease sites. To determine whether termination of transcription occurred at restriction endonuclease sites, the chromatin complex was restricted with either (i) *Eco*RI, (ii) *Eco*RI and *Bam*HI, or (iii) *Eco*RI and *Hae*II. After restriction, the templates were added to the transcription reaction mixture at a concentration of 30 μg/ml and incubated at 30°C for 60 min. The results of this experiment oriented the direction of transcription and demonstrated that transcription terminates at the restriction site (Fig. 4). Transcription of *Eco*RI-restricted chromatin (Fig. 4, lane B) produced dominant RNA bands of 3,450, 1,600, and 1,460 nucleotides. Transcription of *Eco*RI-*Bam*HI-restricted chromatin (Fig. 4, lane C) produced RNA transcripts of 2,700, 1,600, and 1,460 nucleotides. The specific decrease in the size of the 3,450-nucleotide transcript to approximately 2,700 nucleotides suggests not only that termination occurred at the restriction site but also that this RNA transcript originated at the early SV40 promoter and proceeded counterclockwise toward the *Eco*RI site. Transcription of the *Eco*RI-*Hae*II-restricted chromatin generated RNA transcripts of 3,450, 650, and 515 nucleotides (Fig. 4, lane D). The specific decrease in length of the 1,600- and 1,460-nucleotide transcripts suggests that these transcripts originated at late SV40 promoter sites and proceeded clockwise toward the *Eco*RI site. An RNA transcript of approximately 850 nucleotides, which did not correspond to any of the transcripts discussed above, was reproducibly observed in the *Eco*RI-*Hae*II transcription samples. Although the origin of this transcript was not studied in detail, a preliminary analysis suggested that it is transcribed from the early SV40 strand and originates near map position 1,680.

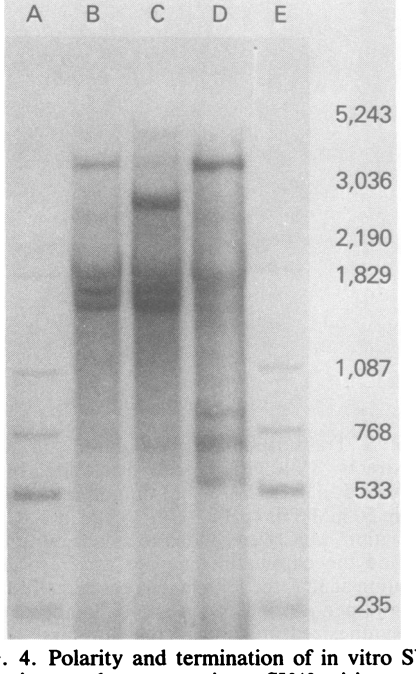

FIG. 4. Polarity and termination of in vitro SV40 chromatin template transcripts. SV40 virions were dissociated as described in the legend to Fig. 3 and restricted with either *Eco*RI (lane B), *Eco*RI and *Bam*HI (lane C), or *Eco*RI and *Hae*II (lane D). [α-^{32}P]UTP-labeled RNAs were synthesized in standard reaction mixtures, purified, and analyzed by glyoxal gel electrophoresis. Lanes A and E contained DNA markers. The lengths of all RNA transcripts have been corrected by 5.6% to compensate for the different migration rates of RNA and DNA molecules in glyoxal gels.

Orientation of RNA transcripts by hybridization, elution, and glyoxal gel electrophoresis. Restriction of SV40 DNA with *Hpa*II and *Bam*HI, followed by strand separation on agarose gels, allows for the separation of the main coding sequences for early and late SV40 mRNAs (3). Hybridization of RNA transcripts synthesized from *Eco*RI-restricted SV40 chromatin to the early/late Southern blot resulted in the pattern shown in Fig. 5, lane A. RNA hybridization was predominantly to the EA and LB DNA fragments, which correspond to the major coding sequences of early and late mRNAs, respectively. When the RNA that hybridized to the four DNA fragments was quantitated, 70 to 80% of the RNA hybridized to the EA and LB DNA fragments. To determine which RNA species hybridized to the specific DNA fragments, RNA was eluted from each fragment, purified, and analyzed by glyoxal gel electrophoresis. Together with data presented above, the distinct hybridization of the 3,450-nucleotide RNA to DNA fragment EA (Fig. 5, lane C) and the hybridization of the 1,600- and 1,460-nucleotide RNAs to the LB DNA fragment (Fig. 5, lane E) demonstrated that these RNAs correspond to initiation of RNA synthesis at early and late SV40 promoters, respectively. The heterogeneous smear of RNA in Fig. 5, lane C, apparently was due to degradation of the 3,450-nucleotide RNA species. The light hybridization of the 3,450-nucleotide RNA to DNA fragment EB and the hybridization of the 1,600- and 1,460-nucleotide RNAs to DNA fragment LA were due to overlaps of

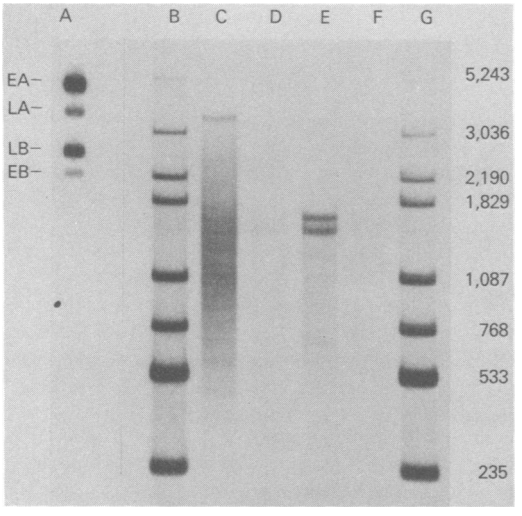

FIG. 5. Hybridization, elution, and glyoxal gel electrophoresis of RNAs synthesized from SV40 chromatin. RNAs were synthesized from an *Eco*RI chromatin reaction mixture and purified. [α-^{32}P]UTP-labeled RNAs were hybridized to the separated strands of a *Bam*HI-*Hpa*II digest of SV40 (3) in hybridization buffer containing 50% formamide, 2 mM EDTA, 0.75 M NaCl, 0.5% SDS, and 200 μg of tRNA per ml in 100 mM Tris (pH 7.4) for 16 h at 37°C. Hybridization strips were washed with 10 successive rinse solutions (5 ml each) containing 0.5% SDS and 2 mM EDTA in 10 mM Tris (pH 8.0). The hybridization strips were dried and exposed to X-ray film. Radioactive bands were localized and cut out, and the radioactive RNA was eluted by incubation at 85°C for 5 min in hybridization wash buffer. RNA transcripts were then purified and analyzed by glyoxal gel electrophoresis. Lane A, Early/late hybridization (EA, early strand, A fragment; LA, late strand, A fragment; LB, late strand, B fragment; EB, early strand, B fragment); lanes C through F, [α-^{32}P]UTP-labeled RNA transcripts hybridized to EA, LA, LB, and EB DNA fragments, respectively; lanes B and G, DNA markers.

RNA sequences at the 3′ end of the early RNA and the 5′ end of the late RNA.

S1 nuclease analysis of RNA transcripts. To determine the position of the initiation site of the RNA transcripts more precisely, we performed S1 nuclease mapping of unlabeled RNA transcripts with several DNA fragments labeled at their 5′ termini (Fig. 6). Hybridization of chromatin RNA to *Ava*II fragment D labeled at both 5′ termini (nucleotides 5,123 and 561) resulted in the protection of three DNA fragments of 115, 230, and 375 (lower band of doublet) nucleotides (Fig. 6a, lanes D and E). The 430-nucleotide DNA fragment was not a reproducible S1 nuclease fragment and appeared to be the result of minor contamination in the probe. From our tentative location of RNA initiation sites (see above), the 115-nucleotide band was consistent with an early RNA transcript that originated at map position 5,240 ± 10, whereas the 230- and 375-nucleotide bands corresponded to late RNA transcripts that originated at map positions 325 ± 10 and 185 ± 10, respectively. These map positions were confirmed by performing a similar S1 nuclease analysis with DNA fragments labeled at the *Taq*I site (Fig. 6a, lanes G through L) (map position 4,741), the *Hpa*II site (Fig. 6b, lanes A through D) (map position 349), or the *Hin*dII site (Fig. 6b, lanes E through H) (map position 473). Briefly, the *Taq*I probe protected two bands of 495 and 410 nucleotides (Fig. 6a, lanes J and K) the *Hpa*II probe protected a band of 170 nucleotides (Fig. 6b, lanes C and D), and the *Hin*dII probe protected bands of 285 and 145 nucleotides (Fig. 6b, lanes G and H). Since the structure of linearized chromatin might allow recognition of promoter sequences which are not accessible in chromatin that contains a covalently closed circular DNA, each analysis included an RNA sample synthesized from uncut SV40 chromatin. Our results demonstrated that specific initiation of transcription occurred on both intact and restricted SV40 chromatin and that these initiation sites corresponded to initiation sites used in vivo (2, 20, 27, 39). It is of interest that whereas the levels of RNA synthesis from the late SV40 promoter were comparable in intact and restricted SV40 chromatin templates, RNA synthesis from the early SV40 promoter appeared to be enhanced in the intact chromatin template. The significance of this observation is presently being investigated.

DISCUSSION

Our results demonstrate that extracellular SV40 virion chromatin is accurately transcribed in the in vitro transcription system of Manley et al. (26). The lengths of RNA transcripts (Fig. 3 and 4), the hybridization patterns (Fig. 5), and the S1 nuclease mapping data (Fig. 6) demonstrate that two major early RNA transcripts, which originate at map positions 5,240 ± 10 and 5,145 ± 10, and two major late transcripts, which originate at map positions 325 ± 10 and 185 ± 10, are synthesized from the chromatin template. These map positions in turn correspond to RNA initiation sites which have been mapped previously by either primer extension or S1 nuclease analysis (2, 20, 27, 39). In contrast to transcription of purified DNA templates, accurate transcription of SV40 chromatin is less dependent upon the addition of specific DNA concentrations to the transcription assay mixture.

Recent analyses of transcriptionally active and inactive eucaryotic genes suggest that one level of transcriptional regulation is by modification of chromatin structure. The nucleosome structure of transcriptionally inactive eucaryotic

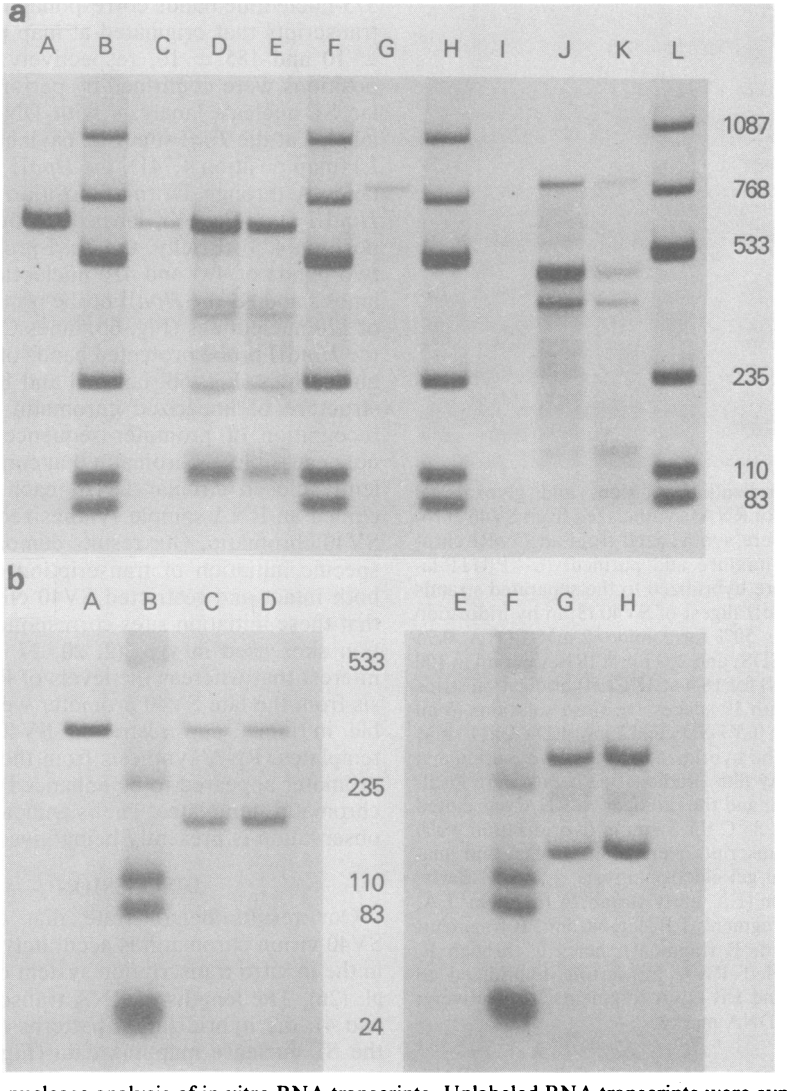

FIG. 6. S1 nuclease analysis of in vitro RNA transcripts. Unlabeled RNA transcripts were synthesized from *Eco*RI-restricted or intact chromatin in standard reaction mixtures, except that [α-^{32}P]UTP was omitted. Purified RNA transcripts and a labeled DNA probe were lyophilized, suspended in 20 μl of 0.4 M NaCl–0.04 M PIPES (pH 6.4)–1.25 mM EDTA–80% formamide, and heat denatured by incubation at 85°C for 5 min. Hybridization samples were transferred immediately to a 50°C water bath and incubated for at least 3 h. Hybridization reactions were stopped by adding 9 volumes of 0.25 M NaCl–1 mM $ZnSO_4$–0.03 M sodium acetate (pH 4.6) pre-equilibrated to 4°C. S1 nuclease was added to a final concentration of 5,000 U/ml, and the reaction mixture was incubated for 45 min at 30°C. Protected DNA fragments were then purified and analyzed by glyoxal electrophoresis in a 2.0% agarose gel (10 mM phosphate buffer, pH 7.0) at 150 V (50 mA) for 2 h. (a) S1 hybridization with *Ava*II fragment D (map positions 5,123 and 561) or *Taq*I (map position 4,741) probe. Lane A, *Ava*II fragment D probe; lane C, *Ava*II fragment D probe hybridization, S1 treated; lane D, *Ava*II fragment D intact chromatin RNA hybridization, S1 treated; lane E, *Ava*II fragment D-*Eco*RI chromatin RNA hybridization, S1 treated; lane G, *Taq*I probe; lane I, *Taq*I probe hybridization, S1 treated; lane J, *Taq*I-intact chromatin RNA hybridization, S1 treated; lane K, *Taq*I-*Eco*RI chromatin RNA hybridization, S1 treated. Lanes B, F, H, and L contained DNA markers. (b) S1 hybridization with *Hpa*II (map position 349) or *Hin*dII (map position 473) probe. Lane A, *Hpa*II probe; lane C, *Hpa*II-intact chromatin RNA hybridization, S1 treated; lane D, *Hpa*II-*Eco*RI chromatin RNA hybridization, S1 treated; lane E, *Hin*dII probe, S1 treated; lane G, *Hin*dII-intact chromatin RNA hybridization, S1 treated; lane H, *Hin*dII-*Eco*RI chromatin RNA hybridization, S1 treated. Lanes B and F contained DNA markers.

genes consists of an octomer of four histones (H_2A, H_2B, H_3, and H_4) which are associated with 145 base pairs of DNA. Histone H_1 interacts with this basic chromatin structure to condense the DNA into 25- to 30-nm higher-order chromatin fibers. (For a review of chromatin structure, see reference 29.) By virtue of the nucleosome structure, chromatin DNA is quite resistant to enzymatic activity and is an inefficient template for either the initiation or elongation reactions of RNA synthesis in vitro (7, 9, 46). Analyses of transcriptionally active eucaryotic genes, such as the genes for globin (44), ovalbumin (16), integrated viral genomes (6, 8, 12, 14, 15, 33), and the *Drosophila* GP-70 heat shock gene (47, 48), have demonstrated that these genetic loci reside within chromatin having an altered structure, as indicated by DNase I hypersensitivity. Wu et al. (47, 48) demonstrated this clearly in their comparison of the transcriptionally active and inactive chromatin structures of the major heat shock protein gene of *Drosophila*. When the GP-70 gene was transcriptionally activated by heat shock, disruption in nucleosomal organization was evidenced by a loss or smearing of discrete DNA fragments in micrococcal nuclease digests. When the cells recovered from the heat shock and the GP-70 gene returned to the inactive state (47, 48), micrococcal nuclease DNA ladder patterns were restored. Further evidence that transcriptionally active chromatin is modified comes from recent studies by Weisbrod and Weintraub (45), who demonstrated that "active" nucleosomes may be separated from "inactive" nucleosomes by affinity chromatography with HMG proteins bound to a solid matrix (45). Although these experiments demonstrated a clear difference in chromatin structure, the basis of nuclease hypersensitivity and differential binding of HMG proteins to transcriptionally active chromatin remains to be established. The contribution of chromatin modifications which include histone acetylation, loss of histone H_1, and nonhistone protein interaction with chromatin all require further analysis to clarify their role in chromatin transcriptional activation. Chromatin modifications may affect promoter selection or function primarily to "open" chromatin to allow entry of the polymerase at the promoter site and subsequent elongation of the RNA chain through nucleosomes.

We have recently reported that extracellular SV40 viral chromatin, which is similar to transcriptionally active eucaryotic genes, has a "relaxed" chromatin structure which makes the viral DNA highly accessible to enzymatic activities (4, 5). The conversion of SV40 chromatin to the relaxed conformation occurs as intracellular 75S chromatin sequentially matures into a 200S previrion, a 240S nuclear virion, and finally an extracellular virion (4, 5, 11, 23). These studies demonstrate that the maturation process is accompanied by significant changes in chromatin composition, including increased acetylation of histones H_3 and H_4, the loss of histone H_1, and the specific association of nonhistone viral protein with the chromatin complex. After in vitro dissociation of nuclear and extracellular SV40 virions, we have shown by comparative enzymatic analysis that extracellular virion chromatin is nuclease hypersensitive and 16- to 20-fold more efficient as a transcriptional template (4). To determine what effect chromatin modifications might have on promoter selection, we compared the transcription of extracellular virion chromatin and purified DNA in three independent systems which included (i) *E. coli* RNA polymerase, (ii) purified polymerase II, and (iii) HeLa cell extracts (Manley extract). Although the utilization of specific promoters differed qualitatively in each system, we found no significant difference in chromatin and purified DNA transcription patterns, which suggests that extracellular SV40 virion chromatin structure regulates neither strand selection nor promoter recognition by the RNA polymerase. Our results suggest that SV40 virion chromatin modifications, rather than regulating promoter selection, function primarily to increase the accessibility of RNA promoter sequences to polymerase II and allow efficient elongation of the RNA chain after the initiation event.

In contrast, intracellular SV40 chromatin, which has quite different structural properties than extracellular SV40 virion chromatin, apparently has the potential to affect SV40 promoter selection. Jakobovits et al. have shown that the initiation specificity of *E. coli* polymerase is altered when transcription is carried out on intracellular SV40 chromatin rather than on purified DNA (22). Initiation of transcription on the viral chromatin occurred preferentially from the late strand in the region close to the in vivo late promoter. This observation is striking since no *E. coli* promoters, which initiate synthesis of RNA on the late strand, have been mapped in this region of the genome (24). Precisely within the later promoter region, approximately 20% of the intracellular chromatin complexes contain a nuclease-sensitive gapped stretch of DNA which is not contained in a typical nucleosome structure. These results may suggest that in vivo transcription initiates within this region on such gapped molecules. The observation that actively transcribed eucaryotic chromatin also displays nucleosome-deficient stretches of DNA makes this model an attractive mechanism for promoter selection for some genes in eucaryotic cells.

The SV40 genome is divided into two sets of

genes which are temporally regulated during the infection cycle. Before DNA replication, stable SV40 RNA transcripts are synthesized predominantly from the early gene which codes for the T-antigens. After expression of the early gene products and subsequent DNA replication, transcription occurs predominantly from the late SV40 genes which code for viral structural proteins. Transcriptional analyses based on in vivo and in vitro studies demonstrate that regulation of the SV40 early gene is under the control of the Goldberg-Hogness-like sequence TATTTA which is located 25 to 30 nucleotides upstream from the major RNA cap site and a 72-base pair tandem DNA repeat sequence located more than 100 nucleotides upstream from the cap site (1, 2, 18–20, 27). Expression of SV40 early mRNA is repressed by its own gene product, large T-antigen (31, 35, 40). The regulatory sequences which control expression of the SV40 late genes and the mechanism by which accumulation of late RNA transcripts is suppressed early in the infection cycle are not clear at present. The observation that both late and early SV40 promoters were efficiently expressed in the in vitro transcription system of Manley et al. suggests that promoter strength could not account for the 20- to 100-fold-lower amount of late mRNA found during the initial stages of infection (34). Since our transcriptional analyses of extracellular virion chromatin demonstrate that chromatin modifications do not regulate promoter selection, it also appears unlikely that chromatin structure can accurately account for the unequal expression of early and late SV40 mRNAs during the early stages of SV40 infection. It appears more plausible, as previously hypothesized by Birkenmeier et al. (3), that initiation of late SV40 RNA synthesis occurs in vivo during the early stages of infection but stable transcripts are not produced due to attenuation of late RNA synthesis by cellular proteins or by specific degradation of late RNA after synthesis.

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