Both *trans*-Acting Factors and Chromatin Structure Are Involved in the Regulation of Transcription from the Early and Late Promoters in Simian Virus 40 Chromosomes

LOIS C. TACK† AND PETER BEARD*

Department of Virology, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland

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We isolated simian virus 40 (SV40) chromosomes from lytically infected CV-1 cells at various times during the late phase and transcribed them *in vitro* with either whole-cell or nuclear extracts of HeLa cells. The late promoter was 3- to 10-fold more active than the early promoter. With bare SV40 DNA templates, the early promoter was up to 10-fold stronger than the late promoter. The relative strengths of the early and late promoters on SV40 chromosomes were essentially independent of template concentration or length of the replicative phase of the infection. When monoclonal antibodies or antisera against T antigen (T Ag) were added to SV40 chromosomes or when T Ag, both free and chromatin bound, was removed by immunoprecipitation with anti-T, the activity of the late promoter remained essentially unchanged. Washing with 0.4 M NaCl removed T Ag from more than 90% of the mature chromosomes associated with T Ag. Transcription from the late promoter still predominated in the salt-washed T Ag-depleted chromosomes, even though there was a marked increase in early promoter activity. The derepression of the early promoter could be reversed by adding the T Ag-containing extract back to the depleted chromosomes. Extraction of SV40 chromosomes with 1.5 M NaCl resulted in a decrease in the activity of the late promoter and a further increase in the activity of the early promoter so that the relative amounts of early and late RNA synthesized were similar to those for bare SV40 DNA templates. Late RNA synthesis from bare SV40 DNA templates was stimulated by high-speed supernatants prepared from nuclear extracts of SV40-infected cells but not from those of uninfected cells. Pretreatment of the supernatants with anti-T did not alter the result. Our findings indicate that the activity of the early and late SV40 promoters is regulated by at least two different mechanisms at the chromosomal level. One is mediated by a subclass of T Ag bound to SV40 chromosomes which represses early SV40 transcription but has no effect on late transcription. A second level of regulation, involving a tightly bound *trans*-acting chromosomal factor and a stable nucleoprotein structure, favors the late promoter over the early promoter by up to 10-fold.

The genome of SV40 encodes two classes of viral mRNA, early and late, which are transcribed during lytic infection. RNA is synthesized from the early SV40 genes throughout the lytic cycle, whereas transcription from the late genes is temporally regulated in that significant activation does not occur until the onset of viral DNA replication. The major and minor 5' termini of early and late mRNA and their cap structures, strand orientation, and posttranscriptional processing have been delineated. The late promoter is usually expressed only in permissive cells. Although the 5' ends of early mRNA are fairly homogeneous, the late 5' ends are heterogeneous, spanning ca. 300 base pairs (bp) from near the origin of replication extending in the late direction (for reviews, see references 13 and 51). The SV40 early promoter consists of a TATA box 30 bp before the RNA cap site and upstream sequences in the region of the GC-rich 21-bp repeats (9, 37, 40). Early RNA expression *in vivo* also requires the 72-bp repeats (5, 21). The SV40 late promoter, unlike other promoters for RNA polymerase II, does not contain a consensus TATA sequence. Late RNA expression is optimal in the presence of the 21- and 72-bp repeats and an intact origin of replication (7, 11, 17, 18). Downstream control elements appear important, as deletions within the late leader region (including the major late start site) result in

altered utilization of 5' termini but no reduction in the amount of late RNA synthesized (20). In addition, an attenuation site located within the proximal late leader region can regulate late RNA synthesis by premature termination (28).

Specific *in vitro* transcription of added DNA templates with HeLa crude whole-cell lysates (36), subcellular fractions supplemented with purified RNA polymerase II (52), or 0.42 M NaCl extracts of purified HeLa nuclei (14) has been described. Whole-cell lysates and 0.42 M NaCl extracts of nuclei of HeLa cells contain RNA polymerase II (responsible for the synthesis of mRNA in eucaryotic cells) and other transcriptional factors (14, 48). These systems accurately initiate, synthesize cap structures, and efficiently elongate RNA (14, 22, 36, 52). *In vitro* transcription studies have defined DNA sequences controlling early (34, 37) and late (11) transcription of SV40 and polyomavirus (31). When linearized SV40 DNA is the template for HeLa whole-cell lysates, the early promoter is predominant (4, 23, 42). Early RNA synthesis is repressed by purified large T antigen (T Ag) when bound to both sites I and II on SV40 DNA templates (25, 40, 42). Furthermore, shifts in the early RNA start sites both *in vivo* and *in vitro* have been correlated with limited binding of T Ag to SV40 DNA (19, 25). Regulation *in vitro* has also been described for adenovirus late promoters which are activated by the addition of HeLa cell extracts prepared during the late phase of adenovirus infection (16). Virion proteins appear to regulate the accessibility of RNA polymerase to promoters in disrupted SV40 virions rather

* Corresponding author.

† Present address: Molecular Biology and Virology Laboratory, The Salk Institute, La Jolla, CA 92037.

than affect promoter selection (8). Accurate polyadenylation of late adenovirus transcripts (10) and specific termination of late SV40 transcripts at an attenuation site (41, 46) occur with nuclear extracts from infected HeLa and CV-1 cells, respectively, without added factors. Thus, these *in vitro* transcription systems allow one to begin to characterize the elements involved in the control of initiation, elongation, termination, and posttranscriptional processes in eucaryotic mRNA production.

Recently, the *in vitro* transcription by HeLa cell lysates of SV40 chromosome templates extracted during lytic infection has been reported (4). The amount of RNA synthesized was substantially greater than that produced by endogenous RNA polymerase bound to SV40 chromosomes and consisted mostly of specific late transcripts. The pattern of transcription was similar to that observed *in vivo* during that phase of the lytic infection. In contrast, RNA synthesis on bare SV40 DNA templates under the same conditions consisted of more early transcripts than late ones (see also references 23 and 42). In the work presented here, we have used antibodies specific for SV40 T Ag and salt washes of SV40 chromosomes to define the factors which determine the activity of the late and early promoters in SV40 chromosomes.

MATERIALS AND METHODS

Cells and virus. CV-1 cells, obtained from H. Turler (Geneva), and SV40 strain 777 were grown as previously described (3). SV40-infected freeze-thawed CV-1 cell lysates were used as virus stock; 0.4 ml was used to infect each 90-mm dish (producing a multiplicity of 10 to 50 PFU per cell) of CV-1 cells at 80 to 90% confluency.

NE preparation. Nuclear extracts (NEs) containing SV40 chromosomes were prepared from SV40-infected CV-1 cells by hypotonic lysis with HBE buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.8], 5 mM KCl, 0.5 mM dithiothreitol, 1 mM disodium EDTA), as previously described by Beard and Nyfeler (4). SV40 DNA was radiolabeled by incubating infected cells with 100 to 200 μ Ci of [*methyl*- 3 H]thymidine (specific activity, 44 Ci/mmol; Amersham Corp.) per 150-mm plate for 5 to 10 h at 37°C before lysis. Unless noted, viral chromosomes were prepared from cells at 36 h postinfection. After cell lysis in a Dounce homogenizer and centrifugation for 2 min at 2,500 rpm with a Sorvall HB4 rotor, nuclei were suspended in 1 or 2 volumes of ice-cold HBE buffer and extracted for 12 h (overnight) at 0°C. For this CV-1 cell line, ca. 20 and 50% of the extractable viral complexes were recovered from nuclei after 1 and 5 h of incubation, respectively. After centrifugation for 10 min at 15,000 rpm with a SS34 rotor, the NE, containing ca. 60 to 80% of the total viral DNA (20 μ g of SV40 DNA per 150-mm plate), was either directly used for *in vitro* transcription assays or further purified. The integrity of the SV40 chromosomal DNA in the NE fraction was assessed by electrophoretic analysis, after the addition of sodium dodecyl sulfate (SDS), on a horizontal 1.5% agarose slab gel in a buffer containing 40 mM Tris, 5 mM sodium acetate, and 1 mM disodium EDTA (pH 7.8) for 4 h at 150 V and room temperature. Gels were stained with ethidium bromide (1 μ g/ml) and photographed with a UV transilluminator. SV40 chromosome concentrations were estimated by coelectrophoresis with standards of known DNA concentrations.

SV40 chromosome purification by sucrose gradient. Newly prepared NE (250 μ l) was layered onto each 5 to 20% sucrose gradient (in HBE buffer) and centrifuged at 40,000

rpm for 85 min at 0°C in a Beckman SW60Ti rotor. Fractions (150 μ l) were collected from the bottom, and aliquots were measured for radioactivity by counting in Aquasol (New England Nuclear Corp.). Greater than 95% of the 3 H label contained in the 70S viral chromosome peak was acid insoluble. About a threefold dilution of SV40 DNA in the peak fraction was observed compared with NE, as judged by either 3 H label or ethidium-stained viral DNA electrophoresed on a 1% agarose minigel at 50 V for 1 to 2 h in a buffer containing 96 mM Tris, 89 mM boric acid, and 2.5 mM disodium EDTA (pH 8.3). Immunoprecipitation and transcription assays were performed immediately on sucrose-containing chromatin pools.

Salt extraction of SV40 chromosomes. Newly prepared NE (200 μ l) was made 0.4, 0.8, or 1.5 M with respect to NaCl and then layered onto a step gradient consisting of 10 and 50% sucrose (containing HBE buffer and the corresponding salt concentration) in the upper and lower levels, respectively. After centrifugation with an SW60Ti rotor at 4°C for 140 min at 40,000 rpm for 0.4 and 0.8 M NaCl concentrations or for 210 min at 50,000 rpm for 1.5 M NaCl extraction, fractions were collected. SV40 complexes sedimented to the interface of the two sucrose solutions. This pool (diluted about twofold compared with NE) and the fractions at the top of the gradient (corresponding to the initial sample volume) were each dialyzed overnight against HBE buffer containing 50 mM KCl. Measurements of T Ag content and template activity were then made.

Purification of SV40 DNA. SV40 DNA was selectively extracted from CV-1 cells (48 h after virus infection) with SDS and high salt (29), purified by extraction with equal volumes of phenol-chloroform (1:1 [vol/vol]) and then chloroform-isoamyl alcohol (24:1 [vol/vol]), and then precipitated with 2.5 volumes of ethanol. The pellet was washed with 70% ethanol, dried, and dissolved in 50 mM Tris (pH 7.9)–5 mM disodium EDTA. It was then digested with RNase, heat denatured, extracted with phenol–0.5 M NaCl to purify supercoiled DNA (35), and ethanol precipitated. The pellet was rinsed with 70% ethanol, dried, and dissolved in 10 mM Tris (pH 7.9)–1 mM disodium EDTA (TE). Supercoiled SV40 DNA was cleaved with the restriction endonuclease *Pst*I (New England Biolabs) by the protocol recommended by the supplier, extracted with phenol-chloroform, and ethanol precipitated in the presence of 0.3 M sodium acetate. The pellet was washed, dried, and dissolved in TE as described above.

***In vitro* transcription.** HeLa whole-cell lysates were prepared as described previously (4) by the methods of Sugden and Keller (48) and Manley et al. (36). HeLa cells (strain S₃) were grown in suspension culture in Dulbecco minimal essential medium with 5% fetal bovine serum. O. Hagenbuchle generously provided most of the whole-cell lysate used. Transcription reactions with HeLa whole-cell lysate were carried out as previously described (4) except that the reaction volume was reduced to 14 μ l. Each reaction (in siliconized Eppendorf tubes) contained 5 μ l of HeLa lysate (added last), 1 μ l of [α - 32 P]UTP (400 Ci/mmol, containing 5 to 10 μ Ci of radioactivity; Amersham), 1 μ l of *Pst*I (11.2 U/ μ l), 1.4 μ l of freshly assembled transcription mix (1.5 parts nucleoside triphosphates, 1 part polydeoxyinosinic-deoxycytidylic acid [1 mg/ml], 2.5 parts 100 mM creatine phosphate), DNA or chromosomal template, and sterile, distilled water to make 14 μ l. Nucleoside triphosphates consisted of equal volumes of 25 mM ATP, 25 mM GTP, 25 mM CTP, and 2.5 mM UTP. All stocks were stored at –25°C except HeLa lysate, which was kept at –70°C in 50- μ l

portions, and SV40 chromatin fractions, which were kept in ice. Up to 5 μ l of viral DNA template was added. This normally consisted of 1 μ l of NE (0.1 to 0.2 μ g of SV40 chromosomal DNA), 3 to 5 μ l of sucrose gradient-purified 70S chromatin, or 0.1 to 0.2 μ g of *Pst*I-cut SV40 DNA (in this case *Pst*I was omitted from the transcription reaction). If more than 2 μ l of template in EDTA-containing buffer was used, an equivalent concentration of $MgCl_2$ was added to the reaction. The reaction was initiated by the addition of HeLa extract, the contents were mixed, and the tube was centrifuged for 3 s in an Eppendorf microfuge to uniformly combine all the reactants and then was incubated for 60 min at 30°C. The reaction was terminated by the addition of 36 μ l of TE, SDS to 0.5%, disodium EDTA to 20 mM, and 1 to 2 μ l of pronase (20 mg/ml, predigested for 30 min at 30°C). This mixture was further incubated for 15 to 30 min at 30°C.

HeLa cell extracts were also prepared with a 0.42 M NaCl wash of isolated nuclei as described by Dignam et al. (14). Transcription reactions with this HeLa extract were carried out with their standard assay except that the final nucleoside triphosphate concentrations were those used above. Optimum KCl and $MgCl_2$ concentrations for specific transcription of SV40 DNA and chromosomal templates with this extract (data not shown) were determined to be 60 and 8 mM, respectively, similar to those observed for specific adenovirus transcription (14). The 0.42 M salt HeLa extract constituted one-half of the final reaction volume.

Purification and analysis of RNA transcripts. Labeled RNA transcripts generated in vitro were immediately purified by chromatography in TE, on Bio-Gel A15 (Bio-Rad Laboratories) columns in siliconized Pasteur pipettes to remove free label. The excluded RNA peak (400 μ l) eluted after ca. 600 μ l of washing. After the addition of 20 μ g of carrier tRNA (Boehringer Mannheim Biochemicals) and 20 μ l of 5 M NaCl, the RNA was precipitated with 2.5 volumes of ethanol, either in an ethanol-dry ice mixture for 10 min or overnight at -25°C. After centrifugation and brief drying, the pellet was dissolved in 20 μ l of sterile water. Glyoxal mix (40 μ l) was added (39), and the samples were incubated for 10 to 20 min at 50°C and then immediately loaded onto a horizontal 2% agarose slab gel containing 10 mM sodium phosphate (pH 6.8). Electrophoresis was carried out in the same buffer plus 0.1% SDS, first at 100 V until the sample had entered the gel and then at 200 V for 4 h. Two side-by-side reservoirs, each containing 0.5 liter of buffer and connected by sponge wicks, were used per electrode to maintain pH in the gel. Gels were dried onto DE 81 paper on top of Whatman 3MM filter paper and autoradiographed with Kodak XR-5 film and a Du Pont Cronex Lightning Plus intensifying screen for 1 to 2 days at -70°C or for 1 week without a screen at room temperature. For quantitation, several exposures were made, and the relative peak heights of the labeled RNA bands were measured from densitometer tracings.

Immunoprecipitation with anti-T. Immunoprecipitations were carried out essentially as described by Tack and DePamphilis (50) with the following modifications. For quantitative analysis of SV40 chromosomes associated with T Ag, either 10 μ l of NE or 50 μ l of gradient-purified SV40 chromatin, labeled in vivo with [³H]thymidine, was reacted with increasing amounts of either hamster tumor anti-T serum (Huntington Research Center, National Institutes of Health contract) or monoclonal anti-T (see below). The reaction volumes were adjusted to 100 μ l with HBE buffer and incubated for 30 min in ice. For transcriptional analysis, HBE buffer was added to 10 μ l of NE to make a fixed final volume of 20 μ l. Controls included hamster preimmune

serum, ascitic fluid containing monoclonal antibody against a human HLA Ia determinant (R. Acolla, Lausanne) or an anti-cytochrome p450 hybridoma supernatant (K. Griffin, Scripps Clinic). For quantitation, 50 μ l of fixed *Staphylococcus aureus* (washed and resuspended at 10% [wt/vol] in HBE; The Enzyme Center) was added. For samples that were to be subsequently transcribed, either 20 μ l of a washed *S. aureus* suspension or 3 μ l of settled, washed protein A-Sepharose (Pharmacia Fine Chemicals) was used per μ l of tumor serum or ascites fluid used. After incubation in ice for 30 min, centrifugation, and washing with HBE buffer, the immunoprecipitate and supernatant fractions were analyzed for radioactivity and by in vitro transcription.

Monoclonal anti-T's 101 and 109 (hybridoma supernatants were provided by E. Gurney, University of Utah) and 402, 405, 416, 423, and 430 (hybridoma cell lines were provided by E. Harlow, Imperial Cancer Research Fund, London; see reference 26) were used. With the help of H. Engers (Lausanne), ascitic fluid was prepared with the Harlow cell lines. BALB/c mice were treated with 1 ml of pristane 10 days before intraperitoneal injection of 1×10^7 to 2×10^7 cells. The ascitic suspension, collected after 6 to 8 days, was centrifuged to remove cells, filter sterilized, and stored at -20°C. Ascites fluid was added to labeled SV40 chromosomal DNA with fixed *S. aureus* alone or together with rabbit anti-mouse immunoglobulin (IgG) as the immunoabsorbent. Hybridoma supernatants 101 and 109, as well as tumor and preimmune serum, were purified on a protein A-Sepharose column by the method of Ey et al. (15) to remove contaminating nuclease activity.

Preparation of high-speed supernatants. NEs were prepared with HBE buffer, isotonic NP-40 (100 mM NaCl, 50 mM Tris-hydrochloride [pH 8.0], 1 mM disodium EDTA, 1% Nonidet P-40 [NP-40]), or 0.3 M salt-NP-40 (300 mM NaCl and 0.1% NP-40 in HBE buffer). Nuclei from infected CV-1 cells (36 to 46 h postinfection), after being lysed in HBE buffer, were suspended in one of the above buffers for 12 h in ice and then centrifuged as described earlier. The resulting extract (usually 400 μ l) was then centrifuged again, after being overlaid with paraffin oil, at 40,000 rpm for 2.5 h at 2°C in a SW60Ti rotor. The supernatant was removed by side puncture of the centrifuge tube with recovery of about 250 μ l. Electrophoretic analysis of these supernatants detected no viral DNA.

Reconstitutions. High-speed supernatants (depleted of SV40 chromosomes), the top fractions of sucrose gradients (on which NE had been fractionated), and unfractionated NE were each added to bare *Pst*I-cut SV40 DNA and incubated on ice for 10 to 30 min. Between 1 and 5 μ l of the subnuclear fraction was added per 0.2 μ g of DNA; parallel incubations were carried out with mock-infected CV-1 nuclear fractions. The mixtures were then assayed in the standard transcription reaction; no *Pst*I was added. Dialyzed salt-depleted chromatin was incubated for 10 min on ice with an equal volume of the corresponding dialyzed top fractions of sucrose gradients and then transcribed (final DNA concentration, 5 to 10 μ g/ml) in the presence of added *Pst*I.

RESULTS

In vitro transcription of SV40 chromosomes: *Pst*I runoff analysis. When restriction endonucleases are added to transcription reactions containing chromosomes and HeLa lysate, the chromosome templates are partially cleaved. Discrete runoff transcripts, synthesized by RNA polymerase in the lysates, terminate at the cutting sites (4). Here, the restriction enzyme *Pst*I was chosen because it cleaves SV40

FIG. 1. Predicted runoff RNA transcripts from *Pst*I-cut SV40 chromosomal and bare DNA templates. The major early (5232 to 5237 bp) and the major late (325, 264, and 170 bp) *in vivo* start sites are shown (*Bgl*I = 0). Cleavage of SV40 chromosomes (30% at each site) with saturating amounts of *Pst*I followed by transcription should generate two unique sets of RNA transcripts: 1.7 and 2.9 kb for the late promoter and 2.0 and 3.3 kb for the early promoter. Cleavage of SV40 DNA with *Pst*I (100% cleavage at both sites) should generate one set of transcripts (a 1.7-kb late transcript and a 2.0-kb early transcript).

DNA at two sites near the replication termination region, partitioning the early region from the late (Fig. 1). The predicted major transcripts from *Pst*I-cut DNA templates, calculated from *in vivo* SV40 early and late lytic RNA start sites (11, 20), are a 2.07-kb early transcript and a group of late transcripts (1.66, 1.72, and 1.82 kb), which extend from near the origin of replication in opposite directions around the genome. For SV40 chromosomes, maximal cutting of the chromosomal DNA at each site is about 30% due to associated nucleosomes (49). From this mixture of cut and uncut chromosomes, four major runoff RNA transcripts are predicted: 1.66 and 2.88 kb from the late promoter and 2.07 and 3.28 kb from the early promoter. Thus, two sets of early and late RNA transcripts, only slightly different in length, can be compared.

The results of *in vitro* transcription of SV40 chromosomes contained in NE, which were cut with *Pst*I and transcribed with HeLa whole-cell lysates, are shown in Fig. 2. The four major ³²P-labeled transcripts predicted were present (Fig. 2A, lane 2). The high-molecular-weight RNAs were assumed to be transcripts of the whole genome synthesized from uncut chromosomal templates. Sufficient *Pst*I was added so that maximal cutting of chromosomes occurred within 5 min, well before generation of transcripts of 1.5 kb or more. No end-to-end transcripts at 1.22 kb were observed. Bare SV40 DNA (Fig. 2A, lane 1) generated a 1.7-kb late and a 2.0-kb early runoff transcript as expected for templates completely cleaved by *Pst*I. Densitometer scans of the autoradiograph for lanes 1 and 2 are shown (Fig. 2B). When SV40 chromo-

somes (NE) were the template, the ratio of late to early RNA was between 3 and 10 for both sets of *Pst*I runoff transcripts (Fig. 2A, lane 2). An equal amount of bare SV40 DNA generated the opposite result; about threefold more early RNA was synthesized than late RNA. As reported earlier (4), these results demonstrate a switch from early to late transcription when viral chromatin, prepared during the late phase of lytic infection, is used as template instead of bare DNA. In addition to the major 1.66-kb late RNA band, a minor RNA band 1.72 kb in length was observed (Fig. 2A, lane 2; see also Fig. 3A, lanes 1 and 6, and Fig. 7, lane 1). Transcription was α -amanitin sensitive (2 μ g/ml) (Fig. 2A, lane 3). The endogenous band at 1.83 kb was insensitive to α -amanitin, was labeled in the absence of *Pst*I, and was presumed to be rRNA contained in the HeLa extract (compare Fig. 2A, lanes 3, 4, and 5; see reference 25). Lane 5 of Fig. 2A indicates the low relative amount of RNA produced by the endogenous SV40 chromatin-bound RNA polymerase without added HeLa lysate. We saw no evidence for processing under these conditions. We cannot comment on attenuation of late SV40 transcripts (28), as our purification procedure removes RNA of that size (i.e., 94 bp).

In subsequent experiments, we used either HeLa whole-cell lysates (36) or 0.42 M NaCl extracts of HeLa cell nuclei (14) as transcription systems. The type of extract used in each experiment is indicated in the figure legends. The relative activities of the early and late promoters in SV40 DNA were similar with the two types of extract. With SV40 chromosomes, however, the 0.42 M salt HeLa extract showed an even more marked preference for the late promoter than did the whole-cell extract. In our study, it gave a lower incorporation of precursor into RNA with all types of template. Besides the major and minor late start sites at 325 and 264 bp observed for the HeLa whole-cell extract, virus-specific transcripts 1.82 and 3.03 kb in length corresponding to a 5' end at nucleotide 170 (this start site is observed *in vivo*; see reference 10) were made by this extract (see Fig. 6).

Variation of template concentration. We examined the effects of varying the SV40 chromosome concentration on the transcription pattern. A 30-fold concentration range of chromosomal DNA for NE (2.5 to 75 μ g/ml) and a 10-fold range for gradient-purified chromatin (2 to 20 μ g/ml) were tested. The transcription pattern of SV40 chromosomes was relatively unaffected by concentration changes, showing a preponderance of late RNA at all concentrations tested. Figure 3A shows a 10-fold range for NE (lanes 1 through 5) and a 3-fold range for chromatin (lanes 6 through 7). A three- to fourfold dilution of either NE or gradient-purified chromosomes had no effect on the ratio of late to early RNA observed for undiluted NE. Greater dilutions resulted in a ratio of late to early RNA of 2 or 3 rather than the usual ratio of between 4 and 10.

In contrast to chromosomes, concentration did affect the transcription pattern of bare DNA; the proportion of late RNA increased as DNA concentration increased (Fig. 3B). For DNA concentrations greater than 60 μ g/ml, the overall length of the RNA synthesized was shorter, as though transcription factors supplied by the HeLa lysate were limiting (not shown). With *Eco*RI-cut DNA as template, equal amounts of early and late RNA were synthesized at high template concentrations (see reference 42). As *Eco*RI-cut DNA generated transcripts of unequal length (1.5-kb late and 3.4-kb early RNAs), the shorter late transcript might be preferred under limiting conditions. Although choice of restriction enzyme may affect the results, it does not account

FIG. 2. In vitro-labeled transcripts from *Pst*I-cut SV40 chromosomes and bare DNA templates. (A) NE (prepared at 36 h postinfection) containing SV40 chromosomes was transcribed as described in the text with HeLa whole-cell lysate. Purified 32 P-labeled RNA transcripts were analyzed after glyoxal treatment by agarose gel electrophoresis and autoradiography. Templates used were bare SV40 DNA (lane 1), NE (lane 2), NE plus α -amanitin (2 μ g/ml) before addition of HeLa lysate (lane 3), and NE with no added HeLa lysate (lane 5). Lane 4 consists of HeLa lysate alone with no added template. (B) Densitometer tracings of lanes 1 and 2 are shown; electrophoresis was from right to left. The background of heterogeneous RNA from uncut chromosomes and from transcripts of less than runoff size was subtracted before the relative peak heights of the specific RNA bands were measured.

for the increase in late RNA at high *Pst*I-cut DNA concentrations. The late promoter may be less dependent than the early promoter on factors present in HeLa lysate. In general, when equal amounts of bare or fresh chromosomal DNA were transcribed in parallel reactions, approximately equal amounts of total viral runoff transcripts were made, suggesting comparable template efficiencies. We conclude that increases in template concentration do not account for the increased proportion of late RNA synthesized in vitro from SV40 chromosome compared with bare DNA templates.

The optimum DNA concentration in the transcription reaction for chromosome and DNA templates that gave full-length transcripts was between 10 and 20 μ g/ml. In general, assays were performed with 1 μ l of undiluted NE as the standard for template concentration (diluted 14-fold in the reaction). Equal amounts of bare DNA or purified chromatin were transcribed in parallel. We estimated that NE prepared from infected cells (see above) had an SV40 chromosomal DNA concentration of about 0.2 μ g/ μ l and was at most a two- to threefold dilution of soluble nuclear contents. From this, the in situ SV40 DNA concentration in the nucleus can be estimated to be at least 0.13 μ M.

NE was also prepared at various times postinfection, and a constant volume was transcribed with HeLa whole-cell lysates to determine if the ratio of late to early RNA changed (Fig. 3C). The times chosen were 24, 34, 38, 40, and 46 h after SV40 virus infection. No difference in the ratio of late to early RNA was observed, even though the amount of viral

chromosomal DNA was increased up to 20-fold at 46 h compared with that observed at 24 h (SV40 DNA replication begins at about 15 h). Thus, the increased ratio of late RNA to early RNA is reached before 24 h, perhaps concomitant with the onset of DNA synthesis, and is maintained throughout the late phase, regardless of DNA concentration. At times before 24 h postinfection, insufficient amounts of SV40 chromosomes were present for transcription in vitro. From 24 to 36 h postinfection the amount of RNA synthesized by NE samples was proportionate to the amount of template present. In contrast, the amount of RNA synthesized by NE at 40 to 46 h was no more than that synthesized by an equal volume of NE at 36 h even though the amount of template was at least twofold greater. This suggests that inhibitory factors, probably chromatin-bound virion proteins, were present at late times.

We found no relationship between the amount or kind of template used and the amount of the endogenous 1.83-kb band.

Regulation of SV40 transcription by T Ag. The proportion of SV40 chromosomes associated with T Ag was measured by immunoprecipitation with increasing volumes of anti-T tumor serum or monoclonal antibodies as described above. The conditions were identical to those in which at least 90% of free T Ag and 96% of early replicating SV40 chromosomes were precipitable (comparative SDS-gel analysis of anti-T precipitates and supernatants, L. Tack, unpublished data; 50). The results of one titration are shown in Fig. 4. An

FIG. 3. Effect of various template concentrations and times of infection on transcriptional specificity. Concentrations were determined as described in the text. (A) Various amounts of NE and gradient-purified 70S SV40 chromosomes (CHR) were transcribed with added *Pst*I. Lane 1 was the same as lane 2 but was exposed for less time. Template concentration is shown as micrograms of chromosomal DNA per milliliter. (B) Various amounts of purified *Pst*I-cut SV40 DNA were transcribed. Lanes 1 and 2 are the same as 3 and 4 but were exposed for less time. (C) NE was prepared at various times postinfection as indicated. Except for lane 1, which contained 5 µl of NE, equal volumes (1 µl) of NE were transcribed with added *Pst*I (lanes 2 through 5). Lane 6 (*Pst*I-cut SV40 DNA) had the same template concentration as lane 5. HeLa whole-cell lysate was used. L, Late transcript; E, early transcript; endo, endogenous band.

average of 23% (five NE preparations) of the mature viral chromosomal DNA in NE labeled for 5 to 6 h with [3 H]thymidine was maximally precipitable. Monoclonal 109 purified with protein A-Sepharose consistently precipitated 1.2- to 1.5-fold more labeled chromosomal DNA than did 101; clone 416 was the most reactive of the ascites fluids. Control values averaged 1.5%. NE preparations were stable for about 5 days at 2°C with respect to the amount of T Ag bound to SV40 DNA.

After sucrose gradient purification (see above), an average of 10% of the mature chromosomes labeled for 5 to 6 h and 13 to 14% of the chromosomes labeled for 30 min were immunoprecipitable with anti-T (data not shown). These results are in good agreement with those previously published (8% of total mature sucrose gradient-purified chromosomal DNA) for experiments with a different CV-1 cell line and virus strain (50). The decrease in bound T Ag detected immediately after sucrose gradient centrifugation (23% compared with 10%) was assumed to be due to loss of a weaker-binding subclass of T Ag and the free T Ag pool with which it was in equilibrium.

The effect of T Ag on the in vitro transcription of SV40 chromosomes was investigated either by directly adding tumor anti-T serum to NE and using this as template or by assaying NE from which chromatin-bound and free T Ag had been removed by immunoprecipitation (Fig. 5). Sufficient anti-T to precipitate the maximum number of chromosomes was used (Fig. 4). As much as 50% of the early RNA synthesis could be inhibited by either the addition of anti-T (Fig. 5A, lane 3) or the removal of T Ag complexes (lane 7) compared with synthesis in control samples (lanes 2 and 6) or in the preparation of NE alone (lane 1). In general, little change in the amount of late RNA was observed with either treatment. Quantitation of labeled RNA bands was determined by densitometric scans of the autoradiograph (Fig. 5B; lanes 2 and 3 are shown). After subtraction of the background of heterogeneous RNA (from uncut chromosomes), late transcription in the presence of anti-T (Fig. 5A, lane 2) was 75% of the control (lane 3); early RNA with

FIG. 4. Immunoprecipitation of SV40 chromosomal DNA with anti-T. Separate 10-µl aliquots of NE (labeled with [3 H]thymidine for 6 h) were incubated with increasing amounts of anti-T tumor serum (α T), protein A-Sepharose-purified tumor serum (PA- α T), monoclonal 109, or ascites 416 and fixed *S. aureus* and incubated as described in the text. HBE was added to make 100 µl. Control reactions consisted of protein A-Sepharose purified preimmune serum (Δ) and ascites anti-human Ia fluid ($\circ$). The amount of total labeled chromosomal DNA precipitated was determined. The values for anti-T 109 were obtained separately from the others.

FIG. 5. Effect of anti-T on the transcriptional specificity of SV40 chromosomes. (A) NE (10 μ l) was reacted with 1 μ l of anti-T tumor serum (T) or preimmune serum (C) in a total volume of 20 μ l (adjusted with HBE). A portion (2 μ l) was transcribed directly (lane 3). Fixed *S. aureus* (2 μ l of a 1:1 suspension in HBE) was added. The total immunoprecipitated samples, suspended in 5 μ l of distilled water (ppt.; lanes 4 and 5), and 2.5 μ l of the supernatant fractions (sup.; lanes 6 and 7), containing approximately equivalent amounts of chromatin template, were transcribed along with 1 μ l of untreated NE (lane 1). *Pst*I was added to the reactions just before HeLa whole-cell lysate was added. (B) Densitometer tracings taken from the autoradiographs of lanes 2 and 3.

added anti-T was 45% of the control. For the anti-T supernatant, late RNA was 86% and early RNA was 56% of the control (scans not shown). Only a small amount, <10% of control NE, of virus-specific RNA was synthesized by an equivalent amount of the immunoprecipitated chromatin template (Fig. 5A, lanes 4 and 5). Normal amounts of RNA were synthesized by NE to which just the immunoabsorbent was added.

The same results were observed with tumor serum, protein A-purified tumor serum, ascites monoclonal 416, or protein A-purified monoclonal 109 as the source of anti-T or with protein A-Sepharose as the immunoabsorbent instead of fixed *S. aureus*. In general, we observed little or no reduction in the amount of late RNA produced, whereas early RNA was reduced about 50% after treatment with anti-T. These results suggest that T Ag is associated with about half of the SV40 templates that synthesized early RNA in the assay. T Ag did not appear to be associated with most of the chromosomal templates responsible for the late transcription. The absence of early RNA synthesis in the anti-T precipitate is not surprising, since the association of anti-T with bound T Ag could be expected to block the early promoter. The endogenous 1.83-kb band was the major RNA species labeled in the anti-T precipitate. SV40 chromosomes immunoprecipitated in this way are competent for DNA synthesis (L. Tack, unpublished data).

Transcriptional analysis of gradient-purified mature SV40 chromosomes indicated that early RNA synthesis was consistently increased about twofold compared with the original NE (data not shown). This correlated with a decrease (from 23 to 10%) in the amount of T Ag associated with the viral chromosomal DNA (see above).

Transcriptional activity and T Ag content of salt-extracted SV40 chromosomes. SV40 chromosomes were purified by centrifugation through discontinuous sucrose gradients containing 0.4, 0.8, or 1.5 M NaCl. In one experiment, 15% of the labeled SV40 chromosomal DNA in the NE sample was maximally precipitable with anti-T. After extraction with 0.4 and 0.8 M salt, about 0.8% (after the control value was subtracted) of the labeled chromosomal DNA was reactive with anti-T. Thus, greater than 90% of the T Ag bound to SV40 chromosomes was extracted with 0.4 M NaCl.

The transcriptional properties of the salt-washed chromatin templates were compared with those of unpurified chromosomes (NE) and bare DNA (Fig. 6). After extraction of SV40 chromosomes with 0.4 M NaCl, there was an increase in the amount of early RNA transcription (Fig. 6, lanes 1 through 4). A similar transcription pattern was also observed for SV40 chromosomes extracted with 0.8 M NaCl (Fig. 6, lane 6). Thus, the increase in early transcription after washing with 0.4 or 0.8 M salt correlated with a decrease in T Ag content. Little transcription in the absence of added HeLa extract was observed for either unpurified or 0.4 M salt-extracted chromosomes (Fig. 6, lanes 2 and 4). Extraction of SV40 chromatin with 1.5 M NaCl resulted in a further marked increase in the amount of early RNA synthesized such that it now was substantially greater than the amount of late RNA transcribed (Fig. 6, lane 11). At the same time, the amount of late RNA transcribed decreased compared with the amount transcribed from unpurified chromosomes in NE (Fig. 6, lane 10). Overall, the relative amounts of early and late RNA transcribed from chromatin templates extracted with 1.5 M salt were similar to those synthesized by bare SV40 DNA (Fig. 6, lane 12).

FIG. 6. Transcriptional analysis of salt-washed SV40 chromosomes. Transcription reactions with 0.42 M salt HeLa extracts contained 5 to 10 μ g of DNA template per ml. Gel electrophoresis of 32 P-labeled RNA synthesized from *Pst*I-cut templates: lane 1, NE; lane 2, same as lane 1 except without added HeLa extract; lane 3, SV40 chromosomes sedimented through sucrose containing 0.4 M NaCl; lane 4, same as lane 3 but without added HeLa extract; lane 5, NE extracted with 0.4 M NaCl; lane 6, NE extracted with 0.8 M NaCl; lane 7, same as lane 5 except preincubated with the corresponding top gradient fraction for 10 min in ice (see the text); lane 8, same as lane 6 except preincubated with the corresponding top gradient fraction for 10 min in ice; lane 9, same as lane 7 except the top fraction (20 μ l) was immunoprecipitated with 1 μ l of anti-T tumor serum (α T) before mixing with the chromatin depleted by 0.4 M salt; lane 10, same as lane 1 but a different preparation; lane 11, SV40 chromatin sedimented through sucrose containing 1.5 M NaCl; lane 12, purified precut SV40 DNA.

Salt-depleted SV40 chromosomes were incubated with the top fractions of the corresponding sucrose gradients before *in vitro* transcription as described above. With chromatin treated with 0.4 and 0.8 M NaCl, the effect of this reconstitution was to reduce early transcription back to the level observed with chromatin before salt treatment (Fig. 6, lanes 5 through 8). Late transcription was little affected and remained predominant. Thus, 0.4 and 0.8 M salt extraction of a negative factor for early transcription (presumably T Ag) is reversible. Pretreatment of the top of the 0.4 M salt-sucrose gradient with anti-T before reconstitution with the salt-washed chromatin resulted in the same transcription pattern as the salt-washed chromatin itself (Fig. 6, lanes 5 and 9). In contrast, preincubation of chromatin extracted with 1.5 M NaCl with its corresponding sucrose gradient top resulted in only partial reversal of the transcription pattern (not shown); the amount of early RNA transcribed was reduced compared with that observed with 1.5 M salt-washed chromatin but not to the extent observed with the original chromosomes in NE. Nucleosomal core histones were resistant to 0.4 and 0.8 M salt but were extracted with 1.5 M salt. Thus, chromatin structure, in addition to T Ag, appears to repress early RNA synthesis.

Mixing different SV40 templates: evidence for a *trans* effect on the late promoter. Equal amounts of uncut SV40 chromosomes (NE) and *Pst*I-cut bare SV40 DNA were mixed and

transcribed simultaneously *in vitro* with HeLa whole-cell lysates without added *Pst*I (Fig. 7). In this way, the relative amounts of early and late runoff RNA transcripts synthesized from bare DNA templates could be distinguished from the heterogeneous RNA transcribed from uncut chromatin templates. The effects of virus- or host-specific factors in the NE on bare DNA templates could be analyzed. The amount of late RNA transcribed from *Pst*I-cut SV40 DNA templates in the presence of uncut NE (Fig. 7, lane 2) was markedly enhanced compared with the amount transcribed by cut DNA alone (lane 3). The amount of late RNA transcript was now similar to that made by NE in the presence of *Pst*I (Fig. 7, lane 1). The amount of early RNA transcribed from cut DNA templates in the presence of uncut NE (Fig. 7, lane 2) was usually the same as from cut DNA alone (lane 3) once the higher background of heterogeneous transcripts was subtracted. Thus, the overall result was an enhancement of late RNA synthesis from bare DNA templates caused by the added NE. Removal of T Ag-containing complexes had no effect on the pattern of RNA transcripts synthesized on DNA templates in the presence of NE (data not shown). Mock-infected CV-1 nuclear extracts, when added to bare SV40 DNA under identical conditions, had no effect on the specificity or amount of the viral RNA produced; there was an inhibition of all transcription when more than 2 μ l was added (not shown). These results suggest the presence of a

positive *trans*-acting factor for late transcription in NE containing SV40 chromosomes. Here, this is likely due to transfer of a specific factor to the SV40 DNA rather than some other aspect of chromatin structure such as nucleosome arrangement (see below).

Stimulation of late transcription from SV40 DNA templates with S100 supernatants. High-speed supernatants (S100), prepared from NE and depleted of SV40 chromosomes by centrifugation (see above), were added to bare DNA and transcribed (Fig. 8). Various salt conditions and times post-infection (up to 46 h), including conditions reported for the isolation and identification of the SV40 agnogene product (32), were used to prepare the NE. The same volumes of supernatant (1 to 2 μ l) and incubation conditions as used for mixing NE and bare DNA templates were used. S100 supernatants added to bare DNA gave a 2- to 10-fold increase in late RNA synthesis with no change in early RNA synthesis compared with DNA alone (Fig. 8, lanes 1, 2, and 6 through 8). Some supernatants failed to stimulate the late promoter and instead gave a slight inhibition of early RNA synthesis. Supernatants extracted at times later than 36 h postinfection more often caused an increase in late RNA transcription from bare DNA templates. In general, extractions with isotonic NP-40 buffer but not 0.3 M NaCl-NP-40 were more stimulatory than those with HBE (Fig. 6, compare lanes 7 and 8).

S100 supernatants were pretreated by immunoabsorption with anti-T before addition to bare DNA templates and transcription (Fig. 8, lane 3). T Ag complexes were also removed by immunoprecipitation after reconstitution of DNA with S100 supernatants; the anti-T supernatant was then added to the transcription mix (Fig. 8, lane 4). Neither of these treatments had much effect on the amount of late

and early transcription compared with that observed with the untreated sample (Fig. 8, lane 2). In control experiments in which S100 supernatants from mock-infected CV-1 nuclear extracts were added to DNA templates (Fig. 8, lane 9), no difference in either the amount or the specificity of SV40 transcripts was observed compared with those observed with DNA alone (lane 10). When more than 2 μ l of the mock-infected supernatant was used, all transcription was inhibited (data not shown). When supernatants from infected nuclei were used which still contained some SV40 chromosomes (from 5 to 30% of the original amount), stimulation of the late promoter in *Pst*I-cut DNA templates was always observed (data not shown). These transcripts were not derived from the SV40 chromosomes, since only the short transcripts associated with the fully cut bare DNA templates were made. It is possible that much of the positive *trans*-acting late factor is bound to SV40 chromosomes or other macromolecular complexes and is removed when they are pelleted.

DISCUSSION

NEs containing SV40 chromosomes were isolated between 24 and 46 h after infection of CV-1 cells. As determined by immunoprecipitation analysis, an average of 23% of these viral chromosomes were complexed with T Ag. The chromosomes were transcribed *in vitro* with HeLa cell extracts. Our results indicate that the activity of the early and late SV40 promoters is regulated by at least two different mechanisms at the chromosomal level. One is mediated by a subclass of T Ag bound to SV40 chromosomes which represses early SV40 transcription but has no effect on late transcription. A second level of regulation, involving a tightly bound *trans*-acting chromosomal factor and a stable nucleoprotein structure, favors the late promoter over the early promoter by up to 10-fold. The positive *trans*-acting factor is present in SV40-infected CV-1 extracts but not in uninfected CV1 extracts.

Although the switch to late transcription in lytically infected cells occurs with the onset of SV40 DNA replication, it is not attributable simply to increasing chromosomal template concentration, and it is seen even toward the beginning of the late phase (24 h postinfection) when the amount of SV40 chromatin is low. Furthermore, a fairly constant ratio of late to early RNA is synthesized from chromosomes prepared throughout the late phase of infection. Together, the results of transcription with various template concentrations and washing of chromatin with 1.5 M salt demonstrate that the effects of protein-DNA interactions are much more dramatic than those of template concentration. The *in vitro* transcription pattern of SV40 chromosomes is fairly constant at different template concentrations. Transcription *in vivo* may therefore be less dependent on template concentration than previous observations with bare SV40 DNA templates have suggested (42). Recent *in vitro* transcription studies with hybridization analysis of RNAs synthesized with increasing amounts of bare polyoma DNA template indicate that a constant ratio of early to late RNA was maintained for most of the concentrations tested (31).

As only one-fourth of newly prepared viral chromosomes were associated with T Ag, another form of early RNA repression is necessary to account for the low level of early RNA synthesis from molecules with no T Ag (77% of chromosomes). The increase in early RNA synthesis which occurs after 1.5 M but not 0.8 M salt extraction is consistent with the dissociation of histone octomers releasing the early

FIG. 7. Mixing of SV40 chromosome and DNA templates. Equal amounts of uncut NE, prepared with HBE buffer, and bare *Pst*I-cut SV40 DNA were combined as described in the text and transcribed together with HeLa whole-cell lysate. Lane 1, NE (1 μ l), cut with added *Pst*I; lane 2, uncut NE (1 μ l) plus cut SV40 DNA (0.1 μ g, precut with *Pst*I and purified); lane 3, cut DNA (same as DNA in lane 2). All three samples were analyzed on the same gel.

FIG. 8. Transcription of SV40 DNA templates reconstituted with subcellular fractions. *Pst*I-cut SV40 DNA was preincubated with high-speed supernatant (S100) fractions prepared from NE isolated at 40 h postinfection as described in the text and used as template for transcription with HeLa whole-cell lysate. Lane 1, DNA; lane 2, DNA plus isotonic NP-40-S100 supernatant (NET SUP.); lane 3, same S100 supernatant as lane 2 except that it was immunoprecipitated with anti-T, and the anti-T supernatant (SUP.-T) was added to the DNA; lane 4, same S100 supernatant as in lane 2 but it was first added to the DNA and then immunoprecipitated (the anti-T supernatant was transcribed); lane 5, isotonic NP-40-S100 supernatant alone (no added DNA); lane 6, DNA; lane 7, DNA plus HBE S100 supernatant (HBE SUP.); lane 8, DNA plus isotonic NP-40-S100 supernatant; lane 9, DNA plus mock-infected S100 supernatant; lane 10, DNA. Lanes 1 through 5, 6 through 8, and 9 and 10 were each separate experiments.

promoter in all SV40 chromosomes. Chromatin structure, in addition to T Ag, could thus act to repress early RNA. Random nucleosome arrangement alone can account for some but not all of the asymmetric expression of SV40 chromosomes. Only two discrete DNA sequences separated by about 30 bp serve as the early RNA initiation site (19, 25), whereas multiple sites, spanning over 155 bp, are used as late RNA initiation sites (11). Random nucleosome location could be expected to block binding of RNA polymerase in the early promoter region 70 to 80% of the time, assuming that accessibility of the promoter sequences to polymerase is precluded by the presence of nucleosomes (see reference 49). In contrast, the major late termini are positioned such that one or more of these sites would always be free of nucleosomes protecting 146 bp of DNA spaced an average of 187 bp apart (SV40 nucleosome repeat length; see reference 45). In this way, early transcription could be inhibited about three- to fourfold relative to late transcription.

Further regulation involving phased nucleosome arrangement is indicated by our results. The subpopulation of SV40 chromosomes which contain T Ag accounts for about half of the early RNA synthesized by SV40 chromosomes. Loss of bound T Ag with increasing time resulted in a marked increase (about fourfold) in the amount of early RNA synthesized from these depleted templates (data not shown). Therefore, it appears that the T Ag-containing chromatin fraction (23% of the total), once T Ag is lost, is capable of

transcribing almost as much of the early RNA as is synthesized by bare SV40 DNA templates. Thus, the promoter in the subclass of chromosomes normally associated with T Ag appears to be accessible, presumably as a result of nucleosome phasing, and is capable of altering its transcription pattern depending on the presence of T Ag. Phasing could occur either by sequence-directed or T Ag-mediated (or both) site-specific histone assembly during replication, forming stable, nonrandomly arranged nucleoprotein complexes for some of the SV40 DNA molecules. Treatment with 1.5 M but not with 0.8 M NaCl inhibits the late promoter, suggesting that another possible mechanism for the activation of the late promoter is nucleosome arrangement.

Our analysis of runoff transcripts from a mixture of uncut chromosomal and cut bare DNA templates indicates that a *trans*-acting control element is important for the activation of late RNA synthesis. Factors associated with SV40 chromosomes act in a dominant manner when added to bare DNA templates in a transcription reaction. An S100 fraction of NE, from which SV40 chromosomes were removed, also stimulated late transcription from bare DNA templates. Much of the factor appears to be bound to viral complexes based on comparisons of adding extracts containing chromosomes and high-speed supernatants to bare DNA templates; it perhaps transfers from chromosomes at the elevated temperature of the transcription reaction. It has been previously shown that nucleosomes do not transfer from SV40

chromosomes to bare DNA under low-salt conditions (3). In addition, these extracts do not contain high levels of free histones (SDS-gel analysis of tops of sucrose gradients; L. Tack, unpublished data). Potential *trans*-acting transcription factors could be the SV40-encoded late polypeptides. There is evidence that the agnogene product (1, 30, 32) and the virion proteins VP1, VP2, and VP3 (35) may affect transcription. It is also possible that the *trans*-acting factor that we detected is a permissive host cell product whose synthesis is stimulated by viral infection.

Addition of anti-T to SV40 chromosomes or removal of T Ag by immunoprecipitation with anti-T results in a 50% decrease in early transcription. Thus, for some of the viral chromosomes which contain T Ag, this protein appears not to associate with binding site II. If it did, complete repression of early RNA would be predicted (25, 42). However, the marked increase (about four- to sixfold) in early RNA synthesis after dissociation of chromatin-bound T Ag (Fig. 6) indicates that significant amounts of T Ag are bound to site II in native SV40 chromosomes.

Preferential late RNA synthesis does not appear to be directly mediated by T Ag, since it is insensitive to either the direct addition of anti-T or the removal of T Ag, both free and chromatin bound, by immunoprecipitation. Chromosomes from which most T Ag was removed by treatment with 0.4 or 0.8 M NaCl still contain the active late promoter structure. In addition, the positive late factor, when dissociated from chromatin, does not react with anti-T (Fig. 8). Nevertheless, is there a subclass of T Ag bound to SV40 chromatin which enhances late RNA synthesis? On the basis of our results, this associated T Ag would have to be unreactive with anti-T, perhaps through masking of antigenic sites. In addition, this subclass would be removed by treatment with 1.5 M but not 0.8 M NaCl. Recently, a subpopulation of T Ag tightly bound to SV40 chromatin was described which was resistant to 0.4 M salt and required extraction with greater than 2 M NaCl or guanidine hydrochloride for its complete removal (43, 47). However, this salt-resistant subclass of chromatin-bound T Ag was measured by its reactivity with anti-T (43, 47). Replicating SV40 DNA appeared to be highly enriched in this form of T Ag (47). Since replicating SV40 DNA makes up only 1.2% of the total SV40 DNA and about 75% of the replicating DNA contains T Ag (50), we predict that about 0.9% of long labeled SV40 complexes should be associated with this subclass of T Ag. Experimentally, we find 0.8%. It should be noted that our results were unchanged when chromosomes washed with 0.4 M salt were immunoprecipitated followed by *in vitro* transcription of the anti-T supernatant (data not shown).

Conflicting results concerning the role of T Ag in regulating late transcription have been reported by others. Results of experiments in which plasmids containing the late SV40 promoter were transfected into Cos cells synthesizing T Ag and into control CV-1 cells suggest that T Ag is necessary for activation of the late promoter; T Ag competent for origin-specific DNA binding is a further requirement (6, 33). It is not clear whether the effect of T Ag is direct, involving interaction of T Ag with known binding sites, or indirect, perhaps involving prior binding of T Ag at the origin during replication. In contrast, other results have suggested that T Ag is not needed for late gene expression (11, 24) or that the type of permissive cell line affects the requirement of T Ag for stimulating late transcription (2). If T Ag is involved in regulation of late transcription, it may do so by rearranging chromatin structure (e.g., positioning nucleosomes), thus

creating a binding site for a stimulatory late factor within some chromatin molecules. Subsequent dissociation of T Ag would leave an accessible site for other factors. We cannot rule out that there may be another permissive cell type in which T Ag is active as a positive late transcription factor and that we used one in which it is not. That the host cell can affect whether T Ag functions as a positive factor for late transcription during lytic infection (2) is suggestive of a more indirect role for T Ag in activation of the late promoter. This is consistent with the fact that our CV-1 cell line is extremely efficient in supporting productive infection. If T Ag directly stimulates late transcription and our CV-1 cells are insensitive to this function, then virus production should be significantly altered.

Based on our experimental findings, we envision that a combination of chromatin arrangement and *trans*-acting chromosomal factors would modulate SV40 transcription, along with known DNA sequence elements. There is evidence for specific changes in chromatin structure in this region. New nuclease-sensitive sites, not present in early lytic infection or SV40-transformed cells, appear at the same time as late transcriptional activation (12). These sites are not present in SV40 virion complexes (27), consistent with the transcription pattern of EGTA-disrupted virions resembling that of early lytic RNA synthesis and bare SV40 DNA templates rather than chromosomes from the late phase of infection (8; C. Degoumois and P. Beard, unpublished data). Experiments with *tsA* and *tsB* mutants of SV40 suggest that the nuclease sensitivity pattern is not directly affected by either T Ag or VP1 (44). The possibility that T Ag may create a stable site for other regulatory factors that bind at later times would be compatible with these observations.

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