Quantitation of Transcribing Native Simian Virus 40 Minichromosomes Extracted from CV1 Cells Late in Infection

RAFAEL LLOPIS,[†] FABIENNE PERRIN, FRANÇOIS BELLARD, AND PATRICIO GARIGLIO^{‡*}

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France

Received 15 September 1980/Accepted 17 December 1980

Simian virus 40 transcriptional complexes could be extracted from CV1 cells late in infection and separated from the bulk of inactive viral chromatin. Sucrose gradient sedimentation, cesium sulfate equilibrium density gradient centrifugation, and electron microscopy indicated that the viral transcriptional complexes corresponded to at least 0.5 to 1% of the viral minichromosomes extracted, about 5,000 copies per cell.

Recombinant DNA technology (7, 9) has facilitated the purification and transcription of specific eucaryotic gene sequences in vitro (8, 29, 40, 41). However, the isolation and study of the corresponding native transcribing chromatin is prevented currently by the complexity of eucaryotic genomes (6). At present, only methods for separating heterogeneous populations of active chromatin and inactive chromatin have been developed (5, 19, 23, 35).

In cells infected with simian virus 40 (SV40), progeny viral DNA is associated late in infection with cellular histones (30) to form nucleoprotein complexes having a structure characteristic of eucaryotic chromatin (3, 22, 31, 37). Late in viral development, the small SV40 genome is highly amplified and actively transcribed in an episomal state (17). These findings suggested the isolation and purification of SV40-nucleoprotein complexes as an approach to obtain defined populations of transcriptionally active native gene sequences. We have established previously (16) that the endogenous RNA polymerase of the SV40 complex can be activated under conditions that do not modify the nucleosomic structure of the chromatin and that the template of the isolated native transcriptional complexes is a minichromosome.

There are about 10^6 SV40 minichromosomes per cell late in infection. Provided that a substantial proportion of the minichromosomes are active in transcription, it should be possible to study the mechanisms that control their expression. Available evidence (16, 17, 20) suggests

that only a low percentage (<10%) of the viral DNA is involved in late transcription, but a more precise quantitation is necessary.

This paper presents evidence indicating that a small fraction of the extracted SV40 nucleoproteins are transcriptional complexes; however, these complexes (approximately 0.5 to 1% of the bulk of the viral minichromosome) are far more abundant (2,000 to 5,000 viral genomes per cell) than the well-characterized active genes of highly specialized cells.

MATERIALS AND METHODS

Chemicals and enzymes. [^3H]thymidine (25 Ci/mmol) was obtained from Commissariat à l'Energie Atomique, Saclay, France, and [α - ^{32}P]CTP (300 Ci/mmol; triethylammonium salt) was from The Radiochemical Centre, Amersham, England. Cs_2SO_4 was purchased from Merck. Sarkosyl NL97 was from Ciba-Geigy. Other chemicals were analytical grade. During all manipulations, RNase activity was prevented as previously described (16).

African green monkey kidney cells (CV1) were grown and infected with SV40 strain 777, and the viral chromatin was extracted about 40 h after infection, as described previously (16). The cells were labeled from about 20 to 40 h postinfection with [^3H]thymidine, as indicated in the legends to the figures.

Assay for RNA polymerase activity. The standard reaction mixture (125 μl) contained 5 to 7 μg of viral chromatin DNA, 88 mM Tris-hydrochloride (pH 7.9), 288 mM NaCl, 0.18% Triton X-100, 0.18 mM phenylmethylsulfonyl fluoride, 150 mM $(\text{NH}_4)_2\text{SO}_4$, 2.2 mM MnCl_2 , 1.7 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 1 mM UTP, and 90 μM [α - ^{32}P]CTP (specific activity, usually 300 to 1,200 cpm/pmol, unless stated otherwise). The reaction was carried out at 32°C for the times indicated in the figure legends, and synthesis was stopped by adding EDTA at 0°C to a final concentration of 10 mM. RNA synthesis was determined by measuring the incorporation of [α - ^{32}P]CMP into the acid-precipitable material retained on Whatman

[†] Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

[‡] Present address: Centro de Investigación y Estudios Avanzados, Instituto Politécnico Nacional, Departamento de Genética y Biología Molecular, Apartado Postal 14-740, México 14, D.F.

3MM filters after they were washed 10 times with 3% trichloroacetic acid containing 20 mM sodium pyrophosphate and twice with ethanol. The filters were dried and counted in a toluene-based scintillation mixture.

Sedimentation analysis of the viral chromatin. After incubation for RNA synthesis, EDTA was adjusted to a final concentration of 10 mM and 0.3-ml samples were layered onto 3.7-ml linear 5 to 20% (wt/vol) sucrose gradients containing 10 mM Tris-hydrochloride [pH 7.0], 0.2 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, and various concentrations of NaCl and centrifuged at 2°C as indicated in the figure legends. Fractions were collected from the bottoms of the tubes onto Whatman 3MM filter paper squares, and acid-precipitable counts were determined as described above.

Cesium sulfate equilibrium density gradient analysis. At the end of the incubation period for RNA synthesis, samples were deproteinized (see legend to Fig. 2), and 0.3-ml portions were centrifuged to equilibrium in preformed Cs_2SO_4 gradients (1 ml each of Cs_2SO_4 solutions having the following densities: 1.6223, 1.5206, and 1.3626 g/ml in 10 mM phosphate buffer [pH 7.1]–1 mM EDTA) for 40 h at 30,000 rpm and 20°C in a Beckman SW60 rotor. Fractions were analyzed for acid-insoluble radioactivity as described above and for density by measuring the refractive index.

Electron microscopy. After Cs_2SO_4 equilibrium density gradient centrifugation (see legend to Fig. 5), samples were spread according to a modification of the method of Davis et al. (11). Briefly, 50 μl of the spreading solution containing 2.5 μg of cytochrome *c* (type VI; Sigma Chemical Co.), 0.015 μg of DNA, 30% deionized formamide, 10 mM EDTA, and 100 mM Tris-hydrochloride (pH 8.5) was spread onto redistilled water and mounted for electron microscopy as described previously (16).

RESULTS

Amount of SV40 transcriptional complex as determined by sucrose density gradient fractionation. As a first attempt to determine the proportion of viral DNA involved in transcription, the cells were labeled with [^3H]thymidine, viral complexes were isolated, and RNA synthesis was carried out in the presence of [^{32}P]CTP as described above. Figure 1a shows that in 5 to 20% neutral sucrose gradients containing 1.5 M NaCl, the bulk of the ^3H -labeled viral chromatin sedimented as a sharp peak at about 21S and was separated from most of the ^{32}P -labeled viral transcriptional complexes. (Gariglio et al. [16] present evidence that the RNA labeled in vitro indicates the location of transcriptionally active SV40 complexes.) After 2 h of in vitro RNA synthesis, the tail of fast-sedimenting [^3H]DNA cosedimented with the bulk of the viral transcriptional complexes (Fig. 1a, fractions 25 through 38, and Fig. 1b [scale amplified 200-fold]) and represented about 1.2%

FIG. 1. Sedimentation analysis of viral transcriptional complexes in sucrose gradients containing 1.5 M NaCl. Monolayer cultures of cells were infected and labeled with [^3H]thymidine (10 $\mu\text{Ci}/\text{ml}$) from 20 to 40 h after infection; the viral chromatin was extracted, and RNA was synthesized with [$\alpha\text{-}^{32}\text{P}$]CTP (300 cpm/pmol) as described in the text. The reaction was carried out for 120 min at 32°C in the absence (a) or in the presence (b) of 10 mM EDTA. At the end of the incubation, the EDTA concentration was adjusted to 10 mM, and both samples (0.3 ml) were sedimented for 60 min at 57,000 rpm in an SW60 rotor in sucrose gradients containing 1.5 M NaCl, as described in the text. Fractions were collected on Whatman 3MM filters, washed, and dried, and radioactivity was measured as described in the text. Symbols: ●—●, [^{32}P]RNA; ▲—▲ and △—△, in vivo [^3H]DNA; △—△—△, in vivo [^3H]DNA from (a), with the scale between fractions 25 and 38 amplified 200-fold; ▲—▲—▲, in vivo [^3H]DNA from (b), with the scale amplified 200-fold.

of all SV40 DNA molecules. The control experiment (Fig. 1b; synthesis blocked by excess EDTA) indicated that only 0.7% of the bulk viral DNA molecules were present in an equivalent fast-sedimenting region of the sucrose gradient. Similar results (data not shown) have been obtained with complexes sedimented in sucrose gradients containing Sarkosyl or with complexes extracted by the Sarkosyl method (17). This result suggests that about 0.5% of the total SV40 DNA can be shifted, together with the viral transcriptional complexes, toward a heavier region of the sucrose gradient after in vitro RNA synthesis.

Proportion of transcriptional complexes determined by cesium sulfate equilibrium density fractionation. Our previous work on viral transcriptional complexes extracted by the Sarkosyl method (17, 18) indicated that a large proportion of the RNA product synthesized *in vitro* bands heterogeneously at positions intermediate between the bulk of SV40 DNA (peak at $\rho = 1.46 \text{ g/cm}^3$) and free viral RNA ($\rho = 1.70 \text{ g/cm}^3$) and, therefore, remains associated with its DNA template after sodium dodecyl sulfate treatment and Cs_2SO_4 equilibrium density gradient centrifugation. Similar results (data not shown) have been obtained for viral chromatin prepared and incubated for RNA synthesis under the conditions used here, which do not alter the structure of the SV40 minichromosomes (16; see above). After deproteinization, the amount of labeled RNA banding at hybrid densities increases with time of RNA synthesis, which is linear for at least 2 h at 32°C (16), suggesting that viral transcriptional complexes can be shifted to hybrid densities in Cs_2SO_4 gradients and thus can be separated from the bulk of the viral DNA. We employed this approach to calculate the amount of SV40 DNA engaged in viral transcription. The cells were labeled with $[^3\text{H}]$ thymidine, the minichromosomes were isolated, $[^{32}\text{P}]$ RNA was synthesized, and the samples were centrifuged to equilibrium in Cs_2SO_4 gradients (see above). As Fig. 2a shows, the ^{32}P -labeled material was separated from the bulk of SV40 DNA banding as a sharp peak at a density of about 1.48 g/cm^3 . After 30 and 120 min of RNA synthesis, the majority of the ^{32}P -labeled RNA banded heterogeneously between the bulk of the $[^3\text{H}]$ DNA and a position near that of free RNA (density = 1.71 g/cm^3). Control samples (EDTA added to samples at zero time of incubation or samples treated with RNase) were centrifuged in parallel Cs_2SO_4 gradients (Fig. 2b). Since the DNA was labeled at a very high specific activity, the bulk of the viral DNA and the region containing DNA-RNA hybrids could not be plotted by using the same scale. Figure 3 shows that when the scale of $[^3\text{H}]$ DNA was expanded 1,000-fold in the region containing most of the DNA-RNA hybrid molecules, the banding pattern of a fraction of the $[^3\text{H}]$ DNA followed closely the pattern of the $[^{32}\text{P}]$ RNA (Fig. 2). We found that approximately 0.7 and 0.9% of the viral DNA were in the hybrid density region of the gradient (Fig. 3, fractions 20 to 32) for samples incubated in the RNA polymerase reaction for 30 and 120 min, respectively. In control experiments, with RNA synthesis for 120 min in the presence of EDTA, α -amanitin, and RNase, only 0.2, 0.2, and 0.03%, respectively, of

FIG. 2. Cs_2SO_4 equilibrium density analysis of deproteinized viral transcriptional complexes after *in vitro* transcription. Viral chromatin was labeled with $[^3\text{H}]$ thymidine ($40 \mu\text{Ci/ml}$) and extracted as described in the legend to Fig. 1. (a) RNA synthesis with $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ (300 cpm/pmol) for 30 min (■—■) and 120 min (●—●). (b) Synthesis for 120 min (see above) in the presence of 10 mM EDTA (●—●) or 3 μg of RNase per ml (○—○). Synthesis was also carried out for 120 min in the presence of 0.2 μg of α -amanitin per ml (data not shown [see Fig. 3]). At the end of the incubation, the reactions were stopped with 10 mM EDTA–2 mM CTP, and the samples were deproteinized by adding 0.1% (final concentration) sodium dodecyl sulfate and then incubated for 10 min at 32°C . Portions (0.3 ml) were centrifuged to equilibrium in Cs_2SO_4 density gradients. The radioactivities of the fractions were measured as described in the text and in the legend to Fig. 1. Symbols: ▲—▲, *in vivo* $[^3\text{H}]$ DNA; ○—○, density, as determined from the refractive index (20°C).

the DNA banded in the hybrid region of the gradient. These results suggest that about 0.5 to 1% of all SV40 DNA molecules extracted were viral transcriptional complexes with RNA chains of variable length. Competition experiments were performed to evaluate artifactual binding of free viral RNA to SV40 DNA. The rationale of this approach was as follows: if some of the SV40 DNA labeled *in vivo* was shifted because artifactual DNA-RNA hybrids were formed, it seems likely that the amount of ^3H -labeled SV40 DNA displaced would decrease when an excess of exogenous SV40 DNA was added. No difference was found for the $[^3\text{H}]$ DNA density patterns of samples incubated for 60 min (RNA polymerase reaction) in the absence and in the presence of a threefold excess of viral $[^{14}\text{C}]$ DNA added at zero time of reaction (data not shown). The same amount of ^3H -labeled SV40 DNA

FIG. 3. Quantitation of viral DNA banding at DNA-RNA hybrid density in Cs_2SO_4 equilibrium density gradients. In the experiment shown in Fig. 2, the scale of the ^3H -labeled SV40 DNA was amplified 1,000-fold for fractions 20 to 32. The arrows indicate the positions of DNA and RNA in the gradient. Symbols: $\blacktriangle$, [^3H]DNA after 120 min of RNA synthesis; $\circ$, [^3H]DNA after 30 min of RNA synthesis (see Fig. 2a); $\triangle$, [^3H]DNA after 120 min of RNA synthesis in the presence of 10 mM EDTA; $\blacksquare$, [^3H]DNA after 120 min of RNA synthesis in the presence of 3 μg of RNase per ml; $\bullet$, [^3H]DNA after 120 min of RNA synthesis in the presence of 0.2 μg of α -amanitin per ml (see Fig. 2b).

(0.5% of the total) was shifted in both cases, suggesting that the increase in density of this minor fraction of the DNA molecules was characteristic of the transcriptionally active viral templates and not due to nonspecific binding of free RNA to ^3H -labeled SV40 DNA.

Our previous work (16) provided a way of obtaining sucrose gradient fractions enriched in SV40 transcriptional complexes after elongation of their RNA chains *in vitro*. The amounts of viral transcriptional templates present in the different regions of such sucrose gradients were also estimated. Samples were prepared as described in the legend to Fig. 1 and were sedimented in a preparative 5 to 20% neutral sucrose gradient containing 0.4 M NaCl (Fig. 4a). Under these conditions most of the transcriptionally active and inactive chromatins are separated from each other (16). The fractions of the preparative sucrose gradient were pooled (Fig. 4a, pools I through III) and then centrifuged in Cs_2SO_4 density gradients (Fig. 4b through d). Pools I, II, and III contained 75, 20, and 3% of the total ^3H -labeled DNA, respectively. The material sedimenting faster (pools II and III) than

FIG. 4. Quantitation of viral transcriptional complexes after *in vitro* RNA synthesis and fractionation in a sucrose gradient containing 0.4 M NaCl. Infected CV1 cells were labeled as described in the legend to Fig. 2, minichromosomes were extracted, and RNA was synthesized with [α - ^{32}P]CTP (1,200 cpm/pmol) for 90 min at 32°C as described in the text. At the end of the incubation, EDTA was adjusted to 10 mM (final concentration), and each sample (0.3 ml) was sedimented for 45 min at 57,000 rpm (SW60 rotor) in a sucrose gradient containing 0.4 M NaCl (see text). (a) Fractions (300 μl) were collected from the top of the gradient, portions (30 μl) were spotted onto DE/81 filters, and radioactivity was determined as previously described (16); fractions were pooled as indicated on the figure and deproteinized (see legend to Fig. 2). Portions (0.3 ml) of pool I (fractions 5 to 7) (b), pool II (fractions 8 to 10) (c), and pool III (fractions 11 to 13) (d) were centrifuged to equilibrium in Cs_2SO_4 density gradients. The radioactivities of the collected fractions were measured as described in the text and in the legend to Fig. 1. Symbols: $\blacktriangle$, *in vivo* [^3H]DNA; $\bullet$, [^{32}P]RNA; $\circ$, density (in grams per cubic centimeter).

the bulk of the viral chromatin (pool I) contained most of the DNA-RNA hybrid molecules (fractions 19 to 24 in Cs_2SO_4 density gradients). We found that approximately 0.03% (250 cpm), 1.0% (2,200 cpm), and 10% (3,100 cpm) of the ^3H DNA present in pools I, II, and III, respectively, sedimented in the hybrid region. Therefore, this experiment indicated that after *in vitro* transcription, the ^3H DNA shifted to a fast-sedimenting region of the sucrose gradient and, presenting the density of a DNA-RNA hybrid, was about 0.5% ($0.03 + 0.2 + 0.3\%$) of the total SV40 DNA extracted from the infected cells. Compared with the material banded in Cs_2SO_4 directly after synthesis (Fig. 2 and 3), there was a decrease in the average density of the hybrids and an increase in the amount of ^{32}P -labeled material banding at the density of free RNA. Such striking differences were also found when the fractionation was performed in sucrose gradients containing 0.2% Sarkosyl or 1.5 M NaCl (data not shown). No significant improvement was obtained when the gradients were collected either from the bottom or from the top, where most of the cellular RNase remained. Taking into account that all glassware was freed of RNase either by heating or by treating with diethylpyrocarbonate and that most of the solutions were autoclaved before use, it seems unlikely that these differences could be explained by the action of contaminating RNases. On the other hand, when the sizes of the RNAs made *in vitro* as described in the legend to Fig. 4 were determined, either immediately after synthesis or after purification of transcriptional complexes (as in Fig. 4a), no significant differences were found (data not shown). Taken together, these results suggest that the different patterns observed in the material banded in Cs_2SO_4 either directly after synthesis (Fig. 2 and 3) or after purification in sucrose gradients (Fig. 4) were not due to a drastic reduction in the size of the RNA bound to the DNA; rather, they can be explained by the release of long RNA chains at any step during the sucrose gradient procedure.

Amount of viral transcriptional complex as determined by electron microscopy. As Fig. 2 to 4 show, a large proportion of the SV40 RNA product remained associated with the viral DNA template and banded heterogeneously at positions intermediate between the positions of the bulk of SV40 DNA and those of free RNA in Cs_2SO_4 equilibrium density gradients. We attempted to correlate these results with electron microscopic determinations of the percentages of viral transcriptional complexes and determinations of the average number and length of the complex-associated RNA chains.

Samples and controls prepared as described in the legend to Fig. 3 were centrifuged to equilibrium in Cs_2SO_4 . For electron microscopic studies, fractions from different regions of these gradients were pooled, dialyzed, incubated in the absence or presence of RNase, diluted, and spread for electron microscopy as described in the legend to Fig. 5. In all cases, pool I (Fig. 3, fractions 29 to 31) contained at least 50% of the SV40 DNA as transcribing molecules that contained long (genome size or longer) RNA chains (Fig. 5a). On some viral transcriptional complexes we observed two RNA chains, and a few had three RNA chains, as shown in Fig. 5b. In pool II (Fig. 3, fractions 22 to 24), at least 50% of the SV40 DNA was also associated with RNA molecules (data not shown). We did not observe such hybrids in pool III (Fig. 2, fractions 14 to 16) or when the samples were treated with RNase before spreading, strongly suggesting that these molecules were viral transcriptional complexes.

The fact that at least 50% of the DNA molecules found in pools I and II (about 1% of the total viral DNA) contained attached RNA chains after spreading in formamide (11) confirmed that 0.5 to 1% of the extracted SV40 chromatin was transcriptionally active *in vitro*.

DISCUSSION

SV40 minichromosomes may provide a model system for the study of transcriptionally active chromatin. This small, well-defined viral genome is highly amplified within cells and must use at least some of the nuclear components that regulate normal chromosomal activity. We have reported previously (17) that SV40 DNA is actively transcribed *in vivo* in an episomal state by RNA polymerase B. More recently (16), we used methods that increase the proportion of transcriptionally active native SV40 complexes to demonstrate that they are in the form of minichromosomes. These data provided direct evidence that nucleosome-like structures are associated with nonribosomal transcriptional units containing active RNA polymerase B.

In the present study, we estimated the proportion of SV40 DNA molecules involved in *in vitro* transcription during late lytic infections. Data obtained by using different extraction and separation methods indicated that 0.5 to 1.0% of the viral DNA is engaged in transcription. Taking into account that late in infection 6×10^6 infected cells yield about 20 μg of SV40 DNA, the data above suggest that there are about 5,000 genomes per cell which are active in transcription.

Our previous results (16, 18) indicated that RNA chains initiated *in vivo* can be elongated

FIG. 5. *Electron microscopic visualization of SV40 transcriptional complexes. After Cs_2SO_4 equilibrium density gradient centrifugation, the fractions were pooled (see text), dialyzed for 15 min at 2°C against 10 mM Tris-hydrochloride (pH 7.5)–200 mM NaCl, and incubated in the absence or presence of 0.2 μg of RNase per ml for 15 min at 20°C ; the samples were diluted and spread by the method of Davis et al. (11), as described in the text. (a) Transcribing SV40 complex from pool I (see text and Fig. 3) after 60 min of RNA synthesis and Cs_2SO_4 equilibrium density gradient fractionation. (b) Viral transcriptional complex from pool I (Fig. 3) with three RNA chains. In the line drawings, DNA is the thicker line, and RNA is the thinner line. Bar = 0.1 μm .*

in vitro and remain associated with the template during synthesis and fractionation in sucrose or Cs_2SO_4 . In the present study we show that highly labeled [^3H]DNA is shifted to the hybrid DNA-RNA density only when RNA synthesis is carried out in the absence of transcriptional inhibitors (Fig. 3). In addition, when the reaction mixtures contained a threefold excess of supercoiled SV40 DNA, the pattern in Cs_2SO_4 gradients of the shifted [^3H]DNA (0.5 to 1% of the viral DNA) was not modified, strongly suggesting that the RNA synthesized in vitro was bound specifically to the [^3H]DNA templates (data not shown).

It has been reported (20) that 80% of the viral transcriptional complexes remain bound to the cellular chromatin after infected cells are extracted with Triton X-100 and NaCl (21). It could be argued that our viral chromatin preparation was not representative of the complexes that generate SV40 RNA in vivo. We think that this potential criticism is not valid since the method of extraction used in this study solubi-

lized 60 to 70% of the viral transcriptional complexes (16), as compared with the Sarkosyl method (17), which is known to release at least 90% of the SV40 transcriptional complexes that generate viral RNA in intact nuclei (4, 17, 34).

On the average, our viral transcriptional complexes had one or two RNA chains. The Sarkosyl-extracted SV40 transcriptional complexes (17) purified by Aloni et al. had several RNA chains per complex (1). Using CV1 cells, we did not observe such complexes, and we do not believe that this was due to a failure to extract transcriptional complexes containing several attached RNA chains. In our hands, transcriptional complexes extracted with Sarkosyl (17) or Triton X-100–NaCl (16) have the same sedimentation patterns after short incubations in vitro for RNA synthesis in the presence of Sarkosyl (data not shown). In addition, titration with [^3H]amanitin of RNA polymerases which transcribe SV40 DNA molecules indicated that there are, on the average, about two RNA polymerase B molecules per SV40 transcriptional complex

(16). On the other hand, although our electron microscopy data support the results of the gradient analysis by showing the presence of DNA-RNA complexes, they are not fully convincing with regard to the actual amount of RNA associated with DNA. It is quite conceivable that some RNA chains are lost by shearing or degradation during collection of the Cs_2SO_4 gradients, dialysis, and preparation of the samples for electron microscopy. The fact that pool I (Fig. 3) contains 50% of the DNA molecules without any evidence for associated RNA chains strongly suggests that this is the case, considering the very high density of the fractions pooled (Fig. 3, fractions 29 to 31). In any case, if it is unlikely that we systemically failed to extract most of the transcriptional complexes containing an elevated number of RNA chains, it is quite possible that we were incapable of visualizing them, even though different electron microscopy technologies were used (data not shown). The fact that up to 10 RNA chains were found by Aloni and co-workers (1, 26) with one population of transcriptional complexes could explain the very high density reached by some $[^3\text{H}]\text{DNA}$ after 2 h of *in vitro* incubation for RNA synthesis (Fig. 3).

At present, we have not completely excluded the possibility that the very high density reached by some molecules of SV40 DNA is an artifact. However, the results obtained with pancreatic RNase, which completely avoid any shift of tritiated DNA toward higher densities, indicate that even if this is the case, only molecules involved in transcription are actually shifted.

We do not know why only 1% of the SV40 DNA is involved in transcription late in lytic infection. It is very unlikely that nuclear RNA polymerase B is the limiting factor in the formation of viral transcriptional complexes. Estimates (10; Bellard, unpublished data) suggest that only about 10% of the RNA polymerase B in an adult oviduct cell has initiated transcription. In SV40-infected cells, there is a major increase in RNA polymerase B activity early in infection (Gariglio, unpublished data), suggesting that there might be a large excess of polymerase during viral development. In fact, we have found that most of the RNA polymerase B molecules present in the Triton X-100-NaCl extracts from SV40-infected cells are free molecules, which are not associated with the fast-sedimenting 55S minichromosomes (16). It is also unlikely that the DNA acting as the template in SV40 transcriptional complexes is different from the bulk of viral DNA. We have analyzed the sensitivity to restriction endonuclease digestion of the DNA present in bulk mini-

chromosomes and viral transcriptional complexes and have been unable to detect any significant differences in the gel electrophoresis patterns of the resulting DNA fragments (P. Gariglio and J. L. Mandel, unpublished data).

Recently, new methods have been developed for isolating intracellular forms of SV40 nucleoprotein complexes late in infection, which yield major viral components sedimenting at about 70S and 180 to 200S (2, 13, 15, 24). Only the slowly sedimenting material (10 to 30% of the total SV40 DNA solubilized by such extraction procedures) is active in synthesizing SV40-specific RNA *in vitro* and *in vivo* (13, 27, 28). Thus, packaging of minichromosomes could regulate the concentration of SV40 templates transcriptionally active *in vivo*. On the other hand, digestion of SV40 minichromosomes with restriction endonucleases has revealed the existence of a sensitive, specific region of about 400 base pairs (37-39), which includes the origin of replication (12), the binding sites for T antigen (36), and the postulated major late SV40 promoter (1, 14, 32). The existence of this exposed, nucleosome-free region in SV40 minichromosomes has been confirmed recently by electron microscopy (25, 33). These findings suggest that the initial step in viral transcription could also be regulated by the structure of a fraction of the minichromosomes.

Using two completely different and complementary approaches, Laub et al. (26, 27) and Llopis and Stark (28) have obtained results that can be explained by the presence of two different kinds of late transcriptional complexes late in infection. These two kinds of complexes may be described by the following working model. There is a minority class (20% of the total), which is free of VP2 or VP3, active in low concentrations of ammonium sulfate *in vitro*, and responsible for late transcription *in vivo*, and there is a majority class (80% of the total), which has VP2 or VP3 bound a few hundred nucleotides downstream from the sites of initiation of late transcription and is inactive at low salt concentrations both *in vitro* and *in vivo* but capable of being activated by high salt concentrations or by Sarkosyl. The Cs_2SO_4 equilibrium test proposed here to determine the amount of viral transcriptional complexes can distinguish between these two postulated populations. When highly labeled viral chromatin is isolated and then centrifuged to equilibrium in Cs_2SO_4 directly or after incubation for RNA synthesis in the presence of EDTA or α -amanitin, only 0.1 to 0.2% of the total SV40 DNA is found at the DNA-RNA hybrid density (Fig. 3). After 1 to 2 h of RNA synthesis at high concentrations of ammonium sulfate or in the presence of Sarko-

syl, up to 1% of the total SV40 DNA is shifted to the same region (Fig. 3).

In conclusion, we present biochemical and electron microscopic evidence indicating that a small fraction (about 1%) of all SV40 nucleoproteins solubilized late in infection initiate RNA synthesis *in vivo*. In addition, our results are consistent with a model in which 80% of all SV40 transcriptional complexes are attenuated *in vivo* (26–28) and only 20% are actively involved in synthesizing late RNA.

ACKNOWLEDGMENTS

We thank Pierre Chambon, George R. Stark, Maria Bellard, and Barry Davison for critically reading this paper. We are grateful to Edith Badzinski and Nancy Stouffer for typing the manuscript and to Christiane Werlé for figure drawings. We acknowledge the excellent technical assistance of K. Dott, D. Mertz, and B. Boulay.

This work was supported by grants CRL 76.5.099.1 and ASR 1/004 from the Institut National de la Santé et de la Recherche Médicale to P. Chambon, by grant 2117 from the Centre National de la Recherche Scientifique to P. Chambon, and by a grant from the Fondation pour la Recherche Médicale Française to P. Chambon.

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