Recently Replicated Simian Virus 40 DNA Is a Preferential Template for Transcription and Replication

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The template activities for the processes of replication and transcription were compared for recently replicated ("new") and uniformly labeled ("old") simian virus 40 (SV40) DNA in infected monkey cells (line TC7). New SV40 DNA (pulse-labeled for 1 h) served as a template for a second round of replication with a relatively high probability (8% of the DNA replicated per h) for a period of 5 h, after which time its template activity rapidly decreased by severalfold. Old SV40 DNA (labeled for 24 h) functioned as a template for replication with a constant probability (1.8% of the DNA replicated per h) for at least 12 h. The proportion of RNA polymerase with nonreplicated and with recently replicated (bromodeoxyuridine-substituted) viral DNA was determined by an assay that used the Triton-soluble SV40 transcription complex. The proportion of RNA polymerase associated with nonreplicated SV40 DNA decreased very slowly (to 50% in 6 h), strongly suggesting that replicating viral genomes are not required as templates for the initiation of late transcription. This hypothesis was supported by the finding that the RNA synthesized *in vitro* was associated with covalently closed circular SV40 DNA. Furthermore, after 9 h in bromodeoxyuridine, the recently replicated viral DNA had nearly three times more RNA polymerase per unit of DNA than did the nonreplicated DNA. We thus conclude that recently replicated SV40 DNA is utilized preferentially as a template for transcription and for replication.

Although it has been recognized for many years that changes occur in the transcriptional program of bacterial and animal viruses after the onset of DNA replication, little is known concerning the mechanism underlying the relationship between replication and the transcriptional program. In approaching this problem, we have devised a Triton extraction method for the isolation of the simian virus 40 (SV40) transcription complex (VTC) from infected monkey cells (11). The VTC is a 55 to 60S complex (11) containing SV40 DNA, protein, and active RNA polymerase. Although essentially all of the Triton-extractable SV40 and polyoma DNA is obtained as a nucleoprotein complex sedimenting at 55 to 60S (11, 14, 16) and possessing a chromatin-like structure (10, 15), only a very small fraction of the viral DNA (~0.3%) is associated with RNA polymerase (our unpublished data). It is nevertheless possible to study the properties of the template by using the VTC to synthesize [³H]RNA *in vitro*, thereby selectively tagging only those DNA molecules engaged in transcription (2, 4, 12).

A plausible model whereby viral replication alters the transcriptional program stipulates that replicating genomes are transcribed differ-

ently than prereplicative templates. This specific hypothesis was negated for SV40 by the recent report of Birkenmeier et al. (2), which strongly suggested that replicating SV40 genomes are not necessary templates for obtaining the altered ("late") pattern of transcription. We have taken a more general approach to this problem by examining whether there is any heterogeneity in the population of intracellular SV40 chromatin with respect to template activity for replication and transcription. Previous studies suggested that there is some biochemical heterogeneity in the intracellular pool of viral chromatin. For example, the SV40 transcription complex was found to require a higher salt concentration for its solubilization during Triton extraction than that required for the bulk of the viral chromatin (11). The polyoma VTC was also less readily solubilized than the bulk of the viral chromatin (34). In the case of replication, Roman and Dulbecco observed that a large but limited fraction (60%) of the pulse-labeled polyoma DNA served as a template for a second round of replication (31). The failure of the remaining viral DNA to act as a template was not explained, but it was probably not due to the DNA having been packaged into mature virions. The present report

demonstrates that intracellular SV40 chromatin is indeed functionally heterogeneous. This conclusion is based on the finding that recently replicated SV40 DNA is utilized preferentially as a template for transcription and for replication.

MATERIALS AND METHODS

Virus and cell culture. The TC7 subline of CV-1 monkey cells (30) was used for the propagation of small-plaque SV40 virus stocks and for all of the experiments. Cells were grown at 37°C on either 60- or 100-mm plastic petri dishes in the modified Eagle medium of Dulbecco supplemented with 10% fetal calf serum. A humidified atmosphere containing 10% CO₂ was used. Subconfluent cells were infected at an input multiplicity of 5 to 10 PFU per cell for the experiments and less than 0.01 PFU per cell for virus propagation.

Labeling and extraction of viral DNA. SV40-infected cells were labeled with [*methyl*-³H]thymidine (20 Ci/mmol) for designated periods of time. To assess the ability of the [³H]DNA to function as a template for a subsequent round of replication, the cells were "chased" in the presence of 5-bromodeoxyuridine (BUdR) and 5-fluorodeoxyuridine (FUdR) for various lengths of time. The labeled cultures were washed twice with warm Tris-buffered saline, then incubated in prewarmed fresh medium at 37°C for 0.5 to 2 h, as indicated. BUdR (1.3×10^{-5} M) and FUdR (6×10^{-5} M) were then added, and incubation was continued until the plates were Triton extracted (in 0.4 M NaCl). All steps in the extraction and concentration of the viral DNA were performed at 0 to 4°C as described previously (11). Recovery of SV40 DNA by this procedure was at least 90% of that obtained by the Hirt method (36; our unpublished data). The viral DNA ("chromatin") present in the Triton supernatant was concentrated by centrifugation into a 50% sucrose cushion (11). Sucrose was dissolved in a solution containing Triton (0.25%), 10 mM Tris (pH 7.9), 0.2 mM EDTA, 0.5 mM dithiothreitol, and either no NaCl (solution designated TTED), or 0.4 M NaCl (TTEDS).

Synthesis of [³H]RNA by endogenous RNA polymerase in the VTC. Concentrated VTC (11) was used to synthesize [³H]RNA as described previously (34). Reaction mixtures containing 0.4% Sarkosyl (wt/vol) and 6.7 μ M [³H]UTP (100 μ Ci/ml) were incubated for 10 min at 22°C. This low concentration of UTP and short reaction time ensured that short RNA chains would be synthesized (4).

Analysis of buoyant density in CsCl. (i) **Density of SV40 DNA.** After Triton extraction of ³H-labeled SV40 DNA, supernatants were adjusted to a density of 1.75 g/cm³ by the addition of saturated CsCl and buffer (10 mM Tris, pH 7.4; 10 mM EDTA) in a final volume of 5 ml. Density of the CsCl solution was determined from its refractive index. Paraffin oil was layered over the CsCl, and the samples were centrifuged to equilibrium (≥ 64 h) at 30,000 rpm (21°C) in an SW50.1 Spinco rotor. Fractions were collected from the bottom of each cellulose nitrate tube directly onto Whatman 3MM paper squares and analyzed for acid-insoluble radioactivity. Density is depicted as increasing from right to left in all CsCl density gradients.

(ii) **Density of SV40 DNA-RNA hybrid.** After the addition of marker SV40 [¹⁴C]DNA, the samples were adjusted to the designated density of CsCl, centrifuged to equilibrium, collected, and analyzed as detailed above for SV40 [³H]DNA.

Sedimentation analysis of SV40 chromatin in sucrose gradients. Samples (0.2 ml) of Triton supernatant containing ¹⁴C- and ³H-labeled SV40 chromatin prepared the same day were layered onto 4.8 ml of 5 to 20% sucrose gradients (in TTEDS). Centrifugation was for 90 min at 45,000 rpm (4°C) in an SW50.1 rotor. Fractions were collected from the bottom of each tube directly onto Whatman 3MM paper squares and analyzed for acid-insoluble radioactivity. Sedimentation is depicted from right to left.

Centrifugation through Sephadex G-100. This procedure was used to separate [³H]RNA-DNA hybrid from [³H]UTP rapidly and with little dilution. The protocol (4) was modified in that Sephadex G-100 instead of G-25 was used for the present experiments. Recovery of [³H]RNA was at least 90% of the acid-insoluble material.

Preparation of SV40 DNA form I. SV40 DNA, labeled for 24 h with [*methyl*-¹⁴C]thymidine (0.5 μ Ci/ml; 40 mCi/mmol) was extracted from infected TC7 cells at approximately 40 h postinfection (p.i.) by the method of Hirt (18). Supercoiled SV40 DNA (form I) was separated from other forms of viral DNA and contaminating cell DNA by equilibrium centrifugation in CsCl containing ethidium bromide, and the dye was removed by three extractions with isopropanol (27).

EcoRI endonuclease digestion of SV40 DNA form I. SV40 [¹⁴C]DNA form I, prepared as described above, was adjusted to 100 mM Tris (pH 7.4), 10 mM MgCl₂, and 50 mM NaCl. The DNA (2 μ g in 0.2 ml) was incubated with approximately 8 U of *EcoRI* endonuclease for 1 h at 37°C. *EcoRI* cuts SV40 DNA at one site under these conditions (24, 25). Digestion was stopped by the addition of 0.1% sodium dodecyl sulfate.

Chemicals and enzymes. BUdR and FUdR were purchased from Calbiochem. *EcoRI* endonuclease was kindly provided by Donald Helinski. Other special reagents were obtained from sources indicated previously (13).

RESULTS

New and old SV40 DNA as templates for replication. The activity of SV40 DNA as a template for subsequent rounds of replication was assessed by first labeling DNA molecules with [³H]thymidine, then removing the radiolabel and chasing in the presence of fresh medium to which BUdR was subsequently added. Since polyoma, and by inference SV40, DNA replicates semi-conservatively (17), the [³H]DNA that functions as a template for another round of replication will become heavy-light (HL-DNA), with one light strand containing [³H]-thymidine and one heavy strand containing BUdR. The cells were labeled and chased in fresh medium for periods of time sufficient to

ensure that essentially all of the labeled molecules had been completely replicated before the addition of BUdR. This is based on the observation that SV40 DNA molecules require about 15 min to replicate (23, 26).

When pulse-labeled for 2 h, 17% of this "new" SV40 DNA replicated during a subsequent 3-h chase in BUdR; it was detected as HL-DNA by CsCl density gradient analysis (Fig. 1A). In contrast, only 3.4% of the "old" SV40 DNA (labeled for 19 h) replicated again (Fig. 1B). In both cases, the incorporation of BUdR occurred from 43 to 46 h p.i., at which time SV40 chromatin was extracted by the Triton method (11). Thus, the length of time used for labeling the DNA, not the time of extraction or the period of BUdR treatment, greatly affected the proportion of [3 H]DNA that functioned as a template for a subsequent round of replication.

The rate at which pulse-labeled, or new, SV40 DNA engaged in a second round of replication to become HL-DNA is shown in Fig. 2. New DNA was labeled with [3 H]thymidine for 1 h, either from 24 to 25 h p.i. or from 40 to 41 h p.i., then chased for different times in unlabeled medium containing BUdR. Viral DNA was obtained by Triton extraction, and the percentage of SV40 HL-[3 H]DNA was determined by CsCl

FIG. 2. Template activity of recently replicated SV40 DNA for subsequent replication. SV40-infected cells were pulse-labeled for 1 h with [3 H]thymidine (2 μ Ci/ml) in three different experiments. Labeling was from 23.5 to 24.5 ($\square$), 24 to 25 ($\bullet$), or 40.5 to 41.5 ($\circ$) h p.i. The plates were washed and chased in fresh medium for 2 h before the addition of BUdR (1.3×10^{-5} M) and FUdR (6×10^{-5} M). Cultures were Triton extracted at the designated times thereafter. The SV40 [3 H]DNA in each sample was analyzed for buoyant density in CsCl as described in the legend to Fig. 1. The percentage of radioactivity in the heavier peak (HL-DNA) was determined from the recovery of counts in the two major peaks.

FIG. 1. Buoyant density analysis of BUdR-containing SV40 [3 H]DNA. Two plates of SV40-infected TC7 cells were labeled at 37°C with [3 H]thymidine as follows: (A) 39 to 41 h p.i. at 2 μ Ci/ml; (B) 22 to 41 h p.i. at 0.5 μ Ci/ml. The cultures were then washed and chased in fresh medium for 2 h at 37°C. BUdR (1.3×10^{-5} M) and FUdR (6×10^{-5} M) were added to each plate; the cells were incubated for 3 h and then Triton extracted. The supernatants, which contained the SV40 [3 H]DNA, were brought to a density of 1.75 g/cm 3 in CsCl and centrifuged for 66 h at 30,000 rpm at 21°C in a Spinco SW50.1 rotor. Fractions were collected from the bottom of the tubes and analyzed for acid-insoluble radioactivity.

gradient analysis as before (Fig. 1). The rate of formation of HL-DNA was considerably greater during the first 3 h in BUdR (8% HL-DNA per h) than subsequently, regardless of when the cells were labeled. Since BUdR was not present during the first 2 h of the chase, the data indicate that new SV40 DNA functioned efficiently as a template for subsequent rounds of replication for a period of about 5 h. After this time, the template activity of new SV40 DNA decreased markedly, approaching that of old SV40 DNA (see below).

The rate at which uniformly labeled, or old, SV40 DNA engaged in a second round of replication is shown in Fig. 3. The same protocol as above was followed, except that the infected cells were labeled with [3 H]thymidine from 16 to 40 h p.i., then chased in unlabeled medium to which BUdR was added 30 min later. Old SV40 DNA had a constant probability of engaging in another round of replication for at least 12 h, forming HL-DNA at the rate of 1.8% HL-DNA per h. Thus, recently replicated (new) SV40 DNA functioned as a template for replication with an efficiency about fourfold greater than that of old SV40 DNA.

We next asked whether the sedimentation rates of the Triton-extractable new and old SV40 DNA-protein complex (chromatin) differed. For

FIG. 3. Template activity of total SV40 DNA for subsequent replication. SV40-infected cells were labeled with [^3H]thymidine (4 $\mu\text{Ci}/\text{ml}$; 1.3×10^{-5} M) from 16 to 40 h p.i. The cultures were then washed and chased for 30 min in fresh medium containing 6×10^{-5} M FUDR. BUdR (1.3×10^{-5} M) was then added to each plate, and the cultures were Triton extracted at the indicated times thereafter. The SV40 [^3H]DNA in each sample was analyzed for buoyant density in CsCl as described in the legend to Fig. 1. The percentage of radioactivity in the heavier peak (HL-DNA) was determined from the recovery of counts in the two major peaks.

example, it might be thought that the basis for the relatively inefficient replicative activity of the old DNA is that a large proportion of it is completely or partially encapsidated. To test this hypothesis, preparations of new and old SV40 chromatin were analyzed for sedimentation rate in sucrose gradients (Fig. 4). Old SV40 chromatin was obtained by Triton extraction either 8 or 20 h after the addition of BUdR to infected cells that previously had been labeled for 1 h with [^3H]thymidine. As seen earlier (Fig. 2), 5 h in BUdR is sufficient time for pulse-labeled SV40 DNA to become old with regard to its template activity for replication. Both preparations of old SV40 chromatin sedimented almost identically with the new chromatin. The slight difference may be due to the replacement of thymidine with BUdR. Old and new chromatin cosedimented in other experiments where BUdR was omitted (our unpublished data). Furthermore, the recovery of [^3H]DNA from in-

FIG. 4. Sedimentation analysis of SV40 chromatin extracted after different periods in BUdR. Two plates of SV40-infected cells were labeled with [^3H]thymidine (10 $\mu\text{Ci}/\text{ml}$) from 23 to 24 h p.i. The cultures were chased in medium containing BUdR (1.3×10^{-5} M) and FUDR (6×10^{-5} M) for either 8 h (A) or 20 h (B) before Triton extraction of the SV40 chromatin. A control culture of infected cells was labeled with [^{14}C]thymidine (2 $\mu\text{Ci}/\text{ml}$) from 31 to 32 h p.i. in the absence of BUdR and FUDR, and SV40 chromatin was obtained by Triton extraction. Samples (0.2 ml) containing ^3H -labeled (○) and ^{14}C -labeled (■) SV40 chromatin were layered onto 4.8 ml of 5 to 20% sucrose gradients (in TTEDS) and centrifuged for 90 min at 45,000 rpm at 4°C in an SW50.1 rotor. Fractions were collected from the bottom and analyzed for acid-precipitable radioactivity.

fecting cells in the form of a 55S complex (11, 14, 16) was nearly identical after an 8- or 20-h chase (data not shown). Since mature SV40 virions are not degraded to a 55S complex by the Triton extraction procedure (M. H. Green, unpublished data), we conclude that the difference in template activity between new and old SV40 DNA is not due to the encapsidation of the DNA into completed virions.

New and old SV40 DNA as templates for transcription. In the following experiment, recently replicated (new) and preexisting (old plus new) SV40 DNA were compared with regard to their relative ability to serve as templates for transcription. After the onset of SV40 replication, infected cells were transferred to BUdR for different periods of time and then Triton extracted to obtain the soluble SV40 transcription complex (11). Each preparation was used to synthesize short molecules of [^3H]RNA as described previously (4). The proportion of [^3H]RNA that remained associated with the non-

replicated, light-light SV40 DNA (old plus new LL-DNA) and with the recently replicated SV40 HL-DNA (new HL-DNA) was determined by CsCl equilibrium density gradient analysis (Fig. 5). With increasing time in BUdR, a greater proportion of the [^3H]RNA was found in the region of the new HL-DNA, clearly separated from the LL-DNA and the free [^3H]RNA that was at the bottom of the tube. In the same

experiment, SV40 DNA was labeled uniformly with [^3H]thymidine (18 to 36 h p.i.), and the proportion of viral HL- and LL-DNA extracted after different periods of time in BUdR was measured as described above (Fig. 1 and 3). By this means, it was possible to determine the ratio of RNA to DNA for both the HL- and LL-DNA as a function of time of replication in BUdR.

The data from both parts of this experiment

FIG. 5. CsCl density gradient analysis of [^3H]RNA associated with SV40 DNA template extracted at different times after addition of BUdR. BUdR (1.3×10^{-5} M) and FUdR (6×10^{-5} M) were added to SV40-infected cells at 36.5 h p.i. The VTC was obtained by Triton extraction after 0, 1, 2, 4, 6, and 9 h (panels A through F, respectively). Four plates were used per time point. The VTC was pooled, concentrated, and used to synthesize low-molecular-weight [^3H]RNA *in vitro* (see text). After sodium dodecyl sulfate treatment and centrifugation through Sephadex G-100, CsCl was added (final ρ , 1.75 g/cm^3) and the samples were centrifuged for 69 h at 30,000 rpm (21°C) in a Spinco SW50.1 rotor. SV40 [^{14}C]DNA form I was included as a density reference. Symbols: ■—■, [^3H]RNA; ●---●, [^{14}C]DNA.

are summarized in Table 1, from which two conclusions can be drawn. First, the proportion of [^3H]RNA, and thus of RNA polymerase, associated with the nonreplicated LL-DNA decreased very slowly, requiring 6 h for 50% of the RNA to become associated with the recently replicated viral HL-DNA. This result suggests that replicating SV40 DNA molecules are not utilized as the major templates for transcription. If only replicating DNA molecules served as templates for transcription, all of the RNA polymerases would have left the nonreplicating LL-DNA within the time required to complete the nascent RNA chains, i.e., probably less than 5 min (1). Further evidence for this conclusion is presented in Fig. 6. The second conclusion is that recently replicated SV40 DNA serves preferentially as a template for transcription. This is evident from the observation that the ratio of the percentages of HL-RNA to HL-DNA increased markedly with time after transfer to BUdR, reaching a value of 2.8 at 9 h. Combining these two conclusions with the results presented earlier, we postulate that recently replicated (new) SV40 DNA serves preferentially as a template for transcription and for replication.

The conformation of the SV40 DNA used as a template for transcription at a late stage of infection was analyzed by the following experiment. The Triton-solubilized VTC was used to synthesize short molecules of [^3H]RNA *in vitro* as described previously (4). The resultant [^3H]RNA-DNA hybrid was analyzed for buoyant density in CsCl gradients containing SV40 [^{14}C]DNA form I (Fig. 6A and C) or form III

(Fig. 6B and D) as a density reference. To obtain resolution of closed circular (form I) and linear (form III) SV40 DNA, ethidium bromide was included in two of the gradients (Fig. 6C and D). Form II DNA (nicked circles) would band at the same density as form III DNA. The [^3H]RNA banded at a density that indicated that it was mostly, if not entirely, associated with closed circular SV40 DNA. Thus, closed circular DNA served as a template for SV40 late transcription. This experiment supports the above conclusion that the templates for SV40 RNA synthesis are not required to be replicating DNA molecules. It does not eliminate the possibility that other forms of the viral DNA are also utilized as templates (see Discussion).

DISCUSSION

The major new finding in this paper is that recently replicated SV40 DNA is utilized preferentially as a template for further replication and for late transcription in monkey cells. Since virtually all of the intracellular SV40 and polyoma DNA-protein complex sediments at about 55S (11, 14, 16) and has a chromatin-like structure (10, 15), it is evident that some heterogeneity must exist in this population of chromatin for the recently replicated viral DNA to be selected preferentially. This selectivity could arise via two distinct, though not mutually exclusive, properties of the chromatin: location and biochemical composition. In the first case, recently replicated DNA may be located at sites that give it a selective advantage in binding to enzymes or other factors required for replication and transcription. Evidence that replicating SV40 DNA is specifically associated with intranuclear sites has been reported (21). The second model proposes that some biochemical property of recently replicated SV40 DNA causes it to remain potentially active as a template. Previous studies in our laboratory have indicated that the polyoma and SV40 transcription complexes are less readily solubilized by Triton extraction than is the bulk of the viral chromatin (11, 34). This implies that some heterogeneity does exist in the intracellular viral chromatin, but does not elucidate which of the above models is applicable.

In considering the possible biochemical properties that might serve to distinguish template-active from -inactive viral chromatin, it is worth noting that the viral-coded A-protein is implicated in both processes requiring a template, i.e., replication and transcription (7, 20, 28, 35). Recently replicated SV40 DNA may retain this protein for a period of time, and thus be utilized preferentially as a template for either of these processes. This would be an example of positive

TABLE 1. *Template activity of SV40 DNA as a function of time since replication*

Time in BUdR (h)	LL-RNA ^a (%)	LL-DNA ^b (%)	LL-RNA/LL-DNA	HL-RNA ^a (%)	HL-DNA ^b (%)	HL-RNA/HL-DNA
1	87	95	0.92	— ^c	5	—
2	84	90	0.94	—	10	—
4	72	80	0.90	28	20	1.4
6	50	73	0.68	50	27	1.9
9	19	71	0.27	81	29	2.8

^a Data for LL-RNA and HL-RNA were obtained from the experiment presented in Fig. 5, ignoring the (constant) percentage of [^3H]RNA at the bottom of the gradient and using the zero-time sample to correct for spillover into the HL-DNA region.

^b Calculated as percentage by weight of total SV40 DNA, assuming HL-DNA has one-half the specific activity of LL-DNA.

^c Values for the HL-RNA at 1 and 2 h are not listed because they are inaccurate because of the large amount of LL-RNA spillover in the CsCl gradient (Fig. 5).

FIG. 6. *CsCl* density gradient analysis of SV40 DNA template associated with [^3H]RNA. SV40 transcription complex isolated at 40 h p.i. was used to synthesize low-molecular-weight [^3H]RNA *in vitro*. Protein was released from the viral template by treatment with sodium dodecyl sulfate (0.1%), and, after removal of [^3H]UTP by centrifugation of the reaction mixture through Sephadex G-100, the sample was divided, marker was added, and the mixtures were centrifuged in *CsCl* for 64 h at 30,000 rpm (21°C) in an SW50.1 rotor. SV40 [^{14}C]DNA (form I, closed circular) was included as a density reference for panels A and C, while *EcoRI*-treated SV40 [^{14}C]DNA (form III, linear) was included for panels B and D. Ethidium bromide (200 $\mu\text{g}/\text{ml}$) was dissolved in the *CsCl* (ρ , 1.6 g/cm^3) for panels C and D, but omitted from the *CsCl* (ρ , 1.7 g/cm^3) for panels A and B. Fractions were analyzed for acid-insoluble [^3H]RNA (■—■) and [^{14}C]DNA (●—●).

regulation. Alternatively, the selectivity might involve negative regulation. For example, some viral capsid protein may interact with recently replicated viral chromatin, causing it to become relatively inactive as a template. Our data (Fig. 4) indicate that the complete maturation of SV40 particles is not responsible for the inactivation of the viral templates. This is also evident from the fact that the inactive viral chromatin can continue to function as a template for replication with a constant probability for at least 12 h after transfer to BUdR (Fig. 3).

The size of the template-active pool of viral chromatin is considerably larger than the pool of chromatin that is actively engaged in replication or transcription at a given time. Assuming that the SV40 DNA doubling time found by Manteuil et al. (23) is applicable in our system, we estimate that at 40 h p.i., 2.5% of the Triton-extractable SV40 chromatin contains replicating DNA molecules. Our experiments indicate that

only 0.3% of the SV40 DNA is associated with one or more molecules of RNA polymerase at this time (unpublished data). In contrast, the size of the template-active pool for replication at 40 h p.i. amounts to nearly 25% of the intracellular SV40 chromatin. This estimate is based on the facts that the accumulation of SV40 DNA is linear from 20 to 40 h p.i. (23; our unpublished data) and that recently replicated SV40 DNA becomes relatively inactive as a template for subsequent rounds after about 5 h (Fig. 2). Assuming that the viral DNA synthesized before 20 h p.i. contributes insignificantly to the total mass of intracellular SV40 DNA, and that little viral DNA is packaged into mature virions (Fig. 4), then 25% (5/20) of the Triton-extractable SV40 chromatin at 40 h p.i. is preferentially template active with regard to replication. The template-active pool for transcription must comprise even more than 25% of the total DNA because of the greater time required for the

active template to become inactive (see below). It should be noted that although recently replicated SV40 DNA is utilized preferentially for both the processes of replication and transcription, we have no knowledge of whether the same or separate pools are involved. Present studies are aimed at distinguishing the template-active from the -inactive SV40 chromatin biochemically.

One apparent difference between the viral chromatin used as template for transcription and that used for replication is the length of time during which recently replicated SV40 DNA remains most active as a template. The half-time for maximal template activity for replication is 2.5 h (Fig. 2), whereas that for transcription is about 6 h (Table 1). This difference may not be as great as it appears, because several assumptions are needed to estimate the half-time of the active template for transcription. Since 50% of the RNA polymerase activity was associated with recently replicated (BUdR-substituted) SV40 DNA after 6 h, and the proportion of activity with this DNA increased linearly with time up to 9 h in BUdR, we have assumed that 100% of the enzyme would be associated with the BUdR-DNA by 12 h. This implies that the only template for transcription is the recently replicated DNA, an as yet untested assumption. A second assumption that affects the estimated half-time of the active template for transcription is that the BUdR-substituted and nonsubstituted DNAs have equal template activities. However, we have observed a twofold decrease in the RNA polymerase activity of the total SV40 transcription complex extracted 8 h after the addition of BUdR (unpublished data). For this reason, we believe that the half-time of the active template for transcription is somewhat less than 6 h.

One may question whether the preferential template activity for transcription observed for the recently replicated SV40 DNA is an artifact caused by the incorporation of BUdR. Two findings argue against this possibility. The first is the above-mentioned decrease in RNA polymerase activity associated with SV40 DNA extracted after the addition of BUdR. If the BUdR-DNA were a better template for transcription than normal DNA, one would expect to find more, not less, RNA polymerase activity associated with the SV40 DNA after the addition of BUdR. Since the RNA polymerase was assayed in the presence of Sarkosyl, the decreased activity suggests that there was less enzyme per unit of DNA, not that the histones interfered more with the polymerase activity (8, 13). Secondly, the increased template activity of

the BUdR-DNA did not occur until 6 h after the addition of BUdR, and was most pronounced at 9 h (Table 1). Because of the method of analysis, this conclusion must be inferred from the actual data, which indicate that the template activity of the nonreplicated SV40 DNA (LL-DNA) remained near unity for 4 h after the addition of BUdR, then decreased markedly with time. If the BUdR-DNA were in fact a better template for transcription, the RNA/DNA ratio of the HL-DNA would have remained constant with time in BUdR.

In contrast to SV40, newly replicated polyoma DNA apparently serves not just preferentially but exclusively as the template for subsequent rounds of replication (31). Only polyoma DNA that has replicated during the previous 4 to 5 h will engage in another round of replication subsequently. The basis for this difference between polyoma and SV40 is as yet unknown but apparently does not involve the encapsidation of viral DNA into mature virions.

The pattern of SV40 gene expression, at least with respect to the stable transcripts, changes after the onset of viral replication in monkey cells (19, 22, 32). This change is dependent on a functional viral A-gene product (7). The initiation and maintenance of SV40 DNA replication also require an active A-protein (6, 35). However, the inactivation of the A-protein does not cause a return of the transcriptional pattern to that of the prereplicative (early) stage of infection (20, 28). It might therefore seem unlikely that replicating DNA molecules are required as templates for the late, or postreplicative, transcriptional pattern. The recent report of Birkenmeier et al. (2) provides strong evidence that nonreplicating SV40 DNA (form I) molecules are utilized as templates for late transcription. The results presented here support that conclusion (Fig. 6). In addition, our experiments permit us to exclude the possibility that RNA polymerase initiates late transcription only on SV40 DNA molecules that have begun to replicate but have become "frozen" in a covalently closed circular configuration (form I) because of their involvement in transcription. As seen in Table 1, 50% of the RNA polymerase activity remained associated with SV40 DNA molecules that had not replicated for at least 6 h. Since RNA polymerase can apparently transcribe the entire SV40 genome in less than 5 min (11), this enzyme would have to traverse at least 72 cycles without detaching from the template during the 6-h period in which nonreplicated DNA remains associated with it. We believe it much more likely that RNA polymerase initiates late transcription preferentially on recently replicated SV40 DNA,

not on replicating form I molecules. The biochemical basis for the preferred use of recently replicated DNA as template may provide some insight into the mechanism for the change in the transcriptional pattern that occurs after the onset of SV40 replication.

It is not certain that nonreplicating, closed circular SV40 DNA (form I) is the only form of DNA used as a template for late transcription. The present experiments as well as those of Birkenmeier et al. (2) would probably not detect other forms of template because the methods used to detect the DNA-RNA hybrid might have caused the RNA to dissociate from the DNA (3, 5, 29). The small amount of free RNA (Fig. 6) suggests, however, that form I DNA is the predominant species employed as a template. A second note of caution regarding this conclusion is that we are analyzing only the viral DNA that is Triton soluble. Although this extraction procedure solubilizes at least 90% of the non-encapsidated intracellular SV40 DNA, a large portion of the viral transcription complex is not solubilized (11). Moreover, we do not share the conclusion of some others (9, 33) that the Sarkosyl extraction procedure (9) solubilizes nearly all of the viral transcriptional activity. Our reasons for this have been presented elsewhere (11). The nature of the SV40 template found in the Triton pellet is as yet unknown, but it does not seem to be transcribed differently than the Triton-soluble DNA (F.-J. Ferdinand, G. Khoury, T. L. Brooks, and M. H. Green, unpublished data).

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LITERATURE CITED

- Aloni, Y. 1974. Biogenesis and characterization of SV40 and polyoma RNAs in productively infected cells. Cold Spring Harbor Symp. Quant. Biol. **39**:165-177.
- Birkenmeier, E. H., M. F. Radonovich, M. Shani, and N. P. Salzman. 1977. The SV40 DNA template for transcription of late mRNA in viral nucleoprotein complexes. Cell **11**:495-504.
- Bremer, H., and M. W. Konrad. 1964. A complex of enzymatically synthesized RNA and template DNA. Proc. Natl. Acad. Sci. U.S.A. **51**:801-808.
- Brooks, T. L., and M. H. Green. 1977. The SV40 transcription complex. I. Effect of viral chromatin proteins on endogenous RNA polymerase activity. Nucleic Acids Res. **4**:4261-4277.
- Champoux, J. J., and B. M. McConaughy. 1975. Priming of superhelical SV40 DNA by *E. coli* RNA polymerase for *in vitro* DNA synthesis. Biochemistry **14**:307-316.
- Chou, J. Y., J. Avila, and R. G. Martin. 1974. Viral DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J. Virol. **14**:116-124.
- Cowan, K., P. Tegtmeier, and D. D. Anthony. 1973. Relationship of replication and transcription of simian virus 40 DNA. Proc. Natl. Acad. Sci. U.S.A. **70**:1927-1930.
- Gariglio, P., J. Buss, and M. H. Green. 1974. Sarkosyl activation of RNA polymerase activity in mitotic mouse cells. FEBS Lett. **44**:330-333.
- Gariglio, P., and S. Mousset. 1975. Isolation and partial characterization of a nuclear RNA polymerase-SV40 DNA complex. FEBS Lett. **56**:149-155.
- Germond, J. E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon. 1975. Folding of the DNA double helix in chromatin-like structure from SV40. Proc. Natl. Acad. Sci. U.S.A. **72**:1843-1847.
- Green, M. H., and T. L. Brooks. 1976. Isolation of two forms of SV40 nucleoprotein containing RNA polymerase from infected monkey cells. Virology **72**:110-120.
- Green, M. H., and T. L. Brooks. 1977. The SV40 transcription complex. II. Non-dissociation of protein from SV40 chromatin during transcription. Nucleic Acids Res. **4**:4279-4289.
- Green, M. H., J. Buss, and P. Gariglio. 1975. Activation of nuclear RNA polymerase by sarkosyl. Eur. J. Biochem. **53**:217-225.
- Green, M. H., H. I. Miller, and S. Hendler. 1971. Isolation of a polyoma nucleoprotein complex from infected mouse cell cultures. Proc. Natl. Acad. Sci. U.S.A. **68**:1032-1036.
- Griffith, J. D. 1975. Chromatin structure: deduced from a minichromosome. Science **187**:1202-1203.
- Hall, M. R., W. Meinke, and D. A. Goldstein. 1973. Nucleoprotein complexes containing replicating simian virus 40 DNA: comparison with polyoma nucleoprotein complexes. J. Virol. **12**:901-908.
- Hirt, B. 1966. Evidence for semiconservative replication of circular polyoma DNA. Proc. Natl. Acad. Sci. U.S.A. **55**:997-1004.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365-369.
- Khoury, G., J. C. Byrne, and M. A. Martin. 1972. Patterns of simian virus 40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Natl. Acad. Sci. U.S.A. **69**:1925-1928.
- Khoury, G., and E. May. 1977. Regulation of early and late simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T-antigen. J. Virol. **23**:167-176.
- LeBlanc, D. J., and M. F. Singer. 1974. Localization of replicating DNA of simian virus 40 in monkey kidney cells. Proc. Natl. Acad. Sci. U.S.A. **71**:2236-2240.
- Lindstrom, D. M., and R. Dulbecco. 1972. Strand orientation of simian virus 40 transcription in productively infected cells. Proc. Natl. Acad. Sci. U.S.A. **69**:1517-1520.
- Manteuil, S., J. Pages, D. Stehelin, and M. Girard. 1973. Replication of simian virus 40 deoxyribonucleic acid: analysis of the one-step growth cycle. J. Virol. **11**:98-106.
- Morrow, J. F., and P. Berg. 1972. Cleavage of simian virus 40 DNA at a unique site by a bacterial restriction enzyme. Proc. Natl. Acad. Sci. U.S.A. **69**:3365-3369.
- Mulder, C., and H. Delius. 1972. Specificity of the break produced by restricting endonuclease *R*₁ in simian virus 40 DNA, as revealed by partial denaturation mapping. Proc. Natl. Acad. Sci. U.S.A. **69**:3215-3219.
- Nathans, D., and K. J. Danna. 1972. Specific origin in SV40 DNA replication. Nature (New Biol.) **236**:200-202.
- Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514-1521.

28. Reed, S. I., G. R. Stark, and J. C. Alwine. 1976. Autoregulation of SV40 gene A by T antigen. *Proc. Natl. Acad. Sci. U.S.A.* 73:3083-3087.
29. Richardson, J. P. 1975. Attachment of nascent RNA molecules to superhelical DNA. *J. Mol. Biol.* 98:565-579.
30. Robb, J. A., and R. G. Martin. 1972. Genetic analysis of simian virus 40. III. Characterization of a temperature-sensitive mutant blocked at an early stage of productive infection in monkey cells. *J. Virol.* 9:956-968.
31. Roman, A., and R. Dulbecco. 1975. Fate of polyoma form I DNA during replication. *J. Virol.* 16:70-74.
32. Sambrook, J., P. A. Sharp, and W. Keller. 1972. Transcription of simian virus 40 DNA and hybridization of the separated strands to RNA extracted from lytically infected and transformed cells. *J. Mol. Biol.* 70:57-71.
33. Shani, M., E. Birkenmeier, E. May, and N. P. Salzman. 1977. Properties of simian virus 40 transcriptional intermediates isolated from nuclei of permissive cells. *J. Virol.* 23:20-28.
34. Shmookler, R. J., J. Buss, and M. H. Green. 1974. Properties of the polyoma virus transcription complex obtained from mouse nuclei. *Virology* 57:122-127.
35. Tegtmeyer, P. 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. *J. Virol.* 10:591-598.
36. White, M., and R. Eason. 1971. Nucleoprotein complexes in simian virus 40-infected cells. *J. Virol.* 8:363-371.