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Two Deletions Within Genes for Simian Virus 40 Structural Proteins VP2 and VP3 Lead to Formation of Abnormal Transcriptional Complexes

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The procedure developed by R. M. Fernandez-Muñoz et al. (J. Virol. **29**:612-623, 1979) for isolating simian virus 40 (SV40) chromatin free of disrupted previrions was optimized for preparing late transcriptional complexes, and these complexes were partially characterized. Transcriptional complexes derived from wild-type virus and from several deletion and temperature-sensitive mutants could be activated more than five-fold either by the anionic detergent Sarkosyl or by 300 mM ammonium sulfate, in agreement with the properties of SV40 transcriptional complexes prepared by other procedures. In contrast, complexes from cells infected with deletion mutants dl1261 or dl1262 were not activated at all by a high salt concentration, even though the extent of their activation by Sarkosyl was normal. Mutants dl1261 and dl1262 carry deletions of 54 and 36 base pairs, respectively, at an approximate map position of 0.91, which is within the overlapping genes for the virion proteins VP2 and VP3. The effects of these deletions on transcription in vitro indicate that VP2 or VP3 or both are bound to late transcriptional complexes in a way that affects the progress of initiated RNA polymerase. The properties of late transcriptional complexes derived from wild-type SV40 can be explained by the presence of the following two different kinds of complexes: (i) a minority class (about 20%), which is free of VP2 or VP3, active at low concentrations of ammonium sulfate in vitro, and responsible for late transcription in vivo, and (ii) a majority class (about 80%) with VP2 or VP3 bound, which is inactive at low salt concentrations both in vitro and in vivo but capable of being activated by high salt concentrations or by Sarkosyl. We propose that mutant VP2 and VP3 proteins from dl1261 and dl1262 bind to the majority class of late transcriptional complexes in a way that can be reversed by Sarkosyl but not by a high salt concentration.

Extraction of nuclei from cells infected with simian virus 40 (SV40) with isotonic or nearly isotonic buffers (17, 18) or with hypotonic buffers (28, 31), followed by velocity sedimentation in sucrose gradients, leads to separation of a 200S pool of previrions (70 to 90% of the SV40 DNA extracted) from a 70S pool of nucleoproteins (10 to 30%), which includes all of the SV40 transcriptional activity extracted. With the availability of these new procedures for isolating transcriptional complexes that are more likely to be native, it seemed attractive to investigate control of SV40 transcription in vitro by using viable mutants affected in control of transcription in vivo. Mutants from complementation groups A and D are altered in control of transcription. tsA mutants make temperature-sensitive large-T antigen (1, 54) and fail to repress early transcription at high temperatures (2, 30, 42). tsD mutants, whose mutations map within the structural genes for VP2 and VP3 (32), have a phenotype that can be explained best by the presence in the virions of a repressor that must be removed before any transcription can begin (3). dl1261 is a D mutant, and the onset of infection with this mutant is delayed (11), as predicted for a tightly binding repressor. Our current observation that the majority of transcriptional complexes isolated late in an infection with dl1261 or dl1262 (which has an overlapping deletion [12]) behave as though they contain a tightly binding repressor indicates that repression of transcription late in infection involves an impediment to the progress of initiated polymerase along the late strand ("attenuation") instead of or in addition to an impediment to initiation. As discussed more fully below, this explanation allows us to understand the large

differences found in several laboratories between the very small expression of late RNA early in infections and the appreciable fraction of isolated transcriptional complexes initiated on the late strand. Our results also complement and extend the recent finding of Laub et al. (34, 35) that synthesis of the majority of late RNA is terminated a few hundred nucleotides downstream from the initiation sites for late transcription.

MATERIALS AND METHODS

Cells and virus. CV1 lines of green monkey kidney cells were obtained from Paul Berg and the American Type Culture Collection and were grown in a CO_2 incubator in Lux plastic plates (100 by 15 mm) in Eagle medium as modified by Dulbecco (GIBCO Laboratories) with 10% fetal calf serum (Microbiological Associates). Stocks of wild-type SV40 strain SVS (51) and all of the mutants were obtained from Paul Berg and were grown by the method of Mertz and Berg (38).

Preparation of SV40 nucleoproteins. CV1 cells (10^6 cells per plate) were grown for 3 days, infected (5 PFU/cell for 90 min at 37°C), and maintained in the presence of 2% fetal calf serum. Viral DNA was labeled with thymidine from 20 to 45 h after infection. Viral nucleoproteins were prepared essentially by the method of Fernandez-Muñoz et al. (17). Briefly, each plate of cells was washed twice with 10 ml of TD buffer (137 mM NaCl, 5.1 mM KCl, 0.7 mM Na_2HPO_4, 50 mM Tris-hydrochloride, pH 7.4), and the cells were scraped into 1 ml of this buffer, spun down, and suspended in TD buffer containing 0.5% Nonidet P-40 and 0.2 mM phenylmethanesulfonyl fluoride (1 ml/plate). After the suspension was mixed briefly, the nuclei were spun down at 4°C and then suspended (6×10^6 nuclei per ml) in cold TD buffer (pH 7.9) containing 0.2 mM phenylmethanesulfonyl fluoride but no Na_2HPO_4. The nuclei were placed into a Wheaton Dounce tissue grinder (type A), agitated with 30 strokes of the pestle, incubated for 60 min at 0°C, and centrifuged for 10 min at 17,000 × *g*. The supernatant solution, containing 5 to 10 μg of SV40 DNA per 6×10^6 cells, was either used directly to assay viral transcriptional complexes or analyzed on sucrose gradients.

Sometimes, for reasons which we do not understand, extraction of the nuclei gave a very poor yield of SV40 DNA and transcriptional complexes (as little as 10% of the amount obtained normally). In these cases, the total amount of viral DNA in the nuclei, the amount of transcriptional complexes that could be extracted with Sarkosyl, and the amount of viral transcription that could be obtained by incubating whole nuclei with ribonucleoside triphosphates were all normal. Adding more Nonidet P-40 to the extraction-resistant nuclei or agitating them with many more strokes of the Dounce apparatus had little effect. Resistance seemed to be a property of a particular batch of cells, and the problem could usually be resolved by using a different batch.

Assays for RNA polymerase activity. The standard reaction mixture (125 μl) contained 0.5 to 1 μg of viral DNA (as nucleoprotein), 84 mM Tris-hydrochloride (pH 7.9), 100 mM NaCl, 0.15 mM phenylmethanesulfonyl fluoride, 1.5 mM $MnCl_2$, 1.7 mM dithiothreitol, 3.7 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 50 μM [^{3}H]UTP (unless stated otherwise; specific activities are given in the figures legends), and 0 to 300 mM $(NH_4)_2SO_4$. When the concentration of $(NH_4)_2SO_4$ was higher than 30 mM, all components were mixed at 0°C in the presence of 30 mM $(NH_4)_2SO_4$, and 4 M $(NH_4)_2SO_4$ was then added. Reactions were carried out at 32°C, as described by Gariglio et al. (19).

Sedimentation analysis of viral chromatin. Before or after incubation for RNA synthesis, 0.25 ml of viral nucleoproteins was layered onto 3.2-ml linear 10 to 40% (wt/vol) sucrose gradients in 50 mM Tris-hydrochloride buffer (pH 7.9)–137 mM NaCl–5.1 mM KCl, with a cushion of 65% sucrose. Centrifugation was for 70 min at 55,000 rpm in a Spinco SW56 rotor at 2°C. Radioactivity was determined by mixing 100 μl of each fraction with 1 ml of water and 10 ml of Aquasol (New England Nuclear Corp.).

RESULTS

Extraction of transcriptional complexes from isolated nuclei. The amount of SV40 transcriptional activity extracted from nuclei in isotonic buffer increased with time of incubation at 0°C, even though the amount of labeled DNA extracted was essentially constant (Table 1). The cytoplasm from which the nuclei were separated contained only about 5% of the total SV40 DNA labeled with [^{3}H]thymidine, and no RNA polymerase activity was detected when cytoplasmic extracts of infected cells were assayed in the presence of 300 mM $(NH_4)_2SO_4$ or 0.2% Sarkosyl.

The amount of RNA polymerase activity extracted from nuclei, assayed at 300 mM $(NH_4)_2SO_4$, increased in parallel with the amount of SV40 DNA between 20 and 50 h after

TABLE 1. *Effect of incubation of nuclei on extraction of transcriptional activity*[a]

Time (h)	Amt of UMP incorporated (pmol/plate)	% of labeled SV40 DNA extracted
0	47	40
1	81	44
3	110	45

[a] Nuclei prepared 46 h after infection from CV1 cells labeled with [^{3}H]thymidine were subjected to Dounce homogenization and incubated in isotonic buffer at 0°C. The supernatant solutions from the zero-time and 1-h incubations were kept at 0°C until the 3-h incubation was finished. All three solutions were then assayed for RNA polymerase activity in parallel; assays were for 1 h at 32°C in 300 mM $(NH_4)_2SO_4$–90 μM [^{3}H]UTP (1,000 cpm/pmol). SV40 DNA was prepared by the method of Hirt (27) from cells infected in parallel.

infection (data not shown), suggesting that the initiated polymerase extracted was transcribing viral DNA. The fraction of RNA synthesized in vitro that was complementary to SV40 DNA immobilized on paper (50) was determined by using nuclei prepared 46 h after infection (Table 2). Almost 90% of the RNA made in the nuclear supernatant in the presence of 300 mM $(NH_4)_2SO_4$ and 40 to 80% of the RNA made at low salt concentrations (three experiments) were complementary to SV40 DNA. Most of the total transcriptional activity present in the nuclei remained there after extraction, but 30% of the SV40-specific transcriptional activity was found in the nuclear supernatant (Table 2). To determine the fraction of extracted SV40 nucleoprotein that carried initiated RNA polymerase, we used the assay described by Llopis et al. (36), in which the RNA-DNA hybrids formed by in vitro transcription are banded in a Cs_2SO_4 gradient. A minimum of 0.3% of all viral DNA molecules extracted by this procedure, or about 1% of the viral DNA in the 70S region of a velocity gradient (see below), were capable of being transcribed by initiated polymerase in vitro in the presence of a high salt concentration.

TABLE 2. *Hybridization to SV40 DNA-paper of RNA synthesized in nuclei or in isotonic nuclear supernatant solutions*[a]

Sample	Source of RNA	Radioactivity of the [^{32}P]-RNA in the hybridization (cpm)	% Of [^{32}P]-RNA hybridized[b]
1	Nuclei before Dounce homogenization	108,740	11
2	Nuclei plus supernatant after Dounce homogenization	126,010	13
3	Isotonic supernatant assayed at 300 mM $(NH_4)_2SO_4$	4,050	87
4	Isotonic supernatant assayed at 30 mM $(NH_4)_2SO_4$	1,410	42
5	Nuclei after isotonic supernatant was removed	90,080	7
6	Nuclei of uninfected cells	108,940	1

[a] Nuclei prepared 45 h after infection from two 90-mm plates of CV1 cells were suspended in 2.2 ml of isotonic buffer at 0°C; 450 μl of the suspension was reserved at 0°C (sample 1), and the rest of the suspension was subjected to Dounce homogenization 35 times. A 450-μl portion of the suspension subjected to Dounce homogenization was reserved at 0°C (sample 2), and the rest of the suspension was kept at 0° for 1 h and then centrifuged. Two 450-μl portions of the supernatant solution were reserved (samples 3 and 4), and the pellet of nuclei was suspended at 0°C in 1.3 ml of TD buffer containing 0.05% Nonidet P-40 (sample 5). Sample 6 was from uninfected cells and was prepared in the same way as sample 1. Assays for RNA synthesis were performed in parallel at 32°C for 1 h by using 50 μM [^{32}P]UTP (1,000 cpm/pmol) in the presence of 300 mM $(NH_4)_2SO_4$, except for sample 4, which was assayed in the presence of 30 mM $(NH_4)_2SO_4$. [^{32}P]RNA was prepared essentially as described by Chirgwin et al. (8) by mixing 625 μl of each sample with 1.6 ml of a solution containing 6 M guanidinium thiocyanate, 0.75% Sarkosyl, 38 mM sodium citrate buffer (pH 7.0), and 150 mM 2-mercaptoethanol. The pellet of RNA obtained after centrifugation through 5.7 M CsCl overnight was washed, dissolved in water, and precipitated with ethanol in the presence of 3 μg of carrier tRNA per ml. The [^{32}P]RNA was mixed with about 60,000 cpm of ^{3}H-labeled SV40 complementary RNA and hybridized to SV40 DNA immobilized on diazo paper as described by Stark and Williams (50).

[b] Corrected for hybridization of the internal standard ^{3}H-labeled SV40 complementary RNA (about 60%).

Characterization of SV40 transcriptional complexes isolated under isotonic conditions. For transcriptional complexes isolated by using nonionic detergents, such as Triton X-100 and Nonidet P-40 (17, 23), it has been proposed that activation by $(NH_4)_2SO_4$ is the result of a complex set of phenomena in which salt-dependent changes in the structures of RNA polymerase, histones, and the regulatory proteins associated with the DNA template may all contribute (7, 19, 60). Figure 1 shows the effects of $(NH_4)_2SO_4$ on SV40 transcriptional complexes extracted from nuclei in isotonic buffer as a function of time of extraction. The activation of complexes extracted immediately was very different from the activation of complexes extracted some time after incubation at 0°C. Note that the amount of transcriptional activity in low-salt assays was essentially independent of the time of extraction and that increasing the time only added to the activity present at high salt concentrations. The amount of SV40-specific RNA transcribed in vitro in low-salt assays was also independent of the time of extraction (data not shown). Together with the data of Table 1, these results suggest that there may be two kinds of SV40 transcriptional complexes, one extracted quickly along with the bulk of the viral DNA and activated only a little or not at all by high salt concentrations and another extracted more slowly, virtually inactive in 30 mM $(NH_4)_2SO_4$, but activated appreciably by salt. In a control experiment (data not shown) exogenous ^{3}H-labeled SV40 RNA was undegraded after incubation with a nuclear supernatant solution under assay conditions in the presence of either low or high salt concentrations, showing that the action of RNases did not affect the results obtained.

The degree of activation by $(NH_4)_2SO_4$ was also a function of the length of the RNA synthesis reaction (Fig. 2). When the reaction was performed for 5 min, activation by 300 mM $(NH_4)_2SO_4$ was only 1.5-fold, whereas it was 6.2-

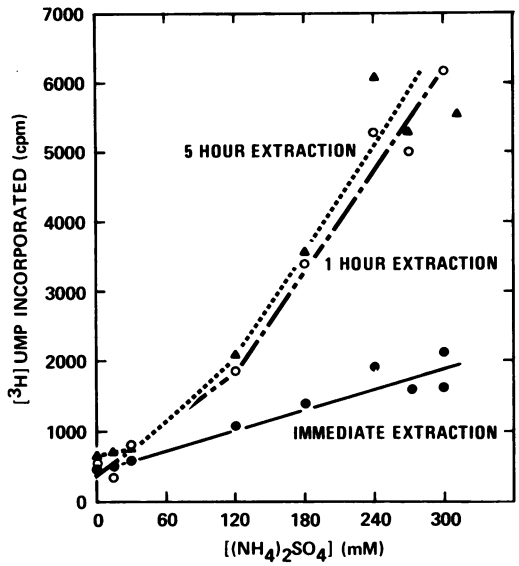

FIG. 1. *Effect of ammonium sulfate on the activity of SV40 transcriptional complexes. Viral nucleoproteins were extracted as described in Table 1, footnote a, and duplicate samples were assayed by using 2 h of incubation and 90 μM [^{3}H]UTP (1,000 cpm/pmol). Symbols: ●, nuclei pelleted immediately after Dounce homogenization; ○, nuclei pelleted 1 h after Dounce homogenization; ▲, nuclei pelleted 5 h after Dounce homogenization.*

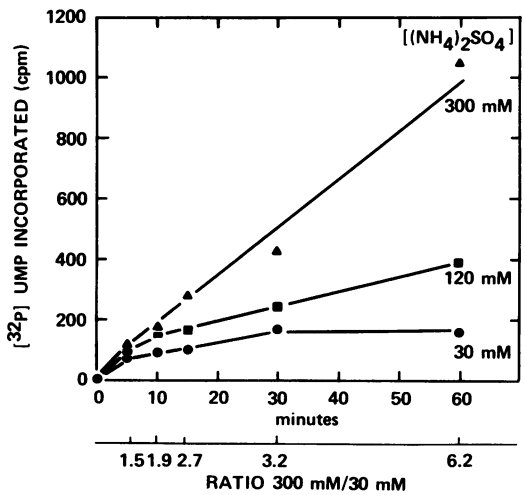

FIG. 2. *Effect of ammonium sulfate on the kinetics of RNA synthesis by SV40 transcriptional complexes. Viral nucleoproteins were isolated from nuclei after 1 h of incubation and assayed by using 50 μM [^{3}H]UTP (9,360 cpm/pmol). At the times indicated, 100-μl portions were placed on DE/81 paper and processed as described in text. Ratios of activity in the presence of 300 mM $(NH_4)_2SO_4$ to activity in the presence of 30 mM $(NH_4)_2SO_4$ are shown at the bottom of the figure as a function of time.*

fold after 60 min of synthesis and reached 10-fold after 2 h. As Fig. 2 shows, this effect was due to rapid cessation of RNA synthesis at low concentrations of $(NH_4)_2SO_4$; at high concentrations, the rate of incorporation was almost linear. If Sarkosyl was included in an assay carried out for 5 min with 300 mM $(NH_4)_2SO_4$, the degree of activation was much higher, about five-fold. A possible explanation for this interesting result is that a minority form of late transcriptional complex (the only form that was active at a low salt concentration) synthesized RNA much faster than the majority form did, even in the presence of a high salt concentration. However, in the presence of a low salt concentration, the minority form stopped quickly, possibly by the normal termination mechanism. This possibility can be examined best by determining the properties of the two kinds of late transcriptional complexes after they are separated.

Table 3 shows the effects of additions to RNA polymerase reactions in the presence of 30 or 300 mM $(NH_4)_2SO_4$. The reactions at the low and high salt concentrations were inhibited by actinomycin D (the template is DNA), by a low concentration of α-amanitin (the polymerase is type II), and by RNase (the product is RNA).

TABLE 3. *Effects of additions on the RNA polymerase reaction in extracts of nuclei from SV40-infected cells*[a]

Addition	% Of incorporation with no addition	
	300 mM $(NH_4)_2SO_4$	30 mM $(NH_4)_2SO_4$
None	—[b]	—
Actinomycin D (7 μg/ml)	0	0
α-Amanitin (0.3 μg/ml)	0	0
RNase A (3 μg/ml)	1.7	0
Sarkosyl (0.2%)	90	830
SV40 DNA (1 μg/ml)	104	430
Rifamycin AF/013 (100 μg/ml)	80	277

[a] Nuclei prepared 46 h after infection from CV1 cells labeled with [^{14}C]thymidine were subjected to Dounce homogenization and incubated in isotonic buffer at 0°C for 1 h. Equivalent portions of a single supernatant solution were preincubated for 10 min at 0°C with the additions shown in the table before the standard components for RNA synthesis were added. The reactions were carried out for 1 h at 32°C with 90 μM [^{3}H]UTP (1,000 cpm/pmol). Under all conditions the reaction depended on the presence of all four ribonucleoside triphosphates and on the presence of divalent cations. The activity with $MnCl_2$ was about five times greater than with $MgCl_2$. Also, the incorporation of labeled UTP into RNA was proportional to the amount of extract used.

[b] For 300 mM $(NH_4)_2SO_4$, 3.8 pmol = 100%, and for 30 mM $(NH_4)_2SO_4$, 0.35 pmol = 100%.

Sarkosyl had little effect at the high salt concentration but stimulated the activity greatly at the low salt concentration. Exogenous SV40 DNA had no effect at the high salt concentration but gave a very appreciable stimulation at the low salt concentration, implying strongly that unbound RNA polymerase was present in the extract and that initiation onto the exogenous DNA could take place in vitro. This conclusion was reinforced by the finding that it was possible to separate on a sucrose gradient the unbound polymerase, which could be assayed with exogenous SV40 DNA at the low salt concentration, from the polymerase already initiated on SV40 nucleoprotein complexes and sedimenting at about 70S, which was not affected by exogenous SV40 DNA (see below). Rifamycin AF/013, which inhibits the initiation of RNA polymerase (37), did not inhibit transcription in vitro at either the low or the high salt concentration, indicating that in the absence of exogenous DNA, reinitiation did not have a major role in the activity observed. This conclusion was supported by the data of Fig. 2, which show that transcription at the low salt concentration stopped quickly. The stimulation with rifamycin AF/013 at the low salt concentration (Table 3) may have been due to an interaction of this drug with proteins of the transcriptional complexes (37).

The possibility that artifactual binding of RNA polymerase to viral nucleoprotein might have contributed to the phenomena which we observed was minimized by an experiment in which rifamycin AF/013 was included during all stages of the preparation of the transcriptional complex, beginning with the washing of the cells and including preparation, Dounce homogenization, and incubation of the nuclei. The amount of viral transcription obtained at the high salt concentration in this case was not significantly different from the amount obtained in a parallel experiment without rifamycin (data not shown).

Sedimentation of SV40 transcriptional complexes extracted by the isotonic procedure. Figure 3 shows the results of experiments in which RNA synthesis was carried out at high and low salt concentrations for 5 min in vitro in the presence of a limiting concentration of [^{32}P]CTP (30% of the maximum rate of incorporation), followed by sedimentation in sucrose gradients. In both cases, there was a single peak of [^{32}P]RNA at about 70S, approximately in the position of a peak of SV40 DNA. The appearance of label in this peak was sensitive to RNase, and the increase in incorporation at 210 mM $(NH_4)_2SO_4$ which was observed (about twofold) was consistent with the degree of activation shown in Fig. 2 for 5 min of synthesis. These

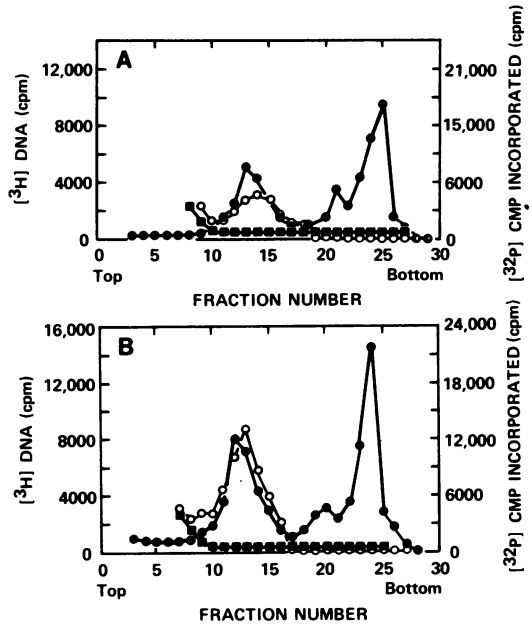

FIG. 3. *Sedimentation of viral nucleoprotein complexes and RNA labeled in vitro. Cells were labeled with [^{3}H]thymidine (50 μCi/plate) 24 h after infection with SV40. At 45 h after infection, an isotonic nuclear supernatant was prepared and then incubated for 1 h. RNA synthesis was carried out for 5 min by using 10 μM [^{32}P]CTP (15,000 cpm/pmol) and 30 mM $(NH_4)_2SO_4$ (A) or 300 mM $(NH_4)_2SO_4$ (B) in the presence or absence of 20 μg of RNase A per ml. RNA synthesis was stopped, and samples (0.25 ml) were sedimented by centrifugation though a 10 to 40% sucrose gradient for 85 min at 55,000 rpm and 4°C in an SW56 rotor. The top seven fractions were not collected since they contained much [^{32}P]CTP. Symbols: ●, [^{3}H]thymidine incorporated in vivo; ○, [^{32}P]CMP incorporated in vitro; ■, [^{32}P]CMP incorporated in vitro in the presence of RNase.*

results indicated that the viral RNA made in vitro under these conditions was not released from the transcriptional complexes and cosedimented with them, a conclusion confirmed by an analysis of the RNA-DNA hybrids in Cs_2SO_4 gradients after removal of the proteins (data not shown).

The sedimentation pattern of the DNA was not changed if no period of RNA synthesis preceded analysis on the gradient (data not shown). The amounts of [^{3}H]DNA sedimenting in fractions 10 to 15, 16 to 20, and 21 to 27 were approximately 35, 15, and 50% of the total, respectively. In this experiment, the extraction was performed 45 h after infection. When viral nucleoproteins prepared 30 h after infection were analyzed, all of the label sedimented at about 70S, and about 70% of the label sedimented in this position after 40 h of infection.

We never observed less than 30% of the labeled SV40 DNA sedimenting at 70S at any time after infection, in contrast with the results of Fernandez-Muñoz et al. (17), who found a much smaller fraction in this position (about 10% after 48 h of infection) and the great majority of the label at 200S, in the position of undisrupted previrions. However, it must be emphasized that different conditions were used in the two laboratories, especially different times of extraction of the nuclei after Dounce homogenization.

We also performed experiments in which the viral nucleoproteins were sedimented in a sucrose gradient first, followed by determinations of transcriptional activity in each fraction at high and low salt concentrations, with and without exogenous SV40 DNA (Fig. 4). Most of the RNA polymerase activity sedimented at about 70S, showing that the transcriptional complexes could be purified in isotonic buffer. The usual degree of activation by 300 mM $(NH_4)_2SO_4$ or by Sarkosyl (data not shown) was observed for material at 70S, and almost all of the polymerase activity found before sedimentation was recovered in the gradient. No RNA polymerase activity was detected in the 200S region of the gradient when the assays were conducted in the presence of Sarkosyl. When SV40 DNA was included in each assay at the low salt concentration, there was a slight activation of the 70S peak, but a major new peak of activity was revealed near the top of the gradient (fractions 5 to 12), probably due to the presence in this region of free RNA polymerase that could initiate SV40 DNA-dependent RNA synthesis in vitro.

To summarize the results described thus far, (i) the extraction procedure solubilized an important fraction (about 30%) of the initiated viral transcriptional complexes, probably in a native state, (ii) the complexes could be separated from free RNA polymerase and 200S previrions on sucrose gradients and retained full activity and ability to be activated by a high salt concentration or by Sarkosyl, and (iii) the differential extraction of complexes active at low and high salt concentrations and the rapid cessation of RNA synthesis at a low salt concentration but not at a high salt concentration suggested that there are two different kinds of viral transcriptional complexes.

Viral transcriptional complexes from SV40 mutants. We used several different viable mutants affected in transcription to obtain more insight into the nature of the SV40 nucleoprotein complexes carrying initiated RNA polymerase and especially to obtain further evidence that there may be two different kinds of complexes. Changes in the properties of the transcriptional

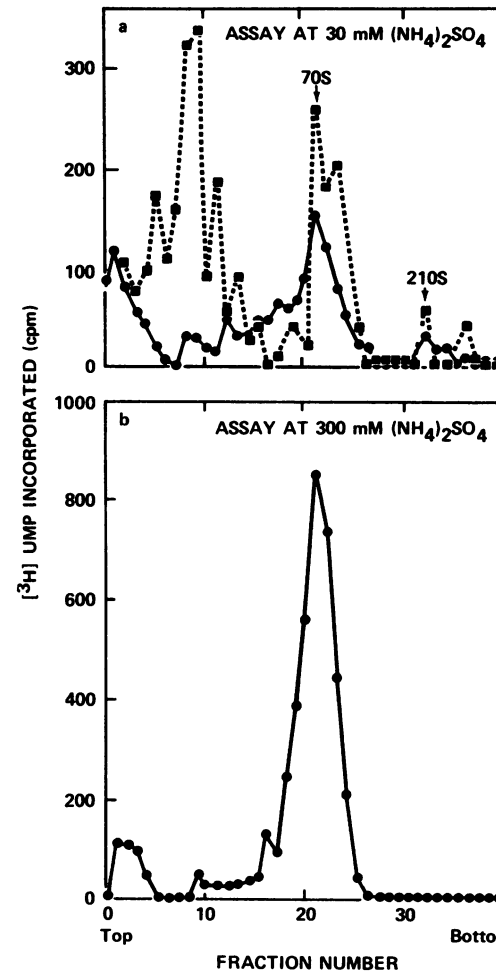

FIG. 4. *Sedimentation of the RNA polymerase activity present in isotonic nuclear supernatants. Supernatants prepared after 1 h of incubation were sedimented, and each fraction (100 μl) was assayed for 1 h by using 90 μM [^{3}H]UMP (1,000 cpm/pmol). (a) Assays at 30 mM $(NH_4)_2SO_4$ in the absence (●) or in the presence (■) of 0.5 μg of SV40 DNA(I) per fraction. (b) Assays at 300 mM $(NH_4)_2SO_4$ in the absence of added DNA.*

complexes, correlated with mutations in a specific viral protein, would indicate involvement of that protein in transcription in vitro and probably also in vivo. As Table 4 shows, the degree of activation of SV40 transcription in vitro by $(NH_4)_2SO_4$ was the same for most of the mutants tested as it was for wild-type SV40. In contrast, mutants dl1261 and dl1262 were activated only slightly or not at all by $(NH_4)_2SO_4$. Similar results were obtained with dl1261 when the assays were performed at 41 instead of 32°C (data not shown). It is known that in infections with these two viable deletion mutants, shortened forms of

TABLE 4. *Salt activation of transcriptional complexes isolated from SV40 mutants*[a]

Virus	Size of deletion (base pairs)	Map position	Reference	Protein(s) affected	Ratios of activities at different concentrations of $(NH_4)_2SO_4$	
					180 mM/ 30 mM	300 mM/ 30 mM
Wild type	None			None	3.2	5.2
dl861	53	0.74	11	None	3.4	5.1
dl884	247	0.54–0.59	60	Small-t antigen	3.2	5.4
dl1265	39	0.18	57	Large-T antigen (C-terminal region)	3.0	ND[b]
dl894	72	0.68–0.71	58	None	3.3	ND
tsB4	None	0.05–0.10	32	VP1	3.2	5.5
dl1261	54	0.91	12	VP2 and VP3	0.8	1.3
dl1262	36	0.91	12	VP2 and VP3	1.4	1.3

[a] Nuclei prepared after 42 to 46 h from CV1 cells infected with wild-type virus or deletion mutants at 37°C or infected with tsB4 at 41°C were subjected to Dounce homogenization and incubated in isotonic buffer at 0°C for 1 h. RNA synthesis was carried out for 1 h at 32°C with 90 μM [^{3}H]UTP (1,000 cpm/pmol). With wild-type SV40 and dl1261, equivalent results were obtained when the synthesis reaction was performed at 41°C.

[b] ND, Not determined.

the virion proteins VP2 and VP3 are synthesized (11).

Failure to activate transcriptional complexes from dl1261 or dl1262 with $(NH_4)_2SO_4$ could have been due either to the absence of a class of transcriptional complexes susceptible to such activation in the pool of material extracted from the nuclei or to a change in the properties of the complexes extracted. These two possibilities could be distinguished by using Sarkosyl, which removes nearly all proteins except initiated RNA polymerase from viral chromatin (21, 25). As Fig. 5 shows, Sarkosyl activated complexes derived from wild-type virus and complexes derived from mutant dl1261, although to somewhat different extents. The ability of Sarkosyl to activate transcription from dl1261 in vitro and the failure of $(NH_4)_2SO_4$ to do so indicated that VP2 or VP3 or both were involved functionally in a major fraction of the transcriptional complexes extracted by the procedure which we used and suggested very strongly that these proteins are also involved somehow in SV40 transcription in vivo. The different final extents of activation shown in Fig. 5 must reflect different relative amounts of complexes susceptible to activation by Sarkosyl in the populations derived from the two viruses. The different extents of activation at low concentrations of Sarkosyl probably indicate that removal of the mutant proteins by Sarkosyl is more difficult than removal of the wild-type proteins.

Partial characterization of transcriptional complexes from dl1261. When CV1 cells were infected in parallel with wild-type SV40 or dl1261 at 37°C and then labeled with [^{14}C]thymidine 20 to 45 h after infection, 40 to 50% of the label incorporated into wild-type DNA was found in dl1261 DNA. A similar experiment with dl1262 gave a different result; the amounts of viral DNA were comparable for the

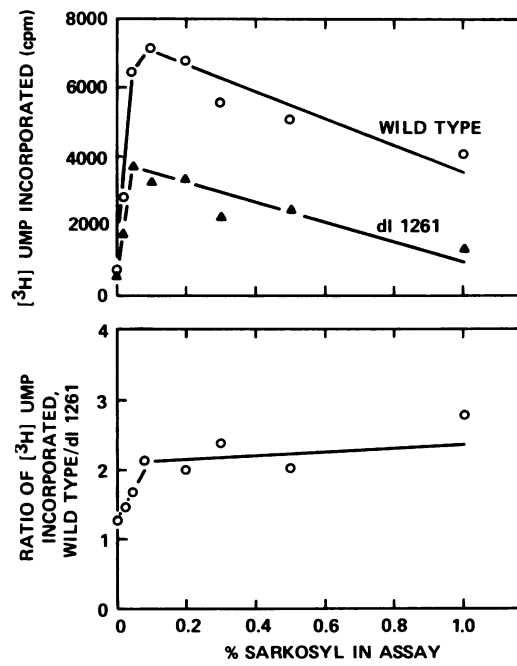

FIG. 5. *Activation of transcriptional complexes by Sarkosyl. Nuclear supernatants prepared after 1 h of incubation were assayed for 30 min in the presence of 30 mM $(NH_4)_2SO_4$ by using 50 μM [^{3}H]UTP (6,000 cpm/pmol) and different concentrations of Sarkosyl. The amount of [^{3}H]UMP incorporated was normalized to the amount of total SV40 DNA in each assay. In this experiment, 45% of the [^{14}C]thymidine incorporated into wild-type DNA 45 h postinfection was incorporated into dl1261 DNA during the same time.*

mutant and the wild-type virus. These results are consistent with the observations of Cole et al. (11), who showed that dl1261 is a D mutant, with delayed onset of infection, whereas dl1262 is not defective. An analysis of the transcriptional complexes derived from dl1261 on a sucrose gradient (data not shown) gave a pattern very similar to the one obtained with wild-type SV40 (Fig. 3). Essentially the same results were obtained when the infections were performed at 32, 37, and 41°C (data not shown). Most of the endogenous RNA polymerase activity present in extracts from infections with dl1261 sedimented at 70S, and in most experiments the fraction of viral DNA sedimenting at this position was greater than in experiments with wild-type SV40 when the times of infection were the same. This result too is consistent with delayed onset of infection by the mutant virus, since the fraction of DNA at 70S with wild-type virus was greater early in infection (see above). The specific transcriptional activities (incorporation of ribonucleoside triphosphates per unit of DNA) at low concentrations of $(NH_4)_2SO_4$ were the same for dl1261 and the wild-type virus (Fig. 5).

DISCUSSION

Isolation of SV40 transcriptional complexes. Complexes have been extracted from the nuclei of infected cells with anionic detergents (Sarkosyl), with nonionic detergents (Triton X-100 or Nonidet P-40), and with no detergent. Extraction with Sarkosyl yields complexes which are stripped of most proteins but retain initiated RNA polymerase (20). Almost all of the SV40 transcriptional activity can be extracted (16, 20, 21, 33, 49), and, upon incubation with ribonucleoside triphosphate, the nascent RNA chains can be elongated (20). Complexes prepared with Sarkosyl are obviously unsuitable for investigating regulatory proteins associated with the viral DNA, but the study of these complexes has uncovered important facts concerning SV40 transcription. The RNA polymerase in Sarkosyl complexes is polymerase II (or polymerase B) (20, 33, 49), and the major template for late transcription is closed circular SV40 DNA (6), not replicative intermediates (5, 21).

Extraction of nuclei with Triton X-100 in the presence of a high salt concentration yields transcriptional complexes which retain the core histones and initiated polymerase II and are very similar by electron microscopy to viral chromatin not active in transcription (19). About 0.5 to 1% of the total viral DNA extracted from nuclei with Triton X-100 or Sarkosyl is engaged in complexes with RNA polymerase II (36). Under optimal conditions in vitro, Triton X-100 complexes can synthesize RNA chains much longer than the viral genome, without irreversible alteration of the chromatin-like structure (7, 19, 24). As with Sarkosyl complexes, about 95% of the RNA made from Triton X-100 complexes isolated late in infection is complementary to the late DNA strand (23). Extraction of complexes in isotonic buffer without appreciable amounts of detergent, as in the present work, has the obvious advantage that, since disruption of previrions is avoided, they can be removed by sedimentation. At least 1% of the 70S complexes obtained in this way contain initiated RNA polymerase active in the presence of 300 mM $(NH_4)_2SO_4$, and the recovery of activity is excellent.

Are there two types of transcriptional complexes? Data in Table 1 and Fig. 1 suggest that one type of complex may be active at 30 mM $(NH_4)_2SO_4$ and another type may be active only in the presence of Sarkosyl or high concentrations of $(NH_4)_2SO_4$. The latter type appears to be extracted from nuclei more slowly than the former, and it may be possible to purify partially the complexes inactive at low salt concentrations by separating the nuclei soon after the Dounce homogenization procedure and incubating them in fresh buffer at 0°C. Green and Brooks (23) have described separation of two types of transcriptional complexes after treatment of SV40-infected CV1 cells with Triton X-100. The relationship between the complexes isolated by the different procedures is unclear, but it is interesting to note that the majority type (about 80% of the total) is extracted with greater difficulty in each case.

The results with the viable deletion mutants dl1261 and dl1262 shown in Table 4 and Fig. 5 also indicate that two types of late transcriptional complexes are present. We propose that the minority type, estimated from the degree of activation by a high salt concentration or by Sarkosyl to be 20% of the total, is actively involved in late transcription in vivo and that the majority type (80%) carries initiated but attenuated RNA polymerase. We propose that the protein(s) involved in attenuation is VP2 or VP3 or both. In vitro, Sarkosyl or high concentrations of $(NH_4)_2SO_4$ can remove the blocking proteins from wild-type attenuated complexes and allow transcription to occur in vitro. This interpretation is in qualitative and quantitative agreement with the recent results of Laub et al. (34, 35), who used a completely different approach to reach the conclusion that attenuated complexes make up the bulk of late SV40 transcriptional complexes late in infection. After very short periods of synthesis in vitro, when transcriptional complexes isolated either with Sarkosyl or in the absence of detergent are used, 80% of

the newly synthesized RNA hybridizes with an SV40 DNA fragment from a region near the origin (between 0.67 and 0.76 map unit), and about 20% hybridizes with the rest of the SV40 genome (35). The interpretation of Laub et al. (35) is that RNA polymerase molecules initiated in vivo are present predominantly in the region between 0.67 and 0.76 map unit.

At low concentrations of $(NH_4)_2SO_4$, RNA polymerase activity in the nuclear supernatant is activated by exogenous SV40 DNA (Table 3), showing that in vitro initiation of polymerase can take place in this extract. For the type of complex active at 30 mM $(NH_4)_2SO_4$, it is unlikely that reinitiation takes place during the assay since, in the absence of exogenous SV40 DNA, incorporation is stimulated by Sarkosyl or by rifamycin AF/013, both inhibitors of initiation. Furthermore, the reaction stops quickly at low salt concentrations (Fig. 2), a result inconsistent with reinitiation. Finally, when the transcriptional complexes are separated from free polymerase by sedimentation, most of the activity at low salt concentrations is recovered in the 70S region. Since including rifamycin AF/013 during all phases of the isolated procedure does not affect the recovery of extracted transcriptional complexes active in high salt concentrations, it is not likely that the majority type of attenuated complex forms only during the isolation procedure. Also, the finding of Laub et al. (35) that RNA complementary to the 0.67- to 0.76-map unit region is pulse-labeled preferentially in vivo in the presence of a drug that accentuates premature termination indicates that most of the initiated polymerase may be present in this region in vivo and is consistent with the existence of the attenuated complexes before isolation.

Structural changes in VP2 and VP3 as a result of the deletions in dl1261 and dl1262. As Contreras et al. (12) showed, the deletion in dl1261 removed 54 nucleotides (nucleotides 1,270 to 1,323) from the region coding for the carboxyl-terminal portions of VP2 and VP3. Since 54 is divisible by 3, the reading frame is unchanged. From the DNA sequence, the decrease in the molecular weight of the proteins should be 2,040, whereas a decrease nearer 6,000 is found in sodium dodecyl sulfate-acrylamide gels (11), probably signifying that the mutant proteins are shortened further by proteolytic digestion in the cells. It will not be possible to rationalize in detail the tight binding of VP2 and VP3 from dl1261 to SV40 DNA until the structures of the mutant proteins have been determined. The deletion in dl1262 removed 36 nucleotides (nucleotides 1,301 to 1,336) from the same region (12). Again, the reading frame is unchanged. The expected decrease in the molecular weight of VP2 and VP3 is 1,450, which compares well with the observed decrease of about 2,000 (11). In this case, the deletion may cause a net change in charge of only +1 (12). If so, it is unlikely that the altered affinities of mutant VP2 and VP3 for SV40 chromatin in the presence of high salt concentrations are due primarily to alterations in ionic interactions.

Possible function of VP2 and VP3 as repressors or attenuators of SV40 transcription. The results with mutants dl1261 and dl1262 suggest that virion protein VP2 or VP3 or both function to inhibit SV 40 transcription late in infection. dl1261 is a D mutant (11), and such mutants are affected primarily at a very early stage of infection. At nonpermissive temperatures, tsD virions do not begin synthesis of early RNA, but tsD DNA can infect cells normally since the mutant protein is not present (10, 45). To appreciate fully the full range of possible effects of alterations in VP2 or VP3 upon infections with SV40, we must consider the consequences of both increases and decreases in the affinities of these proteins for viral DNA at both early and late times in infection. At the beginning of an infection, excessively tight binding should give rise to delayed expression of early RNA, as observed for the D mutants. Conversely, weak binding of a repressor or attenuator might even facilitate the initiation of an infection, perhaps causing the ratio of particles to plaque-forming units to decrease if most repressors are not usually removed in an infection by wild-type virus. Late in infection, binding of a repressor or attenuator is a logical first step in the packaging pathway, serving to channel viral chromatin away from the active processes of transcription and replication and into virions. A tightly binding repressor should have little or no effect at this stage if the interaction between wild-type repressor and viral chromatin is normally irreversible, and it is known that the burst of mutant tsD virions at the end of an infection by tsD DNA is comparable to the burst obtained with wild-type DNA (45). In contrast, a repressor that binds too weakly might cause inefficient packaging and thus smaller bursts of mature virus.

These effects might alter the yields of transcriptional complexes obtained from cells infected with tightly binding or weakly binding mutants. However, in vitro transcription from viral chromatin probably takes place only on templates that were initiated previously in vivo (see above), and thus mutants with altered VP2 or VP3 probably yield transcriptional complexes with altered properties only if these proteins function as attenuators, blocking the progress of

initiated RNA polymerase.

Model for control of SV40 transcription throughout lytic infection. It seems appropriate now to summarize much of the data on control of SV40 transcription into a working model, for the purpose of defining future experiments more clearly. The forms of SV40 nucleoprotein complexes known or postulated to be present late in infection are shown in Fig. 6. Regulation of transcription, DNA replication, and virion assembly is probably due in large part to competition of several viral and nonviral proteins for a central control region near map position 0.67, designated schematically by the dash on each circle. This region includes the origin of bidirectional DNA replication (13) and the initiation regions for both early and late transcription (22, 34). Therefore, both DNA polymerase and RNA polymerase II must bind to each DNA strand within this region. The recent work of DiMaio and Nathans (14) shows that a set of viable, cold-sensitive mutants containing deletions or base substitutions within this central control region are affected in DNA replication and transcription, probably reflecting changes in the protein-DNA interactions required for regulation of these processes.

Direct experiments have shown that large-T antigen binds to isolated viral DNA within the central control region (41, 48) and that the binding of T antigen (48) and the SV40-adenovirus D2 hybrid protein, an analog of SV40 large-T antigen (55), occurs at three well-defined sites near 0.67 map unit. SV40 chromatin isolated from infected cells contains large-T antigen tightly bound at or near the central control region, preferentially in early replicative intermediates (43, 47). Binding of large-T antigen is associated with initiation of DNA replication (52), with repression of early transcription (2, 30, 42), and, less surely, with stimulation of late transcription (39). At nonpermissive temperatures tsA mutants, which make themolabile large-T antigen (1, 54), fail to initiate new rounds of DNA replication (9, 52), fail to repress synthesis of early mRNA's (30, 42), and fail to initiate synthesis of late mRNA's very early (9.5 h) after infection (39). Such a complicated repertoire of functions may require different forms of large-T antigen, which are achieved through post-translational modification (53) or through changes in RNA splicing patterns (36a). The functions of large-T antigen, both as a repressor of early transcription and as an activator of late transcription, may be analogous to the well-defined case of λ repressor, which, in binding to the O_R2 subsite, simultaneously represses transcription from P_R in one direction and stimulates transcription from P_{RM} in the other (40).

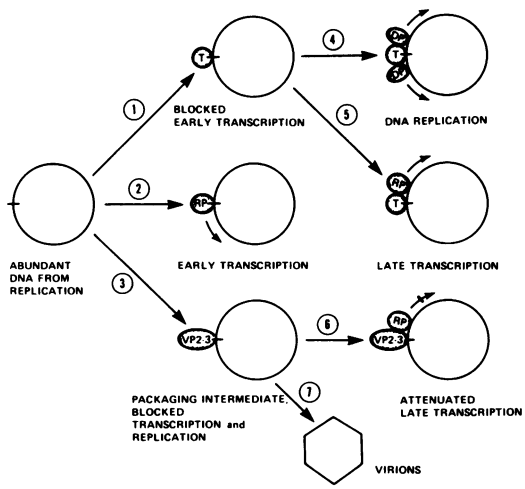

FIG. 6. *Working model for control of SV40 transcription late in infection. The dash on each circle represents the origin of DNA replication at about 0.67 map unit. T, Large-T antigen; RP, RNA polymerase; DP, DNA polymerase; VP2-3, VP2 or VP3.*

Recent work (29, 46, 58) has indicated that the region near 0.67 map unit is usually free of nucleosomes in nucleoprotein complexes isolated from infected cells, which is consistent with the binding of regulatory proteins to a histone-free region of viral DNA. VP2 and VP3 are implicated as competitors for the central control region by the properties of the tsD mutants and dl1261 discussed above. Griffith et al. (26) have shown that nucleoproteins isolated either from infected cells late in infection or from virions contain a "knob" of protein located at approximately 0.67 map unit. It is not clear that VP2 or VP3 is a component of this knob, but such a finding would be consistent with the other results cited above.

Pathway 2 in Fig. 6 shows the binding of RNA polymerase II to an open central control region, which might be present in newly replicated DNA late in infection or might be formed by removal of a repressor from virion DNA at the beginning of an infection. Pathway 1-5 shows the newly postulated role of large-T antigen in late transcription, and pathway 1-4 shows the well-established role of large-T antigen in DNA replication. The blockage of early transcription by large-T antigen could involve a failure of RNA polymerase II to bind to the early strand, possibly because of competition from large-T antigen for the binding site, or it might involve attenuation, with polymerase on the early strand blocked by large-T antigen bound to DNA downstream from the initiation site. Pathway 3-7 shows the binding of VP2 or VP3 to the central

control region as the initial and determining step in the packaging of virions. The advantages of simultaneously shutting off the active processes of transcription and DNA replication for each DNA molecule to be packaged are obvious. Pathway 3-6 shows the formation of attenuated late transcriptional complexes, which may represent as much as 80% of the transcriptional complexes initiated on the late strand, with the active complexes (pathway 1-5) representing perhaps only 20% of the total. Attenuated late transcriptional complexes may be a dead end, unable to participate either in packaging or in transcription, or they may represent a more functional form of control of late transcription.

Figure 7 shows structures which might be present during the initial, uncoating phase of an infection by SV40. Delayed initiation of infection by D mutants, followed by normal progress through the rest of the cycle, seems likely to be due to less probable removal of the tightly bound mutant repressor or attenuator than in the case of their wild-type counterparts, followed by a normal course of events thereafter. If wild-type VP2 or VP3 is ordinarily removed from only a minor fraction of the nucleoprotein complexes in infections with wild-type virus, attenuated late transcriptional complexes similar or identical to those present late in infection might be found on a major fraction of the infecting complexes that never participate actively in the early phase of the infection. At very early times, when late transcription represents only a tiny fraction of total SV40 transcription in vivo, extracted attenuated complexes activated by high salt concentrations or by Sarkosyl could account for the high fraction of SV40 molecules found to be initiated on the late strand, as found in several laboratories (4, 15).

Of course, many features of the model presented in Fig. 6 and 7 are speculative, and many details remain to be worked out. For example, if VP2 or VP3 or both are responsible for attenuation at a point several hundred nucleotides from the region near 0.67 map unit, where late transcription begins, as suggested by the recent work of Laub et al. (34, 35), the detailed representation of the binding of VP2-3 in Fig. 6 and 7 is somewhat incorrect, since it would make more sense for the positions of RNA polymerase and VP2-3 to be reversed in the complex representing attenuated late transcription. The situation may be much more complicated than drawn, with more than one molecule of VP2 or VP3 bound to different regions and functioning either as repressors or as attenuators. It is crucial to separate the attenuated late transcriptional complexes from active complexes and to characterize each of them in detail. Separation might be initiated by selective extraction, followed by the use of specific anti-T and anti-VP3 (44) immunoglobulins to precipitate each subclass specifically. Alternatively, in vitro elongation of RNA chains in low salt concentrations might provide enough of a change in size or density to facilitate separation.

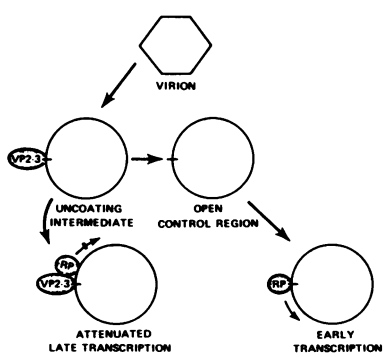

FIG. 7. *Working model for control of SV40 transcription very early in infection. RP, RNA polymerase; VP2-3, VP2 or VP3.*

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