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Relationship of Replication and Transcription of Simian Virus 40 DNA

(gene *A*/temperature-sensitive mutant/RNA–DNA hybridization)

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ABSTRACT **RNA produced by the Simian Virus 40 (SV40) mutant tsA30 during lytic infection of kidney cells of African green monkeys was examined by RNA–DNA competition–hybridization. This mutant is temperature-sensitive in a function (gene *A*) that regulates synthesis of viral DNA. No detectable difference between mutant RNA synthesized at the permissive temperature (33°) and wild-type viral RNA was found. During continuous infection with the mutant at the restrictive temperature (41°) only early viral RNA was produced. When mutant DNA and late RNA synthesis were initiated at the permissive temperature, a shift to the restrictive temperature rapidly terminated synthesis of viral DNA but not that of late viral RNA. The data indicate that the function of gene *A* is required before synthesis of late viral RNA and that after initiation, the production of late RNA continues without further expression of gene *A* or concomittant viral DNA synthesis.**

During lytic infection of African green monkey kidney (AGMK) cells by Simian Virus 40 (SV40), two discrete populations of stable RNA are produced (1–4). Before the onset of viral DNA replication, early RNA is transcribed from 30 to 50% of the minus or E strand of viral DNA and is formed throughout the lytic cycle. After the onset of viral DNA synthesis, late RNA complementary to 50–70% of the plus or L strand is synthesized in addition to early viral RNA (5–7). Furthermore, during lytic infection in the continuous presence of various inhibitors of viral and cellular DNA replication, only early viral RNA synthesis is detected (8–10). This finding has suggested that the regulation of late viral RNA is dependent on viral DNA replication. However, during abortive infection of mouse embryo cells, in which no viral DNA replication has been observed, both early and late viral RNA are found (6). Therefore, the precise role, if any, that viral DNA replication has in the regulation of late viral RNA synthesis remains to be defined.

In the present study, we have examined the relationship of viral DNA replication and transcription using the temperature-sensitive (ts) SV40 mutant tsA30. Previous studies have defined some of the characteristics of this virus (11). The mutant replicates normally in permissive cells at 33° but fails to produce infectious virus, viral DNA, or V antigen (late viral proteins) during continuous infection at 41°. Mutant viral DNA synthesis is rapidly inhibited within 20 min after a shift in temperature from 33° to 41°; and the ts viral gene function (gene *A*) is continuously required for the initiation but not the propagation or completion of viral DNA synthesis.

Abbreviations: AGMK, African green monkey kidney; ts, temperature-sensitive; wt, wild type; SV40, Simian Virus 40.

Our studies of the viral RNA synthesis in AGMK cells infected by this mutant indicate that: the expression of gene *A* is either directly or indirectly required to initiate transcription of late but not of early viral RNA, and the continued expression of gene *A* and the continued synthesis of viral DNA are not required to maintain the transcription of late SV40 genes after the onset of viral DNA replication.

MATERIALS AND METHODS

Virus and Tissue Culture. The mutant tsA30 and the parental clone of wild-type (wt) SV40 were propagated in the TC7 clone of AGMK cells (11, 12). Cells were grown to confluency at 37° in half-gallon roller bottles (840 cm^2 growth area) or in 32-ounce prescription bottles (150 cm^2 growth area) in 50 ml of Basal Medium (Eagle) supplemented with 5% fetal-calf serum.

Preparation of Virus and Viral DNA. Cells were labeled with [^{14}C]thymidine (0.1 μCi/ml, 35 Ci/mol Schwartz–Mann) beginning 24 hr after infection with 1 plaque forming unit (PFU) per ml of wt SV40. Cultures were harvested 7–9 days after infection, and cell membranes were disrupted by freeze–thawing. The combined lysate was adjusted to 0.5 M NaCl–10% (w/v) polyethylene glycol, and the virus was precipitated overnight at 4° (13). The precipitate was pelleted and extracted twice in 8.0 ml of 10 mM Tris·HCl–mM EDTA, pH 7.8 (buffer A) for 10 hr at 4°. The virus was then purified twice by equilibrium density contrifugation in cesium chloride. After dialysis in buffer A, the purified virus solution was adjusted to 0.5% Na dodecyl sulfate. DNA was extracted at 4° with an equal volume of phenol saturated in buffer A and precipitated at −20° by addition of 2.5 volumes of ethanol. The precipitate was resuspended in buffer A. Superhelical form I DNA was isolated by equilibrium density centrifugation in cesium chloride (density of 1.55 g/cm^3) containing 200 μg/ml of ethidium bromide according to Radloff *et al.* (14). Ethidium bromide was extracted with isoamyl alcohol, and the purified DNA was again dialyzed in buffer A. The final concentration of [^{14}C]DNA (5 to 7 × 10^3 cpm/μg) was determined by A_{260}, assuming an extinction coefficient of 20 per mg, and by the diphenylamine reaction as modified by Burton (15).

Radioactive Labeling of Cells and Temperature-Shift Conditions. After incubation of infected cell cultures for the periods designated in the legends to figures, RNA was labeled by addition of [^{3}H]uridine, (10 μCi/ml, 28 Ci/mmol Schwartz–Mann) or with ^{32}P (60 μCi/ml, New England Nuclear Corp.)

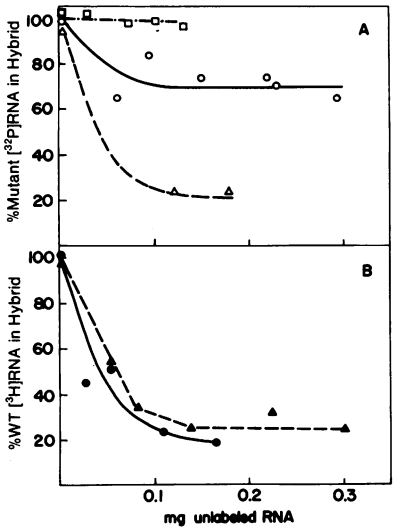

FIG. 1. Mutant RNA synthesized at 33°. (*A*) Hybridization with labeled mutant RNA. Cells were infected with 1 PFU per cell of tsA30 and incubated at 33°. 65 hr after infection the medium was replaced with phosphate-free medium containing ^{32}P (60 μCi/ml), and the cells were labeled for 4 hr. Competition-hybridization reactions contained 24 μg of mutant [^{32}P]RNA (4.15×10^3 cpm/μg), 0.1 μg [^{14}C]DNA on nitrocellulose filters, and increasing amounts of uninfected cell RNA (□·—·□), early wt RNA (○——○), or late wt RNA (△— —△). Radioactivity bound to viral DNA in the absence of competing RNA was 310 cpm. (*B*) Hybridization with labeled wt RNA. Early RNA (5.1×10^4 cpm/μg) was isolated from cells labeled with 10 μCi/ml of [^3H]uridine 4–20 hr after infection at 37° with 10 PFU per cell of wt SV40. Culture medium for this preparation contained 20 μg/ml of cytosine arabinoside. Late RNA (2.2×10^4 cpm/μg) was labeled with 10 μCi/ml of [^3H]uridine 50–70 hr after infection at 37° with 1 PFU per cell of wt SV40. Unlabeled mutant RNA was isolated 65 hr after infection with 1 PFU per cell of tsA30. Annealing reactions contained 30 μg of early wt [^3H]RNA (●——●) or 25 μg of late wt [^3H]RNA (▲— —▲) and increasing amounts of unlabeled mutant RNA as designated. [^3H]RNA bound to viral DNA in the absence of competition was 1,900 cpm (early RNA) and 1200 cpm (late RNA).

after a change to phosphate-free medium; DNA was labeled with [^3H]thymidine (5–100 μCi/ml, 17 Ci/mmol, New England Nuclear Corp.). Conditions used to obtain a rapid transition from permissive to restrictive temperatures and for monitoring of DNA synthesis after the shift in temperature were as described (11).

Preparation of RNA. RNA was isolated from infected cells by a phenol–Na dodecyl sulfate extraction procedure similar to that described by Church and McCarthy (16). Cells were washed with 0.01 M Tris·HCl, pH 7.8–0.14 M NaCl and then lysed in a 0.35% Na dodecyl sulfate solution of 0.1 M NaCl–0.01 M Na Acetate, pH 5.1, containing 200 μg/ml of bentonite, 0.1% diethyl pyrocarbonate, and 15 μg/ml of polyvinyl sulfate (buffer B). RNA was extracted at 4° with an equal volume of phenol saturated with buffer B. The phenol interphase was again extracted at 60°, and the combined aqueous phases were again extracted at 4°. The aqueous phase was then adjusted to 0.2 M NaCl, and RNA was precipitated at −20° with two volumes of ethanol. The precipitate was suspended in 0.01 M Tris·HCl, pH 7.8, containing 5 mM $MgCl_2$. It was incubated for 30 min at 37° in the presence of 30 μg/ml of electrophoretically pure DNase (Schwartz–Mann) and for an additional hour with 100 μg/ml of self-digested Pronase B (Calbiochem). The RNA was then extracted with phenol saturated with buffer A, precipitated in ethanol, and further purified by fractionation on Sephadex G-75. RNA prepared in this manner had an $A_{260/280}$ of 1.9 and an $A_{260/230}$ of 2.2. Analysis by polyacrylamide-gel electrophoresis demonstrated a normal cell RNA profile with no evidence of degradation.

Early wt RNA was isolated from cells infected with 10 PFU per cell of wt SV40 for 20 hr at 37° in the presence of cytosine arabinoside (20 μg/ml, Nutritional Biochemicals); late wt RNA was isolated 72 hr after infection.

RNA–DNA Hybridization was done essentially according to the procedure of Gillespie and Spiegelman (17). Nitrocellulose filters (Schleicher and Schuell, B6) 47 mm in diameter were treated with Denhardt's medium (18) and washed in 0.9 M NaCl–0.09 M Na citrate before use. [^{14}C]-DNA was heat-denatured, bound to filters, and fixed by heating at 80° for 3 hr. Each filter contained 0.1 μg of [^{14}C]-DNA (5 to 7 $\times$ 10^3 cpm/μg), less than 10% of which was lost during hybridization. The annealing reaction was done in 0.3-ml aliquots of 0.3 M NaCl–0.03 M Na citrate–12.5 mM EDTA for 24 hr at 65°. The EDTA was added to prevent breakdown of RNA during its incubation at 65° (19).

Nitrocellulose filters were then washed in 0.3 M NaCl–0.03 M Na citrate–12.5 mM EDTA, treated with pancreatic

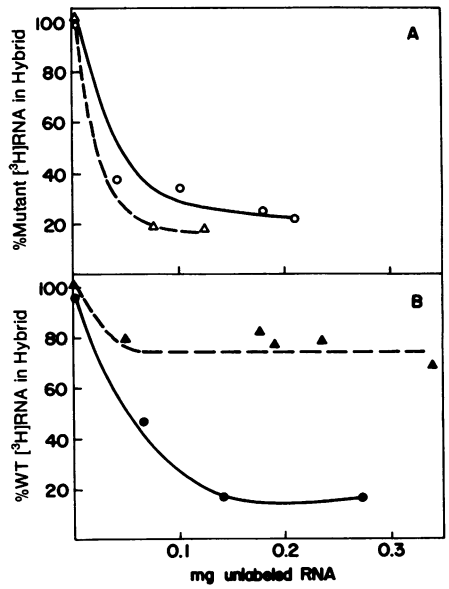

FIG. 2. Mutant RNA synthesized at 41°. (*A*) Hybridization with labeled mutant RNA. Cells were infected with 10 PFU per cell of tsA30 and incubated for 70 hr at 41°. The cultures were labeled 50–70 hr after infection with 20 μCi/ml of [^3H]uridine. Annealing reactions contained 25 μg of mutant [^3H]RNA (1.5×10^5 cpm/μg) and increasing amounts of either unlabeled wt early RNA (○——○) or unlabeled wt late RNA (△——△). Radioactivity bound to viral DNA in the absence of competing RNA was 5020 cpm. (*B*) Hybridization with labeled wt RNA. Unlabeled mutant RNA was isolated 65 hr after infection at 41° with 10 PFU/cell of tsA30. Early and late wt [^3H]RNA and annealing conditions were as described in the legend to Fig. 1*B*. Competition with early wt [^3H]RNA (●—●); competition with late wt [^3H]RNA (▲--▲).

RNase (20 μg/ml, Calbiochem) for 30 min at 37°, and washed again with 0.3 M NaCl–0.03 M Na citrate–12.5 mM EDTA. Radioactivity was monitored in a liquid–dioxane scintillation system. Data were normalized to the radioactivity bound per 0.1 μg of DNA after correction for binding to blank filters.

RESULTS

Mutant RNA Synthesis at 33°. Viral RNA produced in lytic infection by tsA30 at the permissive temperature was compared to standard wt SV40 RNA by the technique of RNA–DNA hybridization. TC7 AGMK cells infected by the mutant were labeled with ^{32}P from 65–69 hr after infection at 33° and the RNA was extracted and analyzed as described in *Methods*. Binding of a near-saturating amount of mutant [^{32}P]RNA to SV40 DNA on nitrocellulose filters was quantitated in the presence of increasing amounts of unlabeled RNA from different sources (Fig. 1*A*). Uninfected cell RNA did not compete significantly with the binding of mutant RNA to viral DNA. In the presence of an excess of early wt RNA, the mutant [^{32}P]RNA bound to SV40 DNA was decreased by 30%. An excess of late wt RNA competed for 80% of the DNA sites that bound mutant [^{32}P]RNA. Under the conditions of the competition–hybridization procedure used in these studies, similar quantities of unlabeled late wt RNA competed with homologous labeled late wt RNA to the extent of 80–90%. This degree of competition is defined as essentially complete. Thus, mutant RNA containing both early and late sequences was produced at 33°.

To confirm that all of the early and late regions of the mutant DNA were transcribed, competition between labeled wt RNA and unlabeled mutant RNA was examined. Fig. 1*B* demonstrates that the competition of unlabeled late mutant RNA with either labeled early or late wt RNA was essentially complete. Thus, within the limits of resolution of the RNA–DNA hybridization technique, these experiments demonstrated that mutant RNA produced at the permissive temperature was indistinguishable from standard wt RNA.

Mutant RNA Synthesis at 41°. When TC7 cells are infected with tsA30 and maintained at 41°, production of virions and replication of viral DNA does not occur. Under these restrictive conditions no late viral RNA was detected. Fig. 2 illustrates the results of competition–hybridization experiments between mutant RNA synthesized during continuous cell growth at 41° and early and late wt RNA. The binding to DNA of mutant [^{3}H]RNA isolated 72 hr after infection was competed to the same extent by both early and late wt RNA (Fig. 2*A*). This result indicates that all species of the mutant RNA were contained in the early wt RNA. When early wt RNA was labeled, its competition by the mutant RNA also approached completion (Fig. 2*B*). However, when the isotope was in late wt RNA, competition with an excess of the mutant RNA was limited to 30–40%. These data further demonstrate that all of the sequences present in early wt RNA were produced in the mutant at 41°. Competition between the late wt [^{3}H]RNA used in Fig. 2*B* and RNA obtained from cells infected with wt virus and maintained for 72 hr at 41° (data not shown) demonstrated that a complete sequence of late wt RNA was synthesized at 41°. Since it is known that complete wt virions are made at this temperature, this was an expected result. The failure of the mutant to synthesize late RNA under these same conditions was therefore clearly a consequence of its temperature-suppressed mutant function. The data are consistent with previous findings showing that immunologically reactive early protein (T antigen) but not late protein (V antigen) was produced in infection at 41° by another member of this group of mutants (20).

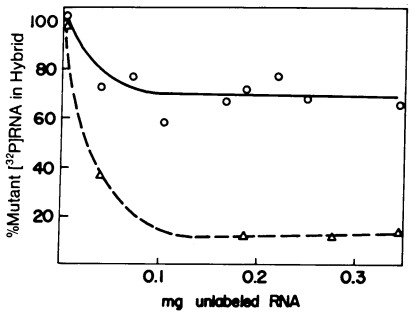

FIG. 3. Mutant RNA synthesized after temperature shift from 33° to 41°. Cells were infected with 1 PFU per cell of tsA30 and incubated at 33° for 65 hr. The medium was replaced with heated phosphate-free medium, and the cells were then placed in a 41° water bath. 2.5 hr after the shift in temperature the cells were labeled for 4 hr with ^{32}P (60 μCi/ml). Labeled mutant RNA (26 μg; 3.1×10^4 cpm/μg) was competed with unlabeled wt early (O——O) or wt late (Δ— —Δ) RNA. Radioactivity bound to viral RNA in the absence of competing RNA was 725 cpm.

Mutant RNA Synthesis after a Shift from 33° to 41°. Temperature shift experiments with tsA30 were then done to determine whether synthesis of late viral RNA required the continued expression of gene *A* and the concurrent replication of viral DNA. Cells were infected with the mutant and incubated at the permissive temperature for 65 hr to allow mutant DNA and late RNA synthesis to occur. At this time the temperature was rapidly raised and maintained at 41°. The incorporation of [^{3}H]thymidine into viral DNA was monitored as described (11). Under these conditions, synthesis of wt SV40 DNA proceeded normally, while replication of mutant DNA was completely inhibited within 30 min after the increase in temperature.

At 2.5 hr after the temperature was increased to 41°, virus-infected cultures were pulse-labeled for 4 hr with ^{32}P. Fig. 3 illustrates the results of competition–hybridization experiments between the mutant [^{32}P]RNA and early and late wt RNA. Competition by an excess of early wt RNA was limited to 30%, while competition by late wt RNA was almost complete. This same pattern of competition was observed in a parallel, temperature-shift experiment that used wt SV40. Thus, the labeled mutant RNA contained sequences that were transcribed from late SV40 genes. Therefore, synthesis of late viral RNA can occur in the absence of both concurrent viral DNA synthesis and the continued expression of gene *A*. Further experiments with the mutant demonstrated that synthesis of late RNA sequences could be detected as long as 20 hr after termination of viral DNA replication by temperature shift. However, while the proportion of late to early mutant sequences synthesized 2.5–6.5 hr after the temperature shift was always constant and similar to wt RNA, the proportion synthesized from late genes after more prolonged incubation at 41° was sometimes decreased.

Mutant RNA identical to late wt RNA is synthesized at 33° (Fig. 1). This RNA would be present after a temperature

shift until its complete turnover had occurred. Therefore, competition experiments, with unlabeled mutant RNA and labeled wt RNA to determine if all species of late RNA were produced after the suppression of the gene *A* function, could not be done. However, the patterns of competition between early wt RNA and labeled mutant RNA synthesized before and after the temperature shift were the same (30%). This result suggests that either all of the late viral genes of the mutant or a constant proportion of both the early and late genes were transcribed after the temperature shift. Since it seems unlikely that the transcription of an equal proportion of both early and late genes would be terminated simultaneously, the former interpretation appears more plausible.

DISCUSSION

Experiments with this mutant have elucidated two fundamental aspects concerning regulation of viral transcription. First, during continuous incubation at 41°, in which no viral DNA replication occurs, only early viral RNA is synthesized. Thus, gene *A* is required either directly or indirectly for transcription of late viral genes. Secondly, the temperature-shift experiments demonstrated that synthesis of late viral RNA is maintained after the activity of gene *A* and viral DNA synthesis are terminated. The data do not indicate whether or not viral DNA replication itself is necessary to initiate late RNA synthesis.

The level at which the gene *A* function interacts to effect the regulation of late RNA has not been determined. Since this same gene is directly required for DNA replication, we have assumed in this paper that the control operates at the transcriptional level. However, we cannot exclude the possibility that its effect could be on the post-transcriptional modification of viral RNA. Recent work by Aloni has suggested that differences in the rates of RNA turnover could be a factor in regulating the composition of SV40 RNA (21).

As has been indicated, tsA30 is temperature sensitive in the initiation of viral DNA synthesis. After a shift to the restrictive temperature, viral DNA replication that had been previously initiated is completed, but further rounds of viral DNA synthesis are not reinitiated. Pulse-chase experiments have demonstrated that almost all of the replicative intermediates formed by tsA30 at 33° can be chased into superhelical form I DNA within 30 min after shift to the restrictive temperature (11). Since late RNA synthesis was consistently detected in infection by the mutant 2.5–6.5 hr after a temperature shift to 41°, the replicative intermediate molecules are apparently not necessary for the continued transcription of late viral genes.

The precise factors involved in the regulation of SV40 transcription remain to be defined. The patterns of RNA synthesis in lytic infection, in abortive infection, and in transforming infection are different. It has not been determined whether a common mechanism regulating transcription is operating in each of these states. Khoury *et al.* (22) and Sambrook *et al.* (7) have suggested that integration of viral DNA into cellular DNA may play an important role in its regulation. Studies of the action of gene *A* in the process of integration and in transcription in abortive infection may suggest a common mechanism to explain the patterns of transcription in each of the infective states. In any case, it is clear that gene *A* is directly or indirectly required to initiate, but not to maintain, synthesis of late RNA during lytic infection. Any model to explain the regulation of SV40 transcription must be compatible with these findings.

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