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The control of SV40 transcription during a lytic infection: late RNA synthesis in the presence of inhibitors of DNA replication

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ABSTRACT

The transition from early to late transcription of SV40 DNA in productively infected BSC-1 cells was analyzed using both inhibitors of DNA replication, and early (Group A) temperature sensitive (ts) mutants of SV40. Late virus-specific cytoplasmic RNA sedimenting at 16S in neutral sucrose gradients and complementary to the plus (L) DNA strand of SV40 was detected in cultures infected in the presence of three inhibitors of DNA replication (Ara-C, FdU, and chloroquine), even though the inhibition of viral DNA replication appeared to be essentially complete. After infection with the early SV40 mutant tsA58, no DNA replication was detected at the restrictive temperature (41°C) and no significant late RNA complementary to the plus (L) strand was found, in either the cytoplasm or nuclei of infected cells. These data support the concept that expression of late viral functions requires the initiation of viral DNA synthesis or a functional gene A protein, or both.

INTRODUCTION

Two discrete populations of viral specific RNA are synthesized during a lytic infection of monkey kidney cells by Simian Virus 40 (SV40) (1-3). Prior to the onset of viral DNA replication, an early 19S RNA complementary to approximately 50% of the minus (E) strand of the viral DNA is found in the cytoplasm (4-9) and is present throughout the lytic cycle. Subsequent to the onset of viral DNA synthesis, late 19S and 16S RNAs complementary to approximately 50% and 25% of the plus (L) strand are found in the cytoplasm in addition to early 19S RNA (4,7,10). These late cytoplasmic RNAs probably represent the product of post-transcriptional processing (8,11). During a lytic infection in the presence of various inhibitors of viral and cellular DNA replication, the detection of only early viral RNA has generally been described (12-15). More recent results supporting the observation that the control of late transcription is dependent on viral DNA replication were reported by Cowan et al. (16). After infection of monkey kidney cells with an early ts mutant of SV40 (tsA30) at 41°C these authors were able to detect the synthesis of only early viral specific RNA. If, however, viral

DNA synthesis was permitted to take place at the permissive temperature (33°C), a subsequent shift of the tsA30-infected cultures to the restrictive temperature did not prevent the continued transcription of late RNA. These findings were confirmed by the studies of Manteuil and Girard (17) who reported that once DNA replication had begun, late RNA synthesis continued even after the introduction of inhibitors of DNA synthesis, or after the shift of early ts mutant-infected cells to the restrictive temperature.

In contrast to the studies which suggested that only early virus-specific SV40 RNA was synthesized in cells pre-treated with inhibitors of DNA replication, there have been other reports suggesting the synthesis of small amounts of late viral RNA in the presence of either Ara-C (16) or FdU (6,18).

A possible explanation for these results was that some viral DNA replication occurred even in the presence of the inhibitors. Alternatively, it seemed possible that the complete replication of SV40 DNA was not a prerequisite for late transcription. The present studies were undertaken in an attempt to determine the requirements for transcription of late viral-specific RNA.

MATERIALS AND METHODS

RNA Preparation: Confluent BSC-1 cell cultures grown in 150 mm Petri dishes at 37°C were infected with plaque purified wild type SV40 virus (at a m.o.i. of 10-20). After adsorption of virus for 2 hr at 37°C, the cultures were refed with serum-free Eagle's medium. In experiments where inhibitors were to be used, cultures were covered after virus adsorption with medium containing the following concentrations of each inhibitor: 15 µg/ml of 5-fluorodeoxyuridine (FdU) (Sigma), 50 µg/ml of 1-β-D-arabinofuranosyl cytosine hydrochloride (Ara-C) (ICN), and 50 µg/ml of 7-chloro-4-(4-diethylamino-1-methylbutyl-amino) quinoline (chloroquine) (Sigma). Cultures were labeled from 45-48 hr post-infection with 1 to 2 ml of medium containing 300-500 µCi/dish of [5,6-^{3}H]-uridine (Amersham-Searle Corp., 40-50 Ci/mmol.) as well as the appropriate inhibitor. For studies involving the temperature-sensitive mutants (tsA30 and tsA58), BSC-1 cultures were grown in 150 cm^{2} plastic tissue culture flasks. Following adsorption at room temperature for 2 hr, cultures were incubated at the restrictive temperature (41°C). At approximately 48 hr, cultures were labeled in 1 to 2 ml of medium with 500 µCi/flask of [5,6-^{3}H]-uridine for 3 hr at 41°C. Following labeling,

cultures were put on ice, washed with 10 ml ice-cold isotonic buffer (0.25 M sucrose, 10 mM triethanolamine (pH 7.4), 25 mM NaCl, 5 mM $MgCl_2$), and then lysed (19) by the addition of a 1% solution of Nonidet P40 (BDH Chemicals, Ltd.) in the same buffer. Phenol extractions of nuclear and cytoplasmic RNA were carried out as previously described (18,20) and the RNA was finally precipitated by the addition of 2.5 volumes of absolute ethanol. The RNA was then resuspended in 10 mM Tris (pH 7.4) containing 0.5 M NaCl and 0.1% sodium dodecyl sulfate and passed over a column containing 5 ml of oligo(dT)-cellulose (P-L Biochemicals). The poly(A)-containing RNA was eluted in 10 mM Tris buffer (pH 7.4), concentrated, and then layered onto linear sucrose gradients (3.9 ml) (10-25%, w/v) prepared in 10 mM Tris-HCl (pH 7.4) containing 0.05 M NaCl and 0.001 M Na_2EDTA. After centrifugation for 2.5 hr at 55,000 rpm in a Spinco SW60 Ti rotor at 10°C, fractions were collected from the bottom of the tube into polyethylene tubes and aliquots of 5-10 µl were spotted on filter paper discs, dried, precipitated with trichloroacetic acid, and the radioactivity counted in a toluene-based scintillation fluid. The remainder of each fraction was adjusted to a concentration of 4 x SSC (SSC:0.15 M NaCl-0.015 M Na_3 citrate-pH 7.4) and hybridized to total SV40 DNA on filters. In other experiments pooled fractions were divided into equal aliquots and hybridized to the ^{32}P-labeled separated strands of sheared SV40 DNA fragments (21,22).

DNA Preparation: BSC-1 cultures were prepared and infected with virus as described above in the presence or absence of the appropriate inhibitor. After 24 hr, cultures were labeled in 1-2 ml of medium with 100 µCi/dish of ^{3}H-methyl thymidine (New England Nuclear Corp., 6.7 Ci/mmol.) in medium also containing the inhibitor. For studies involving inhibition of DNA replication with FdU, cultures were labeled at 24 hr with 100 µCi/dish of [^{3}H(G)]-deoxyadenosine (New England Nuclear Corp., 15-25 Ci/mmol.). At about 48 hr post-infection, the medium was removed from the cultures, and the low-molecular weight DNA was extracted with sodium dodecyl sulfate by the method of Hirt (23). After the supernatant fluid had been extracted with phenol, the nucleic acids were precipitated with ethanol. The precipitate was resuspended in TES buffer (0.01 M Tris, 0.01 M EDTA, and .05 M NaCl, pH 7.5). A portion was centrifuged to equilibrium in cesium chloride-propidium diiodide while another portion was used in DNA-DNA filter hybridization assays (24). The DNA preparations used in filter hybridizations were treated with pancreatic RNase (50 µg/ml) for

30 min at 37°C, phenol extracted, denatured by boiling for 30 min, and finally hybridized to an excess of SV40 DNA immobilized on cellulose nitrate filters.

DNA-RNA Filter Hybridizations: DNA-RNA hybridization used to detect SV40 RNA in sucrose gradients was performed by the following procedure: Purified SV40 DNA I was denatured by boiling and fixed onto membrane filters (Schleicher and Schuell, B6) (25). Two 3.5 mm filters (minifilters) each containing 0.2 µg SV40 DNA, and a blank filter were added to 100 µl of [^{3}H] RNA solution in 4 x SSC in a 300 µl capped polyethylene tube, and incubated with 20 µg/ml of pancreatic RNase (treated at 80°C for 15 min to inactivate possible traces of DNase) in 2 x SSC and dried. The radioactivity was counted in a toluene based scintillation fluid.

DNA-DNA Filter Hybridizations: SV40 DNA minifilters were presoaked in a solution containg 1 M NaCl, .01 M Tris-HCl (pH 7.0), .02% Ficoll, .02% polyvinylpyrrolidone and .02% bovine albumin for 4 hr at 68°C (24). The [^{3}H]-labeled denatured DNAs were then added to a reaction mixture (200 µl to 400 µl) containing .02 M phosphate buffer (pH 6.8), 1.0 M NaCl, and .06% SDS (final concentration). Minifilters were added and the incubation was carried out for 48 hr at 68°C. The filters were finally washed, dried and counted in a toluene-based scintillation fluid.

Hybridizations with Separated Strands of SV40 DNA: Separated strands of SV40 DNA were prepared as previously described (21,22). RNA fractions from sucrose gradients were added to small amounts (0.3 to 1.0 ng) of the minus (E) or plus (L) strands of SV40 DNA in a reaction mixture containing 1.0 M NaCl, .02 M TES buffer (pH 7.5), and .06% SDS. Hybridizations were carried out for 20-24 hr at 68°C and DNA-RNA hybrids were analyzed by hydroxyapatite chromatography as previously described (21,22).

RESULTS

The sedimentation profiles of virus-specific RNA synthesized in the presence of inhibitors of DNA replication and at the restrictive temperature using mutants tsA30 and tsA58: In the first set of experiments, Ara-C, FdU, and chloroquine were added to SV40-infected BSC-1 cultures immediately after adsorption of the virus. Cytoplasmic RNA was prepared 48 hr after infection and purified on oligo(dT)-cellulose columns to select the poly(A)-containing RNA as previously described (18,20). This cytoplasmic poly(A) RNA was then analyzed by sucrose gradient centrifugation and the individual fractions were hybridized to SV40 DNA on filters to determine the profile of SV40-specific RNA. As shown in Fig. 1A, both a

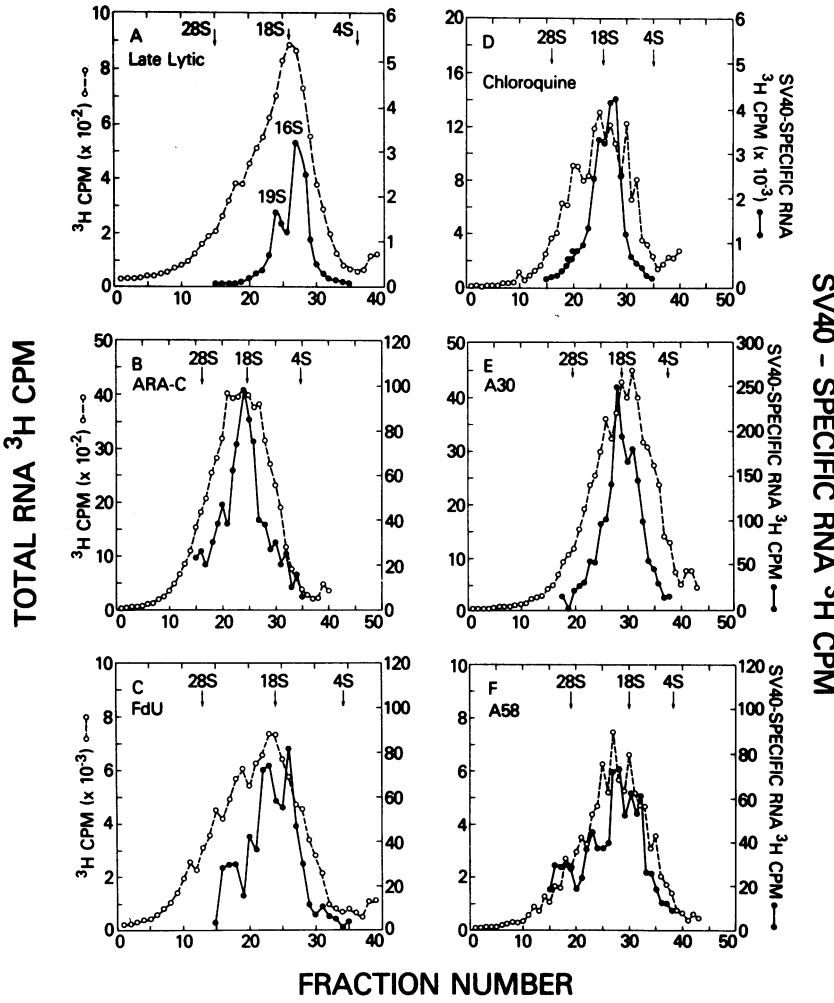

Figure 1: Sucrose gradients sedimentation of oligo(dT)-cellulose selected RNA from SV40 infected cultures. BSC-1 cells were infected with wild type SV40 at 37°C (panel A) in the presence of specific inhibitors of DNA synthesis (B-D) or with early temperature sensitive mutants of SV40 (E, F) at 41°C. RNA which had been ^{3}H-labeled from 45 to 48 hr post-infection was purified as described in Methods and selected on oligo(dT)-cellulose columns. The retained material was sedimented in 10 to 25% neutral sucrose gradients for 2.5 hr at 55,000 rpm, 5°C, in a Spinco SW60 Ti rotor. Fractions were collected drop-wise from the bottom of tubes and aliquots were counted for total radioactivity (0-0). The remainder of the RNA samples were used in hybridization experiments with SV40 DNA immobilized on membrane filters (●---●). Arrows represent the position of migration of internal ^{14}C-uridine-labeled marker ribosomal RNAs. See Materials and Methods for details.

19S and a more abundant 16S viral-specific RNA species were easily detected in a control infection with wild type virus. Several reports have already described the presence of an early 19S RNA which is synthesized throughout the lytic cycle, as well as a late 19S and 16S RNA (4,6,7). These late lytic SV40 RNA species are presumed to be synthesized only after DNA replication has commenced (12-16).

Figures 1B-D present the sedimentation profiles of cytoplasmic RNA from infected cultures in which DNA replication was inhibited by Ara-C, FdU, and chloroquine, respectively. The profile in Fig. 1B in which Ara-C was used to inhibit DNA replication contains a prominent 19S RNA peak and a 16S shoulder. The profile in Fig. 1C in which FdU was used to block DNA replication contains not only a 19S peak, but also a prominent 16S virus-specific RNA peak. Similarly, the RNA profile from chloroquine-treated cultures (Fig. 1D) exhibits both 19S and 16S SV40-specific RNA peaks. The peaks of 16S viral-specific RNA are not nearly as prominent in the inhibitor-treated cultures as they are in the wild-type infection. Nevertheless, their presence suggested that some late SV40-specific RNA might be synthesized during infection in the presence of these inhibitors. In additional experiments the RNA synthesized after infection of BSC-1 cultures at the restrictive temperature (41°C) with tsA30 and tsA58 (Figs. 1E and 1F, respectively) was examined. In both cases, the predominant viral RNA species sedimented at 19S, but a smaller SV40-specific RNA peak in the 16S region of both gradients was also observed.

Hybridization experiments with fragmented, ^{32}P-labeled, separated strands of SV40 DNA: Since the above data do now show whether the 16S viral RNA was late virus-specific RNA, solution hybridization experiments were performed between RNA fractions from neutral sucrose gradients analogous to those shown in Fig. 1 and the separated strands of SV40 DNA. Previous studies had shown that cytoplasmic early and late SV40 RNAs anneal exclusively with the minus (E) and plus (L) strands of viral DNA, respectively. In a control experiments with cytoplasmic RNA prepared from infected cells late in the lytic cycle (Fig. 2A), approximately 25% and 40% of the plus (L) strand DNA annealed with cytoplasmic late-lytic RNA in the 19S and 16S regions, respectively. Ten percent of the minus (E) DNA strand annealed with cytoplasmic late-lytic RNA in the 19S region of the gradient. While the percentage of hybridization in these experiments does not represent a saturation level, it does suggest

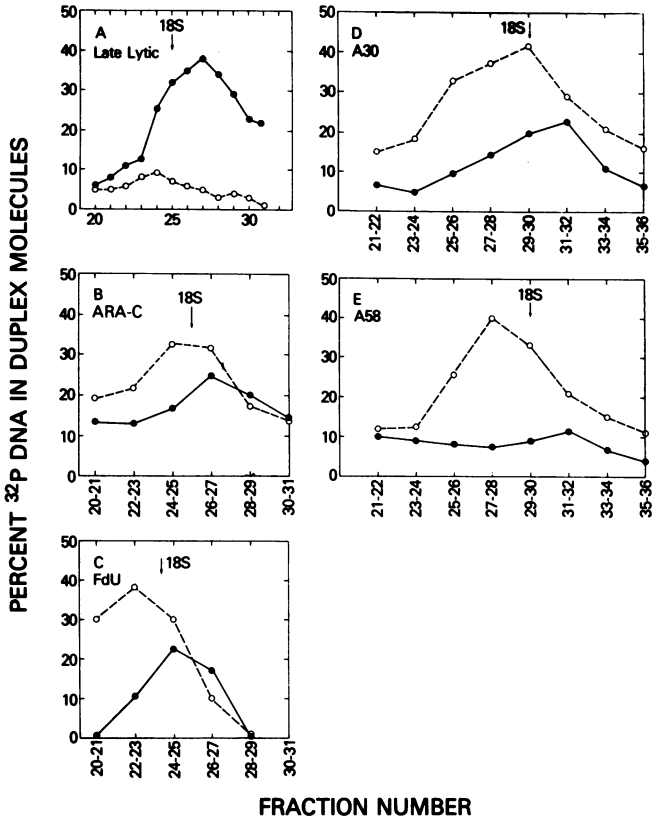

Figure 2: Hybridization of cytoplasmic poly(A) - containing RNA from the sucrose gradients shown in Fig. 1 (A,B,C,E,F) to the fragmented plus (●) and minus (○) strands of ^{32}P-labeled SV40 DNA. RNA from various adjacent fractions of the sucrose gradients shown in Fig. 1 were pooled and annealed with .3 to 1 ng of ^{32}P-labeled SV40 DNA plus or minus strand fragments for 18 hr at 68°C in 1.0 M NaCl, 0.01 M Tris (pH 7.5) and .04% SDS in a volume of 0.1 - 0.2 ml. The percent ^{32}P-labeled DNA in duplex molecules was determined by HA chromatography. It should be noted that the different concentrations of RNA were employed from the various gradients and that the levels of hybridization do not represent saturation values.

that the most abundant cytoplasmic viral RNA species present at 48 hr post-infection is the late 16S RNA.

When analogous studies were performed with cytoplasmic RNAs extracted at 48 hr from cultures infected in the presence of Ara-C (Fig. 2B) and FdU (Fig. 2C), RNA was detected that had been transcribed from both DNA strands. Under Ara-C inhibition at least 33% of the E strand and 25% of the L strand were transcribed; during FdU inhibition, greater than 38% of the E strand and 20% of the L strand were represented in DNA-RNA hybrid

molecules. These studies showed that transcription of RNA from the plus (L) strand of SV40 DNA occurred in the presence of inhibitors employed at levels heretofore assumed to effectively inhibit DNA replication. Furthermore, this RNA sedimented in the 16S region of the neutral sucrose gradients where the predominant late SV40 RNA species has been found. Since the percent hybrids detected in these studies did not represent saturation levels, additional studies were performed (see below) to determine the extent of early and late transcription.

In Figs. 2D-E the control of the late transcription was studied with early mutants tsA30 and tsA58. Viral DNA replication has been reported to be inhibited after infection with these mutants at the restrictive temperature (41°C) (26). Hybridization with fragments of the minus (E) DNA strand was maximal in the 19S region of both gradients (approximately 40%). The RNA obtained from tsA30 infected cells at 41°C showed a prominent peak of hybridization (20%) in the 16S region with ^{32}P-labeled plus (L) strand of SV40 DNA indicating that late transcription had been initiated. This peak of hybridization was not observed with RNA obtained from cells infected with tsA58 at 41°C.

Effect of Inhibitors on Viral DNA Synthesis: It was important to establish if any DNA replication had occurred in cultures infected by wild type SV40 in the presence of Ara-C, FdU, or chloroquine, or in cultures infected by tsA mutants of SV40 at the restrictive temperature since a breakthrough in viral DNA replication would be a possible explanation for the low levels of late transcription observed above (Figs. 1,2). Thus, a series of experiments was performed in which infected cultures were labeled in the presence of inhibitors (or at 41°C with tsA mutants) with ^{3}H-thymidine, (or with ^{3}H-deoxyadenosine in the case of FdU-inhibited cultures). Hirt supernatants from these cultures were prepared as described in Materials and Methods and examined for the presence of viral DNA replication (Fig. 3). The mock-infected cultures (Fig. 3A) showed the presence of a prominent cellular ^{3}H-DNA peak banding at a density similar to SV40 DNA Component II. However, essentially no DNA was found in the region where marker ^{14}C-SV40 Component I DNA bands. The presence of cellular DNA in the Hirt supernatant probably arises from fragmentation of cellular DNA during preparation. In Fig. 3B, which presents the Hirt supernatant fractions from a normal lytic infection, incorporation of significant amounts of label into SV40 DNA Component I was observed. In contrast, in the presence of FdU (Fig. 3D) and chloroquine (Fig. 3E), viral DNA

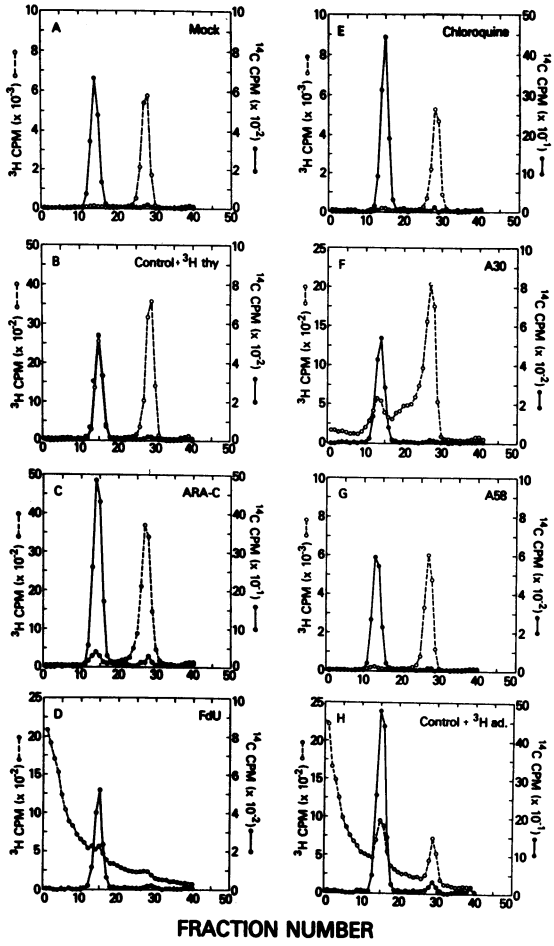

Figure 3: Isopycnic banding of SV40 DNA molecules in propidium diiodide-cesium chloride gradients. BSC-1 cultures uninfected (mock), infected with wild type SV40 in the presence or absence of inhibitors of DNA replication, or infected with temperature sensitive SV40 (A) mutants were labeled with either ^{3}H-deoxyadenosine or ^{3}H-thymidine (see Materials and Methods). Viral DNA, extracted by the method of Hirt (23), was mixed with marker ^{14}C-SV40 DNA I (and a small amount of SV40 DNA II) and centrifuged for 48 hr at 42,000 rpm in a Spinco 50 Ti rotor at 5°C. The direction of increasing density is from right to left. (0--0), ^{3}H DNA; (●--●) ^{14}C DNA.

replication as detected by the incorporation of ^{3}H-labeled nucleotides into DNA Component I was markedly reduced if not eliminated. In the case of Ara-C (Fig. 3C) there was the suggestion of the presence of a small peak in the region where Component I SV40 bands, but subsequent hybridization studies made this observation seem questionable (see Table I).

DNA replication was next examined using the early ts mutants tsA30

and tsA58 (Figs. 3F and 3G, respectively). Incorporation of ^{3}H-thymidine into DNA banding in the region of SV40 Component I was clearly detected after infection with tsA30 but not after infection with tsA58 at 41°C. This finding with tsA30 suggests that some breakthrough of viral DNA replication may have occurred at the restrictive temperature.

Further studies using DNA-DNA filter hybridization methods were employed in an attempt to provide another assay for the effectiveness of inhibitors or early ts mutants in blocking viral DNA replication. These experiments were performed with an excess of unlabeled SV40 DNA on the filters as described in Materials and Methods; the results are presented in Table 1. In control experiments using purified ^{3}H-labeled SV40 DNA, it was shown that as little as 200-250 virus-specific input counts could be registered unequivocally in the filter hybridization assay with an efficiency of about 11%. The level of SV40 DNA filter hybridization in infected cultures labeled with [^{3}H]-deoxyadenosine was approximately 4%, while 13% of the total input DNA in Hirt preparations from infected cells labeled with [^{3}H]-thymidine was found to be SV40 specific. In contrast, the amount of labeled SV40 DNA present in cultures treated with inhibitors of DNA synthesis was extremely small or negligible (.01% to .06%). Mock cultures showed hybridization levels of less than 0.01% of the input radioactivity. Only with the Hirt supernatant material from tsA30-infected

TABLE 1. DNA-DNA FILTER HYBRIDIZATIONS

Sample	Total DNA added (cpm)	Hybridized DNA (cpm)*	% Binding
SV40 DNA	23300	2250	9.7
	2330	300	12.9
	233	26	11.2
Mock Infection	85465	5	0.01
Control[a] + ^{3}H-deoxyadenosine	55357	2130	3.85
Control[a] + ^{3}H-thymidine	61577	8160	13.25
FdU	51134	17	0.03
Chloroquine	60095	39	0.06
Ara-C	88514	12	0.01
A30	46031	512	1.11
A58	91563	37	0.04

[a]Control refers to infection with wild type SV40 at 37°C in the absence of inhibitors

*Background of 20 cpm subtracted from data shown

cultures was there a significant level of hybridization (1.11%). These results confirmed the CsCl isopycnic banding experiments (Fig. 3) in demonstrating that DNA replication had not been effectively terminated in tsA30-infected cultures. The absence of a distinct DNA I peak in the dye-density gradients and the question of significant hybridization to SV40 DNA filters suggests that DNA synthesis, within the limits of detection by these methods, is effectively inhibited or markedly reduced in cells infected with tsA58 or with wild type virus in the presence of Ara-C, FdU, or chloroquine.

A Comparison of Nuclear and Cytoplasmic Virus-Specific RNA Synthesized During Inhibition of Viral DNA Replication: A final set of solution hybridization experiments was performed with the separated strands of SV40 DNA, and both nuclear and cytoplasmic RNAs from cells infected by tsA58 at 41°C or by wild type virus in the presence of Ara-C or FdU (Fig. 4). These experiments were performed in an attempt to determine the extent of trans-

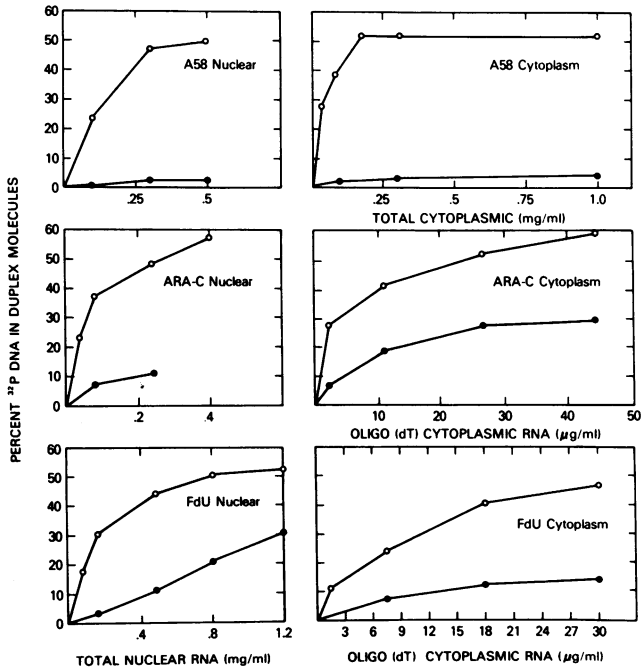

Figure 4: Hybridization of total nuclear RNA and either total cytoplasmic RNA or oligo(dT)-cellulose selected cytoplasmic RNA to the separated fragments of the plus (●) and minus (○) strands of ^{32}P-labeled SV40 DNA. Increasing concentrations of RNA extracted from BSC-1 cultures infected with tsA58 at 41°C or with wild type SV40 in the presence of Ara-C or FdU were incubated with 0.3 to 1.0 ng of SV40 DNA for 18 hr at 68°C in 0.1 to 0.2 ml of 1.0 NaCl, 0.01 M Tris (pH 7.5), 0.04% SDS. The percent ^{32}P-labeled DNA in duplex molecules was determined by HA chromatography.

cription of each viral DNA strand and to evaluate the possible processing of certain RNA species between the nucleus and cytoplasm.

It is clear in all cases that a full complement of early RNA (representing about 50% of the minus strand) is found in both the nucleus and the cytoplasm. There appears to be essentially no RNA complementary to the plus DNA strand in either the nucleus or the cytoplasm of cells infected by tsA58 at 41°C. Therefore, it would seem likely that the absence of late cytoplasmic SV40-specific RNA from these cultures reflects a control at the level of transcription rather than in processing.

In cultures infected in the presence of Ara-C and FdU, RNA complementary to the plus DNA strand can be detected in both the nucleus and cytoplasm. In the case of the nuclear preparations, the extent of transcription is difficult to ascertain since there was insufficient RNA to reach saturation. In the cytoplasm of the Ara-C and FdU treated cultures SV40 RNA complementary to at least 30% and 15% of the plus strand, respectively, was detected. These values clearly represent less than a full complement of late RNA (52% of the plus strand) (7,8,10,27). Studies are presently in progress to determine if this RNA is complementary to a specific and limited portion of the late template.

DISCUSSION

It has been well established that two classes of viral specific RNA are synthesized during the lytic cycle. Prior to the onset of viral DNA replication, which occurs at about 12 hr after infection, only early RNA is synthesized. A number of previous studies suggested that if inhibitors of DNA synthesis were added to infected cultures, early RNA continued to be synthesized in the absence of any late viral specific RNA, even at late times after infection (12-14,16). In contrast, we have found that a decreased but significant amount of late RNA is synthesized in the presence of certain inhibitors of DNA replication (Ara-C, FdU, and chloroquine) when used at concentration equal to or greater than those generally employed. Similar observations have recently been made by others in polyoma virus infected cells grown in the presence of FdU (P. Beard, N. Acheson and I. Maxwell, H. Turler and R. Weil, personal communications). The detection of late viral-specific RNA in our studies (using ^{32}P-labeled separated strands of SV40 DNA) in contrast to previous methods (e.g., competition filter hybridization) might be explained on the basis of an increased sensitivity in the method of detection.

It is clear that the late SV40 RNA found after infection of BSC-1

cells at 41°C by tsA30 can be explained by a breakthrough in viral DNA replication; newly synthesized viral DNA was easily demonstrated both in cesium chloride-propidium diiodide equilibrium gradients and in DNA filter hybridization tests. The leakiness of this particular mutant has also been observed by others (E. May et al., personal communication). On the other hand, there was little or no detectable viral DNA replication or late viral RNA synthesis in cultures infected with tsA58 at 41°C. Furthermore, the absence of any significant amount of late viral-specific RNA from the nuclei of tsA58-infected cells complementary to the plus (L) SV40 DNA strand suggests that late RNA is either not synthesized, synthesized at very low levels, or is rapidly degraded after synthesis. These results are in contrast to the pattern of transcription observed in BSC-1 cells infected with wild type SV40 in the presence of Ara-C, FdU, or chloroquine. In each of the inhibitor-treated cultures the presence of some RNA complementary to the plus (L) strand was detectable in both the nuclei and the cytoplasm. The reason for the synthesis of late viral RNA in cultures infected in the presence of inhibitors of DNA replication (Ara-C, FdU, and chloroquine) is still not clear. A trivial explanation would be that sufficient viral DNA synthesis occurs in spite of the addition of these inhibitors to allow for low levels of late transcription. While a definite peak of newly synthesized DNA co-banding with SV40 form I is seen only after infection with tsA30 (Fig. 3), there is perhaps the suggestion of a very small peak in this region in a number of the other gradients. Although the percentage of input radioactivity from each of these cultures binding to SV40 DNA-containing filters is very low (12 to 39 cpm, or .01 to .06% of the input radioactivity), these levels are slightly higher than those obtained with DNA from the mock infected culture (5 cpm or .01% of input). Thus, we cannot exclude the possibility that a low level of DNA replication may provide the template and/or the necessary factor(s) required for late transcription.

On the other hand, it was noted that the CsCl-propidium diiodide gradient profiles (Fig. 3) and the SV40 DNA filter binding values (Table 1) for tsA58-infected cell supernatant DNA are similar to those of the Hirt supernatant fractions obtained after infection in the presence of the various inhibitors of DNA synthesis. Yet, we did not find late SV40-specific RNA in the nucleus or the cytoplasm of cells infected with tsA58 at 41°C. Thus, the low levels of late transcription detected in cultures infected by SV40 in the presence of inhibitors may relate to

the stage at which viral DNA replication is blocked. Previous studies (see 17) have shown that at least FdU and Ara-C inhibit DNA replication at a step subsequent to initiation while other studies (26) have shown that initiation of viral DNA synthesis is blocked in tsA58 infected cells at 41°C. Therefore, it is possible that the promotion of late SV40 transcription requires only the initiation of viral DNA replication and not chain elongation or segregation or progeny molecules.

An alternative explanation is that the synthesis of small amounts of late RNA might require the presence of a normal, functioning gene A protein and not the subsequent initiation of viral DNA replication, which is itself dependent on the gene A protein. In either case, it is clear that without normal viral DNA replication, the high concentrations of late SV40-specific RNA, which normally accumulate during the lytic cycle, are not found. It seems likely, therefore, that the bulk of late viral-specific RNA is transcribed either from replicative intermediates as suggested by Girard et al., (28) or from progeny viral DNA molecules prior to their encapsidation.

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