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Virus-Specific Transcription in 3T3 Cells Transformed by the ts-a Mutant of Polyoma Virus

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Virus-specific RNA transcription has been measured in 3T3 cells transformed by the ts-a mutant of polyoma virus by RNA-excess hybridization to the separated strands of polyoma DNA. In two cloned sublines maintained at 39°C, the nonpermissive temperature for the A gene function, RNA transcripts of a large fraction of the "early" strand are detected in both nuclear and cytoplasmic RNA fractions, but no "late" strand transcription is detected. Temperature shift to 31.5°C, the permissive temperature, induces viral DNA replication and virus production accompanied by late strand transcription. In two independently derived noninducible cell lines, L strand transcription is never observed, even after cultivation at the permissive temperature. A smaller fraction of the E strand is transcribed in each noninducible cell than in its inducible parent, and this difference is further characterized as a lack of transcripts of portions of *Hpa*II restriction endonuclease fragments 2 and 6.

The ts-a mutant of polyoma virus is a conditional lethal mutant of polyoma (6, 7) which grows normally at the permissive temperature (31°C), but is defective in viral DNA synthesis at the nonpermissive temperature (39°C). Mouse 3T3 cells have been transformed by the ts-a mutant (21) and maintain their transformed phenotype if propagated at the nonpermissive temperature for replication of the virus. If the ts-a 3T3 cells are shifted to 31°C, a variable fraction of the cells begins to produce ts-a virus. Viral DNA synthesis in these temperature-shifted cells is unusual in that much of the viral DNA is larger than superhelical monomer DNA (4). In two cloned sublines of a single ts-a-transformed cell, a defective oligomer (1.54 times the size of a viral monomer in the case of subline 15 and 1.77 times the size of a viral monomer in the case of subline 1) is the major intercellular viral DNA species (13, 21). For each of these sublines, a noninducible derivative has been selected by its ability to survive prolonged cultivation at 31°C. These noninducible derivatives are still superinfectable by polyoma virus, and still retain at least some viral DNA sequences in their high-molecular-weight cellular DNA (Blangy and Vogt, unpublished observations).

Virus-specific transcription has been examined in these two inducible ts-a 3T3 cell sublines maintained at the nonpermissive temperature for the ts-a viral mutant (39°C), or after shift to 31°C, the permissive temperature for replication of ts-a mutant virus.

The extent, cellular localization, and strand orientation of virus-specific transcription have been measured by hybridization of excess cellular RNA to radioactively labeled separated strands of polyoma DNA, or to the strands of specific fragments of the polyoma genome generated by cleavage with *Hpa*II restriction endonuclease. Virus-specific transcription in each inducible transformed line has been compared with that in its noninducible derivative.

MATERIALS AND METHODS

Cells. The derivation and growth of sublines 1 and 15 from the ts-a-transformed 3T3 clone, TRF2, have been previously described (21). Noninducible derivatives of each subline were selected as surviving colonies during continued growth at 31°C in the presence of receptor-destroying enzyme. The ts-a cells were routinely carried at 39°C in the presence of receptor-destroying enzyme (Microbiological Associates, Inc.). Cells for the extraction of RNA were grown in disposable glass roller bottles (Bellco) and harvested when subconfluent. Cells were shifted to 31.5°C when less than 50% confluent and grown at 31.5°C for 40 h.

Viral DNA. Unlabeled viral DNA was extracted from 3T6 clone 10 cells (a clone of 3T6 cells highly susceptible to polyoma infection) 40 to 48 h after infection with 10 to 25 PFU of plaque-purified wild-type polyoma per cell. Viral DNA was selectively extracted by the procedure of Hirt (9). Hirt extracts were phenol and chloroform extracted, ethanol precipitated, and purified by equilibrium density centrifugation in CsCl gradients containing 200 μg of ethidium bromide per ml. Superhelical polyoma DNA was further purified by sedimentation through

5 to 20% sucrose gradients in 1.0 M NaCl, 10 M Tris (pH 8.0), 0.005 M EDTA. The 21S viral DNA was dialyzed against 0.01 M Tris (pH 8.0), 0.001 M EDTA (21).

The ^{3}H-labeled viral DNA was isolated from primary baby mouse kidney cultures infected with 10 to 25 PFU of plaque-purified wild-type polyoma per cell and cultivated in fortified Eagle medium containing 10% dialyzed horse serum, 6×10^{-5} M fluorodeoxyuridine, 2.2×10^{-5} M deoxycytidine, and 3×10^{-4} M uridine. Cells were labeled at 5 and 19 h postinfection with 60 μCi of [^{3}H]thymidine per ml (6.7 Ci/mmol, New England Nuclear).

Viral DNA was purified as described for unlabeled polyoma DNA 40 h after infection.

Iodinated polyoma DNA. Polyoma DNA was iodinated by a modification of the procedure of Commerford (3) to a specific activity of 2.7×10^7 cpm/μg. Iodinated DNA was purified by exhaustive self-annealing and chromatography on hydroxyapatite, and viral DNA strands were separated as described for ^{3}H-labeled DNA. With such iodinated viral DNA, higher ratios of cellular RNA to labeled viral DNA could be achieved.

***Hpa*II restriction enzyme fragments.** Polyoma DNA was digested with *Hpa*II endonuclease (Biolab), and DNA fragments were purified by continuous electroelution as previously described (21). Fragments were further purified by electrophoresis through 1.4% agarose gels (19). Bands were located by staining with 5 μg of ethidium bromide per ml in electrophoresis buffer. The gel was crushed by forcing it through an 18-gauge needle, and DNA was eluted from the crushed gel by incubation at 45°C in 0.2 M NaCl, 0.5% sodium dodecyl sulfate (SDS), 0.01 M Tris, pH 7.4, 0.001 M EDTA, and 50 μg of yeast RNA per ml, for 12 h. The eluted DNA was phenol-chloroform extracted, chloroform extracted, and ethanol precipitated.

Nick translation of restriction enzyme fragments (18). Because chemical iodination of the small amounts of purified restriction enzyme fragments recovered from agarose gels proved difficult, purified fragments were enzymatically labeled in vitro by nick translation. Purified DNA fragments were dialyzed into 0.05 M potassium phosphate buffer, pH 7.5, 0.005 M $MgCl_2$. DNA was labeled in an in vitro reaction mixture containing 5 μg of DNA per ml, 0.2 μg of DNase I per ml (Worthington), 30 μM [^{3}H]dTTP, 60 μM dCTP, 60 μM dGTP, 60 μM dATP, and 30 U of DNA polymerase I per ml (a gift of I. Verma). After incubation at 13°C for 16 to 24 h, the reaction mixture was brought to 0.05 M EDTA and incubated for 10 min at 60°C. The DNA was purified by phenol and chloroform extraction and chromatography on Sephadex G-75. Hairpin structures were removed by passing the DNA over hydroxyapatite immediately after heat denaturation in 0.14 M sodium phosphate buffer, pH 6.8, 0.1% SDS. Specific activities of 5×10^6 to 2×10^7 cpm/μg were achieved.

Preparation of asymmetric cRNA. Complementary RNA (cRNA) was synthesized in a reaction mixture containing 0.04 M Tris, pH 7.9, 0.01 M $MgCl_2$, 0.001 M DTT, 0.001 M each ATP, GTP, CTP, and UTP, 0.45 M KCl, 10 μg of form I polyoma DNA per ml, and 2 U of RNA polymerase (Miles) per μg of form I polyoma DNA. After incubation at 37°C for 2 to 3 h, the reaction mixture was treated with 20 μg of DNase per ml (Worthington) for 30 min. SDS was added to a final concentration of 0.5% and EDTA to 0.02 M, and the RNA was purified by phenol and chloroform extraction. RNA was precipitated by storage at 4°C overnight in 2.0 M NaCl. RNA was redissolved in 2 × SSC and self-annealed at 68°C for 1 to 2 h. The single-stranded RNA was separated from partially double-stranded RNA hybrids by chromatography on CF11 cellulose (5) and was used to separate the strands of polyoma DNA.

Strand separation of polyoma DNA. Polyoma DNA was separated into its complementary strands by hybridization with an excess of asymmetric cRNA and chromatography on hydroxyapatite (17). The cRNA was present in a 50- to 100-fold weight excess for the separation of sonicated polyoma DNA or in 10- to 15-fold sequence excess for the separation of restriction enzyme fragments. Strand separation was performed as described by Kamen except that hydroxyapatite was used in columns rather than batchwise. Self-annealing of the separated strands was accelerated by the inclusion of one-tenth volume of 90% phenol and rapid vortexing according to Kohne et al. (D. E. Kohne, S. A. Levinson, and M. I. Byers, Biochemistry, in press). The resulting phenol emulsion was chloroform extracted before chromatography on hydroxyapatite.

Hybridizations. RNA-DNA hybridizations were performed in 1.0 M NaCl, 0.01 M TES [*N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], 0.001 M EDTA, 0.1% SDS, pH 7.0, at 68°C at RNA concentrations of 10 to 20 mg/ml and DNA concentrations of 1 to 3 ng/ml for variable periods of time. Hybridization was assayed by dilution of appropriate-sized samples (6 to 20 λ) into at least a 10-fold excess of S1 digestion buffer containing 0.25 M NaCl, 0.05 N sodium acetate, 0.0025 M $ZnSO_4$, 25 μg of single-stranded calf thymus DNA per ml, and 2.5 μg of double-stranded calf thymus DNA per ml at pH 4.5. Samples were digested with S1 nuclease (Miles) for 2 h at 45°C, precipitated with 5% trichloroacetic acid after the addition of 50 μg of yeast RNA carrier per ml, trapped on membrane filters (Millipore Corp., type HAWP), and counted in toluene-based liquid scintillation fluor (Liquifluor, New England Nuclear). C_rt values were calculated as the concentration of RNA in moles of nucleotide per liter multiplied by the time of hybridization in seconds. All values have been corrected to standard conditions (0.18 M Na^+) according to Britten and Smith (2).

Cellular RNA extractions. Cells grown in glass roller bottles at 39 or 32°C were removed from the bottles by trypsinization at 4°C and gentle scraping. Cells were collected by centrifugation and washed once with Tris buffer. Cells were resuspended in 20 volumes of isotonic lysing buffer (0.15 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M $MgCl_2$) and lysed by the addition of 0.5% Nonidet P-40. After incubation on ice for 2 to 5 min and rapid vortexing for 30 s, nuclei were pelleted by centrifugation at 3,500 × *g* for 10 min. RNA was extracted from the nuclear and cytoplasmic fractions as previously described (18). The

RNA was redissolved in 0.01 M Tris, pH 7.4, 0.01 M $MgCl_2$, and digested with 20 μg of pancreatic DNase per ml (Worthington) at 37°C for 30 min for cytoplasmic RNA fractions, and 60 min, with the addition of a second 20 μg of DNase per ml after 30 min, for nuclear RNA fractions. The RNA was phenol and chloroform extracted as before, ethanol precipitated, and passed over G-75 in 0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA, after heat denaturation. The excluded RNA was pooled, concentrated by ethanol precipitation and lyophylization, and used in hybridization experiments with the separated strands of polyoma DNA. RNA prepared in this manner was free of contaminating polyoma DNA sequences as judged by the inability of alkaline-hydrolyzed RNA samples to protect labeled polyoma DNA from S1 digestion when hybridized for the longest period of time at the highest RNA concentration used in the experiments reported here.

Double-stranded "symmetric" RNA. The double-stranded RNA fraction of cellular RNA preparations was prepared by self-annealing a portion of each RNA at a concentration of 10 mg/ml for 24 h at 68°C in 0.5 M NaCl, 0.01 M TES, 0.001 M EDTA, 0.1% SDS. The self-annealed RNA was diluted with an equal volume of water, ethanol precipitated, and redissolved in 0.3 M NaCl, 0.01 M TES, pH 7.0, 0.001 M EDTA. Half of each sample was digested for 90 min at 37°C with 10 μg of RNase A per ml and 5 U of RNase T_1 per ml. All RNA samples were brought to 1% SDS and 50 μg of predigested Pronase per ml, and incubation continued for 30 min at 37°C. Samples were phenol and chloroform extracted, precipitated after the addition of yeast RNA carrier, and resuspended at equal volumes for hybridization to separated E and L strands.

RESULTS

DNA strand separation. The complementary strands of radioactively labeled polyoma DNA were separated by hybridization to an excess of asymmetric cRNA followed by fractionation on hydroxyapatite (10, 17). Synthesis of cRNA in vitro using *Escherichia coli* DNA-dependent RNA polymerase was carried out in a reaction mixture containing 0.45 M KCl in order to improve the asymmetry of the RNA product transcribed from polyoma form I DNA (10). Under these conditions, the RNA product is large (average size 16-19S, as measured by velocity sedimentation in gradients containing 99% Me_2SO_4) and predominantly (about 90%) a copy of the L DNA strand.

Contaminating E strand transcripts, which would interfere with strand separation, were removed by exhaustive self-annealing of the cRNA and removal of double-stranded RNA on CF11 cellulose (5). Hybridization of an excess of this purified cRNA with polyoma DNA and fractionation on hydroxyapatite leads to the separation of the polyoma DNA into two fractions, about 50% eluting as single-stranded DNA, designated the E strand, and 50% eluting as an RNA-DNA hybrid, designated the L strand. Table 1 indicates the properties of these two DNA fractions after they have been further purified by extensive self-annealing and refractionation on hydroxyapatite.

RNA transcription in 3T6 cells productively infected with wild-type polyoma. These experiments, which were performed primarily to characterize the preparation of separated DNA strands, in general confirm the results reported by Kamen et al. (10, 11) and Beard et al. (1). Nuclear and cytoplasmic RNA fractions were isolated from 3T6 cells infected at high multiplicity (20 to 30 PFU/cell) with wild-type polyoma virus. Late lytic RNA was isolated 26 to 30 h after infection, whereas early lytic RNA was isolated 18 h after infection from cells treated with 20 μg of cytosine arabinoside per ml after infection. Figure 1 illustrates the results obtained when these RNA preparations are hybridized to the separated strands of polyoma DNA. Polyoma L strand was also hybridized with an excess of pure complementary RNA. Comparison of the $C_rt_{1/2}$ value for this stranded reaction of a single-stranded DNA with its complementary RNA with the $C_rt_{1/2}$ values obtained with cellular RNA fractions allows an estimation of the concentrations of polyoma-specific RNA in various RNA fractions. As has been previously reported (10), at late time after lytic infection, when viral DNA replication has begun, RNA complements to the L or "late" strand of polyoma predominate. In the nucleus, polyoma-specific RNAs complementary to the entire L strand are abundant, amounting to about 0.4% of the nuclear RNA ($C_rt_{1/2}$ = 4.6). Complements to approximately 60% of the L strand are found in total cytoplasmic RNA, amounting to about 0.13% of the cytoplasmic

TABLE 1. *Annealing properties of separated strands of polyoma ^{3}H-labeled DNA*[a]

Additions	% S1 resistant	
	E strand	L strand
None	2.5	2.7
cRNA	2.5	88.8
Polyoma DNA	86.6	89.8

[a] Polyoma ^{3}H-labeled DNA, specific activity 2.4 × 10^6 cpm/μg, was separated into its complementary strands as described in Materials and Methods. E strand at a concentration of 5.3 ng/ml or L strand at a concentration of 5.1 ng/ml was annealed for 24 h at 68°C in 0.5 M Nacl, 0.01 M TES, pH 7.0, 0.001 M EDTA, 0.1% SDS. When present, asymmetric cRNA was present in a 250-fold weight excess and sonicated polyoma DNA was present in a 900-fold weight excess.

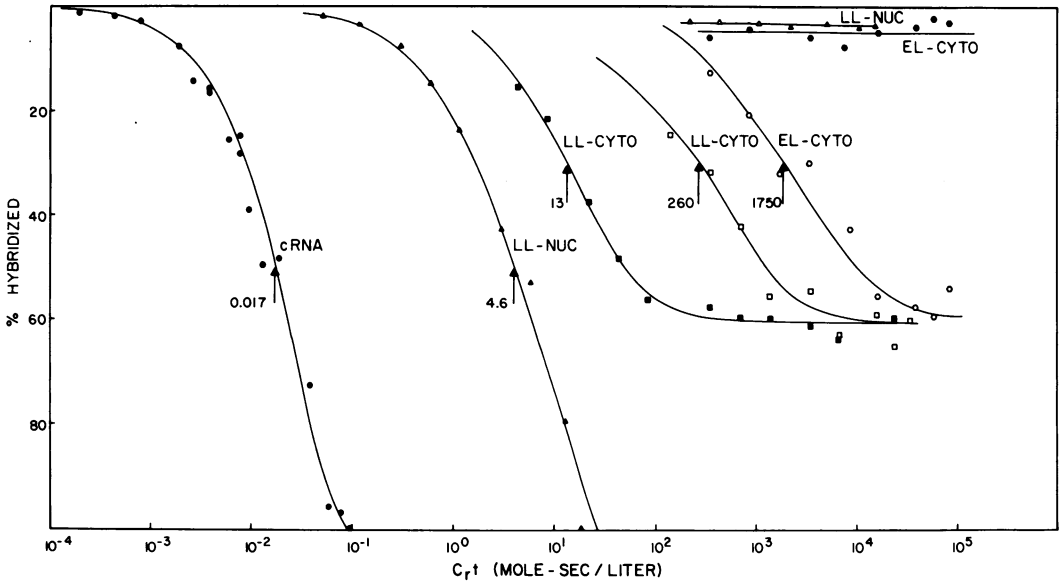

FIG. 1. *Hybridization of RNA from productively infected 3T6 cells to E and L strands of polyoma DNA. L strand DNA (47 ng/ml) was hybridized with: (●) cRNA, 50 and 500 ng/ml; L strand, 2.4 ng/ml; (▲) late lytic nuclear RNA, 17 μg/ml; (■) late lytic cytoplasmic RNA, 1.31 mg/ml; (⬣) early lytic cytoplasmic RNA, 20 mg/ml. E strand DNA (47 ng/ml) was hybridized with: (△) late lytic nuclear RNA, 10 mg/ml; (□) late lytic cytoplasmic RNA, 1.31 mg/ml; (○) early lytic cytoplasmic RNA, 20 mg/ml, and aliquots (6 to 10 λ) were withdrawn at various times. Hybridization was assayed by S1 nuclease digestion. C_0t values are corrected to 0.18 M Na^+ (2).*

RNA. Since our isolation procedure yields roughly four times as much cytoplasmic as nuclear RNA, the total amount of polyoma cytoplasmic RNA is approximately equivalent to the nuclear amounts, although only part of the genome is represented. As reported by Kamen et al. (10), E strand transcripts are undetectable in late lytic nuclear RNA fractions although transcripts of 50 to 60% of the E strand are found at low concentrations (0.006%) in the cytoplasm. This is presumably due to the presence of excess complementary RNA (L strand transcripts) which successfully compete with the labeled DNA probe.

Early after lytic infection, in cells in which viral DNA replication has been blocked by cytosine arabinoside, only E strand transcripts are detected. Complements to 50 to 60% of the E strand are detected in the cytoplasm, comprising about 0.001% of the cytoplasmic RNA.

Transcription in ts-a-transformed 3T3 cells at high and low temperature. The extent and strand orientation of polyoma-specific transcription was measured in two sublines of 3T3 cells transformed by the ts-a mutant of polyoma at 39°C, where free virus or viral DNA is rarely detected, or 40 h after shift to 31.5°C when viral DNA synthesis including the production of characteristic viral oligomers is readily observed. At the nonpermissive temperature (39°C), complements to a large fraction of the E strand can be detected in both the nucleus and cytoplasm of each inducible ts-a-transformed line, but no L strand transcripts are detectable (Fig. 2 and 3). In subline 15, 80% of the E strand sequences are represented in nuclear RNA, whereas 68% are represented in the cytoplasm. RNA extracted from the nuclei of subline 1 cells protects 80% of E strand DNA, whereas cytoplasmic RNA protects at least 65 to 68% of the E strand. A similar fraction of the E strand is protected by RNA isolated from cells shifted to 31.5°C. Mixing experiments in which RNA isolated from the cytoplasm of cells grown at 39 and at 31.5°C was mixed and hybridized to the E strand gave a similar value for the fraction of the E strand protected, suggesting that the same RNA sequences are found in the cytoplasm of cells grown at 39 or 31.5°C (values for the fraction of the E strand protected and the concentration of this RNA are summarized in Table 2).

A striking consequence of the shift to permissive temperature is the appearance, in both nuclear and cytoplasmic fractions, of L strand transcripts which protect up to 80% (nuclear RNA) of the L strand. The exact extent and concentration of these L strand transcripts is

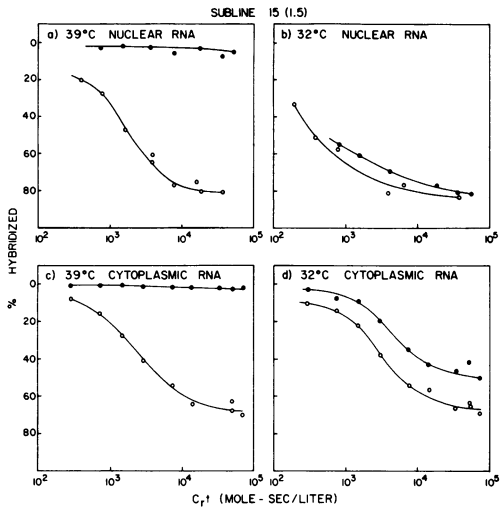

FIG. 2. *Hybridization of RNA from subline 15 to E and L strands of polyoma DNA.* (●) *L strand (47 ng/ml) or* (○) *E strand (47 ng/ml) was hybridized with (a) nuclear RNA from subline 15 cells maintained at 39°C, 10 mg/ml; (b) nuclear RNA from subline 15 cells 40 h after shift to 31°C, 10.6 mg/ml; (c) cytoplasmic RNA from subline 15 cells maintained at 39°C, 20 mg/ml; (d) cytoplasmic RNA from subline 15 cells 40 h after shift to 31°C, 20 mg/ml.*

difficult to determine in the presence of an excess of complementary E strand transcripts.

Transcription in noninducible ts-a-transformed cells. Noninducible derivatives of each of the ts-a-transformed sublines have been selected which are unable to produce virus or replicate viral DNA after a shift to permissive temperatures (31.5°C), even though polyoma DNA sequences are retained in the cellular genome of these cells (D. Blangy and M. Vogt, unpublished observation). The pattern of polyoma-specific transcription in these cells is summarized in Fig. 4 and 5. A large fraction of the E strand is transcribed in the nucleus (70 to 80% for subline 1 and 60% for subline 15) and some of these E strand sequences are also found in the cytoplasm at both high and low temperature. However, L strand transcripts are never detected in these cells, even after cultivation at 31.5°C. In addition, in subline 15, there are differences in the fraction of the E strand transcribed in inducible and noninducible cells. At both high and low temperature, the noninducible cells produce stable nuclear transcripts complementary to only 60% of the E strand, whereas inducible cells transcribe 80%. In the cytoplasm 66 to 70% of the E strand is represented in RNA transcripts in inducible cells, but only 45 (at 31.5°C) to 60% (at 39°C) is represented in noninducible cells. The cytoplasmic RNA in the noninducible cell line appears to contain a subset of the polyoma E strand sequences present in the cytoplasm of inducible cells, since a mixture of these two RNA fractions does not protect any more of the E strand than does inducible cell RNA alone. These hybridization experiments using unfractionated E strands do not reveal any consistent differences between E strand transcripts of inducible or noninducible cells of subline 1. These cells do, of course, differ in their ability to transcribe L strand RNA sequences at permissive temperatures.

Symmetric transcription. No L strand RNA transcripts can be detected in ts-a-transformed cells grown at the nonpermissive temperature or in their noninducible derivatives grown at either temperature. However, it is possible that such late RNAs might be present at low levels in the cells but are prevented from reacting with L strand DNA by the presence of an excess of complementary early strand RNA transcripts. For example, late in lytic infection, early strand transcripts cannot be detected in nuclear or whole cell RNA preparations used in RNA-excess hybridization experiments, presumably because the large amount of complementary L strand transcripts successfully compete with E strand DNA for hybridization to these rare transcripts (1, 10). To ask whether a similar competition was preventing the detection of rare transcripts of the L strand in ts-a 3T3-transformed cell RNA, symmetric RNA-RNA hybrids were prepared from cell RNA fractions by exhaustive self-annealing and RNase digestion of unhybridized RNA. Table 3 shows the results of hybridizing total cellular RNA fractions or the redenatured RNase-resistant RNA-RNA hybrids prepared from an equal amount of RNA to the separated strands of polyoma DNA. As previously described (10), E strand transcripts can be detected in symmetric RNA prepared from late lytic nuclear RNA but not in the total nuclear RNA. No polyoma-specific symmetric RNA can be detected in the cytoplasm late in lytic infection, as is expected, since the cytoplasmic RNAs from the E and L strand have been localized in nonoverlapping regions of the polyoma genome. When RNA from subline 15, grown at the nonpermissive temperature, is tested in the same way, no symmetric polyoma transcripts can be demonstrated, suggesting that no L strand transcription takes place at nonpermissive temperatures. When subline 15 is shifted to 31°C, where L strand transcription is readily detected by conventional RNA excess hybridization experiments, symmetric nuclear RNA transcripts are found. When RNA from the noninducible deriv-

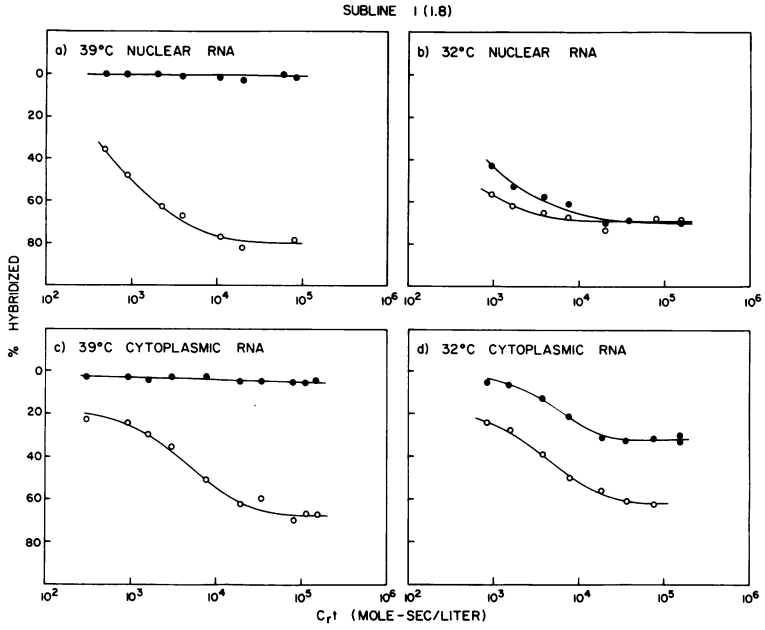

FIG. 3. *Hybridization of RNA from subline 1 to E and L strands of polyoma DNA.* (●) *L strand (a–c, 7.2 ng/ml,* ^{125}*I-labeled; d, 47 ng/ml,* ^{3}*H-labeled);* (○) *E strand (a–c, 6.8 ng/ml,* ^{125}*I-labeled; d, 47 ng/ml,* ^{3}*H-labeled). (a) Nuclear RNA from cells maintained at 39°C, 10 mg/ml; (b) nuclear RNA from cells 40 h after shift to 31.5°C, 10 mg/ml; (c) cytoplasmic RNA from cells maintained at 39°C, 10 mg/ml; (d) cytoplasmic RNA from cells 40 h after shift to 31.5°C, 20 mg/ml.*

ative of subline 15 is hybridized, no evidence for L strand transcripts, either in total RNA or in RNase-resistant hybrids, can be found. This suggests that the observed lack of L strand transcripts does not result from an excess of E strand transcripts and that the noninducible line is in fact incapable of L strand transcription.

Hybridization to isolated restriction enzyme fragments. A more precise measure of the regions of the viral genome transcribed in ts-a-transformed cells can be made by hybridizing transformed cell RNA to the separated strands of individual restriction enzyme fragments prepared from polyoma DNA. The extent of E strand transcription was measured for each of the six largest *Hpa*II restriction enzyme fragments, which together account for 93% of the polyoma genome. The results for cytoplasmic RNAs isolated from both inducible and noninducible ts-a-transformed cells maintained at 39°C, as well as for early lytic RNA, are tabulated in Table 4. A comparison of the cytoplasmic virus-specific RNA present in the cytoplasm of subline 15 and its noninducible derivative, subline 15T, shows that the noninducible cell line contains RNA complementary to only 50% of fragment 2, rather than a complete transcript of this region, and is totally deficient in RNA complementary to fragment 6, which is at least partially represented in the RNA of subline 15. While neither transformed cell contains detectable RNA complementary to fragment 1, both contain "anti-late" complements to fragment 3E. Summation of the fractional lengths of the *Hpa*II fragments represented in RNA indicates that 72% of the E strand has complements in cytoplasmic RNA of subline 15, whereas 55% of the E strand is represented in cytoplasmic RNA from the noninducible subline 15T. Subline 1 differs from subline 15 in the additional presence of cytoplasmic complements to about 40% of fragment 1. Again, the noninducible derivative subline, 1T, differs in the partial (60%) rather than complete transcription of fragment 2 and the lack of transcripts of fragment 6. Mapping with individual restriction fragments suggests that 81% of the E strand is represented in cytoplasmic RNA in subline 1 and only 66% is represented in subline 1T.

These results follow the pattern established by the hybridization experiments performed with the total E strand of polyoma DNA, but yield consistently higher estimates of the fraction of the genome expressed. Complete S1 resistance is difficult to achieve in hybridization experiments which measure reaction of DNA with rare RNA species, where large sequence excesses and sufficiently long times of hybridi-

zation are hard to achieve. Measuring the fraction of the total E strand which becomes S1 resistant therefore probably underestimates the actual extent of virus-specific transcription. Maximum S1 resistance values of 80 to 100% for individual restriction enzyme fragments have been interpreted as complete transcription of the region defined by that fragment. Summation of the fractional lengths of restriction enzyme fragments protected by RNA yields a higher, and probably more accurate, estimate of the extent of E strand transcription.

DISCUSSION

The RNA-excess hybridizations reported here measure the presence of stable RNA species complementary to polyoma DNA in various fractions of ts-a-transformed 3T3 cells. The kinetic analysis of the RNA hybridization was performed primarily to insure that saturation values had been achieved and that RNA was present in sufficient excess. A standard curve was obtained by reacting polyoma L strand DNA with pure complementary RNA and the point at which this reaction was 50% completed ($C_rt_{1/2}$) was measured. Since the kinetics of the RNA-DNA hybridization reaction in RNA excess depend on the concentration of cRNA, the $C_rt_{1/2}$ values obtained for different RNA preparations will be inversely proportional to the concentration of virus-specific RNA in each preparation. By comparison of the $C_rt_{1/2}$ values with the $C_rt_{1/2}$ value for the standard reaction, it can be calculated that virus-specific RNAs are quite rare in these ts-a-transformed 3T3 cells. They comprise 0.001 to 0.01% of the nuclear RNA and 0.0003 to 0.001% of the cytoplasmic RNA fractions. When each of the noninducible cell lines is compared with its parent line, it is clear that the concentration of virus-specific RNA is not significantly reduced in the noninducible derivatives.

If it is assumed that all of the E strand transcription measured is accounted for by one species of RNA molecule, and there are approximately 6 pg of RNA per cell, then the concentration of transcripts of the E strand measured corresponds to 25 to 50 viral RNA molecules per cell. Hybridization at a 10-fold lower concentration to the L strand would have been detected as at least partial protection of the L strand DNA. Since no hybridization to the L strand was detected, there are less than two to five molecules per cell of late-strand RNA when ts-a cells are maintained at the nonpermissive temperature.

When the inducible ts-a-transformed cells are shifted to the permissive temperature for

TABLE 2. *Hybridization of E strand with RNA from ts-a-transformed 3T3 cells*[a]

RNA	% of E strand	$C_rt_{1/2}$ value	% of RNA fraction
Subline 15			
39°C Nuclear	80	1,250	0.0014
39°C Cytoplasmic	68	2,100	0.0008
32°C Nuclear	80	250	0.007
32°C Cytoplasmic	68	2,600	0.0007
Subline 15T			
39°C Nuclear	60	<500	ND[b]
39°C Cytoplasmic	60	800	0.002
32°C Nuclear	60	280	ND
32°C Cytoplasmic	45	1,300	0.0013
Subline 1			
39°C Nuclear	80	600	0.0028
39°C Cytoplasmic	68	2,400	0.0007
32°C Nuclear	70	<300	ND
32°C Cytoplasmic	65	2,000	0.0005
Subline 1T			
39°C Nuclear	75	560	0.003
39°C Cytoplasmic	60	7,600	0.0002
32°C Nuclear	77	510	0.0033
32°C Cytoplasmic	65	3,800	0.0004

[a] The final extent of hybridization for the experiments depicted in Fig. 2 through 5 is indicated, together with the $C_rt_{1/2}$ value where this can be accurately determined. The percentage of each RNA fraction which is virus specific has been calculated by dividing the $C_rt_{1/2}$ value for the reaction of L strand with cRNA (0.017 mol·s/liter) by the observed $C_rt_{1/2}$ values for each cellular RNA fraction.

[b] ND, Not determined.

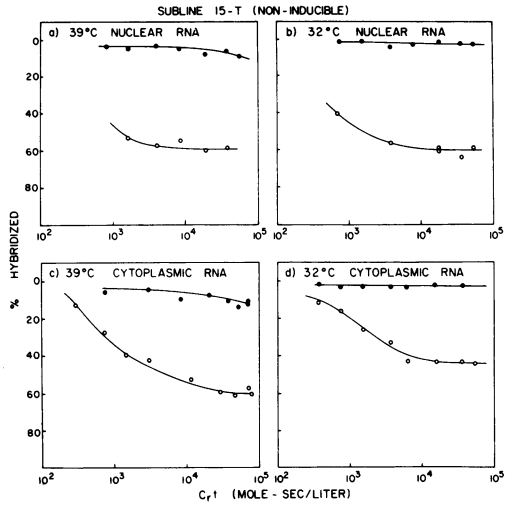

FIG. 4. *Hybridization of RNA from the noninducible subline 15T to E and L strands of polyoma DNA.* (●) *L strand (43 ng/ml) or (O) E strand (37 ng/ml) was hybridized with (a) nuclear RNA from cells maintained at 39°C, 10 mg/ml; (b) nuclear RNA from cells 40 h after shift to 31.5°C, 10 mg/ml; (c) cytoplasmic RNA from cells maintained at 39°C, 20 mg/ml; (d) cytoplasmic RNA from cells 40 h after shift to 31.5°C, 10 mg/ml.*

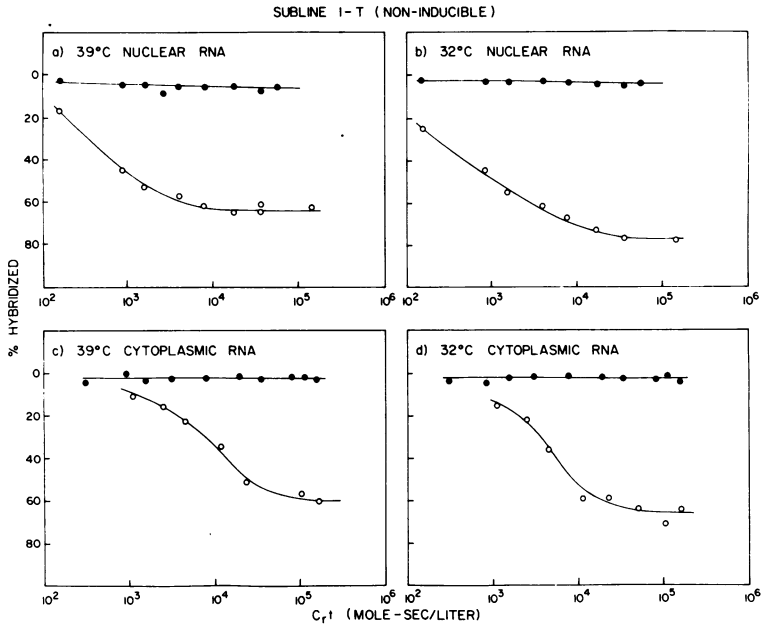

FIG. 5. *Hybridization of RNA from the noninducible subline 1T to E and L strands of polyoma DNA.* (●) *L strand (47 ng/ml for a and b; 28 ng/ml for c and d) or* (○) *E strand (34 ng/ml for a and b, 17 ng/ml in c and d) was hybridized with: (a) nuclear RNA from cells maintained at 39°C, 10 mg/ml; (b) nuclear RNA from cells 40 h after shift to 31.5°C, 10 mg/ml; (c) cytoplasmic RNA from cells maintained at 39°C, 30 mg/ml; (d) cytoplasmic RNA from cells 40 h after shift to 31.5°C, 30 mg/ml.*

TABLE 3. *Symmetric polyoma RNA transcription*[a]

RNA	% S1 resistant			
	E strand		L strand	
	Total RNA	Symmetric RNA	Total RNA	Symmetric RNA
Late lytic				
Nuclear	9.7	42.0	100.0	37.0
Cytoplasmic	45.0	8.5	56	8.4
Subline 15				
39°C Total	38.0	6.8	3.2	8.8
32°C Nuclear	40.0	77.0	32.0	36.0
32°C Cytoplasmic	62.0	6.5	24	4.2
Subline 15-T				
39°C Total	45.0	16.0	5.0	1.7
32°C Total	50.0	12.0	3.5	2.1

[a] Symmetric RNA was prepared and repurified as described in Materials and Methods. RNAs were denatured and hybridized to E or L strand DNA for 72 h.

the ts-a gene function, then L strand transcription is readily detected, as is viral DNA replication, the production of intracellular DNA oligomers, and the release of infectious ts-a virus particles. While it is not completely clear whether this L strand transcription is a direct consequence of the reactivation of the ts-a gene function, or a result of the induction of viral DNA replication, L strand transcription does not follow shift to 31.5°C if DNA synthesis is blocked by cytosine arabinoside. Temperature shift is not accompanied by the appearance of additional E strand RNA sequences, suggesting that the viral defect acting at nonpermissive temperatures is not at the level of viral RNA transcription. Of course, the appearance of RNA molecules of different sizes or with altered arrangements of the same viral sequences cannot be excluded by the experiments reported here. Therefore, it is likely that the ts-a mutation affects some post-transcriptional process, perhaps coding for the synthesis of a

TABLE 4. *Hybridization of ts-a-transformed 3T3 cell cytoplasmic RNAs to the E strand of HpaII restriction enzyme fragments*[a]

RNA	% S1 resistant					
	1[b]	2	3	4	5	6
Early lytic	0	+	0	+	15	30
Subline 15	0	+	+	+	+	60
Subline 15T	0	50	+	+	+	0
Subline 1	40	+	+	+	+	70
Subline 1T	40	60	+	+	+	0

[a] Equimolar amounts of the E strands of fragments 1 through 6 (200 to 1,000 cpm) were hybridized with 300 to 400 μg of RNA to C_0t 5 × 10^5 mol·s/liter and the extent of hybridization was measured by S1 digestion. 0 indicates no hybridization above the self-annealing value of the E strand alone; + indicates maximum S1 resistance. The separated E strands of the restriction enzyme fragments were 5 to 15% S1 resistant after self-annealing, and 80 to 100% S1 resistant after hybridization with excess fragmented polyoma DNA.

[b] *Hpa*II fragment.

temperature-sensitive protein. A likely candidate protein would be the polyoma T antigen, whose expression, as detected by immunofluorescent staining, is temperature sensitive in ts-a-infected cells (14). The inducible ts-a 3T3 cells described in this report are also T antigen negative by immunofluorescent staining at the nonpermissive temperature and become positive after a shift to permissive temperature (15; Bacheler, unpublished data). In addition, Paulin and Cuzin have reported that the T antigen activity of ts-a 3T3 cells is more temperature sensitive in vitro as measured by complement fixation than the T antigen activity of wild-type polyoma-transformed cells (15).

The regions of the early strand present as cytoplasmic RNA at nonpermissive temperatures have been defined by hybridization to purified restriction enzyme fragments. If one assumes that the small region represented by *Hpa*II fragments 7 and 8 (not measured here) is transcribed when the contiguous regions represented by *Hpa*II fragments 2 and 4 are transcribed, then the transcribed regions fall in one contiguous section of the polyoma physical map. In Fig. 6A, the portions of the E strand transcribed are indicated on the map of a 21*S* viral DNA molecule. E strand transcription in these ts-a 3T3 cells includes some "anti-late" transcription of regions not found in cytoplasmic RNA during lytic infection, such as fragments 1 and 3. If transcription proceeds from an integrated viral DNA monomer, this pattern of transcription could result from transcription initiated on a cellular promotor and reading through into viral anti-late sequences. These inducible cell RNAs would then end in the same region of the genome as cytoplasmic early RNA molecules made during lytic infection, perhaps making use of the same signals for termination or processing. Alternatively, transcription may proceed from tandemly arranged viral DNA sequences not present in monomeric DNA molecules. Vogt et al. (21) have proposed detailed structures for the predominant viral DNA oligomers produced by sublines 1 and 15 following temperature-shift induction. It is likely that a viral DNA organization corresponding to the oligomer is present in ts-a-transformed cells maintained at the nonpermissive temperature, since the same oligomer is produced by each subline whenever the cells are shifted to 31°C.

Figure 6B shows a map of the 25.5*S* DNA oligomer produced by subline 15 and the structure of the 27*S* oligomer produced by subline 1. The new *Hpa*II fragment A characteristic of subline 15 probably contains part of the sequences of fragment 2, but not fragments 1 or 6. Fragment O, the characteristic *Hpa*II fragment of subline 1, contains portions of both fragments 1 and 2, but probably not fragment 6. The pattern of E strand transcription observed in inducible cells held at the nonpermissive temperature can be accounted for by an RNA molecule whose 5′ end falls in fragment 5 and whose 3′ end falls within the only fragment 6 in this oligomeric DNA structure. Such a proposed transcript makes use of the same "start and stop" signals, or RNA-processing signals which generate the E strand cytoplasmic transcripts during lytic infections. In this case, RNA transcription from host cell promotors need not be invoked to explain the observed pattern of transcription.

In the two noninducible sublines examined, inability to produce viral DNA, late strand RNA, or virus particles after shift to permissive temperatures was correlated with a lack of transcripts complementary to *Hpa*II restriction enzyme fragment 6 and part of fragment 2. Several mechanisms could account for the lack of RNA transcripts of this region. Transcription might be prematurely terminated in the noninducible lines, or post-transcriptional processing might rapidly degrade transcripts of this region. This portion of the viral genome might be deleted in noninducible cells. It is interesting to note that if the RNA transcription pattern of each noninducible line is mapped onto the viral DNA oligomer of its parent line, transcription through the duplicated defective region (indicated in the figure), containing the new fragments O and A, is sufficient to account for all RNA sequences observed. The intact viral genome tandemly integrated in each oligomer need not be transcribed in the noninducible

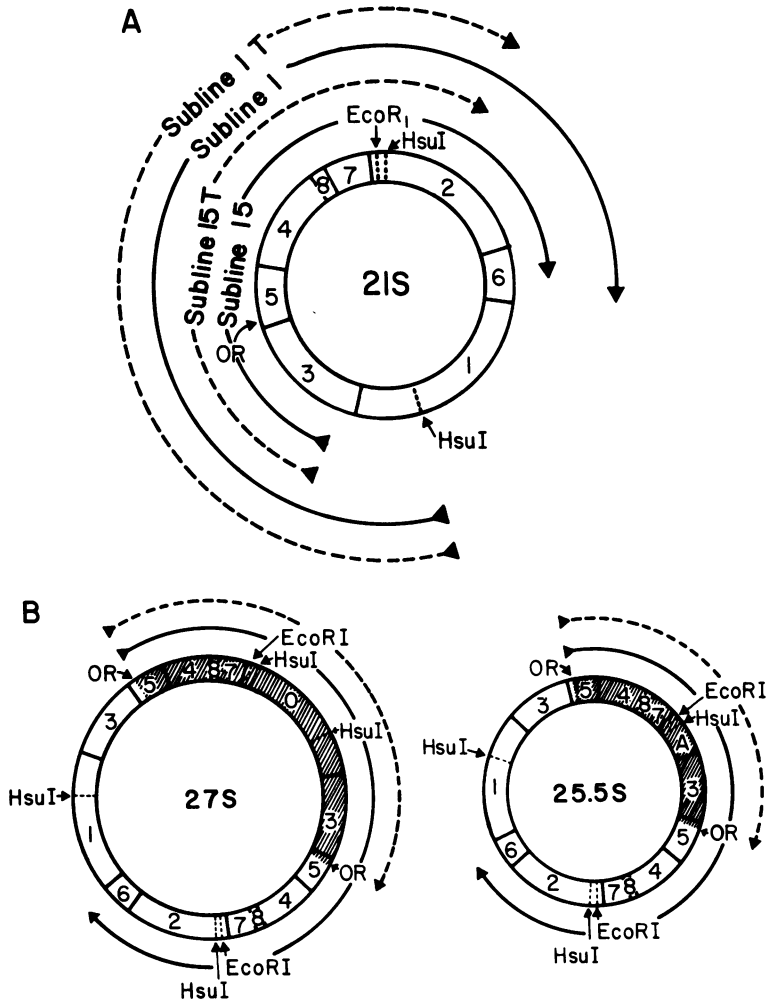

FIG. 6. *Possible orientation of ts-a 3T3 cytoplasmic RNA on the physical map of polyoma DNA and the DNA oligomers characteristic of each subline. (A) 21S polyoma DNA (8); (B) oligomeric polyoma DNAs (21). The 25.5S oligomer is characteristic of subline 1. The defective duplication in each oligomer is indicated by shading.* (——) *Inducible cell RNA;* (-----) *noninducible cell RNA.*

cells, and in fact, need not even be present. Although both inducible and noninducible cells carry DNA sequences complementary to most of the polyoma genome, the number of viral DNA copies is lower in the noninducible cells (Blangy and Vogt, unpublished data), and the deletion of small portions of the genome in the noninducible cells, such as fragment 6 and part of 2, has not been excluded.

The pattern of transcription in noninducible ts-a 3T3 cells is similar to the pattern in a number of polyoma-transformed mouse cells studied by Kamen et al. (10). In most cases, no transcripts of the 3′ OH end of the early region (fragment 6 and a portion of fragment 2) are detected. A number of A group mutants of polyoma have been located in this region (12). Perhaps expression of the information coded for by this region is incompatible with survival as a stably transformed cell. Ts-a 3T3 cells maintained at the nonpermissive temperature are unable to express at least some A gene functions due to the temperature-sensitive lesion. Noninducible ts-a 3T3 cells which survive the thermal reactivation of the A gene function seem to have secondarily prevented expression of at least a portion of the A gene region.

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