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# Translation of polyoma virus T antigens *in vitro*

(immunoprecipitation/mRNA-dependent reticulocyte lysate/tryptic peptide maps/mRNA sizing/cRNA)

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ABSTRACT **Polyoma virus-specific RNA isolated from the cytoplasm of lytically infected cells can be translated *in vitro* to yield three T antigens, of $M_r$s approximately 90,000, 60,000, and 22,000. The tryptic peptide patterns of the T antigens synthesized *in vitro* are similar or identical to the patterns of the corresponding proteins in polyoma-infected cells. All three proteins incorporate methionine donated from initiator tRNA *in vitro*. Polyoma cRNA codes for a protein that is slightly larger than the 22,000 T antigen and that, by other criteria, is similar to the 22,000 T antigen. Translation of cRNA does not yield the 90,000 and 60,000 T antigens, suggesting that the generation of the mRNAs for these T antigens requires the removal of intervening sequences. The mRNA for the 90,000 T antigen is smaller than the mRNAs for the 22,000 and 60,000 proteins. All three proteins share common $NH_2$-terminal sequences, and the 60,000 T antigen may be translated partially in a different reading frame from sequences also coding for the 90,000 T antigen. The demonstration that polyoma virus codes for three different T antigens raises the possibility that all three proteins may be involved in cell transformation.**

Polyoma virus tumor antigens (T antigens) can be identified by immunoprecipitation from extracts of infected cells, using sera from animals bearing polyoma-induced tumors. Polyoma-infected mouse cells contain several species of T antigens (1–5). By tryptic peptide mapping, we have found that three of these species, having apparent $M_r$s of 90,000, 60,000, and 22,000, form a related but not completely overlapping family (5). The organization of the coding sequences for these three T antigens within the early region of the genome has been deduced by analysis of their tryptic peptides and examination of the nucleotide sequence of polyoma DNA (5). All three species share common $NH_2$-terminal regions encoded between 74 and 80 map units on the viral genome. In addition, the 60,000 and the 22,000 species share amino acid sequences encoded between 80 and 85 map units, which are not found in the 90,000 species. The 60,000 species has several unique methionine-containing peptides, probably encoded between 87 and 0/100 map units possibly translated in a different reading frame from the information for the 90,000 species (5). The large and small T antigens encoded in the early region of simian virus 40 (SV40) ($M_r$ 85,000 and 17,000, respectively) are related in a similar way to the 90,000 and 22,000 T antigens of polyoma virus (6–12).

The early region of the polyoma genome encodes two functions required for cell transformation, identified by the temperature-sensitive (tsA) mutants and the host range nontransforming (hr-t) mutants, which complement for transformation (13, 14). The hr-t mutants are located in the region between 80 and 85 map units; the tsA mutants are located in the region between 0 and 25 map units (15, 16). The hr-t mutations affect the 60,000 and 22,000 T antigens but not the 90,000 T antigen (2, 4, 5). The tsA mutants render the 90,000 T antigen thermolabile (1) but do not affect the stability of the 60,000 and 22,000 T antigens (5). Similar functional localizations have been reported for the early region of SV40 (17, 18).

We describe here the *in vitro* translation of polyoma-specific RNA from lytically infected cells. The translation products include the 90,000, 60,000 and 22,000 T antigens, as demonstrated by immunoprecipitation and tryptic peptide mapping. Translation of cRNA results in the synthesis of a protein similar to the 22,000 T antigen but not the 90,000 or the 60,000 T antigens. The mRNA for the 90,000 T antigen appears to be smaller than the mRNAs for the 60,000 and 22,000 T antigens.

## MATERIALS AND METHODS

**Immunoprecipitation.** The procedure used for the extraction and immunoprecipitation of labeled T antigens from infected cells is described in detail elsewhere (5). *In vitro* reactions were precipitated likewise, after 1:10 dilution. The antitumor sera were generously provided by Jon Silver and Ted Friedmann.

**Isolation of Polyoma-Specific RNA from Infected Cells.** Polyoma-specific RNA was selected from total cytoplasmic RNA isolated from polyoma-infected cells 36 hr after infection at 37°C as described (19).

**Synthesis of cRNA.** Polyoma cRNA was prepared by incubation of 10 μg of form I polyoma DNA with either 64 μg of wheat germ RNA polymerase or 30 μg of *Escherichia coli* RNA polymerase (both obtained from Miles) as described (20), except that total cRNA was purified. The RNA was boiled for 30 sec immediately before use.

***In Vitro* Translation.** Polyoma-specific RNA was translated in the mRNA-dependent reticulocyte lysate (21) in the presence of [$^{35}$S]methionine (Amersham/Searle, SJ204; specific activity > 500 Ci/mmol) at a final concentration of 250 μCi/ml.

**Analysis of Immunoreactive Proteins by Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Electrophoresis.** The samples were analyzed on $NaDodSO_4$/polyacrylamide slab gels as described (5).

**Estimation of mRNA Sizes.** Polyoma-specific RNA was sized on composite 2% acrylamide/0.5% agarose gels as described (22).

**Tryptic Peptide Analysis.** Tryptic peptide analysis was carried out as described (5).

## RESULTS

***In Vitro* Translation of Polyoma-Specific RNA from Lytically Infected Cells.** Polyoma-specific RNA was selected from total cytoplasmic RNA isolated from polyoma-infected mouse 3T6 cells during the late stage of infection. This RNA was translated *in vitro* in the mRNA-dependent reticulocyte

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; SV40, simian virus 40; hr-t mutant, host range nontransforming mutant; tsA mutants, temperature-sensitive mutants.

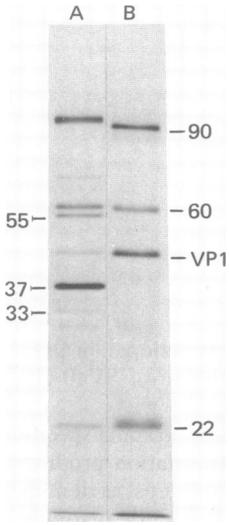


FIG. 1. Comparison of polyoma T antigens synthesized *in vivo* (lane A) and *in vitro* (lane B). The immunoprecipitate in lane A was prepared from polyoma-infected cells labeled with [$^{35}$S]-methionine (5). The immunoprecipitate equivalent to a 5-$\mu$l translation reaction containing 1/50th of the polyoma-specific RNA selected from 2 mg of infected cell cytoplasmic RNA was run in lane B. The fluorogram of the gel was exposed for 72 hr. Numbers show $M_r \times 10^{-3}$. VP1, polyoma major capsid protein.

lysate in the presence of [$^{35}$S]methionine. The patterns of radiolabeled proteins precipitable by anti-T serum from the *in vitro* translation reaction were compared, by NaDodSO$_4$/polyacrylamide gel electrophoresis, with the T antigens precipitated from extracts of infected cells (Fig. 1). The proteins precipitated from infected cell extracts had apparent $M_r$s of 90,000, 60,000, 55,000, 37,000, 33,000, and 22,000 as described (5). Small amounts of the polyoma major capsid protein, VP1, were precipitated also. The immunoprecipitable *in vitro* translation products corresponded to the 90,000, 60,000, and 22,000 T antigens only and to VP1. The band in the region of the 90,000 T antigen produced by *in vitro* translation migrated somewhat faster than the corresponding protein from infected cells. Although the major immunoprecipitable *in vitro* product in the region of the 22,000 T antigen comigrated with the 22,000 T antigen from infected cells, we have observed a variable amount of a protein migrating slightly more slowly than the 22,000 T antigen (Fig. 1, lane B).

We also translated polyoma-specific RNA in the presence of [$^{35}$S]fMet-tRNA$_f^{Met}$, a specific $NH_2$-terminal label (23). The immunoprecipitable *in vitro* products synthesized in the presence of either [$^{35}$S]methionine or [$^{35}$S]fMet-tRNA$_f^{Met}$ were analyzed by polyacrylamide gel electrophoresis (Fig. 2). All three T antigens were labeled with formyl[$^{35}$S]methionine.

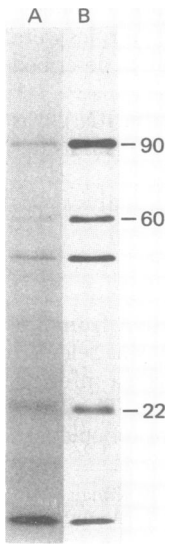


FIG. 2. *In vitro* synthesis of polyoma T antigens in the presence of [$^{35}$S]fMet-tRNA$_f^{Met}$. The immunoprecipitate from a 20-$\mu$l *in vitro* reaction containing $9 \times 10^5$ cpm of [$^{35}$S]fMet-tRNA$_f^{Met}$ and 0.5 mM methionine, using 1/25th of the polyoma-specific RNA selected from 2 mg of infected cell cytoplasmic RNA, was run in lane A. The immunoprecipitate equivalent to a 5-$\mu$l translation reaction, set up as described in the legend to Fig. 1 but with a different batch of RNA, was run in lane B. The gel was subjected to fluorography: lane A was exposed for 21 days and lane B, for 6 days. Numbers show $M_r \times 10^{-3}$.

Therefore, the 90,000, 60,000, and 22,000 T antigens all appear to be primary translation products. We conclude from these experiments that the 90,000, 60,000, and 22,000 T antigens are virus-coded and are the only T antigens detectable by *in vitro* translation.

**Tryptic Peptide Mapping of the *In Vitro* Synthesized T Antigens.** We characterized the *in vitro* products further by tryptic peptide analysis of [$^{35}$S]methionine-labeled material. The patterns of the methionine-containing peptides of the 90,000, 60,000, and 22,000 T antigens produced *in vitro* and in infected cells are shown in Fig. 3. The tryptic peptides of each protein produced *in vitro* were similar to those of the corresponding protein from infected cells. Comparison of the maps of the two 90,000 T antigens shows that most of the major peptides are identical except that spot 7 is missing from the *in vitro* product and a new spot, T, is present. The maps of the 60,000 T antigens synthesized *in vivo* and *in vitro* are essentially identical. The major methionine-containing peptides of the 22,000 T antigen made *in vitro* were the same as those of the 22,000 T antigen from infected cells. There were minor peptides (R and S), however, that were only present in the map of the 22,000 T antigen synthesized *in vitro*. In the map of the 22,000 T antigen in Fig. 3, spots A, B, and C appear to be underrepresented with respect to spots 1 and 4 in the map of the 60,000 T antigen. Quantitation of the amounts of radioactivity in these five spots by elution from the thin-layer plates, however, showed that the ratio of peptides 1 and 4 to peptides A, B, and C was the same in the 60,000 T antigen and the 22,000 T antigen.

**Translation of Polyoma cRNA.** We tested the ability of the *in vitro* system to synthesize T antigens when programmed with cRNA produced by transcription of polyoma DNA with *E. coli* or wheat germ RNA polymerase. The cRNA is a mixture of early and late strand transcripts (24), and the synthesis of the viral capsid proteins VP1, VP2, and VP3 from cRNA can readily be detected in the unprecipitated *in vitro* product (not shown; see ref. 25). Comparison of the immunoprecipitable proteins produced by *in vitro* translation of polyoma cRNA or polyoma-specific RNA from infected cells is shown in Fig. 4. Polyoma cRNA coded for a protein that migrated slightly more slowly than the 22,000 T antigen but did not produce the 90,000 or 60,000 protein. This was true regardless of the RNA polymerase used to synthesize the cRNA. Tryptic peptide analysis of the 22,000 protein product of cRNA showed that it had all the methionine-containing tryptic peptides of the authentic 22,000 T antigen (not shown). Despite the apparently greater molecular weight (by 500–1000) of this protein, it did not contain any major extra methionine-containing peptides. Further analysis of the 22,000 T antigens made from intracellular mRNA and cRNA with a mixture of $^{14}$C-labeled amino acids will be necessary to ascertain whether the cRNA product has extra amino acid sequences. The failure to synthesize the 90,000 and 60,000 T antigens from cRNA is consistent with the notion that the functional mRNAs for these antigens are produced by events that do not occur efficiently in the *in vitro* system.

**Size of the Polyoma T Antigen mRNAs.** We separated polyoma-specific RNAs isolated from infected cells on the basis of size by electrophoresis on nondenaturing polyacrylamide gels (22). By comparison with denaturing gels, it has been shown (22) that this system separates the majority of mRNAs on the basis of their true molecular weights. This system has been used to separate the mRNAs for the SV40 85,000 and 17,000 T antigens and the mRNAs for the polyoma capsid proteins VP2 and VP3 (19). The translation products of the fractions containing the mRNAs coding for the T antigens are shown in Fig. 5. The 90,000 T antigen was produced from an RNA (molecular

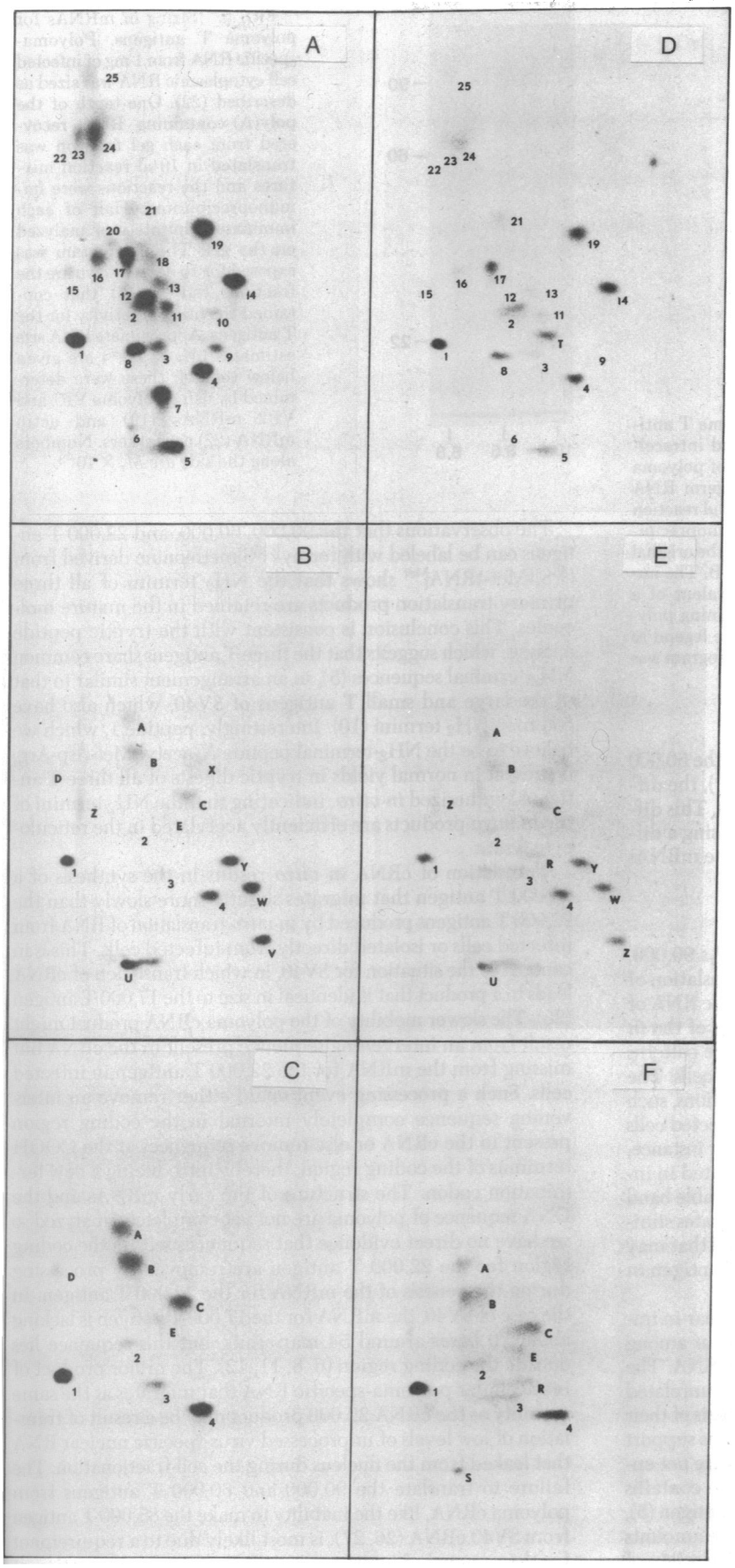


FIG. 3. Comparison of the tryptic peptide maps of [$^{35}$S]methionine-labeled polyoma T antigens synthesized *in vivo* (*Left*) and *in vitro* (*Right*). Tryptic digests of the 90,000, 60,000, and 22,000 T antigens labeled with [$^{35}$S]methionine in infected cells were made as described (5). For preparation of [$^{35}$S]methionine-labeled T antigens synthesized *in vitro*, the total polyoma-specific RNA selected from 1.5 mg of cytoplasmic RNA from polyoma-infected cells was translated in a 400-$\mu$l reaction mixture followed by immunoprecipitation. Tryptic peptides were then prepared from the 90,000, 60,000, and 22,000 T antigens purified by NaDodSO$_4$ gel electrophoresis. The digests were resolved by two-dimensional separation on cellulose thin-layer plates, by electrophoresis at pH 4.7 toward the cathode with the origin on the left followed by ascending chromatography from bottom to top (5). The T antigen, amounts of radioactivity applied for each map, and the length of exposure of each autoradiogram were: (*A*) 90,000 synthesized *in vivo*, 9000 cpm, 22 days; (*B*) 60,000 synthesized *in vivo*, 4400 cpm, 20 days; (*C*) 22,000 synthesized *in vivo*, 2700 cpm, 28 days; (*D*) 90,000 synthesized *in vitro* 3100 cpm, 26 days; (*E*) 60,000 synthesized *in vitro*, 1100 cpm, 66 days; (*F*) 22,000 synthesized *in vitro*, 1400 cpm, 28 days.

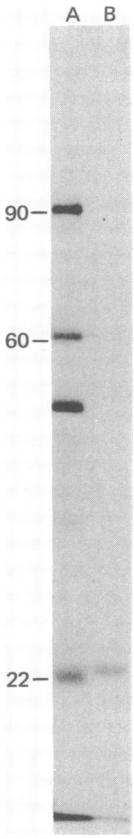


FIG. 4. Comparison of polyoma T antigens synthesized from cRNA and intracellular mRNAs. Two micrograms of polyoma cRNA synthesized with wheat germ RNA polymerase was translated in a 20-$\mu$l reaction mixture. The reaction was immunoprecipitated and the equivalent of 4 $\mu$l of the original reaction mixture was run in lane B. The immunoprecipitate from the equivalent of a 2.5-$\mu$l translation reaction containing polyoma-specific RNA as given in the legend to Fig. 1 was run in lane A. The fluorogram was exposed for 3 days.

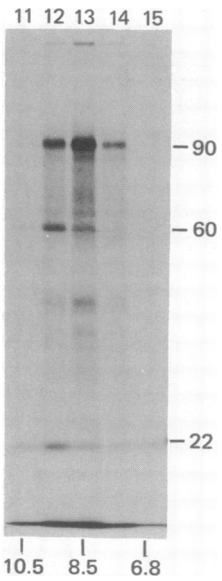


FIG. 5. Sizing of mRNAs for polyoma T antigens. Polyoma-specific RNA from 1 mg of infected cell cytoplasmic RNA was sized as described (22). One-tenth of the poly(A)-containing RNA recovered from each gel fraction was translated in 10-$\mu$l reaction mixtures and the reactions were immunoprecipitated. Half of each immunoprecipitate was analyzed on the gel. The fluorogram was exposed for 15 days. Shown are the fractions (no. 11–15) that contained the mRNA activity for the T antigens. Approximate RNA size estimates ($M_r \times 10^{-5}$) are given below the gel; these were determined by using polyoma VP1 and VP2 mRNAs (19) and actin mRNA (22) as markers. Numbers along the side are $M_r \times 10^{-3}$.

weight, $8.5 \times 10^5$) smaller than the RNAs coding for the 60,000 and 22,000 T antigens (molecular weight, $9.4 \times 10^5$), the difference in size being approximately 280 nucleotides. This difference in mobility has also been observed in a gel using a different crosslinker. We have not been able to resolve the mRNAs for the 60,000 and 22,000 T antigens.

## DISCUSSION

We have shown that three polyoma T antigens ($M_r$s 90,000, 60,000, and 22,000) can be produced by *in vitro* translation of polyoma-specific RNA selected from the cytoplasmic RNA of lytically infected cells. The tryptic peptide patterns of the *in vitro* products are closely similar to the tryptic peptide patterns of the corresponding proteins isolated from infected cells. The differences observed most likely result from modifications, such as phosphorylation, of the proteins synthesized in infected cells that do not occur efficiently in the *in vitro* system. For instance, it is known that the 90,000 T antigen is phosphorylated in infected cells (4). We have observed an immunoprecipitable band in cells pulse-labeled with [$^{35}$S]methionine that migrates similarly to the 90,000 T antigen synthesized *in vitro* and that may represent an unmodified precursor to the 90,000 T antigen *in vivo* (not shown) (see also ref. 1).

The 55,000, 37,000, and 33,000 proteins that appear in immunoprecipitates of infected cell extracts do not appear among the *in vitro* translation products of polyoma-specific RNA. The immunoprecipitable 55,000 and 37,000 proteins are unrelated to the 90,000, 60,000, and 22,000 T antigens, on the basis of their tryptic peptide patterns (5). Our present observations support the conclusion that the 55,000 and 37,000 proteins are not encoded in the polyoma genome. The 33,000 protein contains some tryptic peptides in common with the 60,000 T antigen (5), but the 33,000 protein is not produced in detectable amounts by *in vitro* translation of polyoma-specific RNA. The origin of the 33,000 protein—if it is a discrete protein—is unclear.

The observations that the 90,000, 60,000, and 22,000 T antigens can be labeled with formyl[$^{35}$S]methionine derived from [$^{35}$S]fMet-tRNA$_f^{Met}$ shows that the $NH_2$ termini of all three primary translation products are retained in the mature molecules. This conclusion is consistent with the tryptic peptide analysis, which suggests that the three T antigens share common $NH_2$-terminal sequences (5), in an arrangement similar to that of the large and small T antigens of SV40, which also have common $NH_2$ termini (10). Interestingly, peptide 1, which we believe to be the $NH_2$-terminal peptide *N*-acetyl-Met-Asp-Arg, is present in normal yields in tryptic digests of all three T antigens synthesized *in vitro*, indicating that the $NH_2$ termini of the *in vitro* products are efficiently acetylated in the reticulocyte lysate.

Translation of cRNA *in vitro* results in the synthesis of a 22,000 T antigen that migrates slightly more slowly than the 22,000 T antigens produced by *in vitro* translation of RNA from infected cells or isolated directly from infected cells. This is in contrast to the situation for SV40, in which translation of cRNA leads to a product that is identical in size to the 17,000 T antigen (26). The slower mobility of the polyoma cRNA product might result from an intervening sequence present in the cRNA but missing from the mRNA for the 22,000 T antigen in infected cells. Such a processing event could either remove an intervening sequence completely internal in the coding region present in the cRNA or else remove sequences at the COOH terminus of the coding region, thereby introducing a new termination codon. The structure of the early mRNAs and the DNA sequence of polyoma are not yet completely analyzed so we have no direct evidence that sequences within the coding region for the 22,000 T antigen are removed by processing during the genesis of the mRNA for the 22,000 T antigen. In the case of SV40, the mRNA for the 17,000 T antigen is lacking about 70 bases around 54 map units, but this sequence lies outside the coding region (6, 8, 11, 12). The minor product of intracellular polyoma-specific RNA that migrates at the same mobility as the cRNA 22,000 product may be a result of translation of low levels of unprocessed virus-specific nuclear RNA that leaked from the nucleus during the cell fractionation. The failure to translate the 90,000 and 60,000 T antigens from polyoma cRNA, like the inability to make the 85,000 T antigen from SV40 cRNA (26, 27), is most likely due to a requirement for the removal of intervening sequences in the generation of functional mRNAs for these proteins.

Tryptic peptide analysis of the 90,000, 60,000, and 22,000 T antigens translated *in vitro* supports our previous conclusions about the relatedness of the three T antigens from infected cells, which were based on the nucleotide sequence of part of the early region of polyoma DNA (refs. 5, 28; T. Friedmann, personal communication). The $NH_2$-terminal regions shared by the three proteins have four methionine-containing peptides (spots 1–4 in Fig. 3) encoded between 74 and 79 map units. The 60,000 and 22,000 T antigens have three major methionine-containing peptides not found in the 90,000 protein (spots A, B, and C in Fig. 3) encoded between 80 and 86 map units. The 60,000 protein has at least four methionine-containing peptides (spots U, V, W, and Y) not found in the 90,000 or 22,000 protein. It seems plausible that these unique peptides are encoded between 87 and 0/100 map units, because a polyoma mutant containing a deletion from 98 to 3 map units fails to make a normal-size 60,000 T antigen and the 60,000 T antigen is not affected by tsA mutations (5), which are located clockwise from 0/100 map units. To produce peptides encoded from 87 to 0/100 map units not shared with the 90,000 T antigen, it might be necessary to utilize more than one reading frame. It is interesting to note that there are extensive regions in the sequence between 87 and 0/100 map units where there are two open reading frames (T. Friedmann, personal communication).

The available sequence data suggest that the regions that need to be spliced out of the putative 60,000 T antigen mRNA are not very long, so that the mRNA for the 60,000 T antigen will be a nearly complete copy of the early region. Likewise, the mRNA for the 22,000 T antigen should also be almost a complete transcript of the early region. In contrast, the mRNA for the 90,000 T antigen will presumably lack the sequences between 80 and 86 map units. Our finding that the mRNAs for the 60,000 and 22,000 T antigens are about the same size and both are about 280 nucleotides larger than the mRNA for the 90,000 T antigen is consistent with the genetic organization and mRNA structures outlined above. It is noteworthy that the structures we have deduced for the mRNAs for the 90,000 and 22,000 T antigens of polyoma are strikingly similar to those of the mRNAs for the 85,000 and 17,000 T antigens of SV40 (6, 11, 26).

The properties of the hr-t and tsA mutants of polyoma show that the 90,000 T antigen is required both for viral DNA replication (29) and for cell transformation, whereas the 22,000 or 60,000 T antigens or both are required for transformation but not necessarily for viral replication (13). The 90,000 T antigen is located primarily in the cell nucleus; the 60,000 T antigen is in the cell membrane (2). The demonstration that polyoma virus codes for three different T antigens, with different functions and cellular locations, raises the possibility that all three proteins may play a role in cell transformation.

**Note Added in Proof.** A similar relationship of polyoma T antigens has been shown by Smart and Ito (30) (cf. ref. 5). Use of two different reading frames for a part of the large and middle-size T antigens has also been suggested based on the observation that some early deletion mutants shorten the large and middle-size T antigens proportionately to the sizes of the deletions (Y. Ito, personal communication).

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