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Simian virus 40 large tumor antigen: A "RNA binding protein"?

(electrofocusing/messenger ribonucleoprotein/phosphoprotein/regulation of transcription and translation)

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ABSTRACT **Simian virus 40 large tumor antigen was isolated by immunoaffinity chromatography from monkey or mouse cell cultures undergoing lytic or transforming infection. RNase-treated gel-purified large tumor antigen, on hydrolysis with alkali, gave about equimolar amounts of AMP, GMP, CMP, and UMP. Furthermore, RNA fragments of ≈45 nucleotides could be isolated from large tumor antigen purified by the same procedure. Mapping of the T_1 oligonucleotides showed a high complexity, as indicated by the presence of unique sequences of 15–30 nucleotides and of poly(A). This is compatible with the hypothesis that these RNA fragments are derived from cellular pre-mRNAs or mRNAs. Our results suggest that Simian virus 40 large tumor antigen is a RNA-binding protein and might possibly be involved in regulation of synthesis, maturation, or translation of cellular mRNAs.**

The early region of simian virus 40 (SV40) DNA codes two related proteins, designated large (T) and small (t) tumor antigens, of apparent M_r 88,000 and 19,000, respectively (1–3). They can be isolated by immunoaffinity chromatography (4) from cells undergoing lytic or transforming infection and from tumors induced in animals by inoculation with SV40. The primary structures of the T and t antigens have been established (1, 5–7). T antigen is a phosphoprotein (8–11) that has at least two distinct phosphorylated sites (4), and a fraction of it is further modified by poly(ADP-ribosylation) (12). T antigen is required for initiation of viral DNA replication (1, 13, 14) and binds preferentially to the origin of replication of SV40 DNA (15–19). SV40 T and t antigens play a primordial role in lytic and transforming infection *in vitro* and in tumor formation in animals (1, 20).

In this paper, we show that small RNA fragments, apparently derived from high molecular weight RNA, remain bound to RNase-treated gel-purified SV40 T antigen.

MATERIALS AND METHODS

Confluent primary mouse kidney cultures and cultures of CV-1 (African green monkey) cells were infected with SV40 or mock infected under the conditions described (21, 22). The cultures were labeled 21–25 hr after infection with 100 μCi of [^{35}S]-methionine (500–1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) in 2 ml of methionine-free medium, or 100 μCi of [5-^{3}H]- or [2-^{14}C]uridine (540 mCi/mmol and 50 mCi/mmol, respectively) in 2 ml of medium, or 100 μCi of $^{32}P_i$ (carrier free) in 2 ml of phosphate-free medium. DNA synthesis was inhibited with 1-β-D-arabinofuranosylcytosine (araC; 20 μg/ml) in the medium used to infect and label the cultures. T antigen was extracted with 0.5% Nonidet P40, pH 9, and isolated by immunoaffinity chromatography (4, 23). The immune complexes were eluted with either $NaDodSO_4$ sample buffer (65 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/2% 2-mercaptoethanol/0.01% bromophenol blue/15% glycerol) (24) or urea lysis buffer [9.5 M urea/2% Nonidet P40/5% 2-mercaptoethanol/2% ampholytes (LKB-Produkter AB, Sweden)] (25). Immune complexes, bound to protein A-Sepharose, were incubated in 0.5 ml of 10 mM Tris·HCl, pH 7.0, or in 0.5 ml of 10 mM NaOAc, pH 5.0, containing 100 μg of RNase A, or in 0.5 ml of 10 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$ containing 100 μg of DNase I (RNase free). RNase A and DNase I were purchased from Worthington. After incubation for 30 min at room temperature, the complexes were washed and T antigen was eluted with $NaDodSO_4$ or urea. Isoelectric focusing in sucrose density gradients [5–40% (wt/vol)] containing 2% (wt/vol) ampholytes (pH 3–10) was done in 23-ml columns (ISCO model 212; Lincoln, NB) according to Nelson *et al.* (26). Thereafter, 0.25-ml fractions were collected and radioactivity and pH values were measured. T antigen was assayed in pooled fractions by immunoaffinity chromatography followed by electrophoresis in $NaDodSO_4$/polyacrylamide gels and revealed by fluorography (27). Two-dimensional gel electrophoresis was carried out according to O'Farrell (25).

For chemical analysis and for RNA extraction, T antigen was isolated from 10–12 cultures and purified by preparative electrophoresis in $NaDodSO_4$/7.5% acrylamide gels. The band containing T antigen was localized in the gels by autoradiography or by staining a vertical gel strip with Coomassie blue (or both). T antigen was eluted from the fragmented gel by shaking in 2 ml of 0.25 M $NaHCO_3$/0.1% $NaDodSO_4$ for 20 hr at 37°C. The eluate was then lyophilized. For analysis of phosphate residues, aliquots of the lyophilized material were subjected to either alkaline (0.3 M KOH for 20 hr at 37°C) or acid (2 M HCl for 20 hr at 120°C) hydrolysis. The hydrolysates were neutralized and analyzed by high-voltage paper electrophoresis at pH 3.5 [0.5% pyridine/5% acetic acid (vol/vol)] (28). For RNA extraction, the lyophilized material was incubated in 2 ml of 10 mM Tris·HCl, pH 7.0, containing 500 μg of proteinase K (Merck, Darmstadt) for 2 hr at 37°C. The solution was then extracted with phenol and the RNA was precipitated twice with ethanol in the presence of 0.3 M NaOAc (pH 5.0) as described (29). The pellet was dissolved in 10 mM Tris·HCl, pH 7.4, and incubated in buffer alone or in buffer containing 20 μg of RNase A/ml for 30 min at 22°C. The RNA was analyzed by electrophoresis in 15% acrylamide/7 M urea slab gels (30). For oligonucleotide analysis, unlabeled RNA was extracted as described above and then incubated in 10 μl of 10 mM Tris·HCl, pH 7.4/1 mM EDTA containing 0.5 μg of T1 RNase (Worthington) for 30 min at 37°C. The T1 oligonucleotides were 5'-end labeled with 50 μCi of [γ-^{32}P]ATP (500 mCi/mmol), using T4 polynucleotide kinase (31) and then analyzed by two-dimensional gel electrophoresis (32) followed by autoradiography.

RESULTS

Isolation of SV40 T Antigen. We isolated SV40 T antigen synthesized in CV-1 cultures or primary mouse kidney cultures

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Abbreviations: SV40, simian virus 40; T and t antigen, SV40 large and small tumor antigen, respectively; araC, 1-β-D-arabinofuranosylcytosine.

undergoing lytic or transforming infection, respectively (21). Mock- and SV40-infected cultures were labeled 21–25 hr after infection with [^{35}S]methionine, [^{3}H]- or [^{14}C]uridine, or $^{32}P_i$. At the time of extraction, the percentage of T-antigen-positive nuclei had reached its maximum, as determined by immunofluorescent staining, corresponding to ≈100% and 50% in CV-1 and mouse kidney cultures, respectively. T antigen was isolated by immunoaffinity chromatography. Samples to be used for electrophoresis in $NaDodSO_4$/polyacrylamide gels were eluted from the immune complexes with $NaDodSO_4$ sample buffer (24) and samples to be analyzed by isoelectric focusing were eluted with urea lysis buffer containing 9.5 M urea (25). The two elution methods gave similar recoveries of T antigen, as determined by electrophoresis in $NaDoSO_4$/polyacrylamide gels. The recovery of T antigen and the results reported below were the same whether infection took place in the presence or absence of araC.

Isoelectric Focusing of T Antigen in Sucrose Density Gradients. All attempts to focus in sucrose density gradients (26) ^{35}S-labeled urea-eluted T antigen isolated from CV-1 (Fig. 1) or mouse kidney cultures (not shown) were unsuccessful: T antigen, detected by immunoaffinity chromatography followed by $NaDodSO_4$/polyacrylamide gel electrophoresis, was broadly distributed between pH 4.5 and pH 9 (Fig. 1*A*). The distribution was the same if the sample was included in the gradient and it was independent of the polarity of the electric field. Considering the possibility that T antigen might be complexed to nucleic acids, we treated the immune complexes, while bound to protein A-Sepharose, with RNase A. The results show that RNase-treated T antigen focused in distinct bands at about pH 6.0, 6.5, and 7.0 (Fig. 1*B*); as expected, these bands were absent in extracts of mock-infected cultures (not shown). RNase treatment did not change the migration of T antigen in $NaDodSO_4$/polyacrylamide gels.

Analysis of T Antigen by Two-Dimensional Gel Electrophoresis. ^{35}S-Labeled T antigen extracted from SV40-infected CV-1 cultures (with or without araC) was eluted with urea and immediately analyzed by two-dimensional gel electrophoresis (25). The bulk of T antigen failed to enter the electrofocusing gel (Fig. 2*A*) whereas other proteins (e.g., actin) formed distinct spots at the expected positions. Similar results have been reported by others (33–35). In contrast, the bulk of RNase A-treated T antigen entered the electrofocusing gel, forming major spots at about pH 5.5, 6.0, 6.5, and 7.0 (Fig. 2*B*). During these studies, we noticed that treatment of the immune complexes with DNase I, storage of urea-eluted T antigen for a few days at −20°C, or heating the samples for 1 min at 60°C allowed a variable fraction of ^{35}S-labeled T antigen to enter the electrofocusing gel, forming an extended streak without distinct spots. The same results were obtained with T antigen extracted from SV40-infected mouse kidney cultures (not shown).

Labeling of T Antigen with Radioactive Uridine or $^{32}P_i$. To test whether polyribonucleotides are complexed to T antigen, we labeled mock- or SV40-infected CV-1 or mouse kidney cultures (with or without araC) with [^{3}H]- or [^{14}C]uridine. Aliquots of T antigen bound to protein A-Sepharose were incubated with RNase A or with buffer alone. Extracts of mock-infected cultures were treated in the same way. T antigen was eluted with $NaDodSO_4$ sample buffer, boiled for 90 sec, and then analyzed by electrophoresis in $NaDodSO_4$/polyacrylamide gels. In all samples from infected cultures, whether they had been treated with RNase or not, a single band of radioactivity was observed at the position of T antigen (Fig. 3); this band was absent in extracts of mock-infected cultures. Furthermore, this band was absent when extracts of infected cultures were allowed to react with nonimmune hamster serum or with antiserum produced by inoculation of mice with syngeneic spontaneously transformed cells (T-AL/N; ref. 36) provided by T. Rose (University of Geneva). T-antigen preparations that had not been treated with RNase also contained radioactivity in the stacking gel. Radioactivity was completely removed by treatment of T antigen preparations with 1 M KOH for 1 hr at room temperature before gel electrophoresis (Fig. 3) or by incubating the gels with 5% trichloroacetic acid for 30 min at 90°C (37) after electrophoresis and before fluorography (not shown). T antigen from ^{32}P-labeled CV-1 or mouse kidney cultures formed a single strongly labeled band (refs. 4, 8–11 and Fig. 3). After treatment of the immune complexes with RNase, no ^{32}P was found in the stacking gels and ≈30% of the ^{32}P was removed from T antigen.

To determine the chemical nature of the ^{32}P-labeled residues, T antigen was treated with RNase and subjected to preparative $NaDodSO_4$/polyacrylamide gel electrophoresis. T antigen was eluted from the gels and hydrolyzed with alkali or acid, and the hydrolysates were analyzed by high-voltage paper

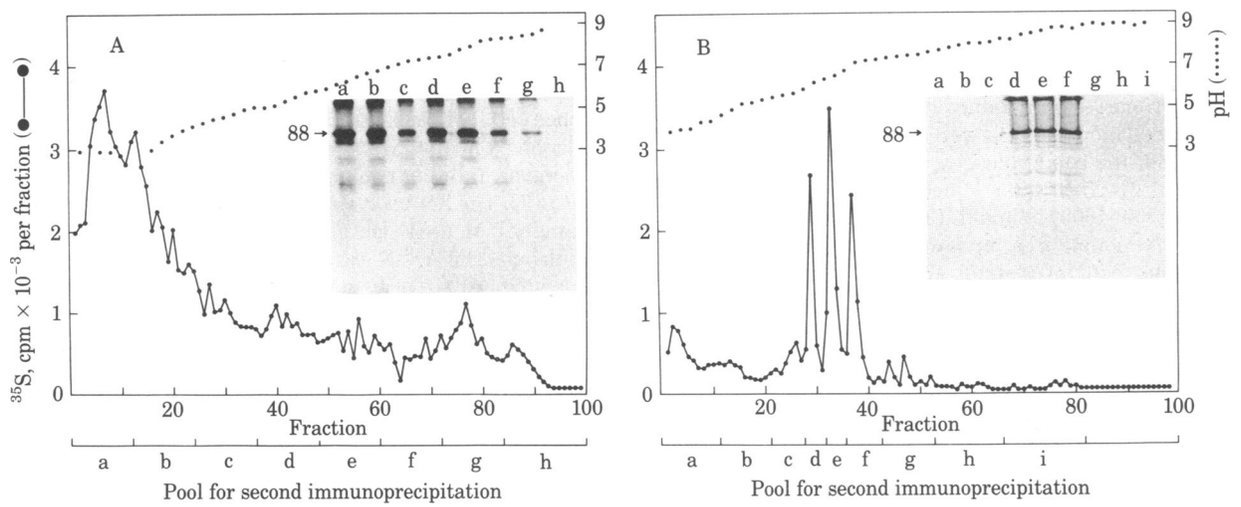

FIG. 1. Isoelectric focusing of ^{35}S-labeled SV40 T antigen in sucrose density gradients. T antigen was isolated from CV-1 cultures by immunoaffinity chromatography. The immune complexes were incubated with buffer alone (*A*) or with buffer containing RNase A (*B*). T antigen was eluted with urea lysis buffer (25) and subjected to isoelectric focusing in sucrose density gradients (26). Radioactivity and pH were measured in 0.25-ml fractions, which were then pooled as indicated. T antigen was assayed by immunoaffinity chromatography followed by $NaDodSO_4$/polyacrylamide (12.5% acrylamide) gel electrophoresis and fluorography for 2 weeks (*Insets*). 88, 88-Kilodalton position.

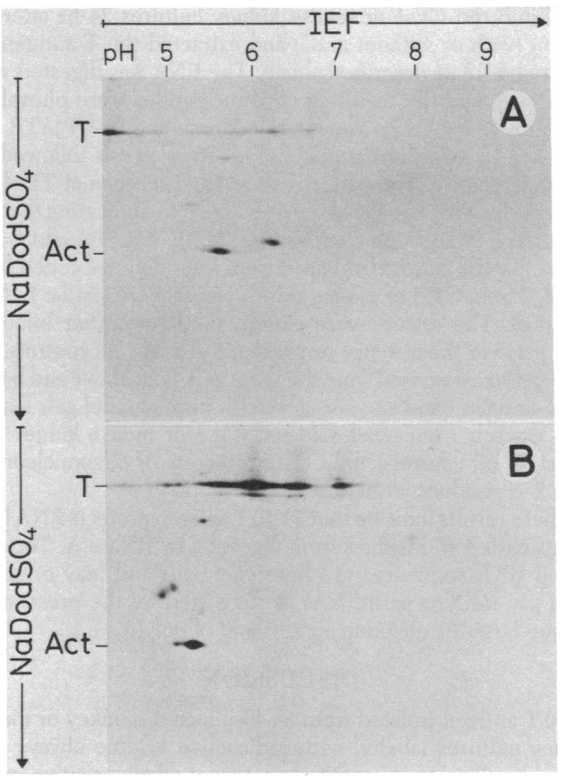

FIG. 2. Analysis of ^{35}S-labeled SV40 T antigen by two-dimensional gel electrophoresis (25). T antigen was isolated from CV-1 cultures. The immune complexes were incubated with buffer alone (*A*) or with buffer containing RNase (*B*). Urea-eluted T antigen was subjected to two-dimensional gel electrophoresis. Fluorography was for 10 days. IEF, isoelectric focusing; T, T antigen; Act, actin.

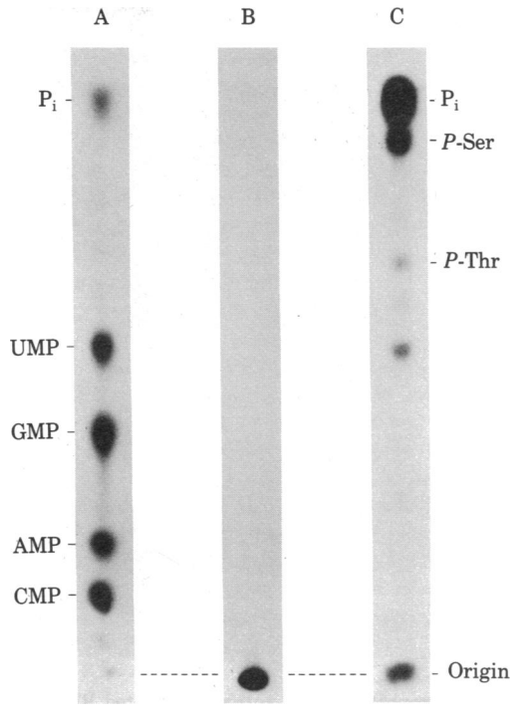

FIG. 4. Analysis of ^{32}P-labeled residues in SV40 T antigen. Immune complexes from CV-1 cultures labeled with $^{32}P_i$ were incubated with RNase A. T antigen was eluted with $NaDodSO_4$ sample buffer and purified by electrophoresis in $NaDodSO_4$/polyacrylamide (7.5% acrylamide) gels. T antigen was eluted from the gel and lyophilized. Aliquots were hydrolyzed with KOH (lane A) or HCl (lane C); one aliquot was not hydrolyzed (lane B). Samples were analyzed by high-voltage paper electrophoresis at pH 3.5 (28) followed by autoradiography. *P*-Ser, phosphoserine; *P*-Thr, phosphothreonine.

electrophoresis. As shown by autoradiography, alkaline hydrolysates gave four spots of similar intensity, corresponding to UMP, GMP, AMP, and CMP (Fig. 4). In accordance with the

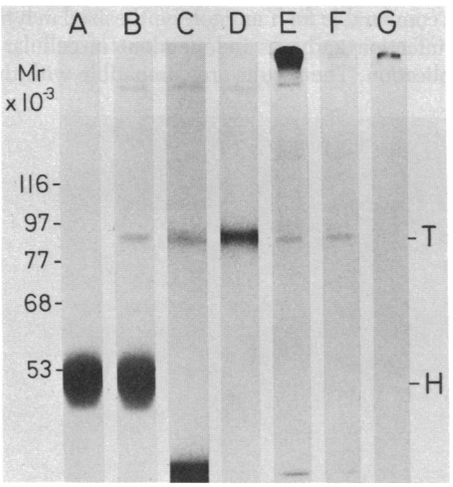

FIG. 3. Labeling of SV40 T antigen with [^{35}S]methionine, $^{32}P_i$, or [^{3}H]uridine. T antigen was isolated by immunoaffinity chromatography from lysates of SV40-infected CV-1 cultures and subjected to electrophoresis in $NaDodSO_4$/polyacrylamide (7.5% acrylamide) gels. Lanes: A and B, Coomassie blue stained mock-infected and SV40-infected extracts, respectively; C and D, autoradiographs of ^{35}S- and ^{32}P-labeled T antigen, respectively, E–G; fluorographs of [^{3}H]uridine-labeled T antigen after incubation of immune complexes in buffer alone (E), with RNase (F), or with 1 M KOH (G) for 60 min. T, SV40 T antigen; H, gamma globulin heavy chain.

results reported by others, acid hydrolysates contained phosphoserine, phosphothreonine (8–11), and a third, unidentified, component. To exclude the possibility that these results were due to fortuitous contamination with RNA comigrating in the gels with T antigen, we carried out the following control experiments. Mock-infected CV-1 or mouse kidney cultures were labeled with $^{32}P_i$ and lysed with Nonidet P40, and the lysates were mixed with lysates from unlabeled SV40-infected CV-1 or mouse kidney cultures. T antigen was isolated and subjected to electrophoresis in $NaDodSO_4$/polyacrylamide gels. No radioactivity comigrating with T antigen could be detected. Under the same electrophoresis conditions (7.5% acrylamide gels), mouse tRNA, 5S RNA, and 5.8S RNA migrated to the bottom of the gel while *Escherichia coli* 16S rRNA remained in the stacking gel. Similar results have been reported by others (37). When SV40-infected CV-1 or mouse kidney cultures were labeled with [^{3}H]thymidine, no radioactivity comigrating in gels with T antigen could be detected by fluorography.

Small RNA Fragments Are Complexed to T Antigen. SV40-infected CV-1 or mouse kidney cultures (with or without araC) were labeled with radioactive uridine or $^{32}P_i$. RNase-treated T antigen was isolated and subjected to preparative electrophoresis in $NaDodSO_4$/polyacrylamide gels. T antigen was eluted from the gel and digested with proteinase K. The digest was extracted with phenol. The ethanol precipitate was dissolved in 10 mM Tris·HCl, pH 7.4; aliquots were incubated with buffer alone or with RNase A and then analyzed by electrophoresis in 15% acrylamide/7 M urea slab gels. Fluorography of [^{3}H]uridine-labeled samples showed a distinct band that had an electrophoretic mobility corresponding to a polyribonucleotide of $\approx$45 residues and was sensitive to RNase; in some preparations, this

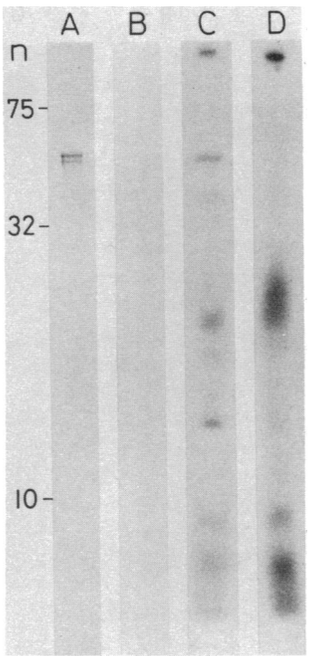

FIG. 5. Electrophoresis of T-antigen-associated RNA. CV-1 cultures were labeled with [^{3}H]uridine or $^{32}P_i$. T antigen was isolated and purified, and RNA was extracted. Aliquots were incubated in buffer alone (lanes A and C) or in buffer containing RNase A (lanes B and D). Samples were analyzed by electrophoresis in polyacrylamide gels containing 15% acrylamide/7 M urea (30). Lanes: A and B, [^{3}H]uridine-labeled material, fluorography for 12 weeks; C and D, ^{32}P-labeled material, autoradiography for 2 weeks. Migration markers were tRNA [75 nucleotides (n)], xylene cyanol (32 n) and bromophenol blue (10 n).

band formed a doublet (Fig. 5). Autoradiography of gels loaded with ^{32}P-labeled samples showed several bands. In all samples that had been incubated with RNase, the band corresponding to that observed by fluorography was absent. In 2 out of 10 preparations, a ^{32}P-labeled band having a mobility corresponding to ≈15 nucleotides was also removed by RNase. The chemical nature of this and the other RNase-resistant ^{32}P-labeled band remains unknown.

In further experiments, we isolated T antigen from unlabeled SV40-infected CV-1 or mouse kidney cultures 24 hr after infection (with or without araC) and extracted the T antigen-associated RNA as described above. The RNA was digested with T1 RNase, and the resulting oligonucleotides were phosphorylated by using T4 polynucleotide kinase and [γ-^{32}P]ATP and analyzed by two-dimensional gel electrophoresis followed by autoradiography. The patterns show the presence of T1 oligonucleotides with 15–30 residues and poly(A), indicating that the RNA has a rather high complexity (Fig. 6). Fig. 6 *A* and *B* also shows that the patterns obtained from T antigen-associated RNA isolated from CV-1 or mouse kidney cultures are similar but not identical. The results were closely similar whether infection took place in the absence or presence of araC. As controls, we used gel strips excised from the same gels 1 cm above and below the T-antigen band and also gel strips from parallel gels loaded with extracts from mock-infected CV-1 or mouse kidney cultures. In all controls, only trace amounts of oligonucleotides with 2–8 residues were observed (Fig. 6*C*).

These results indicate that SV40 T antigen protects RNA fragments with ≈45 residues from digestion by RNase A. The protected RNA sequences are heterogeneous and may originate from pre-mRNAs or mRNAs as suggested by the presence of unique large T1 oligonucleotides and of poly(A).

DISCUSSION

SV40 T antigen isolated from SV40-infected monkey or mouse kidney cultures labeled with radioactive uridine always contained small amounts of RNase-resistant alkali-sensitive radioactivity. Furthermore, alkaline hydrolysates of RNase-treated gel-purified T antigen contained about equimolar amounts of AMP, GMP, CMP, and UMP. These observations and the behavior of T antigen during isoelectric focusing suggested that it might be complexed with RNA. Direct evidence for this assumption was obtained by the isolation of RNA fragments comprising ≈45 nucleotides from RNase-treated gel-purified T antigen. Mapping of the ^{32}P-end-labeled T1 oligonucleotides showed a high sequence complexity, which was emphasized by the presence of unique T1 oligonucleotides with 15–30 residues and poly(A). The degree of complexity of T-antigen-associated RNA was comparable for T antigen synthesized in lytic or transforming infection and was independent of cellular and viral DNA replication. The results are compatible with the hypoth-

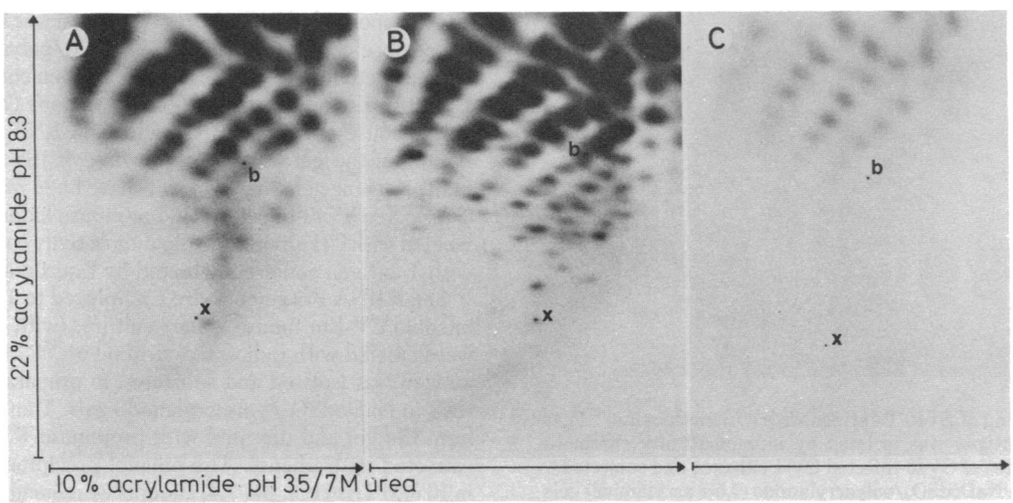

FIG. 6. T1 oligonucleotide maps of T-antigen-associated RNA. T antigen was isolated from CV-1 (*A*) or mouse kidney cultures (*B*) infected with SV40 for 25 hr. T-antigen-associated RNA was extracted and digested with T_1 RNase, and the resulting oligonucleotides were 5′-end labeled with [γ^{32}P]ATP and T4 polynucleotide kinase and analyzed by two-dimensional gel electrophoresis (32) followed by autoradiography for 1 day. (*C*) Material from mock-infected CV-1 cultures was isolated and processed in parallel. Extracts from mock-infected mouse kidney cultures gave essentially the same pattern as shown in *C*. Markers; ×, xylene cyanol (32 nucleotides); b, bromophenol blue (10 nucleotides).

esis that the T-antigen-associated RNA fragments are derived from cellular pre-mRNAs or mRNAs. The electrophoretic migration of proteins in $NaDodSO_4$/polyacrylamide gels is not necessarily changed by bound RNA fragments (37); e.g., p19 of Rous sarcoma virus has the same migration with or without a crosslinked viral RNA fragment of 30–40 nucleotides (unpublished results).

These results and the recent observation that T-antigen molecules are complexed to purified cellular messenger ribonucleoproteins isolated from polyribosomes of SV40-infected monkey or mouse cell cultures (ref. 38; M. R. Michel and M. Schwyzer, personal communication), suggest that SV40 T antigen is a "RNA-binding protein." Earlier observations had shown that treatment of fixed cells with RNase A removed intranuclear SV40 T antigen, judged by the immunofluorescence reaction, suggesting an association of T antigen with RNA (39).

Stimulation of nucleoplasmic transcription and increased synthesis of cellular mRNAs and proteins are observed in both lytic and transforming infection shortly after onset of SV40 or polyoma T-antigen synthesis (ref. 22; unpublished data). As a working hypothesis, we assume that T antigens induce this stimulation by modulating synthesis, maturation, and translation (or combinations thereof) of specific cellular mRNAs. This mode of action might account for the phenotypic reprogramming of the host cell observed in lytic and transforming infection.

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