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Cell-Free Synthesis of Simian Virus 40 T-Antigens

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Polyacrylamide gel electrophoresis and tryptic peptide fingerprint analysis of the proteins made in a cell-free system derived from L-cells and immunoprecipitated with simian virus 40 (SV40) anti-T serum demonstrated that both SV40 large-T and small-t antigens are synthesized in vitro in response to mRNA isolated from productively infected CV1 cells. Sucrose density centrifugation in gradients containing 85% formamide showed that the mRNA's for both forms of T-antigen sediment at about 17.5S, with the mRNA for small-t sedimenting marginally, but reproducibly, ahead of the mRNA for large-T. Hybridization experiments using restriction endonuclease fragments *Hae* III-E and *Hind* II/III-B showed that all fractions active in the cell-free synthesis of both forms of T-antigen hybridized equally to both fragments. This suggests that the mRNA's for SV40 T-antigens are at least partly virus coded and that the bulk of the early SV40 mRNA contains sequence information from both ends of the early region. The data are consistent with the suggestion that the large-T mRNA is spliced. SV40 complementary RNA (the product of transcription of SV40 DNA using *Escherichia coli* RNA polymerase) was also translated in the L-cell system and gave two families of polypeptides which specifically immunoprecipitate with anti-T serum. One family (the small-t family) includes a polypeptide indistinguishable by gel electrophoresis and tryptic peptide fingerprinting from small-t isolated from cells. The other family (the 60K family) has a major component with molecular weight approximately 60,000 and includes other polypeptides with molecular weights ranging from approximately 14,000 to about 70,000. The 60K family has peptides in common with large-T but not with small-t. Together, the peptides of the small-t and 60K families account for virtually all of the methionine peptides of SV40 large-T. We conclude from these results (i) that small-t is probably entirely, and large-T at least predominantly, virus coded; (ii) that the small-t and 60K families represent the translation products of two different portions of the early region of SV40 DNA (approximately 0.65 to 0.55 map units and 0.54 to 0.17 map units); and (iii) that although most, if not all, of the large-T and small-t peptides are present in the cell-free product, some feature of sequence arrangement of SV40 complementary RNA prevents the translation of full-length large-T and results instead in the synthesis of fragments. We suggest that the absence of a splice in the complementary RNA is responsible for this result.

Simian virus 40 (SV40) T-antigen (T-Ag) can be isolated from SV40-induced tumor cells, from SV40-transformed cells, and from productively infected cells, both early and late after infection (3, 35). T-Ag has been implicated as an important control element in the initiation of viral DNA synthesis (5, 49), in the transcription of viral DNA (2, 51), and in the initiation and maintenance of transformation (4, 23, 24, 50).

When extracted from productively infected cells or from SV40-transformed cells under conditions which minimize proteolytic degradation, at least two forms of T-Ag are detected (26, 33). These have apparent molecular weights in the range 90,000 (90K) to 100K and 15K to 20K and are referred to as large-T and small-t, respectively. Fingerprinting data show that small-t contains a subset of the methionine tryptic peptides present in large-T, but that in addition small-t also contains two methionine peptides not present in large-T (26, 32, 33, 48). Presumably, therefore, large-T and small-t are coded for in part by a common DNA sequence.

Early in the productive infection cycle, a 19S cytoplasmic polyadenylic acid [poly(A)]-containing SV40 RNA is detected. This hybridizes with the E strand of the region of SV40 DNA extending counter-clockwise from about 0.67 to

0.17 map units and defines this segment as the early region (16, 41, 53, 54). A similar 19S RNA, which hybridizes with SV40 early region DNA, is also found in transformed cells (17, 24, 53; see 1 and 14 for additional references on early SV40 transcription). The strict correlation found between the presence of early SV40 RNA and the presence of SV40 T-Ags is consistent with the suggestion that the early region codes for an mRNA which in turn codes for T-Ag. Experiments which establish this relationship more directly have recently been reported.

Work with SV40 early region mutants has shown that a specific deletion mutant (*dl1001*) lacking a defined portion of the early region of SV40 produces a shortened form of large-T in mutant-infected cells (39) and that the temperature-sensitive mutant, *tsa58*, overproduces both the early viral mRNA and large-T at the nonpermissive temperature (2, 51). Experiments using cell-free systems have shown that the mRNA's directing the synthesis of both large-T and small-t hybridize to SV40 DNA immobilized on Sepharose (31–33) or cellulose (26; E. Paucha and A. E. Smith, manuscript in preparation) and that SV40 complementary RNA (cRNA) directs the synthesis of a series of T-Ag-related polypeptides (10, 26, 31, 37, 38, 46). Further experiments in which SV40 DNA or SV40 cRNA have been injected into mammalian cells (9) or oocytes (D. Rungger and H. Turler, personal communication) also led to the synthesis of material which specifically reacts with anti-T serum. These experiments establish that SV40 T-Ags are at least partially virus coded.

Analysis of the T-Ags produced in cells infected with several mutants deleted at specific positions in the early region of SV40 DNA (6, 45) have resulted in a model which argues that SV40 T-Ags are entirely virus coded and which also predicts the location of the sequences coding for both large-T and small-t in the early region of SV40 (6). The model suggests that small-t is coded for by the sequences between 0.65 and 0.55 map units and that large-T is coded for by two noncontiguous sequences stretching from 0.65 to 0.59 and 0.54 to 0.17 map units. Strong support for the model comes from sequence studies on the early region of SV40 DNA, which show open reading frames in the predicted regions and terminators at 0.55 and 0.17 map units (7a, 35a, 52, 52a), from studies using the new S1 mapping method which have characterized two spliced SV40 early mRNA's (2a), and from amino acid sequence studies which show that large-T and small-t have identical amino termini mapping at 0.65 map units (27).

The data described here show results obtained on (i) the cell-free synthesis of SV40 large and small T-Ags using mRNA from productively infected cells; (ii) the partial characterization of the active mRNA species by gradient centrifugation and hybridization to fragments of SV40 DNA; and (iii) the translation of SV40 cRNA and analysis of the cell-free products by peptide fingerprinting. The results provide further evidence that both forms of SV40 T-Ag are predominantly, if not entirely, virus coded and are the products of the early mRNA. The results are also consistent with the model outlined above for the arrangement of coding sequences in the early region of SV40 DNA.

MATERIALS AND METHODS

SV40 strain 777 was used throughout these experiments. The virus was propagated on monolayers of CV1 cells. Growth of the cells and their infection has been described in the accompanying manuscript (48).

mRNA preparation and translation in L-cell S30. The preparation of total cytoplasmic RNA and isolation of poly(A)-containing RNA by chromatography on polyuridylic acid-Sepharose (Pharmacia) have been described elsewhere (28, 57).

The preparation of S30 from L-cells growing in suspension culture was carried out as has been described earlier for ascites cells (28). The optimal concentrations of KCl and $MgCl_2$ required for cell-free synthesis were determined for each preparation. Typically, a 25-μl incubation mixture contained 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), 80 mM KCl, 1.2 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 50 μM spermine, 250 μM spermidine (Sigma), 8 mM creatine phosphate, 0.2 mg of creatine kinase (Boehringer) per ml, 0.2 mM of each amino acid except methionine, 15 μCi of high-specific-activity [^{35}S]methionine (Radiochemical Centre, Amersham), and 10 μl of S30 with a protein concentration of about 10 mg/ml. In some cases the system was supplemented with 5 μg of ascites tRNA and 1 μl of reticulocyte ribosomal wash factors prepared as described by Schreier and Staehelin (43). From 1 to 2 μg of poly(A)-containing mRNA or SV40 cRNA was added, and the system was incubated for 90 min at 34°C. Incorporation was found to be linear for at least 60 min under these conditions.

Immunoprecipitation and analysis of cell-free products. The preparation of SV40 anti-T serum has been described (6; 48). The immunoprecipitation method used is a modification of that described by Kessler (15), which uses protein A found on the surface of some *Staphylococcus aureus* strains to recover the immune complexes. At the end of the incubation period 2 μl of each sample was precipitated with trichloroacetic acid to determine the counts incorporated (28). The remainder of the sample was made 10 mM in EDTA and 1 mM in dithiothreitol, chilled, and centrifuged for 5 min at 10,000 × *g* in a Beckman microfuge. Aliquots of 10 μl of the supernatants were diluted with 20 μl of a buffer containing 50 mM Tris (Sigma) (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 7.0), 0.05% Nonidet P-40 (NET buffer), and 1 mg of bovine serum albumin per ml. A 10-μl sample of either

normal hamster serum or hamster anti-T serum was added, and incubation was continued for 1 h at 22°C. The sample was diluted with 200 μl of cold NET buffer, and 20 μl of a twice-washed 10% suspension of heat-killed, formaldehyde-fixed, *S. aureus* Cowan 1 strain (15) was added. After a further 10 min at 22°C, the bacteria were pelleted and washed twice with 500 μl of NET buffer. Immune complexes were dissociated by washing the bacterial pellet with 25 μl of gel sample buffer (57), which contains 2% sodium dodecyl sulfate (SDS; Serva). After boiling for 3 min, samples were loaded directly onto 25-slot SDS-polyacrylamide gels for analysis. The conditions for polyacrylamide gel electrophoresis, drying of the gels, and autoradiography have all been described previously (6, 28, 57). The preparation of SV40 T-Ags from infected CV1 cells and VP1 from purified virions and tryptic peptide fingerprinting of polypeptides eluted from preparative scale gels are all described in the accompanying manuscript (48).

Gradient analysis of RNA. A 25-μg sample of poly(A)-containing mRNA in 25 μl of distilled water was mixed with 50 μl of 85% formamide in 10 mM Tris (pH 7.5)–1 mM EDTA, heated for 15 min at 37°C, then chilled and layered onto a 4.5-ml linear gradient of 2 to 10% (wt/vol) RNase-free sucrose (Schwarz/Mann) in the same formamide-containing buffer (22, 47). Gradients were centrifuged for 20 h at 40,000 rpm in a Beckman SW56 rotor in a Beckman L265B ultracentrifuge at 20°C. Fractions were collected from the bottom of the tubes. Ascites tRNA was added as carrier, and each fraction was ethanol precipitated three times before being translated (47).

Preparation of DNA fragments. SV40 DNA was prepared from productively infected CV1 cells using the method of Hirt (11). Form I DNA was recovered by equilibrium centrifugation in cesium chloride containing ethidium bromide (34) and further purified by velocity sedimentation through sucrose. The viral DNA was routinely checked by digestion with a number of restriction endonucleases (*Eco*RI, *Bam, Hae* III, *Alu,* and *Hind* II/III) and found to be free from defective virus DNA.

A 2-μg sample of gradient-purified SV40 form I DNA was labeled to a specific activity of 5×10^7 cpm/μg using the nick-translation method (36; as modified by R. Kamen and F. Birg, manuscript in preparation). Highly purified DNA polymerase was from Boehringer, and ^{32}P-labeled nucleoside triphosphates were from the Radiochemical Centre, Amersham. The DNA was then digested with *Hind* II/III (a gift from R. Flavell), and the digest was separated on a 1.4% agarose (Seakem) gel in E buffer (44). Bands were detected by autoradiography, and the bands corresponding to the fragments A and B (7) were excised. The fragments were recovered by electrophoresis into one-tenth E buffer containing 0.1% SDS. The eluate was loaded onto a small column of DEAE-cellulose (Whatman) in 50 mM Tris, 150 mM NaCl, and 10 mM EDTA and washed extensively with the same buffer before elution of the fragments with that buffer containing 1.5 M NaCl as described by W. Schaffner (personal communication). *Hind* II/III fragment A was recut with *Hae* III (18), and both the digest and the *Hind* II/III-B fragments were loaded onto a 5% acrylamide gel buffered with Tris-borate as described by Peacock and Dingman (29). Fragments were again detected by autoradiography. The *Hind* II/III-B fragment and that subfragment of *Hind* II/III-A corresponding to the *Hae* III-E fragment were excised and recovered as described above. Samples of the eluted fragments were checked for purity by electrophoresis on analytical 2% agarose slab gels in E buffer containing ethidium bromide. No contaminating bands were visible, and the fragments were judged to be >90% pure.

The strands of the two fragments were separated by annealing to cRNA (prepared as described below) followed by two cycles of chromatography on hydroxyapatite (40). Separated strands were stored at −20°C in 50% ethanol.

Hybridization to gradient fractions. Hybridization conditions were those used by Kamen and Shure (13). Each 20-μl incubation contained fragment DNA equivalent to about 200 pg of full-length SV40 (i.e., 10 pg of the 6% subfragment *Hae* III-E and 30 pg of the 15% fragment *Hind* II/III-B) together with 0.4 μl of the appropriate gradient fraction in hybridization buffer (1 M NaCl, 50 mM Tris [pH 7.5], 1 mM EDTA, 0.1% SDS, and 1 mg of tRNA per ml). The appropriate dilution of the gradient fractions to ensure conditions of DNA excess had been determined in pilot experiments. Incubation was carried out for 5 days at 68°C and corresponds to about 5× the estimated $C_0t_{\frac{1}{2}}$ of the system. Hybrids were quantitated using a standard S1 assay as described (13, 20).

Synthesis of SV40 cRNA. The system used for synthesis of asymmetric SV40 cRNA (55) was similar to that described by Sambrook et al. (40). A 1-ml incubation contained 100 μg of SV40 form I DNA, 60 μl of *Escherichia coli* RNA polymerase (a gift from R. Condit), and 1 mM of each of the four nucleoside triphosphates (PL Biochemicals) in a buffer containing 40 mM Tris (pH 7.5), 140 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. Synthesis was monitored by the addition of small amounts of [^{3}H]UTP (Radiochemical Centre, Amersham). After 1 h at 37°C, EDTA was added to 10 mM, and SDS to 0.1%; the mixture was extracted with phenol saturated with 10 mM Tris–1 mM EDTA, and the supernatant was applied to a column of Sephadex G-100 in a buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.1% SDS. The material in the void volume was collected and concentrated by ethanol precipitation. The precipitate was dissolved in water at a concentration of 1 mg/ml and stored at −70°C. This material was used for translation. For strand separation, the cRNA incubation mixture was treated with DNase before phenol extraction. The void volume fraction was allowed to self-anneal at 68°C in 1 M NaCl for 1 h before being applied to a column of CF-11 cellulose (Whatman) to separate any double-stranded material from the single-stranded RNA (8). The single-stranded RNA was dissolved in 10 mM Tris–1 mM EDTA prior to storage. The procedure can be scaled up 5-fold or scaled down 10-fold without any reduction in yield. Routinely, the amount of cRNA synthesized was four- to fivefold greater than the amount of DNA added. In agreement with the findings of Westphal (55), more than 99% of the product was single stranded as judged by elution from the cellulose column.

RESULTS

Characterization of the cell-free system. Poly(A)-containing cytoplasmic RNA (mRNA) was prepared from CV1 cells 48 to 72 h after infection with SV40. The mRNA was translated in a cell-free system derived from L-cells which was supplemented with initiation factors from rabbit reticulocyte ribosomes. After 90 min of incubation, the cell-free products were immunoprecipitated with anti-T sera using a modification of the protein A method, and the immunoprecipitates were analyzed by polyacrylamide gel electrophoresis.

Figure 1a shows an autoradiograph of a 15% polyacrylamide gel on which the immunoprecipitated products made in response to mRNA from productively infected and uninfected CV1 cells have been separated. Two polypeptides, which were immunoprecipitated by the anti-T sera (track 6) and not by normal hamster sera (track 5), were synthesized when SV40-infected cell mRNA was used. These polypeptides, which migrated with the ^{14}C-labeled carboxy-methylated phosphorylase *a* (94K) marker, and slightly slower than the ^{14}C-labeled carboxy-methylated lysozyme (14K) marker, were not found in the immunoprecipitates of the products made in response to uninfected cell mRNA (tracks 3 and 4). A third major product, synthesized in response to infected cell mRNA, migrated between the ^{14}C-labeled carboxy-methylated glutamic anhydrase (53K) and creatine phosphokinase (40K) markers. This protein comigrated with SV40 VP1 (see Fig. 1a and b), and since it was brought down by both anti-T and normal hamster sera, it probably results from nonspecific precipitation.

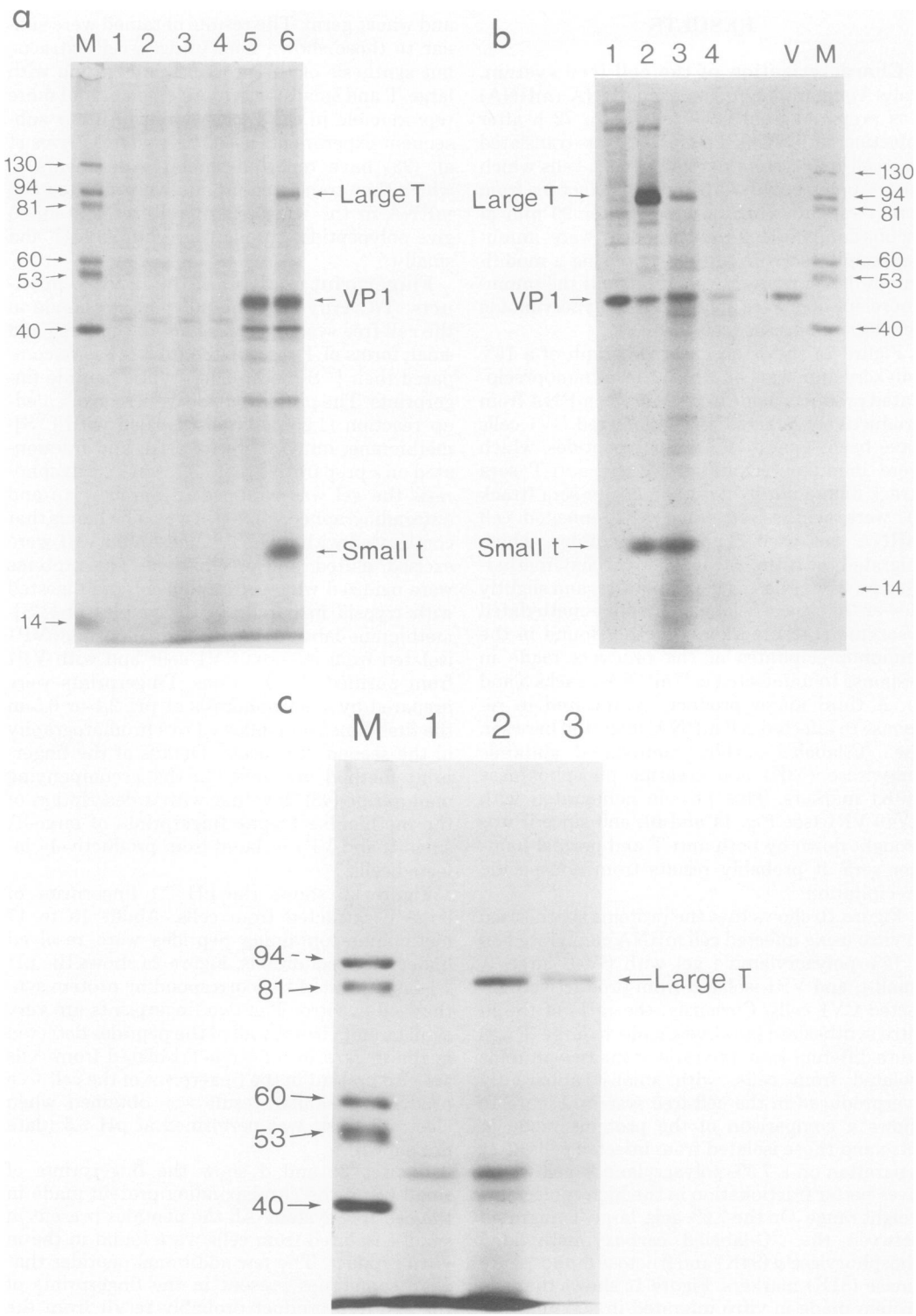
Figure 1b shows that the proteins synthesized in vitro using infected cell mRNA comigrated on a 15% polyacrylamide gel with SV40 large-T, small-t, and VP1 isolated from productively infected CV1 cells. Curiously, the ratio of the in vitro synthesized putative small-t to large-T was quite different from the ratio of the two proteins isolated from cells, with small-t apparently overproduced in the cell-free system. Figure 1c shows a comparison of the proteins made in vitro and those isolated from infected cells after separation on a 7.5% polyacrylamide gel, which gives better fractionation in the high-molecular-weight range. On the 7.5% gels, large-T migrated between the ^{14}C-labeled carboxy-methylated phosphorylase *a* (94K) and fructose 6-phosphate kinase (81K) markers. Figure 1c shows that the protein made in vitro migrated in a similar position.

We have also translated mRNA from infected CV1 cells in extracts from rabbit reticulocytes and wheat germ. The results obtained were similar to those shown here using L-cell extracts, but synthesis of the proteins comigrating with large-T and small-t was more efficient and more reproducible in the L-cell system. All our subsequent experiments used this system. Prives et al. (33) have reported similar experiments in which they translated productively infected cell mRNA in the wheat germ cell-free system to give polypeptides comigrating with large-T and small-t.

Fingerprint analysis of the in vitro products. To verify that the polypeptides made in the cell-free system correspond to the large and small forms of T-Ag isolated from cells, we compared their [^{35}S]methionine tryptic peptide fingerprints. The proteins made in vitro in a scaled-up reaction (1,250 μl) were labeled with [^{35}S]-methionine, immunoprecipitated, and fractionated on a preparative scale gel. After electrophoresis the gel was wrapped in Saran wrap and autoradiographed while still wet. The bands that comigrated with large-T, small-t, and VP1 were excised, eluted, and precipitated. The proteins were oxidized with performic acid and digested with trypsin in parallel with samples of [^{35}S]-methionine-labeled large-T, small-t, and VP1 isolated from infected CV1 cells and with VP1 from purified SV40 virions. Fingerprints were prepared by electrophoresis at pH 2.1 or 6.5 in the first dimension followed by chromatography in the second dimension. Details of the fingerprint method are given in the accompanying manuscript (48), together with a description of the methionine tryptic fingerprints of large-T, small-t, and VP1 isolated from productively infected cells.

Figure 2b shows the pH 2.1 fingerprint of large-T extracted from cells. About 15 to 17 methionine-containing peptides were resolved under these conditions. Figure 2a shows the pH 2.1 fingerprint of the corresponding protein synthesized in vitro. The two fingerprints are very similar, and virtually all of the peptides detected in the fingerprint of large-T isolated from cells are also present in the fingerprint of the cell-free product. A similar result was obtained when electrophoresis was performed at pH 6.5 (data not shown).

Figures 2c and d show the fingerprints of small-t and the corresponding protein made in the cell-free system. All the peptides present in small-t isolated from cells were found in the in vitro product. The few additional peptides that were sometimes present in the fingerprints of the cell-free product probably result from the background of nonspecifically precipitated proteins. This background tends to be higher in the low-molecular-weight region of gels of proteins


a
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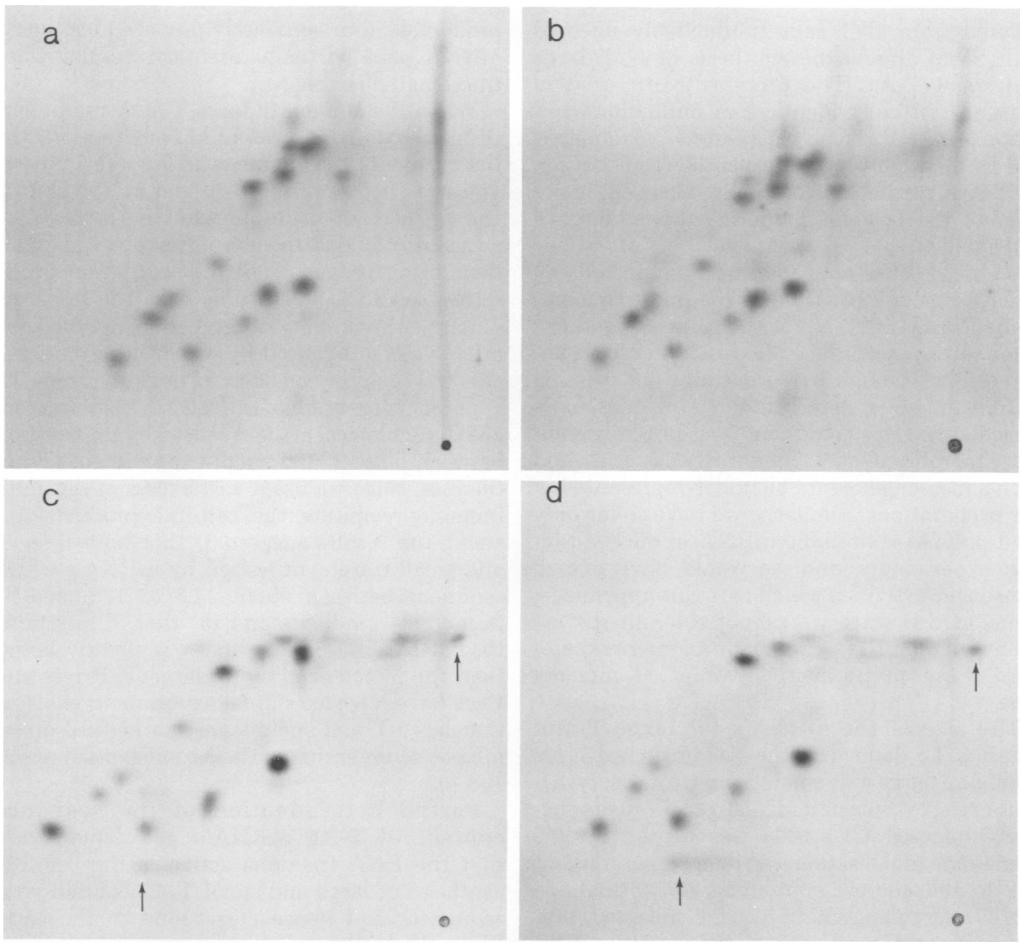

FIG. 2. *[^{35}S]methionine tryptic peptide fingerprints of large-T and small-t synthesized in vitro compared with the corresponding proteins isolated from productively infected CV1 cells. Fingerprints were prepared at pH 2.1 using 2.5 × 10^4 cpm of each sample and autoradiographed for 10 days. (b and d) Large-T and small-t, respectively, isolated from CV1 cells; (a and c) large-T and small-t, respectively, synthesized in vitro.*

made in vitro.

The fingerprints shown in Fig. 2 also confirm that small-t contains a subset of the peptides present in large-T but that, in addition to the shared peptides, small-t contains at least two methionine tryptic peptides not found in large-T. The two unique peptides are also present in the small-t made in vitro. Other experiments have shown that the material from the cell-free product which comigrates with SV40 VP1 has a fingerprint virtually identical to that of VP1 isolated from SV40-infected cells and from purified virions (data not shown). SV40 VP1 has no methionine tryptic peptides in common with SV40 T-Ags.

We conclude from these results that mRNA

FIG. 1. *Synthesis of SV40 large-T and small-t in L-cell extracts. (a) Proteins synthesized in vitro in response to no added mRNA (tracks 1 and 2), uninfected CV1 cell mRNA (3 and 4), and productively infected CV1 cell mRNA (5 and 6), after immunoprecipitation with control (1, 3, and 5) and anti-T (2, 4, and 6) serum. The gel contained 15% polyacrylamide, and autoradiography was for 4 days. The numbers indicate the molecular weight (× 10^{-3}) of the marker proteins (M). (b) Proteins synthesized in vitro (tracks 3 and 4) compared with those isolated from infected cells (1 and 2) after immunoprecipitation with control (1 and 4) and anti-T (2 and 3) serum. V, Purified SV40 virus. The gel contained 15% polyacrylamide; autoradiography was for 4 days. (c) Proteins synthesized in vitro and immunoprecipitated with control (1) and anti-T (2) serum compared with large-T isolated from productively infected CV1 cells (3) after separation on a 7.5% polyacrylamide gel. Autoradiography was for 4 days.*

isolated from CV1 cells productively infected with SV40 directs the synthesis of both large and small T-Ag. The proteins synthesized in vitro are virtually identical in immunoprecipitation properties, in polyacrylamide gel mobility, and in methionine tryptic peptide composition to T-Ags extracted from cells. Henceforth we refer to the proteins made in vitro as large-T and small-t.

So far we have not reproducibly detected any other forms of T-Ag such as the putative membrane form (12, 21, 42) in the cell-free products made in response to mRNA from infected monkey cells. Although our procedure for isolating mRNA involves detergent lysis of cells, it is possible that this treatment does not fully solubilize membrane-associated mRNA species, and such a messenger could be under-represented in our preparations. Similarly, we have so far only used poly(A)-containing mRNA in our translation experiments, and we would have missed messenger activities which lack this appendage. Thus, at this stage we cannot rule out the existence of an mRNA coding for an intermediate-sized T-Ag in productively infected monkey cells.

The size of the mRNA's for large-T and small-t. To determine the size of the mRNA's coding for large and small T-Ags, total poly(A)-containing cytoplasmic RNA from SV40 productively infected CV1 cells was heated in 60% formamide and fractionated on a linear gradient of 2 to 10% sucrose containing 85% formamide (22, 47). Gradient fractions were collected, precipitated, and translated in the L-cell system. The products of translation of each of the gradient fractions were immunoprecipitated with control and anti-T serum and analyzed on 15% polyacrylamide gels.

Figure 3a shows the results of one such experiment. As gradient markers we used mouse cell rRNA run in a parallel gradient and the major late SV40 mRNA, which has a sedimentation coefficient of 16S. The latter was detected in the sucrose gradient fractions because it directs the synthesis of SV40 VP1 (14), and this, as shown above, nonspecifically precipitated during our immunoprecipitation procedure (see tracks 26 to 28 in Fig. 3a). The mRNA fraction coding for large-T sedimented at about 17.5S in the 85% formamide sucrose gradient relative to the 18S and 16S RNA markers (tracks 23 to 26). The mRNA coding for small-t appeared to run slightly ahead of the large-T mRNA, as judged by the slight change in the relative amounts of large-T and small-t synthesized by the different fractions from the 17S to 18S region of the gradient. The difference is small but highly reproducible; four separately prepared batches of mRNA gave virtually identical results when fractionated in this way.

When the amount of large-T and small-t synthesized by mRNA present in each fraction from the gradients was quantitated by densitometry and replotted (as in Fig. 3b and c), the shift in the synthesis of one protein relative to the other was more obvious. In view of the reported highly denaturing properties of the separation procedure used (22), we assume that the apparent difference in sedimentation coefficient of the two mRNA's is not caused by some conformational effect. We also consider it unlikely that the apparent separation of the mRNA's results from an immunological artifact caused by competition between large-T and small-t for the anti-T antibodies, since we use a vast excess of serum to immunoprecipitate the cell-free products. Instead, the results suggest (i) that both large-T and small-t are synthesized by mRNA's which sediment between about 17.5 to 18S on 85% formamide gradients and (ii) that, if anything, the mRNA coding for small-t is slightly larger than the mRNA coding for large-T. Prives and Beck have reported similar experiments showing that large-T and small-t are synthesized on an mRNA sedimenting in their conditions at about 19S (32).

Partial determination of the sequence content of T-Ag mRNA's. To demonstrate that the RNA fractions active in the cell-free synthesis of large and small T-Ag contain viral sequences and hence correspond to the early SV40 RNA that has been detected by several other groups (1, 14, 16, 41, 53), we determined the sequence content of the active fractions by hybridization to the separated strands of restriction endonuclease fragments of SV40 DNA. Furthermore, in the hope of detecting differences in the putative large-T and small-t mRNA's, we used the restriction enzyme fragments *Hind* II/III-B and *Hae* III-E, which are from the extreme ends of the early region of SV40 DNA (Fig. 4a). When the experiments were performed, splicing was unknown to us, and we assumed that if the mRNA's were different in size the difference would be located at one end of the mRNA molecule.

Gradient purified SV40 form I DNA was labeled in vitro to high specific activity (5×10^7 cpm/μg) with ^{32}P using the nick-translation method (36). The DNA was cut with the restriction endonucleases *Hind* II/III and fractionated on an agarose gel, and the fragments A and B were recovered. *Hind* II/III-A was redigested with *Hae* III, and the subfragment corresponding to the *Hae* III-E fragment was isolated on a

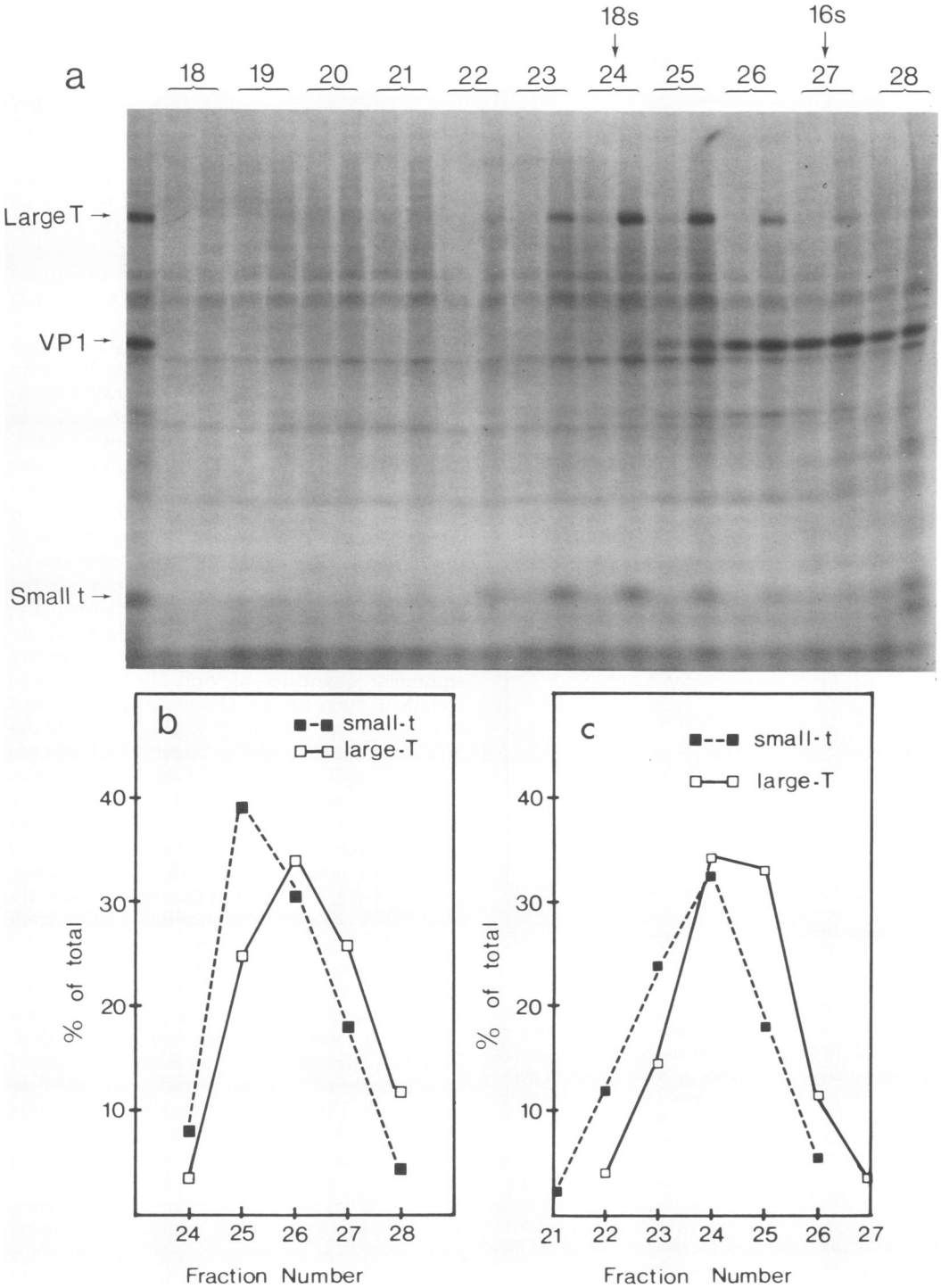

FIG. 3. *Sucrose density gradient fractionation of the mRNA's directing the synthesis of large-T and small-t. (a) mRNA isolated from productively infected CV1 cells was heated in 60% formamide and fractionated on a 2 to 10% sucrose density gradient containing 85% formamide (22). Fractions were collected, precipitated, and translated in L-cell extracts. Each reaction product was immunoprecipitated with control (left-hand track of each pair) and anti-T (right-hand track) serum, subjected to electrophoresis on a 15% polyacrylamide gel, and autoradiographed for 4 days. The extreme left-hand track shows large-T, small-t, and VP1 made in response to unfractionated mRNA. 18S indicates the position of mouse cell ^{3}H-labeled 18S rRNA sedimented in a parallel gradient, and 16S indicates the peak of VP1 coding activity. The autoradiograph shown in Fig. 3 was subjected to densitometry using a Joyce-Loebel densitometer. The peaks corresponding to large-T and small-t were quantitated, and the results are shown as the amount of each protein synthesized in each fraction, expressed as a percentage of the total amount of that protein synthesized in all gradient fractions. (b). The autoradiograph of a similar experiment (see Fig. 5 of ref. 26) was also quantitated by densitometry (c).*

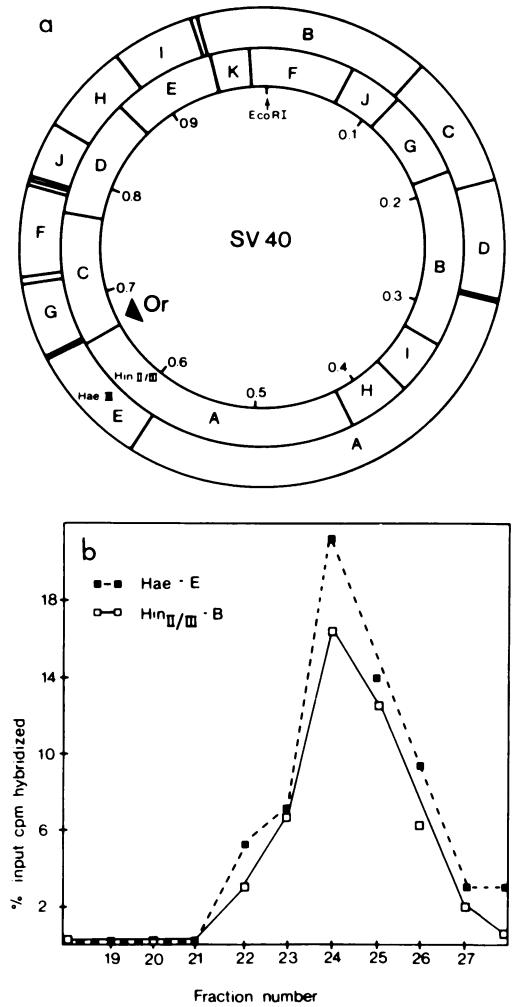

FIG. 4. *(a) Hind II/III and Hae III restriction endonuclease maps of SV40 DNA. Data are taken from references 7 and 18. (b) Hybridization of restriction endonuclease fragments of SV40 DNA to fractionated SV40 mRNA. E-strand DNA of restriction endonuclease fragments Hae-E and Hind II/III-B was hybridized with RNA from each of the fractions of the gradient shown in Fig. 3a and b. The input was adjusted so that both fragments were present in equimolar amounts. Hybridization was at 68°C for 5 days, after which hybrids were quantitated by the S1 assay. The results are expressed as the percentage of the input DNA which entered into hybrid.*

polyacrylamide gel. The positions of these fragments on the conventional SV40 DNA map are shown in Fig. 4a.

The DNA strands of the *Hind* II/III-B and *Hae* III-E fragments were separated by hybridization with an excess of SV40 cRNA, followed by hydroxyapatite chromatography (40). The E DNA strand of both fragments was hybridized to RNA from each fraction of the 85% formamide-containing sucrose gradient shown in Fig. 3c. Appropriate dilution of the gradient fractions was made to ensure that the percentage of the probe hybridized was directly proportional to the amount of viral RNA present; i.e., hybridization was carried out under conditions of DNA excess. The DNA input was adjusted so that the two fragments were present in approximately equimolar amounts. After hybridization at 68°C for 5 days, the RNA:DNA hybrids were quantitated using the S1 nuclease assay (13, 20).

The results of the experiment are shown in Fig. 4b. A single peak of material which hybridized to the E strand of both fragments was detected, and this corresponded exactly with the peak of messenger activity coding for T-Ags in vitro. Presumably this peak also corresponds to the 19S early SV40 RNA detected by others (1, 14, 16, 54). We could detect no difference in the ability of RNA from different fractions of the gradient to hybridize with the two E-strand fragments, and therefore the fractions contained equimolar quantities of viral RNA sequences complementary to viral DNA sequences present in the two end fragments. Thus, although the mRNA for small-t may be larger than that coding for large-T, we could not detect any difference in the sequence content of the mRNA's using these DNA fragments. We deduce from these experiments (i) that the so-called 19S early SV40 RNA includes the mRNA's for both forms of SV40 T-Ag, and (ii) that the bulk of the early SV40 RNA contains sequence information from both ends of the early region.

Translation of SV40 cRNA. The data presented above show that SV40 T-Ags can be synthesized in vitro in response to mRNA which appears to contain sequences from SV40 DNA. However, although these and many other experiments (referred to in the Introduction) indicate that SV40 T-Ags are at least partially virus coded, the evidence that the proteins are entirely virus coded is not yet overwhelming. A possible host-derived component in the T-Ag mRNA's still cannot be excluded. To examine this further, we analyzed the products made in vitro using SV40 cRNA as messenger, arguing that since cRNA is a transcript made up exclusively of viral sequences, any putative host cell sequences could not be present and this should be reflected in the translation products.

The SV40 cRNA used was the product of in vitro transcription of highly purified SV40 form I DNA by *E. coli* RNA polymerase. Under appropriate conditions, transcription is highly asymmetric, with more than 99% of the tran-

script being complementary to the E strand of viral DNA (55). Analysis of the sites on SV40 DNA at which transcription is initiated by *E. coli* RNA polymerase shows that four of the five major initiation sites are in the region between 0.65 and 0.45 map units (19), and gradient analysis of the cRNA demonstates that much of the material is greater than 18S (56; our unpublished data). Experiments using the S1 nuclease method (2a) to assay for interruptions in RNA transcripts indicate that at least some cRNA molecules form contiguous hybrids with SV40 DNA (R. I. Kamen and E. Paucha, unpublished data), and Berk and Sharp have established that SV40 cRNA protects *Hin*d II/III fragment A (0.66 to 0.43 map units) from digestion with S1 nuclease (2a). These experiments establish that SV40 cRNA is closely related to SV40 early mRNA and indicate that at least some molecules are contiguous transcripts of the entire early region. We hoped by translating SV40 cRNA to synthesize SV40 T-Ags in vitro and by fingerprinting the cell-free products to establish which methionine tryptic peptides of T-Ags are virus coded.

Figure 5a shows an autoradiograph of the immunoprecipitated products made in the L-cell system in response to both SV40 cRNA and mRNA from SV40-infected cells. A number of polypeptides were made in response to the added cRNA, and these were specifically precipitated by anti-T serum. The cRNA-directed proteins range in molecular weight from about 14,000 to 70,000, the major bands being numbers 1 to 9 in Fig. 5b. One of the major cRNA-directed cell-free products (band 8) comigrated with small-t made in response to added mRNA, but perhaps the most striking feature of the cRNA product is the absence of polypeptide comigrating with large-T (Fig. 5a).

In an attempt to alter the pattern of polypeptides synthesized and hopefully to synthesize large-T in response to SV40 cRNA, we varied many parameters of the cell-free transcription and translation systems. We tested cRNA's made by both *E. coli* and wheat germ RNA polymerases in other cell-free systems, including wheat germ extracts (38) and the messenger-dependent reticulocyte lysate (30). We also tested synthesis in response to added SV40 DNA using the linked transcription-translation system from wheat germ described by Roberts et al.

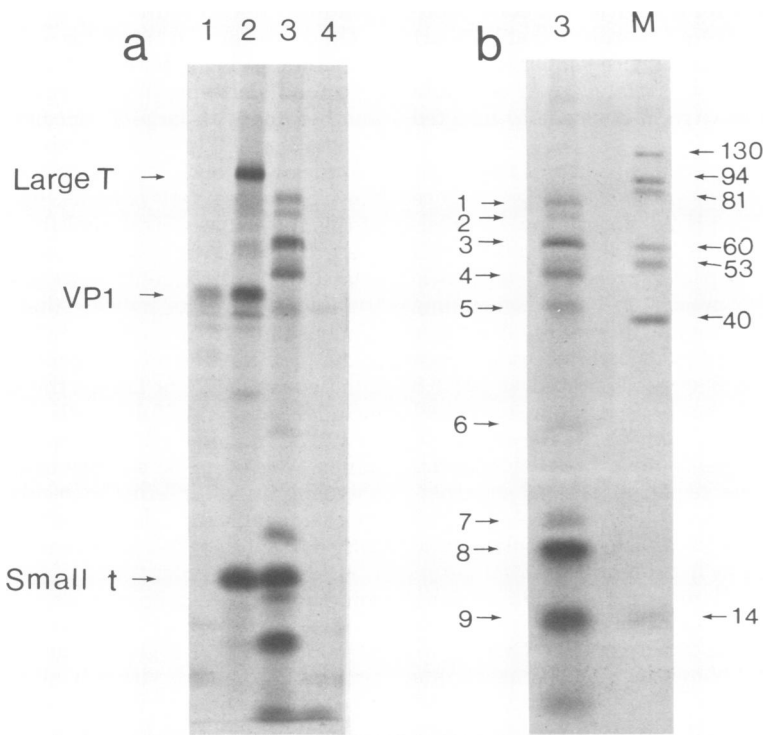

FIG. 5. *Polypeptides synthesized in response to SV40 cRNA. (a) Proteins synthesized in L-cell extracts using infected CV1 cell mRNA (1 and 2) and SV40 cRNA (3 and 4) and immunoprecipitated with control (1 and 4) or anti-T (2 and 3) serum. The gel contained 15% polyacrylamide, and autoradiography was for 3 days. (b) The immunoprecipitated cRNA products (cf. track 3, in a) electrophoresed next to marker proteins (M).*

(38). Although we established in each case that the cell-free extracts made large-T in response to added mRNA from infected cells, the pattern of products directed by cRNA was always remarkably similar to that shown in Fig. 5; one band comigrated with small-t, and the other polypeptides ranged from 14,000 to 70,000 daltons with major bands at 50,000 and 60,000 daltons. No material comigrating with large-T was ever detected. Similar experiments which reached the same conclusion have previously been reported (10, 26, 31, 37, 38, 46).

We considered it most likely that the failure to make large-T in response to SV40 cRNA was either a translational artifact, such as premature termination which resulted from the failure of the transcription system to effect some essential cleavage or modification (e.g., capping or polyadenylation) of the cRNA, or, alternatively, reflected some peculiarity of the sequence arrangement in cRNA.

Fingerprint analysis of SV40 cRNA-directed polypeptides. The cRNA-directed cell-free products were synthesized in the L-cell system in a large-scale reaction, immunoprecipitated, separated on a preparative scale polyacrylamide gel, and prepared for fingerprinting as described above for the mRNA-directed products. Bands 1 to 9 were fingerprinted at both pH 2.1 and pH 6.5 in parallel with similar digests of large-T and small-t (i) isolated from cells and (ii) synthesized in vitro in response to infected cell mRNA.

The pH 2.1 fingerprints of some of the major cRNA-directed bands are shown in Fig. 6. Figure 6d and a show the pH 2.1 fingerprints of small-t from cells and from band 8, respectively. All the methionine peptides present in authentic small-t were also present in band 8, including the two peptides unique to small-t. A similar result was obtained when the peptides from small-t and band 8 were separated at pH 6.5 (Fig. 7a and c). In both cases, a few additional peptides were also present in the fingerprint of band 8; these probably arise from background proteins from this region of the gel or cross-contamination from adjacent cRNA-directed bands. These results establish that a polypeptide similar to small-t by immunoprecipitation properties, by polyacrylamide gel mobility, and by methionine tryptic peptide fingerprinting is made in vitro in response to SV40 cRNA.

Figure 6c and f show the pH 2.1 fingerprints of bands 3 and 4, the cRNA-directed 50,000- and 60,000-dalton proteins. These fingerprints have peptides in common with one another and suggest that these bands are related. The fingerprints also have peptides in common with large-T (Fig. 6e). This was confirmed when the peptides of band 3 were separated at pH 6.5 and compared with a similar fingerprint of large-T (Fig. 7b and d) and when samples of the different digests were mixed with large-T prior to fractionation (data not shown). All the peptides present in the digest of band 3 had their counterparts in the fingerprint of large-T at both pH 2.1 and pH 6.5, and, when mixed together prior to fractionation, the shared peptides comigrated.

A striking feature of the fingerprints of bands 3 and 4 is that although they contain peptides related to large-T they are quite distinct from the fingerprint of band 8 and share no peptides with small-t, with the exception of one which we believe to be Met-Lys (see Fig. 6 and 7). This was most dramatically demonstrated when the digests of the cRNA products band 3 and band 8 were mixed prior to fingerprinting at pH 2.1. Figure 6b shows that the resulting fingerprint was strikingly similar to a fingerprint of large-T (Fig. 6e) isolated from cells, except that the mixture contained in addition the two peptides unique to small-t. The same result was obtained when the mixture was fingerprinted at pH 6.5, which gives better fractionation of the peptides that chromatograph fast in the second dimension (data not shown).

Other fingerprint analyses not shown here have established that the cRNA-directed bands 1 to 6 all share peptides in common with one another and with large-T, but not with small-t. Bands 7 and 9 give fingerprints which share peptides with both large-T and small-t. These fingerprints are very complex for polypeptides of this molecular weight, and this suggests that these bands are mixtures of various fragments related to both large-T and small-t. Together the fingerprint data indicate that the products produced in vitro in response to SV40 cRNA are a complex mixture of polypeptides which fall into two families. One family, the small-t family, includes apparently full-length small-t (band 8) as well as fragments thereof (band 9); the other family appears to be a series of closely related polypeptides, of differing molecular weight and peptide complexity, all of which share peptides with one another and with large-T (bands 1 to 7, 9). Because a polypeptide of 60,000 daltons is commonly the predominant member of this family (10, 26, 31, 37, 38; Fig. 5), we refer to it as the 60K family. Taken together, the small-t and 60K families account for virtually all of the methionine peptides present in small-t and large-T.

We conclude from these experiments (i) that small-t is probably entirely, and large-T at least predominantly, SV40 virus coded, (ii) that the small-t and 60K families represent the transla-

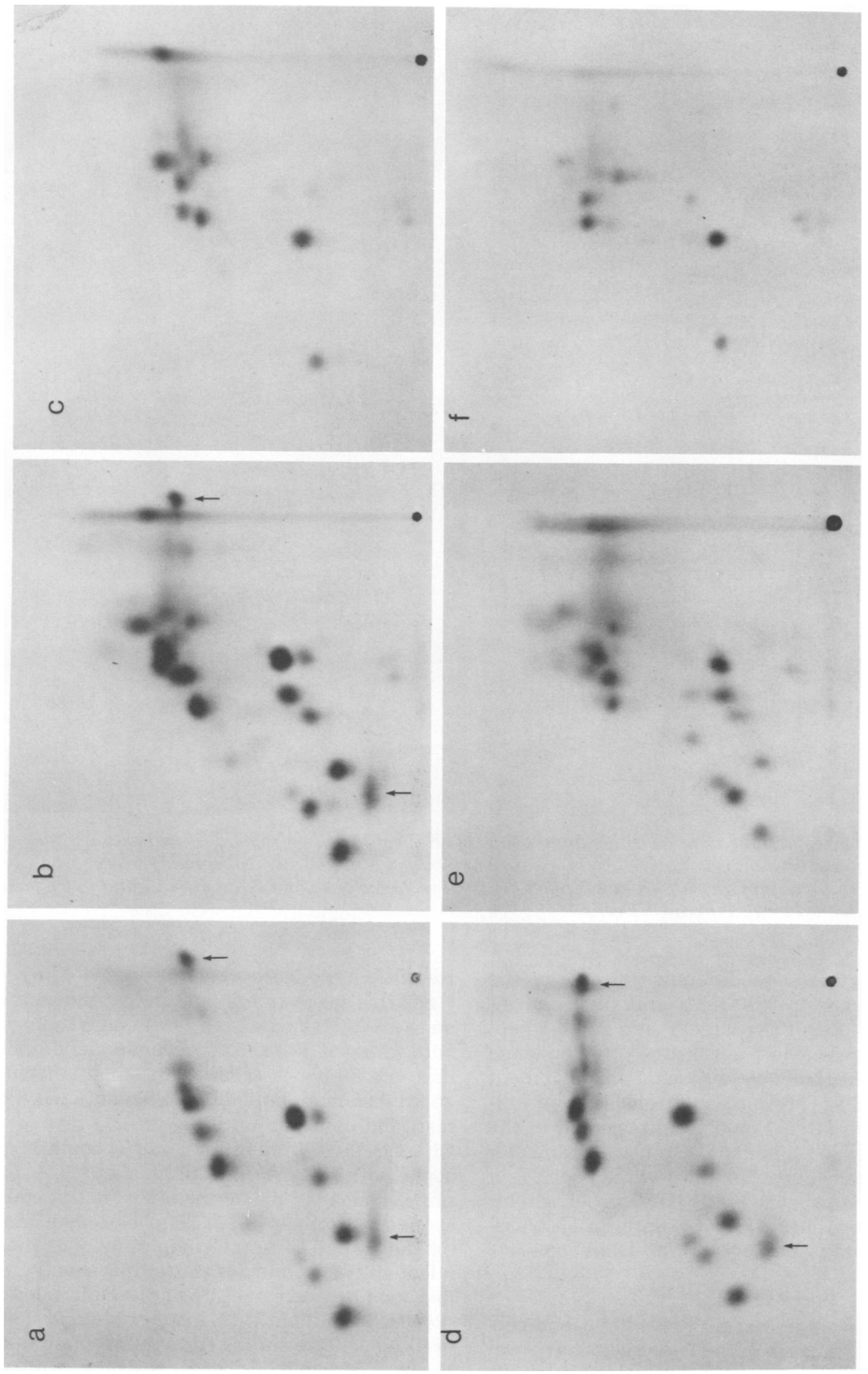

FIG. 6. *[^{35}S]methionine tryptic peptide fingerprints of cRNA-directed polypeptides compared with large-T and small-t from productively infected CV1 cells. Fingerprints (approximately 3 × 10^5 cpm) were prepared at pH 2.1 in the first dimension and autoradiographed for 10 days. (a) cRNA band 8; (b) cRNA band 8 + band 3; (c) cRNA band 3; (d) small-t from CV1 cells; (e) large-T from CV1 cells; (f) cRNA band 4.*

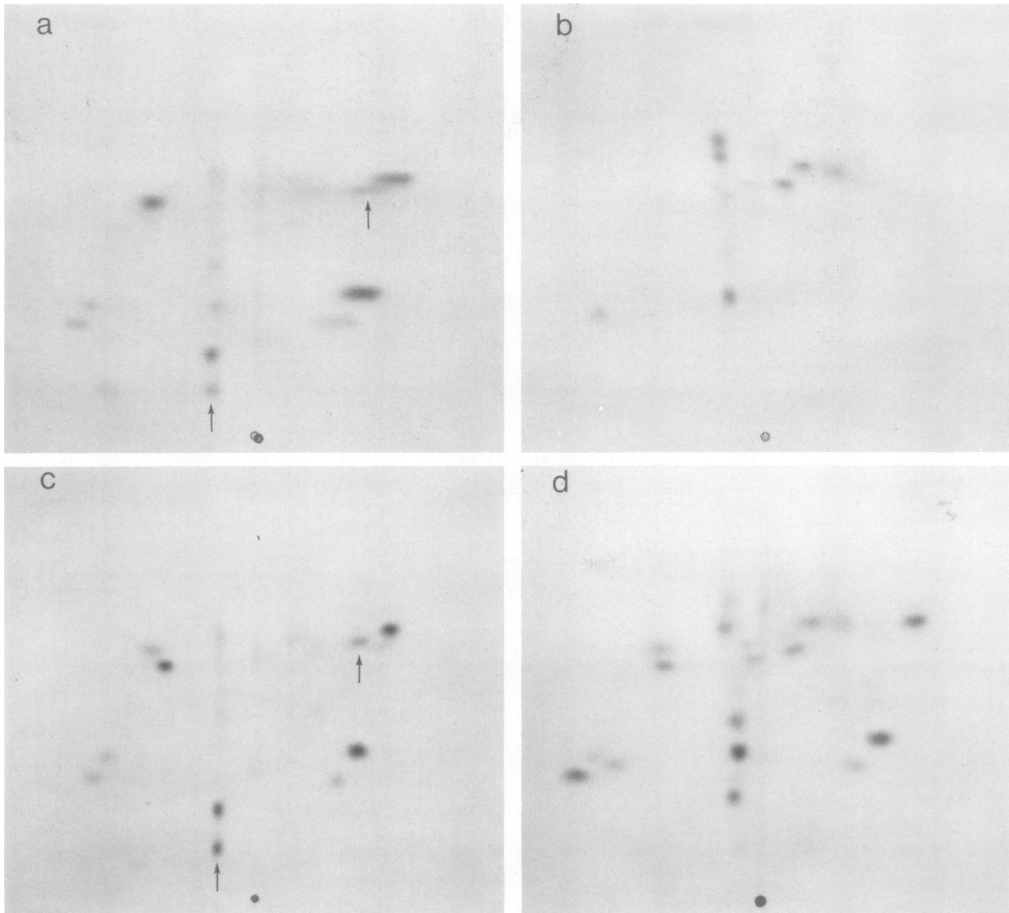

FIG. 7. *[^{35}S]methionine tryptic peptide fingerprints of cRNA-directed polypeptides compared with large-T and small-t from productively infected cells. Fingerprints (approximately 2 × 10^4 cpm) were prepared at pH 6.5 in the first dimension and autoradiographed for 10 days. (a) cRNA band 8; (b) cRNA band 3; (c) small-t from CV1 cells; (d) large-T from CV1 cells.*

tion products of two different portions of the early region of SV40 DNA, and (iii) that, although almost all the large-T and small-t peptides are present in the cell-free product, some feature of the structure or sequence arrangement of SV40 cRNA prevents the translation of full-length SV40 large-T and results instead in the synthesis of fragments. Greenblatt et al. (10) have also concluded that most of the peptides of T-Ag are virus coded on the basis of similar experiments using an *E. coli* transcription-translation system primed with SV40 DNA.

DISCUSSION

A number of studies on the cellular and cell-free synthesis of the SV40 T-antigens have suggested that these proteins are at least partially virus coded (9, 10, 26, 31–33, 37–39, 46, 51). Recently a model has been proposed which extends this suggestion by predicting the location on the SV40 genome of the sequences coding for both forms of T-Ag (6, 27). The model proposes that the sequences coding for small-t begin at about 0.65 map units on the conventional SV40 map and continue until about 0.55 map units, whereas those coding for large-T begin at the same position, continue until about 0.59 map units, and after an interruption in the coding sequence (from about 0.59 to 0.54 map units) extend from 0.54 to 0.17 map units. The model adequately accounts for the finding that large-T and small-t share methionine tryptic peptides (26, 32, 33, 48), but that, in addition, both forms of T-Ag contain some peptides not found in the other. The model is also consistent with the pattern of synthesis of large and small T-Ags in

cells infected with specific SV40 deletion mutants (6, 45) and with the DNA sequence determined for the early region of SV40, which has termination codons in all three phases between 0.54 and 0.55 map units (52, 52a) and also at 0.17 map units (7a, 35a). The results presented here provide further evidence that SV40 T-Ags are virus coded and also support the model for the location of sequences coding for both forms of SV40 T-Ag.

The mRNA's for large-T and small-t. Sucrose gradient analysis of the mRNA's active in the synthesis of both forms of SV40 T-Ag has shown that they cosediment with material which hybridizes with the E strand of SV40 DNA (Fig. 3 and 4). No other size class of RNA hybridizing with the probe was detected. This, together with the finding that the mRNA's for both forms of T-Ag can be selected by hybridization to immobilized SV40 DNA (26, 33; Paucha and Smith, manuscript in preparation) provides direct evidence that the mRNA's for both forms of T-Ag contain at least some sequences from the early region of SV40 DNA.

The experiments to size the mRNA's directing the synthesis of the SV40 T-Ags showed that small-t has an mRNA similar in size to the large-T mRNA and is much larger than the minimum size required to code for a protein of 15,000 to 20,000 daltons. Although the results show a small but reproducible difference in the sedimentation rate of the mRNA's coding for large-T and small-t, all fractions from the peak of early mRNA hybridize equally well to restriction endonuclease fragments of SV40 DNA from both ends of the early region. Assuming that the mRNA's for small-t or large-T are present in roughly equal amounts, the results indicate that the active messenger species for both proteins include sequence information from both ends of the early region.

Further interpretation of our hybridization data is complicated because the two early mRNA's are not well resolved under our gradient conditions, because we do not know the relative amounts of the two mRNA's, and because the gradient fractions do not contain sufficient RNA to determine the saturation level of the probe under conditions of RNA excess. However, using a newly-developed S1 nuclease method for characterizing and mapping mRNA's, Berk and Sharp (2a) have detected two spliced early SV40 mRNA's of roughly equal abundance; one has a splice between 0.59 and 0.54 map units and the second has a splice at 0.54 map units. Our estimates of the sedimentation coefficient of the early mRNA's, the demonstration of a small but reproducible fractionation of the two active mRNA's, and the pattern of hybridization observed with specific early region DNA fragments are all consistent with the data of Berk and Sharp. Both sets of data are consistent with a model predicting that the mRNA coding for small-t is a transcript of virtually all the early region (about 2,600 nucleotides long [7a, 35a]), whereas the large-T mRNA, because it lacks the internal sequences between 0.54 and 0.59 map units, is about 250 nucleotides shorter.

The translation of SV40 cRNA. We and other groups have reported earlier the cell-free translation of asymmetric SV40 cRNA to give polypeptides which specifically immunoprecipitate with anti-T sera (10, 26, 31, 37, 38, 46). In none of these studies was full-sized large-T detected among the cRNA-directed products. All our attempts to produce a radical qualitative change in the pattern of proteins synthesized in response to cRNA were unsuccessful, and we were led to the conclusion that this pattern of synthesis is a property of cRNA rather than an artifact of the cell-free system.

A protein which exactly comigrates with small-t, which is specifically immunoprecipitated with anti-T serum, and which has a methionine tryptic peptide fingerprint similar to authentic small-t is present in the cRNA-directed cell-free product. In addition to showing that small-t is in all probability totally virus coded, this result suggests that the sequences coding for small-t are contiguous on SV40 DNA. Since the N-terminus of small-t is known to be coded at about 0.65 map units (27), this suggestion is consistent with the finding that the DNA sequence between about 0.65 and 0.55 map units has an open reading frame which could code for a protein of about 20,000 daltons. The latter may well be equivalent to small-t.

In addition to small-t, the cRNA-directed cell-free product includes a family of peptides, referred to as the 60K family, which range in molecular weight from 14K to 70K and which share methionine tryptic peptides in common with large-T but not with small-t. Since the region of sequence common to large-T and small-t is known to be located at the N-terminus of large-T (27), the data suggest that the 60K family shares sequences with the C-terminus of large-T. Presumably, therefore, the 60K family results from the translation of the sequences located between the termination codons found in the DNA sequence at 0.54 and 0.17 map units. This region can code for a maximum of about 70,000 daltons of protein.

The polypeptides of the 60K family could represent either a series of polypeptides result-

ing from initiation at a single position on SV40 cRNA, followed by premature termination at various positions along the molecule, or a series which ends at the same termination codon but initiates at a number of alternative positions along the early transcript, or both. We believe the multiple initiation sites model best fits our data, because cRNA made from the DNA of SV40 mutants with deletions in the region from 0.59 to 0.54 map units directs the synthesis of a wild-type-sized 60K family, whereas a mutant with a deletion at 0.21 map units gives a product in which all the bands except band 8 are shortened (Paucha and Smith, manuscript in preparation). This suggests that the 60K family all terminate distal to 0.21 map units, presumably at the terminator at 0.17 map units, and confirms that the sequences proximal to 0.54 map units are not represented in the 60K family.

The failure to synthesize large-T in response to cRNA can be readily explained if the large-T mRNA is spliced. This would mean that, sometime during or after transcription of the SV40 early region, that portion of transcript which contains sequences coding for the C-terminus of small-t, together with the termination codons found in the DNA sequence at about 0.54 map units, is removed to generate an mRNA capable of directing the synthesis of large-T. Berk and Sharp (2a) have shown that SV40 cRNA is not spliced in the region between 0.66 and 0.43 map units during synthesis with *E. coli* RNA polymerase, and presumably extensive splicing does not occur under our conditions in the L-cell or wheat germ cell-free systems. Thus, while cRNA can faithfully direct the synthesis of both portions of large-T, it cannot produce a molecule in which these two portions are joined.

In addition to the data presented here, strong evidence in support of the model for the location of sequences coding for the SV40 T-Ags has recently come from experiments which have shown (i) that large-T and small-t have identical amino-terminal amino acid sequences which map at 0.65 map units (27); (ii) that cRNA made using DNA from mutants with deletions in the region from 0.59 to 0.54 map units directs the synthesis of fragments of small-t, which in some cases lack the peptides unique to small-t (Paucha and Smith, manuscript in preparation); and (iii) that two early SV40 mRNA species, both of which are spliced, can be detected using the new S1 method for characterizing and mapping the sequences present in mRNA's (2a).

The S1 mapping data position the splice in the presumed small-t mRNA just beyond the predicted termination codon for small-t. However, the error limits on the experiment are such that the splice could include a portion of coding sequence. We believe that our data on the cRNA-directed synthesis of small-t favor the interpretation that the splice lies outside the sequence coding for small-t. However, we cannot exclude the possibility that the protein made in response to cRNA differs from authentic small-t in some way not detected by our methods, e.g., by a few amino acids at the C-terminus, nor can we exclude the possibility that some limited RNA processing occurs in the L-cell system. Thus, though unlikely, the possibility of a small splice in the coding region at the carboxy terminal of small-t cannot yet be ruled out. To establish this point rigorously and to determine the exact relationship between the DNA sequence and the amino acid sequence of large-T require further studies on the early proteins and their messengers.

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