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Cell-free translation of simian virus 40 early messenger RNA coding for viral T-antigen

(radioimmunoprecipitations/RNA·DNA-Sepharose hybridization/wheat-germ protein-synthesizing system/sodium dodecyl sulfate polyacrylamide gels)

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ABSTRACT **Simian virus 40 (SV40) mRNA was isolated by hybridization of cytoplasmic RNA, from SV40-infected BS-C-1 monkey cells early in lytic infection, to SV40 DNA immobilized on Sepharose. The early viral mRNA, when added to a wheat-germ translation system, directed the synthesis of a unique class of products including a 90,000 molecular weight (M_r) polypeptide. It was found that this 90,000 M_r product as well as a prominent 17,000 M_r polypeptide could be specifically immunoprecipitated with hamster antiserum to SV40 T-antigen, but not with hamster control serum. Similar immunoprecipitation of extracts of SV40-infected cells with hamster anti-T serum yielded 90,000 M_r and 17,000 M_r polypeptides; these polypeptides were not found in immunoprecipitates of uninfected cell extracts. SV40 cRNA, prepared by asymmetric transcription of plaque-purified SV40 DNA, directed the cell-free synthesis of several products, including a 70,000 M_r polypeptide that could be specifically immunoprecipitated with anti-T serum. However, no T-antigen-related polypeptide was found in infected cells that corresponded in size to the major immunoprecipitated cRNA product.**

Complementation studies with temperature-sensitive mutants of the tumor-causing simian virus 40 (SV40) indicate that it may have as few as three primary gene products (1). Only one of these, the *A* gene product, has been directly linked to the process of virus-induced neoplastic transformation (2–5). Cells in the early phase of productive lytic infection and virus-transformed cells exhibit some common features. In each case, a species of virus-specific RNA is induced, which hybridizes to the same strand of SV40 DNA (6, 7) and to the same fragments of SV40 DNA generated by restriction endonucleases (8, 9). At least three virus-associated antigens, T, U, and TSTA, have been detected both early in lytic infection and in transformed cells (10). The T-antigen has so far been characterized primarily immunologically, employing sera from animals bearing SV40-induced tumors (11–13). T-antigen has been partially purified (14–16) and is now considered to be a DNA-binding protein (17–20) with the molecular weight of the denatured polypeptide estimated to be from 70,000 (21) to 100,000 (22). Comparison of the properties of the T-antigen after infection with either wild-type or *A*-mutant virus at the restrictive temperature has supplied evidence that this antigen (or a part thereof) is encoded by viral DNA (22–24). To demonstrate directly that the information for the SV40 T-antigen is encoded in the viral genome, and to study its expression further, we have investigated the cell-free translation of early SV40 mRNA.

We report that SV40 early mRNA from infected cells, and SV40 cRNA transcripts made *in vitro*, direct the synthesis in the wheat-germ cell-free system of polypeptides that can be specifically immunoprecipitated by antiserum to SV40 T-antigen. The products directed by "*in vivo*" mRNA and synthetic cRNA differ, however, in their electrophoretic mobility on sodium dodecyl sulfate/polyacrylamide gels. The former products correspond to the T-antigen immunoprecipitated from extracts of infected cells and the largest of these has an estimated molecular weight of 90,000. A preliminary description of these findings has been given previously (25).

Abbreviations: SV40, simian virus 40; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; M_r, molecular weight.

MATERIALS AND METHODS

Wheat germ was a gift from Z. Schildhaus of the Bar Rav Mill, Tel-Aviv, Israel. [^{35}S]Methionine (300 Ci/mmol) and [5,6-^{3}H]uridine (40 Ci/mmol) were purchased from Amersham. Antiserum to SV40 T-antigen was obtained from Flow Laboratories. Goat anti-hamster serum was a gift from M. Fogel.

Cells and Viruses. Plaque-purified SV40 (strain 777) was grown in the BS-C-1 line of African green monkey cells as previously described (26).

Cell Extracts. Cultures (10^7 cells) of uninfected or SV40-infected cells (multiplicity of infection = 2×10^9 plaque-forming units/ml) were labeled from 47 to 48 hr post infection with [^{35}S]methionine, (20 μCi/ml of methionine-free medium). After the cells were washed 3 times with cold phosphate-buffered saline, 2 ml of IP extraction buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 8.0, 0.14 M NaCl, 0.5 mM magnesium acetate, and 0.5% Nonidet P-40 was added. The cell extracts were collected and centrifuged at $5000 \times g$ for 2 min to separate nuclear and cytoplasmic portions. Nuclei were suspended in 1 ml of electrophoresis sample buffer [0.1 M Tris·HCl at pH 6.8, 1% sodium dodecyl sulfate, 0.7 M 2-mercaptoethanol, and 10% (vol/vol) glycerol] and sonicated 1 min at half maximum power in a Raytheon sonicator. The cytoplasmic portion was either adjusted to electrophoresis sample buffer conditions or centrifuged for 40 min at 40,000 rpm in a Spinco 40 rotor to obtain a 40,000 post-ribosomal supernatant fraction.

RNA Preparations. To prepare early SV40 RNA, 10^9 BS-C-1 cells were infected with SV40 (multiplicity of infection = 2×10^9 plaque-forming units/ml) in the presence of 20 μg/ml of cytosine arabinonucleoside as well as 5 μCi of [5,6-^{3}H]uridine. Cytoplasmic RNA was extracted 16–18 hr after infection and hybridized to 100 μg of SV40 DNA linked (27) to Sepharose 4B for 24–36 hr. The RNA was eluted by successive treatments with buffered 98% formamide and 1.0 M NaCl as described (27). The two eluate fractions were pooled and precipitated in the presence of 10 μg of rabbit liver tRNA, and collected in a small volume suitable for addition to the translation system (25–50 μl). SV40 cRNA was synthesized as previously described (27) and found to be at least 98% asymmetrically transcribed from SV40 form I (covalently closed, circular, superhelical) DNA.

The Wheat-Germ System. Wheat-germ extract was prepared and cell-free protein synthesis was assayed essentially as

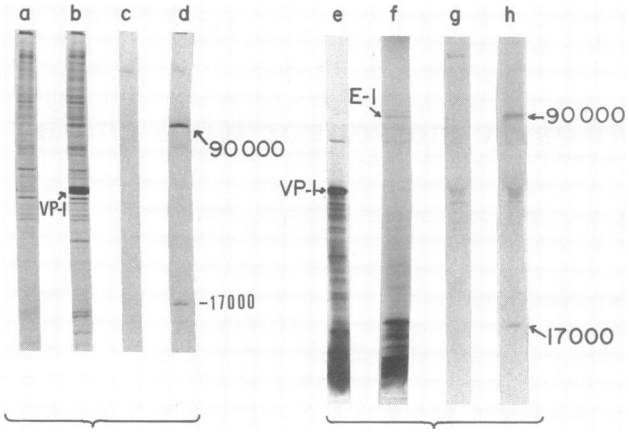

FIG. 1. Autoradiograms of [^{35}S]methionine labeled polypeptides synthesized *in vivo* and *in vitro*. Left-hand group: polypeptides were extracted from (a) uninfected cells, or (b) SV40-infected cells 48 hr post infection and then subjected to immunoprecipitation with hamster anti-SV40-T-serum, yielding (c) from uninfected and (d) from infected cells. Right-hand group: polypeptides synthesized in the wheat-germ system (25 μl assays) directed by (e) 0.5 μg of late SV40 mRNA, 5 μl of reaction mix applied to the gel; (f) early mRNA selected from 2×10^8 SV40-infected cells, 25 μl of reaction mix applied to the gel. The cell-free products were coelectrophoresed with hamster anti-SV40-T immunoprecipitates of cell extracts from (g) uninfected, and (h) SV40-infected cells.

described by Roberts and Paterson (28) with the following modifications: The wheat germ was ground in 15 ml of grinding buffer, the preincubation step was eliminated, the pH of Hepes buffer used in the assays was 8.0, and spermine·HCl was added to a final concentration of 80 μM.

Polyacrylamide Gel Electrophoresis. Cell extracts, cell-free products, or immunoprecipitates which were suspended in electrophoresis sample buffer were subjected to electrophoresis through 10–20% gradient slab polyacrylamide gels as described (29). The gels were dried and exposed to Kodak RP54 x-ray film for various time intervals.

Immunoprecipitations. To 0.3 ml of 40,000 supernatant portions of either cytoplasmic extracts or wheat-germ system reaction mixtures diluted to 0.3 ml with IP extraction buffer, 5 μl anti-SV40-T serum or hamster serum of equivalent gamma globulin content was added. After incubation for 60 min at 30°, 50 μl of goat anti-hamster serum was added and the incubation was continued for 60 min at 30° followed by 16 hr at 4°. The samples were centrifuged at $5000 \times g$ for 2 min and the pellets washed twice in phosphate-buffered saline, suspended in 50 μl of electrophoresis sample buffer, and heated for 10 min at 90°.

RESULTS

Immunoprecipitation of T-antigen from SV40-Infected Cells. The characterization of the cell-free products of early SV40 mRNA relies mainly on immunological procedures, since the early SV40-associated proteins are defined at present by their antigenic specificity. When the proteins of [^{35}S]methionine-labeled SV40-infected monkey cells were reacted with anti-SV40-T-serum and fractionated by gel electrophoresis, a large polypeptide, whose molecular weight (M_r) was estimated at 90,000, as well as another 17,000 M_r polypeptide, was immunoprecipitated from the rest of the cellular and viral proteins (Fig. 1b and d). Other polypeptides close in electrophoretic mobility to the major 90,000 M_r protein are observed in various proportions depending on the cell lines used after immunoprecipitation. Neither immunoprecipitation of a similar quantity of [^{35}S]methionine-labeled cytoplasm of uninfected cells (Fig. 1c and g) with anti-T serum nor immunoprecipitation of the labeled cytoplasm of infected cells with equivalent amounts of control hamster serum yielded the 90,000 M_r polypeptide. Tegtmeyer *et al.* (22) have described a 100,000 M_r polypeptide which is specifically immunoprecipitated from the cytoplasm of infected cells by hamster anti-SV40-T serum, which is most likely the same as or related to the 90,000 M_r polypeptide that we isolate. We consider the 90,000 M_r polypeptide to be the SV40 T-antigen; from its estimated size it appears to represent most of the coding capacity of the early region of the SV40 genome.

Cell-Free Product Directed by Early SV40 mRNA That Had Been Selected by Hybridization. We next analyzed the cell-free products whose synthesis was directed by early SV40 mRNA. BS-C-1 cells were infected with SV40 in the presence of cytosine arabinonucleoside, which inhibits expression of late RNA and associated late functions (30). The RNA was then hybridized to 100 μg of SV40 DNA linked to Sepharose by a previously described procedure (27). In a typical experiment, 5×10^8 cells, of which one-fifth were labeled with 5 mCi of [^{3}H]uridine, yielded 1.8×10^8 cpm total RNA, from which 3.4×10^4 cpm were selected by hybridization (i.e., 0.019% of the total), a value only slightly above the background. However, when a sample of the RNA selected by SV40 DNA-Sepharose was tested by hybridization to SV40 DNA immobilized on filters, 3–5% of the input hybridized, compared to only 0.1% of similarly processed RNA from mock-infected cells.

The hybridized early SV40 RNA was added to the wheat-germ system and the cell-free products were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The products synthesized (Fig. 1f) were different in size and distribution from those directed by late SV40 mRNA (Fig. 1e), in which the major capsid protein VP-1 is predominant (29). The early SV40 mRNA directs a number of products, of which the largest is a prominent polypeptide whose molecular weight was estimated as 90,000, and which we call the E-1 polypeptide. The E-1 polypeptide is not seen among the products directed by uninfected BS-C-1 mRNA (data not shown). The E-1 product was found to migrate with the anti-T-immunoprecipitated 90,000 M_r polypeptide from SV40-infected cells (Fig. 1f and h).

Further identification of the E-1 polypeptide as T-antigen was obtained after immunoprecipitation of the cell-free products of early SV40 mRNA with hamster anti-SV40-T serum or hamster control serum. Fig. 2 shows that the major 90,000 M_r cell-free product is specifically immunoprecipitated with hamster anti-T serum but not with hamster control serum. Another polypeptide product directed by early mRNA, and which is also found in infected cells, is a 17,000 M_r polypeptide. This polypeptide is immunoprecipitated by anti-T serum, although a slight reaction with hamster control serum has been observed. Neither products of the endogenous wheat-germ protein-synthesizing system, nor globin mRNA, tobacco mosaic virus mRNA, nor mRNA from uninfected cells yielded any specific immunoprecipitable material when treated and analyzed as described above.

The efficiency of the hybridization selection of early mRNA was demonstrated in an experiment in which the translation products of SV40-selected and nonselected RNA (that RNA which does not hydridize to SV40 DNA-Sepharose) were compared (Fig. 3). Early mRNA selected by hybridization to SV40 DNA-Sepharose directed the synthesis of a series of products including the prominent E-1 polypeptide. The nonselected RNA gave rise to a different spectrum of products and

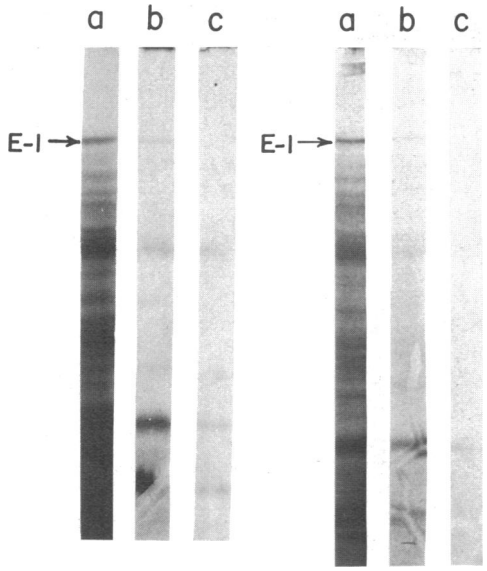

FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled polypeptides synthesized *in vitro*. The two groups represent autoradiograms of two separate experiments. (a) Polypeptides directed by SV40 early mRNA as described in Fig. 1f; (b) immunoprecipitation of (a) with hamster anti-SV40-T serum; (c) immunoprecipitation of (a) with normal hamster serum.

the E-1 polypeptide was totally absent. Thus, the hybridization technique provides an effective means for efficiently selecting and partially purifying early SV40 T-antigen mRNA.

Translation of SV40-Selected mRNA from Transformed Cells. Early mRNA from lytically infected monkey cells was

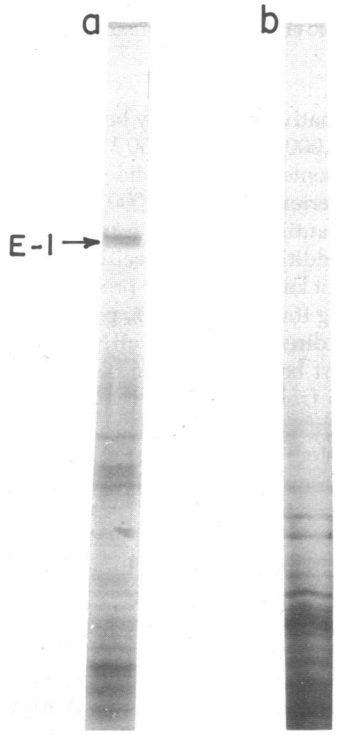

FIG. 3. Autoradiograms of [^{35}S]methionine-labeled polypeptide products of early-SV40-selected and nonselected mRNA (quantities as in Fig. 1f). (a) Polypeptides directed by early mRNA prepared by selective hybridization to SV40 DNA; (b) polypeptides directed by 0.2 μg of poly(A)-containing nonannealed RNA, i.e., that RNA which did not hybridize to SV40 DNA and which had been subjected to oligo(dT)-cellulose chromatography (31).

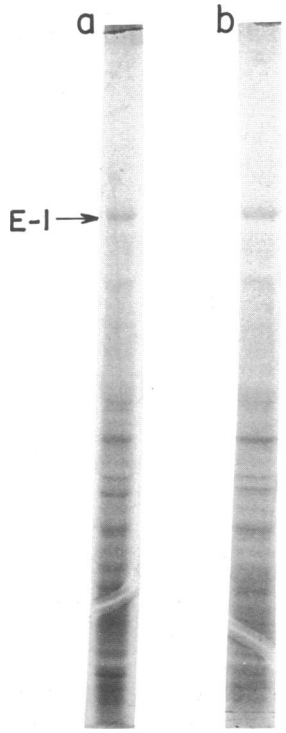

FIG. 4. Cell-free products of SV40 RNA from BS-C-1 cells early in lytic infection and from SV80 transformed cells. Autoradiograms of [^{35}S]methionine-labeled polypeptides directed by (a) SV40 early mRNA from infected BS-C-1 cells (quantities as in Fig. 1f); (b) SV40-specific RNA from SV80 cells. Cytoplasmic RNA from 10^9 SV80 cells was hybridized to 100 μg of SV40 DNA-Sepharose; hybridization-selected RNA from 2×10^8 cells was added per 25 μl of wheat-germ system reaction mixture, all of which was subjected to polyacrylamide gel electrophoresis.

compared to virus-specific RNA from SV40-transformed cells. The transformed line used was SV80, which is reported by Henderson *et al.* (15) to contain a higher proportion of T-antigen than other lines tested, as measured by complement fixation. RNA was extracted from approximately equal numbers of SV80 cells and infected BS-C-1 cells and hybridized to SV40 DNA-Sepharose as described above. Comparison of the cell-free products synthesized showed that both contained similar if not identical products, including the major early E-1 product (Fig. 4). Thus, SV40-selected mRNA from a line of virus-transformed cells directs similar products to that directed by early virus-specific mRNA extracted from permissive cells.

Immunoprecipitation of Cell-Free Products Whose Synthesis Is Directed by SV40 cRNA. An analysis of the cell-free products directed by synthetic SV40 cRNA was carried out in order to compare this alternate source of SV40 early RNA to infected cells. cRNA was prepared as described previously (27), and was found to be 98% asymmetrically transcribed. Analysis on polyacrylamide gels in comparison with known prokaryotic RNA standards showed the cRNA to be heterogeneous in size, the major component being approximately 19 S, with a small proportion of unit length RNA (28 S) (Fig. 5A). The activity of the cRNA in the wheat-germ system was found to be approximately equal to that of other biological mRNAs such as globin mRNA, or mRNA from BS-C-1 cells. The *in vitro* products of cRNA were rarely greater than 70,000 daltons in mass. The cell-free products of SV40 cRNA were subjected to immunoprecipitation with SV40 anti-T serum as described above. It was found (Fig. 5B) that a 70,000 M_r polypeptide, among the

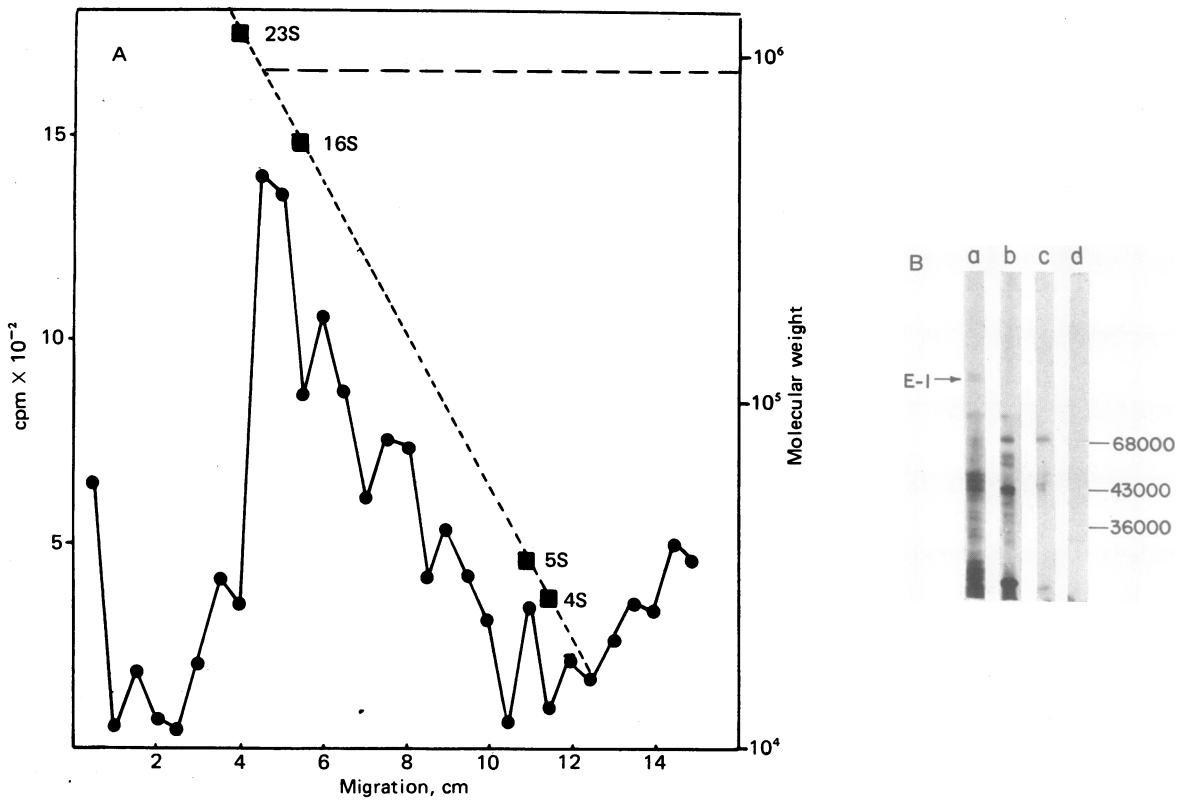

FIG. 5. (A) Size estimation of SV40 cRNA. [5,6-^{3}H]Uridine-labeled SV40 cRNA was prepared as described (27). 15,000 cpm was applied to polyacrylamide agarose slab gels (27) and run concurrently with indicated *Escherichia coli* RNA markers (■ - - - ■). The gels were subjected to 350 V for 3 hr, after which 2 mm slices were dissolved in NCS and radioactivity was measured by liquid scintillation counting (●——●).

(B) Cell-free products of SV40 cRNA. Autoradiograms of [^{35}S]methionine-labeled polypeptides: products directed by (a) SV40 early mRNA as in Fig. 1f; (b) SV40 cRNA whose size distribution is shown in (A); (10 μg was added per 50 μl of wheat-germ system reaction mixture, 5 μl of which was subjected to polyacrylamide gel electrophoresis). Immunoprecipitation of 15 μl of SV40 cRNA cell-free products in (b) with (c) hamster anti-SV40-T serum; (d) hamster control serum.

largest and most prominent of the cRNA products, could be specifically immunoprecipitated with hamster anti-T serum but not with hamster control serum. No counterpart to the 70,000 M_r immunoprecipitated SV40 cRNA products could be detected among the immunoprecipitated polypeptides of SV40-infected cells.

DISCUSSION

We have shown that a preparation of infected cell RNA, 30- to 50-fold enriched for early SV40 mRNA, directs the synthesis of a 90,000 M_r product (the E-1 polypeptide) which can be specifically immunoprecipitated by hamster anti-SV40-T serum and which comigrates electrophoretically with a similarly immunoprecipitated polypeptide isolated from virus-infected cells. The specificity of the immunoprecipitation technique is shown by experiments in which the E-1 product directed by early SV40 mRNA is immunoprecipitated by hamster anti-SV40-T serum and not by hamster control serum, and by the fact that a comigrating cellular 90,000 M_r polypeptide can be immunoprecipitated from infected, but not from uninfected cells. Since both the cellular and cell-free products have the same electrophoretic mobility and are specifically immunoprecipitated by anti T serum, it is likely that they are the same polypeptide and that they represent the primary translation product of the early mRNA. The 17,000 M_r polypeptide, which was similarly immunoprecipitated from the cell-free products of SV40 early mRNA as well as from SV40-infected cells, may be related to the 90,000 M_r product or may represent a nonspecific immunoprecipitation reaction. Evidence in favor of the first alternative has recently been obtained: the tryptic digests of the 17,000 M_r and 90,000 M_r polypeptides, isolated from cells, have some common peptides (C. Prives and L. Lipot, unpublished experiments). The 17,000 M_r product may be part of the native T-antigen complex, a proteolysis product of T-antigen, or an additional virus-specific antigen such as U-antigen (32). It is not known if it is the product of a cistron separate from that coding for the 90,000 M_r polypeptide.

Polypeptides directed by SV40 cRNA in a cell-free translation system must be virus specific because the RNA is transcribed *in vitro* from plaque-purified SV40 DNA. We have observed that different preparations of SV40 cRNA in the wheat-germ system directed the synthesis of products whose electrophoretic mobilities in the gel were somewhat variable. Generally, polypeptides of 60,000–70,000 M_r were specifically immunoprecipitated by SV40 anti-T serum; this class had no size counterpart among similarly immunoprecipitated polypeptides from infected cells. Nevertheless, the fact that SV40 cRNA can direct the cell-free synthesis of a product which can be specifically immunoprecipitated with SV40 anti-T serum provides proof that SV40 T-antigen is at least partly virus coded. Similar findings have been reported for SV40 cRNA in a linked transcription–translation system by Roberts *et al.* (33) and for SV40 cRNA of genome length by Smith *et al.* (34). The discrepancy in size between the cRNA-directed product and that of early SV40 mRNA is not presently understood. Since early SV40 mRNA directs the synthesis of a larger product that corresponds in size and immunospecificity to a polypeptide that can be isolated from infected cells, the translation of viral RNA

isolated from infected cells, rather than synthetic cRNA, may be the preferred approach for studying the metabolism of T-antigen mRNA.

It is noteworthy that it is possible, by hybridization selection, to partially purify and translate an mRNA class that comprises an extremely small proportion of the cells' total RNA. Early mRNA is only 1–2% of late virus mRNA (35), which itself is not greater than 10% of cellular mRNA even late in infection (36). Thus, early SV40 mRNA does not represent more than 0.1% of the total mRNA of the infected cell—a proportion too small to provide detectable products in the cell-free translation system without the prior selection process. Hybridization to immobilized SV40 DNA provides a minimal 20-fold enrichment of SV40-specific sequences in the RNA added to the translation system. Because it is unlikely that all of the nonspecific RNA in this enriched class is mRNA (the source of input RNA being total cytoplasmic RNA from infected cells), the 3–5% SV40 RNA in this fraction (see *Results*) is likely to be an underestimate of the proportion of messenger RNA or other translatable RNA added to the wheat-germ system.

The selection of an early SV40 mRNA class coding for T-antigen, as well as the isolation of T-antigen from cells by immunoprecipitation, opens up several new approaches. In the one example presented herein, the SV40-specific RNA from virus-transformed human cells directs the synthesis of products, including the E-1 90,000 M_r T-antigen polypeptide, similar to those directed by SV40 early mRNA extracted from lytically infected monkey cells. However, T-antigens isolated from different host species have been shown to vary in size (37, 38). Moreover, variations in the virus cell-transformation system have been reported (39) as well as the isolation of revertant offspring of transformed cells, all of which can now be analyzed as to their virus-specific mRNA and related cell-free translation products. The overproduction of the 100,000 M_r. A protein in cells infected by the Ts A SV40 mutant at restrictive temperature (22) can be investigated by the approach described in this paper. Thus, the isolation and translation of SV40 early mRNA coding for SV40 T-antigen can provide an additional approach towards understanding virus-induced cell transformation.

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