Identification and Initial Characterization of a New Low-Molecular-Weight Virus-Encoded T Antigen in a Line of Simian Virus 40-Transformed Cells

GREGORY J. SPANGLER, JAMES D. GRIFFIN, HARVEY RUBIN, AND DAVID M. LIVINGSTON*

The Sidney Farber Cancer Institute and the Departments of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Massachusetts 02115

SV80 cells, a simian virus 40 (SV40)-transformed derivative of a strain of human fibroblasts, synthesize an 8-kilodalton anti-T reactive polypeptide in addition to large T and small t antigens. Although not observed during lytic infection carried out under a variety of conditions, an anti-T reactive molecule which comigrated with the SV80 8-kilodalton protein during sodium dodecyl sulfate-polyacrylamide gel electrophoresis was synthesized by one of five other SV40-transformed cell lines studied. The SV80 8-kilodalton protein was present in lysates of cells exposed to a brief pulse of radioactive methionine and did not accumulate during an extended chase period. This polypeptide could not be generated by mixing an unlabeled extract of SV80 cells with a labeled extract of infected monkey cells. The 8-kilodalton molecule reacts with antibody raised against homogeneous large T antigen, is present only in the cytoplasm, is not complexed with T, lacks DNA-binding properties, and is not phosphorylated. This protein could be translated in a cell-free system programmed by SV40-specific mRNA. At least two messenger species (~19S and ~22S) directed its synthesis. Tryptic peptide analysis of [³⁵S]methionine-labeled proteins demonstrated that the 8-kilodalton protein contains all eight of the common T/t peptides and one additional peptide not present in the maps of t or T. It lacks both of the t-unique peptides. The organization of the integrated viral sequences which encode this molecule was determined by restriction endonuclease analysis. In particular, SV80 cells contain at least two integrated SV40 genomes which are oriented in tandem, with an intervening cellular sequence.

Infection of a eucaryotic cell by simian virus 40 (SV40) results in either lytic viral replication or, in some instances, neoplastic transformation. In both cases, the early viral genes which encode the two known tumor antigens (T and t) are expressed. T is required for the initiation of viral DNA replication (6, 31, 40), and both T and t play a role in the process of SV40-induced transformation (2, 3, 19, 20, 23, 36, 41).

T is an ~80-kilodalton (Kdal) phosphoprotein which migrates with an apparent molecular weight of 96,000 during sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (9, 33, 43). It is present in both the nucleus and plasma membrane of transformed cells and possesses certain DNA-binding and enzymatic properties (8, 10, 13, 27, 31, 32, 39, 44, 45). The t antigen is a 20-Kdal polypeptide which lacks covalently bound phosphate and is a cytoplasmic constituent (29). Little is presently known of its biochemical properties. A third protein(s) of 55 to 57 Kdal (nonviral T antigen) is present in immunoprecipitates of SV40-transformed cell extracts (5, 14, 15, 17, 21). It is a host cell-encoded phos-

phoprotein which binds tightly to T, both in the nucleus and in the plasma membrane (5, 14, 15, 17, 21, 39). Its synthesis is substantially augmented in certain SV40-transformed cells, as well as in other tumor cells (7, 17, 18). Recent evidence strongly suggests that T can, directly or indirectly, promote the production of this protein (18).

Extracts of SV80 cells, an SV40-transformed derivative of a strain of human fibroblasts, contain a fourth anti-T reactive polypeptide. It is smaller than t, migrating in SDS-polyacrylamide gels as an 8-Kdal molecule (9). The data presented here establish it as a virus-encoded polypeptide and define some of its biochemical properties, the gross structure of its messengers, and the location on the viral genome of most of its coding sequences.

MATERIALS AND METHODS

Cells and viruses. A plaque-purified stock of SV40 (strain 777) was grown in BSC-1 cells at a multiplicity of 10^{-4} . SV80 cells (46), an SV40-transformed derivative of a strain of human fibroblasts, and CV1-P cells,

an African green monkey kidney cell line, were grown in 90-mm plastic dishes at 37°C in Dulbecco's modified Eagle medium containing 10% fetal calf serum in a 10% CO₂-containing atmosphere.

Labeling and extraction of cells. Plates (90-mm diameter) of 80 to 90% confluent cells were starved in 10 ml of Hanks balanced salt solution, pH 7.4, for 1 h at 37°C. Three milliliters of Hanks balanced salt solution containing 50 μ Ci of [³⁵S]methionine per ml (400 to 600 Ci/mmol) was added to each plate; cultures were then incubated at 37°C for 4 h. Cells were washed four times with warm phosphate-buffered saline (0.01 M sodium phosphate, pH 7.4–0.14 M NaCl) and then extracted as described previously (9). Radioactive phosphate labeling was performed in a similar manner, except that 250 μ Ci of Na³²PO₄ per ml was substituted for radioactive methionine.

Immunoprecipitation of cell extracts, SDS-polyacrylamide gel electrophoresis, fluorography, and autoradiography. Previously described methods (9) were used. All anti-T serum reactions were carried out in antibody excess.

Immunoprecipitation of proteins isolated from SDS-polyacrylamide gels. Preparative SDS-polyacrylamide gels containing separated T-antigen species were fixed in 5 to 10% acetic acid, dried, and autoradiographed. Bands were localized, excised, and macerated in 1 ml of lysing buffer (20 mM Tris-hydrochloride, pH 9.0, 137 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, and 1% Nonidet P-40). The suspensions were incubated at 37°C for 20 h. After centrifugation for 10 min at 3,000 rpm in an International PR-6000 centrifuge, each supernatant was diluted with an equivalent volume of lysing buffer and then immunoprecipitated with the appropriate sera.

Preparation of polyadenylated RNA from SV80 cells. Lots of 20 g of fresh SV80 cells, grown at the Massachusetts Institute of Technology Cell Culture Center, were washed five times with phosphate-buffered saline and three times with 100 mM Tris-hydrochloride (pH 9.0)–100 mM NaCl–1 mM EDTA. The cells were homogenized in 5 volumes of this buffer for 60 s in a Vortex Omnimixer at 4°C, and the supernatant was collected after centrifugation at 9,000 rpm in the J20 rotor of a Beckman J21B centrifuge. The supernatant was made 1% in SDS and extracted once with phenol-chloroform (1:1). The aqueous fraction was then ethanol precipitated. The precipitate was washed with 70% ethanol, dissolved in 0.5 M NaCl, and applied to a 4-ml column of oligodeoxythymidylate cellulose (Collaborative Research Inc.) at room temperature. The column was washed with 3 volumes of 0.5 M NaCl, after which the bound RNA was eluted with water, quick frozen in a dry ice-acetone bath, and stored at –70°C.

Preparation of SV40 DNA-cellulose. Forty-eight plates of 80% confluent CV1-P cells were infected with SV40, strain 777, at a multiplicity of 1 PFU per cell. Sixty hours after infection, the cells were extracted by the Hirt technique (12). The supernatant was extracted at room temperature with phenol-chloroform-isoamyl alcohol (50:50:1), and the aqueous phase was ethanol precipitated. SV40 (I) DNA was isolated from this fraction by CsCl-ethidium bromide centrifugation followed by neutral sucrose gradient (5

to 20%, wt/vol) centrifugation. Form I DNA was converted to full-length linear DNA by digestion with *Hpa*II. Digestion was monitored by analytical 1% agarose gel electrophoresis and ethidium bromide staining of DNA species (34). *Hpa*II linear DNA was covalently coupled to *m*-amino-benzoyloxymethyl cellulose at a ratio of 25 μ g of DNA per mg of cellulose as described by Noyes and Stark (22).

Preparation of SV40-specific RNA by hybridization to SV40 DNA-cellulose. Total polyadenylated RNA from SV80 cells was ethanol precipitated in the presence of carrier yeast tRNA (500 μ g/ml) and polyadenylic acid (200 μ g/ml), redissolved in 50% formamide–0.1% SDS–0.6 M NaCl–1 mM EDTA–0.1 M Tris-hydrochloride, pH 7.4, and then hybridized to SV40 DNA-cellulose at 37°C for 24 h. After extensive washing, SV40-specific RNA was eluted by suspending the cellulose in 99% formamide–0.1% SDS and heating to 60°C for 1 min. The eluted RNA was ethanol precipitated in the presence of rabbit liver tRNA (10 μ g/ml) and redissolved in water.

Density gradient analysis of SV40 mRNA. Five micrograms of SV80 polyadenylated RNA was suspended in 45 μ l of gradient loading buffer (10 mM Tris-hydrochloride, pH 7.1, 1 mM EDTA, and 85% formamide in 2% sucrose), heated at 37°C for 15 min, chilled to 0°C, and loaded on a 5.0-ml, 2 to 10% (wt/vol) sucrose gradient prepared in this buffer. After centrifugation for 24 h at 40,000 rpm at 20°C in an SW50.1 rotor, samples were collected by bottom needle puncture, ethanol precipitated, vacuum dried, and assayed for translational activity in the same tubes.

Cell-free protein synthesis and immunoprecipitation of products. A micrococcal nuclease-treated, mRNA-dependent rabbit reticulocyte lysate translation system was used for in vitro protein synthesis, as described by Pelham and Jackson (26). Reticulocyte lysates were prepared from erythrocytes isolated from phenylhydrazine-treated rabbits with reticulocyte counts of 90 to 92% as described (26). Translation was carried out in a total volume of 50 μ l, which contained 40 μ l of lysate, 15 μ Ci of [³⁵S]methionine (400 to 500 Ci/mmol), 50 μ g of rabbit liver tRNA per ml, and added messenger as indicated. All assay mixtures were incubated at 35°C for 90 min.

For immunoprecipitation of cell-free synthesized products, 400 μ l of lysing buffer, 50 μ l of protein A Sepharose (50% [vol/vol] in water, Pharmacia Fine Chemicals, Inc.), and 10 μ l of serum were added to each reaction mixture, and the suspension was treated as in other standard immunoprecipitation assays (see prior description).

Tryptic peptide analysis. Each [³⁵S]methionine-labeled protein band was excised from a fixed, dried gel, macerated, and incubated in gel elution buffer (0.05 M NH₄HCO₃, 0.1% SDS, 1% β -mercaptoethanol) for 24 h at 37°C. After centrifugation for 10 min at 3,000 rpm in a PR-6000 centrifuge, the supernatant was collected, frozen in a dry ice-methanol bath, and lyophilized. The dried residue was suspended in 50% trichloroacetic acid and the precipitate was washed twice with acetone and once with diethyl ether. It was then dissolved in 0.2 ml of performic acid (prepared by mixing 1.9 ml of 90% HCOOH with 0.1 ml of 30%

H₂O₂) and incubated for 1 h at 4°C. After the incubation, 1 ml of water was added at 4°C, and the sample was frozen and lyophilized. The dried residue was suspended in 0.2 ml of 0.1 M sodium bicarbonate, pH 8.6, containing 60 µg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin (Worthington Biochemicals Corp.) and incubated at 37°C for 4 h. At that time, another 60 µg of TPCK-trypsin was added to each sample, and the samples were then incubated for 20 h at 37°C. Finally, 1 ml of 0.01 M acetic acid was added to each, and the samples were frozen and lyophilized. The residue in each was dissolved in 10 µl of electrophoresis buffer (90% formic acid-acetic acid-water, 1:4:45, pH 2.1), and electrophoresis was performed on plastic-backed, cellulose thin-layer plates under prescribed conditions (37, 38). Electrophoresis was performed for 60 min at 1,000 V on a water-cooled, glass plate under air pressure. After the plate had dried overnight at room temperature, chromatography was performed in a second dimension in *n*-butanol-acetic acid-water-pyridine (15:3:12:10). Plates were again dried at room temperature and then subjected to autoradiography on Kodak XR-1 film.

Restriction enzyme digestion of cellular DNA, agarose gel electrophoresis, and blotting of restriction fragments. A dextran sulfate modification (47) of the procedure of Botchan et al. (1) was used. ³²P-labeled SV40 DNA (1 × 10⁷ to 5 × 10⁷ cpm/µg) prepared by "nick translation" of SV40 DNA I was used as probe in these experiments.

RESULTS

Immunoprecipitation of *in vivo*-synthesized SV40 T antigens. Immunoprecipitation of [³⁵S]methionine-labeled T antigens from various cell extracts was performed with antisera from individual hamsters bearing SV40-induced tumors. As shown in Fig. 1 (slots B and D), extracts of SV80 cells contained large T antigen, the nonviral mid-size protein(s) (57K), and small t. In addition, these cells contained a very low-molecular-weight species, with an estimated *M_r* of 8,000. Importantly, it was precipitated by anti-T and not by preimmune serum. A variety of SV40-infected monkey cell lines (CV1-P, TC-7, BSC-1, and primary rhesus monkey kidney) were found to synthesize T and t, but in no case was a protein smaller than 20 Kdal observed (Fig. 2). This molecule was not present in 24-, 48-, and 60-h infected cultures of CV1-P cells, labeled for 4 h prior to extraction, nor was it found in extracts of 48-h infected cells labeled for 1 h (data not shown). Several other SV40-transformed cell lines were also examined (Fig. 3). One hamster line (SVCHL 15) synthesized a low-molecular-weight, anti-T reactive polypeptide in small quantities. This protein comigrated with the SV80 8-Kdal protein. Other transformants lacked anti-T reactive proteins of less than 20 Kdal. A transformed monkey kidney cell line (LLcMK2) produced only one anti-T reactive

FIG. 1. Immunoprecipitation of the T-antigen species of SV80 cells. Immunoprecipitation was performed with hamster anti-T tumor serum obtained from each of a series of tumor-bearing animals as described in Materials and Methods. SV80 cells were labeled with [³⁵S]methionine for 4 h, extracted, and then immunoprecipitated. Either preimmune (A) or anti-T serum from individual tumor-bearing hamsters (B-G) was used. Precipitated proteins were eluted and were electrophoresed in a 12% SDS-polyacrylamide gel, which was then autoradiographed.

protein of 55 Kdal. The structure of this polypeptide is not known at present.

Tests of the hypothesis that the 8-Kdal protein is a cleavage product of T or t, or of both. In view of the known susceptibility of T to adventitious proteolysis during extraction from some cells (37, 42), experiments were designed to determine whether the 8-Kdal protein was an intracellular cleavage product of any of the known T antigens or whether it specifically appeared during cell extraction. First, SV80 cells were pulse-labeled (10 min) with [³⁵S]methionine and then extracted and immunoprecipitated. Parallel pulse-labeled cultures were subjected to a chase with nonradioactive methionine-containing medium for up to 8 h and then extracted and immunoprecipitated. The pulse-labeled culture revealed all of the standard anti-T immunoprecipitable proteins, including the 8-Kdal molecule (Fig. 4). During the chase, there

FIG. 2. SV40 T antigens present during lytic infection. From 1×10^7 to 2×10^7 CV1-P (A), TC7 (B), BSC-1 (C), and primary rhesus monkey kidney cells (D) were mock infected or infected with SV40 strain 777, SVS, tsA209, or dl884 at a multiplicity of infection of ~ 5 . Forty-eight hours postinfection, cells were labeled with [35 S]methionine, extracted, and then immunoprecipitated with preimmune (C) or anti-T (T) serum. The eluted immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. tsA209-infected cells were incubated at 34°C .

was no enhancement of the 8-Kdal yield. In fact, the 8-Kdal protein decayed at a faster rate than t, as assessed by densitometric analysis (data not shown). Thus, there is no evidence that the 8-Kdal protein is derived by intracellular cleavage of either of the known T antigens.

Second, mixing extracts of unlabeled SV80 cells and [35 S]methionine-labeled SV40-infected CV1-P cells prior to immunoprecipitation did not result in the appearance of a radioactive 8-Kdal polypeptide (data not shown). Thus, there was no activity evident in an SV80 extract which

FIG. 3. *T* antigens present in various SV40-transformed cell lines. SV80, SV3T3 (C1 16) (mouse), SVT2 (mouse), LLcMK2 (rhesus monkey), SVCHL (Chinese hamster), and H6590B (Syrian hamster) cells were labeled with [35 S]methionine, extracted, and then immunoprecipitated with preimmune (C) or anti-*T* (T) serum. Eluted proteins were then subjected to polyacrylamide gel electrophoresis followed by autoradiography.

FIG. 4. *Pulse-chase analysis of SV80 T antigens.* SV80 cells were pulse-labeled (10 min) with [35 S]methionine (50 μ Ci/ml, 500 Ci/mmol) and then extracted and immunoprecipitated with preimmune (C) or anti-*T* (T) serum. Parallel pulse-labeled cultures were subjected to a chase with nonradioactive methionine-containing (2×10^{-4} M) medium for 0 h (A), 1 h (B), 2 h (C), 4 h (D), or 8 h (E) and then extracted and immunoprecipitated with anti-*T* serum. In addition, an SV80 extract labeled under standard conditions was immunoprecipitated with anti-*T* serum. Eluted proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

promoted the generation of the 8-Kdal molecule from T or t, or both, in vitro.

Antigenic properties of the 8-Kdal mole-

cule. Hamster anti-SV40 tumor sera obtained from each of a series of tumor-bearing animals were assayed for the ability to immunoprecipitate the SV80 T antigens (Fig. 1). Only two of the six sera tested were able to precipitate the 8-Kdal molecule (slots B and D), whereas all precipitated T and nonviral T antigens. One serum (slot G) precipitated only T and nonviral T antigens, failing to bind t and the 8-Kdal protein. All of the others precipitated t, and neither serum which precipitated the 8-Kdal molecule lacked anti-t reactivity. The failure of some sera to react with the 8-Kdal molecule suggests that this protein does not form a stable complex with T or t.

In an effort to discern whether the 8-Kdal polypeptide shares antigenic determinants with T, the gel-purified molecule was incubated with antibody raised against SDS-polyacrylamide gel-purified T (4, 16). This serum was kindly given to us by Robert Carroll. The T antigens of SV80 cells were first precipitated with hamster anti-SV40 tumor serum. The resulting t and 8-Kdal molecules were eluted from the gel and then allowed to react with rabbit anti-T protein serum. Both proteins were efficiently reprecipitated by the antibody and not by preimmune rabbit serum (data not shown). This result demonstrates that the 8-Kdal molecule shares one or more antigenic determinants with T.

Intracellular localization of the various T-antigen species. [35 S]methionine-labeled SV80 cells were subjected to a cellular compartment analysis. Plasma membranes, cytoplasm, and nuclei were each isolated by a differential extraction-sedimentation method (35). The nuclei and plasma membranes were disrupted, and

each of the three preparations was then analyzed by anti-T immunoprecipitation (Fig. 5). Most of the T was found in the nuclear extract, although a significant amount was observed in the cytoplasm, with a trace amount in the plasma membrane. The t antigen and the 8-Kdal protein were present primarily in the cytoplasm.

Phosphorylation and DNA-binding properties of the T antigens of SV80 cells. SV80 cells were incubated in media containing radioactive phosphate and then lysed. The extract

was subjected to anti-T immunoprecipitation. As shown in Fig. 6, both T and nonviral T were labeled under these conditions, whereas t and the 8-Kdal molecule were not.

In another experiment, equivalent samples of a [35 S]methionine-labeled SV80 extract were applied at pH 6.6, in parallel, to identical-size columns of native calf thymus DNA-cellulose or unsubstituted cellulose. The flow-through fractions and 2 M NaCl eluates of each were subjected to anti-T serum precipitation and subsequent SDS-polyacrylamide gel electrophoresis (Fig. 7). T and nonviral T bound specifically to DNA-cellulose and could be eluted in high-ionic-strength (2 M NaCl) buffer. In contrast, neither t nor the 8-Kdal polypeptide bound to DNA-cellulose. None of these proteins bound to the DNA-free cellulose column.

FIG. 5. Intracellular localization of the T-antigen species of SV80 cells. SV80 cells were labeled with [35 S]methionine for 4 h and then collected by use of a rubber policeman. Washed cells were suspended in a hypotonic buffer, swollen for 10 min at 4°C, and Dounce homogenized. After centrifugation at 2,000 $\times$ g for 2 min to pellet the nuclei, the supernatant was centrifuged at 10,000 $\times$ g for 10 min to pellet the plasma membranes. The three fractions—nuclei (A, B), plasma membranes (C, D), and cytoplasm (E, F)—were then extracted with lysing buffer, immunoprecipitated with anti-T (A, C, E) or preimmune (B, D, F) serum, eluted, electrophoresed, and autoradiographed. All operations were performed as described previously (35).

FIG. 6. Analysis of SV80 32 P-labeled T antigens. After incubation with $\text{Na}^{32}\text{PO}_4$ for 4 h (see Materials and Methods), SV80 cells were extracted and then immunoprecipitated with preimmune (A) or anti-T (B) serum. In parallel, a [35 S]methionine-labeled SV80 extract was immunoprecipitated with anti-T serum (C). Precipitates were eluted, electrophoresed, and then autoradiographed.

FIG. 7. DNA binding properties of the T antigens of SV80 cells. SV80 cells were labeled with [35 S]methionine (50 μ Ci/ml) and then extracted. Extracts were applied, in parallel, to identical-size columns of unsubstituted (A, B) or native calf thymus DNA-cellulose (C, D). The flow-through fractions (A, C) and 2 M NaCl eluates (B, D) were immunoprecipitated with anti-T serum, electrophoresed, and then autoradiographed, as described in Materials and Methods.

Genetic origin of the 8-Kdal protein. To elucidate the nature of the messenger(s) for the 8-Kdal protein, translational activity of SV40-specific mRNA was assayed in a micrococcal nuclease-treated, rabbit reticulocyte cell-free translation system (26). SV80 polyadenylate-containing RNA and SV80 polyadenylate-containing RNA further purified by hybridization to SV40 DNA-cellulose directed the synthesis of T, t, and an anti-T reactive 8-Kdal protein (Fig. 8). Neither RNA fraction could direct the synthesis of the nonviral T species, consistent with the results of others (38). SV80 polyadenylate-containing RNA was then subjected to zonal sedimentation in a denaturing sucrose gradient. A sample of each collected fraction was assayed for messenger activity (Fig. 9). The t and T messengers, migrating as approximately 21S and 20S structures, respectively, were identified by this approach, as previously reported by others (24, 28). Of interest was the finding of two discrete peaks of messenger activity for the 8-Kdal protein—one associated with a $\sim$ 22S structure

and a second with a $\sim$ 19S structure. Thus, there are at least two unique messengers for the 8-Kdal protein in these cells, one of which is larger and the other smaller than the two known T-antigen messengers.

A comparative tryptic peptide analysis of T, t, and the 8-Kdal molecule was also performed. [35 S]methionine-labeled SV80 T, t, and 8-Kdal polypeptide were each isolated from SDS-polyacrylamide gels after anti-T immunoprecipitation and digested with trypsin. The digests were subjected first to thin-layer electrophoresis and then to chromatography (Fig. 10). Electrophoresis was performed at pH 2.1, conditions which allow the clear identification of the common amino-terminal tryptic peptide of T and t-N-

FIG. 8. *In vitro* synthesis of the SV80 T antigens. SV80 polyadenylate-containing RNA further purified by hybridization to SV40 DNA-cellulose was translated in a cell-free system. Synthesized proteins were subjected to immunoprecipitation analysis with preimmune (C) or anti-T (T) serum; all operations were performed as described in Materials and Methods.

FIG. 9. Sucrose gradient analysis of the mRNA species directing the synthesis of T, t, and the 8-Kdal protein. SV80 polyadenylate-containing RNA (5 μ g) was centrifuged for 24 h at 40,000 rpm at 20°C through a 2 to 10% (wt/vol) sucrose gradient containing 85% formamide (24). Samples were collected, precipitated, and then assayed for translational activity. Synthesized products were immunoprecipitated, eluted, electrophoresed, autoradiographed, and then analyzed densitometrically. All operations were performed as described in Materials and Methods.

acetyl Met-Asp-Lys (arrowed peptide) (38). The T map contained more than 20 discrete peptides, the t map 10 peptides, and the 8-Kdal map 9 peptides. Each of the 8 peptides common to T and t was present in the 8-Kdal map, including the N-terminal one. The two t peptides not present in the T map (spots A and B) were also absent in the 8-Kdal map. The 8-Kdal fingerprint contained one unique peptide (spot C). The maps of in vivo- and in vitro-synthesized 8-Kdal proteins were identical (data not shown).

Organization of the integrated SV40 sequences in SV80 cells. The fact that the 8-Kdal molecule is, at least in part, a viral gene product, together with the observation that it was not produced by all SV40-transformed cell lines, suggests that the synthesis of this protein may be the result of a unique organization of the integrated viral genome(s) in SV80 cells. Two integrated copies of SV40 DNA were previously detected in these cells by S. J. Flint and G. A. Beltz (Cold Spring Harbor Symp. Quant. Biol., in press). They also found that neither contained an *Eco*RI site. The larger of these contains an apparently intact early region from the *Bgl*II site at 0.67 map units to the *Hind*III site at 0.169 map units. To establish the relative orientation of these two genomes, we subjected high-molecular-weight SV80 DNA to restriction endonucle-

ase analysis. As shown in Fig. 11, *Xba*I, an enzyme which does not cut SV40 DNA, generated two SV40 DNA-containing fragments of 10 and 6.5 kilobases. *Sa*I and *Ava*I, also noncutters, each produced only a single SV40 DNA-containing fragment of 7 and 6 kilobases, respectively. In addition, as shown previously (Flint and Beltz, in press), two *Eco*RI-generated bands were noted. These results suggest that SV80 cells contain at least two integrated SV40 genomes which are, most likely, oriented in tandem, with an intervening cellular sequence.

DISCUSSION

SV80 cells, relative overproducers of the SV40 large T antigen (11), have previously been subjected to anti-T immunoprecipitation analysis (9, 17). In addition to T, nonviral T, and t, a reproducible and unexpected finding in all cases was an 8-Kdal molecule (9). From the intensity of its autoradiographic image, its abundance in SV80 cells appears close to that of T and t. The primary purpose of the work presented here was to determine whether this molecule is a viral gene product and, if so, which segment(s) of the genome constitutes its coding region.

Very low-molecular-weight, SV40-specific polypeptides were not observed during lytic infection under a variety of conditions. Therefore, they are probably not synthesized during the lytic cycle, although it is possible that synthesis is followed by sufficiently rapid degradation to prevent detection after in vivo labeling by the techniques used. However, in addition to SV80, another SV40-transformed cell line among those examined was found to contain a very low-molecular-weight anti-T reactive molecule. Because of its low concentration in this line, compared with SV80, it has not yet been possible to obtain sufficient quantities to permit chemical comparison with the SV80 8-Kdal protein. Hence, although not a property of all SV40-transformed cells, low-molecular-weight anti-T reactive proteins are synthesized in some lines.

Evidence presented here strongly suggests that the 8-Kdal molecule is not a cleavage product of T or t, or both, formed intracellularly or postextraction. It was present in lysates of cells exposed to a brief pulse of radioactive methionine and did not accumulate during an extended chase period. Moreover, it could not be generated by mixing an unlabeled extract of SV80 cells with a labeled extract of infected monkey cells. In addition, the same molecule could be synthesized in a cell-free system programmed by SV40-specific mRNA. The strongest evidence against the 8-Kdal protein being a proteolytic product of T or t, or both, is the existence of specific messengers directing its synthesis. If this

FIG. 10. Tryptic peptide analysis of the T-antigen species of SV80 cells. SV80 cells (10^8) were labeled with [35 S]methionine (50 μ Ci/ml), extracted, and immunoprecipitated with anti-T serum. Precipitated proteins were eluted and electrophoresed in 15% SDS-polyacrylamide gels. T, t, and the 8-Kdal molecule were localized by autoradiography, excised, eluted, digested with trypsin, electrophoresed, and chromatographed as described. The maps of T (1), t (2), and the 8-Kdal protein (3) were then autoradiographed for 3 weeks. A composite map (4) shows the eight peptides common to T, t, and the 8-Kdal protein (filled symbols), including the N-terminal peptide (arrowed). Also shown are the t-unique peptides (A and B) and the 8-Kdal-unique peptide (C).

molecule were a proteolytic product generated in the reticulocyte extract, one would have expected the 8-Kdal protein messenger activity to comigrate in a denaturing density gradient with the peaks of T or t messenger activity, or both.

Much of the coding region for the 8-Kdal protein can be defined from the results presented here. Its tryptic peptide map contains the same amino-terminal peptide, N-acetyl Met-Asp-Lys, present in T and t (25, 38). Thus, from the DNA sequence, it appears that the 8-Kdal cistron commences at the ATG triplet at nucleotides 5080 to 5082 located at 0.647 map units (30), the same initiation site which is operative in the t and T cistrons. Since all of the common T/t methionine-containing tryptic peptides are present in the 8-Kdal map, the 8-Kdal coding segment contains all of the sequence between nucleotides 5080 and 4879 (30). This encompasses 67 amino acids and accounts for most of the mass (~ 7 Kdal) of the 8-Kdal protein. One can only speculate on the origin of the 8-Kdal-unique, methionine-containing tryptic peptide, which may correspond to the C-terminal portion of the protein.

The peptide is not likely derived from the uninterrupted t sequence commencing at nucleotide 4879, since the next methionine codon in this sequence occurs at nucleotide 4800. If, without deletion, enough of this t sequence were present in the 8-Kdal cistron to include this methionine, the protein would have an M_r of $\sim 10,000$ which is higher than the value of 8,000 obtained by SDS-polyacrylamide gel analysis. Similarly, the 8-Kdal-unique peptide is probably not encoded by the uninterrupted T sequence starting from nucleotide 4879, since the next methionine codon occurs at nucleotide 4410. The contribution of this coding region to the 5080 to 4879 sequence would result in a protein product of nearly 12 Kdal.

There are various models which could account for the structure of the 8-Kdal protein and the existence of two messengers directing its synthesis. Since the 8-Kdal protein contains the domain common to T and t, it is possible that the coding sequence for this molecule is derived from an SV40 genome which is fragmented near 0.6 map unit, with its C terminus being encoded

FIG. 11. General organization of the integrated SV40 sequences in SV80 cells. Samples of high-molecular-weight SV80 DNA (20 μ g) were digested with *EcoRI* (B), *AvaI* (C), *SalI* (D), and *XbaI* (E) and were electrophoresed in 1% agarose gel (1). 32 P-labeled lambda DNA digested with *HindIII* (A) was analyzed in parallel. The DNA was transferred to a nitrocellulose sheet and identified by hybridization to 32 P-SV40 DNA and autoradiography as described in Materials and Methods. The unlabeled arrows indicate the migration of full-length linear SV40 DNA in this gel.

by cellular sequences adjoining this partial genome. This would require the presence of at least two sets of integrated viral sequences in SV80 cells, since a genome with an intact early region is necessary for the synthesis of T and t. A variation of this basic model involves the tandem orientation of two SV40 genomes, with an intervening cellular sequence in which splices of segments of transcripts encoded by the fragmented early region and an intact one generate messengers of the observed sizes, which are capable of directing the synthesis of an 8-Kdal molecule with the common T/t domain. The demonstration of the presence of at least two integrated SV40 genomes in SV80 cells (Flint and Beltz, in press) and the localization of these viral sequences to a single restriction fragment (e.g., 6 kilobases with *AvaI*) are consistent with this hypothesis. To further evaluate this model, it will be necessary to analyze the structure of the messengers for the 8-Kdal protein and to isolate and characterize in detail the tandem genomes.

In view of its structure, it is not surprising that

the 8-Kdal protein reacts with antibody raised against homogeneous large T antigen. Like t, it is found only in the cytoplasm, is not complexed with T, lacks DNA-binding properties, and is not phosphorylated. Whether or not the 8-Kdal protein is physiologically active in SV80 cells, it may be a valuable reagent in future studies of T/t function. Because of its unique structure, it might prove to have in vitro functional properties common to both proteins. Once purified, it might also be useful as an adsorbent for common anti-T/t antibodies; their deletion from some anti-SV40 tumor sera could result in domain-specific, anti-t and anti-T antibodies. Furthermore, it might serve as an immunogen in future attempts to generate other domain-specific anti-T/t antibodies.

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