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Characterization of a Fused Protein Specified by the Adenovirus Type 2-Simian Virus 40 Hybrid Ad2$^+$ND1 dp2

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The adenovirus type 2-simian virus 40 (SV40) hybrid virus Ad2$^+$ND1 dp2 (E. Lukanidin, manuscript in preparation) specified two proteins (molecular weights, 24,000 and 23,000) that are, in part, products of an insertion of SV40 early DNA sequences. This was demonstrated by translation in vitro from viral mRNA that had been selected by hybridization to SV40 DNA. These two phosphorylated, nonvirion proteins were produced late in infection in amounts similar to adenovirus 2 structural proteins and were closely related to each other in tryptic peptide composition. The portion of SV40 DNA (map units 0.17 to 0.22 on the SV40 genome) coding for these proteins was joined to sequences coding for the amino-terminal part of the adenovirus type 2 structural protein IV (fiber). The Ad2$^+$ND1 dp2 23,000- and 24,000-molecular-weight proteins were hybrid polypeptides, with about two-thirds of their tryptic peptides contributed by the fiber protein and the remainder contributed by SV40 T-antigen. They shared with T-antigen (molecular weight, 96,000) a carboxy-terminal proline-rich tryptic peptide. Together, the tryptic peptide composition of these proteins and the known SV40 DNA sequences suggested the reading frame for the translation of T-antigen. The carboxy terminus for T-antigen would then be located on the SV40 genome map next to the TAA terminator triplet at position 0.175, 910 bases away from the cleavage site of the restriction endonuclease *Eco*RI. Seven host range mutants from Ad2$^+$ND1 dp2 were isolated that had lost the capacity to propagate on monkey cells. They did not induce detectable levels of the hybrid proteins. Three of these mutants had lost the SV40 DNA insertion that codes in part for these proteins. Thus, in analogy to the Ad2$^+$ND1 30,000-molecular-weight protein, the presence of these proteins correlates with the presence of the helper function for adenovirus replication on monkey cells.

A series of nondefective adenovirus type 2 (Ad2)-simian virus 40 (SV40) hybrid viruses, isolated by Lewis and co-workers (28-31), carry unique segments of SV40 DNA integrated between map positions 0.80 and 0.86 on the Ad2 genome (19, 20, 27, 38). Various members of this series contain overlapping sets of SV40 DNA and have been used in assigning functional domains to the product of SV40 gene *A*, the SV40 T-antigen (30, 32, 33, 56). One of the functions of SV40 expressed by several members of this series is a *trans*-acting helper function, which allows Ad2 to replicate on monkey cells (45). This helper function has been studied by using the hybrid virus Ad2$^+$ND1 (29) and a series of host range mutants derived from it, which have lost the ability to grow on monkey cells (15, 16). The genome structures of Ad2$^+$ND1 and of one host range mutant (H71) are given in Fig. 1.

A new revertant virus, Ad2$^+$ND1 dp2, which can also be propagated efficiently on monkey cells, has been isolated from H71 and extensively characterized (E. Lukanidin, manuscript in preparation). The structure of its genome is shown in Fig. 1b. It has acquired a duplication of part of the H71 genome consisting of the right-hand portion of the SV40 insertion and the left-hand portion of the gene for the Ad2 structural protein IV (fiber). A new species of polyadenylated cytoplasmic RNA is found in cells infected with this hybrid and has been characterized by nucleic acid hybridization to Ad2 and SV40 DNA sequences (Lukanidin, in preparation; A. R. Dunn and J. A. Hassell, in preparation). Here we describe the detection of a protein specific to cells infected with Ad2$^+$ND1 dp2.

From the knowledge of the growth potential of this virus on monkey cells, its genome structure, the hybridization properties of the new RNA species, and the size of the new protein, it

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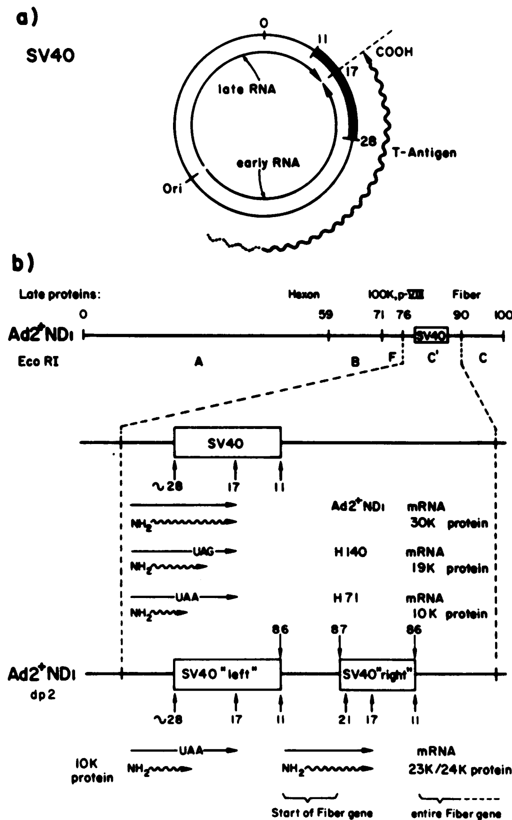

FIG. 1. *Genome structure of SV40, Ad2+ND1, and Ad2+ND1 dp2 and location of SV40-specific proteins. (a) Coordinates on the SV40 genome refer to the EcoRI restriction cleavage site as zero and are expressed as percentages of the genome unit length. The carboxy terminus of T-antigen is drawn close to map position 17. Its exact location is described in the text (see Fig. 6). The dark bar indicates the portion of the genome present in Ad2+ND1 (19, 22, 38). The precise genome arrangement close to map position 0.28 is not yet known. Preliminary data indicate that the sequence is not simply colinear with the SV40 genome in this region (S. Zain and J. Sambrook, personal communication). The origin of viral DNA replication (Ori) and the locations of regions coding for early and late transcripts are indicated (7, 21, 37). (b) The genome structures of Ad2+ND1 (19, 27, 38) and of Ad2+ND1 dp2 (Lukanidin, manuscript in preparation) are shown. The position of several late Ad2 proteins (34) is indicated above the Ad2+ND1 map with reference to the EcoRI restriction endonuclease cleavage sites (49). The SV40 insertions present in these viruses are indicated as boxes in the expanded diagram. The left coordinate can only be given approximately because of the reservation made above. The location of cytoplasmic RNA species is shown below the SV40 coding sequences (12, 22; Dunn and Hassell, personal communication). The 30K protein specified by Ad2+ND1 (15, 35) has been translated in vitro (2) from RNA selected by hybridization to both SV40 and Ad2 DNA. The host range mutant H71 and its corresponding RNA and protein products have been described (13, 16). The new mRNA species encoded in part by the right-hand SV40 insertion in Ad2+ND1 dp2 was characterized by Lukanidin and by Dunn and Hassell (manuscripts in preparation). The 23K/24K proteins are shown as fused proteins with their amino-terminal portions derived from the Ad2 fiber protein and their carboxy-terminal parts from the SV40 T-antigen. The properties of these proteins are described in the text. The Ad2 DNA segment flanked by the two SV40 DNA insertions represents the beginning part of the structural gene for fiber. It is about 390 nucleotides long and carries the cleavage site for the endonuclease Hha (Lukanidin, manuscript in preparation; S. Zain, personal communication; G. Fey, unpublished data). The entire structural gene for fiber is repeated to the right of the smaller SV40 insertion.*

was hypothesized that this protein should contain peptides from the Ad2 fiber protein in its amino-terminal portion and peptides from SV40 T-antigen at its carboxy end. The protein also should mediate the helper function for growth of adenovirus on monkey cells. The characterization of this protein is the subject of the present report.

MATERIALS AND METHODS

Cells and viruses. Our procedures for cell culture and for virus growth and purification have been described previously (16).

Mutagenesis. Ad2+ND1 dp2 virions were treated with nitrous acid as described by Grodzicker et al. (15). The amount of viable virus in samples treated for varying lengths of time was determined by plaque assay, and preparations inactivated to a level of 10^{-4} (6 to 7 min of treatment) were used for plaque isolation on HeLa cells. The screening for host range mutants and complementation assays using coinfection with SV40 or Ad2 were performed as described by Grodzicker et al. (16).

Radioactive labeling of infected cell proteins. Confluent monolayers of HeLa cells were infected with 20 to 50 PFU of virus per cell. Mock-infected cultures were exposed to phosphate-buffered saline (PBS). For adenoviruses and hybrid viruses at 26 h after infection and for SV40 at 36 h after infection, cells were washed twice with methionine-free medium or with Hanks balanced salt solution (GIBCO) and then labeled with $[^{35}S]$methionine at 25 to 100 μCi/ml in Dulbecco modified Eagle medium containing 1/40 of the standard concentration of methionine and calf serum (1.0 ml per 6-cm plate). Proline, lysine, and arginine were used at 0.25 (^{3}H) or 0.02 (^{14}C) mCi/ml in Hanks balanced salt solution at neutral pH. Usually cell extracts were prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Anderson et al. (1) after 1 h of labeling. In those cases, where continuous labeling of the proteins or optimal incorporation of radioactive amino acids was required, 3 ml of complete medium was added to the cultures and

the incubation continued for 2 to 8 h longer. Labeling of SV40-infected CV1 cells with ^{32}P and preparation of 100,000 × g supernatants from cytoplasmic extracts were performed as described previously (18).

Cytoplasmic and nuclear extracts. To prepare cytoplasmic and nuclear extracts from Ad2$^+$ND1- or Ad2$^+$ND1 dp2-infected HeLa cells, the cells were washed with PBS, scraped off the plates, and washed twice again with PBS. They were then suspended in 0.2 ml of PBS per 6-cm plate containing 0.5% (wt/vol) of the nonionic detergent Nonidet P-40 (Shell Co.) and passed twice through a 22-gauge needle. Nuclei were pelleted and extracted two more times with 0.1 ml of the same buffer per 6-cm plate. An equal volume of 2×-concentrated SDS sample buffer (25) was then added to both the cytoplasmic and nuclear extracts. The nuclear extract was then passed three to five times through a 22-gauge needle to shear the DNA and sonically treated for 2 min in a Raytheon model DF101 sonic oscillator. Both samples were then boiled for 2 min and, after a clearance centrifugation (5 min at 10,000 × g), used for electrophoresis. For the hypotonic Dounce procedure, the cellular pellets were suspended in reticulocyte standard buffer (42) and passed 5 to 10 times through a 22-gauge needle. Nuclei were pelleted at low speed and extracted two more times. The extracts were then further treated as above.

Immunoprecipitation and electrophoretic analysis of proteins. Immunoprecipitation of SV40 T-antigen was performed as described previously (18). Typically the immunoprecipitable radioactive material represented about 0.5% of the total radioactivity in the 100,000 × g extracts. About one-fifth of the immunoprecipitated counts were found in T-antigen. Immunoprecipitations with antifiber serum, antihexon serum, and preimmune serum were performed by similar procedures. The antifiber and antihexon sera were the kind gifts of V. Mautner, W. Russell, and L. Philipson. To each 150 μl of cytoplasmic extract was added 20 μl of the first antiserum, followed after 4 h on ice by the second antibody in a 10-fold excess over the first one. After another 8 h at 4°C, the precipitates were treated in the same way as T-antigen precipitates. Electrophoresis was carried out in 15% polyacrylamide slab gels (20 cm by 20 cm by 1.1 mm) as described by Laemmli (25). They were exposed for autoradiography on Kodak No-Screen film or treated for fluoroautoradiography (4, 26).

Chromatography of tryptic peptides on chromobeads P. Proteins were eluted from SDS-polyacrylamide gel slices by electrophoresis in 0.1% SDS containing Tris-glycine buffer (electrophoresis buffer; 25) with 50 μg of bovine serum albumin per ml as carrier in the case of T-antigen. More bovine serum albumin was added to the eluted T-antigen to a final concentration of 1 mg/ml, and the proteins were precipitated with 5 volumes of acetone, washed with 20% trichloroacetic acid to remove the glycine, and then washed with acetone and ether. Proteins were then mixed in pairwise combinations and processed by performic acid oxidation and tryptic digestion as described elsewhere (10), except that 2 × 20 μg of trypsin was used. The samples were suspended in 0.5 ml of buffer A (350 ml of water, 1.5 ml of pyridine, and 140 ml of acetic acid) and applied to chromobeads P in a column (9 mm in diameter, 160 mm high) operated at 51°C (55). The peptides were eluted with a pyridine-acetate gradient, which was generated in a three-chamber gradient mixer. The first two chambers, including the mixing chamber (chamber 1), were filled with 210 ml of buffer A; the last was filled with a mixture of 70 ml of buffer A and 140 ml of buffer B (350 ml of water, 81 ml of pyridine, and 71 ml of acetic acid). The flow rate was adjusted to 30 ml/h with a Vario-perpex pump (LKB). A total of 210 fractions of 3 ml each were collected in glass vials (15 by 45 mm). The column was then regenerated with subsequent washes of buffer B, 6 M pyridine, and buffer A. The samples were dried in an oven at 100°C, resuspended in 200 μl of 10 mM HCl, and counted in 3.5 ml of Aquasol scintillator. The channels of the liquid scintillation spectrometer were set such that there was less than 0.5% spillover of ^3H radioactivity into the second channel and the spill of the second isotope (^{35}S, ^{14}C) into the ^3H channel was less than 20% (17 to 19%) of the actual counts in the narrow second channel. Under these conditions, more than 70% of the counting efficiency of an open channel was retained for the second isotope. The raw counts were corrected for background radioactivity and spillover into the low-energy channel with a programed desk computer.

Isolation of viral DNA, digestion with restriction nuclease, and analysis of DNA fragments on agarose gels. The DNAs of Ad2$^+$ND1 dp2 and the host range mutants H311, H330, and H359 were isolated from purified virus particles (43). The restriction endonuclease digestion and the electrophoretic analysis of the DNA fragments in agarose gels were performed as described by Sambrook et al. (49).

Purification and cell-free translation of RNA. Cytoplasmic RNA was isolated from HeLa cells 27 h after infection with Ad2$^+$ND1 dp2, and SV40-specific RNA was selected by hybridization to SV40 DNA and then translated in a mammalian cell-free protein synthesis system as previously described (2, 34).

RESULTS

Detection of the 23K/24K protein in infected cells. The proteins synthesized in human (HeLa) cells at late times (24 h) after infection with Ad2 and Ad2$^+$ND1 dp2 were compared by electrophoresis in SDS-polyacrylamide gels (Fig. 2, tracks a and b). Both viruses made the expected late viral proteins, ranging from 11,500 molecular weight (11.5K) to 120K (II) (1). Cells infected with Ad2$^+$ND1 dp2 showed an additional protein of 23K not seen in cells infected with Ad2 (track a) or with Ad2$^+$ND1 or H71 (see reference 16). In different experiments, the Ad2$^+$ND1 dp2-specific protein appeared with an electrophoretic mobility of 24K rather than 23K, or as both the 23K and 24K species with variable relative intensities. The proportion of label in the 23K and 24K components remained constant under pulse-chase conditions (data not shown) so that a precursor-product relationship seemed unlikely. More than 70% of the total intracellular

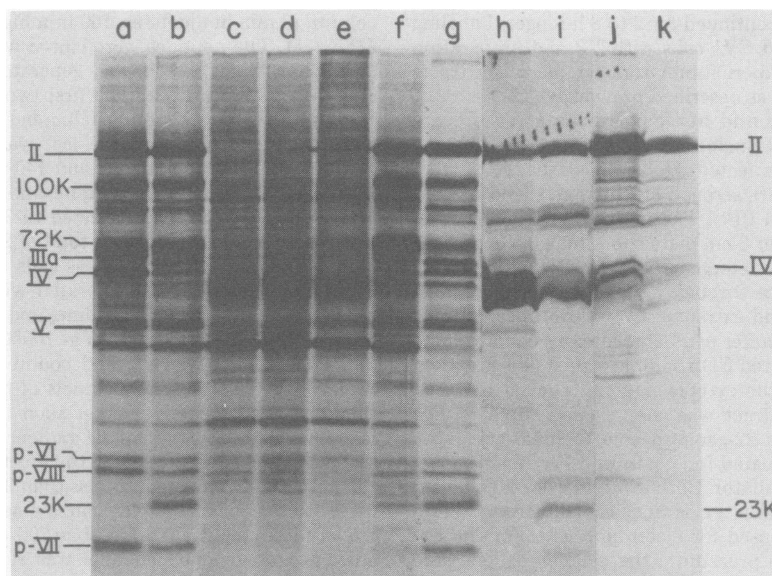

FIG. 2. *Time course of appearance of the 23K/24K proteins and their immunoprecipitation with antifiber serum. HeLa cells infected with 20 to 50 PFU of virus per cell were labeled with [^{35}S]methionine for 1-h pulses at various times after infection, and total infected-cell protein was analyzed by electrophoresis as described in Materials and Methods. (a) Ad2 marker; (b–g) Ad2$^+$ND1 dp2 ([b] 24 h postinfection p.i.), [c] 2 h p.i., [d] 4 h p.i., [e] 8 h p.i., [f] 18 h p.i., [g] 30 h p.i.). Immunoprecipitations from cytoplasmic extracts of infected HeLa cells labeled at 24 h after infection for a 1-h pulse with [^{35}S]methionine were performed as described in Materials and Methods. (h) Precipitate with antifiber serum from Ad2-infected cells; (i) precipitate with antifiber serum from Ad2$^+$ND1 dp2-infected cells; (j) precipitate with antihexon serum from Ad2$^+$ND1 dp2-infected cells; (k) precipitate with preimmune serum from Ad2$^+$ND1 dp2-infected cells.*

23K/24K protein was located in the cytoplasm, regardless of whether a detergent procedure or hypotonic Dounce procedure (see Materials and Methods) was used (data not shown). The 23K/24K proteins were not detected in purified virions (data not shown).

The heterogeneity of the 23K/24K protein was further investigated by two-dimensional isoelectric focusing-polyacrylamide gel electrophoresis, using the method of O'Farrell (41). The 23K/24K protein was observed as a multiplet, with components of isoelectric points 4.5, 4.6, and 4.7, specific to Ad2$^+$ND1 dp2-infected cells (data not shown). Two-dimensional gel analysis of ^{32}P-labeled proteins from Ad2$^+$ND1 dp2-infected cells showed the same characteristic multiplet as seen with [^{35}S]methionine-labeled protein, indicating that 23K/24K protein is phosphorylated, as is T-antigen (52) but not fiber (48). Furthermore, since the ratios of ^{32}P and ^{35}S were similar for each of the 23K/24K species, the heterogeneity of this protein probably was not due to differences in the extent of phosphorylation of the various species (data not shown).

At early times after infection, no significant amounts of the 23K/24K proteins were detected (Fig. 2, tracks c–e), whereas at late times (18 h after infection) these proteins were observed along with the other late proteins (Fig. 2, track f) and continued to accumulate late in increasing amounts (Fig. 2, track g). At very late times, the 23K/24K proteins become a major component in the pattern of Coomassie brilliant blue-stained total infected-cell proteins. Therefore the kinetics of appearance of 23K/24K protein is characteristic of late structural proteins.

To test the hypothesis that the 23K/24K proteins might contain a portion of the Ad2 fiber protein, we attempted to precipitate the proteins with antiserum against purified Ad2 fiber protein. The 23K protein could be precipitated from Ad2$^+$ND1 dp2-infected cells with antifiber serum but not with serum against purified hexon protein nor with preimmune serum (Fig. 2, tracks h–k). Antifiber serum did not precipitate a protein of 23K to 24K from Ad2-infected or mock-infected cells. Of two antifiber sera tested, one was able to precipitate Ad2$^+$ND1 dp2 23K protein. We also repeatedly observed the precipitation with antifiber serum of a strongly labeled infected-cell-specific component of approximately 80K. This product was found in detergent-generated cytoplasmic extracts, but not in extracts produced by the hypotonic Dounce procedure.

Cell-free synthesis of the 23K/24K pro-

teins. To determine whether the 23K/24K proteins are in part the gene products of SV40 sequences in Ad2$^+$ND1 dp2, cytoplasmic RNA was prepared from infected cells, and the RNA species containing SV40 sequences were selected by hybridization to SV40 DNA. The selected RNA was then translated in a cell-free system, and the polypeptides produced were identified by SDS-polyacrylamide gel electrophoresis. For comparison, RNA was also prepared from cells infected with Ad2 or with the host range mutants H140 and H71. Cell-free translation of total cytoplasmic RNA from each of these sources (Fig. 3, tracks a–d) produced an array of Ad2 late proteins (IX through II) similar to those seen in vivo (Fig. 2). In addition, 19K was seen to be specific to H140-infected cells (Fig. 3, track b), 10K was specific to H71-infected cells (Fig. 3, track c), and 23K/24K was specific to Ad2$^+$ND1 dp2-infected cells (Fig. 3, track d). Selection of mRNA complementary to SV40 DNA showed 19K to be the product of the SV40 insertion in H140 (Fig. 3, track f) and 10K to be the product of the SV40 insertion in H71 (Fig. 3, track g), as reported previously (16). In many experiments, the H140 19K was present as two bands (18K and 19.5K). The origin of this variable heterogeneity is unknown. The predominant product synthesized by using Ad2$^+$ND1 dp2 mRNA complementary to SV40 was 23K/24K (Fig. 3, track h), although a small amount of 10K could also have been present. These results are consistent with 23K/24K being the product of the new (right-hand) SV40 insertion in Ad2$^+$ND1 dp2, with 10K arising from the original (left-hand) SV40 insertion present in both H71 and Ad2$^+$ND1 dp2.

Evidence that the 23K/24K proteins contain tryptic peptides derived from the Ad2 fiber protein. Tryptic peptides from fiber and from 23K/24K proteins were first compared by using ^{3}H- and ^{14}C-labeled lysine. Ion-exchange

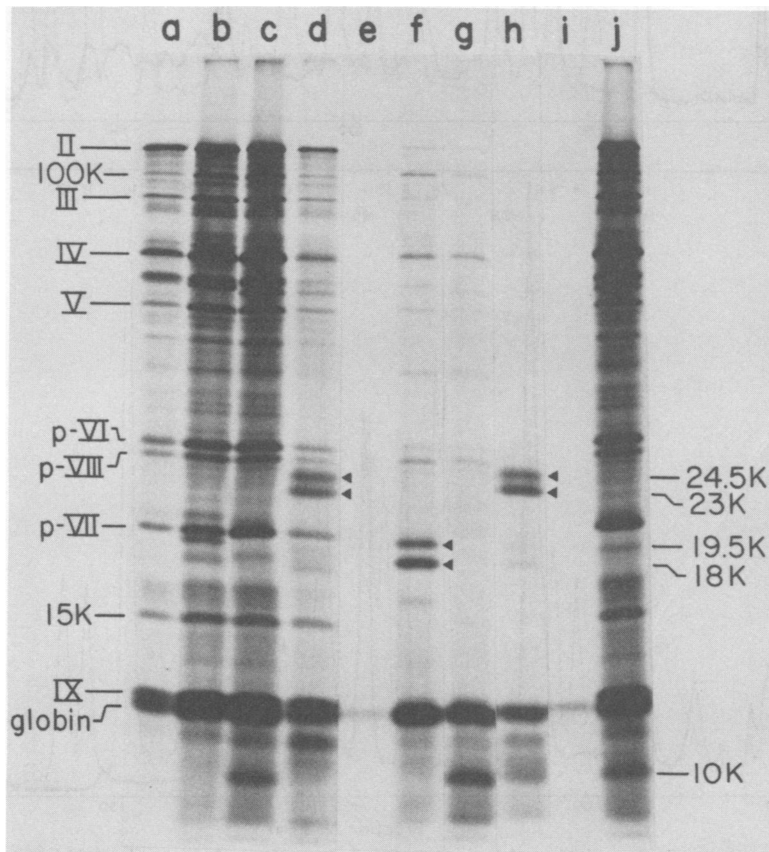

FIG. 3. *Cell-free translation of the 23K/24K proteins. The conditions for the extraction of cytoplasmic RNA from infected HeLa cells, for the hybridization of this RNA to SV40 DNA, for the subsequent chromatography on oligodeoxythymidylic acid-cellulose, and for the final translation in vitro were as described elsewhere (34). Tracks a through d and j represent translation products from crude RNA extracts; tracks f, g, and h represent translation products from RNA after selection by hybridization to SV40 DNA. (a) Ad2; (b) H140; (c) H71; (d) Ad2$^+$ND1 dp2; (e) rRNA from E. coli; (f) H140; (g) H71; (h) Ad2$^+$ND1 dp2; (i) same as (e); (j) same as (c).*

chromatography resolved 18 lysine-containing peptides from fiber and 8 from the combined 23K/24K proteins, of which 6 peptides were common to both fiber and 23K/24K (Fig. 4a). To estimate the extent of random overlap of peptide profiles from two unrelated proteins, we compared the lysine-labeled fiber peptides with the arginine-labeled peptides of the major capsid protein VP1 of SV40 (data not shown). Although VP1 was twice the size of 23K/24K and its

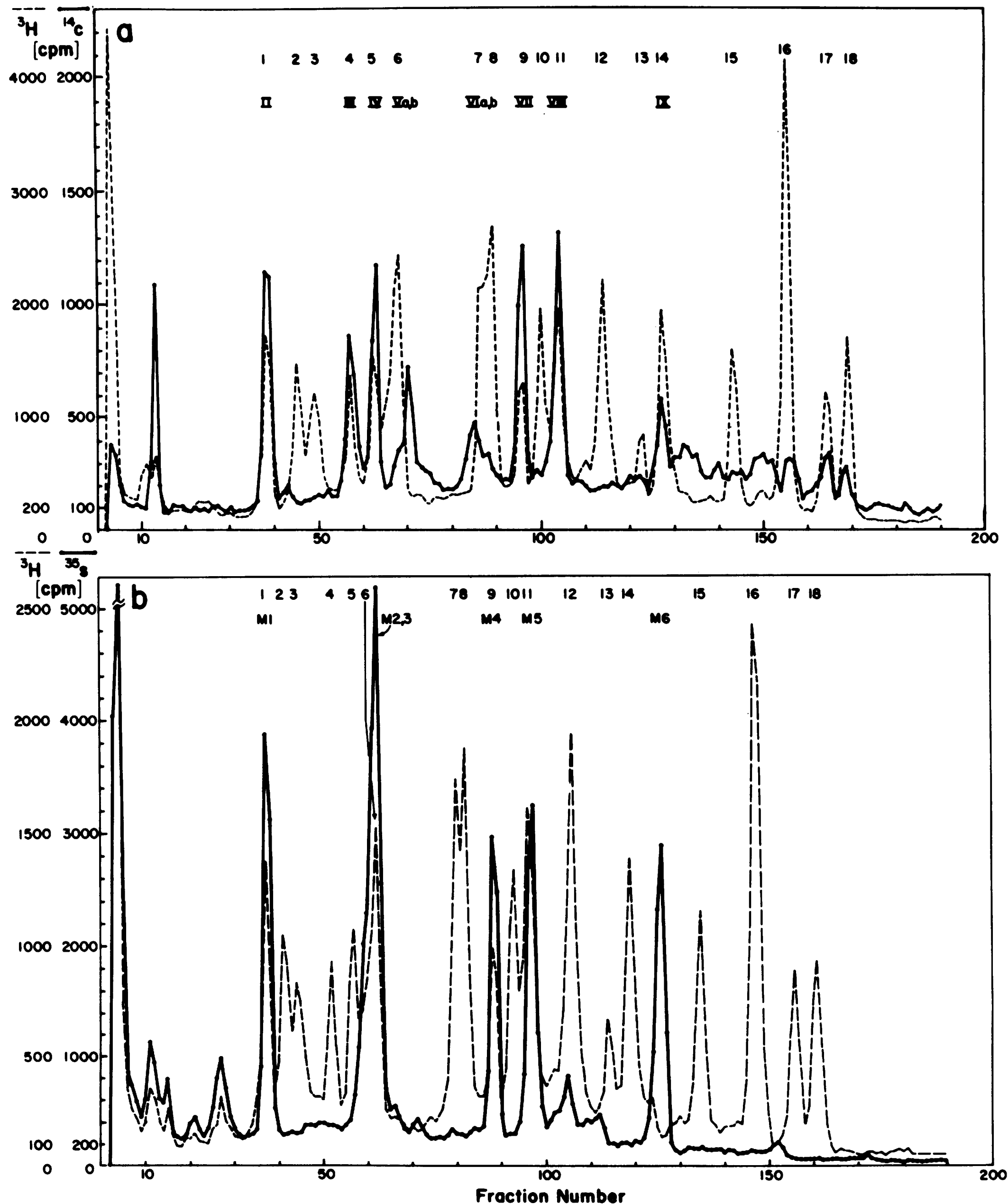

FIG. 4. *Comparison of lysine- and methionine-containing tryptic peptides of the Ad2 fiber protein and the 23K/24K proteins. The recovery of proteins from polyacrylamide gels, their processing, and their analysis on the resin chromobeads P were performed as described in Materials and Methods. (a) Ad2 fiber ([^{3}H]lysine; -----, peptides 1 through 18) and the combined 23K/24K proteins ([^{14}C]lysine; ——, peptides II through IX). (b) Ad2 fiber ([^{3}H]lysine; -----, peptides 1 through 18) and Ad2 fiber ([^{35}S]methionine; ——, peptides M1 through M6).*

peptide profile contained 11 peaks, there was overlap in only one peak. We also compared several other unrelated proteins (e.g., Ad2 hexon, fiber, and 100K proteins). For proteins in the size range between 60 kilodaltons (Kdal) and 120 Kdal, the observed random overlap was never greater than 2 to 3 peaks out of a total of 15 to 20 peaks resolved.

In a similar manner, the methionine-containing tryptic peptides of fiber and the 23K/24K proteins were compared (Fig. 4b and 5). A comparison of lysine- and of methionine-labeled fiber in Fig. 4 shows that the fiber lysine-containing peptides 1, 6, 9, and 11 also contained methionine. In Fig. 5, an indirect comparison of the lysine- and methionine-containing tryptic peptides of 23K/24K, using hexon peptides as marker, shows that 23K/24K lysine peptides II, VII, and VIII corresponded to the 23K/24K methionine-containing peptides 1, 2, and 3. These three 23K/24K peptides II, VII, and VIII were chromatographically identical to the fiber peptides 1, 9, and 11 (Fig. 4a), which contained both lysine and methionine. Thus the methio-

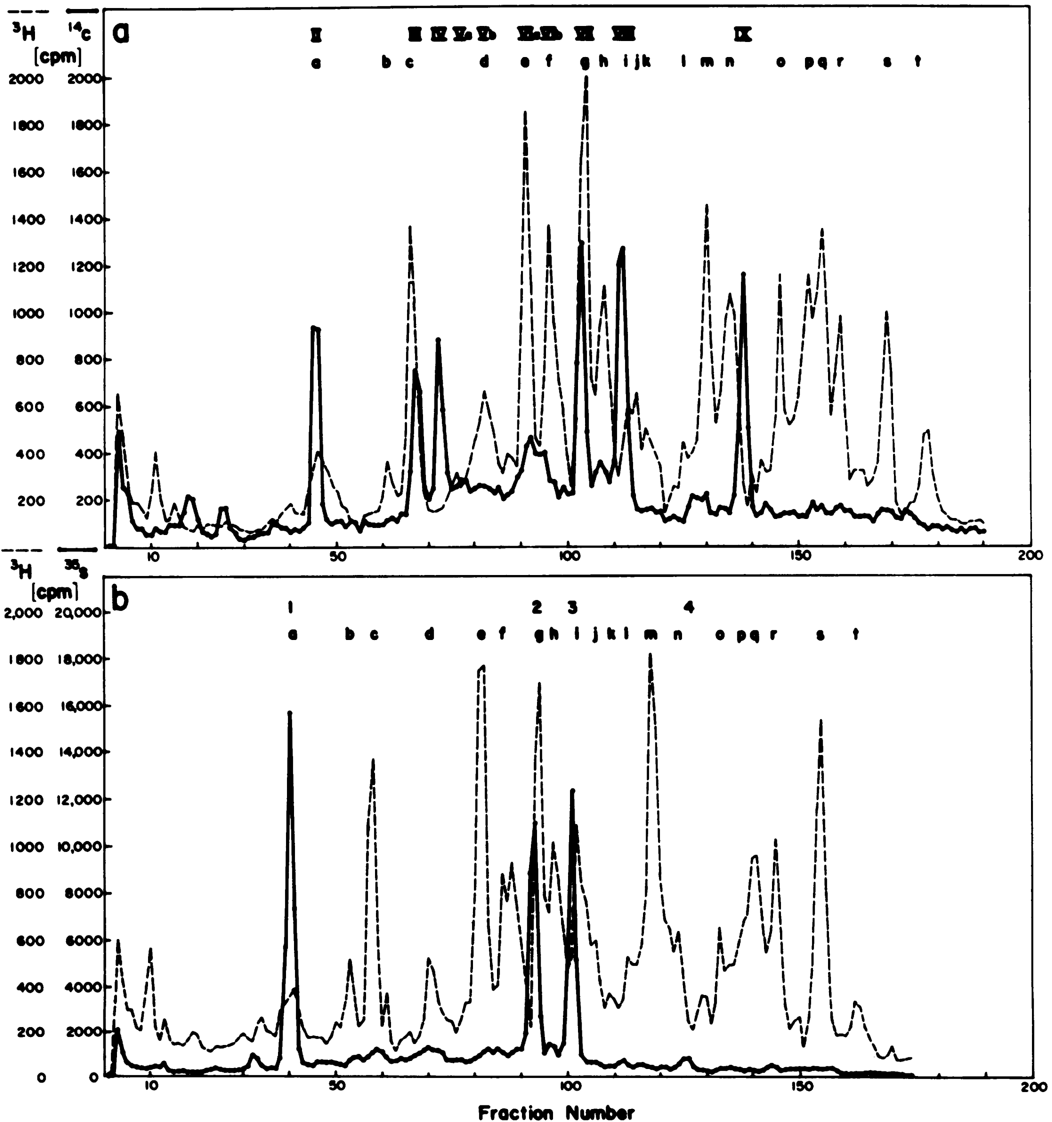

FIG. 5. *Comparison of the methionine-containing tryptic peptides of the Ad2 fiber and the 23K/24K proteins. (a) Ad2 hexon ([^{3}H]lysine; ----, peptides a through t) and the combined 23K/24K proteins ([^{14}C]-lysine; ——, peptides II through IX). (b) Ad2 hexon ([^{3}H]lysine; ----, peptides a through t) and the combined 23K/24K proteins ([^{35}S]methionine; ——, peptides 1 through 4).*

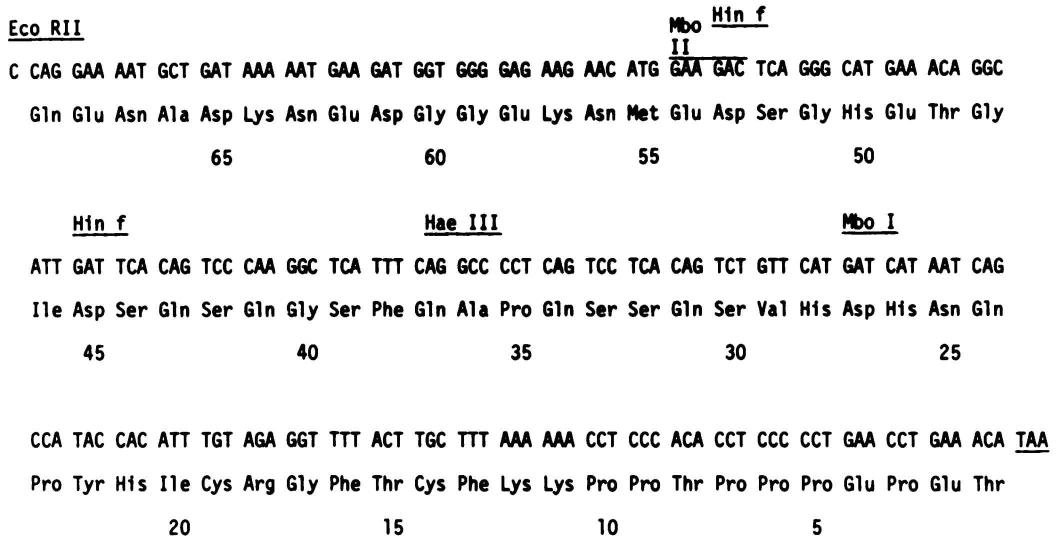

FIG. 6. *SV40 DNA sequences and the prediction of the proline-rich carboxy-terminal peptide of T-antigen. These are nucleotide sequences from the L strand of SV40 DNA. The same sequence appears in early RNA, with U in place of T residues. This sequence corresponds to a portion of the SV40 EcoRII F fragment from the EcoRII cleavage site at map coordinate 0.25 to the termination triplet at map coordinate 0.175 on the SV40 genome, which is located 910 bases away from the EcoRI site which defines the coordinate 0 (11, 46). Several other restriction endonuclease cleavage sites are indicated as reference points. There are 22 additional nucleotides located between the underlined terminator triplet and the HindII cleavage site at the junction of the B and G fragments, which have not been included here. The amino acid sequence is the one predicted in reading frame A (see text). The amino acids are numbered beginning from the carboxy terminus.*

nine-containing tryptic peptides of 23K/24K were derived from fiber. Preliminary data from a comparison of the arginine-labeled peptide profiles of fiber and of 23K/24K indicate that they share two or three arginine tryptic peptides (data not shown).

Evidence that the 23K/24K proteins contain peptides in common with SV40 T-antigen. Two lysine-containing 23K/24K peptides (Va,b and VIa,b in Fig. 4a) were not shared with fiber. To determine whether these could be encoded by SV40 early information, lysine-labeled tryptic peptides from the 23K/24K proteins and SV40 T-antigens were compared as above.

SV40-infected CV1 cells were labeled with [^{3}H]lysine, and SV40 T-antigen was obtained by indirect immunoprecipitation with T-antiserum (see Materials and Methods) followed by electrophoresis in SDS-polyacrylamide gels (5, 6, 18, 47, 51–53). The major immunoprecipitated component migrated with an apparent mobility to 96 Kdal with respect to Ad2 structural proteins (Fig. 7b). A minor component, which was not observed in all experiments, migrated with a mobility of 88 Kdal. Both components were phosphorylated (Fig. 7b, track 3). We did not attempt to compare the 23K/24K proteins with the second early gene product of SV40, a 17K protein, which is detected under conditions different from those used here (44).

Both the 96K and 88K forms of T-antigen yielded a large number of lysine-containing tryptic peptides, two or three of which seemed to be shared with 23K/24K (data not shown). The complexity of the T-antigen pattern made it impossible to determine whether a few peptides were really shared between T-antigen and 23K/24K or whether this correspondence was just random overlap. Accordingly, a less frequent amino acid was used for labeling. Proline was chosen because of a specific prediction made from the SV40 DNA sequence of the early region between map units 0.26 and 0.16 (11, 46). One of the three possible translational reading frames for the region between map units 0.17 and 0.54 was considered most likely because it would allow the synthesis of the longest polypeptide without interruption by translational terminator codons. This reading frame predicted that the carboxy-terminal tryptic peptide of T-antigen would contain 6 proline residues out of 10 amino acids (Fig. 6). This prediction was tested by direct tryptic peptide comparison of proline-labeled 23K/24K proteins and T-antigen.

SV40 T-antigen labeled in vivo with [^{3}H]proline was immunoprecipitated from extracts of SV40-infected CV1 cells and analyzed by SDS-polyacrylamide gel electrophoresis as shown in

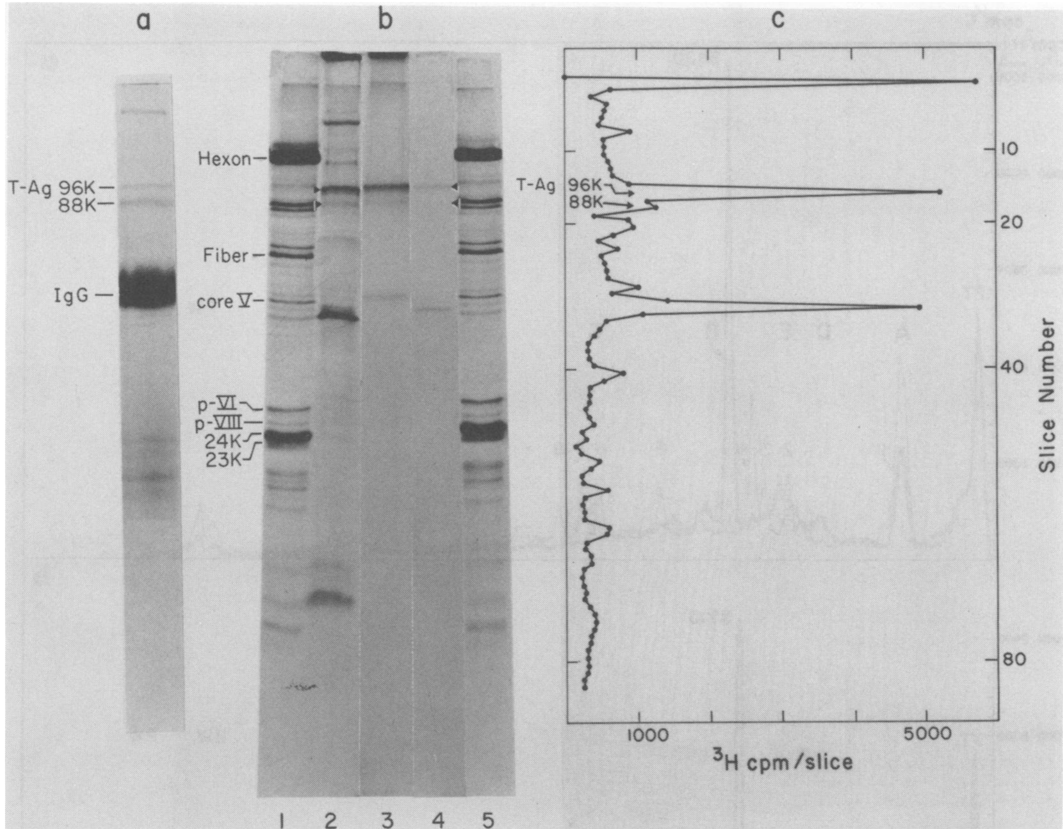

FIG. 7. *Immunoprecipitation of T-antigen and its labeling with [^{3}H]proline. Immunoprecipitated T-antigen from CV1 monkey cells infected with SV40 and labeled with [^{3}H]proline was prepared as described in Materials and Methods. The precipitates were analyzed by electrophoresis in an SDS-polyacrylamide gel in parallel with mobility markers and reference T-antigen precipitates. (a) Proteins stained with Coomassie brilliant blue. The fluoroautoradiography of this lane is shown in panel b, lane 4. (b) Fluoroautoradiography. Lanes 1 and 5: Cytoplasmic proteins from HeLa cells infected with Ad2$^+$ND1 dp2 labeled with [^{35}S]-methionine; lanes 2, 3, and 4: Immunoprecipitated T-antigen from SV40-infected CV1 cells labeled with [^{35}S]methionine (2), $^{32}PO_4$ (3), and [^{3}H]proline (4). (c) Another lane containing the same material as panel a and as panel b, lane 4, was removed from the stained gel, dried on graph paper, and cut into 2-mm slices. These were placed into vials (15 by 45 mm), rehydrated with 50 ml of water, dissolved in 200 ml of H_2O_2 (33%) at 70°C for 4 h, and counted with 3.5 ml of Aquasol scintillation liquid. The arrowheads in panels b and c indicate the positions of the 96K and the 88K forms of T-antigen.*

Fig. 7. The Coomassie brilliant blue-stained pattern of the immunoprecipitated proteins (Fig. 7, track a) revealed the 96K and 88K forms of T-antigen. In this preparation, the 88K form stained more intensely by a factor of 2. In Fig. 7b, the fluoroautoradiography of the same material is shown (track 4) in comparison with molecular weight marker proteins (tracks 1 and 5) and with marker T-antigen precipitates from independent experiments labeled with either [^{35}S]methionine (track 2) or ^{32}P (track 3). Surprisingly, the 96K component carried the majority of the proline label, as substantiated by counting gel slices in a liquid scintillation spectrometer (Fig. 7c). Therefore, there was a significant difference in the proline content of the 96K and 88K components of T-antigen.

The 96K and 23K proteins and the 88K and 24K proteins, all labeled with proline, were compared pairwise (Fig. 8). The 96K form of T-antigen (Fig. 8a) yielded eight proline-containing peptides with a peak-to-background ratio greater than 2 (peptides 1 to 8). If the six peptides (2, 3, 5, 6, 7, and 8) that contain similar amounts of radioactivity are assumed to correspond to one proline residue per peptide, then peptide 4 would correspond to two proline residues and peptide 1 would correspond to about

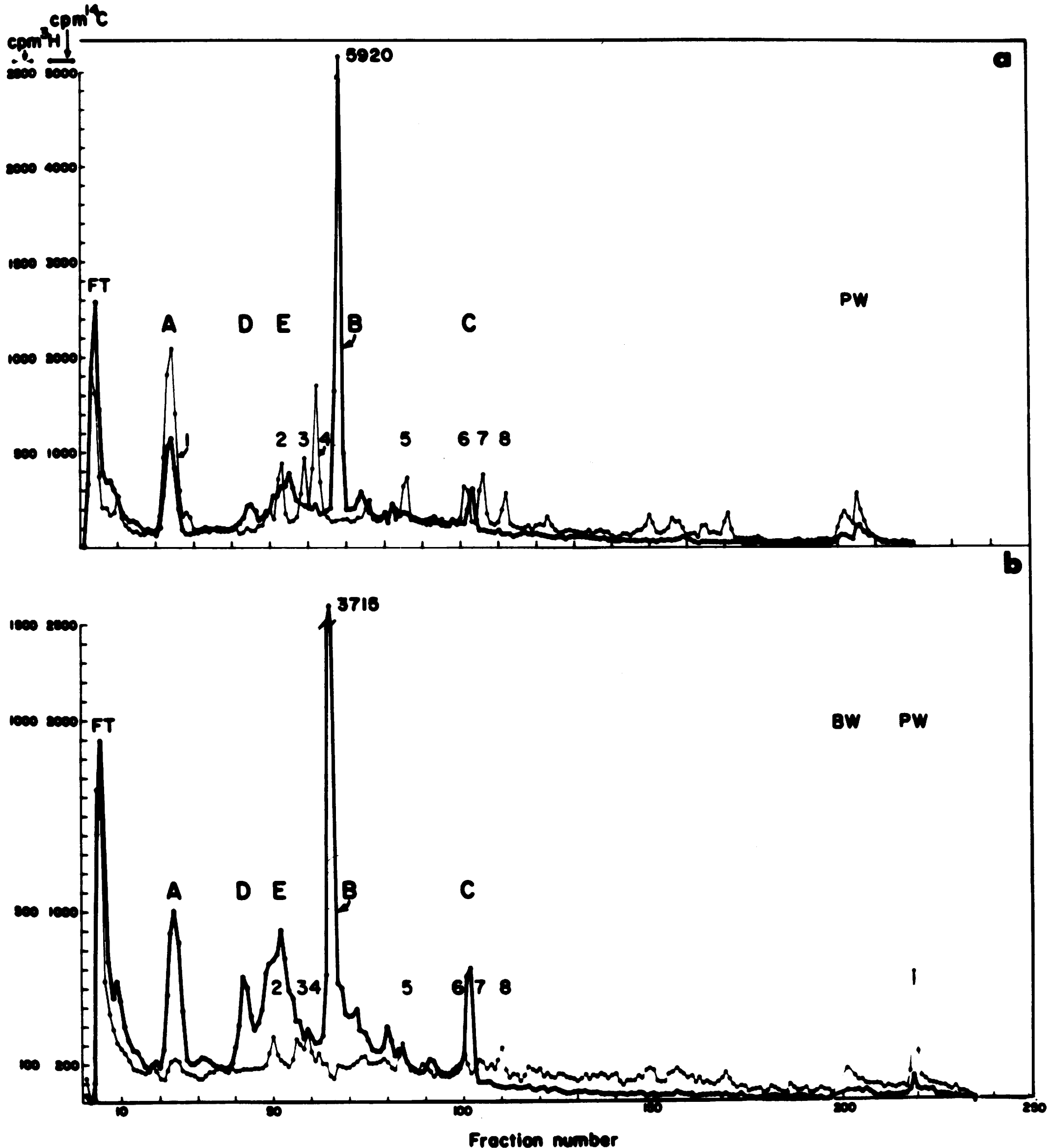

FIG. 8. *Comparison of proline-containing tryptic peptides from T-antigen and the 23K/24K proteins. The proteins were removed from SDS-polyacrylamide gels after their identification using stained side strips as in Fig. 9. They were eluted and processed as described in Materials and Methods and analyzed on chromobeads P. (a) 96K T-antigen ([^{3}H]proline; ——, peptides 1 through 8) and 23K protein ([^{14}C]proline; ——, peptides A through E). (b) 88K T-antigen ([^{3}H]proline; ——, peptides 1 through 8) and 24K protein ([^{14}C]proline; ——, peptides A through E). BW indicates the material that is eluted by the wash with buffer B, PW indicates the material eluted by the wash with 6 M pyridine, and FT refers to the flow-through material, which is not retained on the ion-exchange resin.*

five proline residues. We did not consider six minor peaks with peak-to-background ratios less than 2.

The peptide profile of the 23K protein revealed five proline-containing peptides with peak-to-background ratios greater than 2 (peptides A–E, Fig. 8a). Peptide A coincided with the proline-rich peptide 1 of the 96K form of T-antigen. Peptide C coincided with the methionine- and lysine-containing peptide 11 from Ad2 fiber (Fig. 4). Analysis of proline-labeled fiber (data not shown) shows that fiber peptide 11

also contains proline. Peptides B, D, and E were not shared with either the fiber or the T-antigen profiles. Of these, peptide B was further analyzed by two methods. The first way was to submit it to sequential Edman degradation in an automated sequenator and to score for the release of radioactive proline in each cycle. Instead of the expected all-or-none type of distribution of proline label in subsequent cycles, the radioactivity was found distributed over all of the first 12 cycles. This indicated that peak B might contain more than one proline-rich peptide. A sample of the material was also analyzed by one- and two-dimensional fingerprinting (chromatography and electrophoresis on cellulose thin layers) as described elsewhere (10), resolving B into at least two peptides (data not shown). One possible interpretation of these findings is that peptide B is related to peptide A. It might be generated by incomplete tryptic cleavage, since the terminal tryptic cleavage site is contained in the sequence Phe–Lys–Lys–Pro–Pro, and it is known that trypsin cleaves Lys-Lys and Lys-Pro bonds inefficiently. Peptides A and B might also differ by secondary modifications such as hydroxylation of proline or phosphorylation. We did not identify peptides D or E. None of the 23K or T-antigen peptides in these profiles coincided with free proline, which eluted under these conditions between T-antigen peptides 1 and 2 as determined independently (data not shown).

The profile of the proline-containing peptides of 24K (Fig. 8b) was essentially indistinguishable from that of 23K (Fig. 8a).

The 88K component of T-antigen contained peptides 2 to 8 in common with the 96K form, but lacked the very proline-rich peptide 1 (Fig. 8b). Using the hexon peptides as markers, a comparison of lysine-, arginine-, and proline-containing peptides of 23K/24K shows that the proline-rich peptide shared with 96K T-antigen contained neither lysine nor arginine. From these results we conclude that: (i) the 23K and 24K proteins are very closely related to each other in their primary structure; (ii) the 88K and 96K components of T-antigen are strongly related but not identical to each other; (iii) the 23K/24K proteins share a very proline-rich tryptic peptide with the 96K form of T-antigen, which is absent from the 88K form; (iv) the very proline-rich T-antigen peptide contains severalfold more proline residues than the other proline-containing peptides of T-antigen; (v) peptide 1 is located in the carboxy-terminal portion of T-antigen, because it is shared with the $Ad2^{+}ND1$ dp2 23K/24K proteins and this virus contains only SV40 DNA sequences corresponding to the carboxy-terminal portion of SV40 T-antigen. In addition, this peptide is the carboxy-terminal peptide of the 23K/24K protein since it contains neither lysine nor arginine.

Elimination of the other reading frames. We designate the reading frame that predicts the existence of the proline-rich peptide frame A. The frame in which the sequence shown in Fig. 6 would be translated one nucleotide out of phase to the right of frame A is designated frame B, and the third is designated frame C. Frame B can be eliminated by considering the genome structure of the virus $Ad2^{+}ND1$ dp2. Detailed analysis of the genome structure (Lukanidin, in preparation; S. Zain and G. Fey, unpublished observations) shows the crossover point between Ad2 and SV40 DNA sequences at the left end of the smaller SV40 insertion in $Ad2^{+}ND1$ dp2 (Fig. 1) to be situated 50 ± 20 bases to the left of the *Hae*III restriction endonuclease cleavage site at position 0.21 of the SV40 genome (Fig. 6). In frame B, the right-hand insertion of SV40 DNA sequences in $Ad2^{+}ND1$ dp2 could code for a maximum of about 75 amino acids. Within this segment at least two, and possibly three (depending on the exact location of the crossover point), methionine-containing tryptic peptides, all ending in a lysine residue, would be predicted. One of these should also contain two proline residues. All of these should be present in both 23K/24K proteins and the 30K protein specified by $Ad2^{+}ND1$. The experimental data are incompatible with both of these predictions. First, the 23K/24K proteins consistently showed only three methionine-containing tryptic peptides (numbered 1, 2, and 3 in Fig. 5b and 9a) and a fourth peptide of variable intensity in different preparations (numbered 4). Peptides 1, 2, and 3 were derived from fiber, and only peptide 4 could be specified by SV40 information. Therefore, the higher number of methionine- and lysine-containing peptides predicted by frame B was not observed. Also, as shown by an indirect comparison of the 23K/24K proteins and the 30K protein (Fig. 9a–c), peptide 4 was not found in the 30K protein synthesized either in vivo (Fig. 9b) or in vitro using SV40-specific $Ad2^{+}ND1$ RNA (Fig. 9c). Therefore the 30K and the 23K/24K proteins were not translated in frame B.

In frame C, nine translational terminator codons were predicted for the segment of SV40 DNA early region contained in the right-hand insertion in $Ad2^{+}ND1$ dp2 (map positions 0.21 to 0.17 on the SV40 genome). No two of these terminators would lie further than 17 codons apart. Therefore the 23K/24K proteins could not contain more than 17 amino acids contributed by SV40, equivalent to a maximum of 2 Kdal. With a maximum contribution from fiber of 15 Kdal (based on the $Ad2^{+}ND1$ dp2 DNA

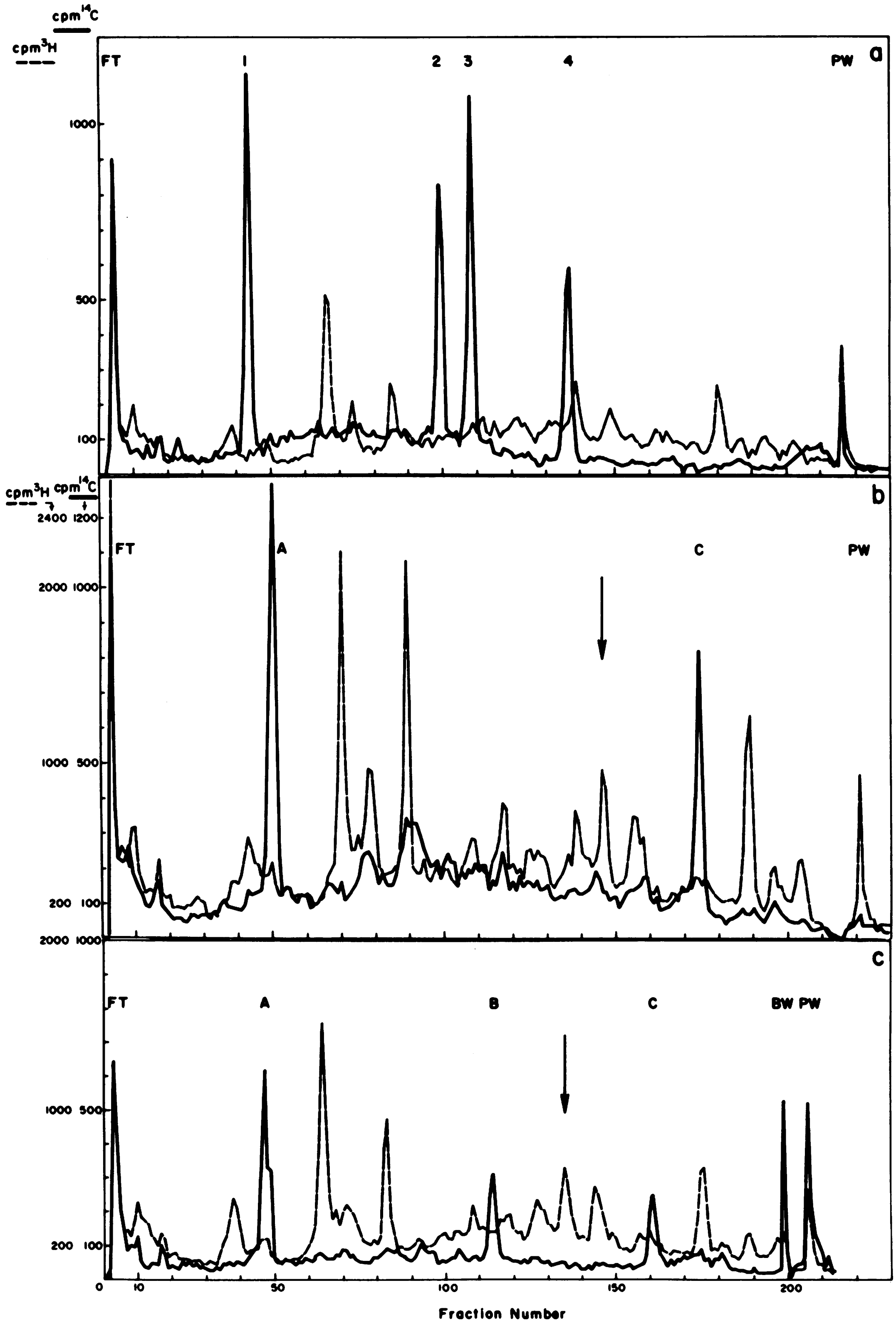

FIG. 9.

structure), the observed size of the protein cannot be explained.

In summary, within the region between 0.17 and 0.54 map units on the SV40 genome, the observed size and peptide composition of the 23K/24K proteins can be explained in reading frame A, but not in reading frame B or C. One concludes that frame A must be used for the translation of SV40 T-antigen, the 23K/24K proteins, and the 30K protein. The carboxy terminus of T-antigen would then be located on the genome map at position 0.175, next to the TAA triplet underlined in Fig. 6.

Evidence that host range mutants of Ad2$^+$ND1 dp2 lack the 23K/24K proteins. To test whether the 23K/24K proteins mediate the helper function for Ad2 growth on monkey cells, host range mutants of Ad2$^+$ND1 dp2 were generated by chemical mutagenesis with nitrous acid as described in Materials and Methods. The mutants were screened for the loss of efficient replication on monkey cells and for simultaneous retention of wild-type propagation on HeLa cells.

Of 200 plaques analyzed, 7 mutants were identified with growth differences of 100-fold or higher. The frequency at which host range mutants were obtained from this virus was about threefold higher than in the corresponding study of Ad2$^+$ND1 (15, 16), suggesting that the mechanism of generation of the mutants is different for the two viruses. Three of the mutants were tested for complementation by Ad2 and by SV40 of their deficiency in propagation on monkey cells. The yields of the mutants on CV1 cells could be enhanced by simultaneous infection with SV40 by factors between 25- and 100-fold, but not by coinfection with Ad2. Therefore these mutants can be complemented by SV40 as if they were affected in the expression of an SV40-specific helper product.

For these seven mutants, the protein patterns were analyzed in cytoplasmic extracts from infected HeLa cells labeled with [^{35}S]methionine. Electrophoresis in SDS-polyacrylamide gels was performed as above. All seven mutants had no detectable levels of the 23K protein seen with wild type and with a positive control virus (no. 337), which had undergone the mutagenization and multiple plaque purification, but continued

TABLE 1. *Growth of virus in monkey cells (CV1) after coinfection with SV40*[a]

Virus	Virus yield (PFU/ml) titrated on HeLa cells	Factor of enhancement
H311	1×10^5	
H330	3×10^4	
H359	2×10^4	
H311 + SV40	2.5×10^6	25
H330 + SV40	1.5×10^6	50
H359 + SV40	2.0×10^6	100
SV40 alone	0	
Mock	0	

[a] Cells were infected simultaneously with 0.5 PFU of the mutants per cell and 10 PFU of SV40 per cell. Controls were either mock infected (exposed to PBS) or infected with 10 PFU of SV40 alone per cell. An independent experiment determined that coinfection with Ad2 does not enhance the yield of these mutants (data not shown). The infected cultures were incubated for 48 h, and the total virus yield was then plaque assayed on HeLa cells.

to replicate equally well on both cell types (Fig. 10). Therefore the loss of growth potential on CV1 cells is accompanied by a loss of the 23K protein, consistent with the hypothesis that the presence of this protein is required for the expression of helper function.

The genomes of three of the seven mutants (H311, H330, and H359) were analyzed by using the restriction enzymes *Bam*HI and *Hha* (data not shown). All three mutants were found to lack specific *Hha* and *Bam*HI fragments characteristic of the right-hand insertion of SV40 DNA sequences (Fig. 1) in Ad2$^+$ND1 dp2. At least these three host range mutants were deletion mutants that had lost the right-hand insertion of SV40 DNA sequences, restoring a phenotype and a genotype very close to or identical to the host range mutant H71, from which Ad2$^+$ND1 dp2 was derived. The mechanism by which these mutants were generated was most likely a spontaneous deletion, which occurred independently of the mutagenization with nitrous acid, possibly explaining why these occurred at a higher frequency than did the original host range mutants of Ad2$^+$ND1.

DISCUSSION

The genome of Ad2$^+$ND1 dp2, a virus that

FIG. 9. *Comparison of methionine-containing tryptic peptides from the 23K/24K proteins and the 30K protein. The chromatograms were generated as described in Materials and Methods. The symbols BW, PW, and FT are defined for Fig. 8. (a) Mixture of the 23K and 24K proteins ([^{35}S]methionine; ——, peptides 1 through 4) and 30K host marker protein from uninfected HeLa cells ([^{3}H]methionine; -----). (b) [^{35}S]methionine-labeled 30K protein from HeLa cells infected with Ad2$^+$ND1 (——, peptides A and C) and [^{3}H]-methionine-labeled 30K host marker protein (-----). (c) [^{35}S]methionine-labeled 30K protein from Ad2$^+$ND1 synthesized in vitro from mRNA selected by hybridization to SV40 DNA (——, peptides A through C) and [^{3}H]methionine-labeled 30K host marker protein (-----). The arrows in (b) and (c) indicate the positions where the fourth methionine peptide from the 23K/24K protein would be expected.*

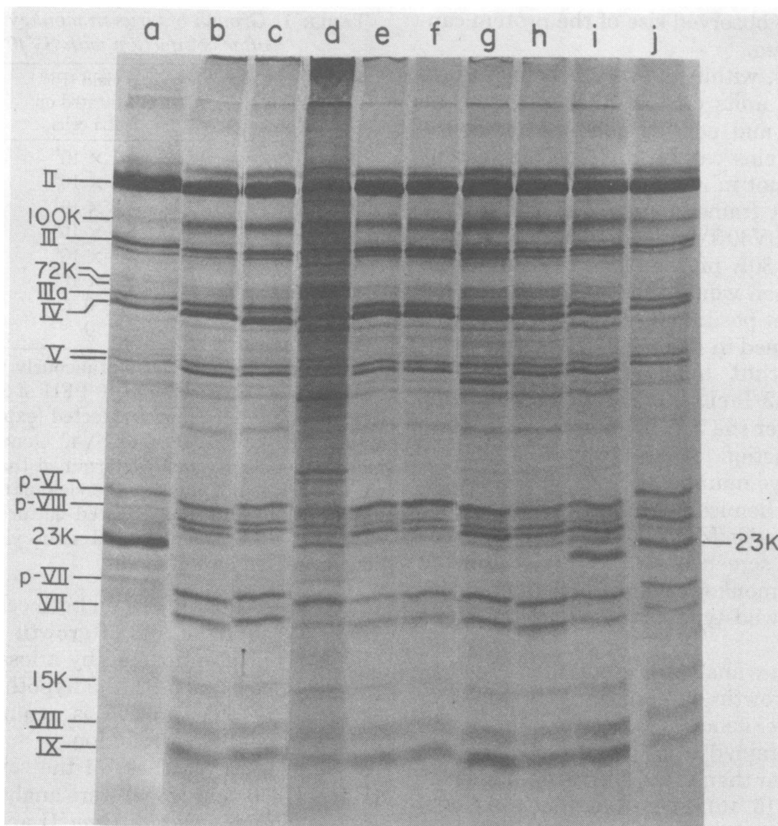

FIG. 10. *Proteins from HeLa cells infected with host range mutants of Ad2⁺ND1 dp2. HeLa cells were infected with equal volumes of virus stocks of the host range mutants and of the positive control no. 337, which had been prepared as described in Materials and Methods. The cells were labeled at 26 h after infection with [^{35}S]methionine, and total cell extracts were analyzed by electrophoresis in 15% polyacrylamide gels. (a and j) Cytoplasmic extracts of HeLa cells infected with the wild-type Ad2⁺ND1 dp2. (b–i) Total cell extracts infected with, from left to right: H289, H296, H311, H359, H383, and the positive control no. 337.*

expresses the helper function for growth of Ad2 on simian cells, contains two insertions of SV40 DNA (Lukanidin, in preparation). The left insertion is functionally silent because of the ochre mutation in this region inherited from H71 (13, 16). The right insertion of SV40 DNA is smaller, presumably deleting the site of the H71 ochre mutation, and is inserted after the region of Ad2 DNA that codes for the amino-terminal portion of the Ad2 fiber gene. Therefore one would expect the helper function in this virus to be coded at least in part by the right SV40 insertion. Consistent with this expectation, we do not find the 30K Ad2⁺ND1-specific helper protein in cells infected with Ad2⁺ND1 dp2, but do find small amounts of the 10K H71 ochre fragment upon cell-free translation of RNA from these cells.

Instead, cells infected with Ad2⁺ND1 dp2 synthesize a prominent virus-specific, nonvirion polypeptide(s) of 23 to 24 Kdal (23K/24K). Cell-free synthesis of 23K/24K using SV40-specific mRNA prepared from cells infected with Ad2⁺ND1 dp2 demonstrates that 23K/24K mRNA is at least partially SV40 coded.

Considerations of the size of 23K/24K and of the detailed genome structure suggested that 23K/24K should be a hybrid protein composed of the amino-terminal portion of fiber and the carboxy-terminal portion of SV40 T-antigen. To test this hypothesis, the tryptic peptides of 23K/24K were compared with those of fiber and of SV40 T-antigen. More than three-fourths of the lysine- and methionine-containing tryptic peptides of 23K/24K were also found among the fiber tryptic peptides. Furthermore, 23K/24K shares a very proline-rich tryptic peptide with SV40 T-antigen. The presence of the amino-terminal portion of the fiber gene in the 23K/24K protein implies that the expression of 23K/24K should be under the same control elements that govern the expression of fiber. This is con-

sistent with our observation that 23K/24K appears late in infection and in large amounts.

Comparison of the tryptic peptide maps with the SV40 DNA sequence establishes that the reading frame for T-antigen must be the one in which the carboxy terminus of T-antigen is a very proline-rich tryptic peptide. Therefore the region of SV40 DNA coding for T-antigen most probably terminates at map position 0.175. This conclusion assumes that the 23K/24K mRNA terminates near position 0.16 on the SV40 map, as has been reported for SV40 early mRNA (11, 46). An alternative reading frame is possible if several segments are spliced together to give an mRNA terminating near position 0.12. An additional proline-rich peptide is predicted in reading frame B, terminating at a TAG triplet 262 nucleotides beyond the TAA at position 0.175, i.e., at about position 0.125. This peptide would contain four prolines and, as with reading frame A, there would be no methionines predicted in this frame within 150 nucleotides of the terminator triplet. Consequently, our results are also consistent with the 23K/24K protein terminating at this point. Presumably a protein terminating near 0.125 map units would be translated from an mRNA in which some sequences between 0.21 and 0.14 map units had been spliced out to maintain a molecular weight for the protein of about 24,000. Indeed, a population of molecules containing both termini could account for the observed heterogeneity of the proline-rich terminal peptide of 23K/24K. However, this possibility would require that at least part of the population of molecules precipitated with SV40 T-antiserum are translated from an mRNA extending more than 200 nucleotides past the reported 3′ end of SV40 early mRNA. Furthermore, it has been demonstrated (39) that helper activity is encoded by the SV40 DNA fragment containing the early region and terminating at the *Bam* I site near position 0.14. Therefore, either any 23K/24K molecules terminating near 0.125 map units are not necessary for helper activity, or else the activity is expressed by a truncated polypeptide lacking 34 C-terminal amino acid residues. Thus further work is required to determine precisely where within this region helper activity is encoded. However, the simplest explanation of the available information is that T-antigen is terminated at position 0.175.

The correlation of 23K/24K with helper function is further strengthened by the observation that seven out of seven host range mutants of Ad2$^+$ND1 dp2, which have lost the ability to grow on monkey cells, also do not express 23K/24K. This correlation has been confirmed by the observation that helper function can be provided by microinjecting purified 23K/24K protein into Ad2-infected monkey cells (54).

Fusion proteins may also be encoded by other Ad2-SV40 hybrid viruses. Preliminary studies of other revertants of H71 have indicated fusion of the carboxy-terminal part of SV40 T-antigen to varying N-terminal portions of IV and other Ad2 proteins (Grodzicker et al., in preparation). Indeed, the Ad2$^+$ND1 30K mRNA itself contains both Ad2 and SV40 sequences (2, 8, 12). Although the methionine-containing tryptic peptides of 30K seem to arise exclusively from SV40 sequences (36), the possibility has not been eliminated that some amino-terminal portion of 30K, perhaps lacking methionine, is Ad2 coded.

Other fused proteins have been described in bacterial systems (40, 50). One of these is a large chimeric protein that contains essentially two entire proteins linked together with only a small deletion at the joint site. In this case, both functions of the constituent products, *lac* repressor and β-galactosidase, are preserved in the fused polypeptide. As far as we know, no such clear case, where the genetic origin of both components is known, has been described in eucaryotic systems. However, multifunctional proteins with functional domains that can be proteolytically separated into fragments that still contain the constituent function are known (e.g., immunoglobulins [9]; for a review on domain structure in multifunctional proteins, see reference 23). These might have originally arisen as fused proteins. T-antigen itself might be visualized as a fused protein since it falls into separate functional domains: the helper function is located at its carboxy terminus (54; this report), and the function responsible for the stimulation of host cell DNA synthesis is located in the remaining part of the molecule (14). A last important class of fused proteins would be expected to exist in some SV40-transformed cells in which the integrated sequences are expressed under a cellular control as they are expressed under Ad2 control in the hybrid Ad2$^+$D2. Proteins larger than 100 Kdal that immunologically cross-react with T-antigen have been observed in cell lines derived from SV40 tumors (T. Chang, personal communication). From these proteins one might obtain information concerning the flanking sequences of integrated SV40 genomes.

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