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A Simian Virus 40-Encoded Protein of M_r 74,000 Daltons Is Structurally Related to the Capsid Proteins of the Virus

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We have demonstrated the synthesis of a 74,000-dalton protein (74K protein) in African green monkey kidney cells infected with simian virus (SV)40. The 74K protein was detected late during the lytic cycle. Its synthesis was inhibited by arabinosyl cytosine as was the synthesis of the capsid proteins. Monospecific antibodies raised against VP1 and VP3 precipitated the structural proteins and the 74K protein. The 74K protein was not found in purified virions. Tryptic peptide analysis demonstrated that the 74K protein shares methionine- and serine-containing peptides with VP1 and VP3 and thus is structurally related to the capsid proteins.

Key words: SV40, structural proteins, immunoprecipitation, tryptic peptide analysis

Simian virus (SV)40 productive infection is divided into early and late stages separated by the onset of viral DNA replication 10-12 hr after infection. The viral capsid proteins VP1, VP2, and VP3 are virus-encoded polypeptides synthesized in the late phase of SV40 replication. The proteins are destined to be incorporated into progeny virions [1]. After viral DNA replication, the late region of the SV40 genome is transcribed into stable mRNAs of 16S and 19S. The major 16S mRNA coding for virion structural protein VP1 consists of a leader segment transcribed clockwise from the L-strand between map coordinates 0.72 and 0.76 and a body segment from the region between map positions 0.94 and 0.17 [2–7]. Late 19S mRNAs that are believed to be translated into virion polypeptides VP2 and VP3 [8] consist of a body segment that is homologous to the DNA between map positions 0.765 and 0.17 spliced to a variety of leader segments derived from the region between map positions 0.67 and 0.76 [7–10]. By tryptic peptide analysis and by in vitro translation studies it has been shown that VP2 and VP3 are unrelated to VP1 but closely related to each other [11–14].

In this study we demonstrate the synthesis of a 74,000-dalton protein in African green monkey kidney (AGMK) cells after lytic infection with SV40. We show that the 74K protein is a virus-encoded protein synthesized in infected permissive cells

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after the onset of viral DNA-replication. Tryptic peptide mapping reveals that the 74K protein shares methionine- and serine-containing peptides with VP1 and VP3. The 74K protein thus seems to be constructed of VP1 and part of VP3.

MATERIALS AND METHODS

Cells and Viruses

Primary African green monkey kidney cells (AGMK) were grown in Eagle minimum essential medium (MEM) supplemented with 5% fetal calf serum [15]. CV-1 cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% newborn calf serum. The SV40 strain 777 propagated in AGMK cells was used in most experiments [16]. The virus stocks were grown at low multiplicity (0.01 tissue culture infective dose $(TCID)_{50}$/cell) from single plaques.

Radioactive Labeling and Purification of Virus

CV-1 cells were incubated with SV40 at a multiplicity of infection of 0.05 $TCID_{50}$/cells. After 2-hr adsorption the virus suspension was replaced by fresh medium. Labeling was carried out by addition of 100 μCi [^{35}S] methionine (600–800 Ci/mmole) per 1.5×10^7 cells. Seven days after infection the cells were harvested. Extraction and purification of SV40 was done exactly as described [17].

Infection and Labeling of Cells

Confluent cultures of primary AGMK cells and CV-1 cells were infected with SV40 at multiplicities of 10–50 $TCID_{50}$/cell and incubated at 37°C. Control cells were incubated with lysates from uninfected cells. For inhibition of viral DNA synthesis, 25 μg cytosine arabinoside (Ara-C) per ml medium was present after infection and during labeling of the cells. After the indicated times cultures were labeled for 12 hr with 300–500 μCi [^{35}S] methionine or with 600 μCi [^{3}H] serine in 3 ml methionine- or serine-free DMEM per 5×10^6 cells.

Preparation of Cell Extracts

At the end of the labeling periods the monolayers were washed three times with TRIS-deficient buffer (TD: 25 mM TRIS-HCl, pH 7.4, 5 mM KCl). The cells were then harvested in TD-buffer containing 0.02% ethylenediamine tetraacetic acid (EDTA), pelleted by low-speed centrifugation, and extracted with 300 μl pH 9.0 extraction buffer (80 mM NaCl, 20 mM EDTA, 1 mM DTT, 20 mM TRIS-HCl, pH 9.0) per 5×10^6 cells [18]. All steps were performed at 4°C. All described buffers contained phenylmethylsulfonyl fluoride (PMSF, 0.3 mg/ml) or Trasylol (0.014 mg/ml).

Production of Antisera

Antiserum against the major capsid protein of SV40 was made by injecting purified sodium dodecyl sulfate (SDS)-denatured VP1 into rabbits and mice. Goat antiserum directed against SV40 virions was purchased from Cappel Laboratories. Guinea pig anti-VP3 serum was kindly supplied by Dr. W. Deppert, University of Ulm.

Immunoprecipitation

Cell extract (100 μl; 2×10^6 cells) was incubated with 5–10 μl of antiserum for 4 hr at 4°C. Some 100 μl of 10% (vol/vol) protein A-Sepharose was then added and

incubated for an additional 4 hr. The immunocomplexes were centrifuged for 5 min at 12,000g, and washed three times with 0.5 ml tris buffered saline, pH 7.4, with the following concentrations of NP40: 0.1%, 0.05%, 0.005%. The complexes were then resuspended in 75 μl electrophoresis buffer to elute precipitated antigens [20]. After heating at 100°C for 5 min the protein A-Sepharose was pelleted by centrifugation.

SDS-Polyacrylamide Gel Electrophoresis

Cell extracts, purified virions, or immunoprecipitates were subjected to electrophoresis through polyacrylamide gels in the presence of $NaDodSO_4$ as described [21, 22]. After drying, gels were fluorographed [23] and exposed to presensitized Kodak X-Omat R films [24].

Peptide Mapping

Labeled proteins were separated through preparative SDS-gels and visualized by autoradiography. The identified protein bands were excised and proteins were eluted by homogenization of the gel. The eluted proteins were mixed with a carrier protein (bovine γ-globulin), concentrated by trichloroacetic acid (TCA)-precipitation and oxidized with performic acid as described [25]. Oxidized proteins were suspended in 0.5 ml of 0.05 M NH_4HCO_3. Some 30 μg L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (1 μg trypsin/1 ml 0.05 M NH_4HCO_3) was added and the samples were incubated for 5 hr at 37°C. Another 20 μg trypsin was added and incubation was continued for another 5 hr. NH_4HCO_3 was removed by three cycles of lyophilization. Two-dimensional mapping was done by electrophoresis at pH 4.7 in the first dimension and ascending chromatography at pH 5.2 in the second [26]. [^{3}H]-labeled peptides were visualized by fluorography with 2-methyl-naphthalene. [^{35}S]-labeled peptides were detected by autoradiography using preflashed films.

Individual [^{35}S]-labeled peptides identified by autoradiography were scraped from the cellulose thin-layer plates and eluted in 300 μl electrophoresis buffer. Lyophilized peptides were resuspended in 6 μl of the same buffer and rerun.

RESULTS

The 74K Protein Is Synthesized Late After Infection

In order to determine the onset of the synthesis of the 74K protein, SV40-infected CV-1 cells were labeled every 2 hr with [^{35}S] methionine. After incubating for 2 hr the cells were extracted and aliquots were analyzed by acrylamide gel electrophoresis. The time course shows that synthesis of VP1 has already started 10 hr postinfection. After another 2–3 hr the 74K protein becomes detectable (Fig. 1).

Synthesis of the 74K Protein Is Inhibited by Ara-C

To see whether the synthesis of the 74K protein depends on the expression of the early or late region of the SV40 genome, viral DNA synthesis was inhibited by Ara-C. The presence of Ara-C from the beginning of infection drastically reduces the rate of late transcription [27, 28]. SV40-infected and mock-infected CV-1 cells were labeled with [^{35}S] methionine from 32 to 44 hr postinfection and extracted as described above. The extracts were analyzed on acrylamide gels and the labeled proteins were detected by autoradiography. Figure 2 shows that the synthesis of the 74K protein in infected cells is clearly suppressed by Ara-C.

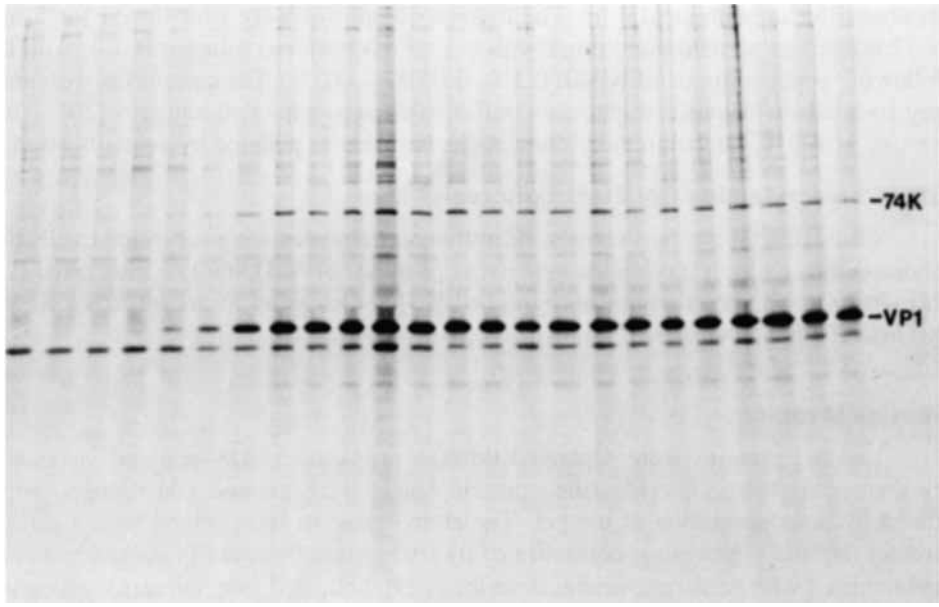

Fig. 1. Time course of protein synthesis of SV40-infected CV-1 cells. Infected cells were labeled in intervals of 2 hr with [^{35}S] methionine (400 μCi/5 × 10^6 cells). Extraction was done as described in Materials and Methods. Proteins labeled from 0 to 2 hr postinfection correspond to the first fraction (left), proteins labeled from 46 to 48 hr postinfection correspond to the last fraction (right). The gel was 10% acrylamide.

Immunoprecipitation With Antisera Against VP1 and VP3

Antibodies directed against SDS-purified capsid proteins VP1 and VP3 were used to examine the immunological relationship of the 74K protein to the capsid proteins. Immunoprecipitation was carried out as described. Rabbit serum directed against denatured VP1 precipitated the capsid protein, the 74K protein, and an 83K protein from labeled extracts of infected cells (Fig. 2G). The proteins were not precipitated from control cells or from infected cells treated with Ara-C (Fig. 2D,E,H,I). The 74K protein could also be immunoprecipitated from lysates of infected cells using guinea pig anti-VP3 serum (Fig. 3B). Neither the capsid proteins nor the 74K protein were identified in immunoprecipitates of SV40 transformed cells (data not shown).

To exclude the possibility that defective viruses were present in our virus stocks that could generate the 74K protein we also infected CV-1 cells with independently isolated plaque-purified SV40 stocks. The 74K protein was found in each case (Fig. 3D,F).

The 74K Protein Is Not Found in SV40 Particles

Although the structural components of SV40 have been described [29–32], it was interesting to see if the 74K protein would be found in isolated virus particles. Radioactive SV40 was purified by cesium chloride banding followed by zonal separation in sucrose gradients. After dialysis, the virus was disrupted in SDS, reduced with β-mercaptoethanol or DTT and analyzed by gel electrophoresis. The 74K protein

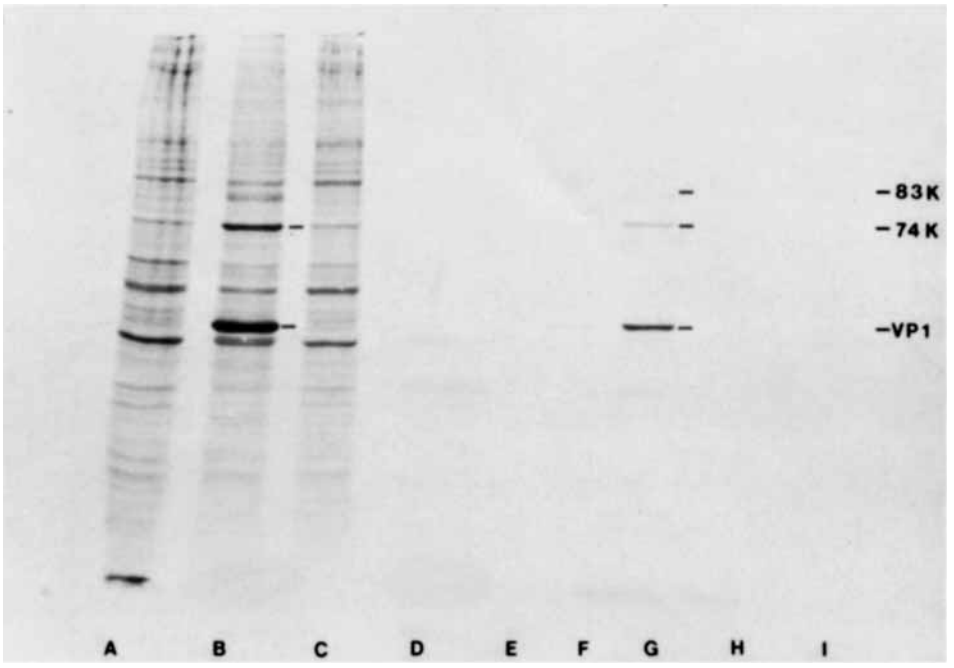

Fig. 2. [^{35}S] Methionine-labeled proteins of pH 9.0 extracts and immunoprecipitates from mock-infected and SV40-infected CV-1 cells labeled 32-44 hr postinfection. Some of the cell cultures were treated with Ara-C. Immunoprecipitation was done with rabbit anti-VP1 serum. The samples were separated on a 10% polyacrylamide gel. A, mock infected; B, infected; C, infected + Ara-C; D, mock-infected, normal serum; E, mock-infected, anti-VP1 serum; F, infected, normal serum; G, infected, anti-VP1 serum; H, infected + Ara-C, normal serum; I, infected + Ara-C, anti-VP1 serum.

could not be demonstrated in these preparations, and is therefore apparently not a component of the virus (data not shown).

Fingerprint Analysis of the 74K Protein and the Capsid Proteins

To examine the structural relationship between the 74K protein and the capsid proteins we compared their peptide fingerprints. Labeled proteins were purified by immunoprecipitation with goat anti-SV40 serum. Details of the preparation of the proteins and peptide mapping have been described in Materials and Methods.

Figure 4 shows that the 74K protein has methionine-containing tryptic peptides comigrating with those found in the maps of VP1. The tryptic peptides of VP1 immunoprecipitated from infected cells and the peptides of VP1 from virus particles were almost identical (Fig. 4A,B). However, two additional peptides of the 74K protein comigrated with the peptides of VP3 (Fig. 4C,D). This finding was confirmed when the eluted peptides were mixed and rerun (Fig. 5A,B).

To study the relationship between the 74K protein and the structural proteins in more detail, [^{3}H] serine-labeled proteins were also analyzed by two-dimensional peptide mapping. Figure 6 shows the serine-containing tryptic peptides of the structural proteins VP1, VP2, VP3, and the 74K protein.

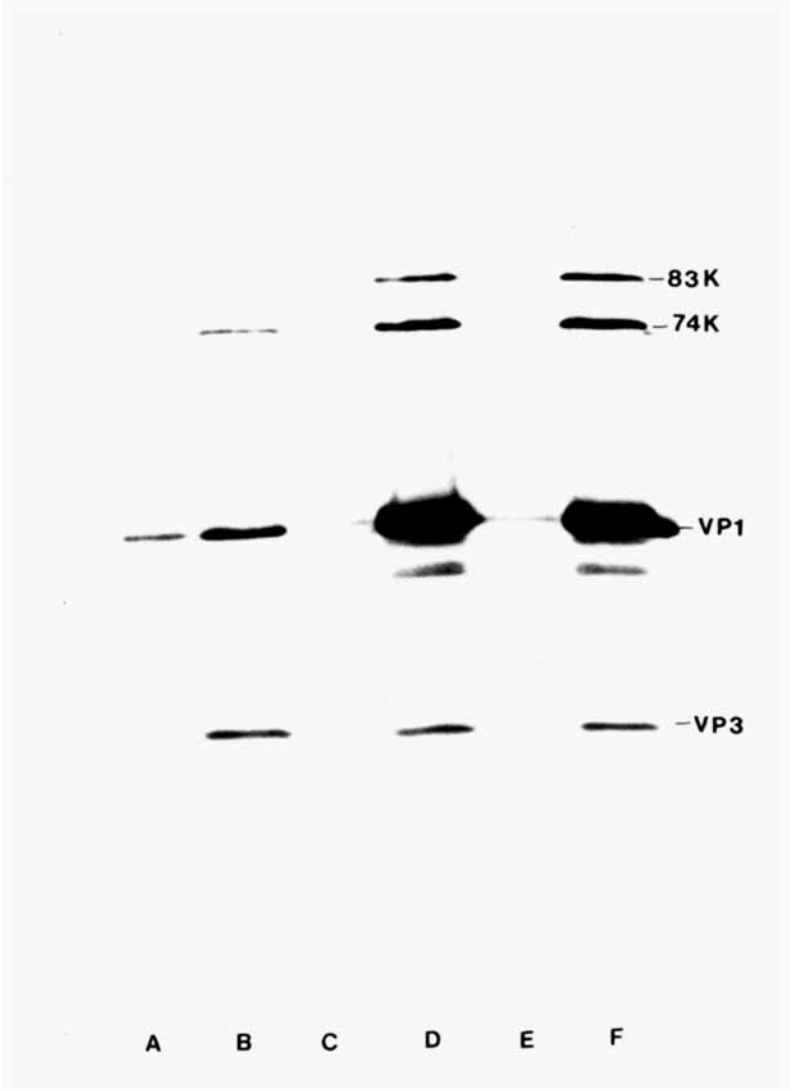

Fig. 3. Immunoprecipitates of pH 9.0 extracts from CV-1 cells infected with different SV40 wild-type stocks that have been grown at low multiplicity from single plaques. Cells were labeled with [^{35}S] methionine 40–43 hr postinfection. Immunoprecipitations were done with guinea pig anti-VP3 serum and mouse anti-VP1 serum. The samples were separated on a 15% polyacrylamide gel. A,B, infected with the SV40 stock used in all experiments described in this paper; A, normal serum; B, anti-VP3 serum; C, D, infected with a SV40 stock given by G. Walter, University of Freiburg; C, normal serum; D, anti-VP1 serum; E, F, infected with a SV40 stock which was kindly supplied by G. Sauer, Deutsches Krebsforschungszentrum Heidelberg; E, normal serum; F, anti-VP1 serum.

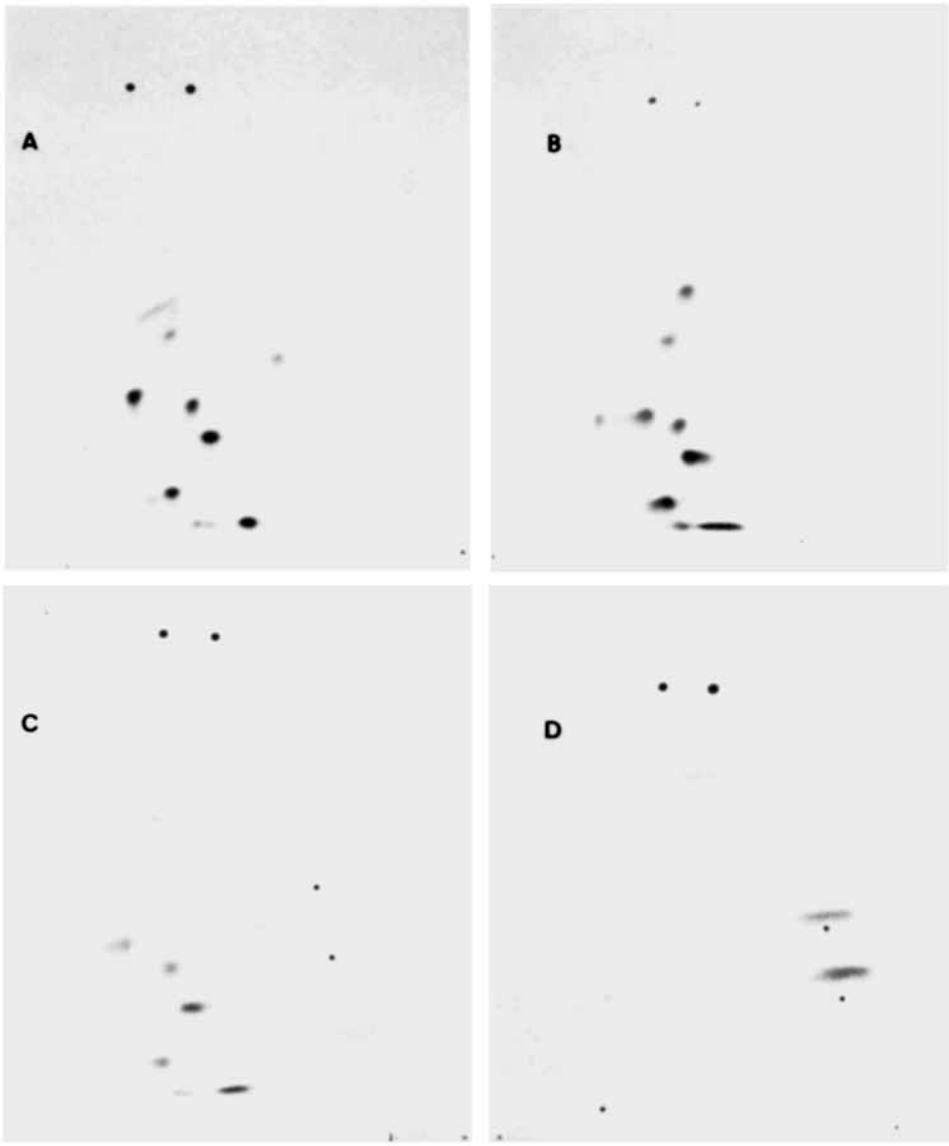

Fig. 4. Tryptic peptide analysis of [^{35}S] methionine-labeled VP1, VP3, and the 74K protein. The amount of radioactivity loaded onto each thin-layer plate and the exposure times are as follows: A, VP1 purified by immunoprecipitation, 70,000 cpm, 4 days; B, VP1 from purified SV40 particles, 64,000 cpm, 3 days; C, 74K protein purified by immunoprecipitation, 60,000 cpm, 4 days; D, VP3 from purified SV40 particles, 5,000 cpm, 14 days.

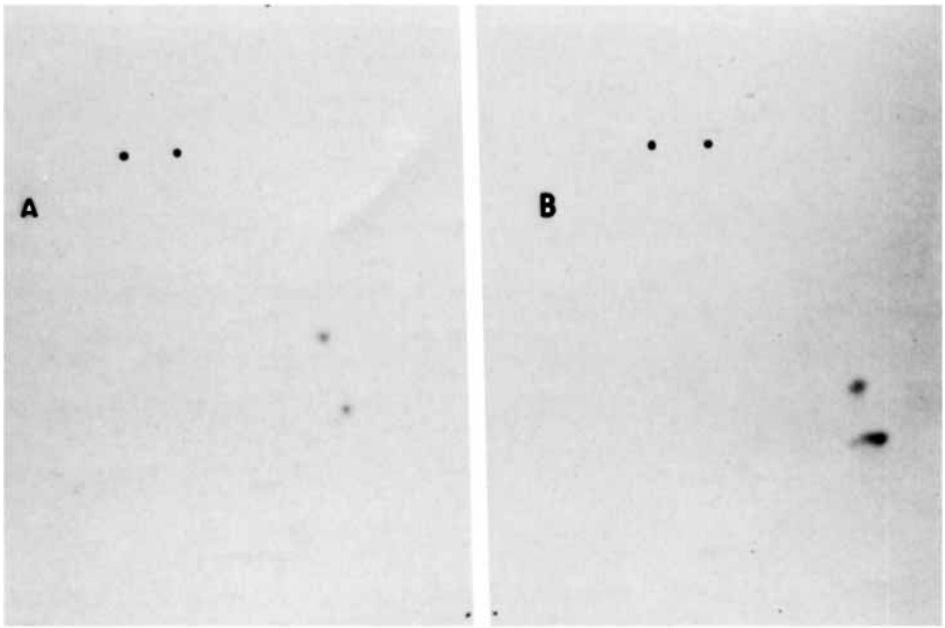

Fig. 5. Rerun of comigration: methionine tryptic peptides of the 74K protein and VP3. Eluted peptides to be analyzed were again separated by electrophoresis and chromatography. The eluted peptides are marked in Figure 4 by dots. A, peptides of VP3, 700 cpm, 22 days; B, mixture of peptides of VP3 and peptides of the 74K protein, 400 cpm of each, 22 days.

The 74K protein shares 13 serine-containing peptides with VP1; the common peptides are indicated by arabic numerals (Fig. 6A,B). Six tryptic peptides of the 74K protein (a–f) are present in the digests of VP2 and VP3 (Fig. 6B–D). As expected, the peptide patterns of VP2 and VP3 are in part identical. The unique peptides of VP2 are indicated by arrows. However, three peptides identified in the maps of VP2 and VP3 are not found in the digest of the 74K protein. The results have been confirmed by mixing equal amounts of the digests and fractionating the mixtures in the same way (data not shown). Taken together these data indicate that the 74K protein is constructed of VP1 and part of VP3.

DISCUSSION

The investigations reported in this paper show that the newly identified 74K protein observed in SV40-infected monkey cells is synthesized after the onset of viral DNA replication. This is based on (1) the finding that the 74K protein was first detectable 12–13 hr postinfection, and (2) the fact that its synthesis was inhibited in the presence of Ara-C, as was the synthesis of the capsid protein. Traces of a 74K protein are present already 2 hr after infection. This protein has been shown to be of cellular origin. It migrates slightly faster in 7.5% acrylamide gels than the viral 74K protein and its synthesis can be stimulated by SV40 and by fetal calf serum [33]. It therefore may well be related to the virus- and heat-shock-inducible 72K cellular protein described by Khandjian and Türler [34].

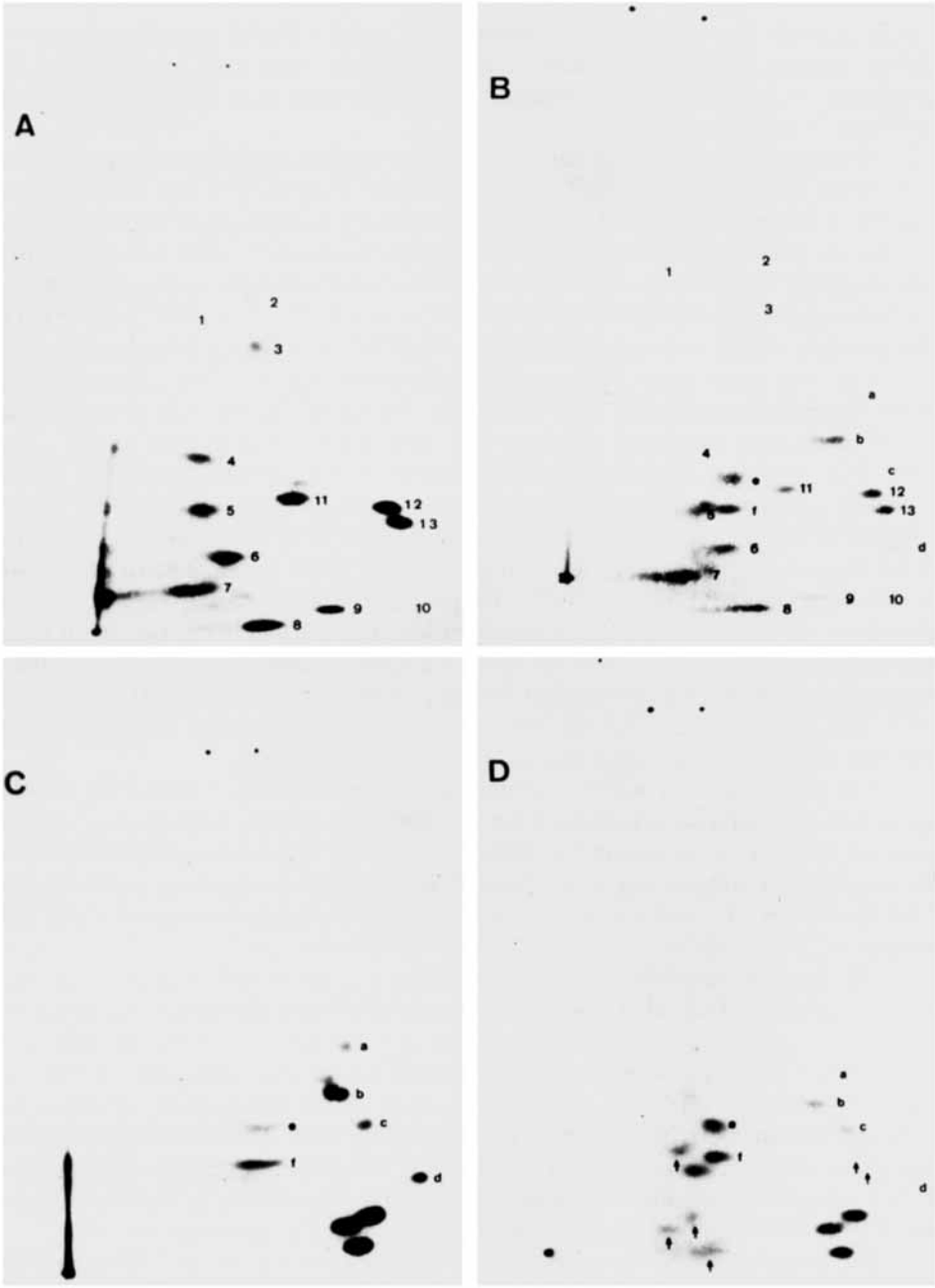

Fig. 6. [^{3}H] serine fingerprints of VP1, VP2, VP3, and the 74K protein. Purification, digestion, and fingerprinting were done as before. The comigrating peptides of VP1 and the 74K protein are indicated by numbers; small letters mark the comigrating peptides of VP2, VP3, and the 74K protein. Arrows indicate the position of the peptides unique to VP2. A, VP1 purified by immunoprecipitation, 30,000 cpm, 17 days; B, 74K protein purified by immunoprecipitation, 6,000 cpm, 8 wk; C, VP3 purified by immunoprecipitation, 30,000 cpm, 17 days; D, VP2 purified by immunoprecipitation, 5,400 cpm, 8 wk.

It could be argued that the 74K protein is made by defective viruses present in our virus stock. However, since this protein was found with different virus stocks and plaque isolates, at about the same concentration in each case, we consider this possibility to be unlikely. In contrast, the 74K protein was not found in SV40-transformed cells.

Our immunoprecipitation data revealed that the 74K protein can be precipitated using monospecific sera directed against the capsid proteins VP1 and VP3. It is reasonable to assume that the 74K protein is specifically recognized by these antibodies because the structural relationship to the capsid protein could be demonstrated by peptide mapping. These results, however, do not exclude the possibility that the 74K protein was coprecipitated with the capsid protein, as was VP3 with VP1 (Fig. 3D,F). The 83K protein, which is also precipitated by anti-VP1, is presently being investigated.

The viral nature of the 74K protein was demonstrated by tryptic peptide analysis of the structural proteins and the 74K protein. We found that the 74K protein shares all the methionine-containing peptides with VP1 and two methionine peptides with VP3. This structural relationship was confirmed by comparison of the [^{3}H] serine-labeled peptides of the 74K protein and those of the capsid proteins. The 74K protein contains 13 serine peptides comigrating with those of VP1; six serine peptides found in the digests of VP2 and VP3 were also identified in the tryptic pattern of the 74K protein. The peptides unique to VP2 were not present in the map of the 74K protein. Moreover, three serine peptides contained in VP2 and VP3 were clearly absent in the tryptic digest of the 74K protein. In view of the two-dimensional mobilities of these three serine peptides we believe that they are derived from the COOH-terminus of VP3. The results indicate that the 74K protein contains the amino acid sequence of VP1 and part of the N-terminal sequence of VP3.

The possibility that the 74K protein is posttranslationally constructed of VP1 and a shortened VP3 is very unlikely for the following reasons: VP3 does not contain cysteine to form an interchain-S-S-linkage with VP1 and noncovalent interactions between the two proteins can almost be excluded because denaturation with 3% SDS, 8 M urea, or 6 M guanidine hydrochloride yields unchanged amounts of the 74K protein.

We therefore consider that the 74K protein is translated from an individual mRNA molecule. This mRNA should be transcribed from the entire late region of SV40 and be spliced in the overlapping region of VP1 and VP3, so that the RNA can be translated in both reading frames. A similar model was considered for the Pr 180$^{gag\text{-}pol}$ protein of RSV [35].The mRNA coding for the 74K protein remains to be identified and characterized to verify the proposed model. The fact that the in vitro translation experiments of Hunter [12] show no 74K protein does not argue against the existence of such a mRNA, because the 74K protein is made in smaller amounts than VP1 and VP3 and therefore its mRNA may not have been found.

The functions of the described protein are not known at present. However, the 74K protein may be involved in virus assembly, although it is not a part of the virus, and could represent some kind of a scaffolding protein, as has been proposed for late adenovirus proteins that are absent from mature virions [36,37].

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