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Cellular Proteins Associated with Simian Virus 40 Early Gene Products in Newly Infected Cells

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Immunoprecipitates of extracts of simian virus 40-infected permissive monkey kidney cells contained two proteins with molecular weights of 56,000 and 32,000 (52K and 32K) in addition to the known viral early gene products. Immunoprecipitates of cells infected with the 0.54–0.59 deletion mutants that lack the viral 17K gene product did not contain the 56K and 32K proteins. The additional proteins appeared in immunoprecipitates of deletion mutant extracts if unlabeled extracts of wild-type-infected cells were added before addition of antiserum. The proteins can also be identified in uninfected cells by co-precipitation with unlabeled viral proteins. Thus, it appears that the 56K and 32K proteins are cellular products that associate with the viral proteins, the 17K in particular, and are indirectly immunoprecipitated by anti-tumor serum.

After infection of permissive CV-1 cells by simian virus 40 (SV40), two known gene products are synthesized from the early region of the SV40 genome (5, 10, 11). These are the nuclear T antigen (*A* gene product) and a smaller nonnuclear protein that has been referred to as small-t or t antigen. The *A* protein is a phosphorylated protein that binds to double-stranded DNA (3, 13) and, specifically, to the origin of replication on the viral chromosome (25). The protein functions both in the initiation of viral DNA synthesis (19) and in cellular transformation (7, 21), and the protein is altered by mutations of the *tsA* class (24). The smaller SV40 gene product is altered in one class of viable deletion mutants of SV40, a class that lacks portions of the genome between 0.54 and 0.59 map units (5, 14). The viability of this class of deletion mutants suggests that intact t antigen is not required for productive growth of the virus. The protein is involved in cellular transformation because the viable deletion mutants are altered in their ability to transform cells to anchorage-independent growth (2, 17).

Both viral proteins have been studied by immunoprecipitation with serum from tumor-bearing hamsters (anti-tumor serum) and electrophoresis on gels containing sodium dodecyl sulfate (SDS). The T antigen has an apparent molecular weight of 94,000 (94K) on SDS gels; the smaller protein has an apparent molecular weight of 17K. Immunoprecipitates of wild-type (WT) SV40-infected CV-1 cells also contain two proteins with apparent molecular weights of 56K and 32K. These proteins are absent in infections with deletion mutants that lack the 17K protein. In this paper, we show evidence that the proteins are cellular in origin and that they are only indirectly recognized by anti-tumor serum. Immunoprecipitation of the 56K and 32K proteins requires the presence of intact viral 17K protein.

MATERIALS AND METHODS

Cells and virus. The experiments described in this paper were performed with the TC7 clone of CV-1 cells (12). Cells were grown in Eagle basal medium containing 5% fetal bovine serum and were infected at confluence. Infections were carried out with a multiplicity of infection of 3 to 10 PFU/cell.

SV40 WT strain VA45-54 (22) and its derived mutant, *tsA58* (20), were obtained from Peter Tegtmeyer. The WT strain SVS (18) and the deletion mutants dl-884, -885, -888, -890, and -1410 (14) were kindly provided by Thomas Shenk. Virus stocks were grown in CV-1 cells at 33 or 37°C from an input multiplicity of 0.01 to 0.1 PFU/cell.

Labeling and extraction of infected cells. Infected cells (10^7 to 2×10^7) were incubated at 39°C for 36 h, washed twice with Hanks salt solution, and then labeled for 1 h with 0.1 mCi of [^{35}S]methionine in 1 ml of methionine-free medium. To label with [^{32}P]phosphate, cells were placed in phosphate-free medium (serum-free) immediately after infection. At 36 h postinfection, cells were washed twice with phosphate-free medium and then pulsed for 1 h with 0.8 mCi of [^{32}P]phosphate in 1 ml of phosphate-free medium.

Radiolabeled cells were washed twice with Tris-buffered saline, pH 8, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then extracted with 0.5% Nonidet P-40 and 0.25 mg of phenylmethylsulfonylfluoride per ml in the same buffer (13). Extraction was at 0°C for 5 to 10 min. The extracts were centrifuged at 45,000 $\times$ *g* for 20 min to remove nuclei and aggregated protein.

Immunoprecipitation. Soluble protein fractions

were incubated on ice with anti-tumor serum (0.01 ml/ml of extract) for 45 min. Immune complexes were isolated after the addition of a 10% suspension of *S. aureus* Cowan strain (0.06 ml/ml of extract) that had been Formalin fixed and heat inactivated (6). After a 30-min incubation on ice, the bacteria and immune complexes were removed by centrifugation at 2,000 rpm and washed three times with Tris-buffered saline, pH 7.4. Buffer containing 2% SDS and 5% 2-mercaptoethanol was added to the bacterial pellet, and the immunoreactive proteins were recovered in soluble form by heating the bacteria at 60°C for 5 min. After removal of the bacteria by centrifugation, the proteins were boiled for 10 min to completely disrupt immune complexes. The proteins were analyzed by electrophoresis on 20% polyacrylamide–SDS gels as described (13, 24).

DNA cellulose chromatography. DNA cellulose was prepared with double-stranded calf thymus DNA by standard procedures (1). Extracts to be analyzed on DNA cellulose were prepared as described above, and then the pH was adjusted to pH 6 by dropwise addition of 0.2 M phosphate buffer, pH 6 (approximately 0.35 ml/ml of extract). Nonbound proteins were washed off the DNA cellulose at pH 6, and a bound protein fraction was recovered with pH 9 buffer as previously described (13). The pH 6 fraction was mixed with an equal volume of the pH 9 column buffer, and each fraction was centrifuged at 45,000 × *g* to remove aggregated material and then immunoprecipitated.

Tryptic peptide analyses. Methionine-labeled proteins were isolated from SDS gels and freed of SDS as described elsewhere (13). After SDS removal, proteins were dialyzed against water, lyophilized, and then oxidized at 0°C in 0.05 ml of performic acid (7). Oxidized proteins were lyophilized and then trypsinized in 0.2 ml of 0.08 M NH_4COOH, pH 8.6, with 0.03 mg of trypsin–tolylsulfonyl phenylalanyl chloromethyl ketone for 8 h and then an additional 0.02 mg for 17 h at 35°C. The peptides were lyophilized three times and then suspended in 0.2 ml of electrophoresis buffer. Insoluble residues were removed by centrifugation at 45,000 × *g*, and soluble peptides were concentrated by lyophilization for analysis. Solvent used for electrophoresis was pyridine–acetic acid–water (289:111:1,600); that for chromatography was pyridine–acetic acid–*n*-butanol–water (28.9:11.1:40:40).

RESULTS

Appearance of the 56K and 32K proteins. CV-1 cells were infected either with WT virus or with the viable deletion mutants dl-885 and dl-888. Infected cells were incubated at 39°C for 36 h, pulsed with [^{35}S]methionine and extracted for immunoprecipitation. The immunoprecipitated proteins observed on SDS–polyacrylamide gels are shown in Fig. 1. The 94K protein was present in cells infected with WT virus and with the deletion mutants. As previously reported, the deletion does not alter the size of the 94K T antigen. The 17K protein was present in WT infections but absent in infections with dl-885, which made a visible deletion fragment, and with dl-888. The 56K and 32K proteins were detected only in extracts of cells infected with WT virus. Although not shown here, extracts of additional deletion mutants (dl-884, dl-890, and dl-1410) did not contain the 56K, 32K, or 17K protein.

Under these radiolabeling conditions, only small amounts of 56K and 32K proteins were present. The amount of [^{35}S]methionine in the 56K protein was 5 to 10% that present in the 94K T antigen. The intermediate bands were detected throughout infection (8 to 72 h), and maximal amounts were present at 32 to 48 h, at which times maximal amounts of viral proteins were also present.

The 94K T antigen is known to be phosphorylated (23). In contrast (Fig. 1G and H), neither

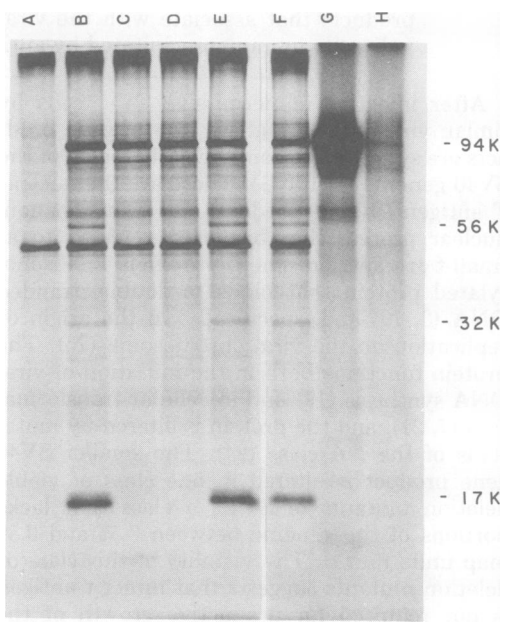

FIG. 1. *Immunoprecipitated proteins from cells infected with WT SV40, dl-885, and dl-888. Cells infected with the WT viruses SVS and VA45-54 and cells infected with the deletion mutants dl-885 and dl-888 were pulsed for 1 h with [^{35}S]methionine or [^{32}P]phosphate at 36 h postinfection at 39°C. Radiolabeled cells were extracted with Nonidet P-40-containing buffer and immunoprecipitated as described in the text. The immunoreactive proteins were analyzed on 20% polyacrylamide–SDS gels. The proteins in lanes A to F were obtained from methionine-labeled cells. Those in lanes G and H were from phosphate-labeled cells. The patterns shown are (A) WT SVS infected, normal serum; (B,E) WT SVS infected, anti-tumor serum; (D) dl-888 infected, anti-tumor serum; (F,G) WT VA45-54 infected, anti-tumor serum; (H) WT VA45-54 infected, normal serum.*

the 17K gene product nor the intermediate proteins were labeled with [^{32}P]phosphate. These results contrast with the presence of ^{32}P in the 85K and 88K protein species. The 85K and 88K proteins are degradation products that are generated during extraction of the infected cells, and the appearance of these products is highly dependent on the conditions of extraction (23). Levels of the 56K and 32K protein bands did not vary with the appearance of the known degradation products.

DNA cellulose chromatography. Infected cells labeled with [^{35}S]methionine were extracted, and a portion of the extract was adjusted to pH 6 and applied to DNA cellulose. Proteins that did not bind the double-stranded DNA were eluted with buffer at pH 6. A bound protein fraction was eluted with buffer at pH 9. The extract and each fraction were immunoprecipitated. As shown in Fig. 2, the 94K protein was present in both the pH 6 and pH 9 fractions. The 56K, 32K, and 17K proteins did not bind DNA, and these proteins were present only in the pH 6 fraction.

In addition to DNA binding studies, experiments have been performed to determine the intracellular localization of the immunoprecipitable proteins. The 56K, 32K, and 17K proteins were consistently found in non-nuclear fractions of extracted cells, even under conditions in which much of the 94K protein remained associated with the nucleus (23, 24). These conditions included extraction with Nonidet P-40 at pH 6 and Dounce homogenization in the presence of 5 mM $CaCl_2$.

Identification of the 56K and 32K proteins in deletion mutant extracts. Radiolabeled extracts prepared from dl-888-infected CV-1 cells were mixed with unlabeled extracts of WT-infected cells (Fig. 3). After incubation of the mixed extracts at 30°C for 30 min, both 56K and 32K protein species were detected in the immunoprecipitates. Incubation of the dl-888 extract alone under the same conditions did not result in the appearance of the intermediate bands. The quantity of the 56K and 32K proteins was small, and the proportion resembled that observed in immunoprecipitates of WT-infected cells. In similar experiments, these proteins have been identified in extracts of cells infected with the other deletion mutants that lack the 17K protein. In each case, the proteins were observed only when extracts of cells infected with the WT virus were supplied before immunoprecipitation. Extracts of mock-infected cells or cells infected with the deletion mutants did not replace the WT extract.

Co-precipitation of the 56K and 32K proteins. Results with mixed extract experiments suggested either that the 56K and 32K proteins were generated proteolytically from the viral 94K proteins or that the proteins interacted with viral proteins such that they were immunoprecipitated indirectly by the antiserum. Several

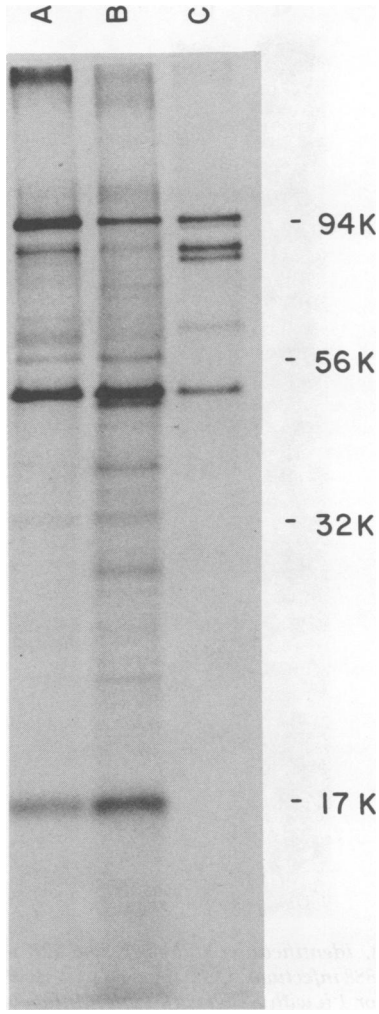

FIG. 2. *Immunoprecipitation of infected-cell proteins after DNA cellulose chromatography. Cells infected with WT strain VA45-54 were pulsed with [^{35}S]methionine for 1 h. After extraction with Nonidet P-40 buffer at pH 8, one portion of the extract was immunoprecipitated. The remainder of the extract was adjusted to pH 6 and applied to DNA cellulose as described in the text. Proteins were washed from the DNA cellulose at pH 6 and then a bound protein fraction was recovered with a pH 9 buffer. The two fractions were immunoprecipitated and analyzed on SDS gels. The patterns shown are from (A) the whole extract; (B) the pH 6 wash; (C) the pH 9 bound fraction.*

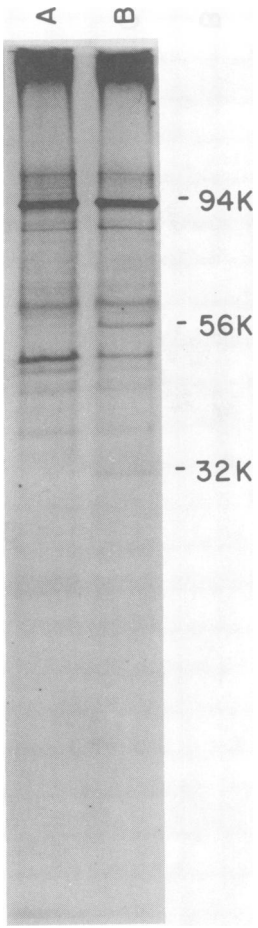

FIG. 3. *Identification of the 56K and 32K proteins from dl-888 infections. Cells infected with dl-888 were pulsed for 1 h with [^{35}S]methionine. Unlabeled cells infected with WT strain VA45-54 were extracted at the same time. Before immunoprecipitation, 0.3 ml of the radiolabeled dl-888 extract was mixed with 0.3 ml of the unlabeled WT extract or with 0.3 ml of buffer. The mixtures were incubated at 30°C for 30 min. After the incubation, the proteins were precipitated with anti-tumor serum. The patterns shown are from dl-888-infected cell extracts incubated (A) without WT extract; (B) with 0.3 ml of WT extract.*

experimental approaches have shown that the 56K and 32K proteins are not proteolytic products of the 94K. Amino acid analogs and protease inhibitors did not prevent the appearance of the 56K in immunoprecipitates. In addition, radiolabeled 94K proteins was completely removed from radiolabeled deletion mutant extracts by pretreatment with anti-tumor serum, yet the 56K and 32K proteins were still identified in the remaining extract by co-precipitation with WT extract (Fig. 4). This experiment ruled out a possible proteolytic origin of the 56K and 32K proteins from the 94K.

The experiment shown in Fig. 4 also showed

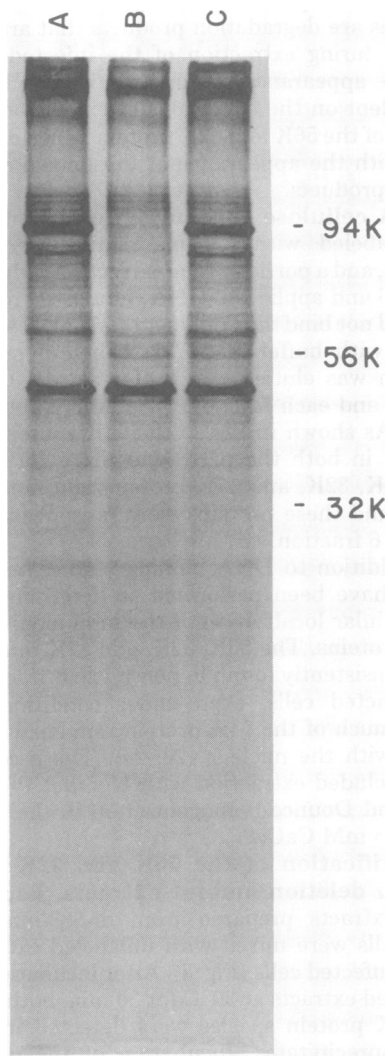

FIG. 4. *Co-precipitation of the 56K and 32K proteins in the absence of radiolabeled 94K. Cells infected with dl-888 were pulsed for 1 h with [^{35}S]-methionine. Unlabeled cells infected with WT SV40 were extracted at the same time. The dl-888 extract was divided into three portions, and then one portion was incubated with anti-tumor serum and Staphylococcus aureus to remove the 94K protein. Proteins remaining from this pre-incubation with anti-tumor serum were then incubated with unlabeled WT extract. A second portion of the dl-888 extract was immunoprecipitated directly, and a third portion was mixed with WT extract before immunoprecipitation. The patterns shown are immunoprecipitates of (A) dl-888; (B) dl-888 pretreated with anti-tumor serum, then mixed with WT extract; (C) dl-888 mixed with WT extract.*

that the 56K and 32K proteins were present in the extracts of deletion mutant-infected cells but were not recognized by the anti-tumor serum. Addition of extracts of WT-infected cells did, however, allow the precipitation of the two proteins. This observation suggested that the 56K and 32K proteins complex with proteins present in infected cells and are immunoprecipitated only as a result of this association. A second observation that indicates complex formation and lack of direct precipitation of the 56K and 32K proteins is that sera that are inefficient in the precipitation of the viral 17K protein are also unable to precipitate the 56K and 32K proteins. These sera, notably those produced in hamsters bearing tumors against the H65-transformed cell line, recognize the 94K protein (data not shown).

Experiments with *N*-ethylmaleimide (NEM) have provided further evidence that the 56K and 32K proteins are not directly immunoprecipitated by anti-tumor serum, but are indirectly immunoprecipitated in association with the viral proteins (Fig. 5). After NEM treatment of WT extracts, only the 94K and 17K viral proteins were detected in immunoprecipitates, even though the 56K and 32K proteins are clearly present in untreated extracts. (A band with a slightly greater mobility than that of the 32K protein appeared in the NEM-treated samples.) This observation suggested that NEM prevented the formation of a complex that contained the two additional proteins and that the proteins were not directly recognized by the antiserum in the immunoprecipitates. The decreased mobility of the 17K protein after NEM treatment has been described (5) and appears to be the result of the high cysteine content of this protein.

Cellular origin of the 56K and 32K proteins. Strong evidence for a cellular origin of the 56K and 32K proteins was obtained by direct demonstration that these proteins are present in uninfected cells (Fig. 6). Radiolabeled extracts of uninfected CV-1 cells were mixed with unlabeled WT extracts before immunoprecipitation. As described for deletion mutant extracts, 56K and 32K proteins were co-precipitated with the unlabeled viral proteins, but were not immunoprecipitated directly from uninfected cell extracts. The requirement for intact 17K protein was again observed, and extracts of deletion mutant-infected cells were unable to replace the WT extract.

The 56K and 32K proteins observed in immunoprecipitates of WT-infected cell extracts are also likely to be cellular in origin. Tryptic peptide analyses of methionine-labeled proteins

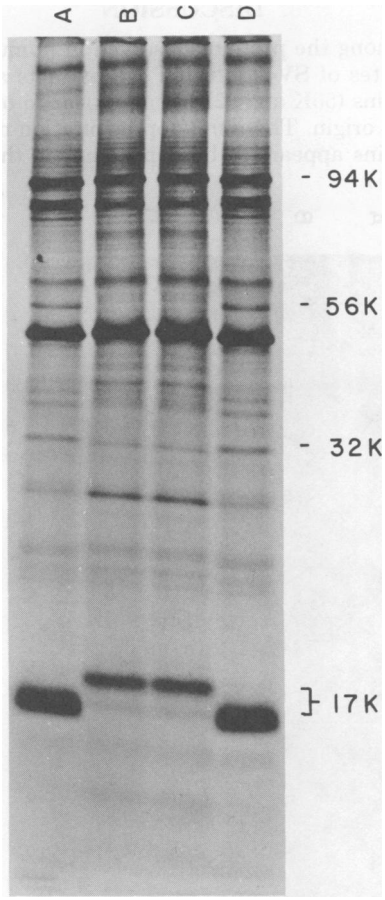

FIG. 5. *Effect of NEM on immunoprecipitate patterns. Cells infected with WT SV40 were pulsed at 36 h postinfection with [^{35}S]methionine for 1 h, and then extracted with Nonidet P-40 at pH 8. The extract was divided into two portions, and then 10 mM NEM was added to one half. After 60 min on ice, 12 mM cysteine, pH 8, was added to both the NEM-treated and control extracts to stop any further reaction of the NEM. The extracts were immunoprecipitated and analyzed on SDS gels. The patterns shown are (A,D) untreated control extract; (B,C) NEM-treated extract.*

(Fig. 7) have shown little, if any, similarity between the viral 94K protein and the 56K and 32K proteins. Analyses of mixtures of the tryptic peptides derived from the 56K and 32K proteins and those of both the 94K and 17K viral proteins have confirmed that the two associated proteins are nonviral in origin (data not shown). Tryptic peptide analyses of the two proteins from uninfected cells have not been done, but it seems highly likely that the proteins will be identical to those observed in infected-cell immunoprecipitates.

DISCUSSION

Among the proteins observed in immunoprecipitates of SV40-infected cell extracts are two proteins (56K and 32K) that appear to be cellular in origin. The immunoprecipitation of these proteins appears to be dependent on the presence of the viral early gene product, t antigen (17K product), because the proteins do not appear in immunoprecipitates of cells infected with deletion mutants that lack the 17K protein. The 56K and 32K proteins are present in uninfected cells and in cells infected with the deletion mutants, but immunoprecipitation of these proteins requires the addition of extracts of WT-infected cells. The WT extracts supply intact 17K protein and allow the co-precipitation of the cellular proteins.

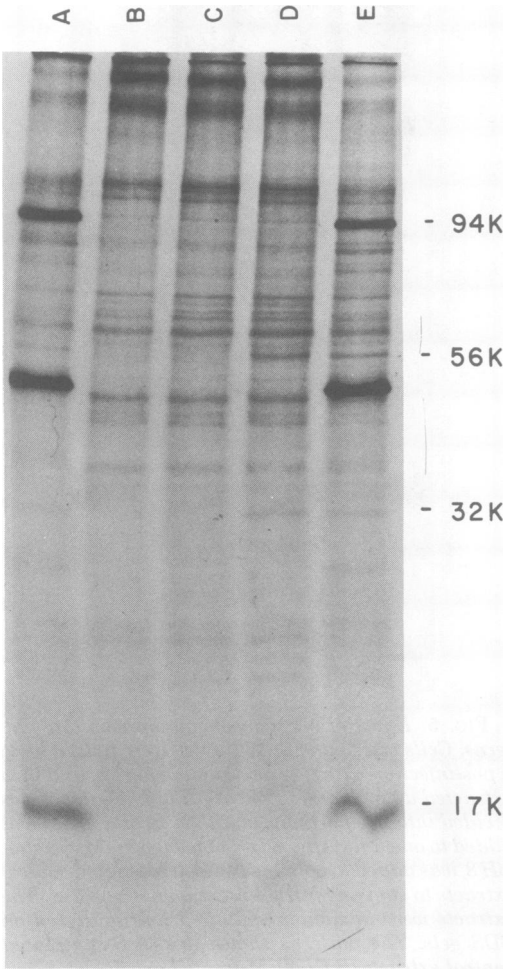

FIG. 6. *56K and 32K proteins of uninfected cells. Confluent cultures of uninfected CV-1 cells were fed with Eagle basal medium containing 5% fetal bovine serum and then pulsed 24 h later with [^{35}S]methionine. Radiolabeled extracts were then mixed with unlabeled extracts of uninfected cells, cells infected with WT virus, or cells infected with dl-888. The mixtures were incubated for 30 min at 30°C and then immunoprecipitated. The patterns shown are immunoprecipitates of radiolabeled uninfected cell extracts mixed with (B) unlabeled uninfected cell extracts; (C) unlabeled dl-888-infected cell extracts; (D) unlabeled WT SV40-infected cell extracts. As a marker, the immunoprecipitated proteins from labeled cells infected with WT virus are shown in (A) and (E).*

It is likely that a complex exists in infected cells with 17K, 32K, and 56K proteins as minimal components. The nature of the complex is not known, but the complex can be disrupted by agents, such as NEM, that irreversibly bind sulfhydryl groups. At present, there is no direct evidence that disulfide interactions are necessary for complex formation. It is possible that NEM disrupts the structure of the 17K protein so that complex formation cannot occur.

The proteins described in this report have been observed in newly infected permissive and nonpermissive cell lines (BSC-1, CV-1P, rat F111) and do not appear to be unique to the CV-1 cell line (unpublished data). In addition, several SV40-transformed cell lines contain a prominent protein, or a pair of proteins, with molecular weights of 50 to 60K. In one report, a cellular origin for a 53K transformed cell protein has been proposed (8). In our experiments with transformed rat F111 cells, a prominent protein has been observed in immunoprecipitates (60K molecular weight). In contrast to the proteins described in this paper, however, the protein is phosphorylated and is present in cells transformed by the 0.54–0.59 deletion mutants (N. Bouck, G. di Mayorca, and K. Rundell, unpublished data). Further structural and biochemical comparisons of the proteins species found in these two types of infections should help to determine whether any relationship exists.

Immunoprecipitates of cells infected with polyoma virus also contain proteins other than the known viral gene products, 100K, 63K, and 21K. In particular, proteins with molecular weights of 56K and 32K are observed in immunoprecipitates of WT-infected cells (15). The 56K and 34K proteins are absent in immunoprecipitates of cells infected with *hr-t* deletion mutants that lack the 21K and 63K gene products. Thus, a similar situation may exist in polyoma virus infections in which the 56K and 34K proteins are associated with viral gene products, but are not viral in origin. In a recent report, tryptic peptide analysis has shown that the 56K protein present in polyoma immunoprecipitates appears to be cellular (16). However, co-extraction of cells infected with WT polyoma and *hr-t* mu-

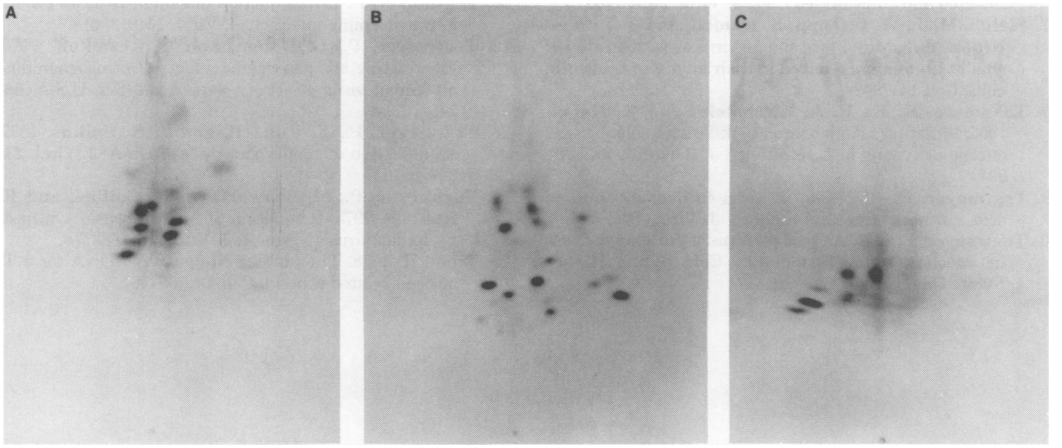

FIG. 7. *Methionine-containing tryptic peptides of 94K, 32K, and 56K proteins. Methionine-labeled proteins were excised from SDS gels, eluted from gel slices, freed of SDS, oxidized, and trypsinized as described in the text. The patterns shown are two-dimensional separations of the tryptic peptides from (A) the 56K protein; (B) the 94K viral protein; (C) the 32K protein.*

tants has not allowed the detection of proteins other than the 100K protein from radiolabeled *hr-t*-infected cell extracts (15).

Demonstration of the 56K and 32K proteins in uninfected cells by co-precipitation with viral extracts will allow direct studies of these proteins and their role in the uninfected cell. For example, experiments are underway to determine whether the expression of these proteins is related to growth state or stage of the cell cycle. In addition, the intracellular location of the proteins in uninfected cells is being determined. With additional information on the behavior of these proteins in uninfected cells, it may be possible to speculate on whether interaction with viral early proteins can explain any of the alterations of the cell that occur as a consequence of infection.

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