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Modification of Simian Virus 40 Protein A

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The A protein of simian virus 40 is phosphorylated in both productive and transforming infection. The phosphorylated amino acid has been identifed as serine and has been localized in a single tryptic peptide of the protein. Because the A protein synthesized in infection by *A* mutants is phosphorylated to the same extent and in the same peptide as in infection by wild-type virus, the functional defect of the *A* mutants is apparently unrelated to phosphorylation. At least three distinct forms of the A protein with apparent molecular weights of 85,000, 88,000, and 100,000 can be identified in extracts of cells infected by wild-type virus. After exposure of cells to Nonidet P-40, the 85,000- and 88,000-dalton proteins were found in varying amounts in extracts of permissive cells but not in extracts of transformed cells. This finding raised the question of the possible functional importance of the smaller proteins in productive infection. However, the virtual absence of the 85,000- and 88,000-dalton proteins in some extracts of the fully permissive CV-1 cell line indicates that a conversion of the larger to the smaller forms of the A protein is not required in significant quantity for productive infection. Furthermore, a study of extraction conditions shows that the smaller proteins are easily generated during extraction and provides an explanation for the appearance of these proteins in some cells after extraction under unfavorable conditions.

Simian virus 40 (SV40) gene *A* plays a central regulatory role in both productive and transforming infection. Studies with temperature-sensitive (*ts*) A mutants have shown that the *A* function is continuously required to initiate each round of viral DNA replication (5, 23) and transiently required to initiate late viral transcription (7, 12) in productive infection of permissive cells. In infection of restrictive cells, the *A* gene is necessary to establish the transformed state and to maintain the growth characteristics of some transformed cell lines (3, 11, 13, 17, 24). Recent direct evidence strongly suggests that gene *A* codes for a 100,000-dalton (100K) protein (21). Because the A protein is overproduced in either productive or transforming infection by the A mutants, the A protein must also regulate its own synthesis in both modes of infection (27).

The specific molecular activity of the A protein is unknown, but the A protein binds to both cellular and viral double-stranded DNA (4, 10) with a preferential binding site on the viral genome, which corresponds to the initiation site for DNA replication (9, 15, 19). Thus, the A protein is in many ways analagous to cellular chromatin proteins, which also bind to specific sites on DNA and are thought to regulate gene activity in animal cells (18). It has been proposed that the action of chromatin proteins is modulated by modifications that include the addition and removal of phosphate groups at specific sites in the protein molecules (22). The studies reported here show that the A protein, like many chromatin proteins, is indeed phosphorylated.

Furthermore, previous studies have shown that, in addition to the 100,000-dalton A protein, minor proteins with molecular weights of 85K and 88K can be immunoprecipitated from extracts of some infected cells with serum from hamsters bearing SV40-induced tumors (27). The smaller proteins are shown to be structurally related to the A protein by the present study. These findings raised the possibility that modification of the A protein by cleavage could have functional significance in infection by SV40. However, we now present evidence that most of the small-molecular-weight forms of the A protein, present in extracts of infected cells, represent artifacts of extraction rather than in vivo protein modification.

The apparent molecular weights of the proteins immunoprecipitated by antitumor sera vary somewhat depending on the method of sodium dodecyl sulfate-polyacrylamide gel electrophoresis used to estimate relative mobilities of proteins (21). The reported molecular weights range from 100K (21) to 88K (1, 21) in the case of the larger form of the A protein and

from 85K (27) to 82K (1) in the case of the most prominent of the smaller forms of the A protein. For convenience, we have designated specific proteins with molecular weights determined by our usual method of gel electrophoresis.

MATERIALS AND METHODS

Cell cultures. The CV-1 (20) and BSC-1 (6) lines of monkey kidney cells were grown in Eagle basal medium containing 5% fetal bovine serum. Transformed lines were derived from Swiss 3T3 cells, New Zealand white rabbit kidney cells, and Syrian hamster embryo cells, as described previously (24), and were cultivated in medium with 10% fetal bovine serum.

Virus. The wild-type (WT) parental clone and the A and BC *ts* mutants were derived from the VA 45-54 strain of SV40 (25-27). Stocks of virus were prepared as described previously. Mutant D202 was derived from the 776 strain of SV40 and was generously supplied by Robert Martin (6).

Productive infection. Confluent monolayers of CV-1 or BSC-1 cells were inoculated with input multiplicities of 10 PFU/cell. After a 2-h adsorption period at room temperature, medium was added to the inoculum, and the infected cells were incubated at 41°C.

Radioactive labeling of proteins. Productively infected or transformed cells, grown in 8-ounce (ca. 0.24-liter) prescription bottles (45-cm^2 cell growing area) were radiolabeled with [^{35}S]methionine (New England Nuclear Corp.; 40 to 60 Ci/mM) or [^{32}P]orthophosphoric acid (New England Nuclear Corp.; carrier free) in complete medium. The precise conditions for the radioactive labeling of proteins are indicated in each figure legend.

Extraction and fractionation of proteins. All extraction and fractionation procedures were carried out at 4°C. For most studies, cells in monolayer cultures were extracted with 1% Nonidet P-40 (NP-40) in 137 mM NaCl, 20 mM Tris-hydrochloride, 1 mM Na_2HPO_4 (Tris-buffered saline) with 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10% glycerol at pH 8 for 20 min. Previous studies have shown that the A protein is efficiently extracted from nuclei by this technique (27). For detergent extraction at pH 6, monolayer cultures were exposed to 1% NP-40 in 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (phosphate-buffered saline) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10% glycerol for 20 min. Extraction by Dounce homogenization was done by the method of Bhorjee and Pederson (2) with slight modifications. After being washed twice with buffered saline, cells in monolayer cultures were swollen for 10 min with RSB (10 mM NaCl, 1.5 mM $MgCl_2$) buffered at pH 6 with 10 mM 2-(*N*-morpholino)ethanesulfonic acid. The swollen cells were scraped from the glass surface and disrupted with a tight-fitting Dounce homogenizer with 10 strokes. In each case, the disrupted cells were spun at 1,000 × *g* to separate the soluble fraction from the nuclear pellet. All extraction buffers contained 1% (by volume) of a freshly prepared solution of phenylmethylsulfonylfluoride in ethanol (25 mg/ml) to inhibit serine protease activity and 1 mM dithioerythritol. All adjustments of buffer pH were made at 23°C.

Immunoprecipitation. The A protein was precipitated from soluble extracts of cells with serum from hamsters bearing SV40-induced tumors. The transformed cells used for tumor induction were virus free, and the antiserum contained no neutralizing activity against SV40. Details of the indirect immunoprecipitation techinique have been described, and previous studies have shown that the precipitation of the A protein is highly specific and more than 80% efficient (27).

Gel electrophoresis. Samples were analyzed by discontinuous polyacrylamide gel electrophoresis using a modification of the method of Maurer and Allen (14) as described previously (27).

Tryptic peptide analysis. Detailed techniques are presented in the accompanying study (21). In brief, radioactive proteins were purified by immunoprecipitation and gel electrophoresis. After trypsinization, concentrated peptides were separated by thin-layer electrophoresis and chromatography and detected by autoradiography.

Identification of amino acids. Purified protein labeled with ^{32}P was prepared by immunoprecipitation and gel electrophoresis as described above. The protein was suspended in 6 N HCl and hydrolyzed under nitrogen in a sealed glass ampoule for 2 h at 110°C. The hydrolysate was dried in vacuo, resuspended in water, and again dried. Hydrolyzed samples in 10 μl of water were applied to MN 300 cellulose plates together with unlabeled marker DL-*O*-phosphoserine and DL-*O*-phosphothreonine (Sigma). Separation of amino acids was carried out by electrophoresis in 2.5% formic acid and 7.8% acetic acid (pH 1.9) for 2.5 h at 320 V. The unlabeled markers were identified with ninhydrin stain, and the ^{32}P-labeled amino acids were identified by autoradiography.

RESULTS

Modification of the A protein in productive infection. The patterns of A protein accumulation and phosphorylation in permissive CV-1 cells infected by WT virus or *ts* mutants at 41°C are shown in Fig. 1. The cells were continuously labeled in complete medium from the onset of infection until the time of extraction, so that the incorporation of isotope would reflect the total accumulation of protein and the total phosphorylation of that accumulated protein. The cells were extracted with 1% NP-40 at pH 8. After exposure to antitumor serum, a predominant protein with an apparent molecular weight of 100,000 was specifically precipitated from extracts of infected but not control cells. The patterns of protein accumulation in infection by WT virus and by the late mutant BC11 were essentially identical. CV-1 cells infected by A58 accumulated less 100K protein than cells infected by WT virus. This finding can be

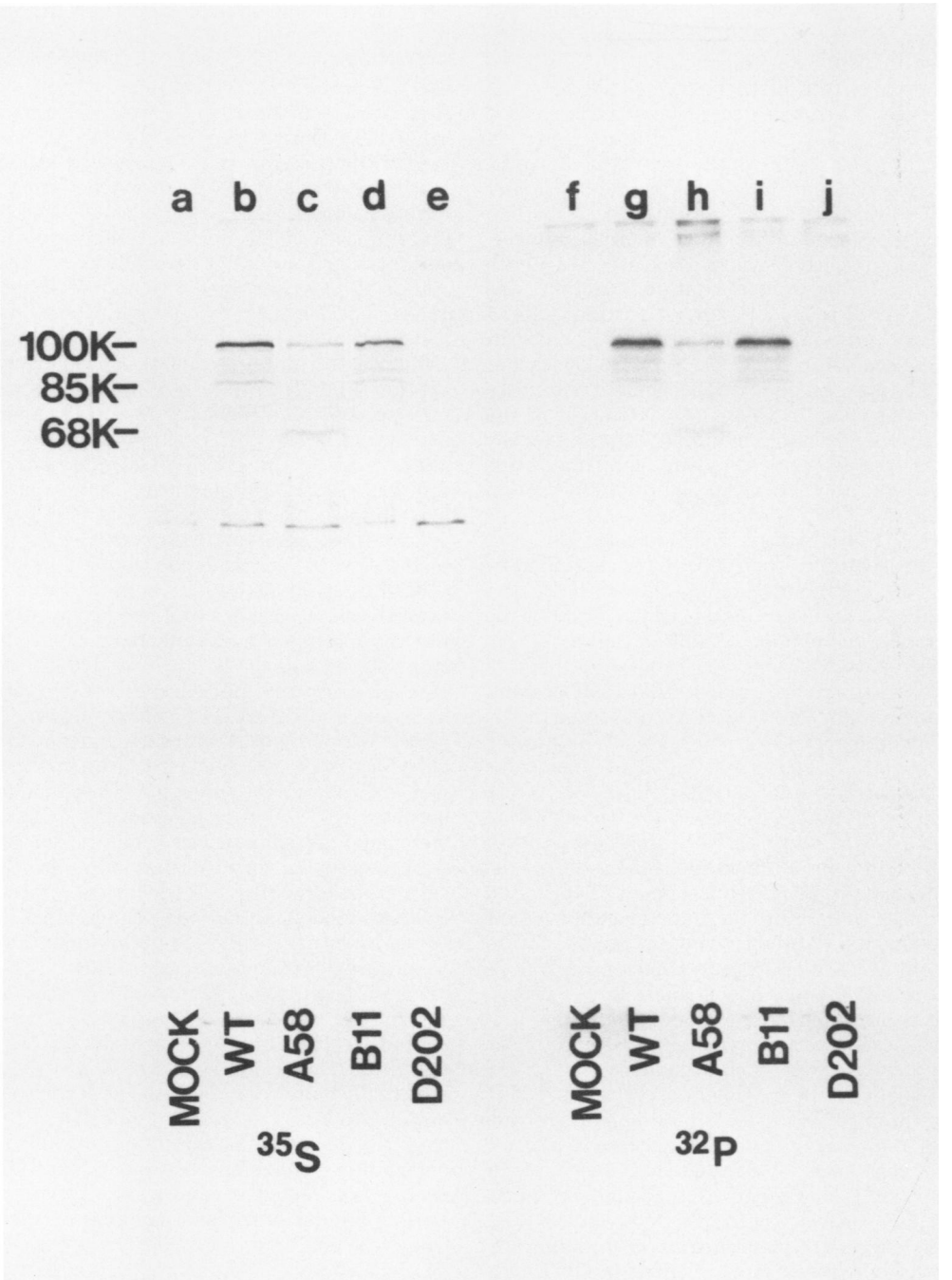

FIG. 1. *Comparison of the accumulation and phosphorylation of the A protein at 41°C. Mock-infected CV-1 cells and cells infected by WT or mutant virus were continuously exposed to either 50 μCi of [^{35}S]methionine or 250 μCi of [^{32}P]phosphoric acid per ml in complete medium from the time of infection until extraction 48 h later. Proteins were extracted with 1% NP-40 at pH 8, immunoprecipitated, subjected to sodium dodecyl sulfate-gel electrophoresis, and autoradiographed as described in Materials and Methods. The sample order is: (a) mock infection, [^{35}S]methionine; (b) WT infection, [^{35}S]methionine; (c) A58 infection, [^{35}S]methionine; (d) BC11 infection, [^{35}S]methionine; (e) D202 infection, [^{35}S]methionine; (f) mock infection, [^{32}P]phosphate; (g) WT infection, [^{32}P]phosphate; (h) A58 infection, [^{32}P]phosphate; (i) BC11 infection, [^{32}P]phosphate; (j) D202 infection, [^{32}P]phosphate.*

explained on the basis of previous studies. Although the A protein is continuously overproduced in A mutant infection at 41°C, an even more rapid rate of turnover results in a net decrease of accumulated protein in comparison to WT infection (27). Little, if any, A protein was found in cells infected with D mutants, which are thought to be blocked in uncoating at 41°C (6).

In infection by either WT or mutant virus, the amount of phosphate associated with each specifically immunoprecipitated protein was proportional to the amount of accumulation of the same protein. The similarity in the patterns of radiolabeling of each protein by either [^{35}S]methionine or [^{32}P]phosphate is immediately apparent in Fig. 1. Quantitation of the amount of radiolabel in individual gel bands by liquid scintillation counting confirmed this close similarity. For example, the 100K protein specified by WT virus contained 4.1 times as much [^{35}S]methionine and 3.8 times as much [^{32}P]phosphate as the 100K protein specified by A58. These findings exclude the existence of a significant block to the phosphorylation of the A protein in infection by A58 at the restrictive temperature.

In addition to the predominant 100K protein, distinct minor proteins were also specifically immunoprecipitated from extracts of infected CV-1 cells. These proteins had molecular weights of 85,000 to 100,000 in infection by WT virus or B mutants and 66,000 to 68,000 in infection by A mutants. The combined quantity of the minor proteins was less than 20% of the 100K protein in this experiment, but varied from less than 5% to as much as 40% in other experiments. When the minor proteins were present in extracts of cells infected by WT virus, the 85K protein was consistently prominent relative to most of the other minor proteins. In some experiments, an 88K protein was also present in significant quantity.

To identify any possible role of the host cell in determining the size of immunoreactive proteins in productive infection, proteins extracted from permissive CV-1 and BSC-1 cells were compared under identical conditions of infection and extraction with 1% NP-40 at pH 8 (Fig. 2). As expected, the predominant immunoreactive protein in extracts of infected CV-1 cells was the 100K protein. In contrast, the predominant protein specifically precipitated from extracts of infected BSC-1 cells had a molecular weight of 85K. When extracts of infected CV-1 cells were mixed with extracts of uninfected BSC-1 cells for 20 min at 30°C and then immunoprecipitated, a significant proportion of the 100K protein appeared to be converted to 85K protein.

The apparent conversion of 100K immunoreactive protein to 85K immunoreactive protein suggested that these proteins are structurally related. To confirm this relationship, the structure of the proteins was compared by the mapping of tryptic peptides from purified proteins. Figure 3 shows that the peptides, derived from proteins 85K to 88K in molecular weight, are very similar to those of the 100K protein. This similarity was confirmed by a map of a mixture of the peptides of the two preparations.

Peptide mapping also showed that the WT 100K protein contains a single phosphorylated peptide (Fig. 3D). Analysis of ^{32}P-labeled A mutant protein gave the same result. To identify the nature of the protein phosphorylation, the amino acids of the 100K WT protein were hydrolyzed under acid conditions and separated by thin-layer electrophoresis. The ^{32}P label migrated either with unlabeled marker phosphoserine or with inorganic phosphate (Fig. 4).

Modification of the A protein in transforming infection. The size and phosphorylation of the A protein were examined in a variety of transformed cells (Fig. 5). The 100K protein was present and phosphorylated in rabbit, hamster, and mouse 3T3 cells transformed by either WT virus or A mutants, but not in untransformed control cells. In contrast, the 85K and 88K phosphoproteins sometimes found in permissive CV-1 cells infected by WT virus were not detected in mouse or hamster cells and were present in very small amounts in rabbit cells transformed by WT virus. As in productive infection, long periods of radiolabeling of transformed cells with [^{35}S]methionine resulted in patterns of radiolabeling similar to the patterns of labeling by [^{32}P]phosphoric acid (data not shown).

Modification of the A protein during extraction. The studies described above show that the A protein can be found in at least three distinctive sizes in extracts of infected cells and that the host cell is an important factor in determining which size is predominant. However, it was not clear whether the size of the A protein is determined before or after extraction. Thus, the effects of the conditions of extraction on the structure of the A protein were investigated more thoroughly.

To determine if the smaller forms of the A protein can be generated during extraction of CV-1 cells under unfavorable conditions, cells infected by WT virus were extracted with NP-40 in a variety of ways. At least two factors had a dramatic effect on the size of the A protein

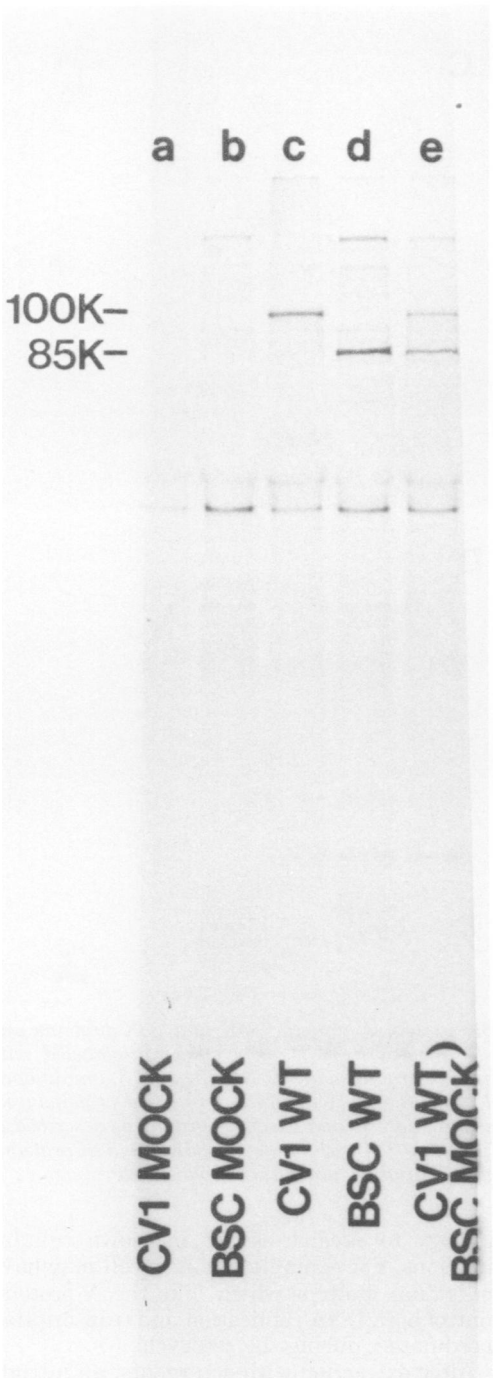

FIG. 2. *Effect of the permissive host cell on the size of the extracted A protein. CV-1 and BSC-1 permissive cells infected by WT virus were continuously exposed to 50 μCi of [^{35}S]methionine per ml. After 48 h, samples were extracted with 1% NP-40 at pH 8, immunoprecipitated, subjected to sodium dodecyl sulfate-gel electrophoresis, and autoradiographed as described in Materials and Methods. The sample order is: (a) mock infection, CV-1; (b) mock infection, BSC-1; (c) WT infection, CV-1; (d) WT infection, BSC-1; and (e) WT infection, CV-1 extract immunoprecipitated after mixture of the extract with an extract of uninfected BSC-1 cells for 20 min at 30°C.*

(Fig. 6). Identical samples were first extracted at pH 8, at pH 8 in the presence of EDTA, or at pH 6. After this extraction, each sample was re-extracted at pH 8, because extraction at pH 6 has been shown to be insufficient to remove all of the protein from nuclei (27). The total radioactivity recovered in the combined extracts of the A protein was approximately the same for each sample. However, a significant portion of the 100K protein was converted to 85K and 88K proteins at pH 8 in the presence of EDTA or at pH 6 in the absence of EDTA. Furthermore, the protein which remained in the nucleus after the first extraction was protected in part from conversion to the smaller forms.

To determine if the predominance of the 85K protein in NP-40 extracts of BSC-1 cells can be prevented by appropriate conditions, alternate methods of extraction were investigated (Fig. 7). When infected BSC-1 cells were first broken by Dounce homogenization at pH 6 rather than by the detergent method, the A protein was not extracted from the nuclei. However, when washed nuclei were subsequently extracted with 1% NP-40 at pH 8, the conversion of 100K protein to smaller forms was blocked to a significant extent. These findings implicate a cytoplasmic factor in the conversion of the 100K protein to 85K protein during the process of extraction.

DISCUSSION

The A protein is synthesized and phosphorylated in a 100,000-molecular-weight form in either productive or transforming infection. The phosphorylation of the protein is simple and specific, inasmuch as a single tryptic peptide contains phosphoserine. It would be premature, however, to exclude the existence of acid-labile phosphate groups, because free phosphate was released after acid hydrolysis of purified 100K protein. In infection by the A mutants the 100K protein accumulates in smaller quantities than in infection by WT virus. Nevertheless, the mutant A protein that does accumulate is phosphorylated to the same extent as the WT A protein. Mapping of the phosphorylated peptides of mutant and WT proteins also confirms a similar location of the phosphate group in each protein. We conclude that the temperature-sensitive defect of the A mutants is not related to protein phosphorylation. Al-

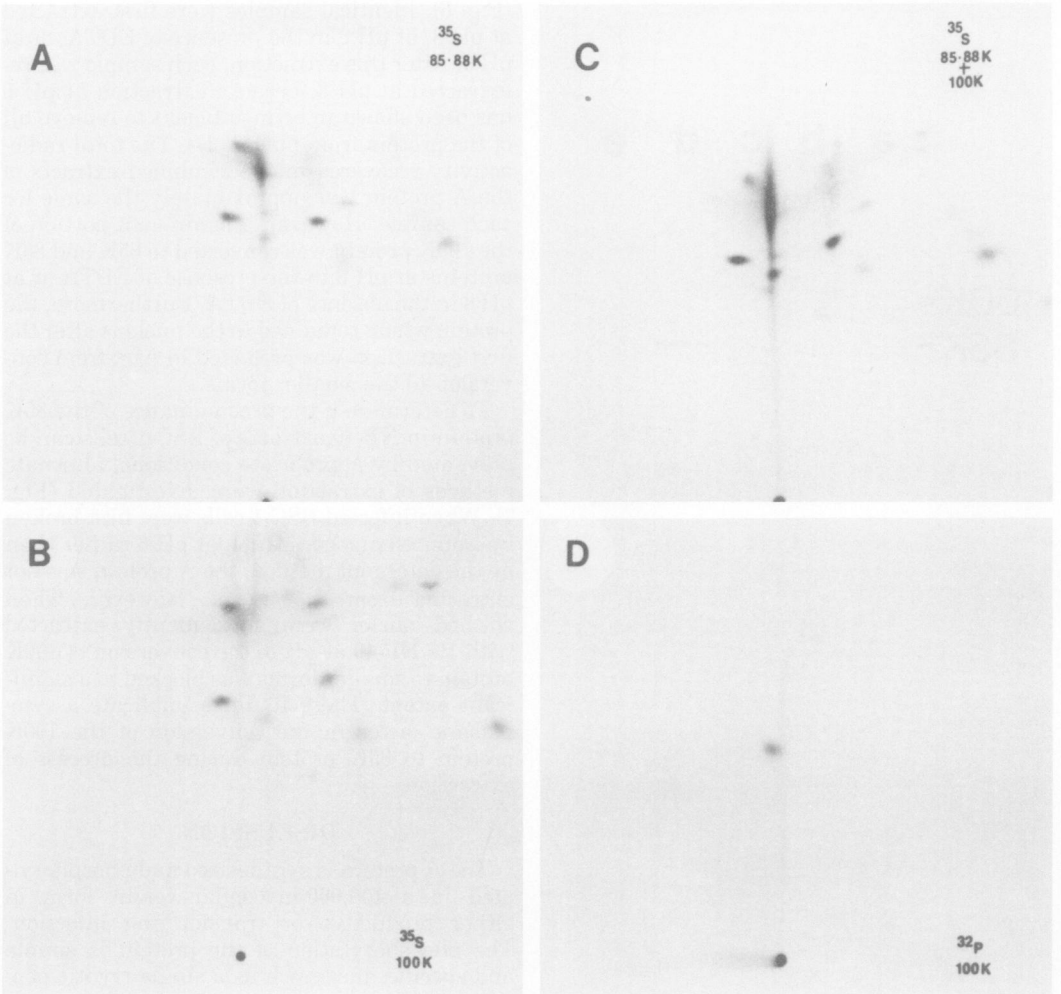

FIG. 3. *Comparison of the tryptic peptides of the 100K protein A and the 85K and 88K proteins and identification of the phosphopeptide. Proteins from cells infected by WT virus were radiolabeled with [^{35}S]methionine or [^{32}P]phosphoric acid as described in Fig. 1, extracted with 1% NP-40 at pH 8, immunoprecipitated, and separated by sodium dodecyl sulfate-gel electrophoresis. After elution, purified proteins were digested with trypsin, and the peptides were mapped by electrophoresis and chromatography as described in Materials and Methods. The sample order is: (a) 100K A protein, [^{35}S]methionine; (b) 85K and 88K proteins, [^{35}S]methionine; (c) a 1/1 mixture of samples a and b; and (d) 100K A protein, [^{32}P]phosphate.*

though this conclusion implies that the protein has multiple functional sites, the role of phosphorylation in protein function is unknown. The phosphorylation is not a consequence of DNA replication or late viral transcription because it cannot be blocked by cytosine arabinoside treatment of infected cells (data not shown).

It is interesting that the analogy between the A protein of SV40 and chromatin proteins of the host cell can be extended to the phosphorylation of the proteins. In view of this continuing similarity, it may be constructive to extend this analogy to predict as yet unknown cellular functions. For example, the host cell may have individual proteins which, like the A protein, control both DNA replication and transcription coordinately during the cell cycle.

After extraction with detergents, an intriguing pattern of protein accumulation in productive and transforming infection was apparent. A distinct 85,000-molecular-weight form of the A protein was present in varying quantities in extracts of productively infected CV-1 or BSC-1 cells but not in transformed cells. Taken alone, these findings provided circumstantial evi-

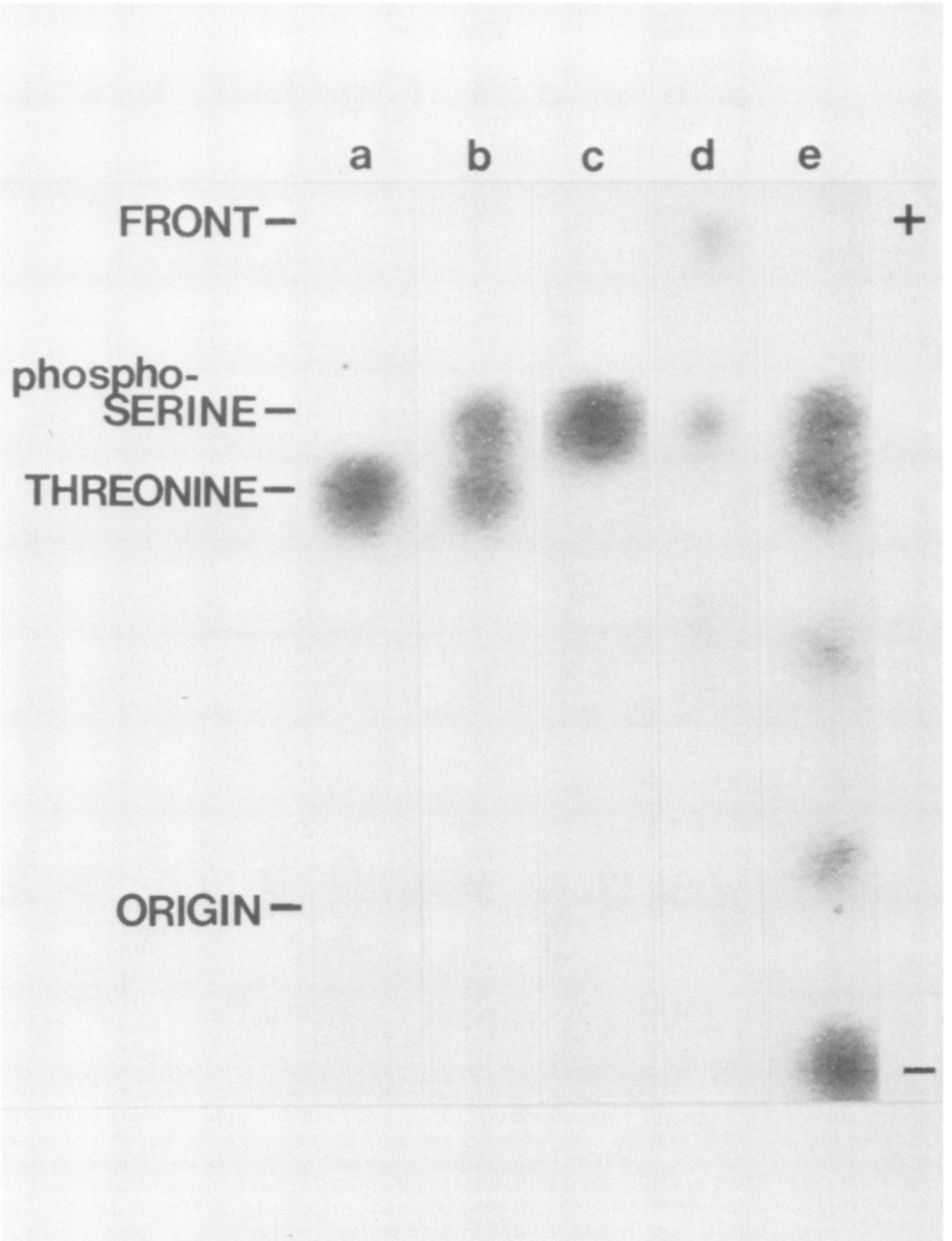

FIG. 4. *Identification of phosphoserine in the A protein. The 100,000-dalton form of the A protein was labeled with [^{32}P]phosphoric acid and purified as described in Fig. 3. The protein was hydrolyzed in 6 N HCl and subjected to electrophoresis with unlabeled marker phosphoamino acids as described in Materials and Methods. The sample order is (a) phosphothreonine, ninhydrin stain; (b) phosphothreonine and phosphoserine, ninhydrin stain; (c) phosphoserine, ninhydrin stain; (d) acid hydrolysate of ^{32}P-labeled 100K protein A with marker unlabeled phosphothreonine and phosphoserine, autoradiography; and (e) same as sample (d), ninhydrin stain.*

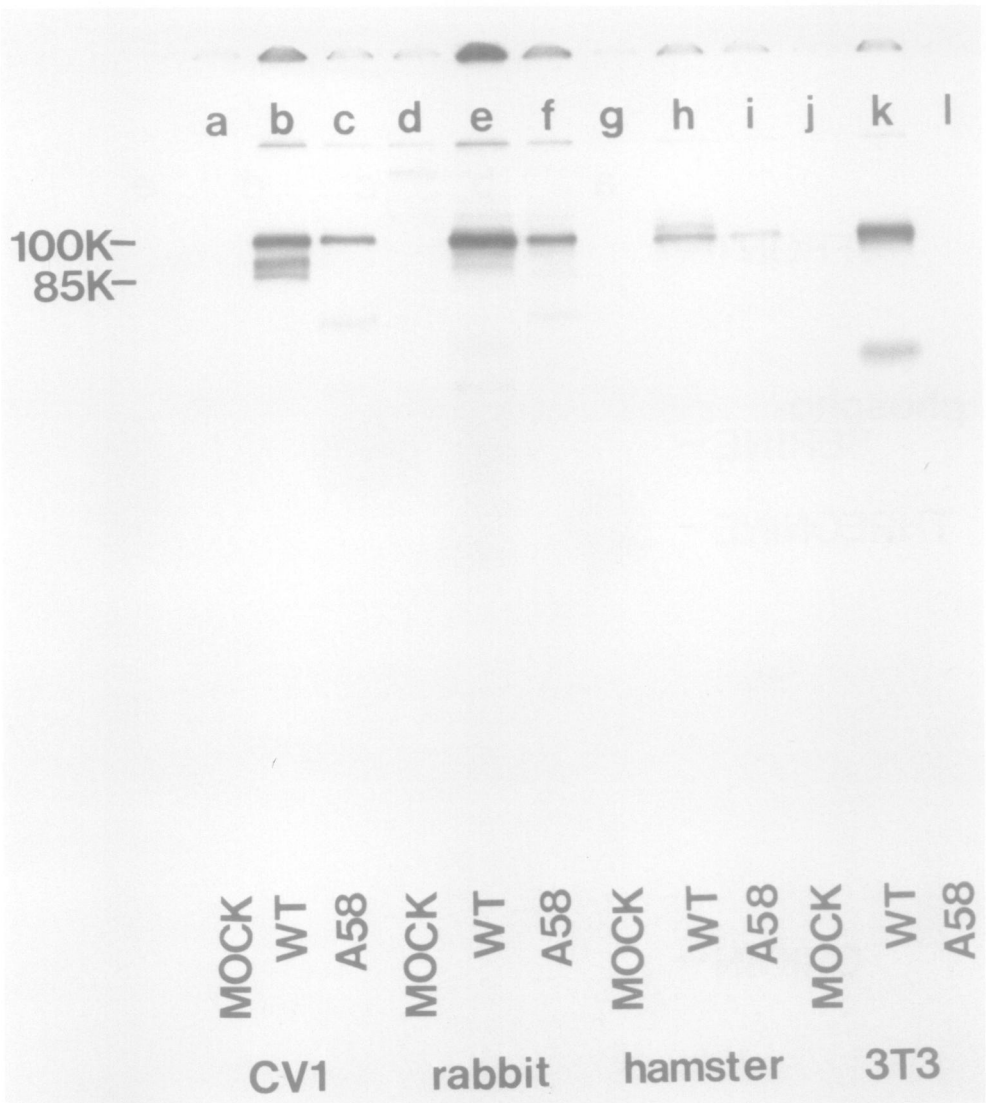

FIG. 5. *Comparison of the molecular weight and phosphorylation of the A protein in productively infected and transformed cells. CV-1 cells infected by WT virus or a variety of cells transformed by WT virus were continuously exposed to 250 μCi of [^{32}P]phosphoric acid per ml for 24 h at 41°C until extraction with 1% NP-40 at pH 8. After 48 h, the extracted proteins were immunoprecipitated, subjected to sodium dodecyl sulfate-gel electrophoresis, and autoradiographed as described in Materials and Methods. The sample order is: (a) mock infection, CV-1 cells; (b) WT infection, CV-1 cells; (c) A58 infection, CV-1 cells; (d) nontransformed, rabbit cells; (e) WT transformation, rabbit cells; (f) A58 transformation, rabbit cells; (g) nontransformed, hamster cells; (h) WT transformation, hamster cells; (i) A58 transformation, hamster cells; (j) spontaneously transformed, 3T3 cells; (k) WT transformation, 3T3 cells; and (l) A58 transformation, 3T3 cells.*

dence for the requirement of the 85K protein in viral replication. However, a thorough study of extraction conditions showed that, for the most part, the 100K protein was converted into an 85K protein after extraction rather than in vivo. Conditions that favor this conversion include the presence of nonionic detergent, acidic pH, chelating agents, and cytoplasmic components. One plausible explanation for these findings is that active, nonserine proteases are released from lysosomes under these conditions. Indeed, lytic infection may even promote the release of lysosomal contents (16). The virtual absence of the 85K and 88K proteins in extracts

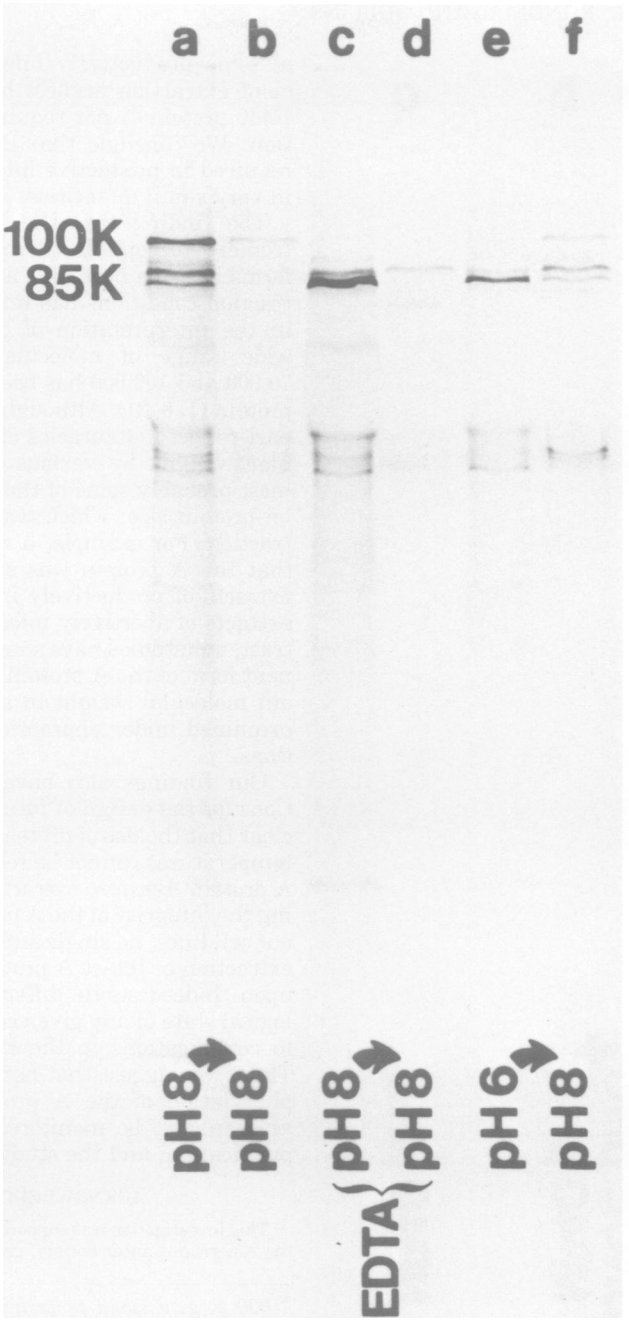

FIG. 6. *Structural alteration of the A protein after extraction. CV-1 cells infected by WT virus were continuously exposed to 50 μCi of [^{35}S]methionine per ml of complete medium from the time of infection until extraction. After 48 h, one infected culture was extracted by the usual technique with 1% NP-40 in Tris-buffered saline (TBS) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 8 (extraction buffer A). A second sample was extracted with 1% NP-40 in TBS with 10 mM EDTA at pH 8 (extraction buffer B). A third sample was extracted with 1% NP-40 in phosphate-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 6 (extraction buffer C). After removal of the first extraction buffer, the nuclei were further extracted with extraction buffer A. Each extraction was carried out for 20 min at 4°C in the presence of 10% glycerol, protease inhibitor, and 0.001 M dithioerythritol. After dialysis against TBS at pH 8 at 4°C, the extracts were immunoprecipitated, subjected to sodium dodecyl sulfate-gel electrophoresis, and autoradiographed as described in Materials and Methods. The sample order is: (a) extraction with buffer A; (b) extraction with buffer A after previous extraction with buffer A; (c) extraction with buffer B; (d) extraction with buffer A after previous extraction with buffer B; (e) extraction with buffer C; and (f) extraction with buffer A after previous extraction with buffer C.*

FIG. 7. *Protection of the A protein during extraction by the removal of the cytoplasm with Dounce homogenization. BSC-1 cells infected with WT virus were continuously exposed to 50 μCi of [^{35}S]methionine per ml of complete medium. After 48 h, one culture was swollen with 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithioerythritol at pH 6. After Dounce homogenization, the nuclei were pelleted at 1,000 × g, washed twice with the swelling buffer, and extracted with 1% NP-40 in Tris-buffered saline (TBS) at pH 8. The other sample was extracted with 1% NP-40 in TBS at pH 8 without previous Dounce homogenization to separate nuclei from the cytoplasm. The samples were immunoprecipitated, subjected to sodium dodecyl sulfate-gel electrophoresis, and autoradiographed as described in Materials and Methods. The sample order is: (a) supernatant fraction after Dounce homogenization; (b) NP-40 extraction of the nuclear pellet remaining after Dounce homogenization; and (c) NP-40 extraction without previous Dounce homogenization.*

of some productively infected cells after efficient extraction argues that processing of the 100K protein is not required for viral replication. We conclude that if the 85K protein is required in productive infection, it is required in very small quantities.

The finding that the 100K protein is extremely susceptible to conversion to smaller forms in some host cells and under certain extraction conditions has important implications in the interpretation of past experiments. A wide range of molecular weights between 70,000 and 100,000 has been reported for the A protein (1, 8, 21). Although, these differences in part reflect inaccuracies of determining molecular weights by various gel techniques (21), most probably some of the estimates are based on protein size, which was altered during extraction. For example, a recent report showed that the A protein was smaller in whole-cell extracts of productively infected cells than in extracts of abortively infected cells (1). In contrast, our studies have shown that the predominent form of the A protein has the same apparent molecular weight in all infected cell lines examined under appropriate extraction conditions.

Our findings also have important implications for the design of future experiments. It is clear that the use of protease inhibitors and low temperatures cannot be relied on to protect the A protein. Because extraction conditions favoring the integrity of the A protein vary for different cell lines, no single strategy for the efficient extraction of intact A protein can be depended upon. Indeed subtle differences in the physiological state of any given cell line may give rise to complications in the handling of extracts. Thus, we suggest that both the size and phosphorylation of the A protein, as well as its antigenicity, be monitored during extraction, purification, and the study of function.

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