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Biosynthesis, Immunological Specificity, and Intracellular Distribution of the Simian Virus 40-Specific Protein Induced by the Nondefective Hybrid Ad2$^+$ND$_1$

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Ad2$^+$ND$_1$, a nondefective hybrid virus containing a segment of the early region of simian virus 40 (SV40) DNA covalently inserted into the human adenovirus 2 genome, enhances the growth of human adenoviruses in simian cells and induces the SV40 U antigen. This hybrid previously has been shown to code for a 28,000 (28K) molecular weight protein not present in wild-type adenovirus 2-infected cells. By radioimmunoprecipitation using sera from hamsters bearing SV40-specific tumors, we have established that the Ad2$^+$ND$_1$-induced 28K protein is SV40-specific. This Ad2$^+$ND$_1$-induced protein is synthesized as a 30K molecular weight precursor, which is detectable only when infected cells are pulse-labeled in the presence of the protease inhibitor tosylamino phenylethyl chloromethyl ketone. Upon fractionation of labeled cell extracts, about 80% of the 28K protein is found in the plasma membrane fraction, whereas the remaining 20% is associated with the outer nuclear membrane. This protein is not detectable either in the nucleus or in the cytoplasm. Blockage of proteolytic cleavage by tosylamino phenylethyl chloromethyl ketone did not alter the topographic distribution of this SV40-specific protein, although the amount of the precursor protein in the outer nuclear membrane increased fourfold while that in the plasma membrane was proportionately decreased. This result suggests that the 28K protein is transferred from the outer nuclear membrane to the plasma membrane after posttranslational cleavage of the 30K precursor polypeptide. These data offer further support to the proposal that the 28K protein contains the determinants for SV40 U antigen and is responsible for SV40 enhancement of adenovirus growth in simian cells.

The isolation of a family of nondefective human adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid viruses, containing varying portions of SV40 DNA covalently inserted into the Ad2 genome (22), has allowed piecemeal study of the expression and function of isolated regions of the SV40 genome. One such hybrid virus, Ad2$^+$ND$_1$, has been shown to contain a segment of the early region of SV40 DNA, mapping from 0.11 to 0.28 unit with respect to the unique endonuclease R·*Eco*RI cleavage site (22).

As a result of its SV40 insertion, Ad2$^+$ND$_1$ has the ability to induce one early SV40-specific immunological activity, termed U antigen; this antigen is also expressed in wild-type SV40-infected cells (18). Furthermore, the SV40 genetic information contained within Ad2$^+$ND$_1$ specifies a function that enhances the growth of human adenoviruses in simian cells (16). It is possible that U antigen is associated with the adenovirus helper function of wild-type SV40, and genetic studies suggest that an SV40-specific protein with an apparent molecular weight of 28,000 (28K), found only in Ad2$^+$ND$_1$ and not in Ad2-infected cells (20), is responsible for this helper function (6).

In an attempt to define the molecular mechanism of action of the 28K protein, we have studied its synthesis, immunological specificity, and intracellular distribution in human KB cells infected by Ad2$^+$ND$_1$.

MATERIALS AND METHODS

Cells and viruses. Stocks of Ad2 and Ad2$^+$ND$_1$ were grown in monolayer cultures of human KB cells in Eagle minimum essential medium (Grand Island Biological Co.) supplemented with 5% fetal calf serum (Grand Island Biological Co.). Viruses were extracted from cell sonic extracts with 1% (vol/vol) *n*-butanol and purified by twice banding on CsCl density gradients (26). Purified viruses were dialyzed against a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 1 mM $MgCl_2$, and 10% (vol/vol) glycerol and stored at

−70°C. Plaque titrations of stock viruses were carried out on monolayers of primary human embryonic kidney cells (17), and all preparations contained 2×10^{11} to 5×10^{11} PFU/ml.

Infection and labeling of cells. Monolayers of human KB cells, routinely tested for contaminating mycoplasma, were infected with purified Ad2 or $Ad2^+ND_1$, at a multiplicity of 20 PFU/cell, in Eagle minimum essential medium supplemented with 2% calf serum. At 24 h postinfection, the monolayers were rinsed with and left in prewarmed Earle balanced salt solution containing 5% Eagle minimum essential medium and 2% fetal calf serum. [^{35}S]methionine (300 to 400 Ci/mmol) was added to a final concentration of 40 μCi/ml. After 20 min, the cells were rinsed with ice-cold phosphate-buffered saline and scraped from the bottles. When L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK; Aldrich) was used, the appropriate amount was added at the same time as [^{35}S]methionine.

Antitumor sera. Sera obtained from hamsters bearing either primary tumors induced by intramuscular injection of purified SV40 of secondary tumors induced by subcutaneous transplant of SV40 tumor cells were screened for their ability to immunoprecipitate the 28K protein from radiolabeled extracts of $Ad2^+ND_1$-infected KB cells. Most of the sera tested were positive, although their titers differed significantly.

Immunoprecipitation. About 1.5×10^7 radiolabeled KB cells were lysed with 0.5 ml of buffer A, made up of Tris-buffered saline (pH 7.4) containing 0.5% Nonidet P-40 and 2 mM phenylmethyl sulfonylfluoride. After 30 min at 4°C, intact nuclei were removed by centrifugation at $1,000 \times g$ for 10 min, and the resulting supernatant was clarified by centrifugation at $30,000 \times g$ for 30 min. Portions (50 μl) of this radiolabeled cell extract were incubated with 2 μl of serum from either normal hamsters or from hamsters bearing SV40-induced tumors. After 1 h at 4°C, 20 μl of a 10% (wt/vol) suspension of inactivated *Staphylococcus aureus* (Cowan I) was added to adsorb out the antigen-antibody complexes (11). The suspensions were agitated occasionally over a period of 15 min at 4°C, and the bacterial adsorbent containing the immune complexes was removed and washed three times with buffer A by centrifugation for 5 min at $2,000 \times g$. Bound radioactivity was recovered by heating the bacterial adsorbent to 100°C for 1 min in a buffer containing 62 mM Tris-hydrochloride (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) sodium dodecyl sulfate (SDS), 2 mM phenylmethyl sulfonylfluoride, and 10% (vol/vol) glycerol.

Subcellular fractionation. Washed cells, suspended in a buffer containing 10 mM Tris-hydrochloride (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, and 2 mM dithiothreitol, were homogenized with a glass Dounce tissue grinder until more than 95% of the cells were seen to be broken under the phase-contrast microscope (eight strokes). The cell lysate was spun at 800 $\times g$ for 15 min, and both the supernatant and the pellet were saved for further fractionation.

(i) Cytoplasmic fraction. The supernatant from the low-speed centrifugation of the cell homogenate was further centrifuged for 30 min at $30,000 \times g$. The resultant supernatant is designated the cytoplasmic fraction.

(ii) Plasma membranes. The plasma membranes and nuclei in the pellet from the low-speed centrifugation were separated by the dextran-polyethylene glycol two-phase system described by Brunette and Till (4), except that 10 mM NaCl was present instead of $ZnCl_2$. Upon separation of the two phases by centrifugation at $11,700 \times g$ for 10 min, the membranes were found at the interphase, and the nuclei were found in a pellet at the bottom of the tube. The two separated phases together with the membranes at the interphase were poured off, mixed thoroughly, and subjected to phase separation by centrifugation a second time. Purified membranes, collected from the interphase, were found completely free of nuclei when examined by phase-contrast microscopy.

(iii) Nuclei and nuclear wash. The nuclear pellet from the first two-phase separation was again suspended with a Dounce tissue grinder in equal volumes of the two phases. Any contaminating plasma membrane was separated from the nuclei upon phase separation by centrifugation. The nuclear pellet was suspended in a buffer composed of 10 mM Tris-hydrochloride (pH 6.8), 150 mM KCl, 5 mM $MgCl_2$, and 1% (vol/vol) Triton X-100, again by homogenization in a Dounce tissue grinder. After 10 min at 4°C, the washed nuclei were separated from the nuclear wash by centrifugation. Suspended nuclei remained fully intact and free of membraneous material as visualized by phase-contrast microscopy.

Polyacrylamide gel electrophoresis. All samples for analysis contained 62 mM Tris-hydrochloride (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) SDS, 2 mM phenylmethyl sulfonylfluoride, and 10% (vol/vol) glycerol and were heated at 100°C for 2 min. The SDS-polyacrylamide gel system used was that of Laemmli and Maizel, described by Laemmli (14), and electrophoresis was performed by the procedure of O'Farrell et al. (25). Radiolabeled protein bands on the gels were detected by fluorography (15).

RESULTS

Identification of the SV40-specific polypeptide induced by $Ad2^+ND_1$. Productive infection of human cells by Ad2 is characterized by the inhibition of host translation and the synthesis of predominently virus-specific polypeptides (2, 28). When parallel cultures of human KB cells, separately infected for 24 h with either Ad2 or $Ad2^+ND_1$, were pulse-labeled with [^{35}S]methionine and the lysates from these labeled cells were analyzed on a 7.5 to 15% SDS-polyacrylamide gel, much of the radiolabel was found incorporated into Ad2-specific polypeptides (Fig. 1a and b). As reported previously (20), the only discernible difference between the proteins induced by Ad2 and $Ad2^+ND_1$ is the synthesis by $Ad2^+ND_1$ of a polypeptide with an apparent molecular weight of 28K.

To demonstrate that the 28K protein induced by $Ad2^+ND_1$ is in fact SV40 specific, we have

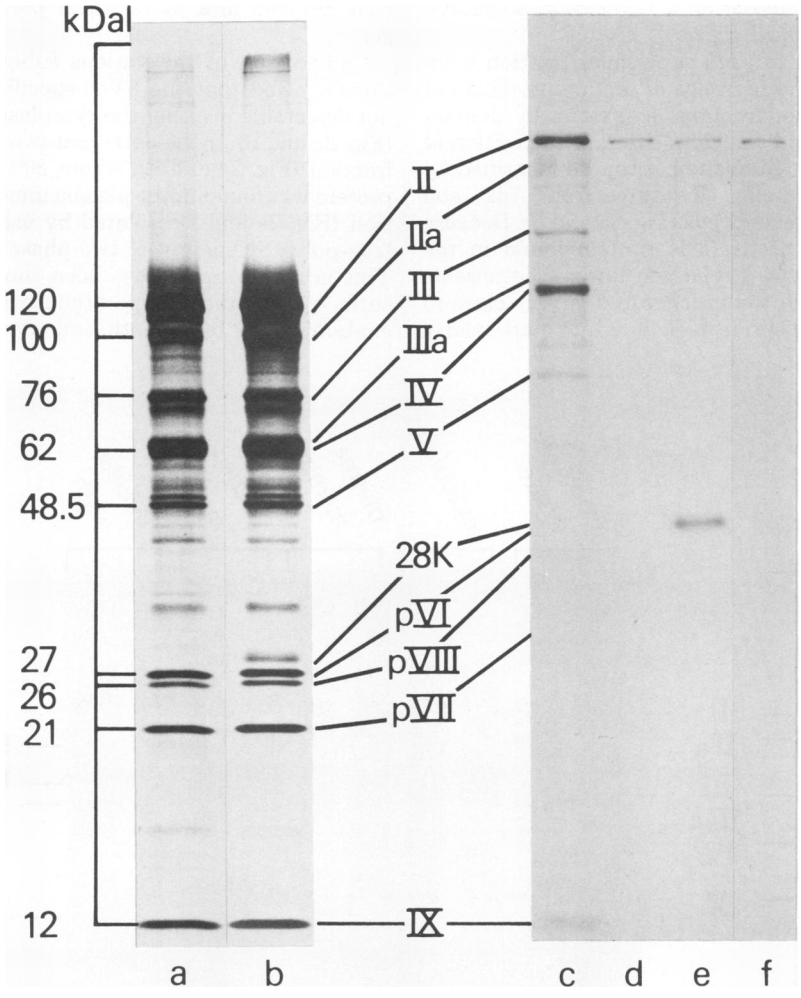

FIG. 1. *Identification of the SV40-specific protein induced by Ad2+ND1. (A) Fluorogram of a 7.5 to 15% SDS-polyacrylamide gel displaying the distribution of proteins from [35S]methionine-labeled extracts of Ad2 (slot a)- and Ad2+ND1 (slot b)-infected KB cells. (B) Fluorogram of a 12.5% SDS-polyacrylamide gel showing immunoprecipitates from a [35S]methionine-labeled Ad2+ND1-infected cell extract, using either serum from a normal hamster (slot d) or serum from a hamster bearing an SV40-induced tumor (slot e). An Ad2-infected cell extract was run in parallel (slot c) as a molecular weight marker. A parallel control is included showing immunoprecipitates from an Ad2-infected cell extract, using the same SV40-specific antitumor serum (slot f).*

studied the immunoreactivity of this protein by using antisera obtained from hamsters bearing SV40-induced tumors. When the antitumor serum was added to the [35S]methionine-labeled extract prepared from Ad2+ND1-infected cells and the resulting immunoprecipitate was analyzed on a 12.5% SDS-polyacrylamide gel, one major radioactive protein band was observed (Fig. 1e). By comparison with molecular weight markers included in the same slab gel (Fig. 1c), this protein has an apparent molecular weight of 28K. When a control serum obtained from a normal hamster was used, this protein was not immunoprecipitated (Fig. 1d). The 28K protein could be detected only in Ad2+ND1-infected cell extracts and not in Ad2-infected cell extracts using the same antitumor serum (Fig. 1f). These data suggest that the 28K protein induced by Ad2+ND1 is SV40-specific.

Subcellular distribution of the Ad2+ND1-induced polypeptide. To study the intracellular distribution of the 28K protein, KB cells infected with either Ad2 or Ad2+ND1 for 24 h were labeled with [35S]methionine for 20 min and subjected to subcellular fractionation. Parallel fractions from Ad2- and Ad2+ND1-infected

cells were analyzed on a 12.5% SDS-polyacrylamide slab gel (Fig. 2).

The purity of each subcellular fraction is indicated by the presence of unique proteins not found in other fractions as previously demonstrated (9) and shown in Fig. 2. The position of the 28K protein in the gel can be identified by comparing whole cell lysates from Ad2- and $Ad2^+ND_1$-infected cells (Fig. 2a and b). Because the amount of the 28K protein found in the whole cell lysate is relatively low and because its molecular size when denatured is very close to that of polypeptide pVI, it is not particularly well resolved in a 12.5% linear polyacrylamide gel.

Comparison of the various subcellular fractions showed that the SV40-specific protein is not detectable in either the cytoplasmic fraction (Fig. 2c and d) or the detergent-washed nuclear fraction (Fig. 2g and h). About 80% of the 28K protein was found in the plasma membrane fraction (Fig. 2e and f), isolated by using the dextran-polyethylene glycol two-phase system (1). This latter procedure has been shown to yield surface membrane preparations with minimal contamination by smooth endoplasmic reticu-

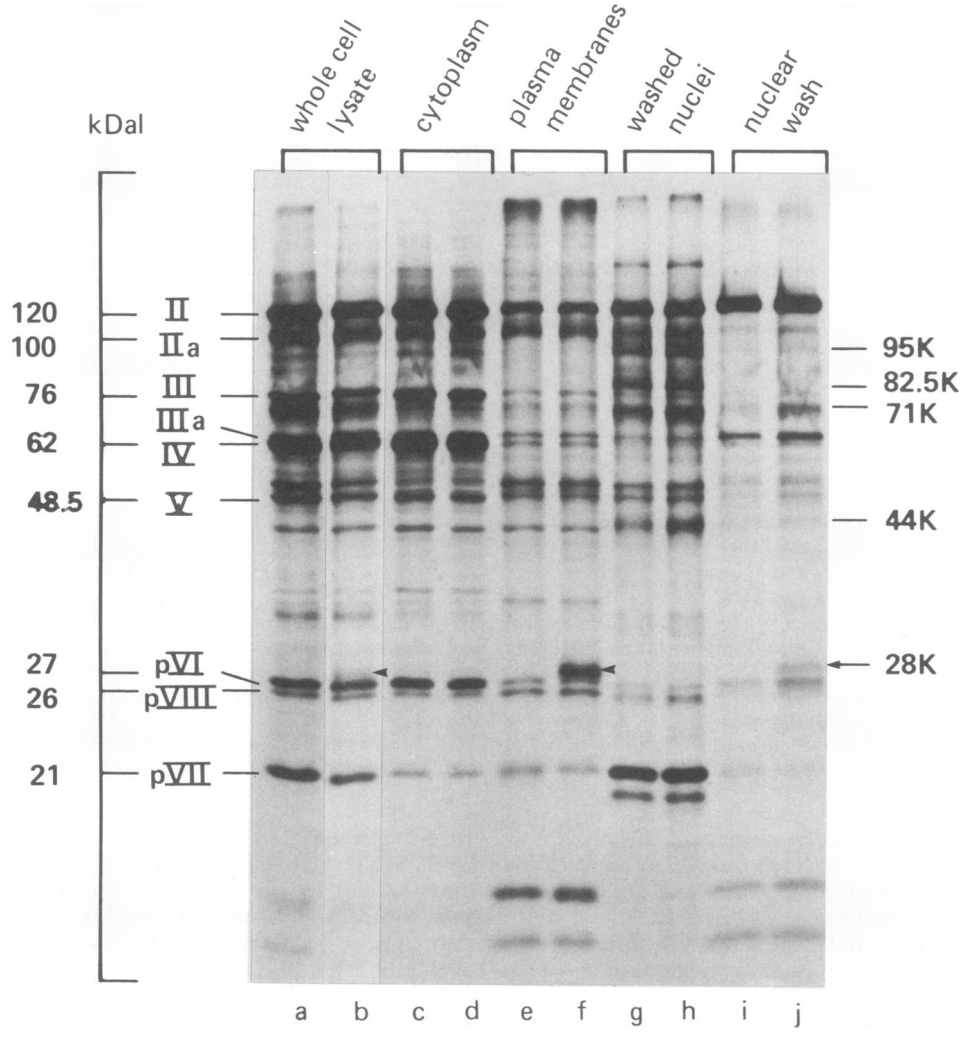

FIG. 2. *Subcellular distribution of the $Ad2^+ND_1$-specific protein. Fluorogram of a 12.5% SDS-polyacrylamide gel displaying the [^{35}S]methionine-labeled proteins found in the various subcellular fractions from KB cells infected with either Ad2 (slots a, c, e, g, and i) or $Ad2^+ND_1$ (slots b, d, f, h, and j). About 15,000 cpm were applied on the gel, and this represented an enrichment of onefold (slots c and d), fivefold (slots e and f), fourfold (slots g and h), and fivefold (slots i and j). The exposure time was 2 days.*

lum, mitochondria, or nuclei (4). The remaining 20% of the 28K protein was found in the nuclear wash (Fig. 2i and j). This fraction is obtained by treating isolated nuclei with 1% Triton X-100 at pH 6.8 to remove the nuclear membrane-associated ribosomes and any remaining cytoplasm that could not be stripped by mechanical means.

No significant qualitative difference in the distribution of the 28K protein could be detected when the 20-min pulse with [^{35}S]methionine was chased with unlabeled methionine for 60 min or when the radioactive pulse was extended to 60 min (data not shown). The 28K protein has not been detected in either the cytoplasmic or the nuclear fraction under any of the labeling conditions used.

Effect of TPCK on the synthesis of the 28K protein. TPCK is a protease inhibitor that specifically blocks serine proteases. When added to KB cells that had been infected with Ad2 for 24 h, the extent of incorporation of [^{35}S]methionine during a 20-min pulse was diminished. At 25 μg/ml, TPCK inhibited the overall incorporation by 15%, and at 50 μg/ml, by as much as 50%. However, analysis of the labeled cell extracts on a 12.5% SDS gel showed no detectable qualitative change in the distribution of Ad2 proteins with increasing concentrations of TPCK (Fig. 3a to c). Although at 25 μg/ml (Fig. 3b) the relative intensities of the protein bands remained the same as those of the control (Fig. 3a), at 50 μg/ml (Fig. 3c) there was a perceptible decrease in the synthesis of some of the minor proteins.

With Ad2+ND1-infected cells, there was again no apparent qualitative effect on each of the Ad2-specific proteins (Fig. 3d to f). However, TPCK had a definite effect on the synthesis of the SV40-specific 28K protein. At 25 μg/ml (Fig. 3e), the relative amount of the 28K protein present in the cell lysate was decreased to about half that found in control cells (Fig. 3d). In addition, a new protein band with an apparent molecular weight of about 30K was detected. When the TPCK concentration was further increased to 50 μg/ml (Fig. 3f), the 28K protein band was barely detectable, whereas the 30K protein band increased to a level comparable to that of the 28K protein in the control cell lysate (Fig. 3d).

Because the 30K protein demonstrated in Ad2+ND1-infected cells with increasing concentrations of TPCK was not seen in Ad2-infected cells under similar conditions, the 30K protein must be Ad2+ND1-specific. The gradual decrease in the synthesis of the 28K protein and the concomitant increase in the accumulation of a 30K protein with increasing concentrations of TPCK suggest that the SV40-specific protein is synthesized as a 30K polypeptide whose rapid conversion to a 28K protein can be blocked by the protease inhibitor TPCK. The 30K polypeptide, therefore, appears to be the precursor of the 28K protein found in Ad2+ND1-infected cells.

Subcellular localization of the precursor polypeptide. Ad2+ND1-infected cells were labeled with [^{35}S]methionine for 20 min in either the absence or presence of 50 μg of TPCK per ml. At the end of the radioactive pulse, the cells were separately harvested and subjected to subcellular fractionation. Parallel fractions from untreated and TPCK-treated cells were analyzed on a 12.5% SDS gel (Fig. 4).

As shown in Fig. 4, the relative amount of the 30K protein accumulated in the presence of TPCK (Fig. 4b) is quite similar to that of the 28K protein present in the control cells (Fig. 4a), when whole cell lysates were compared. The data suggest that there was no selective inhibition by TPCK of the accumulation of the SV40-specific protein during the time of [^{35}S]methionine incorporation and that TPCK quantitatively blocks only the conversion of the 30K protein precursor to its stable 28K product.

A comparison of the various subcellular fractions reveals that the 30K precursor protein is found only with the plasma membranes (Fig. 4f) and the nuclear wash (Fig. 4j). As with the 28K protein, the 30K precursor cannot be detected either in the cytoplasmic fraction (Fig. 4c and d) or in the nuclear fraction (Fig. 4g and h), even when the fluorogram is overexposed (data not shown).

Although both the 28K protein, found in control cells, and the 30K protein, found in TPCK-treated cells, are present exclusively in the plasma membrane fraction and in the nuclear wash, their relative distributions between these two subcellular fractions differ significantly (Fig. 4e, f, i, and j). As indicated earlier (Fig. 2f and j), it can be calculated that there is three to four times as much 28K protein associated with the plasma membranes as with the nuclear wash. However, as shown in Fig. 4, the reverse is true for the 30K protein in TPCK-treated cells. There is about four times as much 30K protein in the nuclear wash as in the plasma membrane fraction.

These data suggest that blockage of the conversion of the 30K precursor to the 28K product greatly diminishes the amount of SV40-specific protein found in the plasma membrane and concomitantly increases the amount of this protein in the nuclear membrane.

DISCUSSION

The maximum coding capacity of the early

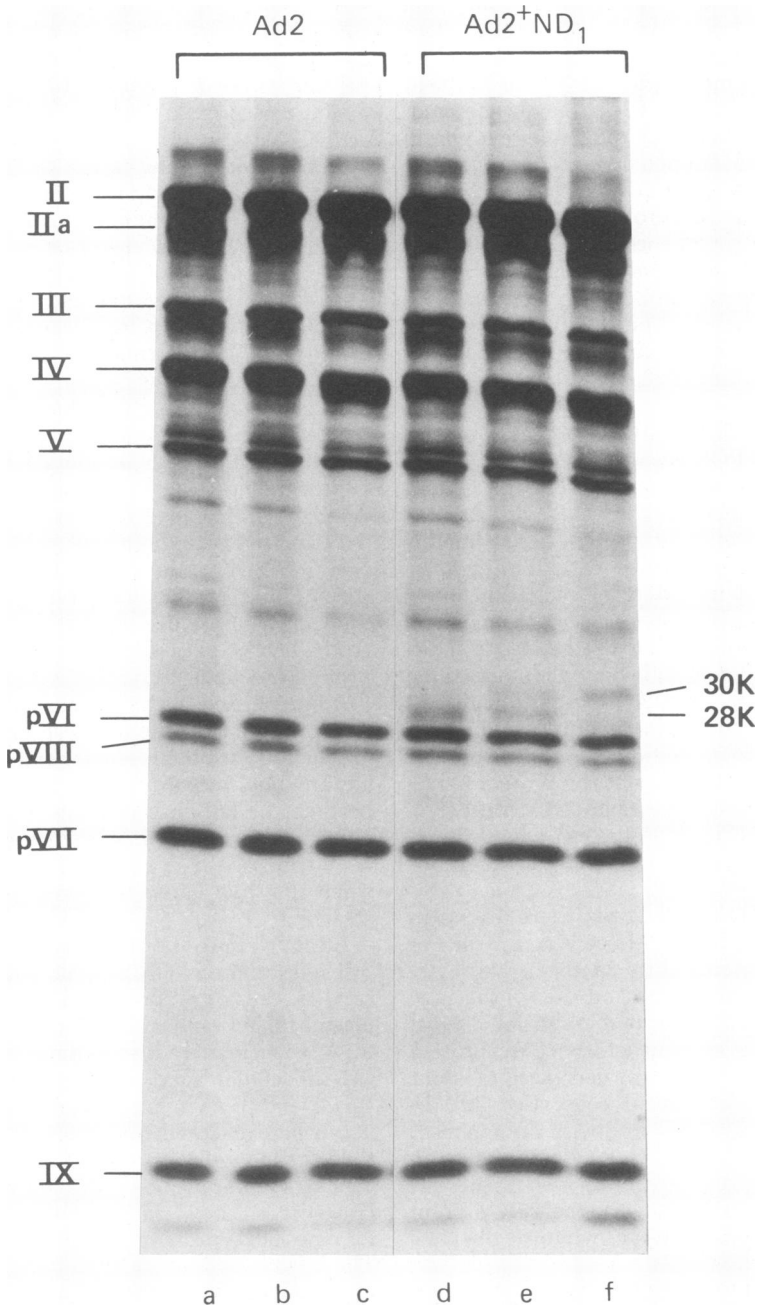

FIG. 3. *Effect of TPCK on the synthesis of the Ad2$^+$ND$_1$-specific 28K protein. Fluorogram of a 12.5% SDS-polyacrylamide gel displaying the distribution of proteins from extracts of Ad2 (slots a to c)- and Ad$^+$ND$_1$ (slots d to f)-infected cells, labeled with [^{35}S]methionine in the presence or absence of TPCK. The TPCK concentrations used were: none (slots a and d), 25 μg/ml (slots b and e), and 50 μg/ml (slots c and f). About 15,000 cpm was applied on the gel, and the exposure time was 2 days.*

SV40 DNA segment present in Ad2$^+$ND$_1$, assuming a molecular weight of 3.5×10^6 for the entire SV40 genome, is equivalent to about 22,200 daltons of protein. When comparing extracts from Ad2-infected and Ad2$^+$ND$_1$-infected cells, a 28K protein can be detected in the latter but not in the former (6, 20). Although this 28K protein can be translated in vitro from Ad2$^+$ND$_1$

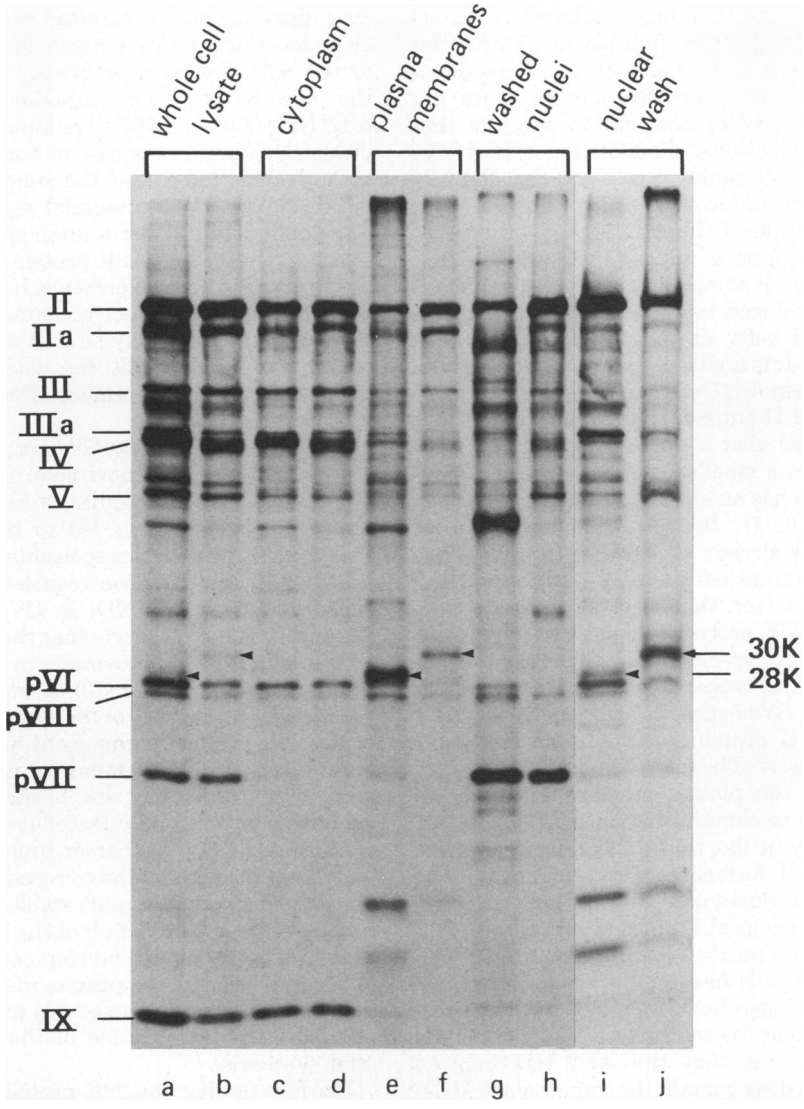

FIG. 4. *Subcellular distribution of the Ad2+ND1-specific 30K precursor protein. Fluorogram of a 12.5% SDS-polyacrylamide gel displaying the proteins found in the various subcellular fractions from KB cells infected with Ad2+ND1 and labeled with [35S]methionine in the absence (slots a, c, e, g, and i) or presence (slots b, d, f, h, and j) of TPCK at a final concentration of 50 μg/ml. About 15,000 cpm was applied on the gel. The exposure time was 2 days.*

mRNA selected by hybridization either to SV40 DNA or Ad2 DNA (3), it has not been established previously that this protein is in fact SV40-coded.

Our finding that the 28K protein can be specifically immunoprecipitated by using antisera from hamsters bearing SV40-induced tumors suggests that this protein must contain SV40-specific antigenic determinants. These data are in agreement with a recent tryptic peptide analysis, demonstrating that the 28K protein shares amino acid sequences in common with the SV40 T antigen (21). Our data do not, however, rule out the possibility of a hybrid protein containing SV40 as well as Ad2-specific amino acid sequences.

Although the stable gene product of the SV40 insertion in Ad2+ND1 is a 28K protein, we have been able to detect, through the use of the protease inhibitor TPCK, a larger precursor polypeptide with an apparent molecular weight of about 30K. It is unlikely that the proteolytic cleavage blocked by TPCK is an artifact generated during cell fractionation. Although addition

of TPCK at the time of pulse-labeling yielded only the 30K protein, addition of TPCK after the radioactive pulse but before cell fractionation yielded only the 28K species (data not shown). This latter observation suggests that the posttranslational modification of the 30K polypeptide is a rapid process and that the 28K protein is the stable gene product in vivo.

The isolation of the $Ad2^+ND_1$ hybrid led to the identification of the SV40 U antigen (18), which has been shown by immunological methods to be induced in SV40-infected and SV40-transformed cells, as well as in $Ad2^+ND_1$-infected cells. It is not clear, however, whether the 28K protein in $Ad2^+ND_1$-infected cells is responsible for the U antigen activity, although it has been claimed that a host-range mutant (H39) that induces a smaller $Ad2^+ND_1$-specific polypeptide also has an altered pattern of U antigen induction (6, 7). Indirect immunofluorescent studies have already shown that U antigen has a distinct perinuclear distribution in $Ad2^+ND_1$-infected cells (18). Our observation that a fraction of the 28K protein is found associated with the outer nuclear membrane, whereas the nuclear and cytoplasmic fractions are completely free of this SV40-specific component, suggests that the 28K protein is responsible for the U antigen activity. The failure to detect U antigen activity on the plasma membrane, which we have shown to contain the majority of the 28K protein, may be due to the shedding of antigens from the cell surface as a consequence of the fixation procedures used (18). Indeed, more recent immunological studies using antisera directed against purified membranes from SV40-transformed cells have demonstrated the presence of U antigen not only at the nuclear envelope but also at the cell surface (19). These data strongly suggest that the $AD2^+ND_1$-induced 28K protein does contain the immunological determinants for the SV40 U antigen.

It should be noted, however, that our data on the subcellular distribution of the 28K protein are not in agreement with that of an earlier report (5) in which it was claimed that the $Ad2^+ND_1$-induced protein is present in stable form in all subcellular fractions, with a particular enrichment in the detergent-washed nuclear fraction. The reason for this discrepancy is not clear. The distribution reported by Deppert et al. (5) suggests that the 28K protein and the U antigen are unrelated, but the subcellular distribution of the 28K protein reported here agrees entirely with the immunofluorescent distribution of U antigen (18). Moreover, in cells infected by $Ad2^+ND_2$, a nondefective hybrid containing the 0.11 to 0.44 SV40 DNA segment, U antigen displays a diffuse cytoplasmic immunofluorescent distribution as contrasted with the perinuclear location of this antigen in $Ad2^+ND_1$-infected cells. We have previously reported that the two SV40-specific proteins induced by $Ad2^+ND_2$ (42K and 56K) are found in the cytoplasm (9). Thus, in the case of both hybrids, we have demonstrated that the subcellular distribution of the SV40 protein(s) agrees with the immunofluorescent distribution of U antigen. It seems likely that the 28K protein is a subset of the 42K and 56K SV40 proteins. If the U antigen determinants are in fact contained within the 28K polypeptide, it may be that as this protein enlarges to 42K or 56K the determinants are mobilized from the perinuclear to the diffuse cytoplasmic location.

It has been shown that SV40 can enhance the growth of human adenoviruses in simian cells (10, 12, 27). Genetic studies of host-range mutants of $Ad2^+ND_1$ have led to the suggestion that the 28K protein is responsible for providing the SV40 helper function required for the efficient growth of $Ad2^+ND_1$ in CV_1 cells (6, 7). Because it is not clear whether the block in the growth of human adenoviruses in monkey cells is at the transcriptional (13) or translational (9, 23, 24) level, knowledge of the exact site of action of the 28K protein is important in defining the mechanism of enhancement. In an attempt to localize the "functional site" of the 28K protein, we have compared its intracellular distribution to that of the 30K precursor protein. We have found that blockage of the conversion of the 30K precursor polypeptide to its stable 28K product results in the accumulation of the SV40-specific protein on the nuclear envelope at the expense of accumulation in the plasma membrane fraction. This observation suggests a temporal order of subcellular mobilization of the 28K protein after synthesis.

Because neither the 28K protein nor its 30K precursor is found in the nuclear fraction, our data do not support the hypothesis that the 28K protein carries out its enhancement function at the transcriptional level (13). However, because of its perinuclear localization, the 28K protein may influence the posttranscriptional maturation or transport of certain RNA species. Alternatively, because the 28K protein is also found in the plasma membrane fraction, which includes the rough endoplasmic reticulum, it is possible that this SV40-specific protein acts as a translational control element while complexed to membrane-bound ribosomes (9, 23, 24). Studies to distinguish between these various possibilities are in progress.

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