JOURNAL OF VIROLOGY, Aug. 1982, p. 608–616
0022-538X/82/080608-09$02.00/0

Vol. 43, No. 2

Identification of a Nonvirion Protein of Aleutian Disease Virus: Mink with Aleutian Disease Have Antibody to Both Virion and Nonvirion Proteins

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Received 22 February 1982/Accepted 15 April 1982

We studied Aleutian disease virus polypeptides in Crandall feline kidney (CRFK) cells. When CRFK cells labeled with [^{35}S]methionine at 60 h postinfection were studied by immunoprecipitation with sera from infected mink, the major Aleutian disease virus virion polypeptides (p85 and p75) were consistently identified, as was a 71,000-dalton nonvirion protein (p71). The peptide maps of p85 and p75 were similar, but the map of p71 was different. p85, p75, and p71 were all precipitated by sera from Aleutian disease virus-infected mink, including those with signs of progressive disease, but heterologous sera raised against purified Aleutian disease virus did not precipitate the nonvirion p71. These results indicated that the nonvirion p71 was unrelated to p85 and p75 and further suggested that mink infected with Aleutian disease virus develop antibody to nonvirion, as well as structural, viral proteins.

Aleutian disease virus (ADV) is a nondefective parvovirus (34) that causes a persistent infection in mink (1, 5, 9, 24, 27, 28, 37). This infection produces a progressive disease associated with a chronic viremia, extremely high levels of antiviral antibodies, and hypergammaglobulinemia (4, 11, 25, 27, 29, 30). Circulating immune complexes (7, 23) are formed and play a prominent role in the pathogenesis of the immune complex renal disease (24, 30, 31). Studies show that some serum complexes contain infectious ADV (26), and viral antigen has been found in glomerular immune deposits by immunofluorescence (24, 27, 29, 30). However, complexes between 9S and 25S (25, 26) can also be demonstrated and are too small to contain entire virus particles (100 to 120S) (34, 40). Furthermore, since no viral particles have been observed in electron microscopic studies of glomerular lesions (24, 27), it is unclear what virion or nonvirion antigens participate in the immune complexes in AD.

A major goal of our work was to define ADV proteins and to determine which of these proteins are immunogenic for mink and occur in immune complexes. We previously found that the purified ADV virion has two major polypeptides (5) (apparent molecular weights, 85,000 [p85] and 75,000 [p75]. In the present report, we have described an additional, distinct nonvirion 71,000-dalton protein (p71) that is demonstrable in immune precipitates of infected cell lysates. Although the two structural proteins had similar peptide maps, the map of the nonvirion p71 was different. Sera from infected mink, including those with progressive AD, had antibodies to all three proteins (p85, p75, p71); however, heterologous antisera prepared against purified ADV lacked detectable antibody to the nonvirion p71. These results suggested that (i) ADV induced a nonvirion protein (p71), (ii) that p71 was unrelated to the major structural proteins, and (iii) that this nonvirion protein may have a role in the genesis of AD.

MATERIALS AND METHODS

Cells and virus. Cultivation of Crandall feline kidney (CRFK) cells, as well as the growth and in vitro assay of the ADV-G isolate (5) of the Utah I strain of ADV (9, 29), have been reported. The permanent mink cell line CCL-64 (38), derived from embryonic lung tissue of sapphire (Aleutian) mink, was obtained from Miles Cloyd and maintained at 37°C. A simian virus 40-transformed line of baby pastel (non-Aleutian) liver cells (SVML) (Bloom, unpublished data) was also maintained at 37°C. Techniques for animal titration of Utah I ADV and Pullman ADV, as well as assay of anti-ADV antibodies by counterimmunoelectrophoresis, have been previously detailed (4, 9, 11).

Metabolic labeling of ADV-G with [^{35}S]methionine. CRFK cells, infected with ADV-G in 150-cm^2 flasks and maintained at 31.8°C were labeled 48 h after infection as follows. Media were decanted from culture flasks, and cultures were preincubated with a prewarmed balanced salt solution (PBS) for 10 min. The PBS was replaced with 5 ml of methionine-deficient minimal essential medium (MEM) containing 50 μCi of [^{35}S]methionine (New England Nuclear

Corp., Boston, Mass.) per ml and incubated for 2 h, at which time 40 ml of complete media was added. Incubation was then continued until virus harvest. ^{35}S-labeled virus was purified and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (5).

Extrinsic labeling of ADV-G with ^{125}I. ADV-G was purified as described previously (5). The CsCl band corresponding to infectious ADV virions was dialyzed for 4 h in PBS and iodinated with 1 mCi of Na[^{125}I] (New England Nuclear), using the Iodo-Gen procedure (21). A 50-μg amount of gelatin was added, and free ^{125}I was removed by extensive dialysis against PBS. The sample was then precipitated with 10 volumes of 90% methanol at −20°C, recovered by centrifugation, boiled in Laemmli sample buffer (4), and stored at −70°C until SDS-PAGE was performed.

Preparation of [^{35}S]methionine-labeled cell lysates and immunoprecipitation. Cultures of infected CRFK cells were labeled at indicated times postinfection for 2 h as described above, at which time cells were scraped from flasks and centrifuged, and the cell pellets were suspended in chilled lysing buffer (16) (0.01 M Tris [pH 7.4], 0.15 M NaCl, 0.001 M EDTA, 1% nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.1% NaN_3). The lysates were adjusted to a concentration of 10^6 cell equivalents per ml, freeze-thawed once, and sonicated on ice. To reduce nonspecific precipitation, samples were treated with normal mink serum (10 μl of serum per ml of lysate) and a 10% suspension of Formalin-inactivated *Staphylococcus aureus* Cowan strain (16) (100 μl of *S. aureus* per ml of lysate) for 30 min on ice and then ultracentrifuged for 45 min in a Beckman type 40 rotor at 35,000 rpm in a Beckman ultracentrifuge. The precleared lysate was stored at −70°C in 1-ml samples until use. Immediately before use, samples were thawed, treated for 30 min as described above, and centrifuged for 5 min in a Beckman Microfuge B. For immunoprecipitation, 0.5-ml samples of lysate were incubated for 3 h at 4°C with 5 μl of antiserum and then reacted for 30 min with 50 μl of *S. aureus*. The precipitates were collected by centrifuging in a microfuge for 1 min, washed three times with chilled lysing buffer, suspended in 75 μl of Laemmli sample buffer, and eluted from the *S. aureus* by boiling for 5 min. The *S. aureus* was pelleted by centrifugation for 5 min, and the eluted proteins were stored at −70°C until analyzed.

For some experiments, cultures of ADV-G were grown in 24-well tissue culture plates seeded with CRFK or with CCL-64 as detailed previously (4). Radiolabeling was done by preincubating with PBS, and incubating with 0.2 ml of methionine-free MEM containing [^{35}S]methionine for periods described in individual experiments. These cultures were then processed for immunoprecipitation as described above.

SDS-PAGE. Techniques for SDS-PAGE and gel fluorography were the same as those used previously (4), except that in most cases resolving gels contained 10% acrylamide.

Two-dimensional peptide mapping. Samples (^{125}I labeled) to be analyzed by peptide mapping were subjected to SDS-PAGE. The gels were soaked briefly in distilled water, dried, marked with dots of ^{14}C-ink (Amersham Corp.), and autoradiographed. The protein bands were located, excised, and swelled in 3 to 5 ml of 0.05 M NH_4HCO_3 (pH 8.1)–1% β-mercaptoethanol–0.1% SDS (22). After removal of paper backing, the slices were crushed in a Dounce homogenizer and incubated overnight at 37°C. The eluate was centrifuged and filtered to remove traces of paper and acrylamide. After addition of 75 μg of ovalbumin as carrier, the proteins were twice precipitated with trichloroacetic acid, and the pellets were washed once with ethanol-ether (70:30) and once with ether and then dried. The samples were dissolved in 170 μl of 0.05 M NH_4HCO_3 (pH 8.1) and incubated at 37°C with α-chymotrypsin (10 μg for 1 h, then two additions of 7 μg for 2 h each; P-L Biochemicals, Milwaukee, Wis.). Digestion was stopped by adding 3 ml of cold water, quick freezing, and lyophilizing. After an additional lyophilization from 3 ml of distilled water samples were suspended in 10 to 20 μl of distilled water, spotted onto plastic thin-layer sheets (Brinkmann Instruments; 0.1-mm cellulose MN300), and electrophoresed for 45 min at 1,200 V in a Savant TLE-20 thin-layer electrophoresis tank equipped with a cooling pump. Before electrophoresis, thin-layer sheets were wetted with electrophoresis buffer (acetic acid-pyridine-distilled water, 100:10:1,890 ml). After thin-layer electrophoresis, the sheets were dried and chromatographed at a 90° angle to the direction of electrophoresis in *n*-butanol–acetic acid–pyridine–distilled water (260:40:200:160 ml) for 90 min. The dried sheets were autoradiographed at room temperature. ^{35}S-labeled samples were processed similarly with several exceptions. After application of sample, the thin-layer sheets were chromatographed first for 60 min, wetted with thin-layer electrophoresis buffer, and then were electrophoresed for 80 min at 600 V (42). The dried sheets were saturated with 0.4% PPO (2,5-diphenyloxazole)–10% toluene in 2-methylnaphthalene and auroradiographed at −70°C (6).

RESULTS

Virion and nonvirion proteins induced by ADV. We immunoprecipitated [^{35}S]methionine-labeled lysates, using a pool of high-titer terminal sera from mink affected with AD (Fig. 1, track c). The two major virion structural polypeptides (p85 and p75) (Fig. 1, tracks a and c), as well as a nonvirion protein at 71,000 (p71), were distinctly resolved. Labeling with ^{14}C-amino acids revealed no additional species (data not shown).

Although ADV does not replicate in vitro in mink cells, the induction of ADV-specific antigen can be demonstrated with fluorescent antibodies (24, 27). To see what ADV proteins were induced in mink cells, a culture of the permanent embryonic mink lung cell line CCL-64 (38) and a culture of a simian virus 40-transformed newborn mink liver cell line were infected and labeled in parallel with the permissive CRFK cells. When the lysates of these cells were studied by immunoprecipitation and SDS-PAGE, the ADV-induced proteins (p85, p75, and p71) (Fig. 2) were noted in all three cell types. These experiments suggested that p71 was, in

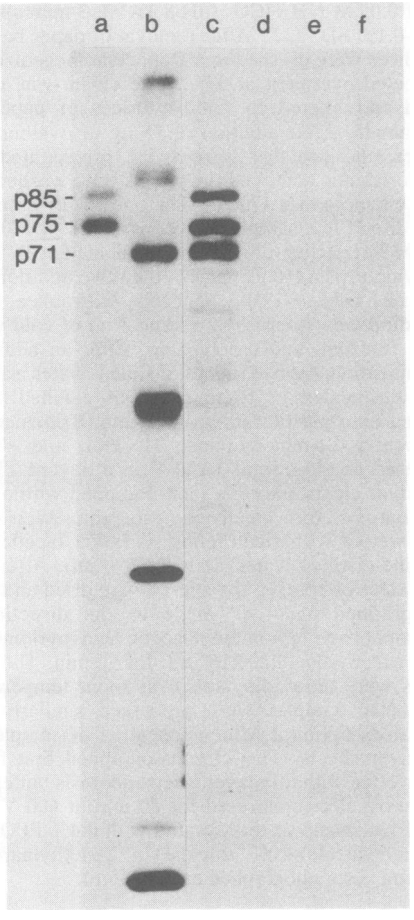

FIG. 1. Polypeptides induced in CRFK cells 60 h after infection with ADV. Cultures of CRFK, either uninfected (e, f) or infected with ADV (c, d) for 60 h, were labeled with [^{35}S]methionine (50 μCi/ml) for 2 h. Cell pellets were lysed, and 0.5 × 10^6 cell equivalents were incubated for 3 h with 5 μl of serum from AD-affected mink (c, e) or from normal mink (d, f). A 50-μl amount of *S. aureus* was then added, and the immunoprecipitates were analyzed as described in the text. Track a, [^{35}S]methionine-labeled ADV virion polypeptides; track b, ^{14}C-labeled protein standards (Amersham), from top: 200,000, 100,000, 92,000, 69,000, 46,000, 30,000, 14,500 daltons.

fact, a virus-specified protein, since the same protein was induced in three different cell types.

To determine when these proteins appeared in infected cells, cultures were labeled at various times after infection and studied. Inspection of the autoradiograph (Fig. 3) revealed that the synthesis of all three virus-induced proteins could first be detected at 24 h and was maximal between 48 and 72 h, but was still detectable at 96 h. The three proteins were always present in the same relative ratios. These results parallel observations made with immunofluorescence, since viral antigens detectable by immunofluorescence also increase to maximum between 48 and 72 h (28).

A series of pulse-chase experiments showed that all three ADV-induced proteins appeared simultaneously with no demonstrable precursor-product relationships evident (data not shown). Furthermore, no incorporation of [^{3}H]glucosamine could be demonstrated (data not shown), indicating that none of the ADV proteins were glycoproteins.

Peptide mapping of ADV proteins. Since the previous results suggested that, in addition to the virion proteins (p85 and p75), an additional ADV-induced nonvirion protein (p71) was present in infected cells, two-dimensional peptide mapping studies were done to examine the relatedness of the three proteins. This was done in two ways. First, autoradiographic bands corresponding to p85 and p75 were isolated from SDS-polyacrylamide gels of ^{125}I-labeled purified ADV and subjected to chymotryptic peptide mapping (Fig. 4). It was evident that these proteins were highly related. All spots resolv-

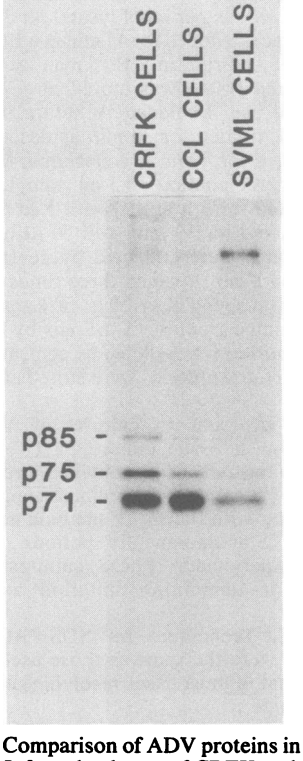

FIG. 2. Comparison of ADV proteins in CRFK and mink cells. Infected cultures of CRFK and mink (CCL and SVML) cells were labeled with [^{35}S]methionine and analyzed by immunoprecipitation as detailed in the text.

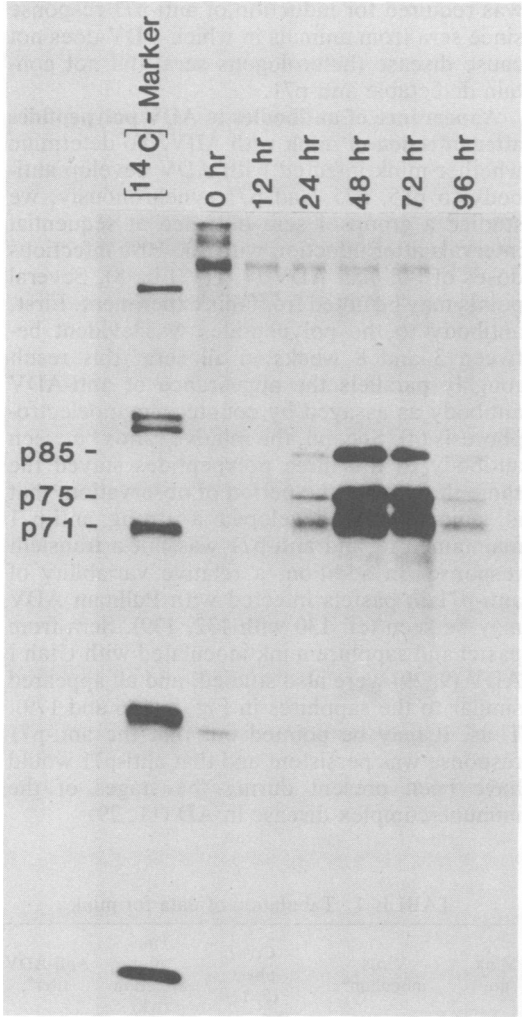

FIG. 3. Appearance of ADV-induced polypeptides after infection of CRFK cells with ADV. Cultures of CRFK cells in a 24-well tissue culture dish were infected with ADV. At the noted times after infection, cultures were labeled with [^{35}S]methionine (50 μCi/ml) for 2 h and 8.1 × 10^4 cell equivalents were immunoprecipitated and analyzed.

able in the map of p75 occurred in the map of p85, and three spots not present in the p75 map could be detected in the p85 map. We next analyzed the three intracellular ADV-induced proteins (p85, p75, p71) present in immune precipitates of [^{35}S]methionine-labeled infected cell lysates (Fig. 5). Homology between the virion proteins p85 and p75 indicated by the ^{125}I-peptide maps was also apparent in [^{35}S]methionine-labeled peptide maps; however, the pattern observed for p71 was markedly different. These results indicated extensive homology between the two virion proteins (p85 and p75), but no obvious homology between the virion and nonvirion proteins.

Antibody to ADV polypeptides in mink sera and heterologous antiviral sera. A panel of mink sera was reacted against samples of a single [^{35}S]methionine-labeled ADV-infected lysate of CRFK cells to determine whether all sera contained antibody to all three ADV-induced proteins. We analyzed a group of sera obtained 8 or 15 weeks after inoculation of mink with 300 50% infectious doses of either Pullman or Utah I ADV. Utah I ADV is highly virulent and causes typical progressive AD with persistent infection, hypergammaglobulinemia, and renal lesions in all sapphire mink and most pastel mink (18, 29), whereas the low-virulence Pullman strain of ADV typically induces AD only in sapphire mink (4, 11). Pastel mink, however, do support limited replication of Pullman ADV and do develop anti-ADV antibodies (4, 11). A total of 18 mink sera with anti-ADV titers between 1/64 and 1/4,096 in counterimmunoelectrophoresis were assayed (Fig. 6 and Table 1). All sera except no. 139 had easily detectable anti-p85 and anti-p75, and all except no. 132 and no. 139 had strong anti-p71. Generally, the level of anti-p71 correlated with anti-ADV titer, although some sera with equivalent titers (no. 128 and no. 130) had different amounts of anti-p71. Thus, it seemed that anti-p71 was present in all sera from mink with AD but that some sera (128, 132, 139) from mink with nonprogressive infection (pastels in-

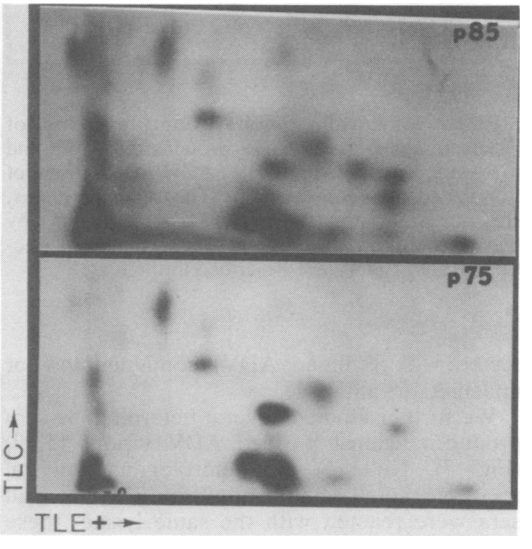

FIG. 4. Two-dimensional peptide maps of ^{125}I-labeled ADV virion proteins. p85 and p75 isolated from SDS-PAGE preparations of ^{125}I-labeled ADV were digested with α-chymotrypsin and analyzed by thin-layer electrophoresis followed by thin-layer chromatography. Details are given in the text.

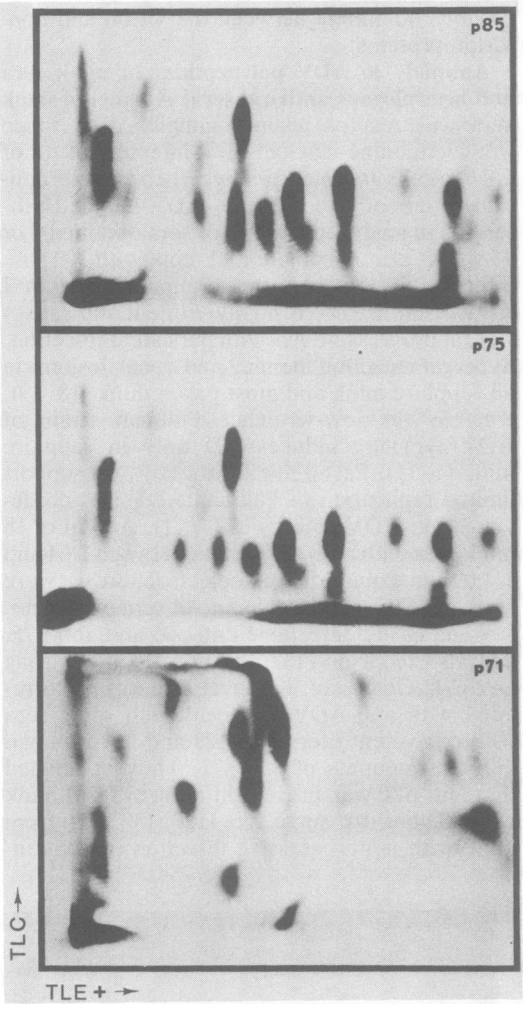

FIG. 5. Two-dimensional peptide maps of [^{35}S]methionine-labeled ADV proteins. p85, p75, and p71 were isolated from SDS-PAGE preparations of ^{35}S-labeled immunoprecipitates. The isolated proteins, after digestion with α-chymotrypsin, were analyzed by thin-layer chromatography and then by thin-layer electrophoresis. Details are described in the text.

fected with Pullman ADV) contained low or undetectable anti-p71.

We further studied several heterologous sera produced against purified ADV virions (5, 9) (Fig. 7). None of the heterologous antiviral antisera reacted with the nonvirion p71. Since all sera were reacted with the same lysate, these data implied that p85 and p75 had antigens not present on p71.

Taken together, these data revealed that progressive AD and persistent infection were associated with a strong anti-p71 response. Furthermore, the data suggested that viral replication was required for induction of anti-p71 response since sera from animals in which ADV does not cause disease (heterologous sera) did not contain detectable anti-p71.

Appearance of antibodies to ADV polypeptides after infection of mink with ADV. To determine whether mink infected with ADV develop antibody to p85, p75, and p71 synchronously, we studied a group of sera obtained at sequential intervals after infection with 300 50% infectious doses of Pullman ADV (4, 11) (Fig. 8). Several points may be noted from this experiment. First, antibody to the polypeptides was evident between 3 and 8 weeks in all sera; this result roughly parallels the appearance of anti-ADV antibody as assayed by counterimmunoelectrophoresis (4). Second, the relative ratios between antibody to the three polypeptides stayed the same throughout the period of observation; that is, animals that developed a strong anti-p71 maintained it, and anti-p71 was not a transient response. In addition, a relative variability of anti-p71 in pastels infected with Pullman ADV may be seen (cf. 130 with 132, 139). Sera from pastel and sapphire mink inoculated with Utah I ADV (9, 29) were also studied, and all appeared similar to the sapphires in Fig. 8 (186 and 179). Thus, it may be pointed out that the anti-p71 response was persistent and that anti-p71 would have been present during the stages of the immune complex disease in AD (11, 29).

TABLE 1. Tabulation of data for mink

Mink no.	Virus inoculum[a]	Color phase (5, 11)	Time after infection (wk)	Anti-ADV titer[b]
128	Pullman ADV	Pastel	15	1/256
130			15	1/256
132			15	1/64
135			15	1/256
139			15	1/64
145			15	1/1,024
179		Sapphire	15	1/1,024
Pool			15	1/4,096
186			15	1/1,024
126	Utah I ADV	Pastel	15	1/1,024
136			15	1/1,024
141			8	1/1,024
142			15	1/4,096
143		Sapphire	15	1/1,024
178			15	1/1,024
184			15	1/1,024
187			15	1/1,024
189			8	1/1,024

[a] Animals were inoculated intraperitoneally with 300 50% infectious doses of either Pullman (5) or Utah I ADV (9, 29).

[b] Anti-ADV antibodies were determined by counterimmunoelectrophoresis (5).

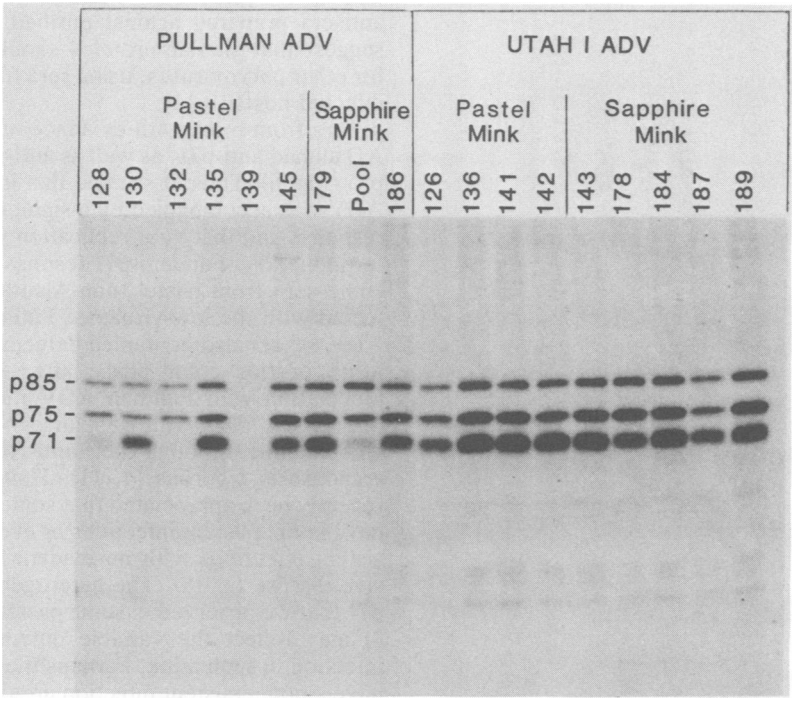

FIG. 6. Immunoprecipitation of ADV-induced polypeptides by sera from mink after inoculation with ADV. Serum samples (5 μl), obtained from pastel or sapphire mink at 8 or 15 weeks postinfection with 300 50% infectious doses of Pullman ADV or Utah I ADV (Table 1) were reacted against samples of a single lysate and analyzed as detailed above.

DISCUSSION

Two important findings have resulted from this study: first, that ADV induced an intracellular protein (p71) not found in purified virions and, second, that sera from mink infected with ADV contained antibody against this protein.

Our studies on the intracellular proteins of ADV that reacted with sera from AD-affected mink revealed that, in addition to the 85,000- and 75,000-dalton structural polypeptides (p85 and p75) (5), a prominent 71,000-dalton species (p71) could be consistently identified (Fig. 1). Heterologous sera raised against purified ADV virions failed to precipitate p71 (Fig. 7), implying that p85 and p75 had antigens not present on p71. In addition, monoclonal antibodies produced against ADV, precipitated p85 and p75, but not p71 (R. E. Race, M. E. Bloom, and B. Chesebro, manuscript in preparation).

Furthermore, our peptide map studies (Fig. 4 and 5) revealed a high degree of relatedness for p85 and p75, indicating that the coding sequences for the ADV virion proteins overlapped. Similar overlapping sequences have been demonstrated for other parvoviruses (15, 41). Since the parvovirus genome can maximally encode about 120,000 daltons of protein in a simple tandem arrangement (34, 40, 41), the sequence overlap between p85 and p75 explains how the ADV genome can code for both proteins. In contrast, the peptide map of p71 had no obvious similarity to those of p85 and p75 (Fig. 5), implying that a different mRNA existed for p71. Since the summed weights of p71 and p85 exceed the theoretical linear coding capacity, an alternative origin for p71 must exist. This could arise from (i) a reading frame shift (17), (ii) extensive mRNA processing (3, 10, 13), or (iii) RNA transcription dependent on the opposite strand of the double-stranded DNA template (35). Of these, only mRNA processing has been identified in parvoviruses (8, 13) and the gene products of these various processed mRNA transcripts have not been identified. Although we have not formally excluded that p71 was cryptic cellular protein induced by ADV infection, this seemed unlikely since ADV induced a p71 of the same molecular weight in different cell lines (Fig. 2).

Nonvirion proteins are well described for other DNA viruses (12, 14, 32, 33, 36, 39). Some of these proteins (e.g., the T-antigen simian virus 40 and the 72,000-dalton protein of adenovirus) have defined functions as DNA-binding proteins involved with DNA replication (12, 14, 32, 35),

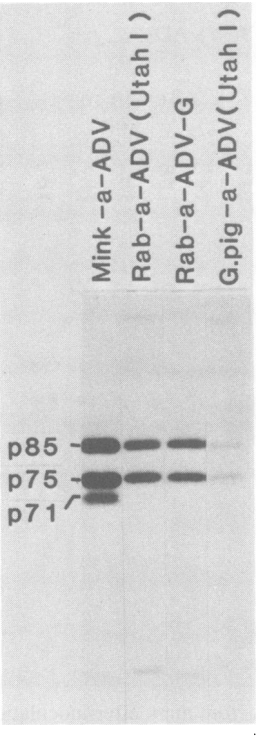

FIG. 7. Immunoprecipitation of ADV-induced polypeptides by heterologous antiviral sera. Sera (5 μl) were reacted in immunoprecipitation with samples of a single lysate prepared 60 h postinfection and then analyzed on SDS-PAGE as described above. In addition to a serum from a mink with AD (mink-a-ADV), sera from rabbits inoculated with CsCl-purified Utah I ADV (9) (Rab-a-Utah I ADV) or cell culture-adapted ADV-G (4) (Rab-a-ADV-G) and serum from a guinea pig inoculated with purified Utah I ADV (9) (GPig-a-ADV) are also displayed.

and T-antigen is a phosphoprotein (36, 39). We have recently observed (M. E. Bloom, data not shown) that p71 could be labeled with ^{32}P, and we are currently investigating the possibility that p71 may also function as a DNA-binding protein or be involved with DNA replication. One recent report described a protein (estimated size of 60,000 to 70,000 daltons) covalently associated with the replicative form DNA of the nondefective parvovirus, H-1. Although the relatedness of this protein to structural proteins of H-1 was not determined, a covalent DNA-protein linkage was not detected in purified virions. Similarly, the existence of replicative form DNA-protein bonds has been postulated for other nondefective parvoviruses (21). Additional studies on p71 are in progress to determine whether p71 is covalently linked to viral replicative form DNA in ADV-infected cells.

Finally, it must be pointed out that p71 would not have been detected, using only heterologous antisera prepared against purified virions. We suggest that similar proteins should be sought for other parvoviruses, using sera from naturally infected hosts.

Sera from mink with evidence of progressive AD all had anti-p71, as well as anti-p85 and anti-p75 (Fig. 6). Thus, it seemed that infection with ADV was accompanied by a significant anti-p71 response and that viral replication was required for induction of an anti-p71 response. However, some sera from pastel (non-Aleutian) mink infected with the low-virulence Pullman strain of ADV (4, 11) also contained detectable anti-p71. Mink of this color phase after experimental inoculation with Pullman ADV rarely develop progressive AD with sustained hypergammaglobulinemia although their anti-ADV antibody response is vigorous (4, 11). However, it has recently been appreciated that some pastels may have nonpersistent infections or even latent persistent infections with no evidence of progressive disease (2, 18). The heterogeneity of anti-p71 that we observed in some pastels (Fig. 6 and 8) may reflect the variable outcome of ADV infection in such mink. Perhaps those mink who have a nonpersistent infection are the mink who fail to develop strong anti-p71. Consequently, further correlation of immunoprecipitating antibody to p71 and clinical status may provide interesting information on this aspect of AD. The observation that anti-p71 was present in sera from mink with progressive AD indicated that p71 was an antigenic viral protein produced during persistent ADV infection. If p71 is a viral gene product expressed throughout the course of AD, the possibility of p71-anti-p71 immune complexes would clearly exist. Perhaps some of the immune complexes found in sera and in renal lesions (24–27, 30, 31) are p71-anti-p71 in composition. Nonstructural viral components might thus have a role in the genesis of the immune complex disease of AD.

The hypergammaglobulinemia of ADV involves increases in serum levels of immunoglobulin G (IgG), as well as IgA (D. D. Porter, H. G. Porter, and A. E. Larsen, Fed. Proc. **36**:1268; J. E. Coe, personal communication). Studies by Portis and Coe (31) revealed that mink IgA is the predominant immunoglobulin that can be identified in glomerular immune complex deposits of mink in terminal stages of AD, and presumably some of this IgA is antiviral antibody. Since *S. aureus* binding to antibodies requires binding to the Fc portion of an IgG molecule (16), our findings did not reflect anti-ADV antibodies of the IgA and IgM classes. Since differential reactivity of IgG and IgM against viral antigens has been reported for murine RNA tumor viruses (19), comparison of ADV proteins recognized by IgG, IgA, and IgM antibodies may also be

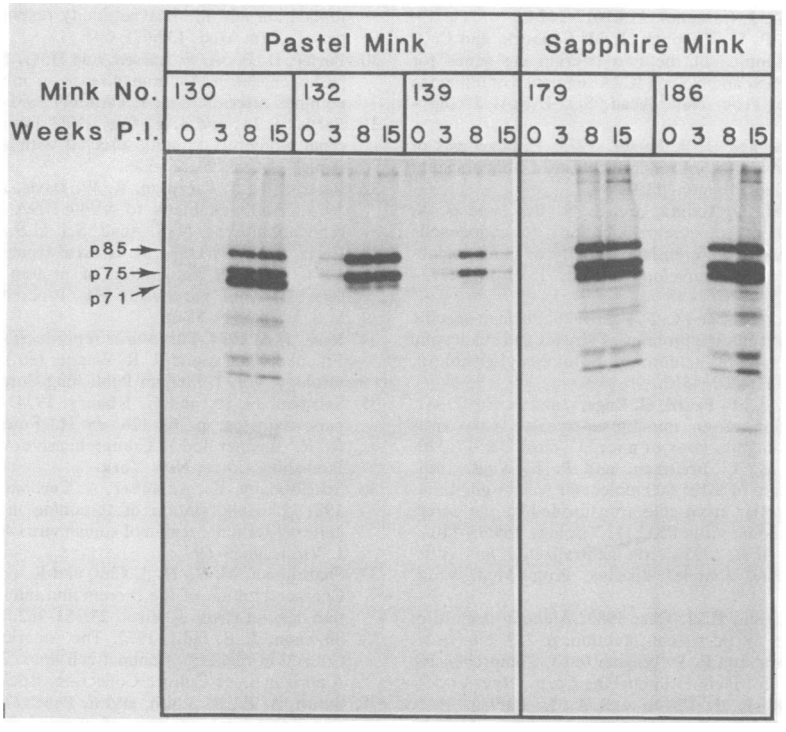

FIG. 8. Antibody response to ADV-induced polypeptides by mink after inoculation with Pullman ADV. Serum samples (5 μl), obtained sequentially from pastel or sapphire mink after inoculation with Pullman ADV, were reacted against samples of a single lysate and then analyzed.

important in understanding the role various antibodies and viral components play in the genesis of immune complex disease (7, 23).

ACKNOWLEDGMENTS

The expert assistance of Helen Blahnik, Helen Donovan, Chuck Taylor, Robert Evans, and John Savochka is gratefully acknowledged. Critical discussion was held with members of the staff at Rocky Mountain Laboratories, and Ralph Judd provided initial instruction in techniques of peptide mapping.

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