JOURNAL OF VIROLOGY, Aug. 1987, p. 2407–2419
0022-538X/87/082407-13$02.00/0

Vol. 61, No. 8

In Situ Molecular Hybridization for Detection of Aleutian Mink Disease Parvovirus DNA by Using Strand-Specific Probes: Identification of Target Cells for Viral Replication in Cell Cultures and in Mink Kits with Virus-Induced Interstitial Pneumonia

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Strand-specific hybridization probes were utilized in in situ molecular hybridization specifically to localize replicative form DNA of Aleutian mink disease parvovirus (ADV). Throughout in vitro infection, duplex replicative form DNA of ADV was located in the cell nuclei. Single-stranded virion DNA and capsid proteins were present in the nuclei early in infection, but were later translocated to the cytoplasm. In neonatal mink, ADV causes acute interstitial pneumonia, and replicative forms of viral DNA were found predominantly in alveolar type II cells of the lung. Viral DNA was also found in other organs, but strand-specific probes made it possible to show that most of this DNA represented virus sequestration. In addition, glomerular immune complexes containing intact virions were detected, suggesting that ADV virions may have a role in the genesis of ADV-induced glomerulonephritis.

Aleutian mink disease parvovirus (Aleutian disease virus; ADV) (10) causes a fatal, acute, interstitial pneumonia in seronegative neonatal mink. The clinical and pathological features of this disease have been described recently (1, 3, 31) and are characterized by severe respiratory distress with costal retractions and grunting, followed by lethargy and death within 24 h. The gross pathological findings in affected kits are limited to the lungs and consist of focal or diffuse reddening, meaty consistency, and moistening of the lung surface. Histologically the pneumonia is characterized by hyaline membranes covering the alveolar surface and hyperplasia and hypertrophia of alveolar type II cells. Viral inclusion bodies and ADV antigen have been demonstrated in nuclei of alveolar type II cells. It has been suggested that the clinical and pathological features of the disease may be a result of direct destruction of alveolar type II cells by ADV infection (1, 3, 31). The type II cells produce the alveolar surfactant that lowers the surface tension in the lung (41), and destruction of these cells results in the loss of this surfactant and development of respiratory distress and hyaline membrane disease. Deficiency of pulmonary surfactant is responsible for the development of a similar hyaline membrane disease in premature human infants; however, this is a result of type II cell immaturity rather than destruction (32).

In seropositive mink kits and in adult mink, ADV infection does not cause pneumonia but results in a persistent infection that leads to chronic immune complex-mediated disease (1, 20, 37, 38). This immune complex disease, classic Aleutian disease, was first described in 1956 (21), and evidence for its viral etiology was presented in the early 1960s (26, 27, 45). Yet, even though the disease has been studied for more than 30 years, the exact mechanism of its development and the cellular site of viral replication are still uncertain. The delineation of target sites is confounded by the fact that ADV is not neutralized by the massive amounts of antibody that are produced during the development of Aleutian disease (38), and consequently, the presence of infectious virus, viral proteins, and viral DNA in an organ or cell type may not be sufficient proof that true replication is occurring there.

Most nondefective parvoviruses, including ADV, encapsidate only one of the two possible single-stranded DNA molecules as the virion genome (22). The complementary strand of DNA is produced during replication when duplex forms of DNA are detectable. Thus, a single-stranded probe with the opposite polarity of the viral genome will hybridize to both virion and replicative form (RF) DNA, whereas a probe with the same polarity as the viral genome will hybridize only to viral RF DNA. Thus, the distinction between sites of viral replication and sites of viral sequestration is facilitated.

We recently described the development of strand-specific hybridization probes and their use in Southern blot hybridizations to localize RF DNA of ADV (3; M. E. Bloom, R. E. Race, and J. B. Wolfinbarger, Intervirology, in press). In the present study we extended the use of these strand-specific probes for in situ hybridization studies of ADV replication in cell cultures and in infected neonatal mink to localize ADV replication to a cellular level. The data obtained from these in situ hybridization studies are correlated with Southern blot quantitation of viral genomes and localization of viral antigen by immunofluorescence assay (IFA). We found that the in situ hybridization technique distinguished between true sites of viral replication and sites of viral sequestration and thus provided the most sensitive approach to study the cellular localization of ADV replication.

MATERIALS AND METHODS

Cells and viruses. Cell culture-adapted ADV-G (10) was grown in feline kidney cells (CRFK cells; 14) in RPMI 1640 supplemented with 10% fetal calf serum at 32°C as previously described (10). Cells were infected at various multiplicities of infection (MOIs) of 0.0025 to 2.5 and harvested 6 to 96 h postinoculation (p.i.). At the time of harvest,

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monolayers were trypsinized for 10 min at 37°C, made 10% in fetal calf serum, and washed once in phosphate-buffered saline. In some experiments, subcellular fractionation was performed according to the procedure described by Tattersall et al. (44).

In vivo-passaged ADV-Utah I virus (11), obtained from United Animal Division, Middleton, Wis., was passaged once in newborn ADV-negative mink kits. An inoculum prepared from their lungs, livers, and spleens was prepared as previously described (1) and was adjusted to contain 10^7 adult mink 50% infective doses per ml.

Animals. All mink used in this study were housed in commercial mink cages and fed a standard mink diet. Eleven adult female and 10 adult male sapphire (Aleutian, genotype *a/a*) mink were obtained from a herd known to be free of ADV infection for at least the previous 3 years. All mink were negative for circulating anti-ADV antibodies when tested by countercurrent electrophoresis (12) and rocket line immunoelectrophoresis (4). Early in March, the 11 female mink were mated twice at intervals of 9 days and then were divided into two groups. Kits born to these females were kept as family groups with their dams until killed.

(i) Group 1. Five litters (15 kits) were inoculated intraperitoneally as newborns (within 16 h after delivery) with 0.1 ml of phosphate-buffered saline. No kits showed clinical signs of disease. One kit was eaten by the dam, and 10 were killed as controls.

(ii) Group 2. Six litters (21 kits) were inoculated intraperitoneally as newborns with 10^6 50% infective doses of the ADV-Utah I inoculum. Fourteen kits showed clinical signs of respiratory disease 7 to 17 days after inoculation. Of these, six were eaten by the dam, three died, and five were killed within 24 h after the first clinical signs were recorded. Of the remaining seven kits, two were eaten by the dam and five were killed before any clinical signs of disease were recorded. No kits survived longer than p.i. day (p.i.d.) 17.

Two kits were randomly selected from each group and exsanguinated under pentobarbital anesthesia on p.i.d. 0 (4 h after inoculation), 4, 8, 12, and 17. Pieces of lung, liver, spleen, kidney, intestine (proximal jejunum), and mesenteric lymph node were snap frozen in liquid nitrogen and kept at −70°C until needed. Other pieces of the same organs (except mesenteric lymph node, which was too small to be divided in two blocks) were fixed for in situ hybridization as described later. The serum was collected and frozen at −70°C. At p.i.d. 12, 1 ml of blood from each kit was stabilized with EDTA, and the leukocytes were purified from the buffy coat.

IFA. Cell suspensions were prepared for IFA by cytocentrifugation of 10^5 cells onto acid-cleaned slides with a Shandon-Southern cytocentrifuge and then fixed in acetone. Frozen sections, 5 to 10 μm thick, of lung, liver, spleen, mesenteric lymph node, and kidney were also placed onto acid-cleaned slides and fixed in acetone. Both direct IFA with fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) from ADV-infected mink (9) and indirect IFA with mouse anti-ADV monoclonal antibodies specific for ADV structural proteins (39) were done as previously described (39). Direct IFA was done on kidney sections, using FITC-conjugated rabbit anti-mink immunoglobulin. The mink immunoglobulin used for immunization of the rabbits was purified on a protein A-Sepharose column as previously described (1).

Quantitation of specific ADV-DNA sequences. Total DNA was extracted from the samples, and 1-μg samples were analyzed by agarose gel electrophoresis and Southern blot hybridization (9; Bloom et al., in press). The number of DNA copies per cell was estimated from known standards included in each blot as described previously (3).

DNA was extracted from sera of kits by two different methods. Method 1 used DNA extraction from serum samples previously treated with DNase as described (Bloom et al., in press). In the other method, 50 μl of serum was diluted to 200 μl with distilled water and ultracentrifuged at 150,000 × *g* for 30 min in an Airfuge (Beckman Instruments, Palo Alto, Calif.). The pellet was dissolved in 50 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and incubated with 1 mg of proteinase K per ml and 1% sodium dodecyl sulfate at room temperature for 5 min. Agarose gel electrophoresis and Southern blots were then done as described (Bloom et al., in press). This method of extraction in low salt was employed to prevent reannealing of complementary strands.

Preparation of probes. Southern blot hybridization was performed using strand-specific RNA probes radiolabeled with [^{32}P]UTP as previously detailed (Bloom et al., in press), using a kit obtained from Promega Biotec, Madison, Wis. For in situ hybridization, the probes were radiolabeled with [^{35}S]UTP (New England Nuclear Corp., Boston, Mass.). The ^{35}S-labeled RNAs were reduced in size to about 200 to 400 bases, to facilitate their diffusion, by partial hydrolysis in 40 mM $NaHCO_3$–60 mM Na_2CO_3 (pH 10.2) (13). The probes were finally neutralized with 0.3 M sodium acetate, ethanol precipitated, and suspended in TE containing 10 mM dithiothreitol. The ^{35}S-labeled RNAs had a specific activity of approximately 5×10^8 cpm/μg, and the ^{32}P-labeled RNAs had a specific activity of approximately 2×10^8 cpm/μg. The size of the final ^{35}S-labeled RNA probes was verified by electrophoresis on a formaldehyde-agarose gel and autoradiography as previously described (Bloom et al., in press). A control template labeled with [^{35}S]UTP was prepared for the in situ hybridizations, utilizing an irrelevant template included in the kit. When infected cell cultures and infected kit tissues were hybridized in situ with this control probe, no grain count over background was observed, indicating specificitiy of the ADV probes.

Probes could be kept at −70°C for up to 2 weeks before use.

In situ hybridization techniques. The in situ hybridizations were performed using a procedure modified from that of Gendelman et al. (18).

(i) Coating of slides. Microscope slides were acid cleaned in 1.0 M HCl for 30 min, washed with tap water for 2 h, and rinsed in distilled water. The slides were then soaked in 0.01% (wt/vol) poly-D-lysine (Sigma p-7886) for 60 min, rinsed well in distilled water, and dried at 45°C for 8 to 12 h. The poly-D-lysine solution could be reused several times and was stored at −20°C.

(ii) Fixation of cells. Cell suspensions (10^5 cells) were cytocentrifuged onto coated slides and air dried. Frozen sections were cut in a cryostat at a nominal thickness of 6 μm, placed on coated slides, and air dried. Such cells or sections were fixed for 20 min at 4°C in a freshly prepared fixative containing periodate-lysine-paraformaldehyde-glutaraldehyde (PLPG). The fixative was made by mixing 6 parts of 0.1 M lysine in 0.05 M sodium phosphate buffer (pH 6.0) with 1 part each of 4% paraformaldehyde and 4% glutaraldehyde. After stirring for 5 min, solid sodium periodate was added to a final concentration of 7 mM. After fixation, the slides were washed twice with distilled water, dehydrated through graded ethanols, and then air dried. Such slides could be kept desiccated at −20°C in a dark box for several months without loss of hybridization efficiency. Tissue blocks (10 by 5 by 2 mm) were immediately placed in

the PLPG fixative for 1 h at 4°C and then for 3 to 4 h at room temperature. The blocks were then washed twice with distilled water, dehydrated through graded ethanols, and embedded in paraffin. Sections 5 μm thick were mounted on poly-D-lysine-coated slides. Before subsequent manipulations, sections were deparaffinized with xylene (twice for 5 min) and hydrated through graded ethanols (5 min in 95%, 5 min in 70%). No difference in hybridization efficiency was noted between frozen sections or sections prepared in this way when PLPG was used as the fixative, but preservation of tissue morphology was much better in paraffin sections, and consequently such deparaffinized sections were used throughout the study.

(iii) Pretreatment of cells. Slides containing cell suspensions or sections were immersed in 0.2 M HCl for 20 min, rinsed in distilled water, and incubated at 37°C in 10 mM Tris (pH 7.4)–2 mM $CaCl_2$, containing 5 μg of predigested proteinase K (Boehringer Mannheim) per ml, for 10 min. These conditions allowed satisfactory penetration of the probe as well as good preservation of cell morphology. Next, the slides were washed twice in 10 mM Tris (pH 7.5)–0.3 M NaCl and then incubated in the same solution containing 100 μg of RNase A and 10 U of RNase T_1 per ml for 30 min at 37°C. After two rinses of 5 min each in 10 mM Tris (pH 7.5)–0.3 M NaCl, the slides were refixed in phosphate-buffered 5% paraformaldehyde (pH 7.2) for 2 h at room temperature. The slides were then rinsed twice in distilled water and acetylated (19, 24) to neutralize positive charges and thus reduce nonspecific electrostatic binding of the probe.

(iv) Prehybridization and hybridization. Slides were prehybridized at 45°C for 2 to 4 h in 2× SSC (1× SSC is 0.015 M sodium citrate, pH 7.4, plus 0.15 M NaCl)–50% formamide–1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll)–200 μg of yeast tRNA per ml. Hybridization was then done under sealed cover slips in 10 mM Tris–0.3 M NaCl–0.5 mM EDTA–0.1% bovine serum albumin–0.02% Ficoll–0.02% polyvinylpyrrolidone–5 mM dithiothreitol–200 μg of tRNA per ml–50% formamide–10% dextran sulfate–10^5 or 10^6 cpm (0.2 or 2 ng) of ^{35}S-labeled RNA probe per μl. A 4-μl sample of probe solution was used for each square centimeter of sample. Hybridizations were done at 45°C for 14 to 16 h.

(v) Posthybridization treatment. The slides were washed as follows: 2× SSC for 10 min, two times, at room temperature; 2× SSC–0.1% Triton X-100–1 mM EDTA–5 mM dithiothreitol, two times at 60°C for 30 min; 0.1× SSC–0.1% Triton X-100–1 mM EDTA–5 mM dithiothreitol at 60°C for 30 min. They were then treated with 40 μg of RNase A and 10 U of RNase T_1 per ml in 10 mM Tris (pH 7.5)–0.3 M NaCl for 40 min at 37°C. The slides were finally washed three times in 2× SSC at 60°C for 15 min, dehydrated in graded ethanols containing 0.3 M ammonium acetate, and air dried.

(vi) Autoradiography. Kodak nuclear track emulsion NTB-2 (Eastman Kodak Co., Rochester, N.Y.) was melted at 45°C and diluted 1:1 with 0.6 M ammonium acetate. Slides were placed in plastic racks containing 25 slides (American Scientific Products, McGaw Park, Ill.), dipped in the emulsion for 1 s, air dried for 2 h, and further exposed in a dry, totally dark environment at 4°C for 1 to 238 h, giving a total exposure time of 3 to 240 h. After exposure, slides were developed for 3 min at room temperature in D-19 developer (Eastman Kodak Co.), rinsed for 1 min in distilled water, and fixed for 3 min in 30% sodium thiosulfate. Finally, the slides were stained with Gill triple-strength hematoxylin (Polysciences, Warrington, Pa.), air dried, and mounted in a water-based mounting medium (Glycergel; Dako, Accurate Chem, Westbury, N.Y.). Photomicrographs were made on Kodak Ektachrome 160 tungsten color slide film with a Leitz Orthoplan microscope equipped with a Leitz fully automatic 35 mm camera.

(vii) Special controls. As a control for nonspecific grain production, uninfected cell cultures and sections from uninfected kits were reacted with ADV probes. Also, DNase treatment was done to show that the probe bound to DNA. The DNase treatments were done at two different stages of the in situ procedure, as follows. First, cells were harvested and suspended in phosphate-buffered saline (pH 7.3)–10 mM $MgCl_2$–1% fetal calf serum–500 μg of DNase per ml for 60 min at 37°C. The samples were then washed with phosphate-buffered saline and processed routinely. Alternatively, fixed cells were treated with 500 μg of DNase per ml in 20 mM Tris (pH 7.4)–10 mM $MgCl_2$ for 60 min at 37°C immediately after proteinase K treatment. This DNase treatment of the cells abolished the signal from both probes (not shown), confirming that the probes hybridized specifically to DNA. Finally, for control of probe binding to single-stranded versus double-stranded DNA, cells were treated with the single-strand-specific mung bean nuclease (Pharmacia, Piscataway, N.J.) at a concentration of 40 U/ml in 50 mM sodium acetate–50 mM NaCl–1 mM $ZnCl_2$ (pH 5.0) at 45°C for 45 min (9).

(vii) Sensitivity of the hybridization. When number of grains was correlated to number of genomes per infected cell containing a large amount of viral genomes (96 h p.i.), the theoretical sensitivity of the assay was approximately 300 genomes per infected cell, assuming that cells containing more than three times background counts were detectable. At low genome content (6 h p.i.), however, it was possible to detect about 20 genomes per positive cell, a level comparable to previously reported finding in other systems that used RNA hybridization probes in situ (13). The difference in sensitivity for samples containing large amounts and small amounts of ADV genomes is probably due to higher background counts in the large-genome-content samples caused by the higher concentration of probe necessary to saturate the sample. Also, it might be partly due to diffusion of sample genomes, as reported for other systems with high levels of genomes per cell (13).

RESULTS

Correlative studies on ADV-infected cell cultures. The replication of ADV in the permissive Crandell feline kidney cell line has been previously studied (9; Bloom et al., in press). However, to provide a direct comparison with the in situ hybridization analyses, some of these experiments were repeated.

(i) Demonstration of ADV antigen in infected cell cultures. Temporal analysis of ADV antigen production by IFA using infected mink sera indicated that ADV antigen was detectable from 24 h after inoculation. At this time only a few cells showed positive immunofluorescence (IF), and the localization was always strictly nuclear. Later, the number of IF-positive cells increased, and by 96 h p.i., both nuclear and cytoplasmic fluorescence was observed (Fig. 1). Similar results were obtained with monoclonal antibodies specific for viral structural components (39); however, these antibodies reacted almost exclusively with cytoplasmic-located antigen in late infection (Fig. 1). These results suggested that ADV structural proteins were found in the nucleus early in infection, but later were largely relocated to the cytoplasm.

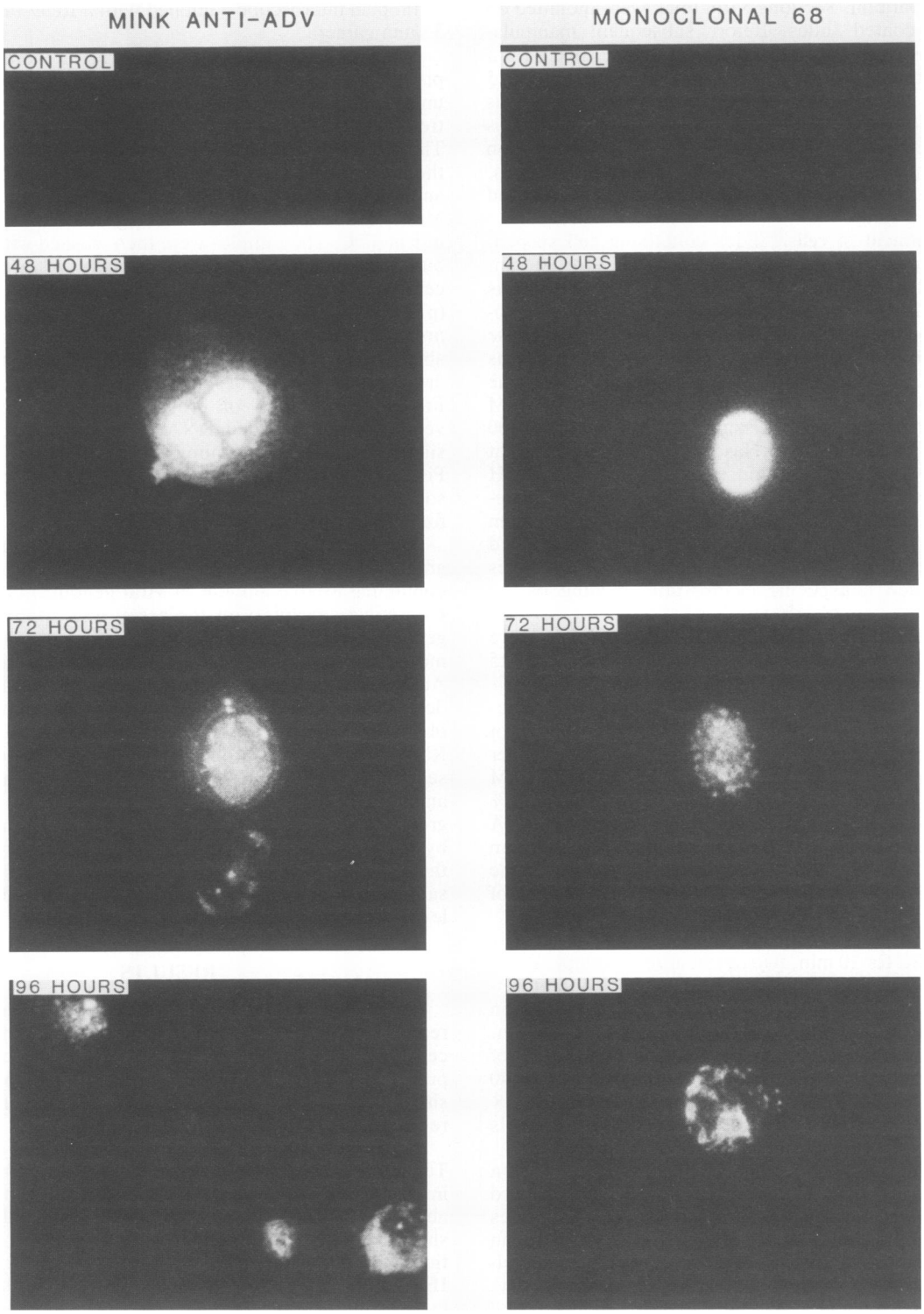


FIG. 1. IFA of uninfected (control) and ADV-G-infected CRFK cells. Cells were cytocentrifuged onto slides as detailed in Materials and Methods at the indicated times after infection. The cells were stained either with FITC-conjugated IgG from infected mink (left) or with a monoclonal antibody specific for ADV structural proteins (right). Forty-eight hours after infection, nuclear fluorescence was predominant with both methods. Seventy-two hours after infection, the fluorescence was both nuclear and cytoplasmic, and at 96 h, the fluorescence was predominantly cytoplasmic, although nuclear fluorescence could be seen in a few cells by using the mink anti-ADV IgG.

(ii) Demonstration of specific ADV DNA in infected cell cultures. We next examined the pattern and intracellular location of ADV DNA sequences by Southern blot hybridization. At high MOI, single-stranded virion DNA was detected 6 h p.i., probably reflecting input virions. From 24 h p.i., both single-stranded virion and duplex RF DNA were detected, and the levels of both increased with time.

When purified nuclear and cytoplasmic cell fractions prepared 72 h after inoculation of the cells were examined to localize DNA species, more than 90% of the single-stranded virion DNA was in the cytoplasmic fraction, while more than 90% of the duplex RF DNA was in the nuclei (not shown). When these findings were correlated with the IFA results, they suggested compartmentalization of virions in the cell cytoplasm and RF DNA in the nuclei.

(iii) In situ hybridization on infected cell cultures. In situ molecular hybridization was next performed on parallel samples, using the strand-specific probes. The "plus"-sense probe hybridizes to both single-stranded virion DNA and duplex RF DNA, while the "minus"-sense probe preferentially reacts with RF DNA (3; Bloom et al., in press).

When cells were infected at a low MOI, no grain count over background could be detected at 6 h p.i. with either probe. At 24 h p.i., grains were found over nuclei by using both probes, indicating that complementary strands of DNA were being produced at this time point and that the bulk of both virion and complementary strands of ADV DNA were localized to the nuclei. Subsequently, the localization of the plus-sense signal became diffuse, covering both nuclei and cytoplasm of the infected cells, while the minus-sense signal remained mainly nuclear (Fig. 2).

Grain counts (after short exposure times) were made 48 and 72 h p.i. (Table 1) and confirmed that the distribution of the plus- and minus-sense signals was significantly different ($P < 0.005$). Treatment with the single-strand-specific mung bean nuclease (9) reduced by about 80% the grains generated by the plus-sense probe in the cytoplasm, but reduced nuclear grains by only 30 to 35%. The same treatment reduced the minus-sense signal by only 25 to 30%, and the distribution was still predominantly nuclear. These findings indicated that the cytoplasmic localization of most of the plus-sense signal was due to single-stranded virion DNA of minus sense and that most of the nuclear minus-sense signal was due to duplex RF DNA resistant to mung bean nuclease treatment. Correlating this information with localization of IF and RF DNA in the nuclei, we concluded that the presence of a signal over the nucleus with the minus-sense probe represented RF ADV and that a reaction with the plus-sense probe over the cytoplasm represented single-stranded virion DNA.

At 24 h and later, the same percentage of cells was recorded positive by using either of the probes (Table 2), suggesting that each infected cell contained both single-stranded virion and duplex RF ADV DNA. The number of positive cells and number of grains per positive cell increased with time (Table 2), and the number of cells scored positive was slightly higher by in situ hybridization than by IFA, probably reflecting a higher sensitivity for the in situ hybridization technique. Nevertheless, these results indicated that the majority of cells in which ADV DNA was present also expressed ADV proteins.

When cells were infected at a high MOI, significant levels of grains were found diffusely over 70 to 80% of cells infected for 6 h when the plus-sense probe was used. The minus-sense probe, however, produced only background levels of grains. When live cells were treated with DNase before analysis, the same grain distribution was observed, suggesting that these early grains seen with the plus-sense probe reflected input virion DNA either inside the cells or bound to the cell surface. Thus the results indicated that input virion DNA can be detected 6 h after infection and that most cells actually have bound or taken up virions when infected at a high MOI. However, at the same high MOI, the final percentage of cells subsequently replicating the virus was much lower (Table 2), suggesting that although most cells initially bind or take up virions, only a fraction are permissive for ADV replication.

At 96 h p.i., the number of cells scored positive by in situ hybridization was correlated to the amount of viral single-stranded DNA and RF DNA as estimated by Southern blot hybridization, and it was concluded that, regardless of the MOI used to infect the cultures, each positive cell contained about 50,000 genomes of virion DNA and about 50,000 genomes of RF DNA (Table 2).

The results from these cell culture experiments indicated that the strand specificity of the probes was maintained in situ. Furthermore, a signal over the nuclei, found with the minus-sense probe, was a good marker for ADV replication, and a cytoplasmic signal with the plus-sense probe represented virion DNA. For convenience, we will hereafter refer to the minus-sense probe as the RF probe and the plus-sense probe as the virion probe.

Analysis of ADV-infected mink kits. The experiments in CRFK cells provided a convenient framework on which to examine the distribution of ADV antigen and DNA in sections from organs of ADV-infected mink kits.

(i) Demonstration of ADV antigen in infected kits. IFA was done on frozen sections of lung, liver, spleen, mesenteric lymph node, and kidney from kits killed at p.i.d. 12. In tissues from control kits, no specific ADV fluorescence was seen (Fig. 3a). In lung tissue from infected kits, viral antigen was observed in cells resembling alveolar type II cells (Fig. 3b). The alveolar type II cell, responsible for the production of alveolar surfactant, is a large, cuboidal cell characteristically situated in corners of the alveoli (41). The intracellular pattern of fluorescence in infected cells was similar to that seen in infected CRFK cells, i.e., nuclear and cytoplasmic. Liver, spleen, and mesenteric lymph node also contained high levels of ADV antigen; however, in marked contrast to lung tissue, the antigen in these organs was always cytoplasmic or intercellular. The antigen was diffusely distributed in liver sections (Fig. 4a), but in mesenteric lymph node and spleen it was concentrated at the periphery of germinal centers (Fig. 5a) in a pattern like that described for infected adult mink (9, 39). In the kidney, viral antigen was found in scattered single cells, probably tubule cells, and in cells lining the renal pelvis. Viral antigen was also found in a granular "lumpy bumpy" pattern in the glomeruli (Fig. 6a), and furthermore, a similar distribution of mink immunoglobulin was also demonstrable (Fig. 6b).

(ii) Demonstration of specific ADV DNA in infected kits. We then examined DNA extracted from selected samples of kit lungs and livers and from serum samples by Southern blot hybridization. The content of ADV-specific DNA sequences in the organs was comparable to that reported previously (3) and will therefore not be reported here.

In the serum of infected kits, small amounts of input single-stranded virion DNA (approximately 10^8 genomes per ml) were detected on p.i.d. 0, and by day 4 a maximum concentration of about 2×10^{10} ADV genomes per ml of serum was detected, a level about 100 times higher than that reported for infected adult mink (Bloom et al., in press).

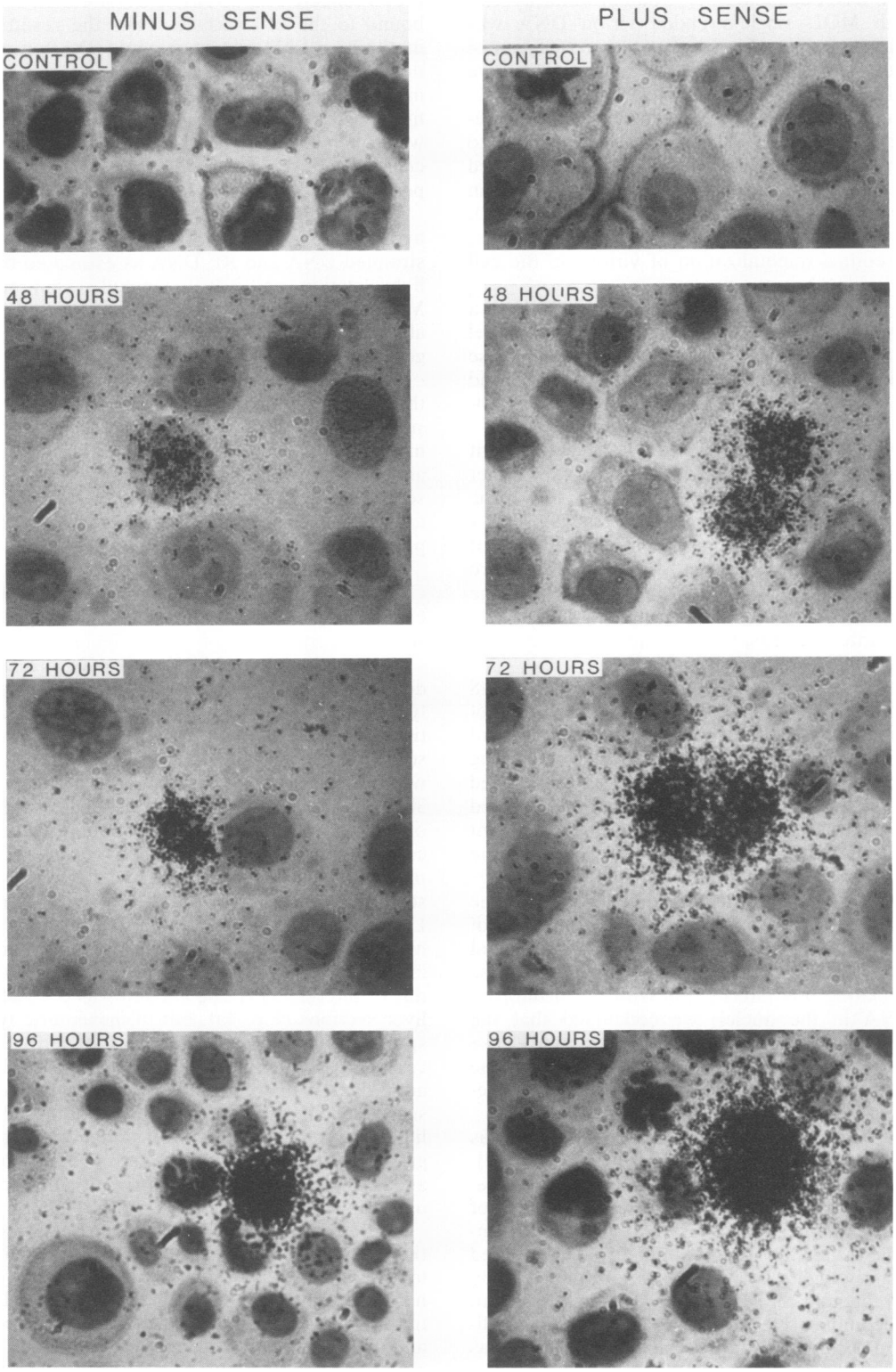


FIG. 2. In situ hybridization on PLPG-fixed CRFK cells. Cytocentrifuged samples of uninfected (control) CRFK cells or cells infected with ADV for the indicated times were hybridized in situ with either the minus sense (RF) probe (left panels) or the plus sense (virion) probe. Forty-eight hours after infection, grains were found over nuclei by both probes; at 72 and 96 h the RF signal remained mainly nuclear, whereas the virion probe signal became diffuse, covering both nuclei and cytoplasm.

TABLE 1. Analysis of grain distribution in infected CRFK cells[a]

Time (h p.i.)	No. of grains					
	Minus-sense probe			Plus-sense probe		
	Nuclei	Cytoplasm	n^b	Nuclei	Cytoplasm	n^b
48	20.88 ± 6.16	9.82 ± 2.21	17	17.24 ± 5.92	26.29 ± 7.20	17
72	38.93 ± 6.46	21.79 ± 4.23	14	27.30 ± 9.47	51.51 ± 10.79	14

[a] Infected CRFK cells were hybridized in situ with either the minus-sense or the plus-sense probe as described in Materials and Methods. Grains developed over nuclei and cytoplasm of individual infected cells were counted, and the mean value ± standard deviation was calculated.

[b] Number of individual positive cells that were counted.

After day 4, the serum content fell to about 10^9 genomes per ml on p.i.d. 8 to 12 and to about 10^8 genomes per ml on p.i.d. 17. This DNA was almost certainly in virions because it was single stranded, DNase resistant, and pelletable by ultracentrifugation.

(iii) In situ hybridization on infected kit tissues. Sections from all kits killed during the experiments were hybridized in situ using the ^{35}S-labeled RNA probes. Sections prepared from control kits consistently gave low levels of grains diffusely distributed over the whole slide and established the background level (Fig. 3c and e). Infected CRFK cells (96 h p.i.) with a known amount of ADV genomes per positive cell were included in each hybridization and served as an internal standard on the basis of which genome number could be calculated. When tissues from mink kits on p.i.d. 0 were examined, no grain counts over background were found, implying that although about 10^8 genomes of input virion DNA could be detected per ml of serum at this time point, no cells had accumulated enough genomes to be detected by in situ hybridization.

From p.i.d. 4, grains in excess of background were formed over cells in all tissues examined using either of the probes. The number of grains per cell and number of positive cells increased until p.i.d. 12 and then decreased to p.i.d. 17. The numbers of positive cells, genomes per positive cell, and genomes per total cell are shown in Tables 3 and 4. Numbers of genomes calculated by this in situ hybridization technique using internal standards are close to earlier published estimates obtained by Southern blot hybridizations (3).

In lung, cells scoring positive with either probe had the same morphology and distribution as those containing ADV antigen and were probably alveolar type II cells (Fig. 3d, f, g, and h). The pattern of grain localization with either probe was the same as for the permissive CRFK cells; that is, the grains could be clearly localized to individual cells. Thus, we concluded that the type II cell was most likely the permissive cell type for ADV replication in lung tissue of infected mink kits. A few sporadic endothelial cells in larger vessels were positive for ADV replication, but this was not a conspicuous feature. The bronchiolar epithelium was always totally negative by using either of the probes. In the last two kits killed in extremis on p.i.d. 17, the level of cells positive for viral replication had fallen considerably. Only 0.2% of the lung cells were positive using the RF probe, although 2% of the cells were positive with the virion probe. These positive cells, found in the lungs in late infection, had a distribution and morphology consistent with those of alveolar macrophages and desquamated type II cells. At p.i.d. 17, the lung was the only organ in which cells scoring positive with the RF probe could be detected.

At all times the other organs, i.e., liver, mesenteric lymph node, spleen, kidney, and intestine, contained 10- to 50-fold fewer positive cells than lung when hybridizations were performed with the RF probe. The number of genomes per positive cell was approximately twofold lower than in lung.

When the virion probe was used on these organs, a totally different picture was seen. One small population of cells had grains clearly localized over the cells, similar to the pattern seen with the RF probe. Another, and larger, population of cells also reacted with the virion probe but had fewer and more diffusely located grains. This pattern was never observed with the RF probe. The numbers of positive cells, genomes per positive cell, and genomes per total cell for organs from kits killed at p.i.d. 12 are tabulated in Table 4. The total number of grains in these organs corresponded to 40 to 650 genomes per total cell, an estimate close to the genome content estimated from Southern blot hybridizations (3).

At p.i.d. 17, no positive cells were found with the RF probe. However, with the virion probe a few cells were positive, indicating that virus could still be found in these organs although there was no evidence of replication.

In the liver, cells positive for viral replication had a morphology and distribution consistent with those of hepatocytes (Fig. 4c). The diffuse localization and distribution of grains over a larger population of cells with the virion probe (Fig. 4b) suggest that this is due to viral sequestration, maybe in phagocytic Kupffer cells.

In the kidney, the cells positive for ADV replication were most often tubule cells (Fig. 6d). Various kinds of tubule cells could be affected without any preferential localization being demonstrable. A few interstitial cells and endothelial cells in larger vessels were also positive for viral replication. Cells in glomeruli were consistently negative for virus repli-

TABLE 2. Summary of the quantitative data of the cell culture experiments[a]

Time (h) p.i.	% of cells positive by IFA[b]	% of cells positive in situ[c] with:		ADV genomes per in-situ positive cell[d]		Relative grain density per positive cell[e]	
		RF probe	Virion probe	RF probe	Virion probe	RF probe	Virion probe
6	<0.001	<0.001	70–80[f]	ND	20	1	3
24	0.1	0.2	0.2	ND	ND	75	105
48	7	10	10	ND	ND	150	225
72	10	15	15	ND	ND	300	410
96	15	20	20	50,000	50,000	360	500
Mock	<0.001	<0.001	<0.001	ND	ND	1	1

[a] Flasks (150 cm^2) of CRFK cells were infected with ADV-G at an MOI of 2.5. At the indicated time points, cultures were trypsinized and portions were subjected to the indicated analysis.

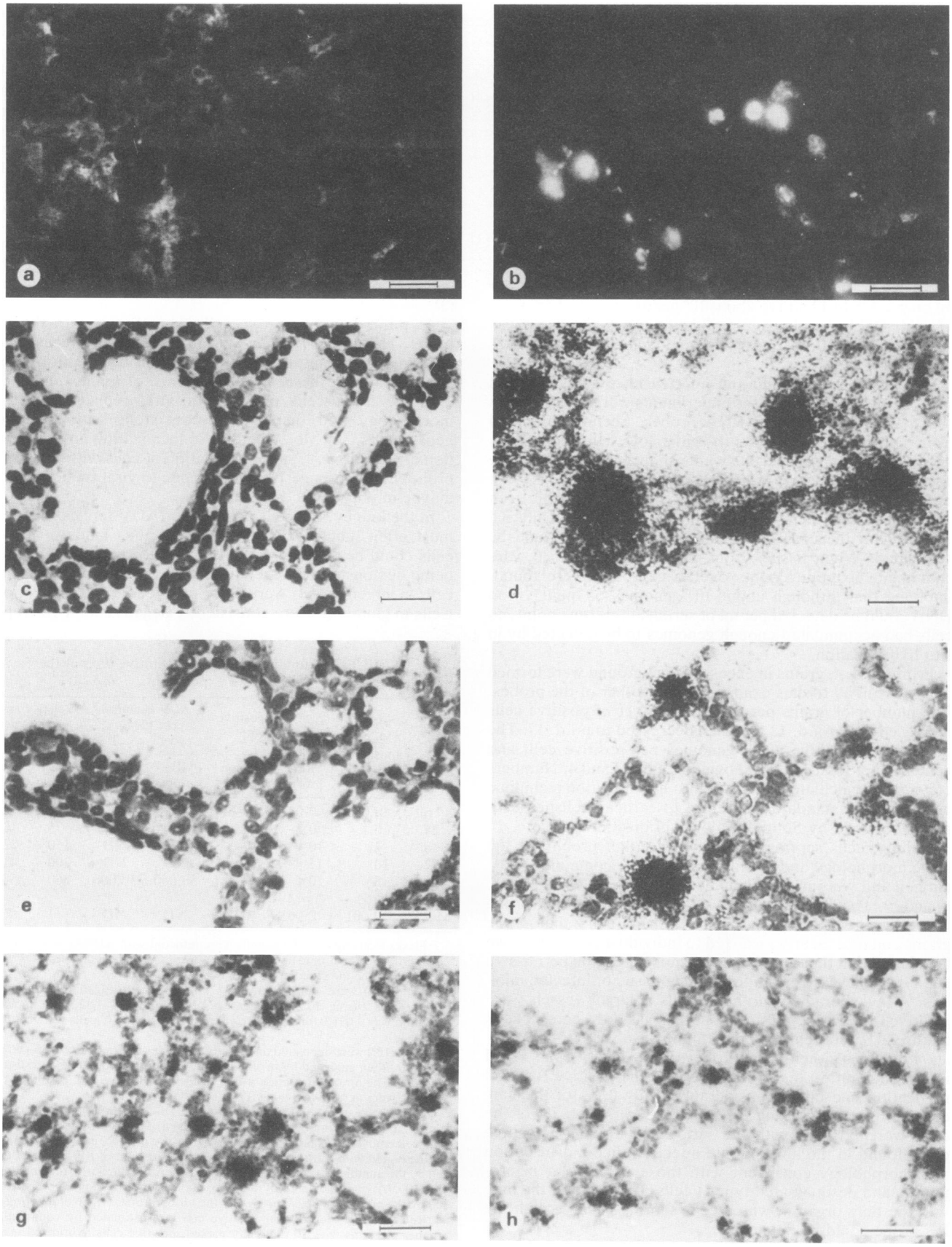
[b] Cells (10^5) were analyzed by IFA after cytocentrifugation onto microscope slides, using FITC-conjugated mink anti-ADV IgG. The limit of detection was 0.001% (one positive cell, assuming that 10^5 cells were actually deposited).

[c] Cells (10^5) were deposited on microscope slides with a cytocentrifuge. The cells were then analyzed by in situ hybridization as detailed in Materials and Methods. The limit of detection was 0.001% (one positive cell, assuming that 10^5 cells were actually deposited).

[d] ADV DNA was estimated in whole-cell DNA by Southern blot hybridization, as detailed in Materials and Methods, and expressed as genomes per total cell equivalent. The amount of RF DNA per positive cell was calculated for the 6- and 96-h-p.i. samples by dividing the amount of RF DNA per total cell by the number of cells positive for the RF probe. Similarly, the amount of virion DNA was calculated by dividing the amount of virion DNA per total cell by the number of cells positive by the virion probe. ND, Not determined.

[e] Relative grain density per positive cell was estimated by counting the number of grains over 20 randomly picked, positive cells hybridized in situ with either the RF probe or the virion probe.

[f] These positive cells probably represent uptake of input virions.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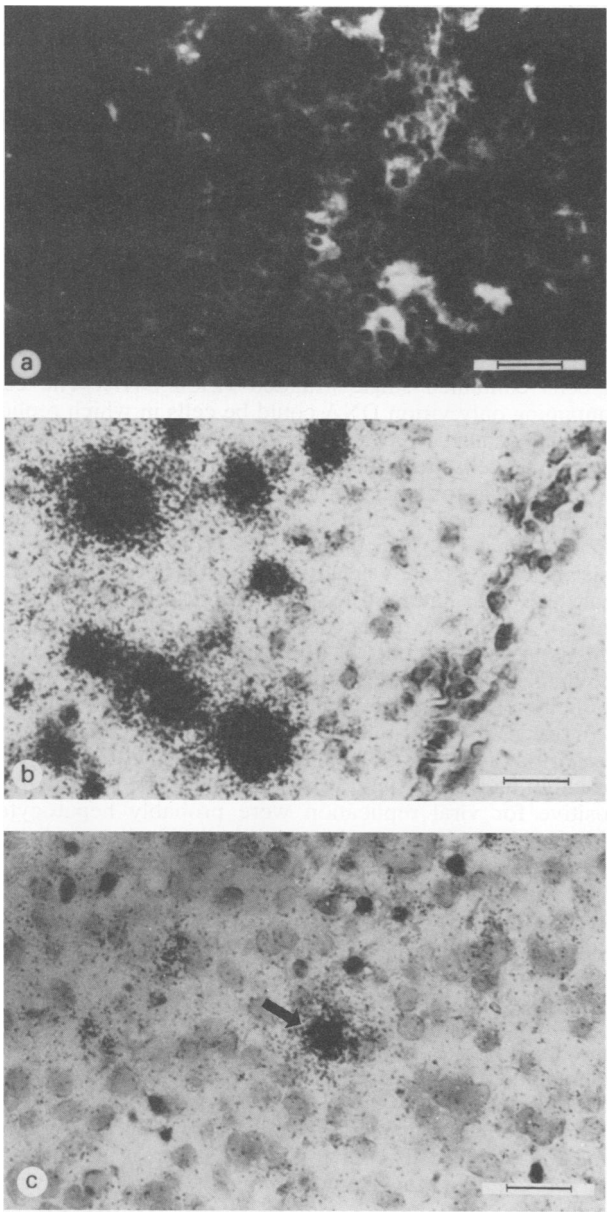

FIG. 4. Analyses of liver tissue from infected mink kits. (a) IFA using FITC-conjugated mink anti-ADV IgG. Acetone-fixed cryostat section of liver from a 12-day-old infected kit is shown. The fluorescence is diffusely distributed over the section and is cytoplasmic or intercellular. No intranuclear fluorescence is present. (b) In situ hybridization on PLPG-fixed paraffin section of liver from a 12-day-old infected kit, using the virion probe. Heavy grain production is seen diffusely over cells scattered throughout the liver parenchyma. (c) In situ hybridization on a parallel liver section, using the RF probe. Heavy grain production is seen densely localized over a single cell, probably a hepatocyte (arrow). Bars, 16 μm.

cation (Fig. 6d), but when the virion probe was used, glomeruli were strongly positive (Fig. 6c), suggesting that virus particles were sequestered in these structures. Such an uptake of virions in glomeruli could be demonstrated as early as 4 days after infection and increased with time after infection. The juxtamedullary glomeruli were always mostly affected. Cells lining the renal pelvis were also frequently positive for viral sequences.

The intestine had the lowest number of positive cells of any organ examined. The cells positive for ADV replication and sequestration were always in the lamina propria of villi or in cells around blood or lymph vessels in the intestinal wall (Fig. 7). The enterocytes were always negative with either probe.

In the spleen and mesenteric lymph node, cells positive for viral replication were detected but could not be characterized with certainty. These cells were most often found in the middle of germinal centers (Fig. 5c). When the virion probe was used, most grains were formed over cells in the periphery of germinal centers (Fig. 5b). This localization of virions correlates well with the finding of viral antigen around germinal centers in the mesenteric lymph node and spleen by IFA. Circulating leukocytes from infected kits were also examined; no cells positive for viral replication could be demonstrated, but a very few cells (<0.005%) had taken up virion DNA.

DISCUSSION

The present paper describes for the first time the successful use of strand-specific in situ hybridization to distinguish between cellular sites of viral replication and sites of viral sequestration during a parvovirus infection. One earlier attempt to address this issue used whole body sections blotted onto nitrocellulose filters (28), but this technique did not provide sufficient details at the cellular level. We studied a naturally occurring, economically very important parvovirus infection in mink, namely, ADV infection. Initially the infection was studied in vitro using a permissive cell culture system. These data showed that the strand specificity of the ADV RNA probes could be extended to include in situ hybridization analyses; that is, it was possible to distinguish the localization of the virion minus strand from that of the complementary plus strand, a marker for viral replication. The experiments indicated that ADV DNA replication occurred in the nuclei of infected cells, but that

FIG. 3. Analyses of lung tissue from 12-day-old infected and uninfected (control) mink kits. (a) IFA, using FITC-conjugated mink anti-ADV IgG. Acetone-fixed cryostat section of lung from a 12-day-old control kit is shown. Only background fluorescence is seen. Bars in panels a through f, 16 μm. (b) IFA performed as for panel a but on lung section from a 12-day-old infected kit. Intranuclear and cytoplasmic fluorescence can be recognized in cells whose morphology and location resemble those of alveolar type II cells. (c) In situ hybridization using ^{35}S-labeled RNA probe. The virion probe was used on a PLPG-fixed paraffin section of lung from a 12-day-old control kit. Only background grains can be observed. (d) In situ hybridization using the virion probe on lung tissue from a 12-day-old infected kit. Extensive grain production is diffusely distributed over cells whose location and morphology resemble those of alveolar type II cells. (e) In situ hybridization using the RF probe. Lung tissue from a 12-day-old control kit is shown. Only background grains can be observed. (f) In situ hybridization using the RF probe on lung tissue from a 12-day-old infected mink kit. Extensive grain production is seen, rather densely located over cells with the location and morphology of alveolar type II cells. (g and h) Same sections and probes as in panels d and f, respectively, but at a lower magnification. The grains are distributed over cells often located at corners of alveoli. This localization of cells is characteristic for the alveolar type II cell (41). Bars in panels g and h, 40 μm.

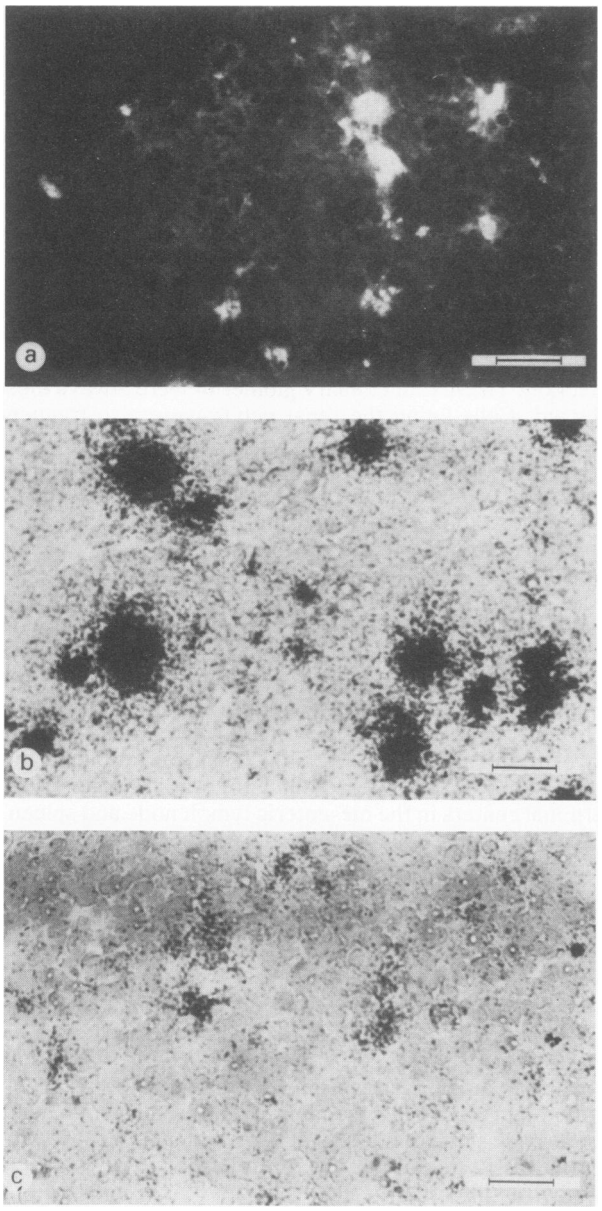

FIG. 5. Analyses of spleen tissue from infected kits. (a) IFA using FITC-conjugated mink anti-ADV IgG. Acetone-fixed cryostat section of spleen from a 12-day-old infected mink kit is shown. Positive fluorescence is intercellular and cytoplasmic in cells at the periphery of a germinal center. (b) In situ hybridization on PLPG-fixed paraffin section of spleen from a 12-day-old infected kit, using the virion probe. Heavy grain production is seen over cells at the periphery of a germinal center. (c) In situ hybridization on a parallel spleen section, using the RF probe. Grain production is seen over cells in the middle of a germinal center. Bars, 16 μm.

late in infection, virions were transported to the cytoplasm. Most of the RF ADV DNA, however, remained located in the nuclei, and the IF pattern suggested that some of the nonstructural p71 protein also remained in the nuclei, since nuclear antigen in late infection reacted only with polyclonal mink antisera and not with monoclonal antibodies specific for ADV structural proteins.

We next analyzed ADV-infected mink kits by using the same methodology. Earlier studies (1–5, 31) suggested that the alveolar type II cell of the lung might be the main primary site of viral replication and cytopathology, although only indirect evidence of viral replication was shown. In contrast, the in situ hybridization technique used in the present study gave direct evidence for viral replication in certain cells. The greatest number of cells supporting viral replication were in lung sections, and their morphology and distribution were consistent with those of alveolar type II cells. Replication of virus in alveolar type II cells probably causes direct cytopathology, followed by decreased surfactant production and development of the characteristic clinical and pathological features of respiratory distress and hyaline membrane disease in affected mink kits. In late infection (p.i.d. 17) only a few lung cells still replicated virus. However, a high number of cells contained single-stranded virion DNA. The cells containing only virion DNA could be cells in which replication had occurred earlier, or, more likely, they could be phagocytic cells attracted to the area by cellular debris. This would be in accordance with the light-microscopic observation of the accumulation of alveolar macrophages in severely affected lungs (1). Interestingly, the bronchiolar epithelium was always completely negative with either probe, indicating that these cells had no role in viral replication and probably did not contribute to the development of the pneumonia. This is in marked contrast to most other viral infections of the lung, in which infection and destruction of bronchiolar epithelium are characteristic features (7, 15–17, 25, 42).

In organs other than lung, only a small population of cells supported actual viral replication, but many more cells probably had sequestered virus. In the liver, the few cells positive for viral replication were probably hepatocytes. This would be in accordance with the earlier finding of intranuclear viral inclusion bodies in hepatocytes (3). However, some positive cells may have been either mononuclear cells migrating through the liver parenchyma or, possibly, cells involved in extramedullary hematopoiesis, both common features in neonatal mink (S. Alexandersen, Ph.D. Thesis, Royal Veterinary and Agricultural University of Copenhagen, Copenhagen, Denmark, 1985). In liver, large amounts of virus were seemingly sequestered in Kupffer cells, similar to the sequestration of ADV reported by IFA for adult mink (36).

In the kidney, replication of virus was found in various kinds of tubule cells, but the glomeruli were negative. In marked contrast, the glomeruli were strongly positive for sequestered virion DNA and were also positive for viral antigen and mink immunoglobulins. This is the first report giving direct evidence for the presence of ADV DNA in glomeruli, and moreover, the coexistence of virion DNA, viral antigen, and mink immunoglobulins suggested an early state of immune complex glomerulonephritis, in which intact virions probably were one of the components. The juxtamedullary glomeruli were most affected, suggesting that filtration mechanisms may be responsible for virion uptake from the bloodstream since these glomeruli are most efficient in renal filtration during kidney maturation (23, 29). Cells lining the renal pelvis were also positive for viral sequestration, suggesting that virions may have been sieving through glomeruli, passing with the urine through the kidney tubules, and being taken up by cells lining the renal pelvis. Furthermore, these findings implied that virus might be shed in voided urine.

In the intestine, cells positive for both replication and sequestration were in the lamina propria of villi and around blood and lymph vessels. The exact identity of these cells could not be established, but lymphocytes, macrophages,

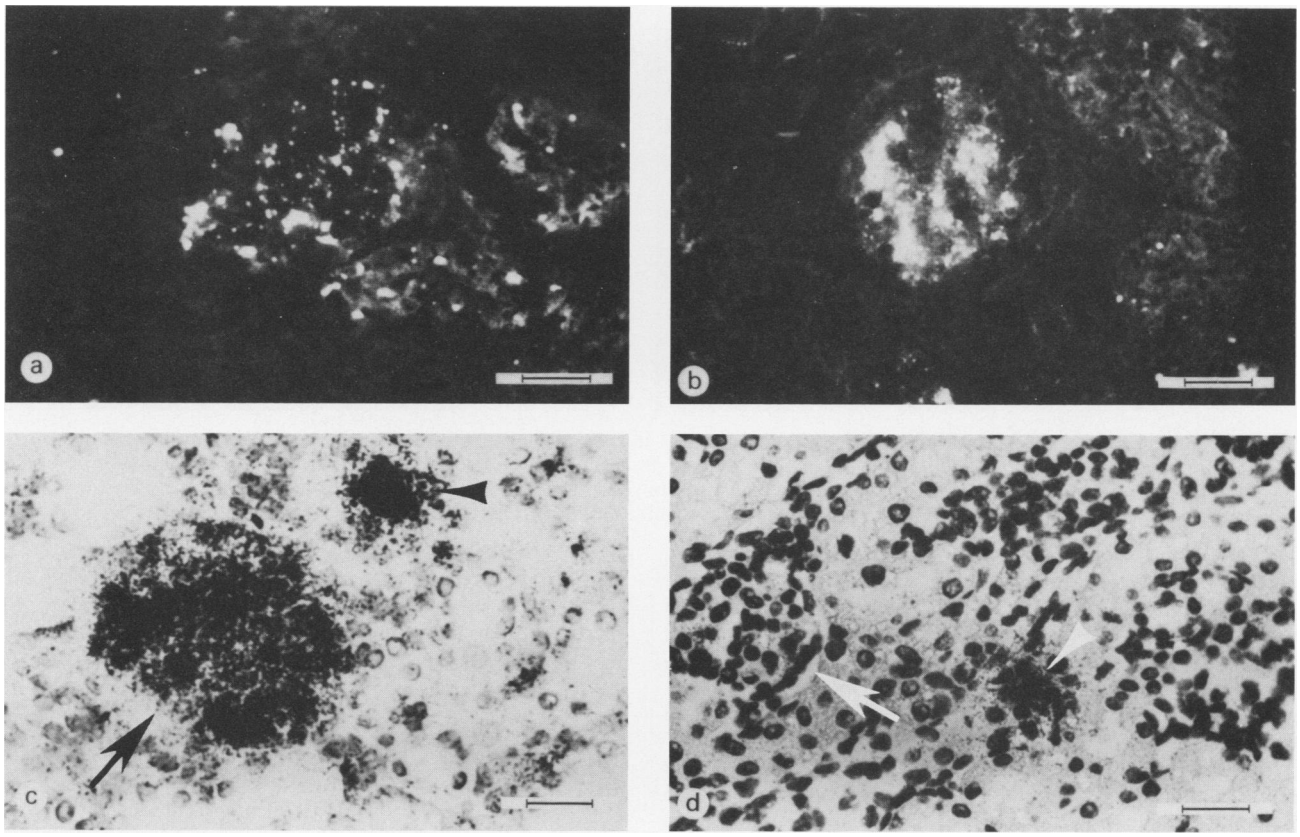

FIG. 6. Analyses of kidney tissue from infected mink kits. (a) IFA on acetone-fixed cryostat section of kidney from a 12-day-old infected kit, using FITC-conjugated mink anti-ADV IgG. Positive fluorescence is seen in a granular, lumpy bumpy pattern in a glomerulus. The pattern of the fluorescence suggests that the ADV antigen is deposited along the glomerular capillaries and not in the mesangium. However, the exact localization of the antigen, as being either subendothelial, in the basal membrane, or subepithelial, cannot be distinguished by this technique. (b) IFA on a parallel section of kidney using FITC-conjugated rabbit anti-mink immunoglobulin. Positive fluorescence is seen in a glomerulus and has the same distribution as ADV antigen (panel a). (c) In situ hybridization on PLPG-fixed paraffin section of kidney from a 12-day-old infected kit, using the virion probe. Heavy grain formation is seen over the capillary tufts of a glomerulus (arrow). A single positive cell outside the glomerulus, probably a tubule cell, is also shown (arrowhead). (d) In situ hybridization on a parallel section of kidney, using the RF probe. A single positive cell, probably a tubule cell, is shown (arrowhead). A glomerulus (arrow) is negative for ADV replication. Bars, 16 μm.

TABLE 3. In situ hybridization analysis of lung tissue from mink kits[a]

Time (days) p.i.	% of cells scored positive		Genomes per positive cell[b]		Genomes per total cell[c]	
	RF probe	Virion probe	RF probe	Virion probe	RF probe	Virion probe
0	0	0	<300	<300	ND	ND
4	0.2	0.2	74,000	55,000	148	110
8	1	1	68,000	60,000	680	600
12	2	2	82,000	100,000	1,640	2,000
17	0.2	2	53,000	63,000	106	1,260
Mock	0	0	<300	<300	ND	ND

[a] Newborn mink kits were inoculated with ADV-Utah I, and two kits were killed at each of the indicated time points. Lung tissue was processed for in situ hybridization analysis as described in Materials and Methods. The number of positive cells and number of grains over each positive cell were recorded for an area containing 20 positive cells or at least 10,000 cells total.

[b] The number of grains per positive cell was correlated to number of genomes by comparison with an included standard of infected CRFK cells containing a known amount of ADV genomes.

[c] The content of ADV genomes per total cell was calculated by multiplying the amount of genomes per positive cell by the percentage of positive cells. ND, Not determined.

and fibroblasts were good candidates because these cells often are found in these locations (6, 8). Enterocytes, however, were consistently negative, suggesting that these cells are not permissive for ADV replication. This was surprising since these cells rapidly divide and, moreover, are permissive for replication of mink enteritis parvovirus (30). The findings suggest that ADV, in common with other parvoviruses (43; 46), needs other factors, in addition to rapidly dividing cells, to replicate.

In the mesenteric lymph node and spleen, the cells positive for viral sequestration were situated around germinal centers, consistent with results of IFA published for adult mink (9, 39). These findings suggested that virus was sequestered in phagocytic, perhaps antigen-presenting, cells often found in the periphery of germinal centers (33, 34, 40). In contrast, cells replicating ADV were located in the middle of germinal centers, suggesting that affected cells may be immature lymphocytes, a cell population characteristically found in this location (40). However, the morphology of the cells was not good enough to distinguish between lymphocytes and nonlymphocytes, and until histochemical markers are developed to distinguish mink cell subpopulations, these cells cannot be classified. When circulating leukocytes from

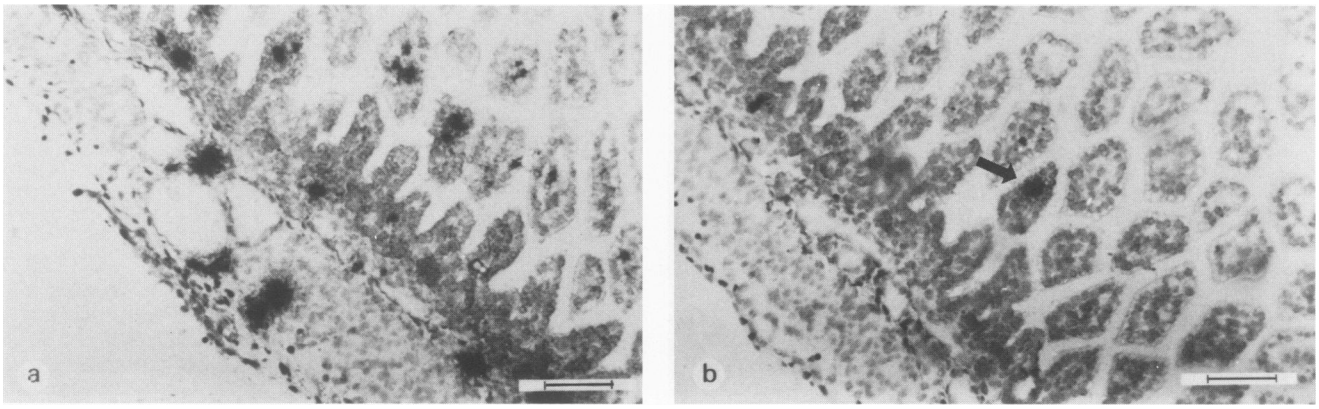

FIG. 7. Analyses of intestinal tissue from infected mink kits. (a) In situ hybridization on PLPG-fixed paraffin section of jejunum from a 12-day-old infected kit, using the virion probe. Heavy, but diffuse, grain production is seen over multiple cells in the lamina propria of villi and around lymph and blood vessels. Enterocytes are negative. (b) In situ hybridization on a parallel section of jejunum, using the RF probe. A single positive cell in the lamina propria of a villus can be recognized (arrow). Enterocytes are negative. Bars, 40 μm.

infected kits were examined, no cells were positive for viral replication, and only a very few were positive for viral sequestration. These data suggested that, although evidence of viral replication was found in lymphatic tissue, the presence of ADV in circulating leukocytes, including lymphocytes, is not a major feature of ADV infection in neonatal mink.

Infectious immune complexes in serum of ADV-infected adult mink have previously been reported (35). In the present study, high levels of virion DNA were found free in serum, and moreover, this DNA probably represented viremia, i.e., free virus particles, because the DNA detected in serum was single stranded, resistant to DNase treatment, and pelletable by ultracentrifugation. These findings suggest that distribution of virions via the bloodstream and subsequent uptake in phagocytic cells or deposition in glomeruli might be an important mechanism for viral sequestration during ADV infection in neonatal mink.

TABLE 4. In situ analysis of organs from infected kits killed at day 12 p.i.[a]

Organ	% of cells scored positive		Genomes per positive cell[b]		Genomes per total cell[c]	
	RF probe	Virion probe	RF probe	Virion[d] probe	RF probe	Virion probe
Liver	0.05	0.6	60,000	30,000	30	180
MLN[e]	0.05	1	65,000	61,000	33	620
Spleen	0.03	0.3	56,000	30,000	17	90
Intestine	0.03	0.1	35,000	32,000	11	32
Kidney	0.03	0.3	47,000	37,000	14	111

[a] Newborn mink kits were inoculated with ADV-Utah I, and two kits were killed 12 days later. Tissue was processed for in situ hybridization analysis as described in Materials and Methods. The number of positive cells and number of grains over each positive cell were recorded for an area containing 20 positive cells or at least 10,000 cells total.

[b] The number of grains per positive cell was correlated to number of genomes by comparison with an included standard of infected CRFK cells containing a known amount of ADV genomes.

[c] The content of ADV genomes per total cell was calculated by multiplying the amount of genomes per positive cell by the percentage of positive cells.

[d] Cells positive with the virion probe consisted of two different populations. One small population, 0.03 to 0.05% of the cells, had a high number of grains densely located over the cells, and another, greater, population, 0.1 to 1% of the cells, had fewer and more diffusely located grains. The calculated number of virion single-stranded genomes per positive cell is calculated as the mean value of both populations considering their relative abundance.

[e] MLN, Mesenteric lymph node.

Further studies using strand-specific in situ hybridization are in progress to elucidate the cellular sites of ADV replication in adult mink and to delineate the subcellular localization of DNA species during other parvovirus infections.

ACKNOWLEDGMENTS

We thank Irene Cook Rodriguez for typing the manuscript, Robert Evans and Gary Hettrick for preparing the figures, William Anderson and Robert Nilles for invaluable assistance in animal care, and Dan Corwin for cutting the paraffin sections. We gratefully acknowledge William Hadlow for the use of histopathology laboratory facilities and Claude Garon for the use of darkroom facilities.

The study was supported in part by the Danish Fur Breeders Association Research Foundation and the Danish Council for Veterinary and Agricultural Research. S.A. is a National Institutes of Health visiting associate on leave from the Institute of Veterinary Pathology, Royal Veterinary and Agricultural University of Copenhagen, Copenhagen, Denmark.

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