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Temporal Synthesis of Proteins and RNAs during Human Astrovirus Infection of Cultured Cells

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Astroviruses are nonenveloped particles with a distinctive star-shaped surface structure that have been detected by electron microscopy in stool samples from humans and animals with gastroenteritis. We examined the patterns of macromolecular synthesis in astrovirus-infected cells with a goal of establishing a molecular basis for taxonomic classification. Trypsin is required for continuous replication of astrovirus in cultured cells; however, during a single cycle of infection, astrovirus antigen was synthesized earlier and at higher levels when serum, rather than trypsin, was included in the growth medium. This enhanced production of antigen, as measured by enzyme immunoassay, was accompanied by the appearance of aggregates of virus particles in the cytoplasm of infected cells. During astrovirus replication in cells cultured in the presence of serum, we detected a single infection-specific protein (90 kDa) beginning at 12 h postinfection. This protein was recognized by antiastrovirus rabbit serum and was sensitive to trypsin digestion in vitro, with the concomitant appearance of three smaller immunoreactive proteins (31, 29, and 20 kDa). We also detected two dactinomycin-resistant RNAs (7.2 and 2.8 kb), both of which were polyadenylated, in the cytoplasm of astrovirus-infected cells. The larger of these two RNAs is presumably the viral genome, whereas the smaller species may be a subgenomic messenger. Comparison of the proteins and RNAs synthesized in astrovirus-infected cells with those of the recognized families of nonenveloped single-stranded RNA animal viruses suggests that astroviruses should not be classified as members of either *Caliciviridae* or *Picornaviridae*.

The term astrovirus was first used by Madeley and Cosgrove in 1975 (24) to describe distinctively shaped virus particles that they detected in fecal specimens from children with gastroenteritis. These particles were approximately 28 nm in diameter and had a smooth circular outline with a characteristic five- or six-pointed star on their surface (23). Viruses with similar morphologic features were subsequently identified in the feces of many species of domestic mammals and birds with gastroenteritis (19).

Through the use of electron microscopy (EM), the principal method for detecting astrovirus in stool samples, astroviruses have been associated with sporadic cases of diarrhea in children younger than 5 years of age, predominantly in the winter (9). Astroviruses have also been associated with outbreaks among children in a hospital (2) and a school (17) and with outbreaks among adults in institutionalized settings such as homes for the elderly (8). The Marin County agent, identified in an outbreak of gastroenteritis in a hospital for the elderly in California (28), has recently been shown to be an astrovirus of serotype 5 (12, 13). While diagnosis of astrovirus is uncommon by existing methods, exposure appears to be universal: 70% of children by age 4 and 77% of young adults in the United Kingdom have detectable levels of antibody to the virus (18). Immune EM and immunofluorescence techniques have been used to identify five human serotypes (22), none of which appear to cross-react with any of the animal strains (19). Two adult volunteer studies have been conducted to amplify the original stool isolates and to examine their pathogenicity in humans. In the only published report, 1 of 17 volunteers had overt symptoms of diarrhea and 13 of 16 volunteers demonstrated serologic evidence of infection (20).

Human astrovirus was first propagated in cell culture by Lee and Kurtz (21), who demonstrated that trypsin is required in the culture medium to sustain virus replication. The initial isolation from stool specimens required passaging in human embryonic kidney (HEK) cells, with subsequent adaptation of some viruses to growth in a continuous monkey kidney cell line (LLCMK2) (21). Adaptation of astrovirus to growth in tissue culture has enabled us to develop a plaque assay for quantifying virus and measuring neutralizing antibodies (16). Virus purified from stool- and tissue culture-grown virus have been used to generate polyclonal and monoclonal antisera (14, 22, 36), which in turn have been used to develop immunoassays to detect both antibody (36) and antigen (15).

In contrast to the recent progress in developing reagents to detect astrovirus in clinical samples, relatively little is known about the genome and capsid proteins of human astroviruses and how they relate to the animal strains. The size of the astrovirus RNA genome has been reported as 7.9 kb for the ovine strain (11) and 34S (~7 kb) for a human strain (9a). Several investigators have analyzed the capsid structure of human and animal astroviruses and have reported considerable variation between the different strains in the patterns of proteins detected. Purified ovine astrovirus has two 33-kDa capsid proteins (11), a recent characterization of a porcine astrovirus identified five capsid proteins ranging from 13 to 39 kDa (33), and the human virus

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(serotype 1) reportedly contains three or more proteins ranging from 32 to 36.5 kDa (19). The lack of detailed information about the basic genome organization, capsid protein structure, and mechanisms of replication of astroviruses has precluded a definitive taxonomic classification for these pathogens. We therefore examined the patterns of macromolecular synthesis in astrovirus-infected cells with a goal of establishing a molecular basis for taxonomic classification of this virus. The protein and RNA patterns that we observed during astrovirus infection are not consistent with those of either of the established families of nonenveloped RNA viruses, *Picornaviridae* and *Caliciviridae*.

MATERIALS AND METHODS

Cells and virus. LLCMK2 cells were obtained from the American Type Culture Collection (ATCC CCL7.1) and were maintained in Earle minimal essential medium (EMEM) supplemented with antibiotics, nonessential amino acids, and 10% fetal bovine serum. Cell culture-adapted astrovirus serotype 2 was obtained from John Kurtz (Oxford, England) and was plaque purified through three consecutive cycles before use. Hyperimmune antiserum to human astrovirus serotype 2 was produced by immunization of rabbits with virus purified by equilibrium sedimentation in cesium chloride gradients (22) and was obtained from John Kurtz.

Virus growth and labeling. For the comparison of culture conditions, LLCMK2 cells in 35-mm wells were infected with astrovirus in EMEM plus 10 μg of trypsin per ml for 1 h at room temperature, after which the inoculum was removed and replaced with 1 ml of either EMEM plus 10 μg of trypsin per ml or EMEM plus 5% fetal bovine serum. At various times after infection, the supernatant medium was removed and the cells were lysed with 1 ml of 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–1 mM EDTA (TNE)–1% Triton X-100. The amount of astrovirus antigen in dilutions of the supernatant medium (released virus) and the cell lysate (cell-associated virus) was measured by enzyme immunoassay (EIA), using poly-L-lysine capture and antiastrovirus monoclonal antibody detector as previously described (14).

For the labeling experiments, LLCMK2 cells in 60-mm dishes were pretreated for 1 h with 2 ml of EMEM plus 20 μg of trypsin per ml and infected with 1 ml of astrovirus in the same medium at a multiplicity of three. After 1 h of incubation at 37°C, 1 ml of EMEM plus 2% fetal bovine serum was added and incubation was continued for various times. For protein labeling, infected cells were rinsed with EMEM minus methionine and then labeled for 2 h with 1 ml of EMEM minus methionine plus 20 μCi of [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.) per ml. For RNA labeling, infected cells were pretreated for 1 h with 1 μg of dactinomycin (Sigma Chemical Co.) per ml and then labeled for 2 h with EMEM plus 1 μg of dactinomycin plus 20 μCi of [^{3}H]uridine (NEN Research Products) per ml. At the end of the labeling period, the supernatant medium was removed and the cells were rinsed twice with TNE and lysed with TNE–0.5% Triton X-100.

EM. Infected cells were scraped from the culture dish, fixed with 2% glutaraldehyde for 1 h on ice, and washed with collidine buffer. The cell pellet was then postfixed in 1% osmium tetroxide and embedded in plastic (25). Sections were cut on a Reichert Ultracut E with a Dupont diamond knife, stained with uranyl acetate and lead citrate, and examined in a Philips 410 EM at 40 kV (25).

Protein gel electrophoresis and radioimmunoprecipitation assay. Proteins were concentrated from cytoplasmic lysates by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% followed by precipitation with 5 volumes of acetone. Samples were boiled in loading buffer containing 2% SDS and 5% 2-mercaptoethanol before analysis on 8 to 20% acrylamide gradient gels with a 4% stacking gel (30). Electrophoresis was at 15 mA constant current for 4.5 to 5.5 h. Following electrophoresis, gels were fixed, impregnated with a fluorographic enhancer (Amplify; Amersham Corp.) under conditions recommended by the manufacturer, dried, and exposed to X-AR2 film (Kodak).

A radioimmunoprecipitation assay was done essentially as described by Sanchez and Kiley (31) with the inclusion of 0.5% bovine serum albumin in the initial washes to reduce nonspecific binding. Lysates from uninfected or astrovirus-infected cells were incubated in the absence or presence of 10 μg of trypsin per ml for 30 min at 37°C, followed by the addition of soybean trypsin inhibitor (Sigma) to 10 μg/ml and phenylmethylsulfonyl fluoride (Sigma) to 1 mM. The treated lysates were reacted with normal or immune rabbit serum at a final concentration of 1% at 4°C overnight. Immune complexes were precipitated with protein A-bearing *Staphylococcus aureus* cells (Boehringer Mannheim Biochemicals). Precipitated proteins were released by boiling in loading buffer and were cleared of the bacteria by centrifugation before analysis on gradient polyacrylamide gels as described above.

RNA purification and gel electrophoresis. Total cytoplasmic RNA was purified from cell lysates by guanidinium isothiocyanate (Boehringer Mannheim)-phenol-chloroform extraction and ethanol precipitation (4). RNA samples were denatured with 1 M glyoxal at 50°C for 1 h before being resolved on a 1% agarose (International Biotechnologies, Inc.) gel prepared in 10 mM sodium phosphate, pH 6.9 (30). After electrophoresis for 6 h at 3 V/cm, the gel was fixed and treated with a fluorographic enhancer (En^{3}Hance; NEN Research Products) as recommended by the manufacturer.

Total cytoplasmic RNA from astrovirus-infected cells was purified by oligo(dT)-cellulose (Pharmacia LKB Biotechnology) chromatography as described previously (30). After chromatography, equivalent percentages of the total RNA loaded onto the column, the poly(A)-negative flowthrough fraction, and the poly(A)-positive bound fraction were analyzed by agarose gel electrophoresis as described above, without denaturation. Following electrophoresis, the gel was first stained with 0.5-μg/ml ethidium bromide (Sigma) and the fluorescent image was recorded with Polaroid Type 57 film. The same gel was subsequently processed for fluorography as described above.

RESULTS

Antigen synthesis. The initial report of the adaptation of astrovirus to growth in cultured cells demonstrated the need for the presence of trypsin in the culture medium to support continuous virus replication, while noting that a single cycle of astrovirus infection occurs in the absence of trypsin (21). As a first step in characterizing the macromolecular synthesis in astrovirus-infected cells, we used an EIA (14) to compare the time course of astrovirus protein synthesis during a single-cycle infection in cells incubated in the presence of trypsin with that of cells incubated in the absence of trypsin with the addition of serum. In the presence of trypsin, antigen was first detectable at 23 h postinfection (p.i.) and increased gradually until 48 h p.i.

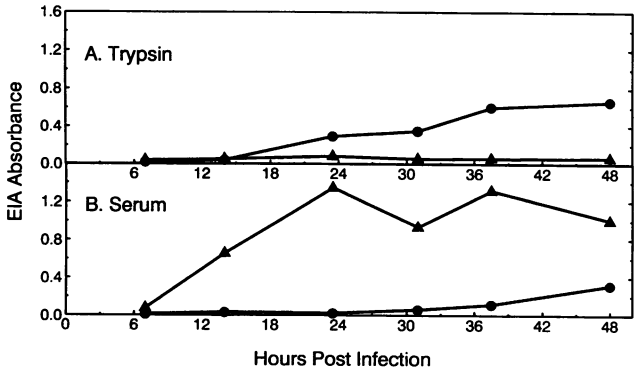

FIG. 1. Time course of astrovirus antigen synthesis. Infected cells were incubated in the presence of 10 μg of trypsin per ml (A) or 5% fetal bovine serum (B). At the indicated times after infection, the amounts of astrovirus antigen released into the culture medium (●) and the antigen remaining associated with the cell monolayer (▲) were determined by EIA.

(Fig. 1A). All the antigen was released into the culture medium, with no detectable antigen remaining associated with the cells. This pattern is consistent with the initial report of astrovirus replication as measured by an immunofluorescence infectivity assay (21). In contrast, when serum was present in the culture medium, quite a different pattern of antigen synthesis was observed. Antigen was detected as early as 7 h p.i. and increased rapidly to a plateau by 23 h p.i. (Fig. 1B). The majority of the antigen remained associated with the cells and relatively little was released into the culture medium until 38 to 48 h p.i. Not only was antigen synthesis detected earlier in the presence of serum, but also the level of antigen production, as measured by the EIA, was 20 to 50 times greater.

Thin-section EM. The EIA detects astrovirus structural protein antigens but probably does not discriminate between individual capsid proteins and complete virions. To determine whether the high level of antigen produced in cells infected in the presence of serum was correlated with virion production, we examined astrovirus-infected cells using thin-section transmission EM. Astrovirus particles were visualized in the cytoplasm of infected cells beginning at 12 h p.i. (Fig. 2) and were also noted at 15 and 18 h p.i. (data not shown). The virus was seen in large cytoplasmic aggregates

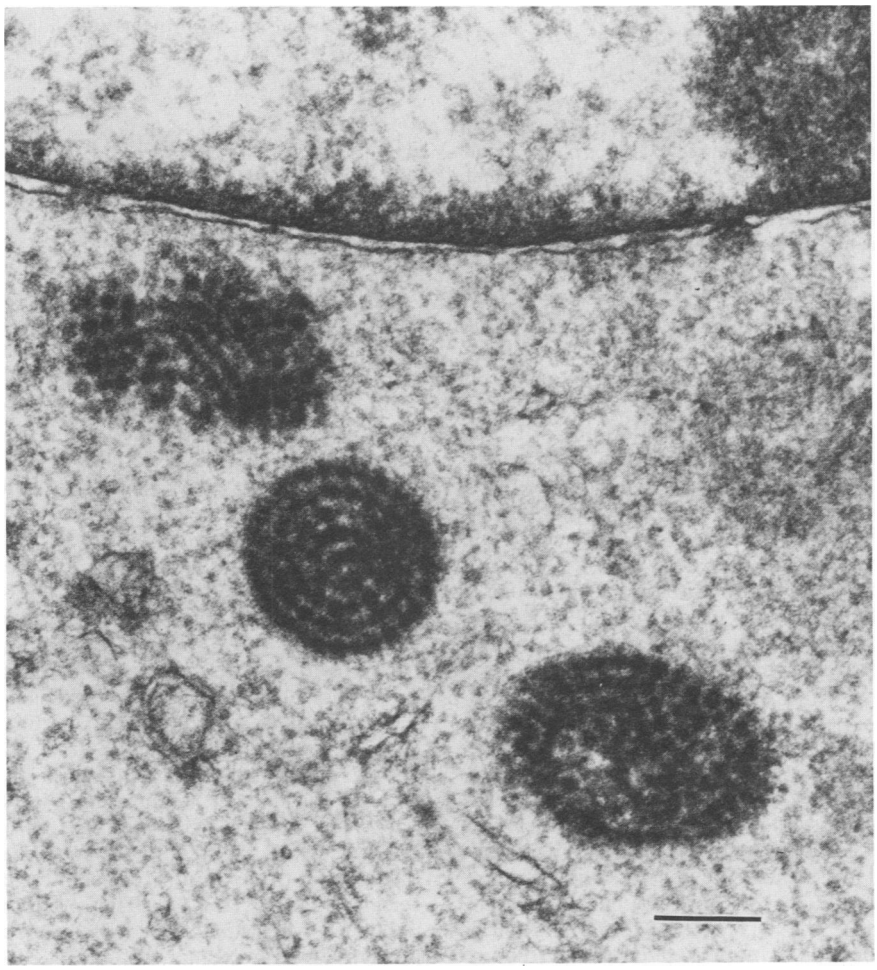

FIG. 2. Electron micrograph of an ultrathin section of LLCMK2 cells 12 h after infection with astrovirus. Aggregates of virus are present in the cytoplasm. The nuclear membrane is near the top of the figure. Bar = 200 nm.

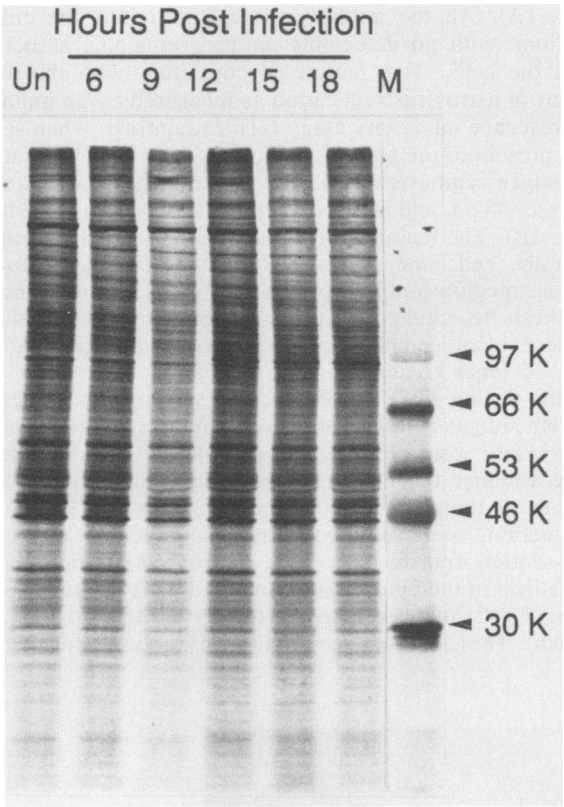

FIG. 3. Time course of astrovirus protein synthesis. Astrovirus-infected cells were labeled with [^{35}S]methionine for 2 h before a total cytoplasmic extract was prepared at the indicated times p.i. Proteins were resolved on an 8 to 20% polyacrylamide gradient gel and detected by fluorography. The lane marked Un is an uninfected cell extract. The masses (in kilodaltons) of the ^{14}C-labeled marker proteins run in lane M are indicated at right.

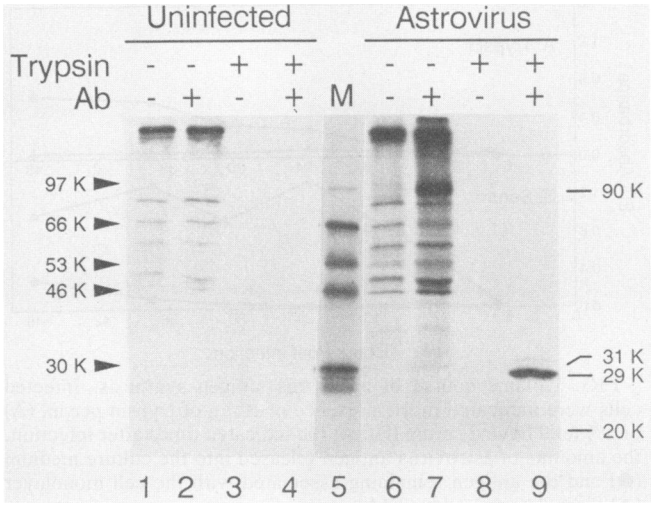

FIG. 4. Immunoreactivity and trypsin sensitivity of astrovirus capsid protein precursor. Lysates from uninfected cells (lanes 1 to 4) or astrovirus-infected cells (lanes 6 to 9) were incubated without (lanes 1, 2, 6, 7) or with (lanes 3, 4, 8, 9) trypsin in vitro, were reacted with normal (lanes 1, 3, 6, 8) or antiastrovirus immune (lanes 2, 4, 7, 9) rabbit serum, and were precipitated with protein A-bearing *S. aureus*. Immune complexes were released from the bacteria, and the precipitated proteins were resolved by gradient gel electrophoresis and detected by fluorography. The masses (in kilodaltons) of the ^{14}C-labeled marker proteins run in lane M are indicated at left. Ab, Antibody.

similar to those previously described in astrovirus-infected primary HEK cells (20).

Protein synthesis. The time sequence of synthesis of astrovirus proteins during infection in the presence of serum was examined by labeling cells with [^{35}S]methionine for 2-h pulses at 3-h intervals p.i. A total cytoplasmic extract was prepared, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Under the conditions of infection used, only 15 to 25% of the cells were productively infected with astrovirus, as measured by immunofluorescence (data not shown), resulting in substantial incorporation of radioactive methionine into cellular proteins. Despite this high background, a 90-kDa infection-specific protein, which migrated slightly faster than the 97-kDa marker, was detected beginning at 12 h p.i. and continuing through 18 h p.i. (Fig. 3).

Previous descriptions of the capsid structure of human, ovine, and porcine astrovirus (11, 19, 33) have reported two or more proteins of 26 to 34 kDa. Although we did not detect proteins of this size, it is possible that they were synthesized during infection but were not readily apparent owing to comigration with cellular proteins. We used a radioimmunoprecipitation assay to reduce the background of cellular proteins and to identify those proteins containing viral structural antigenic domains. Uninfected or astrovirus-infected cells were labeled for 6 h beginning at 12 h p.i., and total cytoplasmic extracts were prepared by detergent lysis. The lysates were incubated with normal rabbit serum or anti-human astrovirus serotype 2 immune rabbit serum, the immune complexes were precipitated with protein A-bearing *S. aureus*, and the labeled proteins were resolved by SDS-PAGE (Fig. 4). This procedure eliminated most, but not all, of the background, and several cellular proteins were precipitated when the lysate from uninfected cells was reacted with either normal (Fig. 4, lane 1) or immune (lane 2) rabbit serum and when the lysate from infected cells was reacted with normal serum (lane 6). The 90-kDa astrovirus-specific protein, in contrast, was detected only when a lysate from infected cells was reacted with immune serum (Fig. 4, lane 7), confirming that the 90-kDa polypeptide contains antigenic domains present on the structural proteins of purified astrovirus.

Since previous reports of the structural proteins of astrovirus analyzed virus grown in the presence of trypsin, we examined the effect of in vitro trypsin treatment on the 90-kDa protein. The lysates from uninfected or astrovirus-infected cells were incubated with 10 μg of trypsin per ml, treated with soybean trypsin inhibitor and phenylmethylsulfonyl fluoride to inactivate the trypsin activity, and reacted with normal or immune rabbit serum as described above. Trypsin treatment hydrolyzed most of the cellular proteins, and no radiolabeled proteins were precipitated from uninfected cells with either normal (Fig. 4, lane 3) or immune (lane 4) serum or from infected cells using normal serum (lane 8). Three proteins were specifically precipitated from the trypsin-treated infected cell lysate by using immune serum (Fig. 4, lane 9). The predominant protein has a molecular weight of 29,000 with faint bands corresponding to proteins with molecular weights of 31,000 and 20,000.

RNA synthesis. We examined the pattern of RNA synthesis during astrovirus infection in the presence of serum by

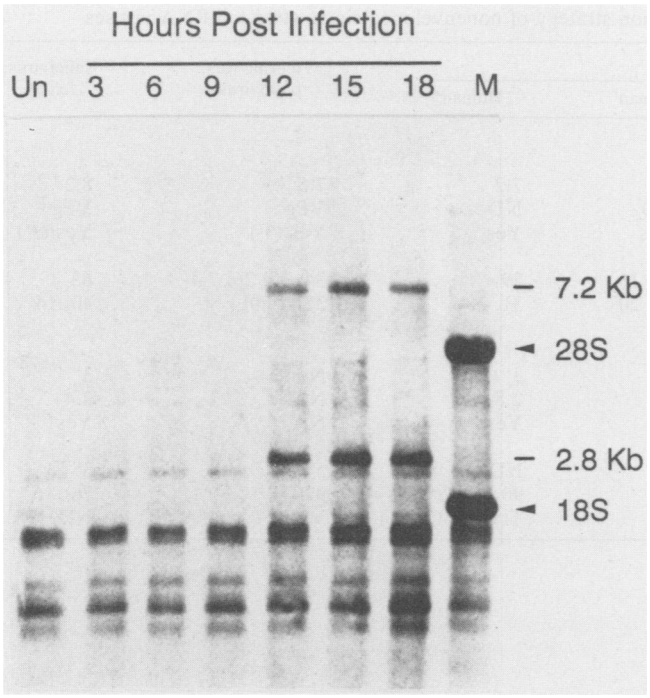

FIG. 5. Time course of astrovirus RNA synthesis. Astrovirus-infected cells were labeled with [^{3}H]uridine in the presence of dactinomycin for 2 h before a cytoplasmic extract was prepared at the indicated times p.i. Total RNA was purified from the extracts, denatured with glyoxal, and resolved by electrophoresis in a 1% agarose gel. The positions of the 28S and 18S rRNAs, labeled in the absence of dactinomycin, are indicated by arrowheads. The calculated sizes of the astrovirus-specific RNAs are indicated at right. Lane Un is an uninfected cell extract.

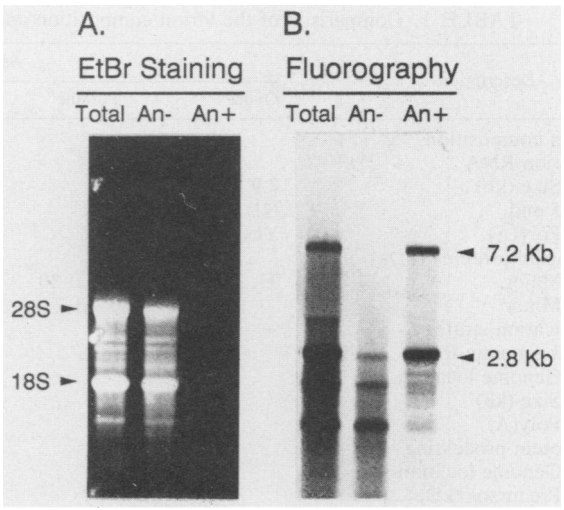

FIG. 6. Oligo(dT)-cellulose chromatography of astrovirus-specific RNAs. Total cytoplasmic RNA was purified from astrovirus-infected cells and fractionated by oligo(dT)-cellulose chromatography. Equivalent percentages of the unfractionated (Total), column flowthrough (An−), and specifically bound (An+) fractions were resolved by nondenaturing agarose gel electrophoresis. Total RNA was detected by ethidium bromide fluorescence (A), after which labeled RNA was detected by fluorography (B) of the same gel.

labeling cells treated with dactinomycin with [^{3}H]uridine for 2-h pulses at 3-h intervals p.i. Total RNA was isolated from cytoplasmic extracts, and the purified RNAs were resolved by agarose gel electrophoresis. Two astrovirus-specific RNA species were detected in cytoplasmic RNA beginning at 12 h p.i. with maximum synthesis at 15 and 18 h p.i. (Fig. 5). The larger RNA has a calculated size of 7.2 kb ($M_r = 2.5 \times 10^6$), similar to the reported size of virion RNA from purified ovine astrovirus (11). The smaller RNA has a calculated size of 2.8 kb ($M_r = 0.95 \times 10^6$) and has not been previously reported.

The astrovirus-specific RNAs were further characterized for the presence of a poly(A) sequence by oligo(dT)-cellulose chromatography. The majority of the mass of cytoplasmic RNA is composed of the 28S and 18S rRNAs, which were readily detected by ethidium bromide fluorescence in both the total RNA and the poly(A)-negative fraction (Fig. 6A). In the presence of dactinomycin, the de novo synthesis of rRNAs is inhibited, and the predominant RNA species labeled by [^{3}H]uridine incorporation were the astrovirus-specific 7.2- and 2.8-kb RNAs (Fig. 6B). Both of these RNA species were retained on the oligo(dT)-cellulose column and specifically eluted in the poly(A)-positive fraction (Fig. 6B).

DISCUSSION

We characterized the temporal sequence of the synthesis of proteins and RNAs in cells infected with human astrovirus (serotype 2), allowing a comparison of macromolecules detected in infected cells with those reported previously from purified virions of other human and animal strains. Our results demonstrate culture conditions for enhanced virus replication, provide insights into the molecular basis for the trypsin requirement for astrovirus propagation, and identify previously unreported intracellular intermediates in the virus replication pathway.

Sustained replication of human (21) and animal (1a, 10, 37) astroviruses in cell culture is dependent on the inclusion of trypsin in the culture medium, although a nonproductive infection occurs in the absence of trypsin (21). Rotavirus, another human and animal enteric pathogen, also requires trypsin for continuous growth in culture, but increased yields of virus can be obtained during a single-cycle infection when serum is included in the medium (1). We compared the kinetics and magnitude of human astrovirus replication in the presence of trypsin with those in the presence of serum to determine whether inclusion of serum would enhance the replication of astrovirus. Our results show that astrovirus replication progresses more rapidly and with synthesis of 20- to 50-fold-higher levels of antigen when serum is included in the medium. We suspect that the increased rate and level of synthesis of astrovirus antigen is a reflection of the increased metabolic capacity of cells cultured in the presence of the growth factors and nutrients found in serum.

Trypsin, or other proteolytic activities, have been shown to be essential for the continuous propagation in cell culture of many groups of enteric viruses, including human group A rotaviruses (35), porcine group C rotavirus (34), and human and porcine enteric caliciviruses (5, 7). The precise nature of the cleavage activation required for infectivity has been identified only for group A rotaviruses (6). Trypsin treatment appears to have two effects on astrovirus replication: it facilitates the release of virus from infected cells into the supernatant medium, and it catalyzes, directly or indirectly, the cleavage of a putative capsid protein precursor to multiple smaller proteins. Further studies will be necessary to

TABLE 1. Comparison of the virion composition and replication strategy of nonenveloped single-stranded RNA viruses

Description	Astrovirus				Picornavirus, poliovirus[e]	Calicivirus, feline[f]
	Ovine[a]	Porcine[b]	Human[c]	Human[d]		
Viron composition						
Virion RNA						
Size (kb)	7.9	ND[g]	~7	7.2	7.5	8.2
5'end	ND	ND	ND	ND	VPg	VPg
Poly(A)	Yes	ND	Yes	Yes	Yes (3')	Yes (3')
Capsid proteins (kDa)						
Major	33, 33	39, 31, 30	31, 16	29	34, 30, 26, 7.4	62
Minor		36, 13	45, 20	31, 20	37 (VP0)	40, 14
Replication strategy						
Subgenomic RNA						
Genome location				3' (?)	None	3'
Size (kb)				2.8	NA[h]	2.4
Poly(A)				Yes	NA	Yes
Protein processing						
Genome location				ND	5'	3'
Precursor (kDa)				90	97	76
Protease(s)				Trypsin (?)	Viral	ND

[a] Herring et al. (12).
[b] Shimizu et al. (33).
[c] Grohmann (10a).
[d] This report.
[e] Rueckert (29).
[f] Carter (3) and Neill and Mengeling (27).
[g] ND, Not determined.
[h] NA, Not applicable.

determine the causal relationship, if any, between these two phenomena.

Previous reports of the capsid structure of animal astroviruses describe surprising variability in the number and sizes of the proteins detected (Table 1). The only consistently reported feature of astrovirus capsid structure has been the presence of at least two proteins of approximately 30 kDa. When we analyzed human astrovirus grown with serum in the absence of trypsin, we failed to detect any infection-specific proteins of approximately 30 kDa and instead observed a single infection-specific protein of 90 kDa. This protein was specifically precipitated by antiastrovirus rabbit serum, suggesting that it is a capsid protein precursor. Synthesis of this putative precursor protein was coincident with the appearance of recognizable virus in the cytoplasm of infected cells, indicating that the precursor form of the protein can be assembled into intact virions. Treatment of the 90-kDa precursor with trypsin in vitro resulted in the appearance of three immunoreactive proteins migrating at 31, 29, and 20 kDa, with the 29-kDa protein being the predominant species labeled with [^{35}S]methionine. The unequal pattern of label incorporation into the three cleavage products may indicate that the 31- and 20-kDa species are not generated stoichiometrically from the 90-kDa precursor or may simply reflect a lower proportion of methionine in these proteins.

We cannot explain the variation in the observed capsid protein composition of human and animal astroviruses (Table 1). Purified ovine astrovirus has two 33-kDa capsid proteins (11), a recent characterization of a porcine astrovirus identified five capsid proteins ranging from 13 to 39 kDa (33), and the human virus (serotype 1) reportedly contains three or more proteins ranging from 32 to 36.5 kDa (19). All recognized picornaviruses contain four capsid proteins which range from 23.3 to 33.5 kDa for VP1, VP2, and VP3 and from 7.2 to 8.5 kDa for VP4 (29). The similarity in size of VP1, VP2, and VP3, particularly in aphthoviruses, often makes it difficult to resolve these proteins by traditional SDS-PAGE (29). The smallest protein, VP4, is often not detected at all (29). The two 33-kDa proteins detected in ovine astrovirus were reportedly not resolved under certain conditions of electrophoresis, so the possibility exists that we and others are underestimating the true number of capsid proteins in astroviruses. The use of different electrophoresis conditions or other separation techniques may help to clarify the number and sizes of astrovirus capsid proteins.

We detected two infection-specific poly(A)-containing RNAs in cells infected with human astrovirus, whereas a previous report had indicated only a single RNA in purified ovine astrovirus particles (11). The presence of two cytoplasmic RNAs during human astrovirus infection could result either from a mixed inoculum containing defective interfering particles or from synthesis of a subgenomic RNA during virus replication. The input virus used for the labeling experiments was only three passages removed from plaque purification, making it unlikely that defective interfering particles were present in the inoculum. The more likely explanation is that the 2.8-kb RNA present in astrovirus-infected cells is a subgenomic mRNA which is not packaged into virions. An mRNA of this size could theoretically encode a polypeptide slightly larger than the 90-kDa precursor protein. Incorporation of [^{3}H]uridine into the smaller RNA was greater than incorporation into the genomic-length RNA, which, together with the 2.5-fold size difference, indicates that the smaller species is synthesized at approximately 5- to 10-fold-higher molar amounts throughout infection. The replication pathways of both caliciviruses and togaviruses involve synthesis of intracellular subgenomic RNAs, which encode the virus structural proteins, at higher levels than those of the genomic RNA (27, 32). We have recently generated astrovirus cDNA clones which contain a poly(T) tract and which hybridize to both the 7.2- and 2.8-kb

RNAs (data not shown), suggesting that the two RNAs have sequences in common and are 3′ coterminal. These clones will enable us to analyze the synthesis of minus-sense RNAs during astrovirus infection, which should indicate whether the 2.8-kb RNA is replicated autonomously or is transcribed from a genome-length minus-strand template. Detailed analysis of the protein-coding and gene expression strategy of astroviruses will require determination of the nucleotide sequence and subsequent mapping of open reading frames of the genomic and subgenomic RNAs.

The classification of a virus is based primarily on genome composition (RNA or DNA, single or double stranded), length, segmentation, and polarity and on the virion capsid structure (enveloped or nonenveloped, and the number and sizes of capsid proteins). We characterized the proteins and RNAs synthesized during astrovirus infection of LLCMK2 cells with a goal of arriving at a definitive classification for this group of human and animal pathogens. There are two families of nonenveloped single-stranded RNA viruses currently recognized by the International Committee on Taxonomy of Viruses, *Picornaviridae* and *Caliciviridae* (26). Viruses in both of these families have a single-stranded RNA genome of 7 to 8 kb with a 3′ poly(A) sequence, features also common to astroviruses (Table 1). Picornaviruses and caliciviruses also have a small protein (VPg) covalently attached to the 5′ end of their genome RNA, while the structure of the 5′ end of astrovirus genome RNA (VPg, m^7G cap, etc.) remains to be determined. What distinguishes picornaviruses from caliciviruses is the number, size, and genome coding location of their capsid proteins. Picornaviruses have four capsid proteins which are encoded at the 5′ end of the genome RNA and which are generated by cleavage of an approximately 97-kDa precursor protein catalyzed by virus-encoded proteases (29). Feline calicivirus, in contrast, has a single major capsid protein (62 kDa) which is encoded at the 3′ end of the genome, is translated from a subgenomic RNA, and is generated by cleavage of a 76-kDa precursor by one or more as yet uncharacterized proteases (3, 27). Thus, while the composition of mature virions of astroviruses resembles that of picornaviruses, the replication strategy of astroviruses more closely resembles that of caliciviruses (Table 1). The differences between astroviruses and the two recognized families of nonenveloped RNA viruses in these defining characteristics are significant and warrant consideration of a new family designation.

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