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Association of the Reovirus S1 Gene with Serotype 3-Induced Biliary Atresia in Mice

GREAME A. R. WILSON,[1]† LYNDA A. MORRISON,[1] AND BERNARD N. FIELDS[1,2,3]*

Department of Microbiology and Molecular Genetics, Harvard Medical School,[1] Department of Medicine, Brigham & Women's Hospital,[2] and Shipley Institute of Medicine,[3] Boston, Massachusetts 02115

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A panel of serotype 3 (T3) reovirus strains was screened to determine their relative capacities to cause lethal infection and hepatobiliary disease following peroral inoculation in newborn mice. A wide range of 50% lethal doses (LD_{50}s) was apparent after peroral inoculation of the different virus strains. Two of the strains, T3 Abney and T3 clone 31, caused mice to develop the oily fur syndrome associated with biliary atresia. The capacity to cause biliary atresia was not related to the capacity to cause lethal infection, however, because the LD_{50}s of T3 Abney and T3 clone 31 were grossly disparate. Examination of liver and bile duct tissues revealed histopathologic evidence of biliary atresia and hepatic necrosis in T3 Abney-infected mice but not in mice inoculated with a T3 strain of similar virulence or with the hepatotropic T1 Lang strain. The consistency with which T3 Abney-infected mice developed biliary atresia-associated oily fur syndrome permitted us to determine the viral genetic basis of reovirus-induced biliary atresia. Analysis of reassortant viruses isolated from an in vitro coinfection with T3 Abney and T1 Lang indicated a strong association of the hepatobiliary disease-producing phenotype with the T3 Abney S1 gene, which encodes the viral cell attachment protein, σ1. Amino acid residues within the σ1 protein that were unique to disease-producing T3 strains were identified by comparative sequence analysis. Specific changes exist within two regions of the protein, one of which is thought to be involved in binding to host cell receptors. We hypothesize that changes within this region of the protein are important in determining the tropism of this virus for bile-ductular epithelium.

Reoviruses are nonenveloped double-stranded RNA viruses with segmented genomes which infect a large variety of hosts and tissues. The outer capsid of the virus consists of a minor component, the σ1 protein, and two major components, the σ3 and μlc proteins (17, 36). σ1 serves as the viral cell attachment protein and hemagglutinin (18, 46), while both σ3 and μlc are involved in various functions in addition to maintaining outer capsid structure (1, 14, 18, 24, 34, 36). Reoviruses are primarily nonpathogenic enteric viruses whose life cycle involves spread to, and infection of, distant tissues and cells in a serotype-dependent manner (16, 19, 45, 47). Their pattern of entry and spread closely resembles that of a number of enteric viruses which are significant human and animal pathogens.

Following peroral (p.o.) inoculation in mice, reoviruses undergo primary replication within the lymphoid tissue of the gastrointestinal tract and subsequently gain access to secondary tissue sites following either hematogenous or direct neural spread from this initial site of entry (16, 21, 33). The genetic bases for cellular tropism within the brain (46) and capacity to spread via neural routes (43) have been linked by reassortant analysis to the viral S1 gene, which encodes the σ1 protein. The development of specific pathology following spread to distant tissues depends not only on various host factors but also on the particular strain of infecting reovirus. Previous researchers have described the development of hepatobiliary disease following intraperitoneal injections of newborn mice with high doses of the reovirus serotype 3 (T3) Dearing (T3D) strain, in which the pathology consists of inflammation and necrosis of the intra- and extrahepatic bile ducts (25, 40). These observations led to the proposal that this type of infection be used as a model for studying neonatal hepatitis (40). Additional research involving T3D infection of weanling mice uncovered histological evidence of a selective tropism of this strain for bile ductular epithelium, resulting in cellular fibrosis following epithelial infection and necrosis (26). However, in both of these models of biliary atresia, virus only sporadically induced disease and had to be artificially inoculated into the peritoneum in high doses, circumventing the natural route of reovirus infection.

The T3D strain does not readily infect mice when inoculated p.o., but several other T3 strains are highly infectious by this route (21). We therefore surveyed T3 strains for the capacity to cause biliary atresia in mice after p.o. inoculation in an effort to identify a strain that would consistently cause disease. We have identified two such strains and have used them in reassortant and sequence analyses to uncover a viral genetic basis for their tissue tropism and capacity for hepatobiliary disease induction.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted mouse L929 (L) cells were grown in either suspension or monolayer cultures in Joklik modified Eagle minimal essential medium (Irvine Scientific, Santa Ana, Calif.) that was supplemented with 2.5% fetal calf serum (Hyclone Laboratories, Logan, Utah), 2.5% viable serum protein agamma sera (Biocell Laboratories, Carson, Calif.), 2 mM glutamine, 1 U of penicillin per ml, and 1 μg of streptomycin (Irvine Scientific) per ml. Reovirus strain T3D was grown from laboratory stocks. T3 clone 9 (T3C9), T3C31, T3C43, T3C60, T3C84, and T3C87 (Abney) were originally isolated by Rosen et al. (29–31) and have been

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Building D1-Room 421, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 432-1935. Fax: (617) 738-7664. Electronic mail address: bfields@warren.med.harvard.edu.

† Present address: Surveillance, Influenza & Viral Exanthemata, Laboratory Center for Disease Control, Ottawa, Ontario, Canada K1A 0L2.

further characterized in our laboratory (6, 13). Reovirus stocks were twice plaque purified before use. Purified virion preparations were generated from second-passage L-cell lysates as previously described (11).

Reassortant virus isolation. Reovirus reassortants were generated from nonmutagenized stocks of reovirus serotype 1 (T1) Lang (T1L) and T3 Abney essentially as previously described (8). Briefly, 2×10^5 L cells were coinfected with parental stocks of T1L and T3 Abney at various multiplicities of infection and incubated at 37°C for 18 h. The coinfections were then frozen and thawed three times before being diluted and inoculated onto L-cell monolayers. Well-isolated plaques were picked into 1 ml of supplemented medium and incubated at 4°C overnight. First-passage stocks were then prepared in 25-cm^2 tissue culture flasks and incubated at 37°C until development of cytopathic effect. The double-stranded RNA content of each isolate was then examined as previously described (27, 35). All potential reassortants were plaque purified twice more with confirmation of their genotypes at each stage and were used as purified virion preparations.

Animals and inoculations. Purified virions were freshly diluted in phosphate-buffered saline (PBS), pH 7.2, prior to p.o. inoculation. Blue food dye (2 μl/ml) was added to virus suspensions to permit monitoring of the inoculations.

Mice were maintained in accordance with standards outlined in both Public Health Service and National Institutes of Health guidelines (5a). Two-day-old NIH (Swiss) mice (National Cancer Institute, Frederick, Md.) were inoculated p.o. with various numbers of PFU of purified virions in a 30-μl volume through a catheter passed into the esophagus (33).

Statistical analyses. At least five groups of 2-day-old mice (8 to 12 mice per group) were inoculated by the p.o. route with various doses of reovirus and observed daily for survival and development of oily fur. Experiments were terminated 30 days postinfection, and 50% lethal dose (LD_{50}) and 50% oily fur dose values for each virus were calculated by the method of Reed and Muench (28).

Tissue collection and virus titer determination. On various days postinoculation, mice were euthanized and the small intestine, liver, and/or brain was dissected. For titer determination, tissues were placed into vials containing 2 ml (or 5 ml for brain samples) of ice-cold gel saline, pH 7.2, and frozen at −20°C. Samples were frozen and thawed once prior to disruption by sonication for 30 on ice as previously described (4). Virus titers were determined by a standard plaque assay (42).

Immunohistochemistry. Tissue samples for histology were immersed in 4% neutral buffered formalin (Fisher Scientific, Pittsburgh, Pa.), pH 7.4, for 24 h and then transferred to 70% ethanol prior to being embedded in paraffin and sectioned. Paraffin was removed from tissue sections by immersion in xylene and graded alcohols, and sections were stained for virus antigen by the immunoperoxidase method with polyclonal rabbit antireovirus antiserum and a Vectastain Elite ABC reagent kit (Vector Laboratories, Burlingame, Calif.) as previously described (21).

Comparison of σ1 amino acid sequences. Multiple amino acid sequence alignment was facilitated by using the multiple sequence alignment program PileUp from the University of Wisconsin Genetics Computer Group (Madison).

RESULTS

Determination of virulence and disease induction following p.o. inoculation. We systematically screened a panel of T3 strains (13), after initiating infection via the natural p.o. route, for their relative capacities to cause lethal infection and to produce the "oily fur" syndrome that results from steatorrhea and has been associated previously with reovirus-induced biliary atresia (25, 26, 39). Groups of mice were inoculated p.o. with at least five dilutions of purified virion preparations and observed daily for general health and survival. The results, summarized in Table 1, indicated a broad range in the LD_{50}s among the T3 strains. LD_{50} values segregated into three groups: T3D, T3C31, and T3C84, a group whose LD_{50}s were greater than 10^8 PFU; T3C43 and T3 Abney, a highly lethal group with LD_{50}s of 2×10^2 to 5×10^2 PFU; and T3C9 and T3C60, an intermediate group that required approximately 3×10^5 to 9×10^5 PFU to produce 50% mortality. Although there was great variation in LD_{50} values among T3 strains, the mean numbers of days to death were not appreciably different, ranging from 10 to 12 days postinoculation (data not shown). The cause of death for each strain was not determined, although the previously reported neurotropic potential of T3D and T3C9 suggests that a fatal encephalitic infection may be involved in at least some cases (21, 43, 45).

TABLE 1. T3 strains

Isolate	Species[a]	LD_{50}[b]	Oily fur[c]
T3D	Human	$>10^7$	−
T3C31	Bovine	$>10^8$	+
T3C84	Human	$>10^9$	−
T3C9	Murine	2×10^6	−
T3C60	Bovine	3×10^5	−
T3C43	Bovine	6×10^2	−
T3 Abney	Human	2×10^2	+

[a] Species from which virus was originally isolated.
[b] Number of PFU resulting in 50% mortality following p.o. inoculation of 2-day-old NIH (Swiss) mice.
[c] Phenotype observed in all mice 10 days after p.o. inoculation. T3D and T3C9 failed to induce oily fur even when inoculated at a higher dose of 10^8 PFU. T3C60 and T3C43 were also negative at the highest dose tested (10^6 PFU).

Of the virus strains examined, only T3C31 and T3 Abney had the capacity to induce oily fur syndrome. The dose at which this syndrome became apparent differed between the strains and reflected their LD_{50} values: T3C31 ($LD_{50} > 10^8$ PFU) required 3×10^6 PFU to induce oily fur in 50% of the mice, while T3 Abney ($LD_{50} = 200$ PFU) was much more efficient at inducing oily fur, requiring as little as 30 PFU. The fact that T3C43 and T3 Abney possessed similar LD_{50} values but only inoculation of T3 Abney produced symptoms of biliary atresia indicated that the capacity of a given strain to cause lethal infection was not a prerequisite for induction of oily fur syndrome.

Reovirus tissue tropism in p.o. inoculated mice. We attempted to determine if there was a difference in the specific tissue tropisms of T3 Abney and T3C43 that could explain their similar lethalities in the face of obvious differences in hepatobiliary disease induction. Because T3 strains generally are highly neurotropic (21, 42) and T3C43 is known to spread rapidly to the central nervous system following p.o. inoculation (20), we compared the growth of T3 Abney with that of T3C43 in the livers and brains of mice following p.o. inoculation. T1L, an hepatotropic strain infectious by the p.o. route (32, 49), was included in this analysis. Two-day-old mice were inoculated p.o. with 10^6 PFU of T1L, T3C43, or T3 Abney, and liver and brain tissues were removed at specified times postinoculation and assayed for infectious virus. Virus was detected in liver samples of T1L-inoculated mice by 2 days postinoculation and reached a peak of 2×10^6 PFU/ml of tissue homogenate on

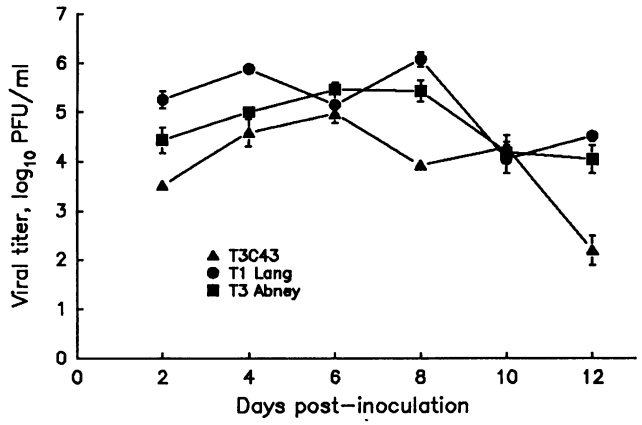

FIG. 1. Reovirus growth in livers of p.o. inoculated mice. Two-day-old mice were inoculated p.o. with 10^6 PFU of T1L, T3C43, or T3 Abney. The viral titers at various times postinoculation were determined by plaque assay of excised tissue. Each point represents the mean ± standard deviation for a minimum of six samples. Viral titers are expressed as $\log_{10}$ PFU per milliliter of tissue homogenate.

day 8 postinoculation (Fig. 1), demonstrating that, as expected, T1L is able to productively infect the liver following inoculation into the gastrointestinal tract. Titers of T3C43 and T3 Abney were lower than the titer of T1L on day 2 postinoculation but reached comparable levels by 6 days postinoculation. Peak titers for all three viruses occurred by 6 to 8 days postinoculation and began to fall thereafter. No virus was recovered from tissue samples assayed 21 days postinoculation (data not shown). Because all three viruses were able to productively infect the livers of p.o. inoculated mice but only T3 Abney infection resulted in signs of hepatic disease, growth alone, as determined by titers of virus in whole liver samples, did not confer the capacity to induce biliary atresia.

Virus titers in brain tissue of mice inoculated with T3C43, T3 Abney, or T1L were comparable through day 8 postinoculation, as shown in Fig. 2. Subsequently, titers of T3C43 increased to a maximum of 3.5×10^7 PFU on day 10

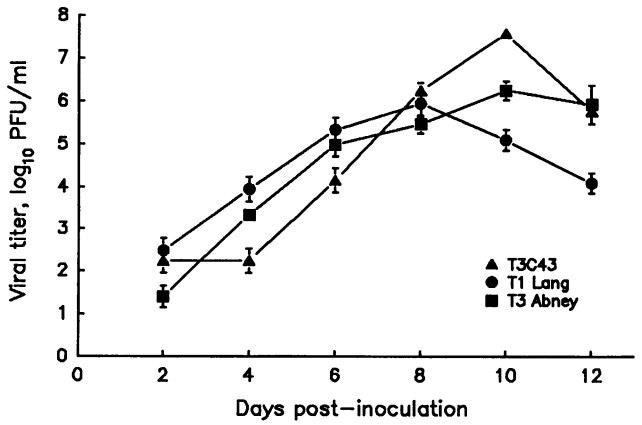

FIG. 2. Reovirus growth in brains of p.o. inoculated mice. Two-day-old mice were inoculated p.o. with 10^6 PFU of T1L, T3C43, or T3 Abney. The viral titers at various times postinoculation were determined by plaque assay of excised tissue. Each point represents the mean ± standard deviation for a minimum of six samples. Viral titers are expressed as $\log_{10}$ PFU per milliliter of tissue homogenate.

postinoculation. Titers for T3 Abney were also maximal on day 10, at 1.7×10^6 PFU, while T1L titers peaked on day 8 postinoculation, at 8.4×10^5 PFU, and declined thereafter. Thus, although the peak titer of T3C43 was 20-fold higher on day 10 postinoculation than that of T3 Abney, both of the T3 strains were able to productively infect the brains of p.o. inoculated mice. Taken together, these results suggest that T3C43 and T3 Abney possess similar capacities to spread to, and replicate in, the brain and liver.

Pathological changes within the livers of infected mice. Two-day-old mice were inoculated p.o. with 10^6 PFU of T1L, T3C43, or T3 Abney or with PBS as a control and were examined daily. The mice infected with T3C43 or T1L showed no physical differences throughout the period of study compared with mice inoculated with PBS. Mice infected with T3 Abney, however, developed the characteristic oily fur syndrome by day 10 postinoculation, were typically smaller than mice infected with T3C43 or T1L, were very lethargic, and showed evidence of jaundice. After mice infected with T3 Abney had developed the oily fur syndrome, we examined their livers and those of uninfected mice or mice infected with T3C43 or T1L for changes pathognomonic for biliary atresia. Histologic sections of livers were prepared and stained with hematoxylin and eosin or for viral antigen by the immunoperoxidase method. The livers from mice inoculated with PBS, T3C43 (Fig. 3A), or T1L (Fig. 3B) were essentially identical and showed few or no pathological changes which would indicate active infection. Some of the portal triads in livers from mice infected with T1L showed a slight mononuclear cell infiltration into surrounding areas (Fig. 3B), but these areas were limited and no other pathological changes were observed. In contrast, livers from T3 Abney-infected mice showed severe pathological changes (Fig. 3C and D) consistent with the previously described model of reovirus-induced biliary atresia (26). The areas surrounding portal triads showed extensive cellular proliferation and mononuclear cell infiltration with loss of bile duct epithelial cells and occlusion of bile ducts. Areas of extensive fibrosis extended between portal triads; these were probably caused by the proliferation of bile-ductular epithelium. We also frequently were able to identify distinct regions of hepatocellular necrosis containing hepatocyte ghosts and large amounts of cellular debris which were typically confined to the margins of the liver sections (data not shown).

We utilized immunoperoxidase staining of infected liver sections to positively identify reovirus antigens within areas of T3 Abney-induced pathology (Fig. 4A and B). The staining indicated the presence of virus antigen in bile duct epithelial cells within areas adjacent to affected portal triads (Fig. 4A) and within parenchymal cells in areas of extensive fibrosis throughout the liver (Fig. 4B). This pattern of antigen staining differed from that observed in the sections of liver from either T1L, T3C43, or other reovirus strains, which showed a more diffuse hepatocellular staining (Fig. 4C) and no specific staining of regions surrounding bile ducts. These results provided histopathologic evidence of the differential abilities of T1L and T3 Abney to induce biliary atresia, even though both were capable of infecting and growing within the livers of infected mice.

Mapping of the viral genetic basis for biliary atresia. The consistent induction of clinical symptoms of biliary atresia that is a feature of infection with T3 Abney permitted us to attempt to identify the gene(s) from T3 Abney associated with the capacity to cause biliary atresia. Reassortant viruses were generated between parental stocks of T3 Abney and T1L. Sixteen reassortants isolated from two independently derived

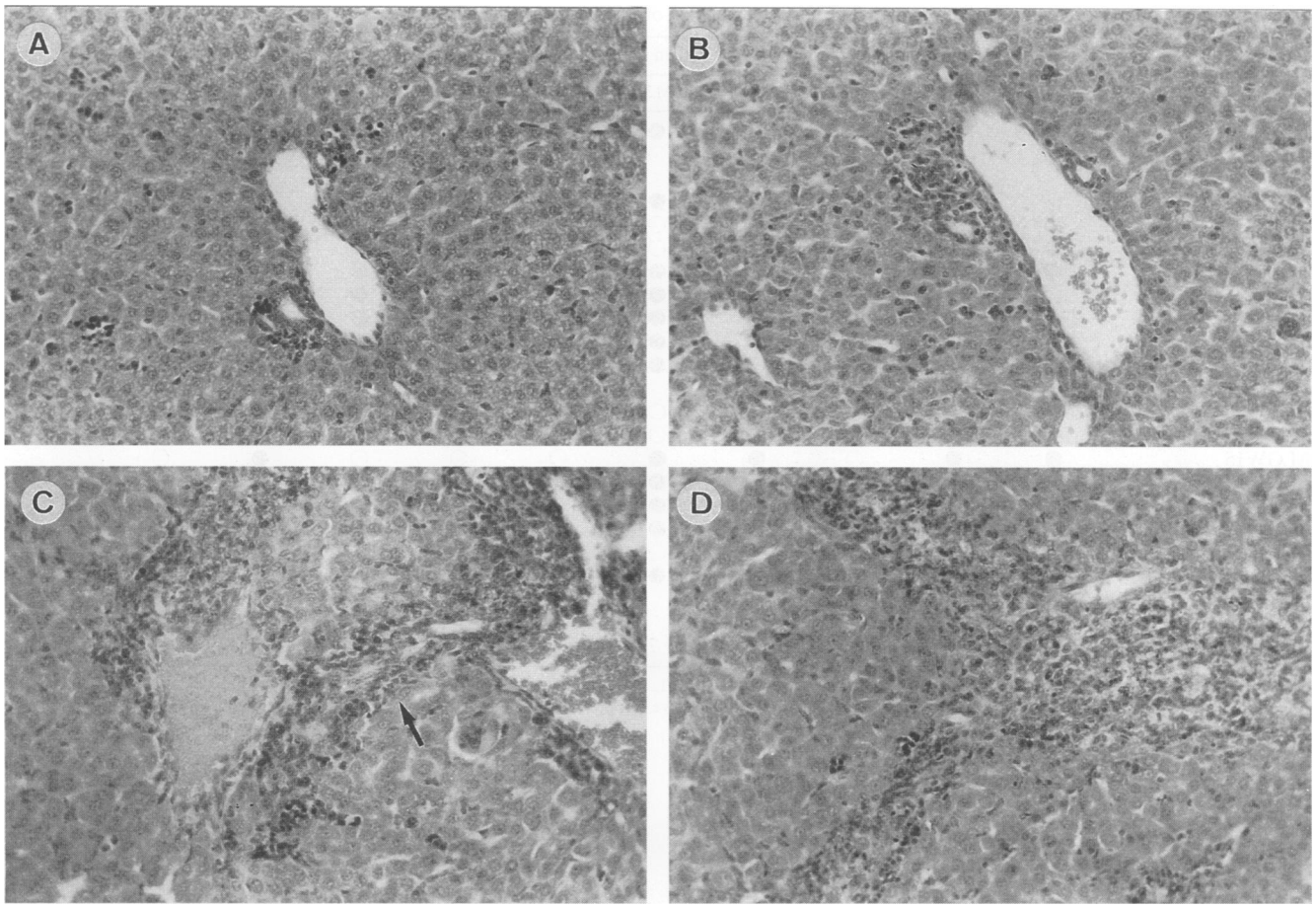

FIG. 3. Liver pathology following p.o. reovirus infection. (A) Portal triad from a mouse showing few pathological changes 10 days after inoculation with 10^6 PFU of T3C43. (B) Portal triad from a mouse inoculated with 10^6 PFU of T1L, showing slight mononuclear cell infiltrate. (C) Portal triad from a mouse inoculated with 10^6 PFU of T3 Abney, showing proliferation of bile-ductular epithelium and mononuclear cell infiltrate. Note the band of fibrosis that joins adjacent portal triads (arrow). (D) Section of liver infected with T3 Abney, showing the extensive fibrosis that extends throughout the liver. Magnification, ×89.

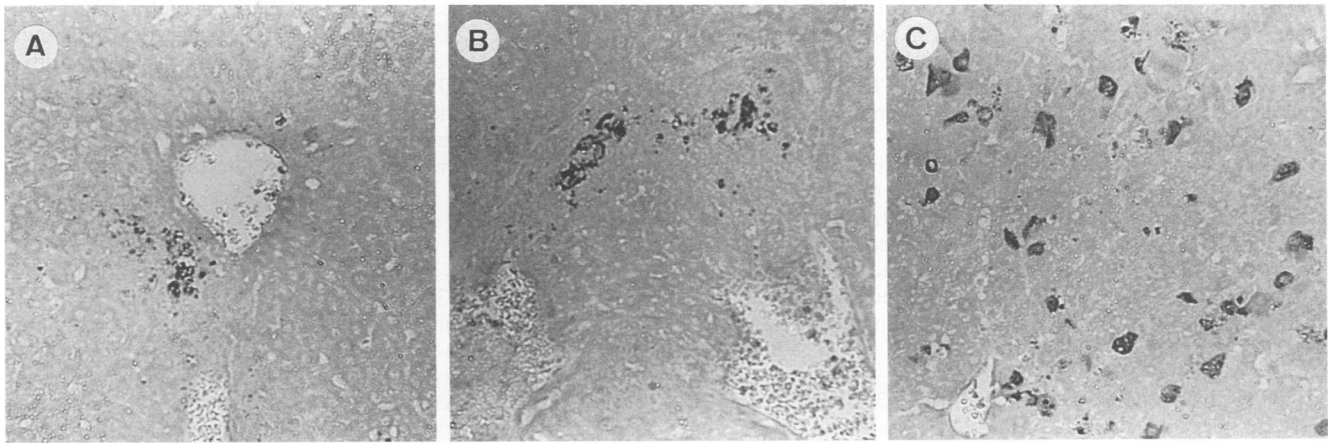

FIG. 4. Immunohistochemical staining of reovirus antigens in livers of infected mice. All mice were inoculated p.o. with 10^6 PFU of virus. (A) T3 Abney antigen staining surrounding a possible bile duct. (B) T3 Abney antigen staining within areas of fibrosis extending throughout the liver. (C) Antigen localization in tissue infected with Abney-Lang reassortant AL100, showing diffuse hepatocyte staining typical of reovirus isolates not causing biliary atresia. Magnification, ×36.

TABLE 2. Electropherotypes of T3 Abney × T1L reassortants

Virus	Origin of gene segment[a]										Oily fur[b]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
T3 Abney	●	●	●	●	●	●	●	●	●	●	+
T1L	○	○	○	○	○	○	○	○	○	○	−
ALGW7	○	○	○	○	●	●	●	○	●	●	+
ALGW12	●	○	○	●	○	●	●	○	●	○	+
ALGW13	●	●	○	●	○	●	●	●	●	●	+
ALGW24	●	○	○	○	●	●	●	●	●	●	+
ALGW30	○	○	○	●	●	○	●	●	●	○	+
ALGW33	●	○	○	○	●	○	●	●	○	●	+
ALGW49	○	○	○	●	●	○	●	●	●	●	+
ALGW928	○	○	○	○	●	○	●	●	○	○	+
ALGW4	●	○	○	○	○	●	○	○	●	●	−
AL8	○	○	●	●	○	○	○	○	○	○	−
ALGW15	●	●	○	●	●	○	○	○	●	○	−
ALGW31	●	○	○	●	●	○	○	●	●	○	−
AL36	●	●	●	○	●	○	○	●	○	●	−
AL100	○	○	○	○	●	○	○	○	○	●	−
AL118	●	●	●	○	○	●	○	●	●	●	−
AL133	●	●	●	●	●	●	○	●	●	●	−
Sum of exceptions	10	11	12	8	7	7	0	6	7	8	

[a] ●, gene segment derived from T3 Abney; ○, gene segment derived from T1L.
[b] Observed 10 days postinoculation. All mice in groups designated positive were symptomatic for oily fur syndrome.

stocks (15, 49), representing different combinations of genes from the two parental strains, were inoculated p.o. into newborn mice. A dose of 10^6 PFU was selected for the genetic analysis since it was well above the level required to produce oily fur in mice inoculated with the T3 Abney parental strain. The mice were examined throughout a 12-day period for the development of oily fur syndrome and biliary atresia. Of the 16 reassortants, 8 were able to induce biliary atresia (Table 2). Analysis of individual genes from each reassortant revealed that only the S1 gene (encoding the viral hemagglutinin and cell attachment protein, σ1) from T3 Abney was always associated with the capacity to confer the disease phenotype. The sum of exceptions, indicating the number of times a gene is not associated with a particular virus phenotype, was ≥6 for all gene segments except the S1 gene from T3 Abney (Table 2). Thus, the S1 gene from T3 Abney was genetically linked to the capacity of this reovirus strain to infect the liver and induce biliary atresia in mice following p.o. inoculation.

Comparative sequence analysis of the T3 strain S1 gene segments. Because the S1 gene of T3 Abney was associated with induction of biliary atresia, we made use of existing S1 sequence data to look for changes in individual amino acid residues or regions of variability within the T3 Abney σ1 protein which were specific to this virus strain. The deduced amino acid sequences of the σ1 protein from 10 T3 strains plus the T3 prototype Dearing strain that had been previously analyzed in our laboratory (7) were compared. These strains included a group of seven whose σl sequences are highly related: T3D, T3C43, T3C44, T3C45, T3C84, T3 Abney, and T3C93. A second group had slightly more variable sequences but identical total lengths (455 residues): T3C8, T3C9, T3C18, and the second biliary atresia-inducing strain, T3C31 (7). There were only six amino acid differences between T3 Abney and T3D throughout the entire σ1 protein. Of those changes, two were not shared with any of the non-biliary atresia-inducing T3 strains. The first specific amino acid difference occured at position 245, an arginine-to-glycine change in a region of the protein with great sequence variability among the T3 strains examined (Fig. 5A). This substitution falls just upstream of a domain of the protein proposed to be involved in carbohydrate binding and hemagglutination (Fig. 6) (6). Although the change at residue 245 in the T3 Abney σ1 protein sequence was unique to the Abney strain, an alanine-to-methionine change at nearby residue 248 is unique to the second biliary atresia-inducing strain, T3C31. The second specific amino acid difference between the σ1 sequence of T3 Abney and those of other T3 strains occured at position 356, a glutamate-to-asparagine change (Fig. 5B). The carboxy-terminal portion of the σ1 protein from approximately amino acid position 311 to position 455 (Fig. 6) previously has been identified as the head of the protein and contains all the information necessary for cell attachment or binding to host cell receptors (22). Significantly, T3C31 also bears a unique amino acid substitution within this region of the protein, at position 363, resulting in a glycine-to-serine change. Thus, there are unique changes at two amino acid positions in the T3 Abney σ1 sequence (and two in nearby positions in the T3C31 sequence), one of which lies in the cell attachment domain of the protein.

DISCUSSION

Two strains of T3 reovirus, T3 Abney and T3C31, have been shown to induce biliary atresia in newborn mice after inoculation of virus into the gastrointestinal tract. Inoculation of these reovirus T3 strains p.o. represents a new model of virus-induced hepatobiliary disease which possesses significant advantages over previous models. One important improvement, the consistent induction of disease, permitted analysis of the viral genetic basis for disease induction. Altered cellular tropism of T3 Abney for bile duct epithelium had been implicated as a cause of disease because ductular epithelial

A

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                230         240         250
T3 Dearing NNLTLKTTVF DSINSRIGAT EQSYVASAVTP
         8 .D........ .......STI ...........
         9 .....A.G.. .P....VSTI ...........
        18 .K........ .PL...ASTI .........A.
        31 .....A.... .PL...VSM. .........A.
        43 .......... .........I ...........
        44 .......... ......T..I ...........
        45 .....A.... .........I .....T.....
        84 .......... .........I ...........
     Abney .......... .....G...I ...........
        93 .......... ....P....I ...........
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B

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                340         350         360
T3 Dearing DIFIVDDYIH ICLPAFDGFS IADGGDLSLNF
         8 ....A..... .........A ...........
         9 .......... .........T ...........
        18 .......... .........T ...........
        31 .......... .........T ...S.......
        43 .......... .......... ...........
        44 .......... .......... ...........
        45 .......... .......... ...........
        84 .......... .......... ...........
     Abney .......... ......N... ...........
        93 .......... .......... ...........
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FIG. 5. Regions of reovirus T3 σ1 protein sequence containing changes specific for disease-producing isolates. Amino acid differences in the σ1 sequences of T3 strains compared with that of T3D are indicated. Specific changes in the sequences of the disease-producing isolates T3 Abney and T3C31 are in boldface. (A) Comparative amino acid sequence analysis from positions 230 to 260. (B) Comparative amino acid sequence analysis from positions 340 to 370.

cells as well as liver parenchymal cells contained viral antigen in T3 Abney- but not T3C43-infected livers. Reassortant analysis revealed that, in fact, the T3 Abney S1 gene encoding the viral cell attachment protein was strongly associated with the capacity of reovirus to induce hepatobiliary disease. The amino acid sequence deduced for the T3 Abney S1 gene contained two unique changes, one of which was located in the head of the protein, the domain known to be involved in host cell-receptor binding. We hypothesize that a single, critical amino acid substitution in the viral cell attachment protein may affect receptor specificity and thus influence hepatocellular tropism of the virus and induction of biliary atresia. The extent to which the second unique amino acid change contributes to the disease phenotype is unknown.

We found no exceptions to the association of the T3 Abney S1 gene with the capacity to induce biliary atresia in newborn mice among the 16 independent reassortants with different genotypes tested. All other viral genes were excluded at least six times. Furthermore, a monoreassortant containing all genes except the S1 gene derived from the T3 Abney parent did not induce disease. Therefore, we conclude that induction of biliary atresia by the T3 Abney strain apparently is genetically associated with a single gene, the S1 gene encoding the viral cell attachment protein. Because a monoreassortant containing the T3 Abney S1 gene in a T1L background was not available, we cannot formally exclude a possible minor contribution to the disease phenotype of another gene or genes acting in concert with the S1 gene. Our results differ from those of a previous study of the genetic basis of liver disease, using T1L and T3D reassortants, which was unable to identify specific genes involved in reovirus-induced biliary atresia (2). This discrepancy may reflect our use of different virus isolates for the generation of reassortants or may reflect differences in administration of virus, since in the previous study T3D was injected intraperitoneally into 21-day-old mice (2).

Comparative sequence analysis has been used successfully to identify conserved and variable domains of the σ1 protein not only across reovirus serotypes but also within a single serotype (6, 7, 9, 23). In this manner, changes in particular regions of highly related sequences or even in individual amino acid residues that influence functional phenotypes have been identified (3, 6). Our comparative sequence analysis of the σ1 proteins from a panel of T3 strains revealed unique amino acid changes in the disease-producing strains T3 Abney and T3C31 (Fig. 5). Of particular interest were the changes at residues 356

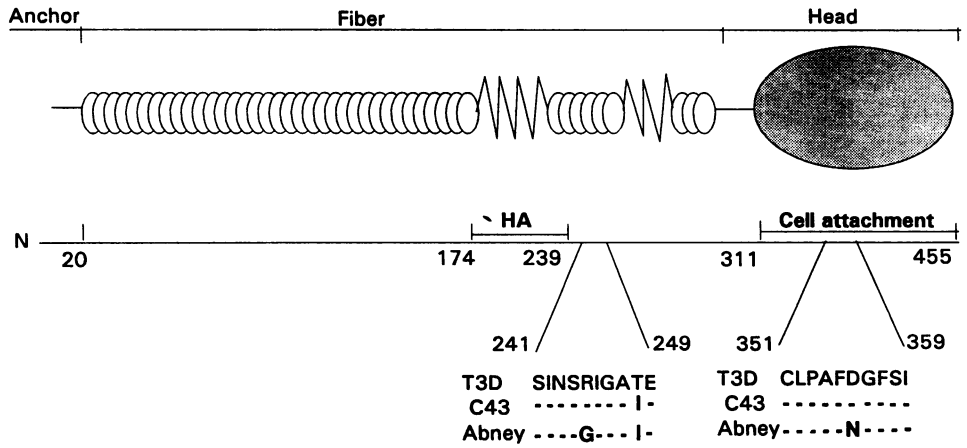

FIG. 6. Schematic of the σ1 protein, showing predicted secondary structure, the location of the hemagglutination (HA) and cell attachment domains, and the locations of specific changes within the T3 Abney σ1 amino acid sequence. The protein can be divided into three discrete regions, the anchor, fiber, and head regions, on the basis of secondary structure predictions (23). The coils indicate predicated α helices, the zigzag lines indicate predicted β sheets, and the stippled area indicates a predicted globular domain. Specific amino acid changes within T3 Abney occurred just carboxy terminal of the region believed to be involved in HA and within the region believed to be involved in cell attachment.

(T3 Abney) and 363 (T3C31) within the head region of the σ1 protein, first, because the head region is highly conserved not only within T3 strains but also between the different serotypes (9, 10) and second, because these changes are in close proximity to a protein domain proposed to be directly involved in binding to host cell receptors (5, 48). T3D variants resistant to anti-σ1 monoclonal antibodies contain single amino acid substitutions within the head of the σ1 protein that affect not only the neurovirulence of these isolates but also the tropism and extent of neuropathology (3, 37, 38). In addition, site-specific as well as deletion mutagenesis within conserved regions of the carboxy-terminal portion of in vitro-expressed σ1 protein results in a loss of binding to L-cell receptors (10, 41). In view of these precedents, a reasonable interpretation is that the unique amino acid changes found in the T3 Abney σ1 protein may influence the ability of the protein to bind to certain host receptors in the bile duct epithelium. Proof, however, awaits the ability to introduce a σ1 protein containing specific amino acid changes into a virus to assess the effect on its capacity to bind to certain host cell receptors.

Another important improvement inherent in our new model of virus-induced biliary atresia is the ability to study infection occurring by the natural route of spread from the gastrointestinal tract. Striking findings were the wide range of LD_{50}s among T3 strains when infecting from the gastrointestinal tract and the apparent lack of association between the capacity of a p.o. inoculated strain to cause lethal infection and its capacity to cause biliary atresia. For example, the LD_{50}s of T3 Abney and T3C31 differed by 5 $\log_{10}$ units, yet both strains induced biliary atresia. Similarly, T3 Abney and T3C43 possessed equivalent lethalities and capacities to replicate in the liver, but only T3 Abney induced biliary atresia. The pathogenesis of reovirus infection via the natural portal of entry from the intestinal lumen involves replication at the primary site of infection, spread to secondary sites of infection, tissue invasion, and replication. Because the capacities to grow in intestinal tissue were similar for all strains except the inefficient T3D strain (49), the differences in capacities of T3 strains to cause lethal infection more likely resulted from differences in secondary tissue or cellular tropisms or from some virulence factor influenced by other viral genes and unrelated to tissue binding. Interestingly, mice inoculated p.o. with T3C9 and depleted of $CD4^+$ and $CD8^+$ T cells develop oily fur syndrome (44). Inoculation of T3C9 p.o. in immunocompetent mice does not result in the oily fur syndrome, however, suggesting that host immune factors in addition to viral determinants may play a role in determining the hepatobiliary disease potentials of some viral isolates.

Although both T3C43 and T3 Abney were highly lethal and grew to similar peak titers in liver and brain tissues, tropisms for cell populations within the infected liver apparently differ since T3C43 did not cause biliary atresia and did not appear to infect bile duct epithelium. Previous research has demonstrated that the genetic basis of cellular tropisms within the nervous system between reovirus strains T1L and T3D lies in the virus S1 gene (46). In these studies it was hypothesized that differential infection of particular cell populations results from the capacity of the σ1 protein of T1L or T3D to specifically interact with receptors on these host cell populations. The pathological changes we observed in the livers of infected animals lend additional support to this hypothesis. T3 Abney was able to selectively infect bile duct epithelial cells, as well as parenchymal hepatocytes, whereas both T1L and T3C43 infected only hepatocytes. Thus, we believe that the capacity of the T3 Abney strain to cause biliary atresia, while the other virus strains (although they are able to infect the liver) cannot, results from T3 Abney's capacity to infect the specialized epithelial cells associated with bile ducts.

The previous model of reovirus-induced biliary atresia used the T3D strain injected intraperitoneally in large doses to induce disease (12, 25, 26). Our T3D isolate failed to induce disease when inoculated by the p.o. route, even with doses of virus exceeding 10^7 PFU. This may reflect an inability of our T3D to grow adequately at the initial site of infection in the gastrointestinal tract and thus to spread to the liver, since a viral titer was only sporadically detected in the livers of mice inoculated p.o. These results are in accord with previous observations concerning the avirulence of T3D following p.o. inoculation in newborn mice (33). In contrast to results with the previous model, we consistently were able to induce biliary atresia in mice by using a natural route of infection by p.o. inoculation of extremely low doses (200 PFU) of the T3 Abney strain. T3 Abney infection of bile duct epithelial cells resulted in liver pathology which was consistent with that described with the previous reovirus model utilizing T3D (25, 26). Our new model has allowed us to gather the first genetic evidence concerning the viral basis of reovirus-induced biliary atresia. Such advances in our understanding of how a virus induces hepatobiliary disease in mice may eventually aid in understanding the mechanisms that underlie biliary atresia, an important human disease whose etiology remains largely unknown.

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