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Replication of Hepatitis Delta Virus RNA in Mice after Intramuscular Injection of Plasmid DNA

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To establish a readily manipulable small-animal system for the study of human hepatitis delta virus (HDV) replication in vivo, plasmid DNAs containing head-to-tail cDNA dimers of HDV were inoculated intramuscularly into mice. Genomic-sense HDV RNA was detected in the injected muscle within 1 week and increased to substantial levels by week 7 postinjection. The intramuscular accumulation of HDV RNA was determined to be the direct result of viral RNA replication by three lines of evidence: (i) injected tissues also accumulated antigenomic-sense HDV RNA, (ii) plasmid DNA that synthesized primary transcripts of antigenomic sense also led to the accumulation of genomic-sense HDV RNA, and (iii) injection of a cDNA dimer defective in antigenomic RNA cleavage failed to produce detectable HDV RNA in muscle. Immunohistochemical analysis of injected muscle demonstrated the presence and nuclear localization of hepatitis delta antigen in myocytes. Finally, sera from DNA-injected mice contained antibodies specific for delta antigen, indicating the induction of an immunological response to the intracellularly expressed antigen. These findings demonstrated the ability of HDV RNA to replicate in skeletal muscle and provide a useful system for the study of HDV replication, delta antigen processing, and its presentation to the immune system in vivo. Furthermore, this system offers an efficiently replicating RNA as a potential vehicle for in vivo gene transfer.

The development of small-animal models for studying human viruses in vivo can facilitate the understanding of the molecular mechanisms of disease induction. Hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus (HBV) that is responsible for a wide range of acute and chronic liver diseases, which often lead to fulminant hepatitis (16). The molecular mechanism of HDV pathogenesis has not been established. Two potential mechanisms have been proposed as the basis for delta hepatitis. The first mechanism suggests that HDV genome replication and/or gene expression results in direct cytopathologic damage to infected hepatocytes. Experimental evidence has both supported (10) and contradicted (15, 25) this model. The second potential mechanism involves an immunologically mediated pathology resulting from specific host responses to HDV infection. Support for this model has come primarily from clinical and histopathological observations on HDV-infected humans and chimpanzees which showed the presence of immune cells in the hepatic lesions (9, 28). However, direct evidence of involvement of the immune system in HDV pathogenesis is still lacking.

The hepatitis delta virion consists of a single-stranded, circular RNA genome of 1.7 kb that is closely associated with the only virus-encoded protein, hepatitis delta antigen (HDAg), as a ribonucleoprotein core (6, 20, 32). The core is enclosed within an envelope consisting of multiple forms of HBV surface antigen (3), which are provided by helper HBV (30). The small form of HBV surface antigen is sufficient for assembly of HDV particles (5, 31, 40), but the large form of HBV surface antigen is necessary for HDV infectivity (35, 36), allowing the virus to enter hepatocytes, presumably by using the same cellular receptor as HBV. HDAg is usually present as two related protein species, a 24-kDa small form and a 27-kDa large form (LHDAg) (2), that are identical except for a 19-amino-acid carboxy-terminal extension in LHDAg (42). This 19-amino-acid extension arises from a specific RNA editing event during genome replication, resulting in elimination of a termination codon and extension of the open reading frame (4, 21, 44). These two proteins have similar biochemical properties and yet have very different functional roles during virus replication. The 24-kDa small HDAg protein is a transactivator of HDV RNA replication (19), while LHDAg inhibits RNA synthesis (7) and initiates virion assembly (5).

Establishment of a readily manipulable small-animal model for HDV has remained elusive, probably because of the lack of a specific receptor for the virus. A mouse model has been described in which HDV replicates in the liver following intraperitoneal or intravenous administration of large quantities of HDV (24). However, the efficiency of HDV infection was very low, and this approach did not lead to chronic infection or hepatitis. This approach also does not permit genetic alteration of viral genomes for studying phenotypic changes in vivo. Recently, we have demonstrated that HDV RNA replicates very efficiently in the skeletal muscle of transgenic mice, once the viral RNA is expressed from an HDV transgene (25). This observation, along with the demonstrations of viral replication and gene expression after various viral DNAs or cDNAs were inoculated into animals (11, 13, 33, 37), prompted us to examine whether HDV RNA can replicate following intramuscular administration of viral cDNA. The success of this approach would allow genetic manipulation of HDV RNA to determine the molecular basis of viral replication in vivo and possible roles of the immune response to viral infection.

HDV RNA expression in skeletal muscle from injected cDNA dimers. To examine whether intramuscular injection of

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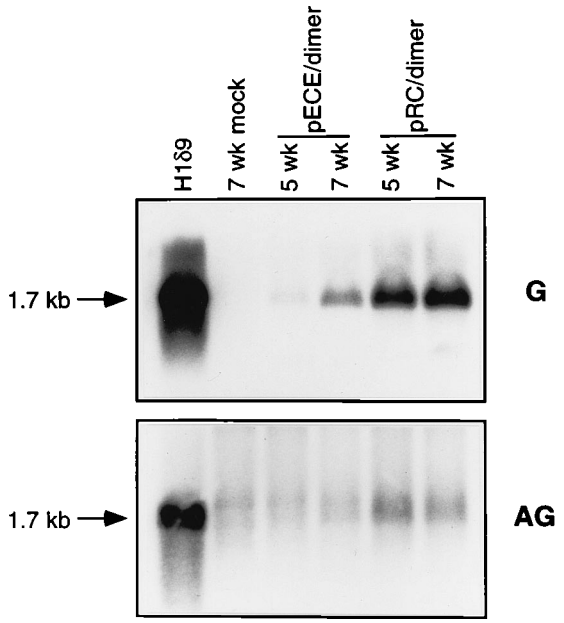

FIG. 1. Expression of HDV RNA following intramuscular injection of HDV cDNA. Six- to eight-week (wk)-old C57BL/6 (*H-2*^{b}) mice (purchased from Jackson Laboratory, Bar Harbor, Maine) were anesthetized with Metofane (Pitmann-Moore), and either pECE/dimer or pRC/dimer plasmid DNA was injected intramuscularly into the gastrocnemius muscles of the hind legs at 100 μg of total DNA per animal (four 25-μl injections; the DNA was suspended at 1 mg/ml in phosphate-buffered saline). Animals were sacrificed at the times indicated above the lanes, the gastrocnemius muscles from both injected legs were removed, and total cellular RNA was isolated with guanidinium isothiocyanate and acid phenol as described previously (8). Equal amounts of RNA (10 μg of each) from each sample were treated with formaldehyde, electrophoresed through 1.2% agarose gels containing formaldehyde, transferred to Hybond-C Extra nitrocellulose membrane (Amersham), and hybridized with ^{32}P-labeled antigenomic-sense HDV RNA as described previously (23). RNA from H1δ9 cells, which express HDV monomeric RNA from an integrated HDV cDNA trimer (22), was used as a positive control. The 7-week mock lane represents animals injected with phosphate-buffered saline and sacrificed at 7 weeks postinoculation. G, HDV genomic RNA; AG, HDV antigenomic RNA.

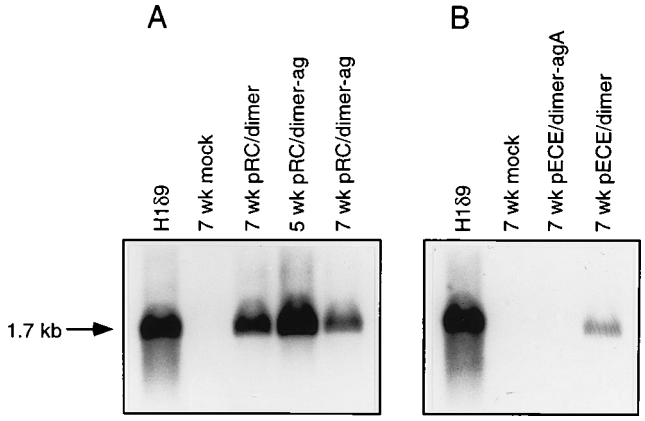

FIG. 2. HDV RNA replication from different HDV cDNA constructs. Mice were injected intramuscularly with a total of 100 μg of the plasmid DNAs indicated. Animals were sacrificed at the postinoculation times indicated, and RNA was analyzed by Northern blotting with an HDV antigenomic-sense RNA probe. Plasmids pRC/dimer and pECE/dimer synthesize genomic-sense primary transcripts, plasmid pRC/dimer-ag synthesizes antigenomic-sense primary transcripts, and plasmid pECE/dimer-agA synthesizes genomic-sense HDV dimer RNA containing a point mutation at the antigenomic autocleavage site (23). H1δ9 is the positive control for HDV RNA, and the 7-week (wk) mock lane represents mice injected with phosphate-buffered saline.

HDV cDNA could lead to RNA replication, a cDNA dimer of HDV was used. Plasmid pECE/dimer contains a head-to-tail cDNA dimer of HDV under the control of a simian virus 40 transcriptional promoter (23). This plasmid transcribes a genomic-sense HDV RNA, which previously has been shown to be replication competent in cultured cells (23). The plasmid DNA was injected into 6- to 8-week-old C57BL/6 (*H-2*^{b}) mice at several sites of the gastrocnemius muscle in each hind leg (20 to 100 μg of DNA per animal). Following injection, animals were sacrificed at weekly intervals, the injected muscles were removed, and total RNA was isolated. Northern (RNA) blot analysis was performed with HDV-specific riboprobes. Preliminary data showed that a 1.7-kb HDV genomic-sense RNA was detectable as early as day 7 postinjection, and the amount of HDV RNA continued to increase throughout the experiment (data not shown and Fig. 1). Since the primary transcript of this plasmid was dimer HDV RNA, detection of the 1.7-kb HDV monomer RNA species suggested that the RNA transcript had been replicated and/or processed.

To increase the level of HDV RNA synthesis, we generated a different cDNA dimer construct which utilizes a cytomegalovirus immediate-early promoter for transcription of the HDV genomic-sense RNA. This plasmid, designated pRC/dimer, was constructed by excising the entire HDV cDNA dimer sequence from plasmid pECE/dimer by digestion with *Hin*dIII and *Xba*I, which cut unique enzyme sites in the polylinker region of the pECE vector (12). The excised fragment was purified from agarose gel, and its *Xba*I site was converted to a *Hin*dIII site by blunt ending with Klenow and ligation with *Hin*dIII linkers, followed by ligation of the *Hin*dIII-flanked fragment into the *Hin*dIII site of plasmid pRC/CMV (Invitrogen, San Diego, Calif.). Mice were injected in parallel with either pECE/dimer or pRC/dimer DNA (100 μg per animal). The injected mice were sacrificed at 5 and 7 weeks postinjection, and total RNA from the injected muscle was analyzed by Northern blotting with strand-specific HDV riboprobes. Figure 1G shows that a 1.7-kb HDV genomic RNA was detected at both time points in both pECE/dimer- and pRC/dimer-injected mice. The amount of HDV RNA was substantially higher in pRC/dimer-injected animals than in those injected with pECE/dimer. Very little HDV dimer RNA, which was the primary transcript from these two plasmids, was detected. Furthermore, a 1.7-kb antigenomic-sense HDV RNA was detected in each of the samples (Fig. 1AG). These results, combined, indicate that HDV RNA indeed replicated in the muscle tissue. Again, the amount of HDV antigenomic RNA in pRC/dimer-injected mice was higher than that in pECE/dimer-injected mice. Interestingly, no 0.8-kb antigenomic-sense RNA, which was postulated to be the mRNA for HDAg (17), was detected.

HDV RNA detected in muscle was the product of RNA replication. To confirm that the RNA detected in the muscle indeed resulted from HDV RNA replication and did not represent primary transcripts from the injected DNA, two additional HDV constructs were tested in animals. The first construct, designated pRC/dimer-ag, transcribes an antigenomic-sense dimer HDV RNA from the cytomegalovirus promoter. This plasmid is similar to the pRC/dimer construct, except that the HDV dimer is in the opposite orientation. As shown in Fig. 2A, muscle tissue injected with this plasmid synthesized as much genomic-sense HDV RNA as did muscle tissue injected with a plasmid transcribing the genomic-sense transcript (pRC/dimer). These results could only be possible if the primary antigenomic-sense transcripts were replicated.

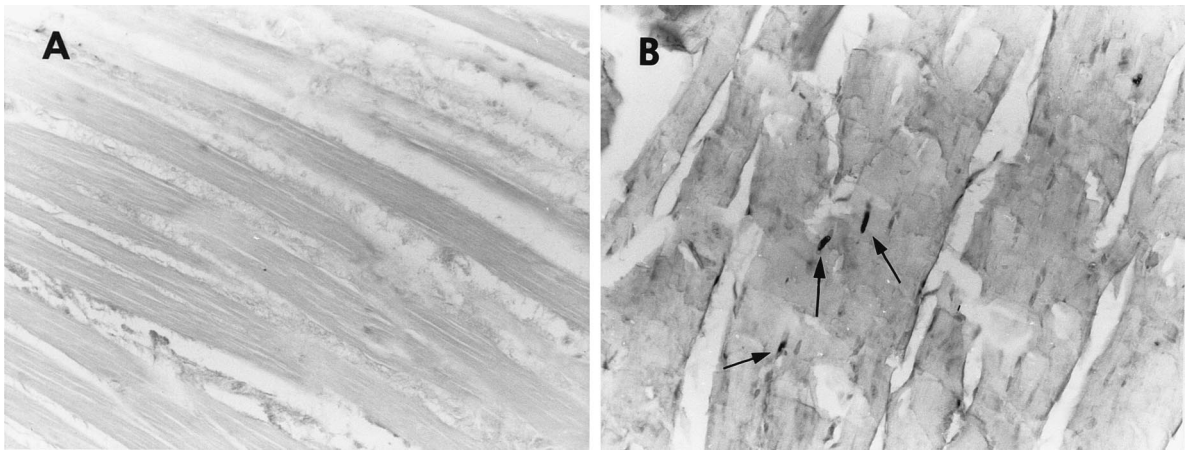

FIG. 3. Histopathology and immunohistochemistry of skeletal muscle from DNA-injected mice. Indirect immunoperoxidase staining of skeletal muscle from normal mice (A; magnification, ×160) and DNA-injected mice (B; magnification, ×320). The arrows indicate HDAg in the nuclei of myocytes.

The second construct, pECE/dimer-agA, contains a single nucleotide substitution (G to A) at the autocatalytic cleavage site of the antigenomic strand of HDV RNA (23). This RNA lost autocleavage activity in vitro (43) and could not replicate when transfected into cultured cells (23). Figure 2B shows that no genomic-sense HDV RNA could be detected in muscle tissue injected with this plasmid. This result suggests that the sequence requirement of HDV RNA replication in animals is similar to that in tissue culture.

These results combined indicate that the genomic-sense RNA detected in muscle tissue following injection of wild-type pECE/dimer or pRC/dimer DNA resulted not from accumulation of the primary transcript but rather from the replication and amplification of RNA transcribed from the input plasmids. Taken together, these data provide conclusive evidence that HDV RNA is expressed and efficiently replicated following direct injection of naked plasmid DNA into skeletal muscle of mice.

Expression of delta antigen during replication in muscle. To demonstrate the expression of HDAg in muscle cells following intramuscular injection of HDV dimer-expressing plasmid DNA, muscle sections were examined immunohistochemically. By using human sera from HDV-infected individuals in an indirect immunoperoxidase system (14), delta antigen was observed in myocytes (Fig. 3). The HDAg-positive cells were clustered in distinct foci within the tissue, probably reflecting the sites of injection and suggesting an inability of the replicating RNA to spread to distant sites. In addition, HDAg was localized to the nuclei of myocytes. Thus, intramuscular HDV replication resulted in production of delta antigen.

Induction of humoral immunity to delta antigen. To determine if DNA-injected animals respond immunologically to the expression of HDAg in muscle, blood samples were obtained from animals prior to sacrifice for RNA studies. Sera were tested for the presence of HDAg-specific antibodies by enzyme-linked immunosorbent assay (ELISA) with purified, baculovirus-expressed HDAg (18) and a horseradish-peroxidase-conjugated goat anti-mouse antibody. As shown in Fig. 4, all of the mice injected with plasmid pRC/dimer or pRC/dimer-ag, or plasmid pRC/δ, which contained only the LHDAg-coding sequence under control of the cytomegalovirus promoter (26), made very high and equivalent levels of antibody specific for HDAg. Therefore, the intramuscular expression of HDAg induced effective immune responses.

The data presented in this report thus show that HDV RNA replication occurred following intramuscular injection of naked HDV cDNA. This provides an efficient murine model for studying in vivo HDV replication in extrahepatic tissues. Previously, studies of HDV replication in vivo had been limited by the narrow host range of the virus, which grows only in chimpanzees and woodchucks (27, 29). Such animal models are difficult to use for studying the in vivo effects of alterations of HDV RNA sequences. The successful replication of HDV

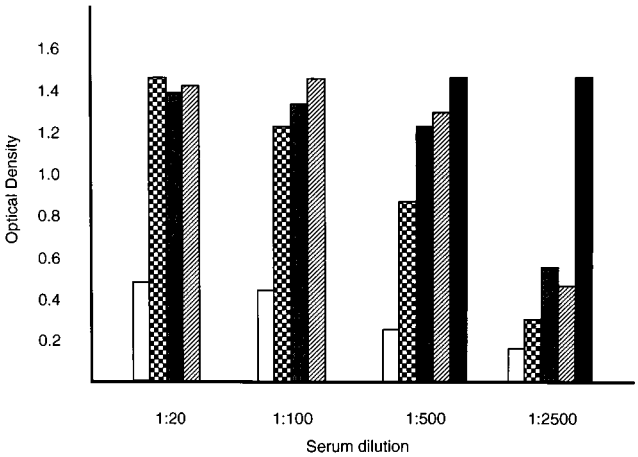

FIG. 4. Detection of HDAg-specific antibody in DNA-injected mice. Antibody specific for HDAg was detected by ELISA in accordance with previously published procedures (34). Purified HDAg, isolated from Sf9 insect cells infected with a recombinant baculovirus expressing LHDAg (18), was adsorbed to wells of a microtiter plate at a concentration of 10 μg/ml (100 μl per well) at 4°C overnight. Plates were washed, mouse sera at different dilutions and no-serum and mock-infected serum controls were added to the wells in duplicate (100 μl per well), and the plates were incubated at room temperature for 4 h. Plates were washed thoroughly, and goat anti-mouse antibody conjugated with horseradish peroxidase was added (1:2,000 dilution) and incubated for 1 h at room temperature. After washing, *o*-phenylenediamine and H_2O_2 were added and colorimetric quantitation was performed with a Dynatech Minireader II ELISA plate reader. The data depicted represent optical densities of the sera of mice injected with different plasmid DNAs minus background level obtained from the no-serum control. Mock-infected serum was obtained from mice injected with phosphate-buffered saline, and polyclonal serum was from mice immunized with purified, baculovirus-expressed HDAg (18). ▭, mock infection; ▨, pRC/dimer; ■, polyclonal serum; ▩, pRC/dimer-ag; ▨, pRC/δ.

RNA after intramuscular injection of cDNA into mice will allow specific alterations of the HDV genome to be made at the cDNA level and the resulting phenotypic effects to be easily determined in vivo. Our data in this report show that an antigenomic cleavage site mutant could not replicate in mice, suggesting that the ribozyme activity is also required for HDV RNA replication in vivo. It is not clear how HDAg was synthesized from the transfected DNA in the muscle. No 0.8-kb subgenomic, antigenomic-sense RNA, which is the putative mRNA encoding HDAg (17), was detected. There likely is an alternative mechanism for the synthesis of HDAg.

Although the replication of HDV RNA in skeletal muscle may not reflect the natural history of HDV infection in humans, HDV replication has been demonstrated in many cell types of diverse origin following transfection of HDV cDNA in cultured cells (19, 23) and in different tissues in transgenic mice expressing HDV RNA (25). Most surprisingly, in these transgenic mice, HDV RNA showed an unusually high propensity to replicate in skeletal muscle. In fact, its replication levels in muscle were more than 100-fold higher than those in liver (25). Thus, the failure of natural HDV infection to involve skeletal muscles is likely the result of tissue tropism imposed by the HBV surface antigen-containing envelope of HDV, which interacts only with liver-specific cell surface molecules. Thus, skeletal muscle may be a legitimate site for studying HDV replication in vivo. To extend this approach to liver, we also attempted to inject HDV cDNA intrahepatically. We detected only a minimum level of HDV RNA replication in a small fraction of injected mice, suggesting either low efficiency of DNA uptake by liver cells or the technical difficulty in delivering DNA precisely into the liver. Our data thus indicate that intramuscular injection of HDV cDNA is the simplest and most reproducible method for studying HDV RNA replication in vivo. This success echoes many published reports that muscle takes up naked DNA very efficiently (38, 39).

This approach also provides opportunities to study the host's immune responses to HDAg. Immunologically mediated pathology is one of the possible mechanisms for HDV-induced liver diseases. Patients infected with HDV have been shown to develop antibodies to several different antigenic sites on the HDAg (1, 41). However, it remains unclear whether HDAg can elicit a cellular immune response. In our previous studies, we infected mice with recombinant vaccinia viruses expressing HDAg. Despite the capacity of these viruses to express very large amounts of HDAg, the infected mice developed humoral antibodies to HDAg very poorly and did not develop detectable cellular immunity specific for HDAg (unpublished observations). In contrast, our current study showed that intramuscular replication of HDV efficiently induced HDAg-specific humoral antibody production. Thus, similar to other systems (38, 39), intramuscular inoculation of HDV cDNA induced good immune responses. In the current study, we also performed experiments with injected mice to detect the possible presence of HDAg-specific cytotoxic T-cell responses by using recombinant vaccinia virus vectors expressing HDAg for in vitro stimulation and target cell infection. However, no cytotoxic T-cell response was detected in the C57BL/6 mice used in these studies (data not shown). The lack of an HDAg-specific cytotoxic T-cell response in these animals does not preclude the possibility of such a response in mice of other major histocompatibility complex haplotypes.

This system also offers a potential application in gene transfer. This HDV RNA undergoes efficient self-amplification via RNA-RNA replication, thus offering a potential vector for delivering foreign genes.

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