Sequence Homology Within the Morbilliviruses

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Double-stranded cDNA synthesized from total polyadenylate-containing mRNA extracted from monkey kidney cells infected with canine distemper virus (CDV) was cloned into the PsI site of Escherichia coli plasmid pBR322. Clones containing CDV DNA were identified by hybridization to a CDV-specific 32P-labeled cDNA. A cDNA clone containing an insert 1,700 base pairs (CDV 364) has been identified as the reverse transcript of the mRNA coding for the nucleocapsid protein. The size of the mRNA species complementary to this insert is 1,850 nucleotides, as determined by the Northern technique. Hybridization experiments and heteroduplex mapping indicated homology between the central region of the CDV and measles virus nucleocapsid gene. The completion of the nucleotide sequence analysis of the measles virus gene allowed the reconstruction of the entire coding region of the measles virus gene and a comparison with the counterpart sequence of CDV. This comparison delineated three regions: (i) a region of high homology (nucleotides 501 to 1215), in which 77% of the nucleotides and 88% of the encoded amino acids are identical; (ii) a region of moderate homology at the 5' end of the message (nucleotides 1 to 500), in which 59% of the nucleotides and 66% of the encoded amino acids are identical; (iii) a region of little or no homology (nucleotides 1216 to 1625) near the 3' end of the message.

Canine distemper virus (CDV) and measles virus (MV) are members of the morbillivirus subgroup of paramyxoviruses. Strong immunological cross-reactivity has been observed among all poly peptides of these viruses, with the possible exception of the hemagglutinin (12, 15, 17, 25). Both viruses can establish persistent infection in cell culture (21, 26), and both can invade the central nervous system of their natural host. A slowly progressing neurological disease known as subacute sclerosing panencephalitis has been directly correlated with the presence of measles virus in the central nervous system of human patients (8, 11, 14, 18). Similarly, CDV has been shown to be the causative agent in a slow neurological disease, old dog encephalitis (1, 9, 23). Both viruses have been implicated as being involved in multiple sclerosis, but the evidence for this has been weak at best.

We have undertaken a comparative study of these two morbilliviruses as an approach to the elucidation of possible molecular events that could lead to the alteration of these viruses from an acute infection to one producing a "slow" virus syndrome. In the initial phase of this work, we have previously cloned three of the MV genes (4, 5, 11, 19). In the present study, we have identified cDNA clones of CDV. One of these clones, containing almost the entire coding region of the CDV nucleocapsid gene, and a measles clone encompassing this same coding region have been completely sequenced and analyzed with respect to nucleotide and amino acid sequence homologies.

MATERIALS AND METHODS

Cells and viruses. The Onderstepoort strain of CDV was obtained from M. Appel (Cornell University, Ithaca, N.Y.) and was plaque purified twice in the CV-1 line of African green monkey kidney cells. A stock was prepared by infecting CV-1 cells at a multiplicity of infection of 1/1,000. After the development of a marked cytopathic effect, virus was harvested (20).

Preparation of RNA. CV-1 cells (3 × 108) were infected with plaque-purified CDV at 0.5 PFU per cell; 18 h later, cytoplasmic RNA was extracted. RNAs were purified by successive phenol-chloroform-isoamyl alcohol extraction followed by LiCl precipitation. Polyadenylated [poly(A)+] mRNAs were then purified by oligodeoxythymidylicate-cellulose chromatography (2).

cDNA libraries. The cDNA library of canine distemper virus was constructed by oligodeoxythymidylicate priming of CDV mRNA. Methods employed for first- and second-strand synthesis, tailing, and insertion into pBR322 at the PsI site have been described previously (11). The constructions of the cDNA library produced by reverse transcription of measles mRNA and the genomic library have been previously described (5, 11).

Detection of CDV-specific clones. Transformants were transferred onto nitrocellulose filters and hybridized with [32P]-labeled cDNA probes (2 × 106 cpm/ml) prepared from poly(A)+ RNA from CDV acutely infected cells. The probe was incubated before hybridization with a 100-fold excess (10 μg) of poly(A)+ RNA from uninfected cells (19, 20). The incubation was performed at 68°C for 4 h in a 100-μl reaction mixture containing 0.27 M sodium citrate (pH 7.2), 0.9 M NaCl, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 150 μg of sonicated salmon sperm DNA. After hybridization, filters were washed and autoradiographed.

Northern blot hybridization. Poly(A)+ RNA (1 to 2 μg) was electrophoretically separated in formaldehyde-agarose (1%) gels by the method of Derman et al. (10). The RNA was transferred onto Zeta probe membrane filters (Bio-Rad Laboratories) which were then baked for 2 h. Hybridization to nick-translated CDV 364 (5 × 106 dpm/μg) was performed at 42°C in 50% formamide (stringent conditions) or in 35% formamide (decreased stringency) for 12 to 15 h. Hybridization solutions, in addition to the formamide, contained 5 × SSPE (0.90 M NaCl, 50 mM sodium phosphate [pH 7.0], 5 mM EDTA), 0.02% (wt/vol) bovine serum albumin, Ficoll 400, polyvinylpyrrolidone, 0.3% sodium dodecyl sulfate (SDS), and 100 μg of sonicated salmon sperm DNA per ml.

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After hybridization, the filters were washed at 45°C with 2× SSPE containing 0.2% SDS (three times for 20 min each). For stringent washing, the final wash was performed at 60°C with 0.2× SSPE containing 0.2% SDS for 30 min. For decreased stringency, the final wash was done at 50°C with 1× SSPE containing 0.2% SDS for 30 min. Filters were exposed to Kodak XR-2 X-ray film with the use of a screen and stored at −70°C until developed.

RESULTS

Construction and identification of cDNA clones. The population of double-stranded cDNA prepared from poly(A)+ RNA extracted from CDV-infected cells was tailed with oligodeoxycytidylylate and inserted into appropriately tailed pBR322 at the PstI site. The circularized hybrid DNA was introduced into Ca2+-sensitized Escherichia coli HB101.

Transformants (4,000 clones) resistant to tetracycline (15 µg/ml) were isolated, and 384 clones containing CDV sequences were identified by a selective hybridization method adapted for screening clones that contain CDV-DNA sequences (11). Characterization of one clone, clone 364, revealed an insert of ca. 1,700 base pairs (bp). The size and nature of the cDNA insert were characterized by two methods. Digestion of CDV 364 DNA with PstI (Fig. 1, lane B) generated three fragments. One corresponded in size to linear pBR322 DNA, a second fragment consisted of 1,100 bp, and a third fragment was 600 bp. The size of the inserted fragment was further confirmed by electron microscopic studies. The lower part of Fig. 1 shows an electron micrograph of heteroduplex molecules formed by reassociation of pBR322 and CDV 364 DNAs that were linearized by digestion with EcoRI which does not cut the insert. The length of the inserted CDV sequence appeared to be 1,750 ± 50 bp, in good agreement with the length determined by agarose gel electrophoresis.

Hybridization of clone CDV 364 with CDV and MV mRNA. For a determination of the size of the CDV mRNA complementary to CDV 364 and an assessment of the relatedness between this clone and MV mRNA, Northern blot analyses were performed. For these studies, mRNA extracted from cells acutely infected with CDV or MV was fractionated on agarose gels under denaturing conditions together with 14C-labeled rRNA markers. Subsequently, the RNA was electroblotted onto Zeta probe membrane. Clone CDV 364 plasmid DNA was nick translated in vitro and used as a hybridization probe, under both stringent conditions and decreased stringency, using duplicate blots. Under stringent conditions of hybridization, CDV 364 DNA hybridized only to mRNA extracted from CDV-infected cells, not to mRNA extracted from MV-infected cells (Fig. 2A). A strong hybridization of clone CDV 364 with CDV and MV mRNA. 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This 605-bp clone was derived from the precise 3′-proximal end of the MV genome, and the nucleotide sequence has been previously reported (6). The last 208 nucleotides of MV P-3 are identical to the first 208 nucleotides of MV CL-15. Clone pWB 9D8 was selected from a measles genomic library and contains the last 207 nucleotides present within MV CL-15 before crossing the intercistronic boundary into the next downstream gene, the phosphoprotein (4). All CDV and MV clones described were completely sequenced. The combined sequences obtained from the MV clones permitted the reconstruction of the entire coding sequence for measles nucleocapsid. This nucleotide sequence, begin-

FIG. 3. Electron microscopy of heteroduplex DNAs. pBR322 and three cloned DNAs (CDV 364), measles 15, and measles 16 (1 μg of each) were linearized with EcoRI, denatured, and annealed (7). (a) pBR322 and CDV 364; (b) MV CL-16 and CDV 364; (c) MV CL-15 and CDV 364. Insets (surrounded by white) show interpretive drawings.

Heteroduplexes of CDV 364 and MV CL-15. The possibility that CDV 364 might contain sequence homology with MV CL-15 was explored by using heteroduplex formation and electron microscopic examination. The pBR plasmid DNA containing CDV 364 insert was linearized with EcoRI and annealed with EcoRI-linearized plasmid MV CL-15 DNA or MV CL-16. The latter MV clones contain the identical 1,400-bp insert of the MV nucleocapsid clone inserted in pBR322 DNA at the PstI site, but in opposite orientation.

Figure 3 shows electron micrographs of heteroduplex molecules formed by reassociation of CDV 364 with MV CL-16 (Fig. 3b) and MV CL-15 (Fig. 3c). A double-stranded region within the insert location was observed only when CDV 364 was annealed with MV CL-15. The length of the double-stranded region was determined to be 681 ± 48 bp based on the statistical analysis of 11 molecules. These results clearly demonstrated a sequence similarity between the MV nucleocapsid clone and a region of CDV 364 and strongly suggested that CDV 364 is a nucleocapsid clone.

Nucleotide sequence of CDV and MV nucleocapsid. Confirmation of the nucleotide homology between CDV 364 and MV CL-15 was established by nucleotide sequencing, done by both the chemical method (16) and the chain termination method (22). The CDV 364- and MV CL-15-related clones employed for establishing the nucleotide sequence are schematically illustrated in Fig. 4. Clones CDV 360 and CDV 93 were selected from the CDV message library, with end-labeled CDV 364 as probe. Clone MV P-3 was obtained from Martin Billeter (University of Zürich, Zürich, Switzerland).

FIG. 4. Schematic representation of the cDNA clones employed in sequencing of (A) CDV and (B) MV nucleocapsid. *, MV P-3 was received from M. Billeter (5).
FIG. 5. Nucleotide sequences of MV (top line) and CDV (bottom line) nucleocapsids. The sequences are displayed as (+) genome sense and represent the putative coding regions. Asterisks represent nucleotide identity; nd, sequence not determined.
amino acid levels (Fig. 5 and 6). Thus, it was concluded that CDV 364 and related clones represent a class of cDNA clones specific for the nucleocapsid gene of CDV.

**DISCUSSION**

In the present report, we have identified cDNA clones specific for the nucleocapsid of CDV. One particular clone (364) has been studied in detail. Northern analysis of CDV and MV mRNA with CDV 364 as probe indicated that, under decreased stringency, an mRNA of ca. 1,850 nucleotides was detected in both mRNA preparations. The size of this mRNA was consistent with the mRNA species detected by a CDV nucleocapsid specific clone reported previously (3) and with the mRNA detected by a measles nucleocapsid clone, MV CL-15 (11). An additional mRNA species of ca. 3,300 bases was detected in both mRNA preparations. This most likely represents a readthrough dicstronic mRNA of the type previously described for negative-strand RNA viruses (13, 27). Heteroduplex mapping between MV CL-15 and CDV 364 indicated sequence homology in the central region of the CDV-MV hybrid molecules, with an average duplex length of 681 ± 48 nucleotides. Clone 364 has been employed in hybrid selection studies not shown here. Subsequent in vitro translation of the selected CDV mRNA resulted in the synthesis of polypeptides ranging from 60,000 to 20,000 in molecular weight. Only the largest polypeptide comigrated

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**FIG. 6.** Predicted amino acid sequences of MV and CDV nucleocapsid protein. Asterisks represent amino acid identity; ×, termination codon; nd, not determined.
with the authentic nucleocapsid protein of CDV. The lower-molecular-weight products are believed to be nucleocapsid-related peptides, possibly a result of premature termination in the in vitro translation system. A similar phenomenon was observed with hybrid-selected mRNA encoding the nucleocapsid protein of MV (11, 19). The most compelling data for the nucleocapsid specificity of CDV 364 and related clones stem from the striking homology with the MV sequence at the nucleotide level and especially at the amino acid level (Fig. 5 and 6).

Alignment of these sequences was done at their respective termination codons at the end of each single open reading frame of the deduced messages. The strongest homology began at nucleotide 501 and extended to nucleotide 1215. This region contains 715 nucleotides, of which 77% of the measles and CDV sequences are identical. Both the central location and the number of nucleotides involved in this partial homology, as identified by nucleotide sequencing, are in excellent agreement with the location and length of the heteroduplexes observed.

A region of moderate homology was observed at the 5′ end of the deduced mRNA sequences. This region, which encompasses nucleotides 28 through 500 of the MV coding sequence, is 59% homologous with that of the sequence derived from CDV 364. Unfortunately, no CDV clone was identified that would allow the analysis of the most 5′ sequences. In contrast to the two regions of homologous sequence identified within the first 1,200 nucleotides, little or no homology could be detected in the last 400 nucleotides toward the 3′ ends of these deduced messages. It should be emphasized that the sequences in the latter regions were derived from three independently isolated clones of CDV and from two clones, one message derived and one genomic derived, of measles nucleocapsid. Thus, the regions of nonhomology represent the best analyzed with respect to nucleotide sequence accuracy.

Measles and CDV antisera very strongly cross-react with respect to the nucleocapsid protein (12, 15, 17). The deduced amino acid sequences (Fig. 6) of the available CDV and MV nucleocapsid proteins suggest that the cross-reactivity of such antisera would likely occur within the regions of moderate and high homology where 66 and 88% of the respective amino acid sequences are identical. These percentages are somewhat higher than those observed at the nucleotide level. Many of the nucleotide changes occur at the central position of the triplet codon and often encode the same amino acid. In many instances in which the nucleotide change encodes a different amino acid, the properties of the substituted amino acid are identical, i.e., non-polar uncharged, polar uncharged, etc. (Fig. 6).

In contrast, the last 107 amino acids of the deduced sequences show only chance homology. The sequence predicts that unique antibody populations reacting exclusively with either MV or CDV nucleocapsid should exist. Antibodies to synthetic oligopeptides constructed to these clearly divergent regions will be powerful tools for assessing the importance of the divergence in terms of protein-protein or protein-nucleic acid interactions, or both.

In summary, the complete nucleotide sequence of the nucleocapsid gene of a third paramyxovirus, Sendai virus, has been reported (24). A direct comparison of the coding region of this sequence with MV and CDV at the amino acid level was performed by aligning the sequences at the putative initiator methionine of Sendai and MV nucleocapsid proteins. This comparison resulted in the identification of two regions of precise identity. The first contained six amino acids, Ala-Gly-Leu-Ala-Ser-Phe (nucleotides 790 to 807), and the second contained eight amino acids, Leu-Trp-Ser-Tyr-Ala-Met-Gly-Val (nucleotides 994 to 1017). It should be stressed that these regions, although small, occur precisely in the same location from the first methionine used to align the sequences. Whether these conserved sequences serve some functional role in RNA binding is currently under investigation.

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LITERATURE CITED


