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Cloning of the Bluetongue Virus L3 Gene

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The genes of the bluetongue virus (BTV) serotype 17 have been cloned into pBR322 by tailing both strands of the double-stranded RNA with polyadenylic acid, transcribing them with reverse transcriptase with an oligodeoxythymidylic acid primer, hybridizing the cDNA products, and completing them into duplex structures with the Klenow fragment of *Escherichia coli* DNA polymerase. After cloning the double-stranded cDNA molecules into pBR322, the complete sequence of the cloned L3 gene was determined. The clone is 2,772 nucleotides long (1.78×10^6 daltons), excluding the 3′ polyadenylic acid sequence, and has an open reading frame which codes for a protein of some 901 amino acids (103,412 daltons). This clone can hybridize L3 RNA segments of three other U.S. BTV serotypes, BTV-10, -11, and -13 in addition to -17 but not the equivalent RNA segment of epizootic hemorrhagic disease virus of deer, an orbivirus related to BTV.

Bluetongue is an arthropod-borne disease of sheep and cattle caused by an orbivirus, bluetongue virus (BTV). The genome of the virus consists of 10 segments of double-stranded (ds) RNA. Based on the information obtained for reovirus, it is probable that each BTV RNA segment includes recognition sites for transcription, translation, replication, and assembly into virions. To identify the basis for these structure-function relationships, we have generated DNA clones of the viral RNA species. We describe in this report the cloning of the genes of BTV-17 into pBR322 and present the complete sequence of the cloned L3 gene which codes for viral polypeptide P3 (14).

MATERIALS AND METHODS

Growth of virus and isolation of ds RNA. BTV-17 was grown in BHK-21 cells, and the ds viral RNA was purified as described by Sugiyama et al. (17). The individual ds segments were isolated by agarose gel electrophoresis as described by Rao et al. (12). Each RNA species was electroeluted from the gel as described by Maniatis et al. (9), with the following modifications. Electroelution was carried out, using only dialysis membranes to trap the eluted RNA. The RNA was collected by washing the membrane three times in 200-μl volumes of a solution containing 2 mM Tris (pH 8.0), 2 mM EDTA, and 0.4 M NaCl.

Polyadenylation of ds RNA. Preparations of ds RNA were denatured in the presence of 0.01 M methyl mercury for 10 min at room temperature. Methyl mercury was then inactivated by the addition of an equal amount of 2-mercaptoethanol. The 3′ termini of both strands of ds RNA were polyadenylated, using ATP and *Escherichia coli* polyadenylic acid polymerase (obtained from Bethesda Research Laboratories, Gaithersburg, Md.) as described by Sippel (15). The polyadenylic acid-tailed RNA was then purified through a Sephadex G-50 column and, before use for cDNA synthesis, again denatured with 10 mM methyl mercury.

Synthesis of cDNA. cDNA copies of the polyadenylated BTV RNA were synthesized with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) and 5′-phosphorylated oligodeoxythymidylic acid [oligo$(dT)_{12-18}$] (Collaborative Research, Waltham, Mass.) as the primer (10). The reaction conditions were similar to those described by Buell et al. (3). The RNA-cDNA hybrids were purified from nucleotides by a G-50 Sephadex column and precipitated with ethanol. The RNA templates were removed from RNA-DNA hybrids by incubating the hybrids in 0.3 M KOH at room temperature overnight. After neutralization, the products were passed through a Sephadex G-50 column and cDNA precipitated with ethanol.

Preparation of full-length ds cDNA. The cDNA, which consisted of transcripts of both polarities, was dissolved in a solution containing 50 μl of 10 mM Tris-hydrochloride (pH 8.0), 0.4 M NaCl, and 1 mM EDTA and self-annealed by incubation at 60°C for 24 h. The annealed cDNA was collected by precipitation with ethanol. To ensure that the cDNA preparations were completely ds, the products were treated with the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs) before cloning (2).

Molecular cloning. The *E. coli* plasmid was restricted with *Hin*dIII; the ends were repaired with the Klenow fragment of *E. coli* DNA polymerase and dephosphorylated with alkaline phosphatase. The cDNA transcripts were blunt-end ligated to the plasmid with T4 DNA ligase (New England Biolabs). By this means the *Hin*dIII site was reconstituted efficiently by the terminal A-T of the BTV cDNA. The hybrid plasmids were transfected into competent *E. coli* strain MC1061 cells (4), and colonies were selected by resistance to ampicillin. The resulting transformants were screened for the presence of BTV-cDNA inserts by the Grunstein-Hogness colony hybridization assay (7) with a ^{32}P-labeled cDNA short copy probe made with the oligodeoxythymidylic acid primer and polyadenylated viral RNA (2). Hybridization-positive colonies were characterized and sized by comparing their *Hin*fI restriction fragments with those of pBR322 (18). The largest clones were subjected to Northern blot and nucleotide sequence analysis.

RNA gel electrophoresis and blotting. RNA was extracted from BTV-17-infected BHK-21 cells and resolved by electrophoresis on 1.0% agarose gel as described previously (17). After electrophoresis, the gels were prepared for blotting as described by Alwine et al. (1). Briefly, the gel was soaked in 50 mM NaOH containing 14 mM mercaptoethanol and 1 μg of ethidium bromide per ml for 20 min. The gel was washed four times (5 min each) in 25 mM sodium phosphate buffer (pH 6.5) and osmotically blotted for 2 h to Genescreen hybridization transfer membrane (New England Nuclear, Boston, Mass.). After blotting, the membranes were air dried and baked in an oven at 80 to 100°C for at least 2 h.

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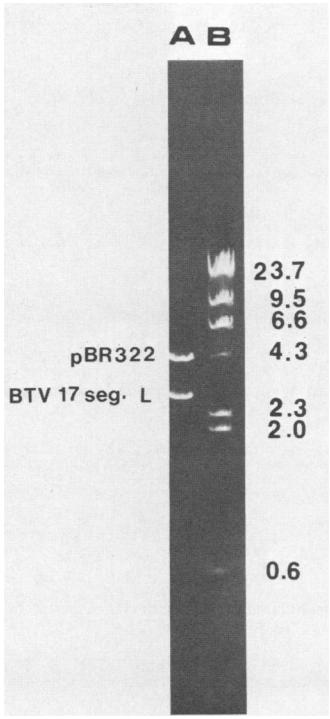

FIG. 1. Migration pattern of clone 7 plasmid DNA cut with *Hin*dIII and run on a 1% agarose gel. (A) Migration pattern of clone 7 DNA *Hin*dIII fragments. The position of the pBR322 vector and the BTV-17 segment 2/3 cDNA are marked on the left side of the figure. (B) *Hin*dIII restriction fragments of cDNA, with the sizes of the fragments in kilobases listed at the right of the figure.

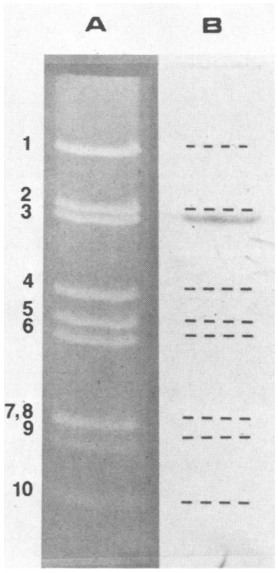

FIG. 2. Nick translation hybridization of clone 7 plasmid DNA. (A) RNA migration pattern of BTV-17 genomic RNA run on an agarose gel. (B) Autoradiograph of clone 7 DNA hybridized to BTV-17 segment 3 RNA. The numbers to the left of the figure represent the numbering system for BTV genomic RNA. Segments 7 and 8 comigrate in agarose gels.

Hybridization to DNA probes was performed by a procedure similar to that described by Denhardt (6). RNA blots were prehybridized at 42°C for 16 h in 50% formamide containing 5× SSC (1× SSC equals 0.15 M NaCl plus 0.15 M sodium citrate), 1% sodium dodecyl sulfate, 100 μg of denatured salmon sperm DNA per ml, and 0.04% each of polyvinyl-pyrrolidone, Ficoll, and bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.). The membranes were then hybridized for 16 h at 42°C to nick-translated, ^{32}P-labeled DNA samples in the same formamide mixture. After hybridization, the membranes were washed twice at 25°C for 5 min in 2× SSC, twice at 65°C for 30 min each in 2× SSC containing 0.5% sodium dodecyl sulfate, and twice for 30 min each at 25°C in 0.1× SSC. After washing, the membranes were air dried and autoradiographed.

DNA sequence analyses. DNA nucleotide sequences were determined on strand-separated, end-labeled restriction fragments containing viral DNA insert sequences by the method of Maxam and Gilbert (11), using the formic acid protocol for the A+G reactions as described previously (2).

RESULTS

Synthesis of full-length ds cDNA and strategy for cloning BTV genes. The strategy used to clone the BTV genes was similar to the strategy for reovirus gene cloning as described by Cashdollar et al. (5), with some modifications. Briefly, individual ds RNA species were isolated from an agarose gel and were polyadenylated at the 3′ ends of both plus and minus strands by first melting them in 10 mM methyl mercury and then by using *E. coli* polyadenylic acid polymerase to add polyadenylic acid tails 20 to 30 residues long. cDNA copies of both strands were then synthesized with oligodeoxythymidylic acid and reverse transcriptase (3). After the removal of the RNA template strands by digestion with KOH, the cDNA products were self-annealed, and to ensure that all strands were full length, their 3′ ends were repaired with the Klenow fragment of *E. coli* DNA polymerase I. For the segment L2 and L3 RNA species, the estimated size of the derived cDNA copies (2.8 kilobases)

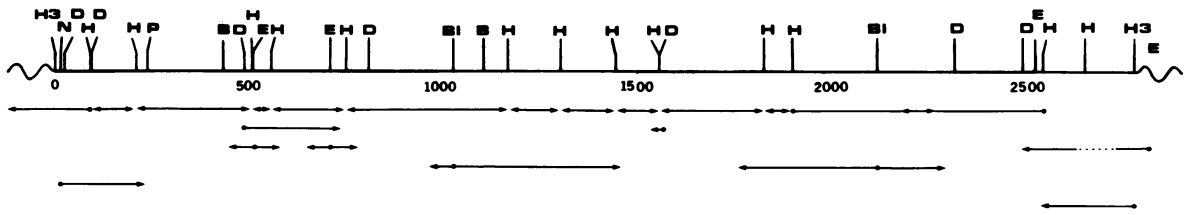

FIG. 3. Sequence strategy used to determine the sequence of the cloned BTV-17 segment 3 gene. The restriction enzymes used to generate the restriction fragments are shown on the right side of the figure. The distances and directions in which individual strands were sequenced are shown by the solid arrows. Restriction site symbols used are as follows: H3, *Hin*dIII; N, *Nco*I; D, *Dde*I; H, *Hin*fI; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; and BI, *Bgl*II.

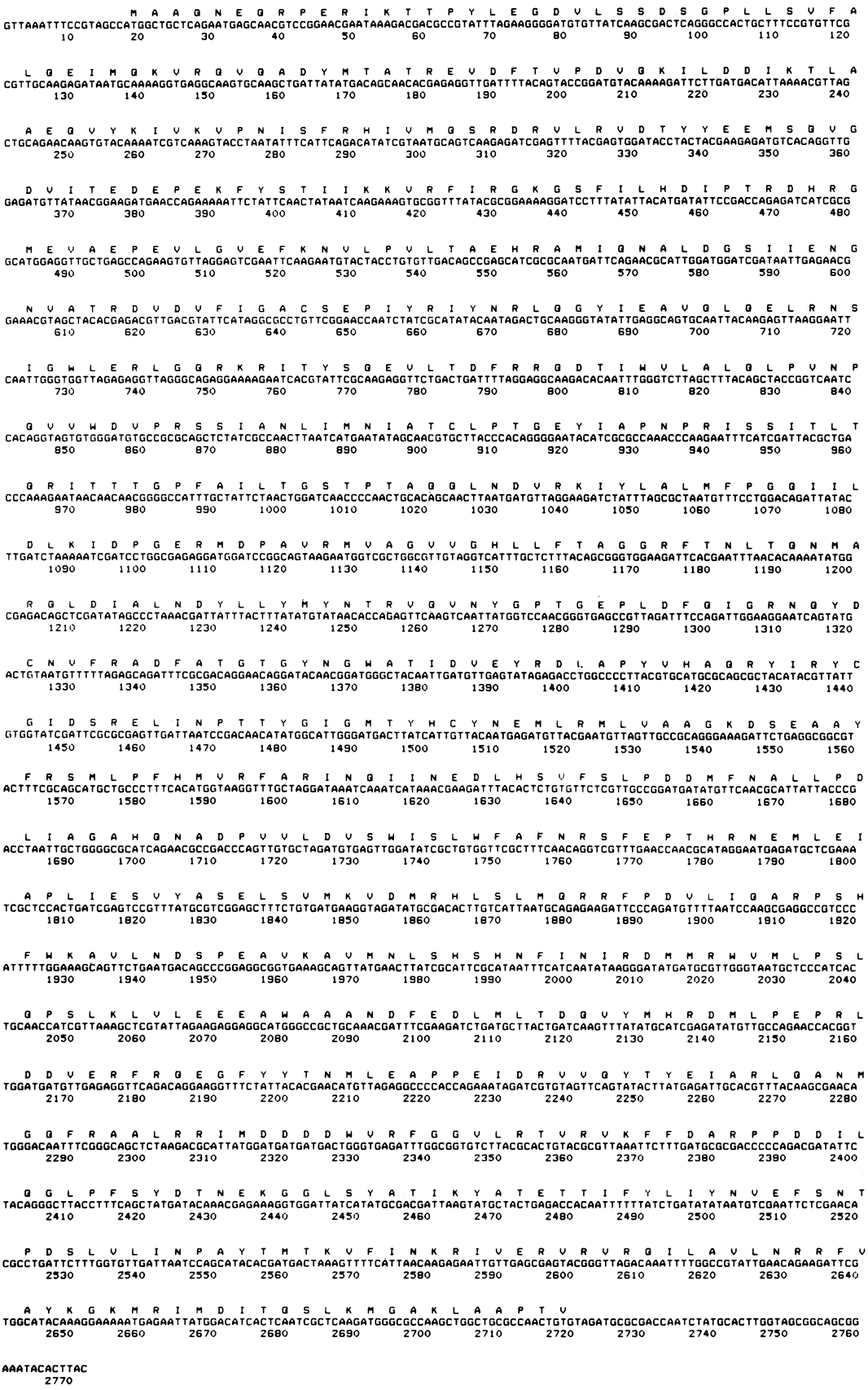

FIG. 4. The nucleotide sequence of the cloned L3 gene of BTV-17. The open reading frame begins at position 18 to 20 and is terminated by the TAG codon at position 2721 to 2723. The single-letter amino acid codes are shown above their respective DNA codons.

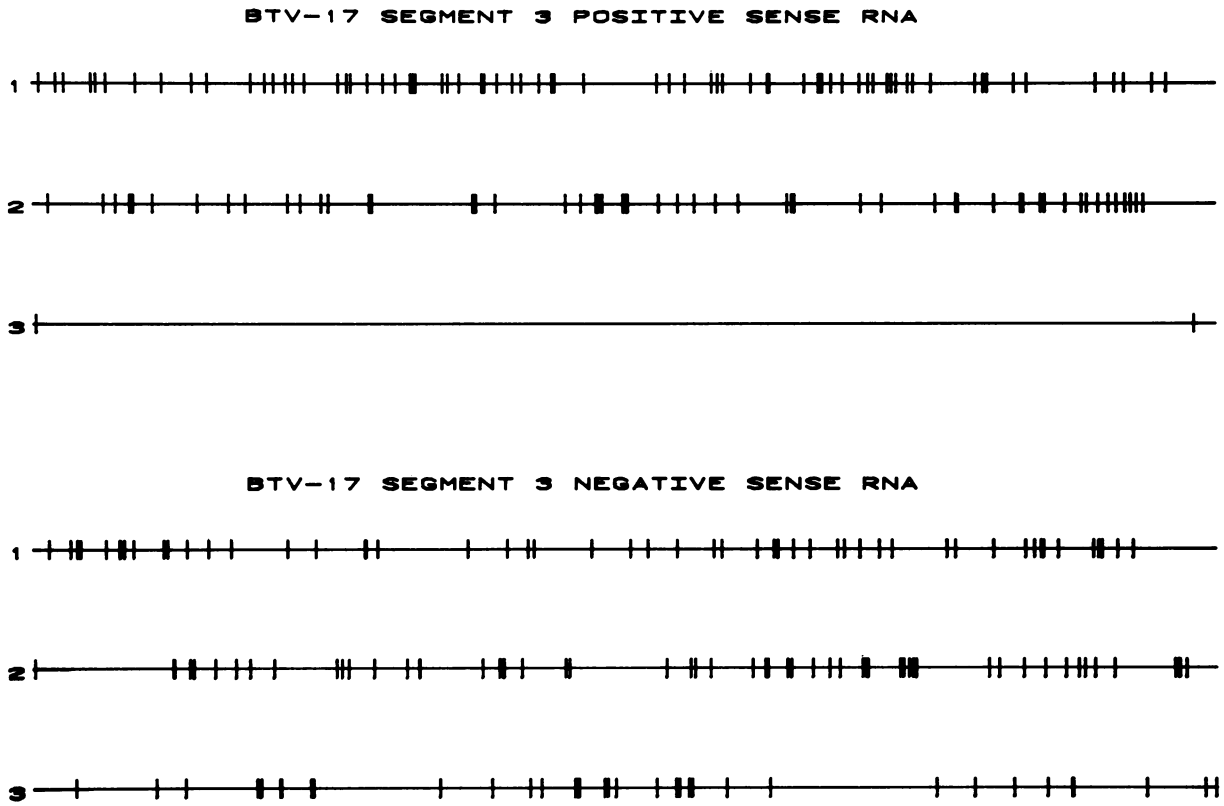

FIG. 5. The positions of stop codons in the segment 3 gene of BTV-17 for all possible reading frames. The numbers to the left of each horizontal line denote the reading frame. The vertical lines represent the positions of the stop codons for a given reading frame.

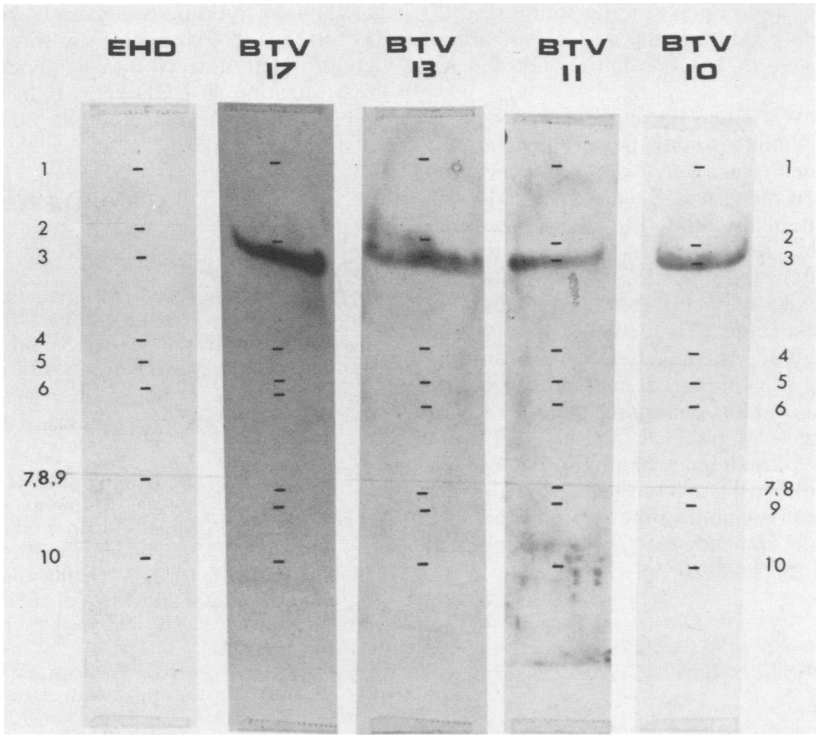

FIG. 6. Hybridization of BTV clone 7 to BTV and EHD L3 RNA segments; autoradiograms of nick-translated, ^{32}P-labeled clone 7 DNA hybridized to genomic RNAs of four U.S. BTV serotypes and EHD-1, purified from infected cells. The numbers to the sides of the figure represent the numbering systems for the genomic RNA segments. Segments 7 and 8 (BTV) and segments 7 to 9 (EHD) comigrate in agarose gels.

suggested that they were complete or almost complete transcripts of the BTV L2 or L3 segments. The cDNA duplexes were cloned into the *E. coli* plasmid pBR322 as described above.

The size of one of the L gene clones (clone 7) was determined as shown in Fig. 1. The DNA insert in the recombinant clone was excised with *Hin*dIII from plasmid DNA and run in a 1% agarose gel in parallel to molecular markers of λ DNA. The size of the DNA fragment, 2.8 kilobases, suggested that it contained a full-length L3 gene. By sequence analysis, it was determined to be 2,772 base pairs in length, excluding the homopolymeric tails.

Since BTV cDNA L2 and L3 segments comigrated in agarose gels, confirmation of the identity of the derived BTV DNA clone was obtained by determining the ds RNA genome segments to which they were capable of hybridizing. Figure 2A shows a Northern blot of total BTV-17 RNA after electrophoresis in a 1% agarose gel, and Fig. 2B demonstrates that nick-translated, ^{32}P-labeled probes from the clone hybridized specifically and only to the BTV-17 L3 RNA segment.

Nucleotide sequences and predicted gene products. The sequence of the cloned L3 gene was determined on strand-separated, end-labeled restriction DNA samples of one L3-specific clone (clone 7) by a standard technique, using the restriction endonuclease fragments as shown in Fig. 3. The distance each DNA strand was sequenced is indicated by an unbroken arrow. The complete nucleotide sequence of the DNA clone of the L3 gene is presented in Fig. 4 with the predicted amino acid sequence of the single extensive open reading frame. About 80% of the gene was analyzed in both directions. Excluding the homopolymeric tails, the entire sequence is 2,772 nucleotides long. The L3 ds RNA has a corresponding size of 1.78×10^6 daltons in agreement with previous reports (19). A single open reading frame (Fig. 5) codes for a primary gene product composed of 901 amino acids with a calculated size of 103,412 daltons and −4 net charge.

Hybridization capability of clone L3 segment with equivalent segments of U.S. BTV and epizootic hemorrhagic disease (EHD) virus, a related orbivirus. Since our previous work (13, 16, 17) had shown that the four U.S. serotypes, BTV-10, -11, -13, and -17, are genetically related to each other (e.g., they have comparable RNA fingerprints and can reassort their viral RNA species), the question of whether any sequence homologies exist between the L3 segments of these viruses was investigated. To detect any common sequences, a nick-translated, ^{32}P-labeled cDNA probe of the L3 segment of BTV-17 was prepared and hybridized (6) under stringent conditions to blots containing genomic RNAs purified from cells infected with either BTV-10, -11, -13, or -17, or EHD-1 virus. The ^{32}P-labeled probe hybridized to the L3 RNA segments of all four U.S. serotypes but did not hybridize to the equivalent segment of the related orbivirus, EHD (Fig. 6). The results indicate that there are common nucleotide sequences in this gene for all U.S. BTVs.

DISCUSSION

The nucleotide sequence of the L3 gene of BTV-17 is 2,772 base pairs long (Fig. 4), in good agreement with the 2.8-kb length calculated from agarose gels. The clone represents the complete RNA sequence as shown by the correspondence of the terminal DNA nucleotide sequences with those determined for the termini of segment 3 genomic RNA (12).

The DNA sequence of the L3 gene has one long open reading frame, beginning with an ATG at position 18 to 20 and terminating at position 2721 to 2723 with a TAG codon. This reading frame codes for a protein of 901 amino acids. The first ATG codon is the most likely initiation point for the L3 gene product. In addition to being the nearest ATG to the end of the RNA, it has a guanine at position +4 and another at position −3 (8). The second ATG (at position 31 to 33) only codes for a protein seven amino acids long.

The reading frame that generates the 901-amino acid proteins is assumed to be the correct reading frame, as all other reading frames (Fig. 5) fail to generate any amino acid sequences of significant length. The L3 protein has a size of 103,412 daltons with a net charge of −4 at neutral pH.

When the original sequencing of the BTV RNA termini was reported, it was not possible to determine which strand of the ds RNA contained the information that coded for the BTV proteins (12). The strands were referred to as the slow or fast strands by their migration rates in a 5% polyacrylamide strand-separating gel. The present work shows that for the L3 gene of BTV-17, the slow strand codes for protein. It will be of interest, as further BTV genes are sequenced, to determine whether the strands with the corresponding conserved-end sequences to the slow strand of L3 also code for proteins.

Our extensive studies on the oligonucleotide fingerprinting comparisons of isolates representing the U.S. BTV serotypes have shown previously that both genetic drift (involving the incidence and accumulation of point mutations in the viral genome) and recombination (involving RNA segment reassortment) occur frequently among these viruses (13, 16, 17). The hyridization data, which show that the L3 segment of BTV-17 hybridizes efficiently under stringent conditions, as do L3 RNA species of three other BTV serotypes, confirm that these viruses are genetically similar. The lack of hybridization to EHD virus is in agreement with the serotypic data that indicate that this orbivirus is genetically distinct.

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