A Comparison of Genomic Homologies among the Coxsackievirus B Group: Use of Fragments of the Cloned Coxsackievirus B3 Genome as Probes

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SUMMARY

Using fragments of the cloned coxsackievirus B3 (CB3) genome as hybridization probes, regions in the CB3 genome with widely varying homology to heterologous CB serotype genomes have been demonstrated. A region composed of about 3.2 to 3.5 kbp of the CB3 genome in the 5' half of the map is essentially unique to CB3 and exhibits little or no homology with heterologous CB serotype RNAs. The non-coding terminal 5' end of the CB3 genome is well-conserved among the CB serotype RNAs tested. A sequence in the P3 region of the CB3 genome is also well conserved among heterologous CB serotypes.

INTRODUCTION

The coxsackie B viruses (CB viruses) are ordered in six serotypes (CB1 to CB6) and are classified as enteroviruses in the Picornaviridae family. The RNA genome is single-stranded, approximately 7.4 kb long and polyadenylated at the 3' end (Chatterjee & Tuchowski, 1981). The CB viruses have been linked to numerous diseases, among them being various myocardial cardiopathies (Grist et al., 1978; Woodruff, 1980). Genomic relationships among the CB group have not been extensively defined. Hewlett & Florkiewicz (1980) showed that the terminal ten bases at the 5' end of CB1 were identical to the 5' end terminal ten bases in poliovirus types 1 and 2 genomic RNA. Brown et al. (1976) and Harris et al. (1977), using RNA/RNA hybridization, showed considerable divergence among the RNA genomes of strains of CB5. Similarly, Young (1973) compared the homology of CB4 RNA with some enterovirus RNAs. The genome of CB3, strain Nancy, has been molecularly cloned, restriction-mapped and the 5' genomic terminal region quenched (S. Tracy, N. M. Chapman & H.-L. Liu, unpublished). While this work was in preparation, Hyypia et al. (1984) reported the cloning of, but did not describe, a fragment of the CB3 genome.

This paper presents results from a study which specifically compared the homologies in the genomes of the CB group of viruses to regions of the CB3 genome defined by restriction fragments of the cloned genome. In this manner, a sequence in the CB3 genome representing approximately 45% of the coding region has been identified which shares little or no homology to heterotypic CB genomes. This sequence may prove useful identifying clinical isolates.

METHODS

Viruses and isolation of viral RNA. All viruses were obtained from the American Type Culture Collection with the exception of CB3 which was the gift of J. Galpin. Serotypes and strains used were CB1 (Conn-5), CB2 (Ohio-1), CB3 (Nancy), CB4 (JVB, Benschoten), CB5 (Faulkner) and CB6 (Schmitt). Viruses were propagated in Vero cells.

Viral RNA was isolated by proteinase K digestion and phenol/chloroform extraction (S. Tracy, unpublished method). One µg of viral RNA was the equivalent of 0.025 A_{260} units.

Isolation and nick translation of double-stranded DNA. Restriction endonucleases were obtained from several sources and used as suggested by the supplier. Restriction endonuclease-generated DNA fragments for use as hybridization probes were separated in neutral agarose gels and isolated from the gels by electroelution onto dialysis
membranes or using hydroxyapatite (Tracy, 1981). Electroeluted fragments were passed through 0.45 µm cellulose acetate filters prior to concentration by ethanol precipitation.

Double-stranded recombinant DNAs were radioactively labelled with $^{32}$P to high specific activity by nick translation (Rigby et al., 1977). Nick-translated fragments were separated from free nucleotides on Sephadex G-100 (Pharmacia). Double-stranded DNA was denatured in 0.4 M-NaOH at 68 °C for 5 min, chilled on ice-slush for 10 min, then neutralized immediately prior to hybridization.

**Preparation and hybridization of RNA dot blots.** Viral RNA was denatured in 50% formamide at 60 °C for 20 min and chilled in ice-slush. RNA was diluted with ice-cold 20 × SSC (3 M-NaCl, 0.3 M-sodium citrate) to 1% formamide and immediately applied to nitrocellulose (BA85, Schleicher & Schüll) which had been saturated previously with 20 × SSC and dried. A Minifold apparatus (Schleicher & Schüll) was used for uniformity of dot size. Forty ng of RNA was applied to each dot. Filters were baked in a vacuum at 80 °C for 2 h and used within 2 weeks.

Blots were prehybridized for 4 h, then hybridized with radioactive probe for 16 to 30 h at various temperatures in 500 mM-NaCl, 50 mM-sodium phosphate buffer pH 6.8, 10 mM-EDTA, 0.1% SDS, 1 × Denhardt’s buffer (0.02% each bovine serum albumin, polyvinylpyrrolidone and Ficoll), and 100 µg/ml denatured *Escherichia coli* DNA. Blots were washed in 2 × SSC, 0.5% SDS at room temperature for 30 min with two changes of buffer, then for 2 h at 62 °C in 0.2 × SSC, 0.2% SDS with four changes of buffer. Filters were exposed to Kodak XAR5 film with intensifying screens at −70 °C.

**RESULTS**

The genome of CB3 (Nancy) has been molecularly cloned (S. Tracy, N. M. Chapman & H.-L. Liu, unpublished). Briefly, six partially overlapping clones (pCBIII51, 29, 50, 33, 35 and 210) containing 7.20 kbp of specific CB3 information were isolated and characterized from a small library of recombinants. The library had been constructed by insertion of dC-tailed CB3 cDNA : RNA hybrids into the dG-tailed *PstI* site of pBR322. Fig. 1 shows the restriction map for the viral genome and the overlapping clones which comprise it. Approximately 250 bases, comprising the terminal 3' sequence, were not located in any clone examined. Hybridization of each clone separately as probe to CB1 to CB6 RNAs showed different extents of hybridization with heterologous RNAs, suggesting that specific regions of the CB3 genome varied significantly in homology with respect to other CB RNAs.

To explore this observation further, 17 restriction fragments, A to Q (Fig. 1), were isolated from agarose gels, radioactively labelled by nick translation and used to probe RNA dot blots at 68 °C and 81 °C. The fragments varied in ability to label by nick translation; in general, from 10⁶ to 8 × 10⁶ c.p.m. of nick-translated fragments were applied to each blot. The autoradiograms from these experiments are shown in Fig. 2. In all cases, the fragments hybridized most prominently to the homologous CB3 RNA.

Fragment A (pCBIII210) lacked significant homology to CB4 RNA while fragment B (pCBIII35), a much smaller fragment contained within fragment A, showed poor hybridization to CB4 and 6 RNA at 68 °C. The similarity between CB3 and 5 RNAs is conserved in this region of the CB3 genome, for CB5 RNA hybridized well with both probes. The fragment C (pCBIII35) hybridized well with CB5 RNA at 68 °C. CB1 and 2 RNAs showed greater homology than did CB4 and 6 RNAs to fragment C but all were significantly less homologous than CB5. Fragment D (pCBIII35) hybridized to similar extents with all heterologous RNAs at 68 °C. Fragments A to D hybridized well only to CB3 RNA at 81 °C.

The fragments E and F (pCBIII35) are contained within fragment D. Fragment E hybridized to the greatest extent with CB3 and 5 RNAs, then to CB1 and 2 and CB4 and 6 RNAs. At 81 °C, the extensive hybridization to CB5 RNA was reduced dramatically, as were hybridizations to the other heterologous RNAs. It is noteworthy, however, that fragment E did hybridize to a low but significant extent at 81 °C to CB1, 2 and 5 RNAs whereas fragments A, B and C hybridized essentially not at all to these RNAs at 81 °C. Fragment F also hybridized well to CB5 RNA at 68 °C but not as strongly as fragment E; CB1 and 2 RNAs showed much greater homology with fragment F than did CB4 and 6 RNAs.

Fragment G (pCBIII35) exhibited little homology with RNAs of CB4 and 6 but maintained the strong hybridization with CB5 RNA as observed in fragment D. The pattern from the 81 °C hybridization was similar but the annealing was to a lesser extent than that seen with fragment D and E.
**Fig. 1.** Restriction endonuclease cleavage map for the CB3 genome and location of the restriction fragments isolated for use as probes. Orientation with the viral RNA genome is depicted with 5’ to the left. Arrows orient insert by pointing to the unique EcoRI site in pBR322 vector. Subscript on each fragment denotes recombinant plasmid of origin. Fragments O51 and B35 were isolated with adjoining pBR322 sequence attached. Restriction endonuclease abbreviations are: D, HindIII; G, XhoI; H, HincII; K, EcoRV; L, BssHII; M, SphI; N, NcoI; P, PstI; R, EcoRI; S, SstI; V, PvulI; X, XbaI.

**Fig. 2.** Hybridization of restriction fragments of the CB3 genome to CB1 to CB6 RNAs at two different temperatures. Temperatures, name of the fragment used as probe, and CB serotypes from which the RNA came are shown. The fragments are arranged as ordered in the genome (Fig. 1); a line representing the viral RNA orientation is between the two sets of data. Blots were exposed for 21 to 26 h.

Beginning with fragment H (pCBIII33), which overlaps the 5’ end of fragment G, a greatly decreased hybridization to heterologous RNAs at 68 °C was evident. This low to undetectable level of hybridization to essentially all but CB3 RNA extended as far as fragment O. This region, about 3.2 to 3.5 kbp long or about 45% of the viral genome, is at best only distantly related to
heterologous RNAs when assayed at the lower criterion. By comparison to the gene map for poliovirus (Kitamura et al., 1981; Pallansch et al., 1984), the part of the CB3 genome described by fragments I to O constitutes what is likely to be the coding region for the viral capsid proteins VP2, VP3 and VP1 in addition to the N-terminal portion of the P2 cleavage product region. The region of CB3 specificity was most pronounced in and delineated by fragments L (pCBIII50) and N (pCBIII29). Even the faint hybridization seen previously at 81 °C to CB1, 2 and 5 RNAs in fragments A to G entirely disappeared in this region.

Fragments O and P (pCBIII51) showed hybridization at 68 °C to heterologous RNAs. The homology of CB3 DNA to CB5 RNA was no more extensive than to other RNAs in this region in contrast to fragments A to C and E to G. Fragment Q, representing the terr-inal 5' 550 bases of the viral genome, hybridized exceptionally well to all RNAs, showing an almost identical extent of hybridization to CB3 at 68 °C as to heterologous RNAs. At 81 °C, the difference in sequence homology was discerned, with fragment Q hybridizing most prominently with the CB3 RNA. Interestingly, this fragment hybridized extremely well at 81 °C to CB4 and 6 RNAs whereas it hybridized to a very low extent with CB1 and 2 RNAs. The region encompassed by fragment Q represents a large portion of the non-coding 5' end region of the viral RNA. Fragment P, which lies 3' to fragment Q, represents the coding region for the start of the open reading frame and for capsid protein VP4 (data not shown).

DISCUSSION

Fragments of the cloned CB3 genome have been used as hybridization probes to examine the homologies between the CB3 genome and those of heterologous CB genomes. As expected, the restriction fragments hybridized to the greatest extent with the homologous CB3 RNA in all cases. Except for the generally well-observed regions in pCBIII51 (fragment Q) and pCBIII35 (fragment D), the CB3 DNA hybridized to the least extent with CB4 and 6 RNAs, consistent with results obtained with CB cDNA/RNA hybridization (S. Tracy, unpublished). The CB3 genomic fragments A to G and Q hybridized well to the heterologous CB1, 2 and 5 RNAs at 68 °C whereas only fragments E (contained within fragment D) and Q hybridized well to CB4 and 6 RNAs. The region represented by fragments A to G spans about 3-2 kbp on the 3' end of the viral genome; fragment E represents 1-25 kbp located approximately 1-2 kbp in from the 3' end of the viral restriction map. Orienting the protein-processing regions of poliovirus (Kitamura et al., 1981; Pallansch et al., 1984) from the 5' end of the CB3 genome, fragments A to G would lie in the 3' half of the P2 region and in the entire P3 region. Fragment E likewise would represent primarily the N-terminal third of the P3 region where the viral protease is encoded. Fragment Q, which hybridized well at 68 °C to all CB RNAs, represents the 5'-terminal 550 base sequence in the untranslated region of the viral RNA. Thus, CB3 shares a well-conserved homology in the N-terminal third of the P3 region (fragment E) and in the 5' end region (fragment Q) with all the heterologous CB RNAs tested. There is good homology between CB3 and both CB4 and 6 RNAs only in these regions whereas CB1, 2 and 5 share nearly 50% of the CB3 sequences with good homology.

The differences in CB genomic primary structure apparently are distributed much less evenly throughout the CB genomes than the variations which have been observed with the most-studied member of the enterovirus group, poliovirus. Total nucleotide sequences have been deduced for poliovirus RNA from serotypes 1 and 3 (Stanway et al., 1984; Cann et al., 1983; Kitamura et al., 1981; Nomoto et al., 1982; Racaniello & Baltimore, 1981). Because of the generally dispersed genetic variation in the poliovirus genomes, any single region greater than 400 to 500 bases long could be expected to hybridize well to and detect other poliovirus RNAs even at the stringent (81 °C, 0-5 M-NaCl) criterion used in this study. This was not the case for CB3 (Nancy) RNA.

These observations imply significant divergence among the CB genomes and the possibility of discrete regions of serotype-specific sequence homology in other CB genomes as well. Such areas of conservation are likely to be in the 5' terminal 500 to 600 bases (the non-coding region) and in the P3 region of the genome in which the protease is encoded. Different isolates of CB3 obtained over 20 years are being examined for relatedness to the cloned fragments of the CB3 (Nancy)
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...genome in an effort to determine in which genomic region(s) active sequence alteration may be occurring.

These sequence homology studies have defined at least two specific regions of the CB3 genome useful for hybridization probes in detection assays and identification of clinical CB isolates. The 2.05 kbp sequence contained in pCBIII29 can be used as a CB3-specific probe. The conditions used to hybridize blots (0-5 M-NaCl, 68 °C) allowed CB3 cDNA to detect readily all CB RNAs tested and raising the temperature of the hybridization reaction to 81 °C essentially obliterated hybridization of the cDNA to all but the CB3 RNA. The total plasmid pCBIII29 hybridized only to CB3 RNA even at 58 °C (data not shown). The 5' terminal sequence (fragment Q of pCBIII51) can be used as a general probe as can the region in fragment E (pCBIII135).

While it is unlikely that strain variations within the serotypes would void the utility of these probes, experiments are now in progress to test these sequences as probes against RNAs from CB3 and CB5 isolated over a span of 20 years. For example, the Faulkner strain of CB5, used in this study, may not be the best candidate for a prototype CB5 strain. Brown et al. (1976) and Harris et al. (1977) noted a closer homology between CB5 (Faulkner) and an isolate of swine vesicular disease virus than between CB5 (Faulkner) and two other strains of CB5. It is known that human isolates of poliovirus show a large variation over time (Nottay et al., 1981) and that considerable variation in the CB5 genome has occurred since its initial isolation in 1952 (Brown et al., 1976). Thus, the good homology between CB3 (Nancy) and CB5 (Faulkner) observed here might be a strain-specific phenomenon.

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REFERENCES


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