Comparison of Genomic Homologies in the Coxsackievirus B Group by Use of cDNA:RNA Dot-Blot Hybridization

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Radioactive cDNAs to coxsackievirus B (CB) genomic RNAs were used to probe genomic homologies between RNAs of the CB group serotypes. CB5 shared approximately equivalent homology with the other five CB serotypes, CB1, CB2, and CB3 hybridized preferentially to CB5 in turn, suggesting that these three have diversified in one direction from CB5. CB4 and CB6 were more related to each other than to the other serotypes at a lower criterion, but at a more stringent criterion they did not demonstrate apparent homology with each other. This suggests that they have diversified from CB5 as well but perhaps earlier in time or at a greater rate than did CB1, CB2, or CB3. Some potential applications of these relationships to clinical CB detection assays based on nucleic acid hybridization are discussed.

The coxsackievirus B (CB) group is composed of six serotypes (CB1 through CB6) and is classified as enteroviruses in the Picornaviridae. The viral genome is a single-stranded RNA molecule of about 7,400 bases which is polyadenylated at the 3' end. The CB viruses are implicated in several human diseases, and there is convincing evidence for a link between various myocardio pathies and CB infection (12). At present there exists no rapid and clinically usable technique for the identification of the CB viruses or picornaviruses in general. To identify a specific CB serotype in a clinical assay by using nucleic acid hybridization, knowledge of the homologies between the genome for which one is searching and other, related viral genomes is vital.

Little comparative data exist for the CB group. Young (13) demonstrated varying homology between CB4 and other, non-CB enteroviruses by using RNA:RNA hybridization. More recently, Hewlett and Florkiewicz (6) showed that CB1 and poliovirus RNAs have an identical 10-base sequence, UUAAGACGAC, at the 5' end of the RNA. Tracy et al. (S. Tracy, N. M. Chapman, and H.-L. Lin, Arch. Virol., in press) have molecularly cloned and partially characterized the CB3 genome and have shown this sequence to be maintained intact in the 5' terminus of the CB3 genome as well. Brown et al. (1) and Harris et al. (5) demonstrated that wide divergence can occur among the CB5 strains. Hyyppä et al. (7) reported that they had cloned the 3' half of the CB3 genome and that it would hybridize with entervoiral RNAs.

This study reports the results of using representative cDNA from each of the six CB serotypes as probes to hybridize with nitrocellulose-bound heterologous CB RNAs to examine the homologies among the CB serotypes.

MATERIALS AND METHODS

Isolation of viral RNA. CB3 (Nancy) was a gift from J. Galpin; all other viruses were obtained from the American Type Culture Collection. 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. The virus was pelleted from cleared tissue culture medium in a Beckman 60Ti rotor at 252,000 × g and 4°C for 2.5 h. The virus used as the source of target RNA on nitrocellulose filters was resuspended in 10 mM NaCl-50 mM EDTA-10 mM Tris-hydrochloride (pH 8.3)-10 mM β-mercaptoethanol, lysed in 0.2% sodium dodecyl sulfate and 500 μg of proteinase K (Boehringer-Mannheim) per ml, and incubated at 55°C for 30 min. The viral RNA was extracted twice with an equal volume of redistilled phenol-chloroform (1:9, vol/vol) and extracted once with chloroform only, and the RNA was ethanol precipitated at -20°C in the presence of 20 μg of highly purified oyster glycogen per ml as carrier and 0.5 M NaCl. Purified RNA was stored as aliquots in sterile water at -70°C; the A260/A280 ratio was greater than 1.9 for all preparations. Virus used for preparation of RNA for cDNA synthesis was centrifuged to equilibrium in CsCl before extraction of RNA as described above.

Synthesis of radiolabeled cDNA. cDNA was synthesized essentially as described by Kacian and Myers (8). RNasin (Promega/Biotech), a human placental ribonuclease inhibitor, was included in the reverse transcriptase reactions at 3,000 U/ml; oligmeric calf thymus (OCT) DNA primer (9) was used at 80 μg/ml; nonradioactive dCTP and dTTP were used at 0.2 mM; and [α-32P]dATP and [α-32P]dGTP (800 Ci/mmol; Amersham Corp.) were used at 0.0025 mM in the reaction. Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences. Genome-length cDNA was synthesized as the primary product and in high yield when oligo(dT)10 was used as primer (2 μg/ml), from which it may be inferred that the viral RNA template was largely intact. Oligo(dT)-primed cDNAs were synthesized at a lower specific activity, as the presence of large amounts of high-specific-radioactivity deoxynucleoside triphosphate tends to reduce both yield and size of the cDNA (S. Tracy and D. E. Kohne, unpublished observation); all four nonradioactive deoxynucleoside triphosphates were present at 0.2 mM, and [α-32P]dGTP and [α-32P]dATP were included at 5 × 10-4 to 10-3 mM each. OCT-primed reactions proceeded for 45 min, and oligo(dT)-primed reactions proceeded for 65 min at 40°C, after which the reactions were made 50 mM in EDTA. The cDNA was separated from free nucleotides on Sephadex G-100 (Pharmacia Fine Chemicals, Inc.). After ethanol precipitation and resuspension, DNA was incubated in 0.4 M NaOH at 68°C for 1 h before hybridization to hydrolyze viral RNA. The specific radioactivity of OCT-primed cDNA was approximately 106 to 2 × 108 dpm/μg.

RNA dot-blot filters and hybridization. Nitrocellulose (BA85; Schleicher & Schuell, Inc.) was soaked first in water,
Results

Blots containing viral RNA from CB1 through CB6 were hybridized with OCT-primed CB3 cDNA at 68, 75, 78, 81, and 83°C as described above (Fig. 1). Previous work (S. Tracy, In D. T. Kingsbury and S. Falkow (ed.), Proceedings of NRL-UCB International Symposium on Rapid Detection and Identification of Infectious Agents, in press) had shown that hybridization at 68°C provides the optimal rate of hybridization under these conditions and that the lower limit of detection in this buffer at 68°C with a Minifold-generated dot size of 5-mm diameter is 1 to 5 pg of viral RNA applied per dot. As expected, the cDNA annealed best with the homologous CB3 RNA at all temperatures. The CB3 cDNA also hybridized well to CB5 RNA even at 81°C, close to the melting temperature of well-matched hybrids of entoviral RNA and cDNA (11). Reaction at 83°C, however, significantly reduced hybridization to heterologous RNAs.

The preceding experiments showed that at 68°C and 0.5 M NaCl, hybridization of distantly related sequences was possible, whereas a temperature of 81°C was sufficiently stringent to allow only that hybridization based on close nucleotide homology. To examine the relationship of one CB serotype to another, complementary OCT-primed DNAs were made as described for the CB serotypes CB1 through CB6. These were hybridized with RNA blots identical to those shown in Fig. 1 at both 68 and 81°C (Fig. 2). In every case, each CB cDNA hybridized best to its template RNA at both criteria. At 68°C (Fig. 2a), CB1, CB2, and CB3 cDNAs each hybridized next best to CB3 RNA, whereas CB4 cDNA hybridized next best to CB6 RNA and CB6 cDNA hybridized next best to CB4 RNA. CB5 cDNA hybridized essentially equally well to all heterologous RNAs at 68°C. At 81°C (Fig. 2b), CB1, CB2, and CB3 cDNAs also detected CB5 preferentially to other RNAs, suggesting regions of close homology in these genomes. CB4 cDNA hybridized to an almost equal extent with heterologous CB RNAs, no longer preferentially to CB6 as was seen at 68°C. The same can be seen for CB6 cDNA, suggesting that the homology observed at 68°C between CB4 and CB6 is relatively less well conserved than that observed between CB1, CB2, and CB3 cDNAs and CB5 RNA. As at 68°C, CB5 cDNA hybridized slightly better to CB4 and CB3 RNAs than to other RNAs at 81°C. This might be interpreted as short sequences of relatively good homology held in common with CB3 and CB4 RNAs and which may be distinguished from the background of general CB relatedness observed at lower temperature by the small but stable amount of hybrid formation observed at a more stringent criterion.

It is generally assumed that cDNA synthesized with OCT primers is "representative" of the template RNA. Representation, in this sense, implies that all sequences in the template are present in the cDNA at the same frequency as in the template. However, it has been argued elsewhere that cDNA obtained with OCT primers may not be ideally representative and could therefore give misleading hybridization data (S. Tracy, Ph.D. dissertation, University of....
California, San Diego, 1979). A full-length DNA copy of a polyadenylated RNA template, which may be obtained with an oligo(dT) primer and reverse transcriptase, would be the ideal representative cDNA. To examine the general validity of the results gained with an OCT-primed cDNA, a comparison of data obtained with a fully representative genomelength cDNA would be useful. Hybridization at the more stringent criterion would be able to point out differences perhaps obscured at the lower-criterion conditions.

Full-genome-length cDNA was transcribed for all six CB RNAs by using oligo(dT)10 primer. Figure 3 shows the results of using an alkaline agarose gel to analyze the size of the transcripts. Although some smear of lower-molecular-weight material is seen, the predominant fraction occurs in the 7- to 7.5-kilobase pair region as a neat band, strongly implying full-length, or essentially full-length, cDNA copies of the template RNAs. These cDNAs were incubated at 68°C in 0.4 M NaOH for 2 h to hydrolyze RNA as well as to partially degrade the large cDNAs to smaller sizes.

RNA dot blots containing all six CB serotype RNAs were then hybridized to the cDNAs at the more stringent 81°C criterion (Fig. 4). Owing to the lower specific radioactivity of these probes, the blots required a much longer exposure time. As observed with the OCT-primed cDNAs, CB1, CB2, and CB3 cDNAs were more closely related to CB3 RNA than to other RNAs. CB4 cDNA hybridized better to CB5 and CB6 RNAs. CB5 cDNA hybridized well to CB3 RNA, as had been observed with OCT-primed cDNA at 81°C (Fig. 2), but generally to an equivalent extent with the RNAs tested. CB6 cDNA also hybridized to an approximately equivalent extent with the heterologous RNAs. These results are in accord with those observed for OCT-primed cDNAs and therefore serve as a useful control for the reliability of those results.

**DISCUSSION**

cDNAs to six CB serotype RNAs were used to study the nucleotide homologies among the six serotype RNAs of the CB group. The results of Young (13) and Hyypiä et al. (7) showed that some sequence homology is conserved not only among the CB viruses but also among enteroviruses in general. The results here significantly extend these data by specifically analyzing the homologies within the CB group. At a relatively nonstringent criterion (68°C and 0.5 M NaCl), any CB cDNA hybridized to essentially all the CB RNAs tested. Raising the temperature of the hybridization reaction to 81°C, near the melting temperature of well-matched enteroviral cDNA-RNA hybrids, significantly reduced the interserotype hybridization and allowed a finer definition of CB serotype relatedness to be made.

The cDNA hybridization data may be used to evaluate the overall relatedness of the CB viral genomes used in this study. At the lower criterion, CB5 cDNA hybridized generally to an equivalent extent with all heterologous CB viral RNAs, suggesting an equivalent degree of homology and divergence from these RNAs. CB1, CB2, and CB3 cDNAs hybridized preferentially to CB5 RNA at both 68 and 81°C, suggesting that these genomes share a reasonably well conserved sequence with CB5 RNA. On the other hand, CB4 and CB6 cDNAs preferentially hybridized to CB6 and CB4 RNAs, respectively, at 68°C. At 81°C, the relationship between CB4 and CB6 was greatly diminished or obliterated, suggesting that the commonality between these two genomes is not well conserved and may be due to earlier or more rapid divergence from a common ancestral genome.

To demonstrate the utility of OCT-primed cDNA for clinical use as well as the study of genomic relationships, control experiments with oligo(dT)-primed, genomelength cDNAs were also performed. These two sets of data were in agreement. The use of DNA oligomers as primers for cDNA synthesis is widespread and is generally assumed to generate a reasonably representative cDNA population. The value of a truly representative cDNA is of lesser importance in clinical detection blot experiments. This is due in large part
to the relatively high concentrations of probe commonly used in such experiments. It is also due in part to the usual design of such experiments, which is to detect viral sequences present in the sample, but does not necessarily require the detection of all viral sequences present in the sample. However, a nonrepresentative probe could lead to false conclusions when comparative genomic homologies are evaluated. This could occur if a sequence in the target genome were not represented or were under-represented in the probe. Clearly, if the template RNA can be obtained intact and if a full-length cDNA could be transcribed, then such a probe or complete molecular clones of the sequence are preferable in such cases. It can be extremely difficult to make either of these criteria for certain viruses, however, and many viral genomes of interest have not been molecularly cloned. In such cases, and if required, a cDNA primed with DNA oligomers can be shown at some cDNA/RNA mass ratio to be representative by RNA "protection" experiments (2; Tracy, Ph.D. dissertation).

It has been shown that individual CB serotype cDNAs can be used to detect specific CB serotypes or can be used as pan-CB probes to detect essentially all CB serotype RNAs. In other work with fragments of the cloned CB3 genome, I have shown that some regions of the CB3 genome are highly conserved and other regions share good homology with heterologous CB RNAs (S. Tracy, J. Gen. Virol., in press). These results are encouraging for the eventual widespread use of nucleic-acid-hybridization picornavirus probes in many fields. A sensitive method to detect and identify picornaviruses in human feces, urine, and tissue would be of great clinical utility. The potential presence of picornaviruses in processed sewage water, so-called "gray water," is one reason why many municipalities and countries do not use reclaimed water for irrigation. Nucleic acid hybridization represents an ideal method, in connection with filtration-concentration methodology (4), for the rapid identification and quantitation of potentially infectious picornaviruses in such large volumes. Recent reports suggesting a relationship between insect and mammalian picornaviruses (10) and the data linking swine vesicular disease virus to a strain of CB5 (1) point out the widespread presence of picornaviruses and their ability to infect, and potentially shelter in, hosts other than mammalian hosts. The definition of serotype and strain-specific hybridization probes in the CB group, as well as research into the possibility of constructing a pan-picornavirus hybridization probe, should prove useful in seeking out new related viruses in hitherto unsuspected hosts.

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