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Replicase Gene of Coxsackievirus B3

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A cDNA copy covering two-thirds of the coxsackievirus B3 genome was cloned in the *Pst*I site of the pBR322 vector. A nucleotide sequence containing the gene for the viral replicase and the 3′ noncoding region of the coxsackievirus B3 genome was determined. The predicted amino acid sequence of the coxsackievirus B3 replicase was shown to be remarkably similar to that of the poliovirus 1 replicase. The 3′ noncoding region, in contrast, was only weakly homologous to the poliovirus 1 sequence but showed a close relationship to the sequence of swine vesicular disease virus, a variant of coxsackievirus B5. A 13-nucleotide-long segment located near the polyadenylic acid junction is conserved in several members of the enterovirus group and may thus serve an important function during replication of viral RNA.

The enterovirus group includes a number of viruses, some of which cause severe infections in humans (15). The polioviruses have been the most intensively studied members, although their medical significance in the developed world has decreased because of efficient vaccination programs. Other members of the enterovirus group, such as the coxsackieviruses, still present an important medical problem in both undeveloped and developed parts of the world.

The complete nucleotide sequence of the poliovirus 1 (PV1) genome has been established for both the virulent Mahoney strain (12, 19) and its attenuated counterpart, the Sabin 1 strain (17). These sequences have provided valuable information concerning genome structure and how attenuation may be determined by alterations in the VP1 protein, claimed to be the most important antigen in the enterovirus coat (2, 6, 7, 21, 25). Surprisingly little sequence information about the genomes of different enteroviruses is available, presumably because RNA sequencing methods are laborious. Molecular cloning circumvents this problem as it makes possible the conversion of the single-stranded RNA genome to a double-stranded cDNA copy, which can be produced in large quantities. This approach has been used successfully to study the genomes of two important picornaviruses, poliovirus and foot-and-mouth disease virus (12, 13, 19, 23). We have initiated a study of the coxsackievirus B3 (CB3) genome, the ultimate goal of the project being to explain the molecular basis of enterovirus pathogenicity. Racaniello and Baltimore have made the important observation that cloned cDNA copies of the poliovirus genome are infectious (20). This finding opens interesting possibilities for studying enterovirus genetics. We have chosen CB3 as a model because of its medical significance and because of the availability of simple animal models for studying virulence.

MATERIALS AND METHODS

Virus and cells. CB3 strain Nancy (4) was obtained from Hans Diderholm, Department of Medical Virology, Uppsala University. The virus was grown in suspension cultures of HeLa cells at a density of 5×10^5 cells per ml. The cells were harvested 8 h postinfection, and virus was purified by the method of Crowell and Philipson (5).

Purification of RNA. Purified virus was subjected to phenol-chloroform-isoamylalcohol (25:24:1) extraction twice. The water phase was then reextracted twice before the RNA was precipitated with 2.5 volumes of ethanol. The RNA was subsequently dissolved in TE buffer (0.01 M Tris-hydrochloride [pH 7.9], 1 mM EDTA) at a convenient concentration.

RNA analysis. Separation of RNA in formamide-containing gels was performed by the method of Vennstrom et al. (24). The RNA was subsequently transferred to nitrocellulose by the method of Thomas (22), and viral RNA was detected by hybridization with ^{32}P-labeled viral cDNA prepared by random priming (10).

Molecular cloning. cDNA synthesis was performed by the method of Land et al. (14). Briefly, 10 μg of purified viral RNA was primed with oligodeoxythymidylic acid, and the first strand was synthesized in a buffer containing 100 μg of actinomycin D per ml to prevent intramolecular base pairing. The RNA moiety of the resulting RNA-DNA hybrid was removed by treatment with 0.3 M NaOH at 42°C for 2 h. The single-stranded cDNA was then tailed with deoxyribosylcytosine by using terminal deoxytransferase (14). The second strand was synthesized by using avian myeloblastosis virus polymerase and oligodeoxyguanylic acid as primer. The double-stranded cDNA and the pBR322 vector (1) previously cleaved with *Pst*I were tailed with deoxyribosylcytosine and deoxyribosylguanine, respectively. The cDNA was purified by centrifugation in linear 5 to 20% sucrose gradients, and material from the leading edge of the cDNA peak was annealed with the vector and transformed into the *Escherichia coli* strain HB101.

Several hundred colonies were obtained, and the cDNA inserts in 100 individual clones were checked by agarose gel electrophoresis. Plasmid DNA was prepared by the method of Holmes and Quigley (9).

DNA sequencing. DNA sequencing was performed in accordance with the protocol of Maxam and Gilbert (16).

RESULTS

Cloning of the CB3 genome. Virus was purified by cesium chloride gradient centrifugation, and the viral RNA was extracted and analyzed by electrophoresis in an agarose gel under denaturing conditions. The results showed that the RNA preparation contained CB3 RNA of the expected size. Double-stranded cDNA copies were prepared and inserted into the *Pst*I cleavage site of the pBR322 vector as described above. Several hundred clones were obtained after transformation, and quick lysates were prepared from 100 randomly

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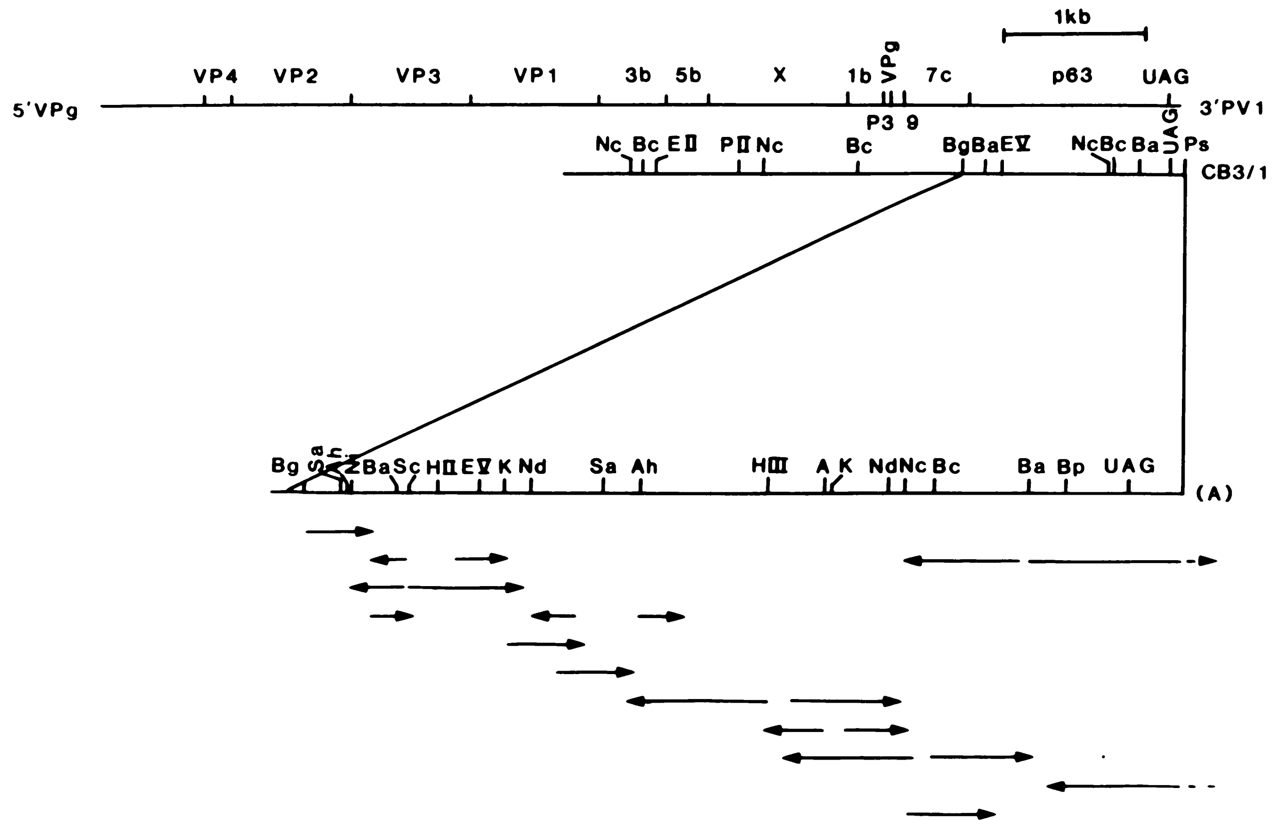

FIG. 1. Organization of the enterovirus genome, as deduced from the PV1 sequence (12, 17, 19). The PV1 genes (VP4 to p63) are positioned according to the sequence of the PV1 genome (12, 17, 19). The cDNA insert in clone pCB3/1 is also shown (CB3/1), together with cleavage sites for selected restriction enzymes. The arrows indicate the amount of sequence information that was derived from individual restriction enzyme cleavage sites. A, *Ava*I; Ah, *Aha*II; Ba, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*I; Bp, *Bsp*1286; EII, *Eco*RI; EV, *Eco*RV; HII, *Hinc*II; HIII, *Hind*III; K, *Kpn*I; Nc, *Nco*I; Nd, *Nde*I; Ni, *Nci*I; PII, *Pvu*II; Ps, *Pst*I; Sa, *Sac*I; and Sc, *Sca*I. kb, Kilobase; b, base.

chosen colonies. One clone, designated pCB3/1, was chosen for further analysis because of its large insert (ca. 4,500 base pairs). Further analysis with ^{32}P-labeled oligodeoxythymidylic acid$_{24}$ as a probe for colony hybridization revealed that clone pCB3/1 contained a polyadenylic acid [poly(A)] tract. The clone was used for sequence analysis. Figure 1 shows a map of the clone and the strategy used for the sequencing of the 3'-terminal part of the CB3 genome. This part was chosen for analysis as we expected that it would contain the gene for the viral replicase (12) and that the results thus would provide structural information about this interesting enzyme. We also wanted to collect sequence information from the 3'-terminal end of the RNA to compare it with the corresponding regions of other picornaviruses, as this part of the genome should contain important regulatory elements. Figure 2 shows the established sequence, which comprises 1,514 nucleotides from the 3'-terminal end of the CB3 genome.

Comparison with other picornavirus sequences. The established sequence could easily be aligned with the corresponding PV1 sequence (12, 19). A comparison of the two sequences revealed an unexpectedly high degree of sequence homology (Fig. 2 and 3). For the replicase gene the homologies were found to be 68 and 74% at the nucleotide and amino acid sequence levels, respectively. The homologous regions were distributed along the replicase gene, and only

TABLE 1. Predicted amino acid compositions of replicases from CB3 (Nancy) and PV1 (Mahoney)

Amino acid	No. of amino acids in replicase of[a]:	
	CB3	PV1[b]
Lys	35	38
His	15	12
Arg	20	18
Asp	29	31
Thr	27	27
Ser	30	30
Glu	28	31
Pro	22	21
Gly	32	28
Ala	20	30
Val	27	25
Met	14	17
Ile	27	25
Leu	46	46
Tyr	22	25
Phe	21	19
Trp	7	7
Cys	7	5
Asn	19	18
Gln	6	8

[a] The molecular weights of CB3 and PV1 were 52,329 and 52,524, respectively.
[b] Data are from Racaniello and Baltimore (19).

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     CUCAAACACUAUUUUAAUGAUGGGCAAGGUGAAAUAGAAUUUAUUGAGAGCUCAAAGGACGCCGGGUUUC
5960 AAGCGAUCAUACUUCACUCAGAGUCAAGGUGAAAUCCAGUGGAUGAGACCUUCGAAGGAAGUGGGA[illegible]UC

  71 CAGUCAUCAACACACCAAGUAAAACAAAGUUGGAGCCUAGUGUUUUCCACCAAGUCUUUGUGGGGAACAA
6030 CAAUCAUAAAUGCCCCGUCCAAAACCAAGCUUGAACCCAGUGCUUUCCACUAUGUGUUUGAAGGGGUGAA

 141 AGAACCAGCAGUACUCAGGAGUGGGGAUCCUCGUCUCAAGGCCAAUUUUGAAGAGGCUAUAUUUUCCAAG
6100 GGAACCAGCAGUCCUCACUAAAAACGAUCCCAGGCUUAAGACAGACUUUGAGGAGGCAAUUUUCUCCAAG

 211 UAUAUAGGAAAUGUCAACACACACGUGGAUGAGUACAUGCUGGAAGCGGUGGACCACUACGCAGGCCAAC
6170 UACGUGGGUAACAAAAUUACUGAAGUGGAUGAGUACAUGAAAGAGGCAGUAGACCACUAUGCUGGCCAGC

 281 UAGCCACCCUAGAUAUCAGCACUGAACCAAUGAAACUGGAGGACGCAAGGUACGGUACCGAGGGUCUUGA
6240 UCAUGUCACUAGACAUCAACACAGAACAAAUGUGCUUGGAGGAUGCCAUGUAUGGCACUGAUGGUCUAGA

 351 GGCGCUUGAUCUUACAACGAGUGCCGGUUACCCAUAUGUUGCACUGGGUAUCAAGAAGAGGGACAUCCUC
6310 AGCACUUGAUUUGUCCACCAGUGCUGGCUACCCUUAUGUAGCAAUGGGAAAGAAGAAGAGAGACAUCUUG

 421 UCUAAGAAGACUAAGGACCUAACAAAGUUAAAGGAACGUAUGGACAAGUAUGGCCUGAACCUACCAAUGG
6380 AACAAACAAACCAGAGACACUAAGGAAAUGCAAAAACUGCUCGACACAUAUGGAAUCAACCUCCCACUGG

 491 UGACUCAUGUAAAAGAUGAGCUCAGGUCCAUAGAGAAGGUAGCGAAAGGAAAGUCUAGGCUGAUUGAGGC
6450 UGACUUAUGUAAAGGAUGAACUUAGAUCCAAAACAAAGGUUGAGCAGGGGAAAUCCAGAUUAAUUGAAGC

 561 GUCCAGUUUGAAUGAUUCAGUGGCGAUGAGACAGACAUUUGGUAAUCUGUACAAAACUUUCCACCUAAAC
6520 UUCUAGUUUGAAUGACUCAGUGGCAAUGAGAAUGGCUUUUGGGAACCUAUAUGCUGCUUUUCACAAAAAC

 631 CCAGGGGUUGUGACUGGUAGUGCUGUUGGGUGUGACCCAGACCUCUUUUGGAGCAAGAUACCAGUGAUGU
6590 CCAGGAGUGAUAACAGGUUCAGCAGUGGGGUGCGAUCCAGAUUUGUUUUGGAGCAAAAUUCCGGUAUUGA

 701 UAGAUGGACAUCUCAUAGCAUUUGAUUACUCUGGGUACGAUGCUAGCUUAAGCCCUGUCUGGUUUGCUUG
6660 UGGAAGAGAAGCUGUUUGCUUUUGACUACACAGGGUAUGAUGCAUCUCUCAGCCCUGCUUGGUUCGAGGC

 771 CCUAAAAAUGUUACUUGAGAAGCUUGGAUACACGCACAAAGAGACAAACUACAUUGACUACUUGUGCAAC
6730 ACUAAAGAUGGUGCUUGAGAAAAUCGGAUUCGGAGACAGAGUU---GACUACAUCGACUACCUAAACCAC

 841 UCCCAUCACCUGUACAGGAAUAAACAUUACUUUGUGAGGGGUGGCAUGCCCUCGGGAUGUUCUGGUACCA
6797 UCACACCACCUGUACAAGAAUAAAACAUACUGUGUCAAGGGCGGUAUGCCAUCUGGCUGCUCAGGCACUU

 911 GUAUUUUCAACUCAAUGAUUAACAAUAUCAUAAUUAGGACACUAAUGCUAAAAGUGUACAAAGGGAUUGA
6867 CAAUUUUUAACUCAAUGAUUAACAACUUGAUUAUCAGGACACUCUUACUGAAAACCUACAAGGGCAUAGA

 981 CUUGGACCAAUUCAGGAUGAUCGCAUAUGGUGAUGAUGUGAUCGCAUCGUACCCAUGGCCUAUAGAUGCA
6937 UUUAGACCACCUAAAAAUGAUUGCCUAUGGUGAUGAUGUAAUUGCUUCCUACCCCCAUGAAGUUGACGCU

1051 UCUUUACUCGCUGAAGCUGGUAAGGGUUACGGGCUGAUCAUGACACCAGCAGAUAAGGGAGAGUGCUUUA
7007 AGUCUCCUAGCCCAAUCAGGAAAAGACUAUGGACUAACUAUGACUCCAGCUGACAAAUCAGCUACAUUUG

1121 ACGAAGUUACCUGGACCAACGUCACUUUCCUAAAGAGGUAUUUUAGAGCAGAUGAACAGUACCCCUUCUU
7077 AAACAGUCACAUGGGAGAAUGUAACAUUCUUGAAGAGAUUCUUCAGGGCAGACGAGAAAUACCCAUUUCU

1191 GGUGCAUCCUGCCAUGCCCAUGAAAGACAUACACGAAUCAAUUAGAUGGACCAAGGAUCCAAAGAACACC
7147 UAUUCAUCCAGUAAUGCCAAUGAAGGAAAUUCAUGAAUCAAUUAGAUGGACUAAAGAUCCUAGGAACACU

1261 CAAGAUCACGUGCGCUCACUGUGUCUAUUAGCUUGGCAUAACGGGGAGCACGAAUAUGAGGAGUUCAUCC
7217 CAGGAUCACGUUCGCUCUCUGUGCCUUUUAGCUUGGCACAAUGGCGAAGAAGAAUAUAACAAAUUCCUAG

1331 GUAAAAUUAGAAGCGUCCCAGUCGGACGUUGUUUGACCCUCCCCGCGUUUACAACUCUAGGCAGGAAGUG
7287 CUAAAAUCAGGAGUGUGCCAAUUGGAAGAGCUUUAUUGCUCCCAGAGUACUCAACAUUGUACCGCCGUUG

                   END
1401 GUUGGACUCCUUUUAGAUUAGAGACAAUUGAAACAAUUUAGAUUGGCACAACCCUACUGUGCUAACCGAA
7357 GCUUGACUCAUUUUAGUAA-------------------------------CCCUACCUCAGUCGAAUUG
                   ENDEND

1471 CCAGAUAACGG---UAC---AGUAGGGGUAAAUUCUCCGC--AUUCGGUGCG
                                                         Poly(A)
7395 GAUU----GGGUCAUACUGUUGUAGGGGUAAAUUUUUCUUUAAUUCGG--AG
```

FIG. 2. Nucleotide sequence of the 3′-terminal end of the CB3 genome. The sequence was compared with the corresponding PV1 sequence (19). Matching nucleotides are indicated with asterisks. Deleted nucleotides are indicated with dashes. The arrow shows the beginning of the replicase gene. "END" indicates stop codons which terminate the replicase gene.

one single amino acid insertion was noticed. Some biochemical properties of the replicases from CB3 and PV1 are summarized in Table 1.

A comparison of the 3′ noncoding sequences of CB3, PV1, and swine vesicular disease virus (SVDV) (18) is shown in Fig. 4. CB3 was more closely related to SVDV than to PV1 in this region, and CB3 and PV1 showed a much weaker homology in the noncoding part than in the replicase gene itself. An outstanding feature was a 32-nucleotide-long insertion in the CB3 sequence immediately after the termination codon (Fig. 1). Three regions, 6 to 13 nucleotides long each, were completely conserved in CB3, PV1, and SVDV (Fig. 4).

DISCUSSION

Enteroviruses have been neglected by molecular virologists even though they are of tremendous medical importance in both human and veterinary medicine. Questions concerning the molecular basis of pathogenicity and tissue tropism are of utmost medical importance, and improved methods for diagnosis of enterovirus infections and for the production of polyvalent enterovirus vaccines are obviously needed. Recombinant DNA technology is likely to have a great impact on enterovirus research in the near future. The possibility of converting the RNA genome into a cDNA copy makes it possible to study the structure of enterovirus genomes and to make comparisons between virulent and attenuated strains. This should ultimately lead to a better understanding of enterovirus evolution and also to the identification of the precise molecular changes which are responsible for attenuation.

Of particular significance is the discovery by Racaniello and Baltimore (20) that cloned DNA copies of the entire poliovirus genome are infectious, a property which is likely to be shared by other enterovirus genomes. This should make it possible to introduce specific mutations and to study the phenotypic consequences of these. The approach may, moreover, make it possible to construct a novel type of vaccine, based on deletion mutants which are nonvirulent and unable to revert to the wild-type phenotype.

In the present communication we have reported on the cloning of the CB3 genome. A cDNA copy covering two-thirds of the viral genome was cloned in *E. coli*, and the complete nucleotide sequence of the replicase gene and the 3′ noncoding region was established. The sequence showed a remarkably high degree of homology to the corresponding PV1 sequence. This was unexpected as previous studies in which nucleic acid hybridization techniques were used (26) indicated a weak relationship among different members of the enterovirus group. The results shown in Fig. 2 clearly demonstrate that conserved regions can be detected along the entire replicase gene. The similarity is particularly striking when the amino acid sequences are compared (Fig. 3). Homologous regions are present in all parts of the replicase, although the N-terminal end is slightly less conserved than the other parts. One single amino acid insertion has occurred near the middle of the 462-amino-acid-long polypeptide chain (residue 262 in Fig. 3). The PV1 replicase is terminated by two nonsense codons, UAGUAA, in contrast to the CB3 replicase, which uses a single termination codon, UAG. The predicted amino acid compositions of the two proteins are very similar, one difference being a lower net charge (+6) in the PV1 replicase than in the CB3 replicase (+13). The N-terminal sequence of the CB3 replicase has not yet been determined experimentally. Its position can, however, be

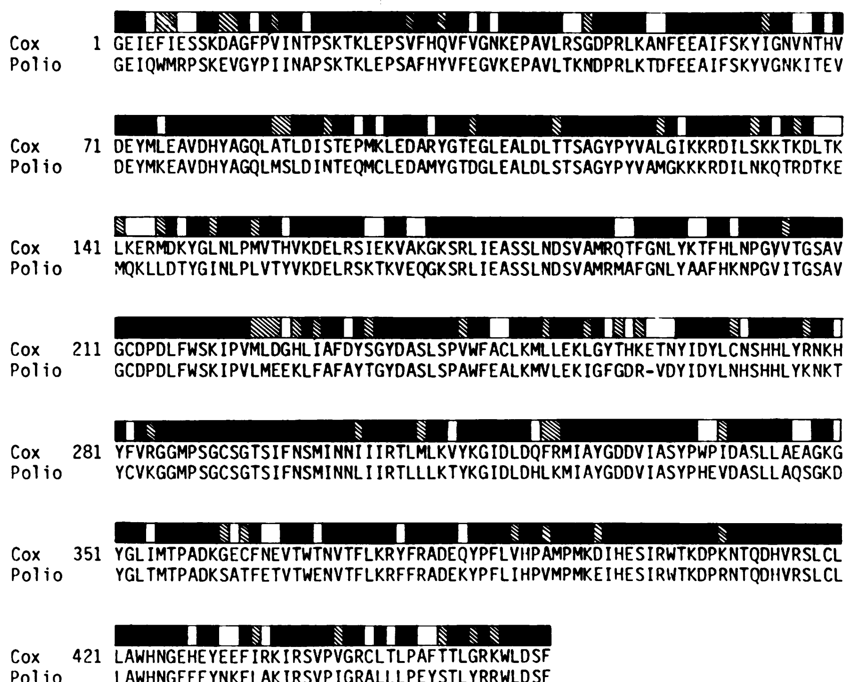

FIG. 3. Comparison of the amino acid sequences of CB3 (Cox) and PV1 (Polio) replicases. The PV1 sequence is from Racaniello and Baltimore (19). Solid boxes represent identical amino acids, and crosshatched boxes represent functionally related amino acids designated by the following scheme: Nonpolar: G, A, V, L, I, F, W, P, and M; polar neutral: G, S, T, Y, C, N, Q, and N; acidic: D and E; and basic: K, R, and H. Amino acids are given as their one-letter codes. Open boxes represent nonhomologous amino acids.

deduced from the position of the PV1 sequence, as it appears that the cleavage takes place between Gln-Gly residues (12).

The 3′ noncoding part of the CB3 sequence showed a much lower degree of homology to the 3′ noncoding part of the sequence (Fig. 2). A conserved hexanucleotide is found 29 nucleotides downstream from the termination codon in the CB3 sequence. An interesting conserved region is found further downstream near the poly(A) junction; a 13-nucleotide-long sequence is shared between the CB3 and the PV1 genomes, and yet another conserved hexanucleotide is found closer to the poly(A) junction (Fig. 4). Of particular interest is the finding that all of these conserved sequences are also present in the 3′ noncoding region of the SVDV genome, suggesting that they have an important function, possibly as recognition signals for the viral replicase. It is noteworthy that the region immediately after the termination codon in the CB3 sequence is absent in the PV1 sequence, indicating that it has been deleted from or inserted into one of the two genomes. A comparison of the 3′ noncoding parts of PV1, SVDV, and CB3 revealed some interesting properties (Fig. 4). The polyadenylation signal AAUAAA is absent from all three sequences. It is striking to note that the 3′ noncoding regions of CB3 and SVDV are more related to each other than to the PV1 3′ noncoding region. In this respect it is interesting that SVDV was found to have a close antigenic relationship to CB5 (3, 8) and thus does belong to the coxsackievirus B subgroup. It is also noteworthy that at least part of the sequence which is absent from the PV1 genome (only 75 nucleotides of SVDV have so far been sequenced) is present in the SVDV genome, suggesting that it may be specific for the coxsackievirus B group.

The availability of cDNA clones makes it possible to use nucleic acid hybridization as a tool for virus identification. We have recently shown that the cDNA clone pCB3/1 can be used as a convenient tool for identifying different enteroviruses in clinical specimens (11). The unique sequences which

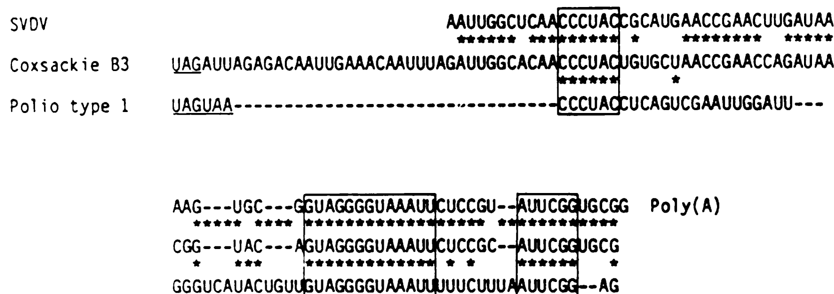

FIG. 4. Comparison of the 3′ noncoding regions of CB3 (Coxsackie B3), PV1 (Polio type 1) (19), and SVDV. Sequences which are common to all three viruses are indicated with boxes. Matching nucleotides are indicated with asterisks. Deleted nucleotides are indicated with dashes. Only 75 nucleotides of the SVDV genome have been sequenced.

are present in the 3′ noncoding part could possibly be used for further subgrouping by utilizing short oligonucleotides for identification.

We are currently constructing a full-length clone which could be used for further genetic studies and for expression of proteins which are important in coxsackievirus neutralization.

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