An Apparent Deletion of an Oligonucleotide Detected by RNA Fingerprint in the Nondiabetogenic B Variant of Encephalomyocarditis Virus Is Caused by a Point Mutation

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The diabetogenic D variant of encephalomyocarditis virus (EMC-D) was previously shown to be different from the nondiabetogenic B variant of encephalomyocarditis virus (EMC-B) by a single spot in an oligonucleotide fingerprint after RNase T₁ digestion of their genomic RNAs. An oligoribonucleotide was missing from EMC-B but was present in EMC-D. The oligoribonucleotide specific to EMC-D was isolated from a two-dimensional polyacrylamide gel and sequenced as 5'-ACAAUCUCACUUUCCAACAACAG-3'. Molecular hybridizations of EMC-D and EMC-B genomic RNAs with a DNA primer complementary to the EMC-D-specific oligoribonucleotide revealed that the absence of a corresponding spot in EMC-B was due to a point mutation rather than a deletion. By sequencing a cloned cDNA of EMC-B corresponding to the EMC-D-specific oligoribonucleotide, the point mutation was identified as a G for EMC-B and an A for EMC-D transversion at base 9 of the oligonucleotide. Comparative sequence analysis of eight randomly picked RNA segments around the EMC-D-specific oligoribonucleotide revealed that there were no base changes between EMC-D and EMC-B. It is concluded that the diabetogenic EMC-D viral genome differs from the nondiabetogenic EMC-B viral genome by at least a point mutation.

Encephalomyocarditis virus (EMC) infects and destroys pancreatic beta cells when inoculated into mice (8, 19). Several years ago, we showed that our EMC pool contained two antigenically similar variants which could not be distinguished by hyperimmune sera and competitive radioimmunoassays (16, 18). One of the variants is highly diabetogenic (EMC-D), and the other is nondiabetogenic (EMC-B). When EMC-D was inoculated into SJL/J male mice, diabetes developed in over 90% of the animals. In contrast, none of the mice inoculated with EMC-B developed diabetes (18). Molecular hybridization studies with radiolabeled cDNAs complementary to EMC-D and EMC-B RNAs also failed to distinguish EMC-D and EMC-B. However, oligonucleotide fingerprinting after RNase T₁ digestion of the genomic RNAs from these two variants revealed a difference in one spot (12). An oligonucleotide was missing in the RNA of EMC-B, but it was present in the RNA of EMC-D. The present investigation was initiated to isolate the oligonucleotide unique to EMC-D, to sequence it, and to identify its genomic localization.

Viruses were purified from the supernatant of L929 cell cultures infected with EMC-D or EMC-B (multiplicity of infection, 10) by sucrose and CsCl gradient centrifugation (17, 20). The diabetogenic activities of purified EMC-D and EMC-B were rechecked by infecting SJL/J male mice (18). All 30 mice infected with EMC-D (5×10^5 PFU per mouse) developed diabetes (glucose level in blood, 425 ± 36 mg/dl), whereas none of the 20 mice infected with EMC-B (5×10^5 PFU per mouse) became diabetic (149 ± 17 mg/dl). EMC-D and EMC-B genomic RNAs were obtained from the purified virions after phenol extraction (12) and completely digested with RNase T₁, which is specific for guanosine base. Total oligonucleotides in the digestion mixture were end labeled

with [α -³²P]ATP and separated by two-dimensional polyacrylamide gel electrophoresis (12), which revealed a distinctive fingerprint pattern for each viral genome. The fingerprint shown in Fig. 1a is identical between EMC-D and EMC-B except for the unique appearance of spot A in EMC-D. Although the experiment was repeated several times with different RNA preparations, the result was unchanged. The position of spot A is in excellent agreement with that of spot 8a described in the previous report (12). This oligonucleotide was eluted from the gel and sequenced enzymatically (1, 3). Briefly, the oligoribonucleotides were extracted from the gel by using 650 μ l of elution buffer composed of 0.5 M ammonium acetate, 10 mM magnesium acetate, 10 mM vanadyl ribonucleoside complex, and 40 μ g of carrier *Saccharomyces cerevisiae* tRNA. The eluate was then extracted with phenol to remove the vanadyl ribonucleoside complex. The yield of each labeled fragment was at least 10^5 cpm as determined by Cerenkov counting in water. The enzymatic RNA sequencing reactions were carried out as described elsewhere (1, 3). The EMC-D-specific oligonucleotide was 24 bases long, with the sequence 5'-ACAAUCUCACUUUCCAACAACAG-3' (Fig. 1b). To see whether other spots with similar mobility in both EMC-D and EMC-B fingerprints were identical in sequence composition, eight randomly picked spots near spot A were also sequenced. There were no base changes in the sequences of these spots between EMC-D and EMC-B (Table 1).

Data from the RNA fingerprints did not tell whether the appearance of spot A was the consequence of a point mutation in either viral genome (EMC-D or EMC-B). To find whether the absence in the EMC-B RNA fingerprint of an oligonucleotide corresponding to spot A in EMC-D was due to a deletion, a DNA primer complementary to the 23 bases from the 5' end of the EMC-D-specific RNA sequence was synthesized (2, 7). After the 5' end was labeled with [³²P], it

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was hybridized to the Northern blot of EMC-D and EMC-B genomic RNAs. The primer hybridized equally well to both EMC-D and EMC-B genomic RNAs, indicating that the corresponding RNA segment was not deleted in the EMC-B variant (Fig. 1c). The oligonucleotide probe remained hybridized upon sequential washings at up to 56°C with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate, but it was washed off at 60°C from both of the genomic RNAs. Based on these hybridization data and observations on the resolution limit of

the oligoribonucleotide fingerprints, the corresponding EMC-B RNA segment most likely contained a guanosine residue in the middle, thus rendering it digestible by RNase T₁ to fragments smaller than the resolution limit of approximately 14 bases. The mismatch between the EMC-D-specific primer and EMC-B RNA was presumed to be a T-G pair, which has been shown to be too stable to be distinguished from a perfectly matched complementary hybrid, even under the most stringent washing conditions (9).

The point mutation was further confirmed by the sequences of cloned cDNA of EMC-B corresponding to the EMC-D-specific oligoribonucleotide (Fig. 2). Briefly, for cloning of the EMC-B viral genome covering the genomic area which corresponds to the EMC-D-specific oligoribonucleotide, DNA primer (29 bases) was chemically synthesized from the RNA sequences of fingerprint spot E (genomic position 4972), as shown in Fig. 1a and Table 1 (2, 7). Double-stranded cDNAs were synthesized by the method of Gubler and Hoffman (5), tailed with deoxycytidine (about 20 nucleotides) by using terminal deoxynucleotidyl transferase, annealed to *Pst*I-cut oligo(dG)-tailed pBR322, and transformed in *Escherichia coli* RR1. After purification of the recombinant plasmids, cDNA inserts were excised by *Pst*I digestion, and a restriction map was constructed by using restriction enzymes, including *Tag*I and *Rsa*I. The restriction fragments were then subcloned into matching sites of a pTZ18R (or pTZ19R) vector. After transformation into *E. coli* JM109, single-stranded template DNA was isolated and the nucleotides were sequenced by the dideoxy nucleotide chain termination method (13) with 17-base M13 reverse primer and [³⁵S]dATP (500 Ci/mmol). The nucleotide sequence of EMC-B cDNA corresponding to the EMC-D-specific oligonucleotide was ACAATCTCGCTTTTCCAA-CAACAG (RNA form: 5'-ACAAUCUCGCUUUUCCAAC-AACAG-3'). Thus, base 9 of this oligonucleotide was changed from A for EMC-D to G for EMC-B.

Based on the genomic sequences of EMC-RRR (11), a laboratory strain of EMC, the EMC-D-specific oligonucleotide was mapped with 92% sequence homology to the 3' end of the I(2B) protein (Fig. 2). The role of the I(2B) protein in EMC-D or EMC-B is not known. Previous work with poliovirus clones has shown that viral peptide G(2A) has proteolytic activity and is probably the agent that catalyze the first cleavage within this virus at the P1 and G(2A) sites (15). The other study suggested that cardioviral G(2A) peptide is not functionally equivalent to that of poliovirus (4, 14). The primary cleavage within cardioviral polyproteins occurs at a site different from that of poliovirus (2A-2B rather than P1-2A), and peptide I(2B) is considered to be an alternative candidate for the G(2A) equivalent in this virus (10). Whether a point mutation at the polyprotein cleavage junction of a proteolytic enzyme would significantly alter the biological activity of the virus remains to be determined.

The diabetogenic EMC-D and nondiabetogenic EMC-B variants have been shown to be extremely similar in their proteins and nucleic acids (12, 19). Recently, Jordan et al. also showed that these two variants are very similar at the molecular level, except within the poly(C) tract (6). The poly(C) tract of EMC-D is estimated to be 144 bases, whereas that of EMC-B is 141 bases long. The untranslated 5'-terminal 103 nucleotides are identical for both EMC-D and EMC-B. The restriction maps of 12 different enzymes (*Pst*I, *Apa*I, *Sma*I, *Bam*HI, *Hind*III, *Stu*I, *Bgl*II, *Eco*RI, *Kpn*I, *Xba*I, *Bho*I, and *Hinc*II) in the entire open reading frames of both variants are also identical. Although available data show these two variants to be indistinguishable at the

FIG. 1. (a) Fingerprints of EMC-D RNA. Genomic RNA was digested with RNase T₁, precipitated with ethanol, and terminally labeled with [γ -³²P]ATP by using T4 polynucleotide kinase. The labeled oligonucleotides were separated by two-dimensional gel electrophoresis. Spot A is the oligoribonucleotide specific to EMC-D. Spots B through I were also sequenced (Table 1). (b) RNA sequences of the oligoribonucleotide, spot A. (c) A Northern (RNA) blot of EMC-D and EMC-B RNAs. A Northern blot of both EMC RNAs was hybridized with a ³²P end-labeled DNA primer complementary to the 23 bases of spot A oligoribonucleotide. The control markers are 16S and 23S rRNAs derived from *E. coli*.

FIG. 2. Structure of the EMC genome. Viral RNA and encoded peptides are schematically illustrated. The position of the oligonucleotide unique to diabetogenic EMC-D was identified near the 3' end of the I(2B) protein. The nucleotide sequence of EMC-B RNA was converted from the cDNA corresponding to the EMC-D-specific oligonucleotide. The different bases in EMC-D and EMC-B are underlined.

TABLE 1. Nucleotide sequences of EMC-D and EMC-B RNA fragments from RNase T₁ fingerprints^a

RNase T ₁ No. finger- print of bases spot		Sequence in EMC-D	% Homo- logy match with EMC- RRR	Ge- nomic posi- tion ^b
A	24	A C A A U C U C A C U U U U C C A A C A A C A G	92 ^c	4392
B	26	A A A A U C U U C A C C A C U C C C C C C C C C G	76	4359
C	27	C A A A C A A A A C C U C C A C C U U U C C U C U U G	81	7414
D	28	U U C C U U U C A U C U C C C C C A C A C A C U U C C G	82	2497
E	29	U U U C A A C U A C A A A U U U C C U U C C U A A C A U G	79	4972
F	43	A U U A U C A A U A C U U U U A U U C U A A C C A A U A U C A A A A U U C U A U U G	88	1092
G	21	U C A C A A A C A C C C A A U C A A C C G	90	1312
H	23	A C U A C C C A C U C C U A C U U U C A A U G	86	64
I	22	A A U U C C U C C U A U U C C U U U A C C G	77	2475

^a The nucleotide sequences of the EMC-D and EMC-B RNA fragments from the RNase T₁ fingerprints were determined as described in the text.

^b The EMC-D and EMC-B oligoribonucleotides were compared with and mapped to the site of highest sequence homology with the complete 7,835-base sequence of the EMC-RRR variant (11).

^c The corresponding EMC-B oligoribonucleotide spot was absent in the RNase T₁ fingerprint. The other EMC-B oligoribonucleotide sequences were identical to the corresponding EMC-D sequences.

molecular level, aside from the poly(C) tract, they show distinct differences in diabetogenicity and interferon inducibility (18). The only biochemical difference found in this study was one base change in the 3'-terminal region of the I(2B) gene. One speculation for the biological role of this point mutation is that it may alter either ability to induce interferon or the ability of the virus to replicate in cells. It is unlikely to affect attachment of the virus to cells, since the site of mutation is not located in any of the outer capsid proteins. Only complete sequences of both genomes, now under investigation, will elucidate the entire genomic differences between diabetogenic EMC-D and nondiabetogenic EMC-B. This information is needed to determine the functional consequences of genomic changes in both variants. From our present studies, it is concluded that the diabetogenic EMC-D genome differs from the nondiabetogenic EMC-B genome by at least a point mutation identified as G for EMC-B and A for EMC-D transversion near the 3' end of the I(2B) gene.

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