Development of a recombinant RNA technique for the construction of chimeric RNA with a long poly(C) tract

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ABSTRACT

The murine cardioviruses and bovine aphthoviruses are distinguished from other (+) strand RNA viruses by their long poly(C) tract in the 5'-noncoding region. The presence of this poly(C) tract has long hampered the construction of full-length cDNA with the complete poly(C) tract, because long poly(dC-dG) homopolymer-containing plasmids are difficult to amplify in bacterial systems. To overcome this problem, we constructed a chimeric RNA by joining the poly(C) region of the viral RNA to the 5'-truncated RNA transcript of the encephalomyocarditis (EMC) virus cDNA. The non-chimeric, recombinant EMC virus with a short poly(C) tract produces recombinant progeny virus, but this is not pathogenic *in vivo*. On the other hand, the EMC viral RNA chimera with the complete poly(C) tract produces recombinant progeny virus that is pathogenic *in vivo*. This method of viral RNA construction will be invaluable for functional studies of other cardioviruses and aphthoviruses, as well as for recombinant RNA manipulations.

INTRODUCTION

Genetic analysis of the RNA virus has long been hampered by a lack of defined mutants. The discovery that full-length cDNA of viral RNA can produce authentic progeny viruses after transfection of mammalian cells with recombinant plasmid (1–4) or its RNA transcript (5–10) has made it possible to construct and characterize mutation in RNA genomes at the DNA level. Infectious cDNA clones have been constructed and used for analysis of viral genes (e.g. cardioviruses and aphthoviruses) through site-specific mutagenesis. However, cardioviruses and aphthoviruses are distinct because of their long poly(C) tract (60 to 350C). This poly(C) tract has impeded the construction of full-length cDNA clones, because long poly(dC-dG) homopolymer-containing plasmids are difficult to amplify in bacterial systems.

To circumvent the problem of a long poly(C) region, cDNAs containing short oligomeric dC-dG sections were constructed and progeny viruses were obtained after transfection of cells. However, these recombinant progeny viruses containing short

poly(C) tracts showed a dramatically attenuated pathogenicity in mice (11–16). For example, viral cDNA clones, containing a short poly(dC-dG) homopolymer (20C, 30C, or 40C) instead of a 130C long polymer, were constructed from the diabetogenic variant of the encephalomyocarditis (EMC-D) virus (14). However, the progeny virus, produced by transfecting mammalian cells with RNA transcripts from the short (dC-dG) homopolymer-containing cDNA, did not show pathogenicity *in vivo* (14). As suggested in a report on Mengo virus (15), the lack of pathogenicity of the recombinant progeny virus *in vivo* may be due to the short length of the poly(C) tract. As such recombinant progeny viruses cannot be used for functional studies of specific loci, this homopolymer problem needs to be solved so that progeny viruses that have the same pathogenicity as does the wild type (*in vivo*) might be produced. We report here a new method: construction of a RNA chimera of the EMC-D virus by joining the authentic poly(C) region of viral RNA (first 476 bases) to a 5'-truncated RNA transcript (bases 477–7830).

MATERIALS AND METHODS

Virus and viral RNA

The EMC-D virus was grown in L929 cells and the viral RNA was obtained from the purified virus as described elsewhere (16). The RNA, showing a ratio of A260/A280 of around 1.8 and a single band on the agarose gel, was used for this experiment.

Construction of 5'-truncated cDNA clone of EMC-D

Full-length EMC-D cDNA, which contains a short poly (dC-dG) homopolymer at the site of long poly(C) tract, was assembled from 7 overlapping partial cDNA clones as described previously (14). The cDNA fragment between the restriction sites of *Hind*III and *Pst*I (from base 479 to the 3'-end poly(A) tail) of the full-length cDNA was joined into the corresponding restriction sites in pTZ19/R vector (Pharmacia Biotech, Uppsala, Sweden), which contains a T7 promoter site. This recombinant vector was named pEDfH (Fig. 1). Bases 477–478 (GG) of the EMC-D cDNA were provided by the two GG bases of the transcription initiation site of the vector.

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Figure 1. Schematic illustration of a method for construction of RNA chimera. EMC-D viral RNA is annealed with a chimeric oligonucleotide (bases 467–491) as shown at the top, digested with *RNaseH*, and separated on an agarose gel. The fragment of the first 476 bases, containing intact poly(C) tract, is eluted from agarose gel and annealed with 5'-truncated RNA transcript covering the viral genome from base 477 to the 3'-end poly(A) tail in the presence of a brace oligonucleotide (bases 460–501). The gap between the bases at positions 476 and 477 is joined by RNA ligase. Following heat denaturation, the RNA chimera is introduced into mammalian cells to produce recombinant progeny viruses. The progeny virus is tested for its diabetogenicity.

Preparation of 5'-viral RNA fragment (1–476) containing intact poly(C) tract

Three pmole (10 μ g) of viral RNA was annealed with 50 pmole of chimeric oligonucleotide primer (bases 467–491), which consisted of deoxyribonucleotides (d) and 2'-o-methyl-ribonucleotides (mr) [5'-mr(CUU CAA GAA GCU UCC) d (AGAG) mr (GAACUG)-3'] in *RNaseH* buffer [0.1 M KCl, 20 mM HEPES buffer (pH 7.6), 10 mM MgCl₂, and 1 mM dithiothreitol (DTT)]. The annealing mixture was incubated at 65°C for 5 min and then slowly cooled to 37°C. Five units of *RNaseH* were added to the annealing mixture, and the mixture was incubated at 37°C for 1 hr. Heat denaturation (70°C for 10 min) was followed by application of the sample to a 1.2% low-melting agarose gel. The viral RNA band of the first 476 bases, which includes the intact poly(C) tract, was extracted from the agarose gel and used as a source of intact poly(C) tract.

Construction of chimeric viral RNA

We constructed a recombinant plasmid, containing 5'-truncated cDNA (from base 479 to the 3'-end poly(A) tail), and the plasmid was linearized by *SalI* digestion. The resulting linear DNA was clarified by repeated phenol-chloroform extraction and ethanol precipitation, and transcribed by T7 RNA polymerase at 37°C for 1 hr in a reaction mixture containing 40 mM Tris–HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine, 25 mM NaCl, 5 mM DTT, 1 unit/ μ l RNasin (Pharmacia Biotech), 2 mM each of ATP, GTP, CTP, and UTP, 0.1 μ g/ μ l linearized plasmid DNA, and 5 units/ μ l T7 RNA polymerase (BRL, Gaithersburg, MD). *DNase I* (Pharmacia Biotech) was added at a rate of 15 μ g/50 μ l reaction mixture and incubation was continued for an additional 15 min at 37°C. The reaction mixture was clarified as mentioned above and precipitated with ethanol. The RNA transcript, containing triphosphate at its 5'-end, was modified by dephosphorylation

Figure 2. Preparation of 5' portion of viral RNA fragment (bases 1–476) containing the intact poly(C) tract. EMC-D RNA was digested with *RNaseH* after annealing with chimeric oligonucleotide primer (bases 467–491) and then separated on a 1.2% low-melting agarose gel (a). Arrowhead indicates the first 476 base-RNA fragment. This RNA fragment was eluted and tested for its integrity by annealing with ^{32}P -(1–23) (lane 2); ^{32}P -(301–330) (lane 3); ^{32}P -(460–476) (lane 4); and ^{32}P -(477–501) (lane 5) together with intact viral RNA annealed with ^{32}P -(301–330) (lane 1), and then separated on a 1% agarose gel (b). m1 and m2 are *Hind*III-digested λ DNA and 123 bp ladder size markers.

Figure 3. Northern blot hybridization of chimeric RNA. RNA samples were separated on a 1.2% agarose/formamide RNA denaturing gel and blotted to a nitrocellulose membrane (Micron Separations Inc.). The Northern blot of RNA samples was hybridized with ^{32}P -labelled primer complementary to the EMC-D genomic sequence of 301–330. Lane 1, gel-eluted viral RNA fragment (1–476); lane 2, 5'-truncated RNA transcript (477–7830); lane 3, ligated chimeric RNA; lane 4, 2 μg of control EMC-D RNA.

and phosphorylation to attach monophosphate at its 5'-end as follows. The RNA transcript in TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) was applied to a Sephadex G-50 column and treated with bacterial alkaline phosphatase (BRL) at 45°C for 20 min. Dephosphorylated RNA was phosphorylated at its 5'-end at 37°C for 1 hr in a reaction mixture containing 10 pmole RNA transcript, 1 mM ATP, 1 unit/ μl of RNasin, 50 mM Tris–HCl (pH 7.5), 7 mM MgCl_2 , 10 mM 2-mercaptoethanol, and 3 units of T4 polynucleotide kinase (Pharmacia Biotech).

The gel-eluted viral RNA fragment containing bases 1–476 and the 5'-truncated RNA transcript were annealed in the presence of a brace primer (bases 460–501). One pmole of each

Figure 4. Analysis of the poly(C) tract of chimeric progeny virus. RNA from each chimeric progeny virus or wild type virus was digested with *RNase T₁* and *RNase U₂*, clarified, labelled with ^{32}P at the 5'-end, and separated by polyacrylamide gel electrophoresis. Abbreviations indicate wild type EMC-D (WD), homochimeric EMC-D (D/D), wild type EMC-B (WB), and heterochimeric EMC-B/D (B/D). M is the known T-lane of the pTZ18R sequence using M13 reverse primer.

of the three fragments was mixed in an annealing buffer (250 mM KCl and 10 mM Tris–HCl, pH 7.6), incubated at 65°C for 5 min, and then slowly cooled to 35°C. The annealant was precipitated and redissolved in RNA ligation buffer containing 50 mM Tris–HCl (pH 7.6), 10 mM MgCl_2 , 8 mM 2-mercaptoethanol, 1 mM ATP, 2 mM DTT, 1 unit/ μl RNasin, and 0.4 $\mu\text{g}/\mu\text{l}$ of T4 RNA ligase (BRL). The mixture was incubated at 10°C for over 10 hrs and clarified by phenol-chloroform extraction and ethanol precipitation. This final product was named chimeric RNA. A schematic illustration of the method for constructing the RNA chimera is shown in Fig. 1.

This ligated chimeric RNA, as well as the gel-eluted RNA fragment (bases 1–476) and the 5'-truncated transcript (bases 477–7830), were examined by Northern blot hybridization (17). The samples were heat denatured (65°C for 10 min) in a sample buffer (50% formamide and 6.5% formaldehyde in 1× MOPS buffer), separated in 1.2% agarose/formamide RNA denaturing gel (17), transferred to a nitrocellulose membrane and hybridized with ^{32}P -labelled primer (bases 301–330) in 6× SSC containing 1× Denhart's solution, 100 $\mu\text{g}/\mu\text{l}$ yeast tRNA, and 0.05% sodium pyrophosphate.

Biological activity of chimeric RNA

The ligated chimeric RNA, gel-eluted viral RNA fragment (bases 1–476), 5'-truncated RNA transcript (477–7830), chimeric RNA ligation mixture without brace primer (460–501), chimeric RNA ligation mixture without RNA ligase, or EMC-D RNA with *RNaseH* was introduced into L929 cells to check infectivity *in vitro* by the calcium-phosphate transfection procedures described by Chen and Okayama (18). The progeny viruses produced from the *in vitro* transfection experiment were tested for their diabetogenicity by inoculating SJL/J male mice and determining the blood glucose level in infected mice as described elsewhere (19, 20).

Table 1. Transfection efficiency and diabetogenicity of chimeric RNA

RNA	Infectivity ^a (<i>in vitro</i>)	pfu/ μ g	Serum Glucose ^b (mg/dl)	Diabetes ^c %
Chimeric RNA ^d	+	10–100	408 $\pm$ 27	90
Viral RNA fragment (1–476), gel eluted	–	0	ND ^e	ND
5'-truncated RNA transcript (477–7830)	–	0	ND	ND
Chimeric RNA ligation mixture without RNA ligase ^f	–	0	ND	ND
Chimeric RNA ligation mixture without brace primer (460–501) ^f	$\pm$ ^g	≤ 1	ND	ND
EMC-D RNA/ <i>RNaseH</i> ^h	–	0	ND	ND
Ho EMC-D ⁱ	+	10 ⁵	406 $\pm$ 36	80
He EMC-B/D ⁱ	+	10 ⁵	411 $\pm$ 30	90
EMC-D ⁱ	+	10 ⁵	420 $\pm$ 36	100
EMC-B ⁱ	+	10 ⁴	169 $\pm$ 17	0

a. Data expressed from transfection of L929 cells with 1 μ g of RNA by Ca-phosphate method.

b. Ten SJL/J male mice were inoculated with each progeny virus using 3×10^4 pfu/mouse; data are expressed as glucose index.

c. The mean glucose index of 10 uninfected SJL/J male mice was 168 ± 16 mg/dl. Any mouse with a glucose index greater than 248 mg/dl, which was 5 standard deviations above the mean, was classified as diabetic.

d. EMC-D viral RNA fragment, 5'-truncated RNA transcript, RNA ligase and brace primer.

e. ND, not determined

f. Reactions were performed without RNA ligase or brace primer.

g. Showed slight infectivity in only 1 out of 10 trials.

h. *RNaseH* digested EMC-D RNA after annealing with chimeric and opening primers.

i. Homochimeric and heterochimeric progeny viral RNAs.

j. Wild type EMC-D or EMC-B viral RNA.

Analysis of RNA from chimeric progeny virus

Viral RNA was purified from progeny virus obtained from transfection of L929 cells with the ligated chimeric RNA as described elsewhere (16). Following phosphorylation of a synthetic primer (bases 505–530), the sequence was determined by RNA-directed DNA sequencing (16). We also determined the length of the poly(C) tracts of wild type EMC-D and EMC-B virus and the progeny virus produced from transfection of homochimeric (5' EMC-D and 3' EMC-D) or heterochimeric (5' EMC-B and 3' EMC-D) RNA. Two μ g of viral RNA prepared from each wild type or chimeric progeny virus were digested with 100 units of *RNase T*₁ (G-specific) and 100 units of *RNase U*₂ (A-specific). The cleaved RNA fragments were clarified using a Sephadex G-50 spin column (Boehringer Mannheim, Mannheim, Germany), labelled with ³²P at their 5'-end, and separated on an 8% polyacrylamide gel containing 7M urea. In addition, the biological activity of the homochimeric and heterochimeric progeny virus and the wild type EMC-D and EMC-B virus was determined as described above.

RESULTS

The chimeric oligonucleotide primer, containing deoxy-ribonucleotides (d) and 2'-o-methylribonucleotides (mr) [5'-mr(CUUCAAGAAGCUCC) d(AGAG) mr (GAA-CUG)-3'] (22), was used to cut a specific site at position 476/477 with *RNaseH* (Fig. 1). The RNA fragment containing the first 476 bases was eluted from an agarose gel (Fig. 2a), and its integrity was roughly confirmed using several ³²P-labelled primers that were complementary to some portions of the viral RNA. As shown in Fig. 2b, the hybridization efficiencies of the eluted viral RNA fragment to two different primers (one complementary to bases 1–23 and the other complementary to bases 460–476 of EMC-D viral RNA) are almost identical to

that of the primer complementary to the internal portion (bases 301–330) of the eluted RNA fragment. In contrast, a primer complementary to bases 477–501 was not hybridized to the eluted fragment. These data suggest that the eluted RNA fragment was not damaged in its 5'- and 3'-end portion by *RNaseH* digestion, but was cleaved at or just beside the expected site.

The gel-eluted RNA fragment (bases 1–476, which contains the intact poly(C) tract) was attached to the 5'-truncated RNA transcript (monophosphate at its 5'-end) in the presence of another oligonucleotide as a brace (bases 460–501) using T4 RNA ligase. Prior to annealing and ligation, the RNA transcript was dephosphorylated and then phosphorylated to attach monophosphate in lieu of triphosphate at its 5'-end. To determine the efficiency of this RNA ligation method, northern blots of RNA samples from each step were hybridized with a ³²P-labelled primer complementary to the genomic sequence between bases 301–330. As shown in Fig. 3, about half of the poly(C)-containing viral RNA fragment was ligated to the 5'-truncated RNA transcript in this experiment.

The ligated chimeric RNA was introduced into L929 cells by the calcium-phosphate transfection method (18) and produced progeny virus with an efficiency of 10–100 pfu/ μ g of ligation mixture. The RNA prepared from this progeny virus exhibited the same infectivity to L929 cells as that of parental virus. The progeny virus induced diabetes in 9 out of 10 infected SJL/J male mice. In contrast neither RNA fragments (5'-viral RNA fragment or 5'-truncated RNA transcript) nor the unligated RNA mixture was infectious to L929 cells even at a high titer *in vitro* (Table 1).

To confirm that the RNA from the chimeric progeny virus produced by transfection *in vitro*, had the same sequence as the prototype viral RNA, especially at the joining region, we checked the sequence of the joining portion of chimeric progeny virus. We found exactly the same sequence at the joining region in the progeny viral RNA as in the EMC-D viral RNA.

To rule out the possibility of contamination by wild type RNA and to confirm the general applicability of this method, a heterochimeric RNA (containing distinctive marker sequences) also was constructed by joining 5'-RNA fragment from EMC-B and 3'-RNA transcript from EMC-D cDNA and its progeny virus was compared to the wild type virus and the progeny virus of the homochimeric RNA with regard to the length of the poly(C) tract and biological activity. As shown in Fig. 4, homochimeric progeny virus (5'-D and 3'-D) and heterochimeric progeny virus (5'-B and 3'-D) maintained the same length of poly(C) tract as their respective 5'-RNA fragment donors. In addition, both of the chimeric progeny viruses caused diabetes in mice to the same degree as mice infected with wild type EMC-D virus (Table I). Particularly noteworthy is the finding that heterochimeric progeny virus containing the poly(C) tract of EMC-B caused diabetes in susceptible animals, suggesting firstly that the diabetogenic viral gene(s) is located at least downstream from the joining part of the chimeric RNA, and secondly that the production of progeny virus by transfection of ligated chimeric RNA mixture was not due to the contamination of wild type viral RNAs or artifacts.

DISCUSSION

In this study, we have demonstrated for the first time that a chimeric viral RNA with a long poly(C) region can be constructed by joining the poly(C) region of the authentic viral RNA to the 5'-truncated RNA transcript of the viral cDNA.

Recently, it was shown that *RNaseH* preferentially recognizes the 4 base pairs of the RNA-DNA heteroduplex and cleaves it at its 3'-end of RNA (22–25). On the basis of this information, we designed and synthesized a chimeric oligonucleotide to provide a cutting site for *RNaseH*. This chimeric oligonucleotide, containing deoxyribonucleotides (d) and 2'-o-methyl-ribonucleotides (mr) [5'-mr(CUUCAAGAAGCUUCC) d(AGA-G) mr (GAACUG)-3'], was used to cut a specific site at position 476/477 with *RNaseH*. The RNA fragment of the first 476 bases containing the intact poly(C) tract was attached to the 5'-end of the 5'-truncated RNA transcript in the presence of another oligonucleotide primer that acted as a brace (bases 460–501). This chimeric RNA was transfected into L929 cells to produce an intact recombinant virus with a long poly(C) tract.

During the course of constructing the RNA chimera, we found that the region downstream from the cutting site (bases 476/477) had a strong hairpin structure. Thus, a chimeric oligonucleotide covering a small portion of this area did not anneal efficiently. Therefore, our chimeric oligonucleotide was designed to have additional bases at the cutting site (resulting in 15 bases from the 5' end to the cutting site and 10 bases from the cutting site to the 3' end). We also added one additional oligonucleotide, containing 2'-o-methylribonucleotides (bases 495–511) as an opener, to unfold the hairpin structure and to improve the annealing efficiency of the chimeric oligonucleotide primer. These two modifications resulted in a substantial improvement of the annealing efficiency. Despite the strong hairpin structure, we selected the cutting site at the 476/477 nucleotide position, because our transcription vector (pTZ19/R) has a *HindIII* site immediately after the T7 promoter and promoter originated 2G residues (transcription initiation site) could be used as the first two residues of the 5'-truncated RNA transcript (477–478).

According to the Northern blot experiments, up to 50% of the 5'-poly(C)-containing RNA fragment (1–476) was ligated to the 5'-truncated RNA transcript (477–7830) through this method.

However, the ligation mixture revealed 10^3 to 10^4 fold lower infectivity *in vitro* than the parental virion RNA (10^5 pfu/ μ g for EMC-D) as shown in Table 1. The reason for this is unknown, but we propose two possibilities. First, during the repeated extraction and purification procedure, each fragment and ligated RNA might have been damaged at 5'-VPg and/or 3'-poly(A) tail region, resulting in lower infectivity to L929 cells. Second, the remaining unligated RNA fragments (50%) in the reaction mixture might impede the infectivity of ligated RNA by acting as anti-sense to negative (–) strand during viral RNA replication.

Cardioviruses and aphthoviruses are distinct from other picornaviruses in having a long poly(C) tract (60–350C). This long poly(C) tract has impeded the construction of full-length clones, because long poly(dC-dG) homopolymer plasmids are difficult to amplify in bacterial systems. In functional studies, the long poly(C) tract is required for the pathogenicity of the progeny virus *in vivo* (14, 15). Recently, Duke et al. (15) constructed a full-length cDNA of Mengo virus, containing the natural form ($C_{50}UC_{10}$) of poly(C) tract, and infectious RNA synthesized from this cDNA produced progeny virus that showed the same biological activities as those of wild type Mengo virus *in vivo*. This system, however, could not be applied to EMC virus (14) or aphthovirus, since these viruses contain poly(C) tracts at least twice as long as that of Mengo virus. Therefore, the method reported here was designed to overcome this poly(C) limitation by the construction of an RNA chimera to produce progeny virus with the full-length poly(C) tract. This method will be invaluable for functional studies of cardioviruses and aphthoviruses, as well as for recombinant RNA manipulations.

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