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Facile PCR Cloning of Full-length Sendai Virus mRNAs

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Paramyxoviruses, which cause severe childhood diseases like measles, mumps, pneumonia, and acute upper respiratory tract illnesses, are characterized by a single-strand of RNA genome of negative polarity.[1] Recently, mRNA editing during viral transcription has been discovered in several paramyxoviruses.[2,3] Sendai virus is a prototype paramyxovirus and its genome consists of six genes (NP, P, M, HN, F, and L) which are transcribed into seven subgenomic-size mRNA species. Except for P, all of the genes are transcribed linearly into single mRNA species. The P gene gives rise to two major mRNA species, P and V, as a result of editing during transcription. The functions of various Sendai virus proteins in replication and pathogenesis are poorly understood.[1,4]

The entire 15.4-kb genome of Sendai virus has been cloned and sequenced.[1] The cDNA clones, however, represent either partial or overlapping fragments of both the genomic RNA and mRNAs, and often several manipulations are required to create the entire coding region of an mRNA from these clones.[5,6] Thus, obtaining full-length clones for all of the Sendai virus mRNAs to study viral proteins and their interactions may require an inordinately long time. In this communication, we describe a method that allows generation of full-length mRNA clones (NP, P, V, M, F, and HN), including putatively regulatory 5′ and 3′ untranslated regions (UTR) in a relatively short time using the polymerase chain reaction (PCR). We believe that this approach will also be suitable for cloning other viral and cellular mRNAs.

MATERIALS AND METHODS

Preparation of Total RNA from Sendai Virus Infected Cells

COS-1 cells grown in DMEM supplemented with 10% fetal bovine serum in 100-cm dishes were infected with the Enders strain of Sendai virus at a

TABLE 1. PCR Primers for Sendai Virus mRNAs Cloning

NAME	POSITION	SEQUENCE	Tm
F.A1	Left	HindIII 1 CTT AAGCTT AGG GAT AAA GTC CC 14	69
F.A2	Right	KpnI 1816 ACC GGTACC CTT ATT ATA TAC AGG TCT C 1797	74
HN.A1	Left	HindIII 1 CTT AGGCTT AGG GTG AAA GTG AGG 15	74
HN.A2	Right	Bam HI 1883 TCC GGATCC CTT AAT ACT GTG AGA GAC 1866	78
M.A1	Left	NheI 1 CTT GCTAGC AGG GTG AAA GAA ATT TCA C 19	80
M.A2	Right	Bam HI 1170 TCC GGATCC TTC TTA TTT AAG ACA AGG 1153	78
NP.A3	Left	NheI 21 GCTT GCTAGC GAG GAG CAG GTT CCA GAC 38	87
NP.B2	Right	Bam HI 1668 TCC GGATCC GGA TCA AGT ACC TTG AAG CCT C 1646	90
P.A1	Left	HindIII 1 CTT AAGCTT AGG GTG AAA GTT CAT C 16	71
P.A2	Right	Bam HI 1888 TCC GGATCC CTT AAT CTT TAC TGG CTG 1871	82

Restriction sites in primers have been underlined. Number at the top of a nucleotide corresponds to the nucleotide in mRNA.

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multiplicity of 5 pfu/cell. Infected cells were harvested 48 hr post-infection and total cellular RNA was prepared as described previously.[7] Briefly, cells were lysed in a low-salt buffer containing 10 mM Tris-HCl (pH 7.2), 10 mM NaCl, 5 mM Na_2EDTA, 0.5% sodium deoxycholate, and 1% NP-40 at 2×10^6 cells/ml. Nuclei were pelleted, and the cytoplasmic extract was made 1% with SDS (1/10 volume of 10% SDS), mixed with an equal volume of buffer-saturated phenol, and extracted at 56°C for 5 min. The aqueous phase was reextracted with phenol/chloroform/isoamyl alcohol (25:24:1) at room temperature, and the RNA was precipitated with 2.5 volumes of ethanol. To enrich for high-molecular-weight RNA and to eliminate any DNA contamination, the total RNA was subjected to high-salt (1.5 M NaCl) precipitation. The total RNA was finally dissolved in sterile water and used for synthesis of the first strand of cDNA by reverse transcription.

Synthesis of the First Strand of cDNA

Two strategies were used to synthesize the first strand of cDNA to Sendai virus mRNAs. In the first, PCR right primers (Table 1) specific to 3′ ends of each of the six (NP, P, V, M, F, and HN) mRNAs were used, and a separate reaction was performed for each mRNA-specific cDNA synthesis. Alternatively, cDNAs to all polyadenylated mRNAs contained in the total RNA were synthesized using oligo$(dT)_{12-18}$ primer. In either case, cDNA was synthesized in a 25-μl reaction mixture containing 40 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 50 mM Tris-Cl (pH 8.3), 1 mM each of dATP, dCTP, dGTP, and dTTP, 2 μg total RNA, and 1 μg (200 pmoles) oligo(dT) or 100 pmoles right primer. Prior to mixing the reaction components, template-primer was prepared by heating total RNA and primer in 10–15 μl of water to 95°C for 3 min and immediately cooling on ice. Reverse transcription was initiated by adding 20 units of AMV reverse transcriptase (Promega Biotech). Reactions were incubated at 42°C for 90 min and stopped by freezing on dry ice. Aliquots of reverse transcription reactions were used directly for PCR amplification.

Design of PCR Primers

Primers were synthesized to contain the exact 5′ and 3′ termini of the Sendai virus mRNAs and unique restriction sites at their 5′ ends (Table 1) for cloning of the PCR products in a multipurpose plasmid vector glfl[6] or its derivatives (our unpublished results). The primers ranged in size from 23 to 31 nucleotides and the T_m ranged from 69°C to 90°C, as calculated by the Primer software (Scientific & Educational Software).

Polymerase Chain Reaction

An optimized PCR reaction (100 μl) contained 1 μl of reverse transcription reaction, 100 pmoles each of left and right primers, 200 μM each of dATP, CTP, dGTP, dTTP, 2 units of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer Cetus), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-Cl (pH 8.3).[8] Each reaction was overlaid with 100 μl of paraffin oil. Reactions were initiated by heating the reactions to 94°C. Thirty amplification cycles were carried out at 94°C for 1 min, 40°C for 1 min, and 72°C for 3 min in a Perkin-Elmer DNA thermal cycler. PCR products (4-μl aliquots) were analyzed in a 0.8% agarose gel containing 0.5 μg/ml ethidium bromide. Amplified DNA was purified using Gene-Clean (Bio-101), digested with appropriate restriction enzymes, resolved in agarose gel, and purified again with Gene-Clean. The purified DNA was eluted from glass beads in 30–50 μl of H_20.

Cloning of PCR Products

Approximately 100–200 ng of PCR product was ligated to an equimolar amount of the appropriately digested vector, glfl, or its derivatives. Transformation of *E. coli* HB10l with each ligation reaction resulted in several ampicillin-resistant colonies. Twelve colonies from each of the ligation reactions were grown as minicultures (4 ml), and plasmid DNA from these cultures was analyzed for cDNA insertion, size, and restriction pattern. Clones for the V mRNA were identified by selective hybridization of V1 oligonucleotide (5′-CTC CTA TGC CCC TTT TTT GTT G) to clones generated with P-PCR primers. Clones were further characterized by double-stranded DNA sequencing[9] (Sequenase kit, ver. 2, USB) using either SP6, T7 promoter primers (Promega Biotech), and/or the PCR cloning primers (Table 1).

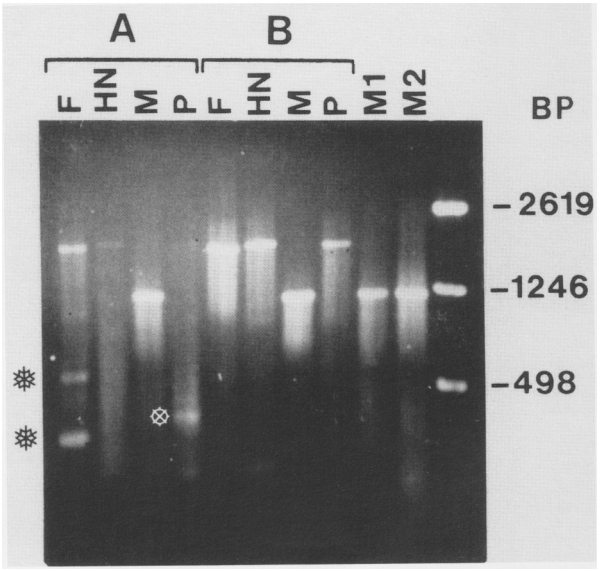

FIGURE 1 Agarose (0.8%) gel electrophoresis of PCR products synthesized using the first-strand of cDNA which was synthesized from total cytoplasmic RNA with mRNA-specific primers (*A*) or with oligo(dT) primer (*B*). Abortive PCR products are marked with asterisks. Product specific to each of the amplified mRNA is designated at the top of the figure. M1 and M2 represent PCR positive control products for the M mRNA. Size markers were prepared after digesting pBR322 with *Apa*LI.

RESULTS AND DISCUSSION

Synthesis of the First Strand of cDNA

Since Sendai virus mRNAs contain edited mRNAs in addition to the faithful copies of the genome, total cytoplasmic RNA from Sendai virus-infected cells was used instead of the viral genomic RNA to obtain clones of both edited and unedited mRNAs. Starting with 2 μg of total RNA in a 25-μl reverse transcription reaction, enough first-strand cDNA was synthesized to amplify six of the seven Sendai virus mRNAs. It is estimated that 2 μg of total RNA from Sendai virus-infected cells would contain about 100–200 ng (5–10% of total RNA) of viral mRNAs.[10] Each PCR reaction was initiated with 1 μl of the reverse transcription reaction, which is equivalent to 4–8 ng of viral mRNAs. Although we have not systematically examined the lower limit of the total RNA that can be used for reverse transcription, our preliminary experiments indicate that we can start with as low as 200 ng of total RNA (i.e., 0.4–0.8 ng of viral mRNAs) and obtain good amplification.

Synthesis and Analysis of PCR Products

PCR products of NP, P, V, M, F, and HN mRNAs were expected to be 1665, 1894, 1894, 1177, 1823, and 1890 bp, respectively (GenBank). When cDNA synthesized from reverse transcription of individual mRNAs with a right primer (Table 1) was used for PCR, only NP, M, and F PCR reactions gave the correct size products (Fig. 1A). Moreover, the final yield of product (except for the M mRNA) was less than 1 μg per reaction; F, P, and NP reactions generated products much shorter (<600 bp) than expected (Fig. 1A; data for NP not shown). In contrast, when cDNA synthesized from reverse transcription of mRNAs with oligo(dT) was used for amplification, each of the mRNA-specific PCR reactions yielded product of the expected size and the yield was greater than 4 μg per reaction (Fig. 1). There was no detectable shorter size product in any of these reactions. It is interesting to note that, despite wide range of difference in the T_m of the PCR primers (Table 1), all PCR reactions worked well under the

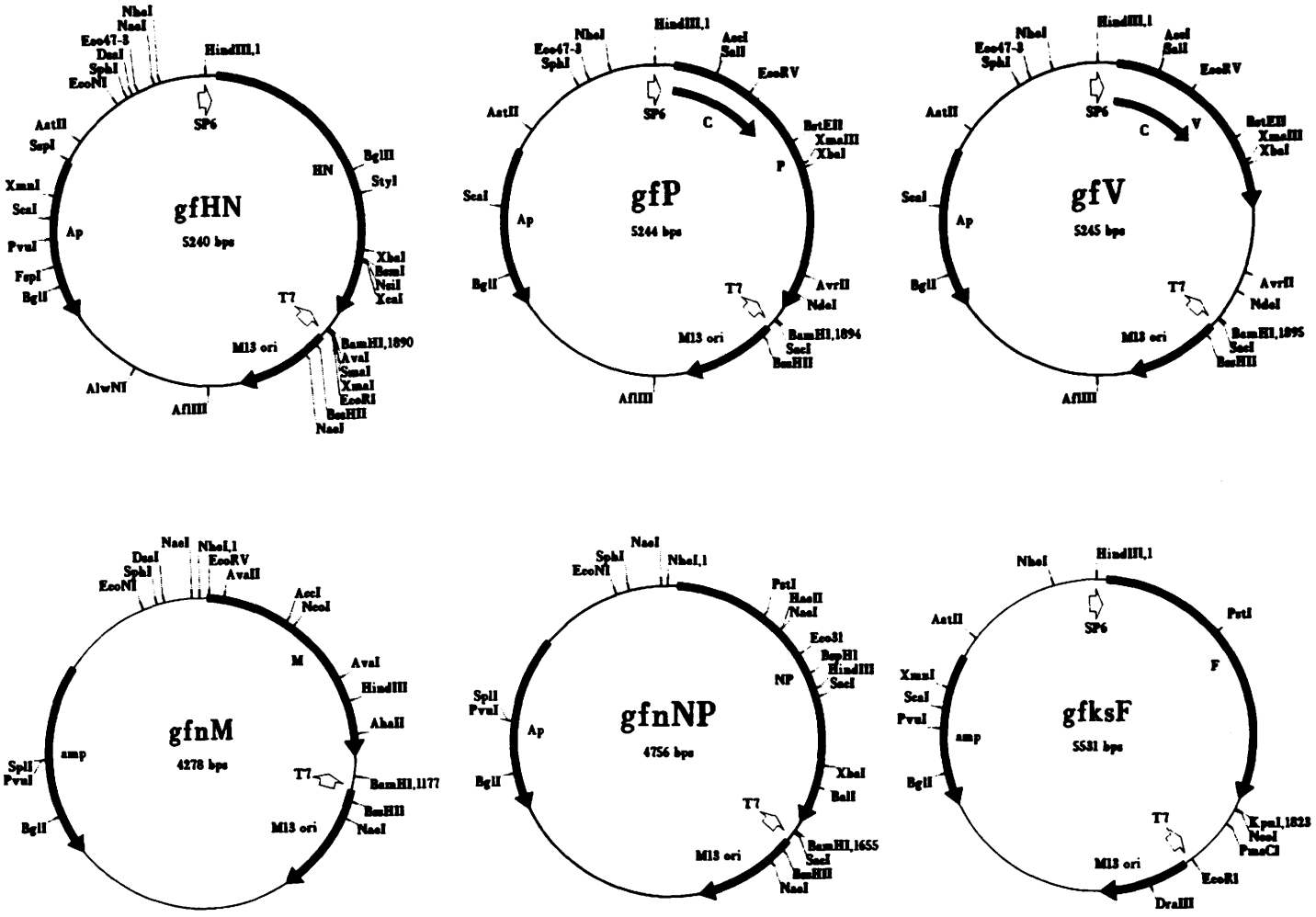

FIGURE 2 Genetic maps, sizes, and deduced partial restriction sites of the recombinant plasmids obtained by the cloning of the PCR products. The HN, P, and V genes were cloned at *Hin*dIII and *Bam*HI sites of glfl. The M and NP genes were cloned at *Nhe*I and *Bam*HI sites of glfl, while the F gene was cloned at *Hin*dIII and *Kpn*I sites of gfkss.

identical conditions of amplification. Perhaps the oligo(dT) primer allowed better priming because the poly(A) tail of mRNA was not expected to have any structure that might hinder annealing.[10]

Cloning of PCR Products

Purified and appropriately digested (Table 1) PCR DNA was cloned in the vector glfl (Fig. 2). Minipreparations of PCR clones were screened initially by restriction analysis and finally by sequencing the cloned DNA. At least 300 nucleotides from each end of the cloned DNA were sequenced, as the primers were available in our laboratory. Two clones for each of the Sendai virus mRNAs were sequenced. P (gfP), V (gfV), and HN (gfHN) clones were sequenced using SP6 and T7 promoter primers. F (ksF) clones were sequenced using SP6 promoter and F.A2 PCR primers. M (gfnM) clones were sequenced using T7 promoter and M.A1 PCR primers, whereas NP (gfnNP) clones were sequenced using T7 promoter and NP.A3 PCR primers. In total, more than 10,000 nucleotides were sequenced. No sequence errors as compared to published (Genbank) sequences were observed, indicating that under our reaction conditions *Taq* polymerase faithfully copied the template. On the basis of the total number of nucleotides we sequenced, we presume that the PCR-cloned Sendai virus mRNAs do not contain any errors.

Identification of P and V mRNA Clones

The P gene of Sendai virus is transcribed into at least two biologically active mRNA species, viz. P and V.[4] The only difference between the two mRNAs is the presence of an additional G base at position 1053 in the V mRNA. Since the 5′ and 3′ ends of these two mRNAs are identical, the same primer pair (P.A1 and P.A2) amplified cDNAs to these mRNAs. Clones for the V mRNA were selected by hybridization to a primer (V1) specific for this mRNA. P and V clones were further confirmed by sequencing the variant regions in these mRNAs using a sequencing primer complementary to P and V mRNAs from nucleotides 1210 through 1228 (data not presented). Except for the difference in the edited region, no other difference was found between P and V clones. Further, it may be possible to discover other edited mRNAs in Sendai and other related viruses by translating the PCR cloned mRNAs.

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