Molecular cloning and characterization of a Sendai virus internal deletion defective RNA

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A small defective Sendai virus RNA was selectively amplified from a virus preparation obtained after serial undiluted passages in embryonated eggs. Preliminary characterization showed that this defective RNA was a true internal deletion defective RNA, containing the 5' and 3' ends of the non-defective viral genomic RNA. Cloning of this RNA after reverse transcription and polymerase chain reaction amplification was performed in such a way that an exact copy of the defective RNA could be obtained by transcription of the plasmid with T7 RNA polymerase. Sequence analysis of the plasmid allowed further characterization of the defective RNA. It was shown potentially to encode a C-terminally truncated nucleocapsid (NP) protein of 162 amino acids. This truncated NP protein was identified in cells naturally infected with the defective virus preparation. Moreover the protein produced was shown to correspond to the protein synthesized in vitro from the T7 polymerase transcript of the cloned defective genome.

Defective interfering (DI) viruses are ubiquitous in animal and plant viruses. They contain deleted genomes (hence they are defective) which are generated at low frequency during viral nucleic acid replication (for reviews see Perrault, 1981; Schlesinger, 1987). DI viruses have been shown to modulate virus infections in cell culture as well as in animals (reviewed by Holland et al., 1985; Holland, 1987; Huang, 1987; Barrett & Dimmock, 1989). Two major types of DI genomes have been described for the non-segmented minus strand RNA viruses. Copy-back DI genomes lack the genomic 3' end sequences, but have sequences complementary to the 5' end at the 3' end. These complementary ends have been shown to vary from 50 to 200 nucleotides in length (for reviews see Perrault, 1981; Re, 1991; see also Enami et al., 1989). The RNA structure restricts the copy-back genome so that it can serve only as a template for its own replication. Indeed, since it lacks the non-defective (ND) genomic 3' end sequences, which contain the signals for transcription (Kolakofsky & Roux, 1987), it is unable to express an mRNA from even a fused gene. On the other hand, 'internal deletion' DI genomes conserve the ND genomic 3' and 5' ends, and therefore retain replication and transcription signals. The ability of vesicular stomatitis virus (VSV) and Sendai virus internal deletion DI genomes to encode viral proteins has been reported (Colonno et al., 1977; Chow et al., 1977; Johnson & Lazzarini, 1977, Re et al., 1985; Hsu et al., 1985). The transcriptional potential of internal deletion DI genomes is interesting for many reasons. First, it may provide information on the interference mechanisms exerted by deletion DI viruses. These have not been satisfactorily explained, especially in the case of internal deletion DI genomes (discussed in Re, 1991). Second, internal deletion DI RNAs of a small size may represent suitable tools for the study of the various signals involved in transcription. Finally, since they represent mini-genomes capable of replication and transcription, their use as vectors to express foreign genes potentially can be exploited. In fact, internal deletion DI genomes, constructed artificially and expressed from plasmids, have already been used for the expression of reporter genes in influenza, Sendai and respiratory syncytial virus infections (Luytjes et al., 1989; Park et al., 1991; Collins et al., 1991).

To obtain a virus preparation containing a deletion DI RNA of suitable size, seven Sendai virus preparations of different origins (strains) were repeatedly passaged in embryonated eggs. After six or seven serial passages, each strain was used to infect BHK cells. Intracellular nucleocapsid (NC) RNAs were purified and subjected to Northern blot analysis using probes specific for the 3' and 5' genomic ends to distinguish copy-back defective RNAs from potential internal deletion RNAs. A fifth passage sample of the E9072 strain (Curran & Kolakofsky, 1988) was chosen because it contained, along with larger subgenomic RNA species, the smallest RNA (estimated size of about 2 to 3 kb) reacting with both the 3' and 5' end probes (not shown). To enrich for this small
defective RNA, the larger subgenomic RNAs were inactivated by u.v. treatment. Fig. 1 (a and b) shows that the u.v. treatment gradually prevented the replication of the intermediate size defective RNAs, and allowed, after 30 min exposure, almost exclusive, replication of the smaller species. Therefore virus stocks were prepared by an initial 30 min u.v. exposure followed by passage in eggs, and then by a subsequent 30 or 40 min additional u.v. treatment.

The stocks produced, E307 and E407 (seven passages in egg, plus a 30 or 40 min u.v. exposure), were finally characterized for their genomic RNA content (Fig. 1 c and d). The repeated u.v. treatments had completely abolished replication of the intermediate size RNAs (compare a with c, lanes 4 and 5), and the two virus stocks prepared contained a unique deletion DI RNA (d, lanes 4 and 5). However, hybridization with the 5′ ex probe (c) also detected two small RNA species not seen with the 3′ ex probe. These presumably represented copy-back RNA species which had not been eliminated by u.v. treatment owing to their small size. Stock E307, contained a putative unique deletion defective RNA which replicated efficiently upon infection of cells; this stock was used in subsequent experiments (the 40 min u.v. treatment of stock E407 had a detrimental effect on subgenomic RNA replication).

Preliminary characterization was undertaken to estimate the content of the primary sequences present in E307 RNA as well as to map the deletion point. This included reverse transcriptase primer extension using an oligonucleotide from the NP gene (positions 14 to 48 of the minus strand), and Northern blot analysis using probes covering entire (or portions of) genes or gene-specific oligonucleotides (not shown). In summary, this characterization showed that (i) no extra sequences were present at the ND or DI 3′ ends and (ii) no sequences in the phosphoprotein (P/C), matrix protein (M), fusion protein (F0) and haemagglutinin-neuraminidase (HN) genes could be detected. Only a portion of the NP gene proximal to the genomic 3′ end extending from approximately nucleotide 157 to nucleotide 624 was identified. No sequence corresponding to the first 3462 nucleotides of the large protein (L) was detected. Positive results were obtained with probes covering that portion of the L gene proximal to the genomic 5′ end, possibly extending from nucleotide 5671. From these results, the maximum and minimum sizes of the E307 RNA were deduced to be 1862 and 1158 nucleotides including the 3′ and 5′ leader sequences.

Owing to the small size of the E307 RNA, reverse polymerase chain reaction (PCR) amplification was performed using primers complementary to the 3′ and 5′ genomic ends. In principle, this strategy could also amplify full-size ND RNA. However, its 15 kb size limits this possibility. In contrast, such a strategy would not amplify copy-back DI RNA. Another feature of this strategy was the possibility of introducing non-viral sequences, i.e. flanking SalI and BamHI sites to allow convenient cloning of the PCR product. A T7 polymerase promoter and a BsmI restriction site, properly positioned, allowed T7 polymerase transcription of a viral RNA containing the correct 5′ and 3′ ends (for details of the cloning procedure, see Calain et al., 1992). PCR amplification yielded a unique DNA species of about 1.7 kb, present only when the NC RNA templates were extracted from E307 virus-infected cells, but absent when reverse transcriptase was omitted (not shown). Using a ND RNA of large size as the template or RNA transcripts of smaller size but lacking the genomic 5′ end
Fig. 2. Primary structure of pSP65-E307. The PCR product amplified from E307 NP RNAs and cloned into pSP65 (see text) was sequenced using a L5662-5682 primer of negative polarity. (a) Relevant portion of the sequencing gel showing the junction (T residue marked with an asterisk) between the L (to right) and NP genes. (b) Outline of the E307 RNA sequence, presented as RNA and aligned with the L and NP gene sequences. Upper case and lower case letters show, respectively, base similarity and divergence. The boxed U residue (corresponding to the T residue marked in (a) could originate from either gene. The fusion of the two genes generates a UAG codon on the positive RNA strand which interrupts an ORF that would otherwise continue into the L gene. In addition to the sequence of the NP/L fusion region, the sequences of both ends of the insert were obtained (not shown). They confirmed the integrity of the 3' and 5' end genomic sequences and their proper cloning into pSP65.

also gave negative results. This PCR product was finally cloned into the SalI/BamHI sites of pSP65 to generate the plasmid pSP65-E307 (Fig. 2.d). Using the oligonucleotide primer L5662-5682, a sequence was amplified which corresponded to the junction between the NP and L genes (Fig. 2.a). The two genes are fused by a U residue which could have originated from either gene. The fusion of the two genes generates a UAG codon on the positive RNA strand which interrupts the NP protein open reading frame (ORF) after glycine 162 (Fig. 2.c). This stop codon interrupts an ORF that would otherwise continue into the L gene. In addition to the sequence of the NP/L fusion region, the sequences of both ends of the insert were obtained (not shown). They confirmed the integrity of the 3' and 5' end genomic sequences and their proper cloning into pSP65. The total calculated size of

Fig. 3. E307 genomic RNA is transcribed into mRNA. Intracellular NC RNA (lanes 1 to 3) was isolated from mock- (lane 1), ND- (lane 2) or E307- (lane 3) infected BHK cells (see legend to Fig. 1). In vivo or mock, ND or E307 mRNAs (lanes 6 to 8) were recovered in the CsCl gradient pellets by resuspension in 2 mM-EDTA, 20 mM-Tris–HCl pH 7.0, 0.5% SDS, phenol–chloroform extraction and ethanol precipitation. T7 and SP6 transcripts (lanes 4 and 5) were synthesized in vitro from pSP65-E307 using the T7 and SP6 RNA polymerases (Promega) according to the manufacturer's instructions. Northern blot analysis was performed using oligonucleotide probes of positive or negative polarity specific for the 3' (NP) or 5' end (L) of the viral genome. Numbers refer to the position of the oligonucleotides in the respective genes.
The insert is 1794 nucleotides, in good agreement with the size of the PCR product.

The ability of E307 RNA to be transcribed into mRNA was then investigated. This RNA was predicted to be found in the viral mRNA fraction (i.e. not encapsidated), to be about 1-7 kb long and to hydridize not only to genomic NP sequences but also to genomic L sequences. Northern blot characterization of the transcripts synthesized in E307 virus-infected cells confirmed this prediction (Fig. 3). The relevant data are presented in Fig. 3(d), in which a band (lanes 3 and 8), the size of which corresponds to that of the in vitro plus transcript (lane 5), was detected with an L gene probe of negative polarity. In (a) and (b), probes of positive polarity did not identify any non-encapsidated transcripts produced in vivo, confirming the exclusively positive polarity of the in vivo E307 transcript, and strongly suggesting that this transcript is a true mRNA.

To confirm this, immunoprecipitations were performed on infected cell extracts after labelling of the cells with [35S]methionine. As mentioned above (Fig. 2c), the sequence of E307 RNA predicts that the fused NP/L transcript is able to encode a truncated NP protein of 162 amino acids (18K). Fig. 4 shows that rabbit antisera against NP (Rab-NP, b), precipitate a low Mr protein (NPTR, estimated to be about 16K) only from E307 virus-infected cells (lane 3). A protein of the same Mr was made in vitro from in vitro T7 polymerase transcripts originating from pSP65-E307 (Fig. 4c). As shown by overexposure of the autoradiograms, the amount of NPTR is very low relative to that of NP (even after using a 4-3 correction factor to account for the difference in the methionine content of the two proteins). Rough estimates indicate a molar ratio of 1:200 to 300, which is apparently in contradiction to the relative abundance of the E307 and NP RNA transcripts (see Fig. 3c). However, pulse-chase experiments showed a marked difference in the turnover of the two proteins (half-life of more than 2 h for NP and of 12 to 14 min for NPTR, not shown).

Therefore, E307 RNA appears to be a bona fide internal deletion DI RNA. Its transcription ability in a natural infection has been confirmed by the presence of a specific transcript, which has been shown to encode a truncated NP protein. Therefore, E307 RNA must contain both the replication and transcription signals present on the ND genomic RNA. Its small size allowed its cloning by reverse PCR. The cloned E307 insert, when transcribed by T7 RNA polymerase, yields a transcript corresponding to the in vivo transcript in size and coding capacity. As the cloning procedure was designed to generate by T7 RNA polymerase transcription an exact copy of the natural E307 DI RNA (of positive polarity), this transcript potentially represents an adequate substrate for encapsidation and replication by Sendai virus proteins. Recently, encapsidation and replication of a Sendai virus copy-back DI RNA expressed in a similar way has been achieved (Calain et al., 1992). Using a similar system, transfection of pSP65-E307 into cells simultaneously infected with a vaccinia virus recombinant expressing the T7 RNA polymerase resulted in production of the NPTR protein (not shown). Preliminary experiments have shown that encapsidation of the E307 RNA transcript of positive polarity takes place efficiently. However, evidence for the replication of this encapsidated RNA has not been obtained. Attempts to demonstrate a possible effect of NPTR expression on ND RNA replication have also been unsuccessful.

In conclusion, the characterization and cloning of an internal deletion DI RNA has been achieved. The DI RNA corresponds to a subgenomic RNA generated and amplified in a natural infection, so it probably contains all the features for efficient transcription, encapsidation and replication (and possibly interference). Attempts to obtain a defective genome originating from pSP65-E307 are under way. This would provide us with a viral "mini-genome" open to genetic manipulation, as well as with a potential vector to express foreign proteins.
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References


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