Complete sequences of the intergenic and mRNA start signals in the Sendai virus genome: homologies with the genome of vesicular stomatitis virus

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ABSTRACT

All of the consensus intergenic and transcription initiation sequences of the genome of Sendai virus, a paramyxovirus, have been determined. The boundary between the intergenic sequence, 3'-GAA, and the mRNA start signal, 3'-UCCCANUUUC, was identified by sequencing the 5' termini of specific viral mRNA molecules. One of the five intergenic trinucleotides differed from the rest, consisting of 3'-GGG, and single base substitutions were observed in two of the mRNA start signals. The Sendai virus intergenic sequence was similar to the analogous sequence (3'-GA) of vesicular stomatitis virus (VSV), a member of another family of negative-strand RNA viruses, the rhabdoviruses, but there was no sequence homology between the mRNA start signals of the two viruses. Nevertheless, these mRNA start signals were organized in the same way, being ten bases long and possessing two consensus regions, divided by one (Sendai virus) or two (VSV) variable internal nucleotides. These findings extend the evidence that both families of negative-strand RNA viruses descended from a common ancestor and that an archetypal mechanism of transcriptional regulation has been conserved in their evolution.

INTRODUCTION

The genome of Sendai virus, a useful laboratory model of the medically important paramyxovirus family, is a single-stranded RNA molecule of negative polarity, consisting of about 15,000 bases (1). At the beginning of infection, the virus genome serves as template for the transcription of six monocistronic mRNAs, specifying the internal proteins NP, L and P, and the envelope proteins HN, F₀ and M. Subsequently, virus-specific proteins change the transcriptive complex into a replicative complex, which produces full-length positive-strand copies of the genome (2). These full-length positive strands serve as templates for the synthesis of genome replicas.

As in the case of the other widely-studied negative strand virus model, vesicular stomatitis virus (VSV), a member of the rhabdovirus family, little is known about the mechanism that differentiates transcriptive and replicative viral RNA synthesis. Indeed, the events that lead to production of monocistronic mRNA species during transcription are matters of persistent controversy

(3). To obtain information about these points, we have been examining the nucleotide sequences at the boundaries of Sendai virus genes (Fig. 5). Previously, we identified the consensus sequences at the 5' ends (region E, Fig. 5) of all of the virus genes, and observed that they were markedly similar to the analogous sequences of VSV (4, 18, 19). Now, we have determined the sequences in the intergenic (I) regions and mRNA start (S) signals (Fig. 5) of all Sendai virus genes.

MATERIALS AND METHODS

Sendai Virus Genomic RNA and mRNAs

Virus particles were purified from allantoic fluid of infected chicken embryos and viral genomic (50S) RNA was isolated by sucrose gradient centrifugation (5). Polyadenylated mRNAs from infected chick embryo lung cell cultures were selected by oligo(dT)-cellulose chromatography. VSV mRNAs were synthesized in vitro at 30°C for three hours and purified as described by Gupta et al. (6). All RNA preparations were precipitated at least twice with ethanol, with the last precipitation performed in the presence of 0.4 M NaCl. The final RNA pellet was rinsed with 95% ethanol, dissolved in sterile distilled H₂O and stored at -20°C.

Primers

The deoxyribonucleotide phasing primer, 5'-(dT)₁₀dCdT, was a product of PL Biochemicals, Inc. A set of specific 12 or 14 base deoxyribonucleotide primers complementary to sequences near the 5' ends of five of the virus genes was made with an Applied Biosystems oligonucleotide synthesizer. Another series of primers, complementary to the 5'-proximal regions of three viral mRNAs, was prepared the same way. Primers were radiolabeled enzymatically (T4 polynucleotide kinase) at their 5'-termini with [γ -³²P]ATP. Reaction products were separated by electrophoresis in a 20% polyacrylamide gel and eluted from the gel by soaking in 20 mM Tris-Cl (pH 7.5), 2 mM Na₂EDTA and 0.2% SDS in 300 μ l volumes at 37°C overnight. The fluid was withdrawn and filtered through a 0.45 μ m nitrocellulose filter in a microfilter assembly (Bioanalytical Systems, Inc.). Primers were precipitated with ethanol after addition of 30 μ g of tRNA and NaCl to 0.4 M.

RESULTS

3'-terminal Sequences of Viral mRNAs

Our strategy for sequencing intergenic (I) and mRNA start (S) signals in the Sendai virus genome (Fig. 5) was to use the genomic RNA as template for extension of oligonucleotide primers complementary to regions near the 5' end

of each gene. Our previous work on Sendai virus gene-terminal RNase T1-resistant oligonucleotides gave us sufficiently long sequences at the termini of two genes (4) to specify such primers, but it was necessary to obtain more extensive upstream sequence information for the other genes. To accomplish this, we exploited the fact that Sendai virus mRNAs are polyadenylated and our finding that all of the genes terminate in the mRNA end (E) sequence 3'-AUUC(U)₅, the oligo(U) sequence representing the initiation site for polyadenylation (4). A commercially available oligodeoxynucleotide, 5'-(dT)₁₀dCdT, was chosen as a phasing primer for making cDNA copies of the viral mRNAs, because of its correspondence to the 3'-UC(U)₅ of the E sequence and the expectation that the remaining five dT residues would base-pair with A residues in the mRNA poly(A) tracts. Since cellular mRNA species were expected to be present in the mixture of poly(A)-containing RNA species from virus-infected cells to be used as template (7), we hoped that this phasing primer would be selective for the reverse transcription of virus-specific mRNA species. We also expected that the heterogeneity of cellular mRNAs would work in our favor, allowing cDNA copies of the six viral messages to stand out above any cellular background. We still had the task of discriminating among the virus-specific cDNA transcripts. Our approach was to treat the mixture of cDNAs with restriction endonucleases capable of cleaving single-stranded DNA (8) and subject the digest to gel electrophoresis. We expected to obtain a series of 5'-terminally labeled DNA fragments, each one complementary to the 3' end of a specific mRNA species.

As shown in Fig. 1A, lane C, the untreated cDNA preparation was heterogeneous, but it apparently had a high average molecular weight. The restriction enzymes Alu I and Ava II did not yield distinct bands of labeled cDNA, but bands were obtained with the enzymes Hae III and Hinf I. Since the discrete bands produced by Hae III were more intense than the bands produced by Hinf I, consistent with the reported greater efficiency of Hae III in cleaving single-stranded DNA (8), the digestion products of Hae III were chosen for further analysis.

We attempted to sequence the cDNA species produced by Hae III digestion by the chemical cleavage method (9) directly after extraction from the polyacrylamide gel (Fig. 1A), but the sequence ladders were too heterogeneous to be read. Therefore, we electrophoresed each of the cDNA species again, using a strand-separating polyacrylamide gel system (9) to increase the likelihood that contaminants would be separated from the species of interest (Fig. 1B). Each of the cDNAs migrated as a distinct band (Fig. 1B) of sufficient purity

Fig. 1. Isolation of cDNA segments complementary to the 3' ends of Sendai virus mRNA species. Mixed poly(A)-rich RNAs from Sendai virus-infected cells were reverse-transcribed with a phasing primer and the products were treated with restriction endonucleases and separated by polyacrylamide gel electrophoresis (Materials and Methods). Panel A shows the first step in the separation, which employed a 6% polyacrylamide gel. Lane C is undigested product and the restriction enzymes used are designated above the other lanes. Arrowheads point to bands in the *Hinf* I digest that presumably represent virus-specific cDNAs, but were not studied further. Panel B depicts the second step in the isolation. *Hae* III bands excised from the first gel were electrophoresed in a 7% strand-separating polyacrylamide gel. Bands marked M, NP and HN were identified as virus gene-specific by sequencing (see Results). Band X did not yield a readable sequence.

for chemical sequencing. The sharpness of the bands in Fig. 1B indicates that there is little or no heterogeneity at the 3' ends of the mRNA species that they represent. The sequences obtained from the three smaller *Hae* III bands were Sendai virus-specific, since they all contained the consensus tetranucleotide, 3'-AUUC, previously found in each E sequence (4). This tetranucleotide immediately preceded an oligo(dT) tract contributed by the phasing primer. At the 3' side of the tetranucleotide, each of the bands exhibited

Fig. 2. Nucleotide sequences (mRNA sense) deduced from chemical cleavage sequencing (9) of the cDNAs shown in Fig. 1. Numbering is relative to the initiating nucleotide of the following gene in the transcriptional map (see Fig. 3).

homology to one of the gene-terminal RNase T1 oligonucleotides, numbered 46 to 48, analysed previously (4). These results and other data (Fig. 3) indicated that the three Hae III bands correspond to the 3'-terminal regions of the mRNAs for the viral NP, M and HN proteins (Fig. 1). These sequences are presented in the mRNA sense in Fig. 2.

The band marked X in Fig. 1 is probably also virus-specific, but it remained too contaminated by other cDNA species even after the second electrophoretic step to yield useful sequence information. Failure to observe cDNA corresponding to the full set of six Sendai virus genes presumably reflects the distribution of Hae III sites.

Sequences of Intergenic and mRNA Start Signals

The sequences at the 3' ends of the Sendai virus mRNAs described above and the previously determined 3'-terminal sequences of two other Sendai virus genes (4) provided the information needed to specify the following five synthetic oligodeoxynucleotide primers, designated by numbers corresponding to the RNase T1 oligonucleotides previously identified at the 5' ends of Sendai virus genes (4). (The nucleotide positions given below are relative to the start of each gene downstream, as determined later; Fig. 3).

- 44: $\overline{d}^{22}[\text{CGGTATATAATAA}^{\overline{3}}\text{G}]-3'$
 45: $\overline{d}^{22}[\text{CAGTAAAGATT}^{\overline{1}}\text{A}]\text{A}$
 46: $\overline{d}^{26}[\text{GTCTCTCACGGT}^{\overline{15}}\text{T}]\text{A}$
 47: $\overline{d}^{18}[\text{CTTAAATAAGAA}^{\overline{7}}\text{A}]\text{A}$
 48: $\overline{d}^{24}[\text{CTTGATCCGTAG}^{\overline{13}}\text{G}]\text{A}$

Fig. 3. Nucleotide sequences (genome sense) at Sendai virus gene junctions. Numbers in parentheses represent T1 RNase oligonucleotides previously found at the termini of Sendai virus genes (4) homologous to the more extensive sequences obtained in the present study. Above these numbers, the genes bordering the junction are designated, with the arrows pointing in the direction of transcription. These gene assignments were made as follows: (a) the sequence including that of oligonucleotide 48 was identified as NP gene-specific from the sequence of a molecular clone representing NP (30); (b) oligonucleotide 45 is in the published sequence of the P gene (25); (c) we previously identified oligonucleotide 47 in the M gene (4); (d) we have now assigned oligonucleotides 45 and 46 to the genes for the F₀ and HN proteins, respectively, based on their hybridizations to mRNA species with molecular weights appropriate for the two proteins (4); accordingly, these two identifications are tentative. Each sequence is numbered relative to the A residue (position 1) that begins each mRNA species. Consensus sequences are set off by spaces and are emphasized. UAC triplets that might represent protein initiation anticodons are underlined.

Extension of these primers by reverse transcriptase, employing the virus genome RNA as template, was used to identify the intergenic (I) and mRNA start (S) signals of adjacent genes. As observed by others (10), the primers varied in effectiveness for dideoxynucleotide sequencing, although each was extended

to some degree. When this method did not give a readable sequence, we extended the primers with the four deoxyribonucleoside triphosphates and sequenced the extension products by chemical cleavage (9).

The sequencing gels revealed consensus sequences, identified as I and S regions, immediately following an oligo(A) tract thought to represent the polyadenylation site in the mRNA end (E) signal (4). The I sequence was 3'-GAA (genome sense) in four cases and 3'-GGG in one case (Figs. 3 and 5). Next was seen a 10 base conserved region, 5'-AGGG^{UG}_{AU}AA^A_UG, the upper nucleotide in relative positions 5, 6, and 10 being represented in four of five cases; this region appeared to represent the S signal. We based the boundary between I and S on analogy with the intergenic sequences of VSV (see Discussion) and the knowledge that most eukaryotic mRNA species initiate with a 5'-terminal purine nucleotide (11). Adjacent downstream sequences (which revealed no further homologies) are also presented in Fig. 3.

Identification of the I/S Boundary

To verify the identity of the initiating nucleotide in Sendai virus mRNA species, we used the mRNAs as templates in dideoxynucleotide sequencing reactions (12) primed by oligonucleotides that represented regions about 60 to 90 nucleotides downstream from the putative 5' mRNA terminus, based on the information in Fig. 3. The sequences of this second series of primers, and the sequence of a primer specific for VSV NS protein mRNA, which was synthesized on the basis of literature data (14), are as follows (nucleotides are numbered relative to the start of each gene):

	78	67
M:	5'-d[CGTTATCCTCAT]	
	75	64
L:	d[GGATAGACTATG]	
	88	77
F ₀ :	d[GAGATGCACTGT]	
	103	91
VSV NS:	d[CGTTGTGCTTCG]	

A typical result is given in Fig. 4B, where the primer was specific for the L gene. The sequence ladders contain the consensus S region in complementary form. This is followed by a heavy band of radioactivity in all of the lanes, indicating a reverse transcription termination event. However, all four ladders continue beyond this point into the upstream gene.

Extension of the sequence ladders can be explained by the presence of virus-specific polytranscripts (our unpublished observations) in the template RNA preparation; contamination of the template RNA by positive-stranded virus antigenomes (13) seems less likely, since the template RNA was selected by

Fig. 4. Dideoxynucleotide sequencing ladders representing the 5' termini of Sendai virus and VSV mRNAs. Panel A shows the results obtained with a primer specific for VSV mRNA encoding the NS protein; lane O shows the product of a reaction in which dideoxynucleotides were not added. Panel B shows the results obtained with a primer specific for the mRNA specifying the Sendai virus L protein. Arrowheads point to strong stop signals one nucleotide beyond the known initiating nucleotide of VSV mRNAs and the deduced initiating nucleotide of Sendai virus mRNAs. Note that reverse transcription of Sendai virus L mRNA did not terminate completely at that site (see Results).

oligo(dT) chromatography. More puzzling is the strong stop signal one nucleotide upstream from where we expected it. If this represents the true terminus of the mRNA, it is a 5'-terminal C residue, which would be specified by the third G in the I sequence between the HN and L genes (Fig. 3). To test the

Fig. 5. Map of the Sendai virus genome (-), showing the positions of intergenic (I) and adjacent mRNA end (E) and start (S) sequences. The region designated + is the 3'-terminal 51 base positive-strand leader RNA template (23), and virus genes are designated above the map in the most probable order (17, 22). One or two additional genes, designated C and C', are embedded within the P gene (22). The E sequence of the L gene has been reported previously (31).

possibility that reverse transcriptase adds a nucleotide when copying capped mRNAs, we performed a similar dideoxynucleotide sequencing experiment using a primer specific for the mRNA that specifies the NS protein of VSV (14). In this case, also, a second strong stop signal was seen one nucleotide beyond the known 5' terminus of the mRNA (Fig. 4A). Indeed, this signal predominated when dideoxynucleotides were omitted from the reaction and reverse transcripts were permitted to extend completely (Fig 4, lane 0). These results confirmed our suspicion that reverse transcriptase adds a nucleotide to the reverse transcript of a capped mRNA molecule and they show that this reaction is favored over termination at the preceding nucleotide, which initiates the mRNA. Results similar to those obtained with the L gene mRNA primer were also obtained with the other primers shown above. Thus, we conclude that Sendai virus mRNAs initiate with an A residue and that we were correct in defining of the I and S regions as shown in Figs. 3 and 5.

DISCUSSION

There are strong analogies between Sendai virus and VSV in virion structure, genome organization and expression, and the numbers and functions of virus-specified proteins (15-17). Therefore, we expected to find analogous nucleotide sequences at gene boundaries, where signals that specify the beginning and end of each mRNA reside. However, in our previous work on the Sendai virus mRNA end (E) signals, we were surprised at the extent of homology with the corresponding VSV sequences: in each case a consensus tetranucleotide

preceded an oligo(U) tract; the tetranucleotides of the two viral genomes differed in only one base; and the oligo(U) tracts were five and seven bases long in Sendai virus and VSV, respectively (4, 18, 19). Now we have observed additional strong homology in the intergenic (I) regions of both viruses: compare the 3'-GAA of Sendai virus with the 3'-GA of VSV (18, 19). In contrast to the foregoing, the mRNA start (S) signals of these viruses exhibit divergence, although both sequences have a similar organization, starting with a U residue, consisting of the same number of nucleotides and possessing two conserved regions that span a single heterogeneous nucleotide (Sendai virus) or a pair of heterogeneous nucleotides (VSV) (18, 19). Considering that VSV is a pathogen of cattle and swine, whereas Sendai virus is a pathogen of mice, and the likelihood that viruses evolve along with their hosts (20), it is noteworthy that there are such strong similarities in the E, I and S sequences of these viruses. Evidently, the mechanism of transcriptional regulation by these sequences has not been amenable to much improvement in the millennia since these two viruses arose from their common ancestor.

Fig. 5 also displays the start of the first gene in the transcriptional map of Sendai virus, that specifying the NP protein, and the three A residues that precede it (21, 22). This triplet is part of a group of four A residues that may represent an unexpressed region separating the 51 base positive strand leader RNA template (23) from the beginning of the first gene; we show this triplet to illustrate its similarity to the I sequences. The homology between the NP gene S sequence and the other S sequences is evident, in keeping with a common mechanism for initiation of each transcript. Because the NP S sequence contains an A residue at position 5, in common with four of the five other S sequences, we consider the fifth position to be part of the consensus, whereas we consider the sixth position to be heterogeneous, since two S sequences differed at position 6. This distinction is, of course, arbitrary, since we do not know how each nucleotide is recognized within the sequence by the viral RNA polymerase.

Our identification of the demarcation between the I and S sequences rested on sequencing the 5' termini of viral mRNAs. In these determinations, we encountered the addition of an extra nucleotide by reverse transcriptase, presumably because of the presence of a methylated G cap residue. Moore *et al.* (24) found that reverse transcriptase terminated transcription of base-modified DNA at the site of the lesion, whereas other DNA polymerases terminated at the nucleotide preceding the lesion. Thus, we may be observing a similar tendency of reverse transcriptase to recognize and attempt to copy

abnormal nucleotides. Independent evidence that the first nucleotide in the Sendai virus S sequence is U has recently been obtained and the I and S signals of three viral genes have been reported (25).

The genetic map of Sendai virus in Fig. 5 shows the locations of the E, I and S sequences (4). We have now recognized a consensus U residue in the second position upstream of the 3'-AUUC tetranucleotide in the E signal. Thus, the total number of bases involved in E, I and S signals, including the S signal in the NP gene at the 3' end of the genome and the E signal in the L gene at the opposite end, is 141. The only other regulatory sequences in the genome appear to reside at its termini: about 50 bases at each end are not represented in any mRNAs (23). Thus, less than 300 bases may represent the entire complement of sequences that regulate the expression of the remaining 15,000 bases in the viral genome.

What do our results reveal about the process by which monocistronic mRNA species are generated in the transcription of the polycistronic genome of a nonsegmented negative-strand RNA virus? This process has been a matter of controversy since it was shown that there is a single entry point for transcriptase at the 3' end of such a genome, each gene being transcribed sequentially and with decreasing efficiency according to its distance from that entry point (15-17). This process has been considered to occur either by post-transcriptional endonucleolytic cleavage of polytranscripts, with poly(A) addition occurring either before or after separation of the mRNAs (cleavage model), or by a more conventional termination and reinitiation mechanism (stop-start model). We believe that the available data on the E, I and S sequences of VSV and Sendai virus support the stop-start model with greater parsimony, and that polytranscripts (Fig. 4) are aberrant transcription products, for the following reasons:

(a) The E sequences of both viruses contain oligo(U) tracts, which are suitable sites for the transcriptase to initiate and repetitively extend a poly(A) sequence at the 3' end of each mRNA. Compare the influenza virus genome, which is also negative-stranded, but segmented: oligo(U) tracts of five to seven bases that are initiation sites for poly(A) synthesis are found close to the 5' termini of influenza virus genomic RNA segments (26), but the possibility of polytranscript formation does not arise with a segmented genome.

(b) The I sequence is an embarrassment to a cleavage model, since it is two (VSV) or three (Sendai virus) bases long. Two endonucleolytic cleavages or one endonucleolytic cleavage followed by precisely limited exonucleolytic

trimming are complications that must be supported by this model.

(c) The S sequence, 10 nucleotides long for both VSV and Sendai virus, is far more complex than the single nucleotides that are recognized by conventional endoribonucleases, and the E and S sequences of both viruses are significantly more complex than the canonical dinucleotides in eukaryotic mRNA splicing signals (27). Instead, the E and S sequences correspond in length to the transcriptional regulatory signals of prokaryotic and eukaryotic genomes (28, 29).

The 24 base span of the combined Sendai virus E, I and S sequences is about twice as long as the sequence homology seen at the 3' termini of Sendai virus genomes and positive-stranded antigenomes (21). This suggests that the viral RNA polymerase recognizes the E sequence, 11 bases long, and the S sequence, 10 bases long, separately as mRNA stop and start signals, if the viral RNA polymerase has a single template scanning site for initiating RNA synthesis at the 3' termini of its templates and when it operates as a transcriptase. However, the I sequence may also play a role in initiation and termination of transcription (32). Further understanding of the importance of sequence length and composition in the regulatory signals of nonsegmented negative-strand viruses may come from sequence examinations of additional virus species.

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