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Biochemistry

Molecular cloning of the 3′-proximal third of Sendai virus genome

(recombinant DNA/cloning Sendai virus/gene order/translation *in vitro*)

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ABSTRACT **Portions of the Sendai virus genome were randomly cloned by using virion 50S RNA and calf thymus DNA pentanucleotides as primers. The recombinant clones were probed first with radiolabeled products of an *in vitro* virion RNA polymerase reaction to locate early message clones and then with a probe from the viral genome 3′ end to locate the most 3′-proximal clones. Clones were then ordered from the 3′ end of the genome and used to construct a genetic map of the 3′-proximal third of the genome by hybrid-selection of mRNAs. We report that the gene order for this region is 3′-NP–P+C–M-5′ and that the genetic loci of the viral P and C proteins cannot be separated by these techniques.**

Sendai virus, a prototype of the parainfluenza virus family, contains all of its genetic information in a single 15-kilobase (kb) RNA chain of negative polarity (−) (1, 2). The genetic information of this virus appears to be expressed via monocistronic mRNAs transcribed from the (−) genome because Sendai virus-infected cells contain multiple mRNA species of opposite polarity to the viral genome that correlate roughly with the expected coding capacity of the major virion proteins (3, 4). To date, six virion structural proteins have been clearly defined: NP [60 kilodaltons (kDa)], P (79 kDa), and L (≈200 kDa), which are associated with the nucleocapsid core, and HN (72 kDa), Fo (65 kDa), and M (34 kDa), which are associated with the viral envelope (1, 5).

In addition to the structural proteins of the virion, there exists a protein that is present in virus-infected cells but is not found in mature virions (5). This nonstructural protein (C; 22 kDa) has been shown to be virus specific and to contain a peptide map different from the maps of the other viral proteins (6, 7). Because the Sendai virus C protein is not a structural component of the virion itself, it seems likely that it functions in a control capacity during intracellular virus replication. For this reason, the C protein is of particular interest.

In a previous paper on this subject, *in vitro* translation was used to study the relative sizes of the Sendai virus mRNAs (8). The mRNA for the 22-kDa C protein could not be separated by sucrose gradient velocity sedimentation from that of the 79-kDa P protein. In order to resolve this anomaly, and to directly locate the positions of the various genes on the viral genome, we have undertaken the molecular cloning of the Sendai virus genome.

MATERIALS AND METHODS

Synthesis of Sendai Virus Double-Stranded DNA. Three micrograms of Sendai virion 50S RNA (9) in water was treated with 8 mM CH_3HgOH and then incubated with 8 μg of pentadeoxynucleotides from calf thymus DNA (P-L Biochemicals) in a 40-μl reaction mixture containing 80 mM Tris·HCl at pH 8.0, 10 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 50 mM NaCl, 0.5 mM dNTPs including [α-^{32}P]dATP, and 30 units of reverse transcriptase from avian myeloblastosis virus. The mixture was incubated for 10 min at 37°C and then 60 min at 43°C. The mixture was chromatographed on a Sephadex G-50 column and the excluded [^{32}P]cDNA fractions were precipitated with ethanol. For second-strand synthesis, the [^{32}P]cDNA was resuspended in a 20-μl reaction mixture containing 40 mM Tris·HCl at pH 8.0, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 80 mM NaCl, and boiled for 2 min. An equal volume (20 μl) of 0.2 M Hepes, pH 6.9, and 0.5 mM dNTPs containing 4 units of Klenow fragment DNA polymerase 1 was then added and the sample was incubated for 2 hr at 15°C. The entire reaction mixture was precipitated with ethanol, the precipitate was vigorously suspended in 80 μl of H_2O, and then 20 μl of 1.5 M NaCl/0.15 M sodium acetate, pH 4.5/0.03 M $ZnCl_2$ was added. The nonbase-paired regions of the DNA were then digested with 1 unit of S1 nuclease for 30 min at 37°C. This reaction mixture was then extracted with phenol/chloroform and the double-stranded DNA was recovered after Sephadex G-50 chromatography. We calculate that 520 ng of S1-resistant cDNA was produced.

Construction of Hybrid Plasmids, Transformation, and Identification of Recombinant Clones. Poly(dC) and poly(dG) extensions were added onto Sendai virus double-stranded cDNA and *Pst* I-digested pBR322, respectively, by using calf thymus terminal transferase (10). The tailed vector and cDNA were annealed and used to transform competent *Escherichia coli* HB101 by standard techniques (11). Tetracycline-resistant ampicillin-sensitive transformants were isolated and screened by the method of Grunstein and Hogness (12), using partially alkali-digested Sendai 50S RNA radiolabeled with [γ-^{32}P]ATP and polynucleotide kinase or [α-^{32}P]GTP-labeled virion polymerase products as probes (8).

Hybrid Selection of Sendai Virus mRNA. Fifteen micrograms of various plasmid DNAs were boiled in 0.25 M NaOH for 10 min, neutralized to pH 4.5 with acetic acid, and directly fixed to 1-cm discs of aminobenzyloxymethyl (ABM) paper (Schleicher & Schuell). The filters were combined in a single vial and used to hybrid-select 300 μg of CsCl pellet RNA (9) from Sendai virus-infected BHK (baby hamster kidney) cells according to Long *et al.* (13). The hybrid-selected RNA was recovered by boiling and then dissolved in 10 μl of H_2O, and 2 μl of each sample was translated in a reticulocyte lysate kit (New England Nuclear).

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Abbreviations: kb, kilobase(s); kDa, kilodalton(s); bp, base pair(s); VSV, vesicular stomatitis virus.

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RESULTS

Cloning of the Sendai Virus Genome. In order to obtain DNA clones of the Sendai virus genome that contained intercistronic sequences, we started with virion (−) 50S RNA. cDNA was transcribed from this RNA template by using reverse transcriptase and calf thymus DNA pentanucleotides at high concentrations to maximize the randomness of the cDNA synthesis (14). The resulting cDNA was then converted to double-stranded DNA, digested with nuclease S1, and tailed with oligo(dC) by using phage T4 terminal transferase. These DNA fragments were inserted into the *Pst* I site of pBR322 that had been tailed with oligo(dG) and the hybrid DNA was used to transform *E. coli* HB101. Starting with an estimated 90 ng of oligo(dC)-tailed insert DNA, a total of 6,000 tetracycline-resistant ampicillin-sensitive colonies was obtained.

Identification of 3′-Proximal Clones. Ideally, we would like to identify a clone that contains the exact 3′ end of the 50S (−) genome. This clone should contain the sequences of the leader RNA or RNAs, followed by the beginning of the first cistron. In order to locate a 3′ end clone from the library of randomly generated genome clones, we first screened for clones that would hybridize to the RNA products of an *in vitro* virion RNA polymerase reaction. Transcripts from the 3′ end of the (−) genome are thought to be over-represented in the *in vitro* reactions. In addition, short transcripts or leader RNAs, complementary to the exact 3′ end of the (−) genome, are known to be synthesized by detergent-activated virions *in vitro* (9), as well as mRNAs for the viral NP, P, C, and M proteins (8).

The ^{32}P-labeled *in vitro* virion RNA polymerase products were fractionated by agarose gel electrophoresis into leader RNAs and mRNAs and used separately to screen 1,000 tetracycline-resistant ampicillin-sensitive colonies. Fourteen of the colonies showing the strongest signals from each series were chosen and their DNAs were prepared and dotted on aminobenzyloxymethyl paper (15). A more rigorous probe for the 3′ end of (−) genome was then constructed by radiolabeling this end of virion 50S RNA with [^{32}P]pCp and RNA ligase (9). The 3′-end-labeled RNA was again reselected as 50S RNA after CH_3HgOH denaturation and then extensively fragmented by controlled alkaline hydrolysis. The 0–2S and 2–4S end-labeled genome fragments, again selected by sedimentation velocity in sucrose gradients, were then hybridized to DNA dots of the 14 leader-positive (SL series) and early message-positive (SN series) clones. Two of the 28 candidate clones (pSL3 and pSL9) hybridized strongly to both the 0–2S and 2–4S genome probes (data not shown). Restriction analysis demonstrated that both pSL3 [approximately 435-base-pair (bp) insert] and pSL9 (approximately 330-bp insert) contained common internal *Hae* III fragments of 86 and 36 bp and that all of the SL9 insert was contained in the insert of pSL3. pSL3 was therefore chosen for further analysis.

The sequence of the 3′ end of 50S (−) genome RNA was determined and compared to the DNA sequence of pSL3 in order to lcoate pSL3 on the 50S (−) genome. Fig. 1 shows the nucleotide sequence of the first 74 bases of the 3′ end of the 50S (−) genome, determined by direct analysis of the RNA (16), and the corresponding DNA sequence from the cloned insert of pSL3. Note that the DNA sequence from the first C of the poly(C) tail inward corresponds to the RNA sequence between nucleotides 22 and 74 except for nucleotides 58 and 64, which probably represent RNA sequencing errors. The RNA sequence reported here agrees well with that reported by Re *et al.* (19) for the first 25 bases of the 3′ end of the Sendai genome except for two positions (5 and 15), which may represent strain-specific differences. Thus pSL3 starts at nucleotide 22 and contains approximately 400 bp of the 3′ end of the Sendai virus (−) genome.

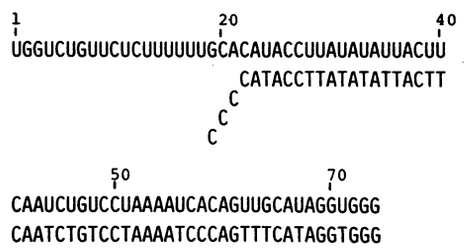

FIG. 1. Location of pSL3 within the 3′ end of the Sendai virus genome. The sequence of the 3′ end of the Sendai virus (−) genome RNA was determined as described (16) by using [^{32}P]pCp-labeled RNA (upper line). The sequences of the ends of the insert of pSL3 were determined by 3′-end-labeling the *Pst* I-digested DNA with [α-^{32}P]cordecypin triphosphate (17) followed by *Hae* III digestion and isolation by polyacrylamide gel electrophoresis of the 132- and 178-bp *Pst* I/*Hae* III fragments. The DNA sequences were determined by the chemical method (18) and that part of the 132-bp fragment corresponding to the RNA sequence is shown (lower line).

Ordering the Sendai Virus DNA Clones from the 3′ End of the Genome. As a prerequisite for determining the genetic map of Sendai virus, we next ordered the remaining DNA clones relative to pSL3. These clones were first screened for insert size by *Pst* I digestion, and those clones containing inserts of more than 500 bp were analyzed further by digestion with *Dde* I, *Rsa* I, *Hae* III, and *Hin*fI, followed by polyacrylamide gel electrophoresis. Fragments internal to the inserts were identified by performing duplicate digestions in the presence and absence of *Pst* I. We eliminated from consideration fragments cut by *Pst* I and parts of the plasmid vector. Overlaps were tentatively considered as having been demonstrated when clones shared at least two internal restriction fragments. This criterion failed in the case of pSL11/pSN11 due to the presence of internal *Pst* I cleavage sites; however, the presence of these rare internal sites served to tentatively identify this overlap. In addition, the restriction fragments from one of the longest DNA clones, pSN5, were ordered by taking advantage of the fact that this clone contains a unique *Eco*RI site, which divides it into fragments of approximately 1,000 and 700 bp. Both the *Pst* I and *Eco*RI ends of these fragments were individually labeled, and the *Hae* III and *Hin*fI fragments were then ordered by partial digestion (20). This ordering led to a restriction map of the first 5 kb from the 3′ end of the Sendai virus (−) genome (Fig. 2).

However, ordering of clones by the sharing of restriction fragments is based only on the comparative electrophoretic mobilities of these fragments in polyacrylamide gels and thus must be considered tentative. The order was therefore confirmed by dot blot analysis. The inserts of nine plasmids were digested with *Pst* I, isolated by electroelution, nick-translated, and hybridized, in turn, with filters dotted identically with DNA from each of the plasmids (Fig. 3). We found that the signal from the 50-bp overlap between pSN10 and pSL6 indicated on the restriction map is barely visible in dot 7 of frame 4 and dot 4 of frame 7 and defines the lower limit of detection of this assay.

Genetic Map of the 3′ End of the Sendai Virus Genome. A genetic map of the 3′ third of the Sendai virus genome was constructed by analyzing the translation products of mRNAs hybrid-selected by the ordered clones. Equal amounts of DNA from plasmids pSL3, pSL11, pSN10, pSL6, pSN5, pSL2, and as a control, pBR322, were each fixed to aminobenzyloxymethyl paper and used to select mRNA from Sendai virus-infected BHK cells. The hybrid-selected mRNAs were translated *in vitro* and the results of the sodium dodecyl sulfate/polyacryl-

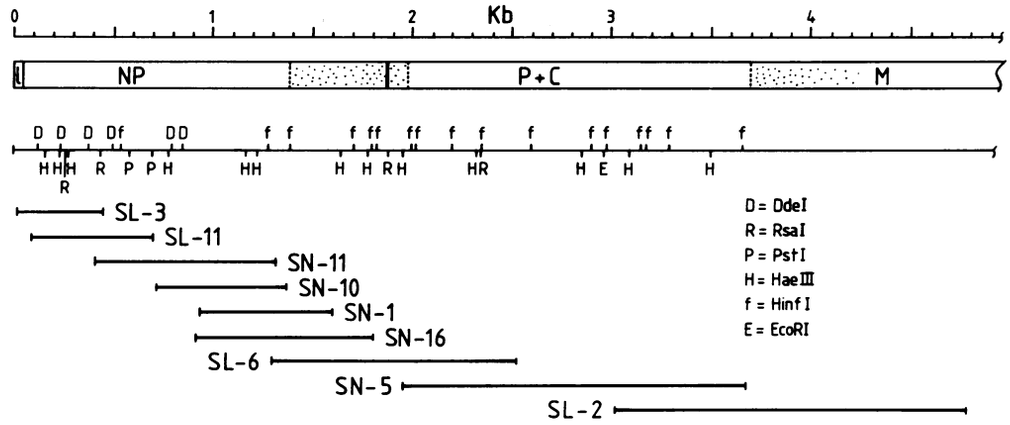

FIG. 2. Map of the 3′ proximal third of the Sendai virus genome. The largest virion RNA polymerase product-positive clones were digested with various restriction enzymes in the presence and absence of *Pst* I and compared by polyacrylamide gel electrophoresis. The order of the clones, their position on the (−) genome, and the resulting restriction map are shown. The restriction map is incomplete but sufficient to give the clone order. The approximate position of the gene boundaries derived from the data in Fig. 4 are shown as speckled areas. The heavy vertical bar shows the position of the NP/P plus C junction determined from primer extension studies (data not presented). The position of the leader/NP junction was derived from previous work (9) and by primer extension (data not presented).

amide gel electrophoresis analysis of the translation products are shown in Fig. 4. Translation from the control plasmid, pBR322, yielded two bands (marked r) which were present even when no exogenous RNA was added to the lysate (not shown) and a small amount of the viral NP protein. Clones nearest the 3′ end, pSL3, pSL11, and pSN10, all selected mRNA coding for viral NP protein (60 kDa) as well as a number of smaller bands marked N*, each of which is present in reduced amounts relative to NP and which probably represent the translation products of degraded NP mRNA. Although the largest N* band appears to migrate in the position expected for the nonglycosylated form of the Fo protein (55 kDa) from tunicamycin-treated cells (23), the 55-kDa N* band was found to immunoprecipitate with NP-specific antisera but not with Fo-specific antisera (not shown). The identity of the viral NP band and smaller N* bands was confirmed by immunoprecipitation with antisera monospecific for Sendai virus protein NP. Another antiserum specific for Sendai Fo protein failed to precipitate any of these [^{35}S]-methionine-labeled bands. pSL6, the first intercistronic clone, selected mRNA for NP protein bands as well as P protein (79 kDa) and the nonstructural C proteins (C and C′; 22 kDa). The next ordered clone, pSN5, selected mRNA for the viral P and C proteins. Note that the small amount of NP protein translated here is similar in amount to the pBR322 control lane within the experimental error of the technique. The second intercistronic clone, pSL2, selected mRNA for the viral P and C proteins as well as the viral M and B proteins [B is a phosphorylated form of M (24)]. Note that although the M protein has an electrophoretic mobility similar to that of one of the N* bands, no other N* band is selected by this clone. Thus, the data in Fig. 4 indicate that the genetic map of the 3′-proximal third of the Sendai (−) genome is 3′-NP–P and C–M-5′. The limits of the boundaries of these genes from the data shown in Fig. 4 are drawn schematically in Fig. 2. The boundary between the NP and P plus C genes was more precisely determined by primer extension of several *Hinf*I restriction fragments from the insert of pSN5 on viral mRNA (not shown) and the putative start of the P plus C genes was located approximately 125 bp to the left of the end of the insert of pSN5 (also shown in Fig. 2).

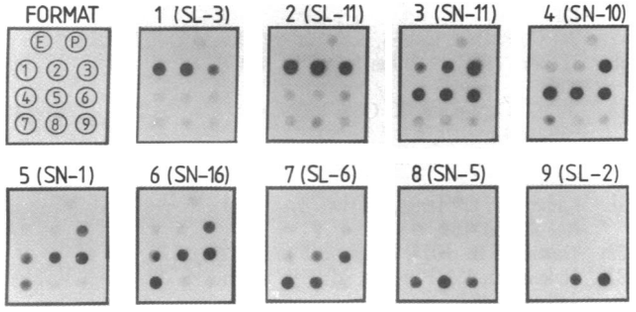

FIG. 3. Dot hybridization analysis of the Sendai virus-specific clones. Each numbered frame shows the results of hybridization of the nick-translated *Pst* I insert of the indicated clone with a nitrocellulose filter dotted with plasmid DNA from each of the clones, numbered identically to the probes. Equal amounts of *E. coli* (E) and pBR322 (P) DNA were also dotted as controls. Filters were prepared according to Kafatos *et al.* (21) and hybridized according to Dawid (22).

DISCUSSION

The use of random pentanucleotides as primers on the Sendai virus (−) genome RNA has allowed the construction of a library

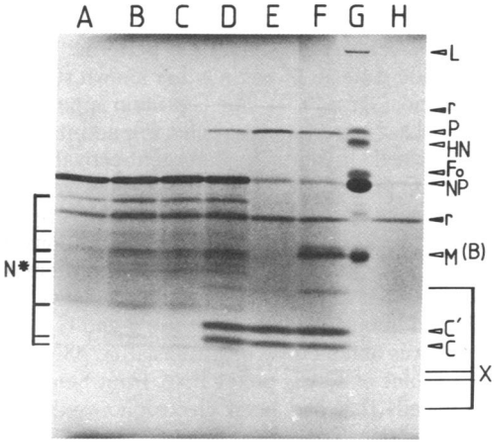

FIG. 4. Sendai virus gene order by hybrid selection of mRNA. DNAs from plasmids pSL3, pSL11, pSN10, pSL6, pSN5, pSL2 (lanes A–F, respectively), and, as a control, pBR322 (lane H) were immobilized on aminobenzyloxymethyl paper and used together to select mRNA by hybridization from total cytoplasmic RNA from Sendai virus-infected BHK cells. Two microliters of each of the hybrid-selected RNAs was then translated *in vitro* with [^{35}S]methionine and the translation products were separated on a sodium dodecyl sulfate/11.5% polyacrylamide gel along with [^{35}S]methionine-labeled Sendai virus as marker (lane G). The various protein bands are denoted at the margins.

of viral clones sufficient to determine the genetic map of the 3′-proximal third of the Sendai virus genome. The viral clones were ordered by first identifying a clone (pSL3) that hybridized to a probe of the 3′ end of the genomic RNA, and the identity of this clone was verified by matching the DNA sequence of its insert with the RNA sequence of the 3′ end of the viral genome. Other large clones were then ordered relative to pSL3 by restriction enzyme analysis of their inserts and by dot hybridization, leading to a restriction map of the 3′-proximal third (5 kb) of the viral (−) genome. Translation of viral mRNAs selected by hybridization to the ordered DNA clones then led to the partial genetic map 3′-NP–P+C–M-5′. Assuming by analogy to vesicular stomatitis virus (VSV) that the gene for the Sendai virus L protein is the most 3′ distal, a more complete genetic map can be drawn as 3′-NP–P+C–M–F+HN–L-5′.

This map differs somewhat from the genetic map previously suggested from the UV inactivation kinetics of the putative mRNAs for the viral proteins (3′-NP–F–M–P–HN–L-5′) (25). However, the genetic map derived from the UV inactivation studies depends on the assumption that the viral transcripts result from a RNA polymerase that can enter the genome template only near its 3′ end, such that the UV target size of each gene is the sum of that gene plus all other 3′-proximal genes. This approach has been used successfully to order the genes of VSV (26), another nonsegmented negative-strand virus, but a member of the rhabdovirus family. The genetic map from our work using ordered DNA clones does not depend on assumptions concerning the mechanism of action of the viral transcriptase. Considering the differences obtained by using these alternative approaches to determining the genetic map of Sendai virus, and the relatively high molar rate of synthesis of the Sendai viral F protein (19, 27), the single-entry promoter, a hallmark of VSV transcription, may not apply to Sendai virus. In the case of Newcastle disease virus, another parainfluenza virus, Collins *et al.* (28) determined the gene order to be 3′-NP–P–(F+M)–HN–L-5′. (Thus Sendai virus and Newcastle disease virus appear to have the same gene order, which is also similar to that of VSV.)

The genetic location of the nonstructural C protein is perhaps the most unexpected finding of this work. Little is known about the role of this nonstructural protein during virus replication. The C protein is known to exist in two electrophoretic forms (C and C′) with similar peptide patterns, which differ from the peptide patterns of all the other known virus-encoded proteins of Sendai virus (6, 7). The C protein appears to be stable in pulse–chase experiments *in vivo* (5), and its mRNA can also be synthesized efficiently by detergent-activated virions *in vitro* (8). The C protein (22 kDa) mRNA, however, could not be separated from the P protein (79 kDa) mRNA by sedimentation velocity in sucrose gradients under conditions in which the P protein, NP protein (60 kDa), and M protein (34 kDa) mRNAs sedimented as expected from their relative molecular weights (8). Additional evidence that the P and C gene cannot be separated was obtained by nick-translating pSN5 and using it to probe a blot of $CsCl_2$ pellet RNA from Sendai virus-infected Vero cells that had been electrophoresed under fully denaturing conditions in a 1.5% agarose/CH_3HgOH gel. A very strong signal was seen against a single RNA species that bands just under 18S RNA. When the intercistronic clone pSL2 was used, it hybridized to the same 18S band and hybridized even more strongly to a second lower molecular weight RNA species that represents the M message. The results of the experiments reported here offer a clue to this anomaly. The genetic location of the C protein similarly cannot be separated from that of the P protein by hybrid-selection of mRNAs under conditions in which the loci of the NP and M protein genes can be clearly resolved. The results of our experiments suggest that the P and C proteins are translated from the same mRNA, either as a tandem nonoverlapping dicistronic RNA or as a mRNA with overlapping reading frames. This problem can be examined by determining the exact start of the M protein mRNA on the restriction map in order to determine whether the P and C transcriptional unit is large enough to accommodate nonoverlapping genes.

Should the suggestion that P and C proteins are coded for by the same transcriptional unit be borne out by such experiments, this will pose yet an additional problem for the interpretation of UV inactivation kinetics for parainfluenza virus genomes.

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