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Molecular analysis of the Sendai virus genome

Park, Kyeong Hoon, Ph.D.

City University of New York, 1992

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MOLECULAR ANALYSIS OF THE SENDAI VIRUS GENOME

by

Kyeong H. Park

A dissertation submitted to the Graduate Faculty in
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ABSTRACT

MOLECULAR ANALYSIS OF THE SENDAI VIRUS GENOME

by

Kyeong H. Park

Advisor: Dr. Mark Krystal

In contrast to DNA viruses, retroviruses and positive-strand RNA viruses, whose genomes are infectious and thus amenable to conventional molecular approaches, negative-strand RNA viruses such as the orthomyxoviruses and the paramyxoviruses do not allow a simple dissection and mutational analysis of their genomes. In addition to the negative polarity, the large genome size and the nonsegmented nature of paramyxoviruses (~15 kilobases) make it very difficult to directly manipulate or modify them for studies.

In the first part of this thesis, an artificial mini-genome of Sendai virus, a prototype paramyxovirus, was developed as an initial step to get around the difficult problems imposed by the intrinsic nature of the viral genome. The mini-genome RNA, called Send-CAT, was synthesized via *in vitro* T7 phage RNA polymerase transcription from a cDNA containing the antisense coding region of chloramphenicol acetyltransferase (CAT) gene flanked by 5' 145 and 3' 119 terminal sequences of the viral genome. When introduced into cells and followed by Sendai virus infection, the RNA was transcribed and translated, as detected by CAT activity. The efficiency of CAT expression was markedly increased by pre-incubating the RNA with cytoplasmic cell extract derived from either Sendai virus-infected or uninfected cells, suggesting that a host cell component(s) may enhance viral gene expression. Furthermore, the Send-CAT RNA was also readily replicated and packaged into progeny virions. Once packaged, the Send-CAT RNA could be stably passaged and even be grown to a very high titer. These studies showed that the existing length of 5' and 3' viral sequences were necessary and sufficient for transcription, replication, and packaging by Sendai virus. More importantly, it now provided a well-suited system for examining various *cis*-acting signals of the viral genome.

In the second part of the thesis, an ***in vivo*** model for pseudo-templated transcription of Sendai virus was developed. During transcription of the *P/C* gene, the Sendai virus transcriptase inserts one or more nontemplated guanine nucleotides at position 1053. Based on the Send-CAT mini-genome, a derivative construct was made that contained a stretch of nucleotide sequence spanning the editing site of the *P/C* gene. When the resulting construct, called PC-CAT, was transfected into cells which were then infected with Sendai virus, 6.5% of its mRNAs contained a nontemplated G nucleotide at the expected position, indicating that the sequence normally responsible for promoting RNA editing in Sendai virus genome was now functionally residing in the PC-CAT construct. By progressively deleting the *P/C* gene sequence from the 5' end (in the genome sense), 24 bases were shown to be sufficient for the pseudo-templated transcription in the model system. In addition, mutational analysis of the consensus sequence showed that the three C residues preceding the insertion site were essential and could not be substituted by G residues. This system will now permit precise delineation of the *cis*-acting element(s) required for various aspects of RNA editing.

ACKNOWLEDGEMENTS

I would like to thank Dr. Mark Krystal for his guidance, teachings, and friendship. Thanks for lifting me to the cutting edge of molecular biology. Thank you Jackie for making my life more bearable and for all that you've done over the years. This thesis is dedicated to my parents who have been, and will be, the source of my inspiration and dedication.

FORMAT OF THESIS

This thesis is prepared according to the new guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has a general introduction, and chapters have specific introductory statements. Materials & Methods and Results sections are in each individual chapter. Also, each chapter has a specific Discussion section, and there is a general discussion as the final chapter of the thesis. The references for all chapters are pooled, in order to avoid redundancy.

Chapter II is not copy protected by the publisher. Chapter III has been submitted for publication, and the copyright release consent will be obtained from the publisher.

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PUBLICATIONS

Park, K.H., Huang, T., Correia, F. F., and Krystal, M. (1991). Rescue of a foreign gene by Sendai virus. Proc. Natl. Acad. Sci. U.S.A. ***88***: 5537-5541.

Park, K.H. and Krystal, M. (1992). An *In vivo* model for pseudo-templated transcription in Sendai virus. Submitted to J. Virol.

Levin, H.L., **Park, K.**, and Schachman, H.K. (1989). Attenuation in the regulation of the *pyr*BI operon in *Echerichia coli*. J. Biol. Chem. ***264***: 14638-14646.

TABLES

FIGURES

I. INTRODUCTION

A. The family *Paramyxoviridae* is comprised of three genera, *Morbillivirus*, *Paramyxovirus* and *Pneumovirus*, that include a number of animal pathogens as well as many important human pathogens. For example, measles virus, mumps virus, respiratory syncytial virus, and various types of parainfluenza viruses all belong to this family. Although some members of the family are more closely related than others, they are all enveloped viruses with a nonsegmented, negative-strand RNA genome that exhibits substantial homology in their nucleotide sequence, genetic organization, and morphology (Kingsbury *et al., 1978*; Matthews, 1982; Pringle, 1987).

Although various biochemical analyses have elucidated the functions of many of the proteins encoded by paramyxoviruses, detailed studies have been hampered by an inability to apply the conventional genetic approaches that were applicable to the studies of other viruses possessing DNA or positive-strand RNA genomes. Unfortunately, it is still not possible to introduce a site-specific mutation into the paramyxovirus genome and rescue the resulting mutant in the absence of a selectable marker. Thus far, the molecular analyses of paramyxoviruses have been limited to the naturally-occurring mutants, monoclonal antibody escape mutants, or mutants isolated serendipitously in laboratories. The needed progress awaits an experimental amenity that would overcome the technical limitation imposed by the large size and negative polarity of the paramyxovirus genome. An ideal model system would allow convenient manipulations of the viral genome *in vitro* and efficient rescue into infectious virions. With that in mind, this thesis focuses on the development of a system which allows modification and mutagenesis of an artificial genome that is biologically active and closely mirrors the true viral genome in terms of its function and behavior. The significance of this approach is highlighted by the fact that various *cis*-acting sequences can now be identified and studied by using traditional recombinant DNA technology.

B. PARAMYXOVIRUS STRUCTURE

1. VIRION MORPHOLOGY

The paramyxovirus virions appear generally spherical under the electron microscope with sizes ranging from 150~300 nm in diameter (Hosaka *et al.*, 1966). The outer surface is made up of a lipid membrane containing viral glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) proteins, which are seen by electron microscopy as "spikes" evenly distributed 8~10 nm apart. The matrix (M) protein is thought to underlie the membrane, surrounding the nucleocapsid core that consists of the RNA genome, nucleoprotein (NP), large protein (L), and phosphoprotein (P).

2. GENOME STRUCTURE

The nonsegmented, single-stranded RNA genome is tightly encapsidated by ~2600 NP molecules into a nuclease-resistant helical ribonucleoprotein (RNP) complex associated with ~250-300 P and ~20-30 L protein molecules (Galinski and Wechsler, 1991). The L and P proteins are thought to comprise the RNA-dependent RNA polymerase complex, responsible for both transcription and replication of the genome (Lamb *et al.*, 1976; Matthews, 1982). By immunogold labeling studies, the P and L proteins appear co-localized in clusters on nucleocapsids during active transcription and replication in infected cells (Portner and Murti, 1986; Portner *et al.*, 1988). Figure 1 shows the genome organization for Sendai virus, which follows the general pattern found in other paramyxoviruses. The exceptions to this scheme are the mumps virus and SV5, where there is a unique additional small gene, termed small hydrophobic or "SH", placed between the *F* and *HN* cistrons (Elango *et al.*, 1989; Hiebert *et al.*, 1985).

During transcription, the viral polymerase transcribes the leader gene (*ℓ*) and six transcription units (starting from the 3' end, *NP, P/C, M, F, HN*, and *L*). It is thought that the polymerase recognizes a single entry site at or near the 3' end and transcribes towards the 5' end, terminating and re-starting transcription at each of the gene junctions. There is a conserved consensus sequence for the stop-start signals within the genome as well as among paramyxoviruses. The transcription termination and polyadenylylation signal (3'-UNAUUCUUUUU-5', denoted as "E" in Fig. 1) is followed by a non-transcribed intergenic trinucleotide sequence (3'-GAA-5', denoted as "I"), which precedes the start signal for the downstream gene (3'-UCCCANUUUC-5', denoted as "S"). The junction sequence between the leader gene (*ℓ*+) and *NP* lacks a polyadenylylation signal, and thus the leader transcript does not normally have a poly (A) tail. The signal for termination of the leader transcript appears unique for most paramyxoviruses (Gupta and Kingsbury, 1984). Following the *L* gene, only the "E" sequence is present, and the "S" sequence is missing. Not all viral polymerases that enters at the 3' end is thought to continue and terminate at the 5' end of the genome, thus generating a transcription polarity gradient. Therefore, the relative abundance of the mRNAs generally follows in decreasing order 3'-*NP*>*P/C*>*M*>*F*>*HN*>*L*-5'.

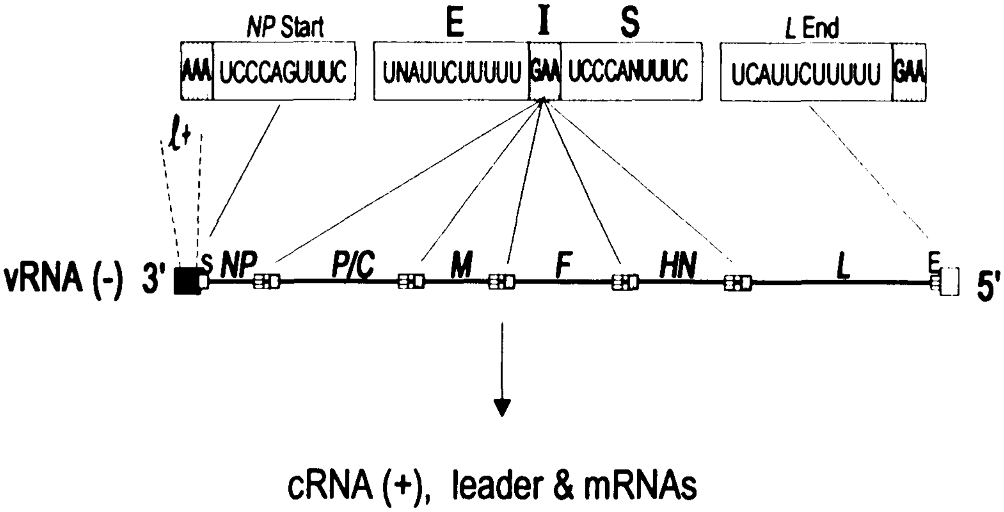


Figure 1. SCHEMATIC REPRESENTATION OF THE SENDAI VIRUS GENOME ORGANIZATION. The positive-strand leader RNA, (ℓ+), and the sequences at the gene boundaries are indicated above the map. Adapted and modified from Kingsbury (1986).

C. STRUCTURAL PROTEINS

1. HN AND F PROTEINS

The initial attachment and entry into a susceptible host cell is mediated by two viral surface glycoproteins, HN and F. The HN surface glycoprotein of paramyxoviruses is a bifunctional molecule with two apparently opposing activities: binding to the sialic acid-containing receptors and the enzymatic cleavage of those receptors. The role of hemagglutination is very clear-- bringing the viral membrane in close proximity to the host cell membrane so that the F protein can mediate the fusion of the two membranes. The neuraminidase activity, although its role is less clear, is probably involved in later stages of infection, promoting progeny virus release from the cell membrane and preventing self-aggregation of the viral particles (Scheid and Choppin, 1974).

The HN proteins are 565-582 amino acids long and classified as type II integral membrane proteins having the N-terminal cytoplasmic tail (variable lengths among paramyxoviruses) and C-terminal ectodomain-- $N_{in}C_{out}$ topology (Blobel, 1980). Interestingly, the neuraminidase (NA) protein of influenza virus also has a type II topology, but the hemagglutinin (HA) has a type I topology ($N_{out}C_{in}$) (Fields *et al.*, 1981; Blok *et al.*, 1982). At the infected cell surface, the HN protein is expressed as a tetramer consisting of a pair of disulfide-linked dimers, which is the active wildtype oligomer form. In many mutant molecules, this structure may become lost and are found as inactive dimers (Parks and Lamb, 1990).

Recent studies on the HN protein have provided evidence for a third likely role served by the molecule: a function involved in promoting membrane fusion. For example, with some HN variants of mumps and parainfluenza type 3 viruses, the formation of syncytium was increased (Shibuta *et al.*, 1983; Merz and Wolinsky, 1983). Also, monoclonal antibodies against Sendai or mumps virus HN can block virus-induced fusion without affecting hemagglutination or the neuraminidase activity (Miura, *et al.*, 1982; Portner *et al.*, 1987; Tsurudome *et al.*, 1986). More compelling is that reconstituted lipid membranes containing Sendai virus F protein (with inactivated HN) are unable to undergo membrane fusion, while those with both F and HN retain the fusogenic activity (Ozawa *et al.*, 1979; Citovsky *et al.*, 1986). Recent characterization of cells persistently infected by human parainfluenza virus type 3 indicates that neuraminic acid must be present on the cell surface for syncytium formation, again indicating the need for HN molecules in fusion process (Moscona and Peluso, 1991). One exception to this general requirement of HN protein for fusion may be simian virus 5 (SV 5), in which the F protein alone seems to be sufficient to induce cell fusion (Paterson *et al.*, 1985).

Unlike the orthomyxovirus counterpart whose hemagglutinin carries the "fusion peptide" embedded in hydrophobic amino terminus of HA2, the F glycoprotein of paramyxovirus is a separate entity, over 500 amino acids long. The F protein requires an activation step by a proteolytic cleavage. The precursor form, F_0 is processed into a large F_1 and smaller F_2 subunits. F_1 has a hydrophobic amino terminus, which is thought to be the fusogenically active center that penetrates into cell membrane. The other subunit, F_2, is smaller than F_1 with no obvious function and remains attached to F_1 by a disulfide linkage (Richardson *et al.*, 1980). The virus-cell membrane fusion occurs directly at the surface and does not involve an endosome stage as is the case for the influenza viruses. Appropriately, the fusion proceeds readily at neutral pH (Harris and Watkins, 1965).

2. M PROTEIN

The matrix protein, about 350 amino acids long, is thought to mediate the interaction between viral membrane proteins and nucleocapsids during virion assembly. *In vitro*, the association between purified Sendai virus nucleocapsids and purified glycoproteins takes place only if the M protein is present in the mixture (Yoshida *et al.*, 1976). The M protein is both basic and hydrophobic, and the basic region may interact with the acidic nucleocapsids whereas the hydrophobic region may associate with the membrane component (s) (Galinski and Wechsler, 1991). Immunogold labeling studies have shown that the M protein is distributed in large quantities along the entire length of the nucleocapsids in cytosol-purified RNPs, while in virion-purified RNPs, fewer M were observed (Portner and Murti, 1986). This suggests that the M protein may regulate transcription.

Other yet undefined functions of the M protein may also exist. A number of studies suggest that the M proteins may be involved in modulating virus-host cell interactions. First, the M protein of certain paramyxoviruses such as NDV and measles virus has been shown to associate with cell cytoskeleton (Morrison and McGinnes, 1985; Bohn *et al.*, 1986). The actin filaments, which are found in the paramyxovirus virions (Wang *et al.*, 1976), might associate physically with the M proteins (Giuffre *et al.*, 1982). Second, staining of Newcastle disease virus (NDV) infected cells with monoclonal antibodies specific for the M protein has shown that the protein may migrate to nucleoli at early times during infection (Faaberg and Peeples, 1988; Peeples, 1991), indicating that it may regulate host-cell gene expression early after infection. Finally, hypermutations affecting the *M* gene have been implicated in the establishment and maintenance of subacute sclerosing panencephalitis (SSPE) by measles virus (Cattaneo *et al.*, 1988), as discussed in more detail later.

3. THE CORE PROTEINS, NP, P, AND L

The NP protein, about 500 amino acids long, is the primary structural building block of nucleopcapsid cores, present at approximately 2600 molecules per viral genome (Lamb *et al.*, 1976). The manner by which NP encapsidates the genome distinguishes paramyxovirus RNPs from that of influenza viruses--the paramyxovirus genome has a very tight structural integrity that is resistant to digestion by nucleases. The genomic sequences required for encapsidation are likely to reside at the 3' terminus, since mRNAs lacking these sequences are not encapsidated but the antigenomic complementary RNA (cRNA) and defective-interfering (DI) RNA which possess them are efficiently encapsidated (Amesse *et al.*, 1982; Re *et al.*, 1984; Re and Kingsbury, 1986). In chapter II, we extend this observation by showing that the vRNAs and cRNAs of an artificial mini-genome possessing the 3' and 5' terminal sequences become encapsidated during replication *in vivo*. It should be noted that there are no available approaches to encapsidating paramyxovirus genomes and DI genomes *in vitro*, although the DI RNAs of vesicular stomatitis virus (VSV) has been successfully encapsidated through incubation with infected cell extract (Mirakhur and Peluso, 1988). Encapsidation is also thought to occur when the influenza virus segments or artificial genomes are mixed with purified virus polymerase preparations (Enami and Palese, 1991).

Aside from constituting the structural basis of the viral genome, the NP protein is likely involved in regulating transcription and replication. For example, the switch from transcription to replication by the viral polymerase complex is thought to be regulated by the availability of free intracellular NP proteins. For Sendai virus, the free NP appears to modulate the chain elongation process (Vidal and Kolakofsky, 1989). Furthermore, since the NP protein also binds to P (Ryan and Kingsbury, 1988), it probably regulates the interaction between the polymerase complex (P and L) and the template.

Post-translational phosphorylation occurs in all Sendai virus protein species except the L protein (Hsu and Kingsbury, 1982). The P (phospho) protein, as the name implies, is the most heavily phosphorylated viral protein, representing ~40% of virion protein-bound phosphates. Most of the phosphates reside in the amino terminus-- an estimated 80% of the total P protein phosphates (Lamb and Choppin, 1977; Hsu and Kingsbury, 1982; Vidal *et al.*, 1988). The P protein is also the most divergent protein among paramyxoviruses, with its molecular size ranging from 391 to 603 amino acids and very little sequence conservation (Galinski and Wechsler, 1991). The P protein is expressed from unedited (Sendai and measles virus) and edited (SV5 and mumps virus) messages of the *P/C* gene that are generated by insertion of nontemplated G nucleotides during transcription (see Fig. 2). The proposed mechanism for RNA editing and one consequence of such gene expression are discussed in the following section. Although the exact function of the P protein is not well understood, it is clearly required for the RNA synthesis *in vitro* (Hamaguchi *et al.*, 1983,). Monoclonal antibodies directed against the P protein carboxy terminus inhibit viral transcription (Deshpande and Portner, 1985; Ryan and Kingsbury, 1988; Vidal *et al.*, 1988). The functional significance of N-terminal phosphorylation is unclear however, since the P proteins treated with *S. aureus* V8 protease, which removes the amino terminal portion and therefore most of the phosphorylations, can synthesize and modify mRNAs *in vitro* (Chinchar and Portner, 1981). In contrast, the level of phosphorylation of analogous NS protein of vesicular stomatitis virus has been shown to dictate the rate of viral transcription (Kingsbury *et al.*, 1981).

Most of the monoclonal antibodies raised against the P proteins recognize determinants within the carboxy terminus (Vidal *et al.*, 1988; Ryan *et al.*, 1991). The C-terminal residues are highly hydrophilic (Shioda *et al.*, 1983; Giorgi *et al.*, 1983), and it is thought that this region of the P protein forms the outer surface of the folded protein. The functional importance as well as the preferential antibody recognition of this domain can be explained on this basis. The amino acids responsible for nucleocapsid binding are at the C-terminus and separated into two domains that appear to form a single binding site and a single epitope in the folded protein (Ryan and Portner, 1990). Internally deleted P proteins synthesized *in vitro* that only contain the two binding domains can still bind to nucleocapsids (Ryan *et al.*, 1991).

Recently, Curran and Kolakofsky (1988) have detected a 10-kDa protein, termed "X" in Sendai virus-infected cells (See Fig. 2). This small protein represents C-terminal ~ 95 amino acids of the P protein (568 a.a.) that appears to be synthesized by internal ribosomal initiation at 1523. The X protein is not present abundantly in infected cells but nevertheless detected *in vivo.* It is probably not derived from a cleavage of P protein precursor. Four out of five monoclonals raised against the P protein also react with the X protein, suggesting a conformational similarity between the P and the X proteins. In light of the functional importance of the P protein C-terminus, it will be interesting to see what regulatory role the X protein serves during viral transcription and replication.

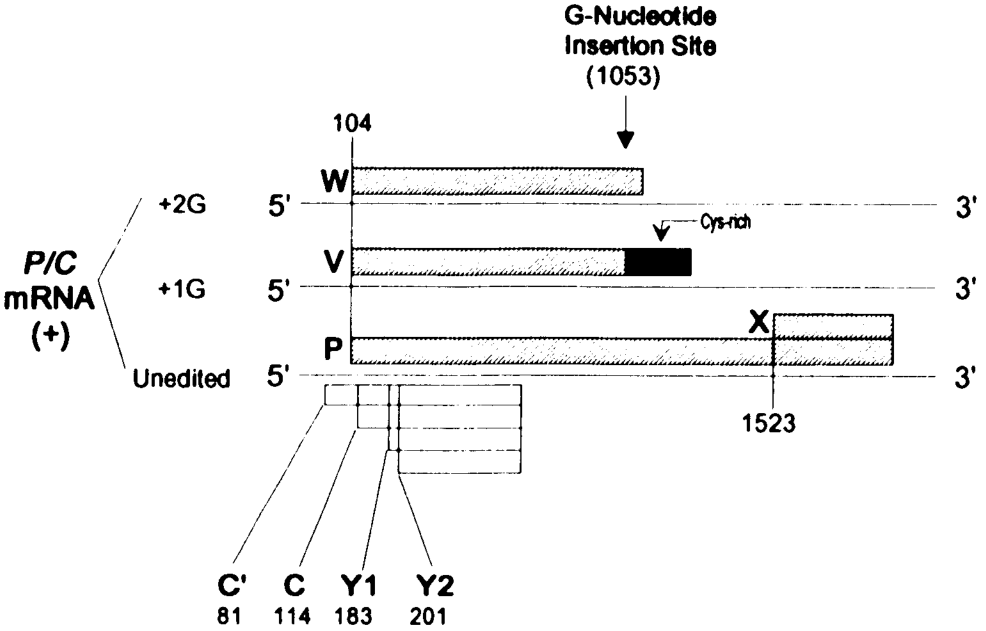


Figure 2. PROTEIN CODING REGIONS OF THE *P/C* GENE mRNAS. Three of the possible mRNAs transcribed are shown as "Unedited", "+1G", and "+2G". The C proteins (C', C, Y1, and Y2) are indicated by open boxes, the proteins sharing the P ORF (P, V, W, and X) are indicated by hatched boxes, and the unique cysteine-rich domain of the V protein is indicated by a cross-hatched box. Adapted and modified from Curran *et al.* (1991).

The L (large) protein is about 2200 amino acids long and has a number of enzymatic activities. Together with the P protein, it is the RNA-dependent RNA polymerase which carries out the synthesis of all viral RNAs including vRNA, cRNA, and mRNAs (Portner and Murti, 1986), and probably responsible for various modifications such as capping and methylation (Galinski and Wechsler, 1991; Chinchar and Portner, 1981). In studies of closely related rhabdovirus VSV, it was shown that a mutant phenotype that abnormally synthesizes long poly(A) tails was associated with the L protein (Hunt *et al.*, 1984). By inference, it is likely that the L protein of the paramyxoviruses is also responsible for polyadenylylation of mRNAs. The L protein is found in infected cells least abundantly, and only about 30 molecules of the L protein are associated with each nucleocapsid, compared with approximately 300 molecules of the P protein (Lamb *et al.*, 1976).

D. TRANSCRIPTION

The paramyxovirus transcription is thought to initiate from a single transcriptase entry site near the 3' end of the viral genome. The polymerase terminates and reinitiates at each gene junction--

the "stop-start" model (Galinski, 1991). The (+)-leader RNA is barely or not at all detectable *in vivo* for Sendai virus (Vidal and Kolakofsky, 1989) or measles virus (Crowley *et al.*, 1988; Billeter *et al.*, 1984; Chan *et al.*, 1989), respectively. In contrast, the (+)-leader RNA is readily detected in similar studies done for VSV (Leppert *et al.*, 1979; Leppert and Kolakofsky, 1980). Based on these findings, it has been suggested that although the start site for replication is at the 3' end of the viral genome, the promoter for transcription may reside more internally (Blumberg *et al.*, 1991). Once initiated, the transcriptase complex proceeds to the transcription termination and polyadenylylation site ("E" in Fig. 1) (Pridgen and Kingsbury, 1972; Gupta and Kingsbury, 1982) and reinitiates at the next start signal ("S" in Fig. 1), with proper capping and methylation (Colono and Stone, 1975; Yoshikawa *et al.*, 1986). The reinitiation event at the junction is always less than 100% efficient, resulting in a transcription gradient with the *NP* gene (3' end) being expressed at the highest levels and the *L* gene (5' end) the lowest levels (Glazier *et al.*, 1977). Similar to VSV, which contains a nontranscribed dinucleotide intergenic sequence (3'-GA-5'), the paramyxovirus transcriptase complex crosses over a trinucleotide sequence before reinitiation ("I" in Fig. 1). Alterations in this spacer region found in some Sendai and human parainfluenza virus type 3 (HPIV-3) variants are associated with aberrant read-through transcription (Gupta and Kingsbury, 1985; Galinski *et al.*, 1987).

Paramyxovirus transcription and replication, like that of other DNA and RNA viruses, may require specific host-cell factor(s). A number of reports suggest a specific virus-cell protein interaction. For example, the presence of host cell extracts containing cytoskeletal proteins such as tubulin and actin, has been shown to specifically stimulate virus transcription and replication both *in vitro* and *in vivo* (Hamaguchi *et al.*, 1985; Yoshida *et al.*, 1986; Chan *et al.*, 1989; Moyer *et al.*, 1986, 1990; De *et al.*, 1990, 1991). In this respect, actin has been found in the virions of paramyxoviruses as a part of the structural proteins (Wang *et al.*, 1976). It should be stressed that not all paramyxovirus transcriptions are stimulated by the same host factors. For Sendai virus (and VSV), *in vitro* transcription using detergent-disrupted purified virions was entirely dependent on the addition of purified tubulin and completely inhibited by monoclonal antibodies against β-tubulin (Moyer *et al.*, 1986). On the other hand, 43-kDa purified actin, but not tubulin, had a considerable stimulatory effect on HPIV-3 *in vitro* RNA synthesis (De *et al.*, 1991). In the latter study, it was noted that the source of the purified actin was not important, since a number of cell lines including CV-1, HeLa, and BHK could be used successfully. It was suggested that perhaps a general type of interaction takes place such as targeting or transport. For VSV, the L protein appears to directly associate with tubulin, since anti-tubulin antibody can co-precipitate in infected cells (Moyer *et al.*, 1986). For HPIV-3, De *et al.* (1991) suggested that actin monomers bind to HPIV-3 nucleocapsids and then polymerize, the process of which facilitates viral transcription. In Chapter II, we have shown that cytoplasmic extract made from either infected or uninfected cells can similarly stimulate the expression and rescue of an artificial genome of Sendai virus. We have suggested that a host cell factor probably assists the model RNA in proper cytoplasmic targeting after transfection.

1. *P/C* GENE EXPRESSION

In recent years, a number of mechanisms have been identified as controlling the processing and modification of eukaryotic mRNA transcripts. The first and most common process is splicing, which generates an intramolecular rearrangement of nuclear RNA at a post-transcriptional level, removing intron sequences and joining exons (Padgett *et al.*, 1986). For viral transcription, splicing appears to be limited to those having a nuclear phase, such as the retroviruses, DNA viruses (SV40, adenoviruses, etc.) and influenza viruses (Lamb *et al.*, 1981). Also recognized is the *trans* splicing phenomenon, which involves an intermolecular rearrangement between two separate messages (Wissinger *et al.*, 1991). A less frequent and recently discovered phenomenon found to occur in various cells is termed RNA editing. Here, specific changes are introduced into a target RNA sequence, such that the mature RNA contains information that is not directed by the template. RNA editing can involve an addition or deletion of nontemplated nucleotides, or specific sequence changes. The most well-characterized trypanosome mitochondrial RNA editing, for example, inserts or deletes a stretch of uridine residues in the transcribed message (Beene *et al.*, 1986; Shaw *et al.*, 1988) by transesterification reactions with guide RNA (gRNA) (Blum *et al.*, 1991; Cech, 1991). In other systems, editing is not as extensive but some base specificity and even tissue specificity are noted. In plant mitochondria (wheat, maze, pea, carrots, and evening primrose), specific C to U and U to C transitions have been found (Covello and Gray, 1989, 1990; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989; Wissinger *et al.*, 1990; Schuster *et al.*, 1990). In human and rabbit small intestines, the tissue-specific expression of a second form of apolipoprotein (apo-B48) requires a precise single C to U change that introduces a translational stop codon (Powell, *et al.*, 1987). A more recent observation has extended the occurrence of RNA editing in the brain where transcripts for the glutamate receptor channels are selectively modified by a single A to G transition (Sommer *et al.*, 1991). In all these studies documented thus far, the observed changes are considered "true" editing in the sense that the event occurs post-transcriptionally. In addition, RNA editing can occur co-transcriptionally, which would be more correctly described as pseudo-templated transcription as discussed below.

The *P/C* gene of Sendai virus is usually transcribed into two distinct transcripts that differ by one inserted G nucleotide, and these two mature messages are translated into at least seven recognized proteins, P, C, C', Y1, Y2, X, and V (Fig. 2). Additionally, a third minor species of *P/C* mRNA containing two G-nucleotide insertions has been detected. This mRNA would encode an eighth protein, W, as discussed later. The C, C', Y1, and Y2 proteins, which together are called the C proteins, represent an in-frame set of polypeptides with staggered N termini (Fig. 2). The C proteins result from alternate ribosomal initiations at multiple start sites (Curran and Kolakofsky, 1988a, 1988b, 1989). The C' protein is the largest of the C proteins and is unusual in that its translation initiates with a non-methionine residue, such as threonine (ACG, Sendai virus) or valine (GUG, parainfluenza virus type 1) (Curran and Kolakofsky, 1988a; Gupta and Patwardhan, 1988; Boeck *et al.*, 1992). The C proteins are all in -1 reading frame relative to the P ORF, which is translated from the first AUG codon at position 104 (Sendai virus). The C proteins are not commonly expressed in all paramyxoviruses, and for some (SV5, mumps virus, and Newcastle disease virus) the C protein reading frame appears to be absent entirely (Sato *et al.*, 1987; Takeuchi, *et al.*, 1988; Thomas *et al.*, 1988).

In addition to P and C proteins, the *P/C* gene expresses the cysteine-rich V protein, which shares a common amino terminus with the P protein but differs at the C terminus. The presence of V protein in infected cells was originally detected in SV5 (Peluso *et al.*, 1977). For SV5, the V open reading frame (ORF) at the amino terminus is accessible in unedited mRNA. On the other hand, no ORFs were large enough to encode the P protein (Thomas *et al.*, 1988). A second species of mRNA, which differs by two nontemplated G residues inserted between nucleotides 548 and 551, allows access to a fused ORF in the -2 reading frame and gives rise to a 44-kDa P protein (Thomas *et al.*, 1988). When a total of 22 cDNA clones were isolated from SV5-infected cells and sequenced, 12 clones were faithful copies of the genome (no insertions), and 10 clones contained nontemplated 2 G nucleotide insertions precisely at the same position. None of the clones examined had one or more than two G nucleotides inserted. The P protein expressed from edited mRNA lacks the cyteine-rich C-terminal domain of the V protein that resembles the zinc metal-binding region found in the steroid hormone receptor superfamily (Evans, 1988), the eukaryotic initiation factor eIF2 (Donahue *et al.*, 1988), and other transcription regulatory proteins (Thomas *et al.*, 1988). The P and V proteins are identical in their first 164 amino acid residues with a large number of proline residues (17 of 164, Thomas *et al.*, 1988). The V protein appears to be present in virions complexed with L, NP, and P in some paramyxoviruses (Randall *et al.*, 1987; Thomas *et al.*, 1988). The cysteine-rich region of the V protein is highly conserved, which led to the prediction that other paramyxoviruses-- measles virus, canine distemper virus, Sendai virus, human parainfluenza virus type 3, Newcastle disease virus, and mumps virus-- would also express the V protein (Thomas, *et al.*, 1988; Cattaneo, *et al.*, 1989). This implied that co-expression of the P and V proteins in these viruses would require RNA editing as well.

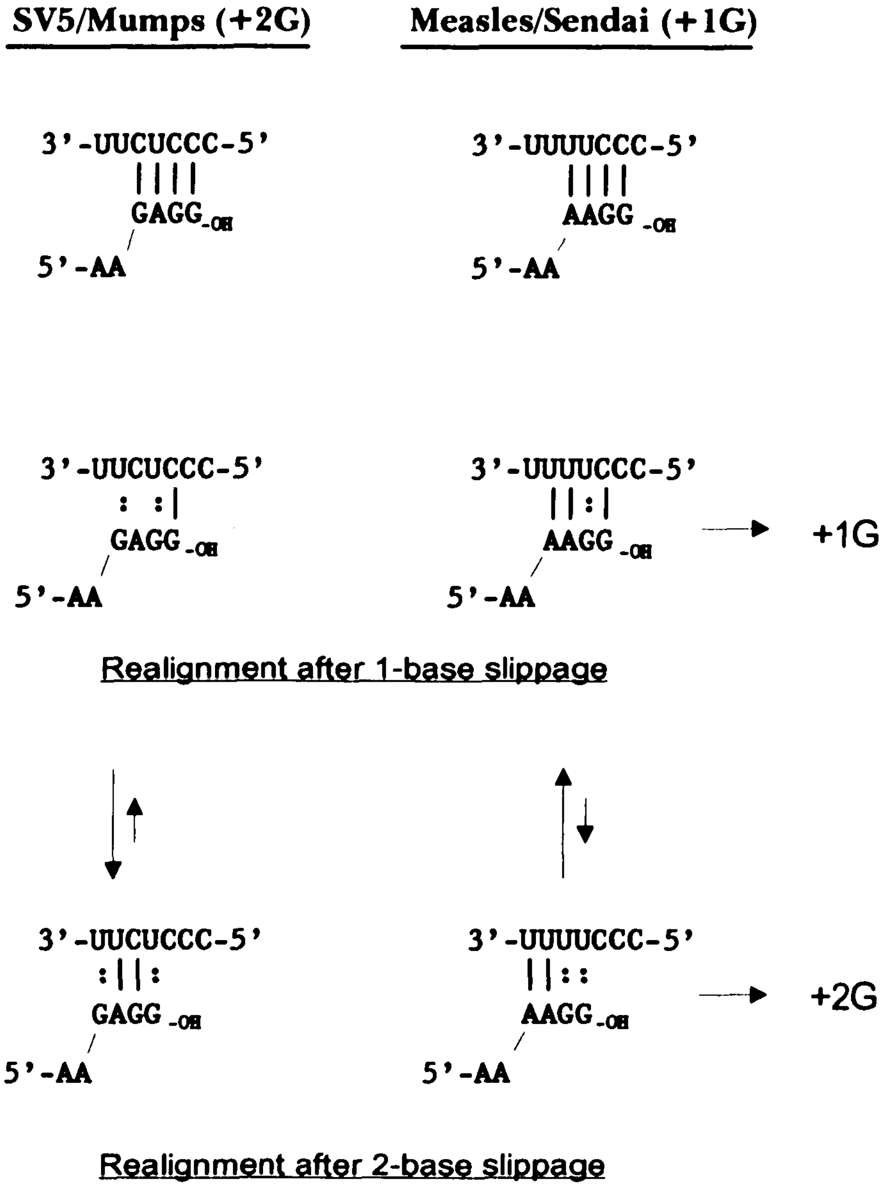
In contrast to SV5, the Sendai and measles virus P protein is expressed from an unedited mRNA-- *i.e.*, the exact complement of the genomic sequence encodes the P protein. The predicted V protein expression comes from a second, edited mRNA that contains a single nontemplated G nucleotide insertion (Cattaneo *et al.*, 1989; Vidal *et al.*, 1990). In measles virus, the inserted G nucleotide causes a substitution of the last 277 amino acids of the P protein with a 68-amino acid sequence that contains seven cysteines (Cattaneo *et al.*, 1989). Of the nineteen measles virus *P* gene cDNA clones examined by sequencing, seven clones had no insertion, and ten clones contained an extra G nucleotide at the predicted position. Interestingly, in addition to single G-nucleotide insertions, two clones contained three extra G nucleotides that were not encoded by the viral genome. The three G-nucleotide insertion resulted in an in-frame addition of a glycine residue to the P protein. It should be noted that the samples examined included not only those from the prototype measles virus (Edmonston strain) infection, but also from two subacute sclerosing panencephalitis (SSPE) cell lines and the brains of three patients who died of SSPE (Cattaneo *et al.*, 1989). Of the four *P* clones from the Edmonston infection sample, one had no insertions, the other three had one G nucleotide inserted, and no clones had more than one extra G nucleotides. Whether the three G-nucleotide insertions were unique to SSPE and not normally seen during a productive infection by the wildtype measles virus remains unclear. Although the number of clones examined is far too few, it was predicted that the P:V ratio (both at the mRNA and protein level) would be about 1:1 (Cattaneo *et al.*, 1989).

Despite the earlier conflicting molecular cloning and sequence studies of the mumps virus *P* gene, which had suggested that the genomic sequence encoded the P protein (Takeuchi *et al.*, 1988; Cattaneo *et al.*, 1989), Paterson and Lamb (1990) showed that the mumps virus *P* gene expression is similar to SV5. Namely, the unedited *P* gene message of mumps virus encodes the V protein, and the switch to the P ORF requires an insertion of two G residues. In a sequence study of 54 *P* gene cDNA clones, 34 were faithful copies of the genome, 10, 7, 1, and 2 clones had 2, 3, 4, and 5 G-nucleotide insertions, respectively (Paterson and Lamb, 1990). Clearly, the viral insertion of nontemplated nucleotides is less precise than predicted from the SV5 findings, which showed only two G-nucleotide insertions. The ratio of P to V cDNA clones approximately correlated with the relative amounts of the two proteins present in mumps virus infected cells, indicating that the number of edited clones detected correctly represented the true frequency of editing in mumps virus (Paterson and Lamb, 1990). Uniquely, the mRNA containing four nontemplated G nucleotides is translated into an additional protein that shares the amino-terminal 155 amino acids in common with the P and V proteins, and this novel protein, called "I", was detected in infected cells.

Although the presence of V protein in Sendai virus infected cells was not clear, sequence studies showed that the CYS-rich ORF in the *P/C* gene could be accessed in the +1 reading frame relative to the P ORF. Upon screening cDNA clones from virus infected cells, Vidal *et al.* (1990a) found that about 31% and 7.5% of the mRNA contained one and two G-nucleotide insertions, respectively, precisely between nucleotides 1051 and 1053. In addition, editing was shown to take place during *in vitro* transcription reactions using purified virion nucleocapsids. The finding that the editing occurs *in vitro* strongly indicated that viral structural proteins are responsible for generating edited mRNAs. Furthermore, there were no evidence of editing in 150 clones examined when the *P/C* gene was expressed from a vaccinia virus recombinant with Sendai virus co-infection. Only the *P/C* mRNA expressed from the Sendai virus was exclusively found to be edited. This provided a strong argument that the mechanism of editing was co-transcriptional and that the viral polymerase complex could not insert G nucleotides on pre-formed mRNAs made by the vaccinia recombinant post-transcriptionally.

Using *in vitro* transcription reactions with purified Sendai virus virions and by varying certain reaction conditions, Vidal *et al.* (1990b) have proposed a RNA polymerase stuttering model (Fig. 3). In this stuttering model, it was proposed that during transcription, the viral polymerase first pauses at or near the editing site due to an intrinsic property associated with the template. When the pausing time is sufficiently long (which occurs at a certain frequency), it allows 3' end of the nascent mRNA chain to slip on the template and to re-align before the next base is incorporated. The outcome after the slippage is then the polymerase reiteratively adds one or more G nucleotides at this site. A displacement of the nascent chain by 1 (Sendai and measles viruses) or 2 bases (SV5 and mumps virus) generates the most stable re-alignment, accounting for the predilection for the respective number of insertions by those viruses. The template slippage and the nucleotide insertion(s) are thought to relieve the pausing constraint and allow transcription to continue past this region.

Figure 3. STUTTERING MODEL FOR PARAMYXOVIRUS PSEUDO-TEMPLATED TRANSCRIPTION OF THE *P* GENE. The insertion site in the *P* gene for each virus group are shown with normal pairs indicated by [|] and U:G pairs with [:]. The top and bottom lines are the genome and the nascent mRNA sequences, respectively. (Adapted and modified from Vidal *et al.*, 1990b).


SV5/Mumps (+2G)
Measles/Sendai (+1G)
3'-UUCUCCC-5'
GAGG-OH
5'-AA
3'-UUUUCCC-5'
AAGG-OH
5'-AA
+1G
Realignment after 1-base slippage
+2G
Realignment after 2-base slippage

In previous studies Vidal *et al.* (1990b) tested this model. Decreasing the concentration of GTP in the *in vitro* transcription reaction would theoretically promote longer pausing time at the editing region. Indeed, when examined, the frequency of greater than one G-nucleotide insertions was significantly increased. Similarly, the substitution of inosine for guanine nucleotide, which increases the likelihood of slippage due to decreased stability in base pairing, also increased the number of multiple G-nucleotide insertions. These results were found to be entirely consistent with the proposed stuttering model for Sendai virus, and the re-examination of sequence elements surrounding the editing region in other paramyxoviruses also supported the correct slippage and re-alignment predictions (Vidal *et al.*, 1990b). In further support of the proposed stuttering model for paramyxovirus RNA editing, it was noted that this viral model bears much resemblance to the proposed telomerase model, in which repeat sequences of 3'-TTGGGG-5' are inserted after the polymerase pausing and slippage of the template (Greider and Blackburn, 1989; Vidal *et al.*, 1990b).

In Chapter III, as a next step in the study of RNA editing in paramyxoviruses, we have successfully reconstituted this unique viral event *in vivo*, using a model genome that contains the sequence surrounding the editing site of Sendai virus *P/C* gene. We also determined that a sequence as little as 24-nucleotides spanning the G-nucleotide insertion site can be placed away from the normal *P/C* gene sequence context and still promote the nontemplated transcription. In addition, we showed that replacing the three C residues in the consensus sequence with three G residues completely eliminated editing. More significantly, the development of this *in vivo* model for editing in Sendai virus now provides a simple approach to completely dissect the sequence requirement.

As discussed previously, in addition to P, V, and C proteins, there are two other proteins that are expressed from Sendai virus *P/C* mRNA. The X protein, which can be expressed from both edited and unedited messages by ribosomal initiation at AUG/1523, is about ~95 amino acids long and represents the C-terminus of the P protein (Curran and Kolakofsky, 1988). Since the C-terminal portion of the P protein is responsible for binding to the nucleoprotein core (Ryan and Portner, 1990), the X protein may also have this function. The other protein, W, can only be expressed from edited mRNAs containing two G-nucleotide insertions that introduce a stop codon at position 1061. The W protein is expected to be in very low abundance, since the +2G mRNAs comprise only ~5% of the total *P/C* mRNA pool. The W protein represents about 320 N-terminal amino acids of the P protein and is colinear the V protein except for the absence of C-terminal ~51 amino acids of the V protein.

2. Reverse Genetics

Recent advances in molecular biology have led to development of *in vivo* systems in which various negative-strand virus RNA synthesis is totally driven by protein expression through either phage T7-based vectors or recombinant vaccinia vectors containing individual cDNAs of the viral genes. This includes influenza virus (Smith *et al.*, 1987), VSV (Pattnaik and Wertz, 1990), and Sendai virus (Curran *et al.*, 1991). Such a system offers powerful tools to study the roles of various *trans*-acting proteins at various stages of the virus replication. For example, the vaccinia-based influenza system led to identification of the minimal subset of viral proteins necessary for genome replication (Huang *et al.*, 1990). The T7-based method for VSV allowed similar success in reconstitution of a replication system for DI RNAs (Pattnaik and Wertz, 1990).

For Sendai virus, the T7-based system has also proved very fruitful. By expressing a subset of Sendai virus proteins through

T7-based plasmids, it was shown that the NP, P, and L proteins were necessary and sufficient for replication of DI RNAs (Curran *et al.*, 1991). The four C proteins, however, were neither required for replication nor able to substitute for P. Interestingly, expression of the V protein had a dose-dependent inhibitory effect on the replication of DI RNAs, and the inhibition could be overcome by overexpression of the L protein. The inhibition did not appear to be due to the Cys-rich domain since the W protein, which does not have the Cys-rich domain, had a similar inhibitory effect. The X protein did not appear to have any effect on the DI RNA replication. Combined together, Curran *et al.* (1991) proposed that the V and W proteins exert their inhibitory effects by binding to and sequestering the L protein. The L protein is thus kept from interacting with the P protein. Since the V and W proteins of Sendai virus are expressed from edited mRNAs containing one and two G-nucleotide insertions, respectively, the down-regulation of genome replication by these proteins could be directly controlled by the frequency of RNA editing during transcription. There still remains a number of unanswered questions regarding the functional significance of various proteins and their domains. For instance, it is still not clear why the Cys-rich domain of the V proteins is so well conserved across most paramyxoviruses (with the exception of pneumoviruses and HPIV-1). Also, the role of other proteins such as X, is not yet understood.

Clearly, the current T7-based replication system for Sendai virus is limited in that very few aspects of the viral transcription can be addressed. In this regard, a more advanced system can be devised in which the T7-expression system is combined with an artificial genome (discussed in Chapter II) that mimics the viral genome in both transcription and replication. This way, it may be possible to focus on whether the X protein or the Cys-rich domain of the V protein is important for regulation of viral transcription.

3. ABNORMAL *M* GENE TRANSCRIPTION AND SSPE

The possibility that some of the viral proteins involved in down-regulating genome replication may facilitate the establishment and/or maintenance of persistent infection by paramyxoviruses in the specific tissue, such as the brain, was raised by Curran *et al.* (1991). As discussed previously, various host cell cytoskeletal proteins have been implicated in playing a role during viral transcription replication, and some differences in viral gene expression in a different host background have been noted (Cattaneo *et al.*, 1987; Schneider-Schaulies *et al.*, 1989).

Combined, these observations and the recent studies using the *in vivo* replication system may shed some light on the pathogenesis of paramyxovirus persistent infection.

Subacute sclerosing panencephalitis (SSPE) represents an example of a class of true slow virus infection that also includes progressive multifocal leukoencephalopathy (PML) and progressive rubella panencephalitis. SSPE almost always affects children, although sometimes it involves adolescents and young adults following an attack of measles or previous measles immunization. Symptoms and signs generally develop 5 to 10 years after the initial exposure to measles virus, and these include personality changes and involuntary movements as well as characteristic high-voltage activity in EEG. Death eventually ensues, from months to years after the initial onset of the symptoms.

Measles virus-like inclusion bodies were first seen under the electron microscope in SSPE brain sections in neurons and oligodendroglia (Wechsler and Fields, 1978; Hall, *et al.*, 1978). Other studies, including epidemiological studies and physical and serological findings of SSPE virions from cocultivation experiments indicated measles virus (MV) as the probable etiologic agent of SSPE. Since there were no regional outbreaks of SSPE, it was assumed that an abnormal host immune response or a rare mutation affecting the parental MV genome, rather than a distinct second viral agent, was responsible for the establishment of SSPE.

Analysis of polypeptides from infected cells indicated that, of the six major viral-specific polypeptides, only the M protein showed a discernible biochemical alteration in SSPE isolates, indicated by aberrant electrophoretic migration patterns (Wechsler and Fields, 1978). This was true for all five SSPE strains examined, and consequently, it was hypothesized that an abnormal M protein may be responsible for SSPE. In this hypothesis, a defective M protein would no longer allow normal recognition of the membrane proteins by the viral nucleocapsids and result in an abortive lytic cycle. Consistently, the M protein from SSPE strains was antigenically distinguishable from the normal measle virus M protein (Hall *et al.*, 1978). Furthermore, when a similar type of analysis was extended to viral mRNAs, again, the mRNAs for the M protein in 2 SSPE strains exhibited different mobilities compared to that of two normal MV strains.

In a later study, Hall *et al.* (1979) showed that the M protein abnormality was not always detected in SSPE isolates (for example, LEC SSPE) and that even within different MV strains, the M protein mobility could vary. In fact, a better biochemical marker for SSPE was the lack of antibodies to the M protein in SSPE patients in the context of hyperimmunity against the other MV polypeptides. When Hall and Choppin (1981) analyzed proteins prepared from brain tissues of SSPE patients, they found a lack of M protein synthesis in these samples whereas the other MV polypeptides were apparently made normally. This was in agreement with their previous finding that SSPE patients did not produce anti-M antibodies. ter Meulen and colleagues (1985) examined another SSPE cell line and found that the *M* mRNA from this cell line could not be translated *in vitro*. This suggested that the defect was in the viral mRNA, and the inability to translate this message could lead to persistence.

Other abnormalities in M protein expression were subsequently described. Sheppard *et al.* (1986) found an SSPE cell line, IP-3-Ca, which could synthesize the M protein but rapidly degraded it. This type of restriction might be expected if the viral genome had undergone a mutational event, affecting the coding region of M protein as well as the non-coding regulatory regions. Immunohistological and biochemical studies of the brains of four SSPE patients showed that although the nucleocapsid proteins and phosphoproteins were found in all brain areas examined, the

H and F proteins, as well as the M protein, were not always detectable. Here, their mRNAs often failed to translate *in vitro* (Cattaneo *et al.*, 1986). But the exact molecular mechanism restricting the expression of the *M* gene (as well as of the *H* and *F* genes) remained obscure.

The sequence studies of cDNA isolated from an SSPE patient (K) showed two remarkable features (Cattaneo *et al.*, 1988a). First, there were multiple point mutations, one of which introduced a stop codon in the M protein reading frame. This would explain the lack of M protein expression in this patient. Second, there was great sequence variation between two sibling clones of the same patient, which could not be explained by cloning errors alone. More specifically, it was found that up to 1% of the nucleotides differed between the two cDNAs of the *M* gene. It appeared that the high mutability of the genome was associated with this RNA virus persistence.

Other sequencing studies of the SSPE strain cDNAs or cDNAs directly obtained from the brains of SSPE patients revealed the nature of the nucleotide changes (Cattaneo *et al.*, 1988b; Wong *et al.*, 1989). For instance, the *M* gene sequence of the IP-3-Ca SSPE cell line differed from the wildtype Edmonston strain, and the differences were distributed over the entire gene, especially in the untranslated region (Cattaneo *et al.*, 1988). The changes affecting the coding regions were more likely to introduce an amino acid alterations (~50%) than predicted. Similar observations were made for *P*, *H*, *N*, and *F* genes, with a decreasing order of variability (M> H> N> P).

In ensuing studies, it was found that a localized biased hypermutation of U to C changes were affecting the SSPE strain genome, and this was most pronounced in the *M* gene. (Wong *et al.*, 1989). Interestingly, the U to C changes occurred at a higher frequency when the SSPE strain was passaged in human neuroblastoma cells, than when passaged in African green monkey kidney cells. This apparent host dependency suggested that a host mutational activity contributed to the observed mutations in addition to the viral polymerase errors. In support of this finding, Bass and Weintraub (1988) have found that various cell types (from *C. elegance* to *X. laevis*, and to mammalian tissue culture cells) possessed a double-stranded RNA unwinding activity that can introduce U to C changes by deamination of the adenine residues. It would appear that, until there is a system in which one is able to recover infectious MV from cloned cDNA and allow introduction of specific mutations into the lytic viral genome, it will be difficult to identify definitively the type of mutations that can lead to SSPE. Ultimately, one would like to develop an animal model via transgenics by manipulation of the SSPE cDNA.

In light of these goals, work done in this thesis may represent an initial progress. The encouraging aspect is that understanding of the influenza virus genome structure and methodology underlying the manipulation and rescue of an artificial genome led to introduction of site-specific mutations and rescue of the mutated genome (Enami *et al.*, 1990). Chapter 1 of this thesis entails the development of a similar artificial replication and rescue system for Sendai virus, and although there is a fundamental difference in genome size between the influenza viruses and the paramyxoviruses, it is hoped that such development would lay down the initial stepping stone and eventually lead to an efficient rescue system for recombinant paramyxoviruses.

E. REPLICATION

Unlike transcription, Sendai virus genome replication is completely dependent on *de novo* protein synthesis (Robinson, 1971; Carlsen *et al.*, 1985). In infected cells, the genome and antigenome are always found encapsidated by the NP protein.

The switch from transcription to replication requires a sufficient intracellular pool of free, unassembled NP proteins available for encapsidation of nascent chains. In VSV, it is thought that the encapsidation of nascent leader chains allows antitermination at the first junction and continued readthrough to the 5' end of the genome without recognizing any of the junctional signals (Blumberg *et al.*, 1983). In the absence of encapsidation, the leader RNA terminates at the first junction, and the short, terminated product has been detected *in vivo* (Leppert *et al.*, 1979). In Sendai virus, the role of encapsidation is less clear, but at least in one strain (Z strain), it appears to be important for polymerase elongation past the first junction. By estimating the relative levels of naked plus-strand RNAs that have crossed the leader-*NP* junction to the level of *NP* mRNA levels, it was found that in Z strain of Sendai virus, the polymerase would read through the first junction in the absence of encapsidation, but terminate sharply somewhere within the *NP* gene (Vidal and Kolakofsky, 1989). It was concluded therefore, the role of encapsidation during replication is more important for promoting continued elongation past the leader-*NP* junction than for conferring antitermination at the junction. It should be noted that although a completely reconstituted *in vitro* replication system has been developed for VSV, such facility is not available for Sendai virus or other paramyxoviruses. Only recently, an *in vivo* replication system for DI RNAs with expression of viral proteins from cDNAs has been successful, and it will provide much needed understanding of the precise functions of various viral proteins (Curran *et al.*, 1991).

F. DEFECTIVE-INTERFERING (DI) RNAs

DI RNAs of Sendai virus are generally divided into two classes, the copy-back and the fusion types. Both RNAs have the same polarity (negative) as the wildtype virus genome but differ in the manner by which they are generated. The copy-back DI RNA, which is the most common type described for paramyxoviruses and VSV (Amesse *et al.*, 1982; Perrault *et al.*, 1978), contains only the 5'-terminal and part of the *L* gene sequences. The 3' end of this RNA is complementary to the 5' end (110-150 nucleotides) and forms a panhandled circular structure (Kolakofsky, 1976). This novel 3' end sequence is somewhat similar to that of the nondefective (ND) genome, since 15 of the first 25 nucleotides are identical between the two genomes (Re *et al.*, 1983). On the other hand, the much less frequently observed fusion DI RNA is a deletion mutant that has conserved 3' and 5' termini and extensive internal deletions. The deleted genes are usually *P, M, F,* and *HN*. In the majority of the original isolates of fusion DI RNAs, the 3' part of the *NP* gene was fused to 5' sequences of the *L* gene (Amesse *et al.*, 1982). The fusion DI RNAs usually retain the *NP* transcription start signal at the 3' end as well as the *L* transcription termination signal. In contrast, the copy-back type lacks the *NP* transcription start sequence but usually retain the *L* gene termination sequence. The largest and smallest Sendai virus copy-back DI RNA studied thus far contain ~2600 and ~450 nucleotides, respectively (Re, 1991). For fusion DI's, genomes as large as 4600 and as little as 1600 nucleotides have been isolated.

By definition, both types of DI particles require nondefective particles for growth and more importantly, interfere with nondefective RNA replication and productive infection. For this reason, Huang and Baltimore (1970) postulated that DI particles may mediate the establishment and maintenance of persistent viral infections such as SSPE. DI particles have been observed and described for a number of paramyxoviruses including HPIV-3, mumps virus, NDV, measles virus, canine distemper virus, SV5, and respiratory syncytial virus (Re, 1991). In general, these DI particles characteristically exhibit the von Magnus effect (von Magnus, 1952), which is the cyclic growth pattern (infectious titer) of nondefective particles when co-passaged undiluted with DI particles. As is the case for the nondefective genome, the Sendai virus DI genomes possess the proper encapsidation signal and thus are protected from ribonuclease digestion (Lynch and Kolakofsky, 1978).

Certain structural features must be present in DI RNAs, and the presence of other features can further increase their chances of survival. For example, in Sendai virus DI genomes, the 5' end of the nondefective genome is invariably retained in all DI RNAs examined (Re, 1991), suggesting that this sequence must be present for replication and packaging. By the same token, sequences at the 3' end are also important, although some variations are allowed as seen in copy-back genomes. A lack of a transcription initiation signal also increases the chances of survival. This may be due to the fact that all polymerase complexes that enter at the 3' end would be exclusively devoted to replication and not diverted to transcription (Re, 1991). This may explain why the transcriptionally inert copy-back DI genomes are more abundant than the transcriptionally active fusion DI genomes. Also, when a mixture of the two types of DI RNAs are co-passaged multiple times, the copy-back DI RNAs eventually eliminate the fusion DI RNAs in the population (Re and Kingsbury, 1986). The optimal size of DI RNAs appear to have a range, between 4 kb and 450 nucleotides (nt). Generally, the smaller the RNA, the better it replicates, and the larger the RNA, the better it packages. If a DI RNA is too large (> 4kb), the replicative advantage over the nondefective genome is lost, whereas if it is too small (< 450 nt), it is lost due to the fact that small nucleocapsids are relatively inefficiently packaged into virions (Re, 1991). These parameters must be taken into consideration when designing an artificial DI genome.

In Chapter II, we have constructed an artificial genome that is very much like a fusion DI RNA. Like the fusion DI RNA, our artificial genome, Send-CAT, contains intact 3' and 5' terminal sequences of the nondefective Sendai genome. To optimize for its survival, we have chosen a size of 924 nucleotides. The transcription start and termination signals of *NP* and *L*, respectively, are preserved as well as the leader gene. We have shown that this fusion DI-like genome is transcriptionally active, since a reporter gene (chloramphenicol acetyltransferase), is efficiently expressed. Furthermore, the Send-CAT RNA is encapsidated and replicated, as assayed by the presence of its nuclease-resistant anti-genomes (cRNA), and packaged into progeny virions. In Chapter III, we further show that the Send-CAT RNA behaves like a DI genome in that it can be grown to a very high titer after undiluted serial passage through embryonated eggs. Thus, our Send-CAT RNA represents a simplified and replicating model of the virus genome, which can be optimally used to study the effects of specific mutation on transcription, replication, and packaging.

II.

Rescue of a Foreign Gene by Sendai Virus

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A. INTRODUCTION

Sendai virus is a prototype paramyxovirus containing a non-segmented, negative-sense RNA genome of 15.3 kilobases (kb). During transcription, the genome directs the synthesis of a leader RNA and six transcription units (*NP, P/C, M, F, HN,* and *L*) (Kolakofsky and Roux, 1987). After active viral protein synthesis, replication ensues, resulting in the synthesis of full-length positive-strand complementary RNA (cRNA) which is used to create new genomes. The mRNAs are capped and polyadenylylated by the viral polymerase, whereas the leader RNA and the cRNA are unmodified. In the viral genome, various *cis*-acting signals have been identified or proposed to function during mRNA synthesis. These include the transcriptase entry signal at or near the 3' end, transcription termination and re-start sequences at the intergenic junctions (Gupta and Kinsbury, 1984), and the site in the *P/C* gene at which RNA editing by G-nucleotide insertion occurs (Vidal *et al.,* 1990). In contrast, sequences required for replication, encapsidation, and packaging of the genome have not been clearly defined, although they probably reside within the terminal sequences. In this regard, efforts to study such signal(s) have been hampered by the large size of the viral genome and the difficulty in performing reverse genetics, due to the negative-sense polarity of the genome.

Only recently have methods been developed that enable molecular studies of negative strand RNA viruses *in vivo*. For influenza viruses, Luytjes *et al.* (1989) have described a system whereby purified nucleocapsid proteins and synthetic RNA (Parvin *et al.*, 1989) are used to assemble a synthetic viral gene-like RNA into biologically active ribonucleoprotein (RNP). Subsequently, this system allowed introduction of mutations into the genome of influenza virus (Enami *et al.*, 1990) and led to the determination of the minimal subset of viral proteins required for genome replication (Huang *et al.*, 1990). In the rhabdovirus system, DI particles of vesicular stomatitis virus (VSV) have been successfully replicated (Pattnaik and Wertz, 1990) and assembled (Pattnaik and Wertz, 1991) through the use of viral proteins expressed from recombinant sources. Recombinant infectious virus systems have also been developed for double-stranded RNA viruses such as bacteriophage $\phi 6$ (Olkkoen *et al.*, 1990) and reovirus (Roner *et al.*, 1990). Together, these studies provide examples of alternative and rewarding approaches to manipulate and understand negative-sense RNA virus genomes and their gene products. Unfortunately, no such advances have been made for paramyxoviruses. Regretfully, earlier reports of infectious measles virus (MV) being generated from cloned MV cDNA by microinjecting "committed transcription complexes" into helper cells (Ballart *et al.*, 1990) have been retracted.

In the present study, we describe a simple system similar to that developed for influenza virus which allows for efficient expression, replication, and packaging of a foreign gene by Sendai virus. In contrast to the studies described above, we find that expression and rescue of synthetic RNA can be achieved by transfection of naked virus-like RNA followed by helper virus infection. Therefore, no encapsidation or modification of the synthetic RNA is needed prior to transfection. However, the addition of host cell factor(s) can markedly enhance the levels of expression and rescue of the gene. This system will now allow us to examine the various *cis*-acting RNA signals and to identify possible host cell auxiliary proteins and their role during viral gene expression and replication.

B. MATERIALS AND METHODS

1. VIRUS AND CELLS

Sendai virus (Sendai/52) was grown in embryonated chicken eggs as described (Parvin *et al.*, 1989). Madin-Darby bovine kidney (MDBK) cells were used for transfection experiments and plaque assays. Cytoplasmic extracts were prepared from baby hamster kidney (BHK) cells.

2. CONSTRUCTION OF pSEND-CAT

Plasmid pSend-CAT was constructed through a series of 3 polymerase chain reactions (PCR) using a total of 5 primers. Each primer was 60-70 bases long. Primers 1 and 2 contained CAT gene sequence fused with partial sequence of Sendai virus 5' or 3' termini. The original PCR reaction was accomplished using primers 1 and 2 on plasmid pCM7 (Pharmacia). This PCR product was then used as template for another PCR reaction using primers 3 and 4. Primer 3 overlaps with the 5' end of primer 1 and also contained the sequence of the remaining 3' terminus of the Sendai genome bounded by the engineered *Ear*I site and a *Sac*I restriction site useful for cloning (Fig. 4). Primer 4 overlaps with the 5' end of primer 1 and contains additional sequence from the

5' end of Sendai virus, but does not complete the 5' terminal Sendai virus sequence. This PCR product was then used as template for a reaction with primers 3 and 5. Primer 5 overlaps with the 5' end of primer 4, completing the 5' terminus of the Sendai virus sequence which abuts the T7 promoter sequence and a *Hind*III site for cloning (Fig. 4). The final PCR product was cloned into the *Hind*III/*Sac*I window of pUC19.

3. PREPARATION OF CYTOPLASMIC EXTRACTS

Cytoplasmic extracts were prepared essentially as described (Mirakhur and Peluso, 1988), except that leupeptin (0.3 µg/ml), PMSF (1 mM), and antipain (1 µg/ml) were included in the extraction buffer. For infected cell extracts, subconfluent monolayers of BHK cells in 150-mm dishes were infected with Sendai virus at moi≅10 and incubated for 16 hours at 33°C. Extracts were prepared at a final concentration of approximately 10^8 cells/ml with protein concentrations in the range of 1-2 mg/ml. RNasin (10 units/ml) was added to the final supernatant.

4. PREPARATION OF RNA AND TRANSFECTIONS

Run-off transcription of *Ear*I-digested pSend-CAT (Fig. 4) was performed using standard T7 polymerase reaction procedures. The RNA was purified and quantitated as described (Parvin *et al.*, 1989). For synthesis of riboprobes, 100 μCi of [α-^{32}P]CTP was added in the presence of 4 mM each of ATP, UTP, and GTP. The full-length product was gel purified (Ausubel *et al.*, 1988). Approximately 0.5- 1 μg of the purified transcript was used for transfection with or without cytoplasmic extract as described (Luytjes *et al.*, 1989).

5. MICROCOCCAL NUCLEASE (MN) DIGESTION

RNA samples were digested with 10 units of MN per 1 μg of RNA in 10 mM Tris, pH 8.1/ 1mM $CaCl_2$ at 37° for 1 hour. For encapsidation analysis, duplicate reactions were carried out with labelled RNA as described (Mirakhur and Peluso, 1988) prior to digestion with MN. Resulting RNA samples were either used directly for transfection or extracted with phenol:chloroform and analyzed on a 1% agarose gel, which was dried and autoradiographed.

6. RIBONUCLEASE (RNase) PROTECTION AND CAT ASSAYS

For RNase protection, cytoplasmic extract was prepared from a 100-mm dishcontaining approximately 2 x 10^7 MDBK cells that had been transfected with an appropriate mix containing 20μg of Send-CAT transcript as described above. The extract was treated with approximately 1000 units of MN to digest mRNA and the resulting nuclease-resistant RNA species were isolated by multiple phenol:chloroform extractions, followed by ethanol precipitation. Hybridization and nuclease treatment with ribonuclease T1 were carried out as described (Ausubel *et al.*, 1988). CAT assays were performed 16-18 hours post-infection as detailed (Gorman *et al.*, 1982).

7. PASSAGING OF VIRUS AFTER TRANSFECTION

After MDBK cells were transfected and infected as described, serum-free medium containing bovine albumin (0.2%)/ trypsin (5 μg/ml) was added. After 24 hours, medium was collected, cleared of cells, and 100 μl of this supernatant was used to infect fresh MDBK cells. For antibody inhibition studies, anti-Sendai virus antiserum or anti-influenza virus (A/Hong Kong/68) antiserum was added to the inoculum prior to infection and incubated for 30 minutes at 37°C. At 18 hours post-infection, cells were harvested and assayed for CAT activity.

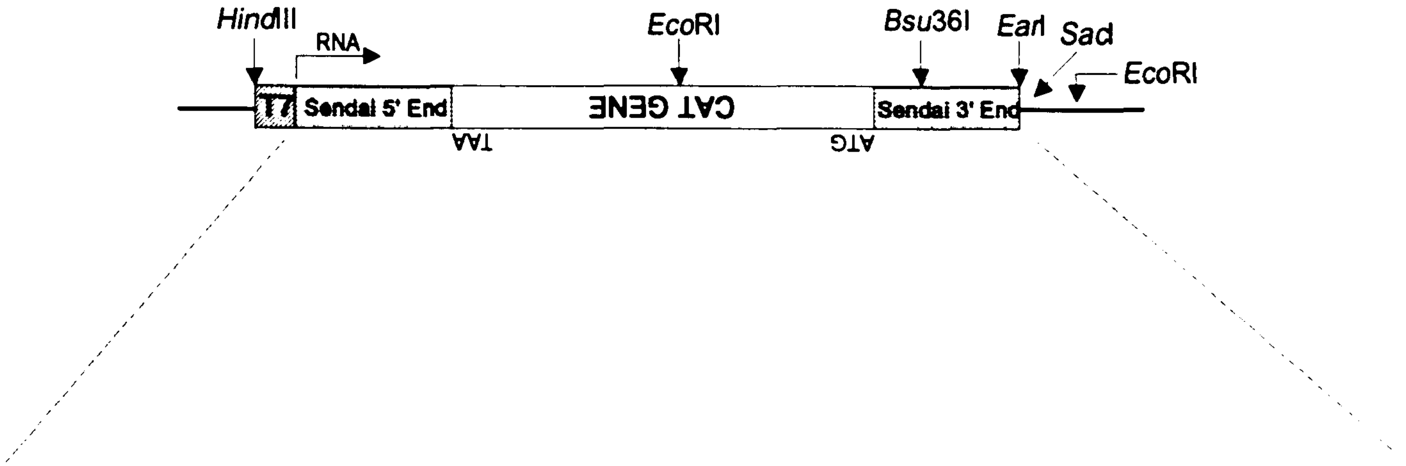
C.RESULTS

1. EXPRESSION OF SEND-CAT RNA BY SENDAI VIRUS

A plasmid, pSend-CAT, was constructed through multiple rounds of PCR reaction. This plasmid could direct the synthesis of a Sendai virus-like RNA in which the coding region of the genome was replaced by the antisense coding region of the chloramphenicol acetyltransferase (CAT) gene. Thus, the CAT gene is flanked by 145 and 119 non-coding nucleotides corresponding to the Sendai virus genome 5' and 3' termini, respectively (see Fig. 4). Some known signals present in these sequences include the entire leader RNA, the 5' noncoding region of the *NP* gene and the transcription termination/ poly-A addition signal of the *L* gene. The construction was such that, after *Ear*I digestion of the plasmid, run-off transcription by T7 RNA polymerase generates a 924-nucleotide RNA molecule sharing the exact 5' and 3' ends of the viral genome. When this recombinant CAT RNA was transfected into MDBK cells, no detectable CAT activity was observed (Fig. 5, lane 1). Since this RNA is of negative polarity, CAT activity would be present only if the Send-CAT RNA is transcribed *in vivo.* When the cells were subsequently infected with Sendai virus, significant CAT activity was observed (Fig. 5, lane 2). This expression was specific to Send-CAT RNA since a related RNA, IVACAT-1, which contains the anti-sense of CAT gene flanked by the 5' and 3' terminal sequences derived from the *NS* gene of influenza A virus (Luytjes *et al.*, 1989) was not transcribed (Fig. 5B, lane 2). Additionally, RNA derived from transcription of *Bsu*I-digested pSend-CAT did not result in significant CAT expression (Fig. 5B, lane 1). *Bsu*I cuts within the 3' Sendai sequence but leaves the CAT sequence intact. Furthermore, if the Send-CAT RNA was preincubated with cytoplasmic extract prepared from Sendai virus-infected cells, CAT expression was markedly enhanced (Fig. 5A, compare lanes 2 and 3). Addition of increasing amounts of the extract resulted in only a minimal increase of CAT activity (Fig. 5A, lanes 4 to 7). However, continued addition of extract at high concentrations eventually resulted in complete inhibition of CAT activity in an extract-dependent manner (see below).

Figure 4. SCHEMATIC REPRESENTATION OF THE RELEVANT REGION OF PLASMID pSEND-CAT. The plasmid contains the CAT gene in anti-sense orientation flanked by the 5' and 3' terminal sequences of the Sendai virus genome. The Send-CAT RNA, including the partial sequence of the Sendai virus termini and the viral sequences immediately adjacent to the CAT gene, is shown below the clone. Note that *BsuI* cuts within the 3' end of Sendai sequence so that the resulting transcript has imperfect 3' terminal sequence but leaves intact the anti-sense coding region of CAT gene as well as the complete 5' terminal sequence.

pSend-CAT



5' ACCAGACAAGAGUUUAA___AGGGACUAAUUAUUA ----- CAT gene ------ CGUGAACUUUGGCAGCAA___AUGUUUUUUCUCUUGUUUGGU 3'

145 bases 660 bases 119 bases

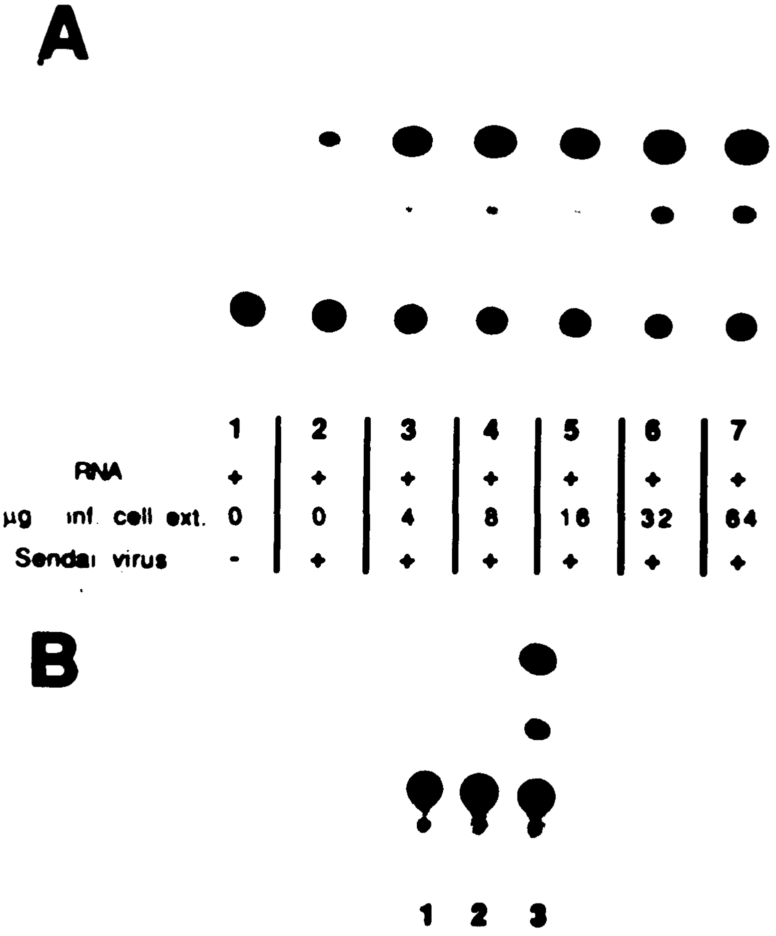
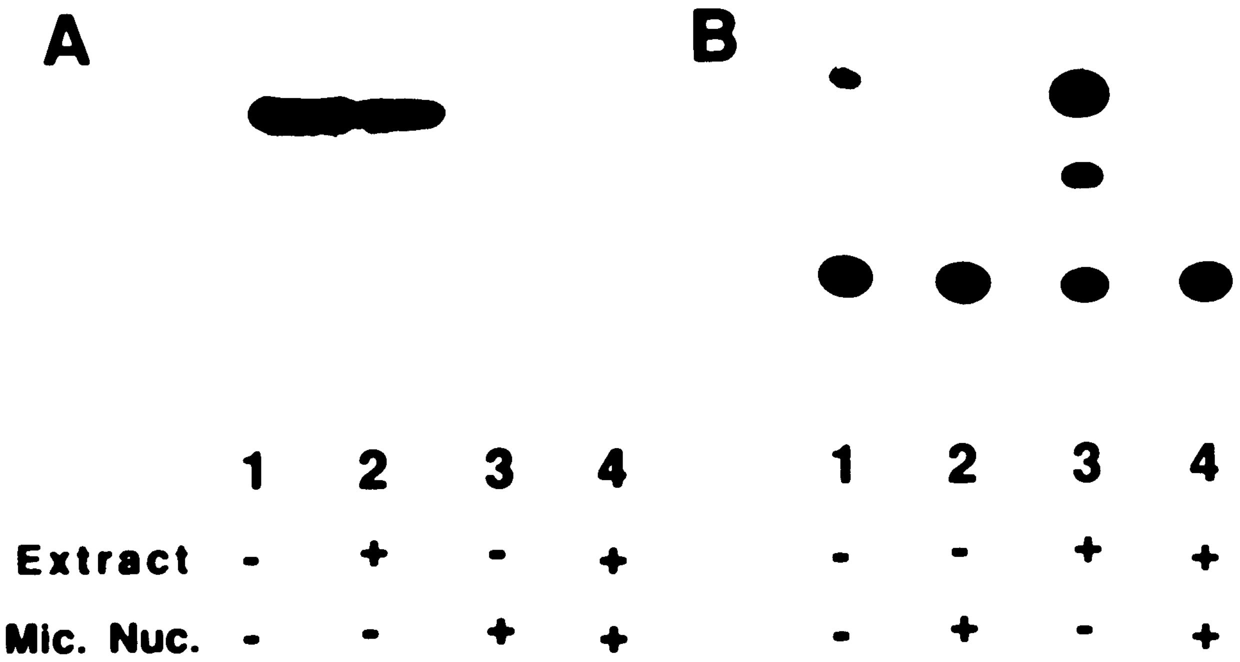


Figure 5. SPECIFIC CAT EXPRESSION AFTER SEND-CAT RNA TRANSFECTION. (*A*) Conditions used for transfections are indicated below each lane. (*B*) CAT activities were measured for different RNAs used in transfections. 1 µg of the indicated RNA was pre-incubated with 32 µg of ICE and transfected, followed by helper virus infection. Lane 1, RNA derived after transcription of *BsuI*-digested pSend-CAT; lane 2, IVACAT-1; lane 3, Send-CAT RNA.

2. THE SEND-CAT RNA IS NOT ENCAPSIDATED *IN VITRO*

Previously, it was shown that defective-interfering (DI) RNA of VSV can be encapsidated *in vitro* through incubation with an infected cell extract (Mirakhur and Peluso, 1988). As incubation of Send-CAT RNA with infected cell extract (ICE) enhances CAT expression, it was postulated that the Send-CAT RNA was assembled into ribonucleoprotein (RNP) by the viral proteins present in the extract. Since a characteristic of paramyxovirus nucleocapsids is their resistance to nuclease treatment, radiolabelled Send-CAT RNA was incubated with/without extract and with/without micrococcal nuclease and analyzed by PAGE. As shown in Fig. 6A, the labelled RNA was completely digested in the absence of added extract (lane 3) and only trace amounts are present when extract is added (lane 4). In order to determine whether the trace amounts of RNA are sufficient to induce CAT expression, duplicate samples of nuclease treated RNA was used to transfect Sendai virus-infected cells. Fig. 6B, lane 4 shows that RNA pre-incubated with ICE and then treated with nuclease does not induce CAT activity in virus infected cells. Therefore, the enhancement of expression through incubation with ICE is not due to the assembly of nucleocapsids *in vitro*.

Figure 6. ANALYSIS OF ENCAPSIDATION REACTIONS. In duplicate samples, labelled Send-CAT RNA was incubated with either ICE or extraction buffer. (*A*) Autoradiography of MN-treated RNAs after electorphoresis. Incubation with ICE and MN is indicated by (+). (*B*) CAT activities observed after transfection of the samples following encapsidation and MN reactions. All transfections were followed by helper virus infection.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Extract - + - + - - + +
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3. A CELLULAR FACTOR(S) INTERACTS WITH SEND-CAT RNA

Since the biologically active RNA construct was found not to be encapsidated, a cellular factor rather than a viral component may account for the enhancement of CAT expression. To address this, we prepared cytoplasmic extracts in parallel from uninfected cells and infected cells. In the presence of helper virus infection, 5 and 10 μg of uninfected cell extract (UCE) clearly increased Send-CAT expression (Fig. 7A, lanes 6 and 7, respectively). Interestingly, 20 and 40 μg of this UCE reproducibly suppressed Send-CAT RNA expression below the level detectable with RNA alone (compare lanes 8 & 9 to lane 1). Inhibition was also observed with ICE, but at much higher protein levels (Fig. 7A, lane 5), with complete inhibition occurring with over 100 μg of ICE (data not shown). This inhibition appeared to be due to a non-specific RNA binding protein(s) since the inhibition could be partially overcome by the addition of tRNA during pre-incubation (Data not shown).

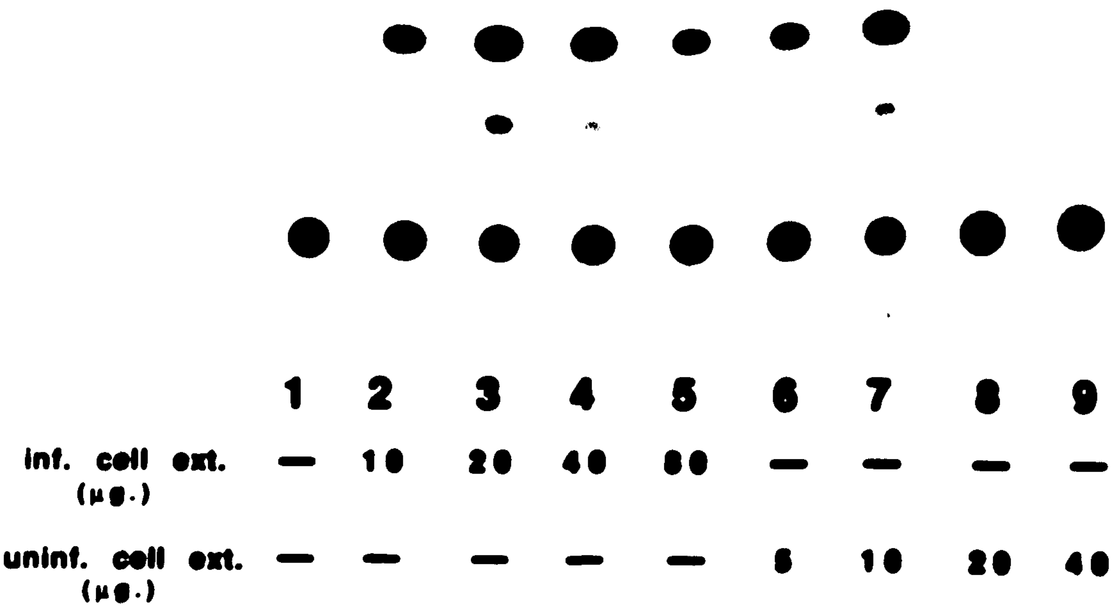


Figure 7. CAT ACTIVITIES OBSERVED AFTER INCUBATION OF THE SEND-CAT RNA WITH INCREASING AMOUNTS OF ICE OR UCE. Typical transfections were performed under conditions indicated. Helper virus infection followed all transfections. Lane 1, RNA only; lanes 2-5, increasing amounts of ICE as shown; lanes 6-9, increasing amounts of UCE as shown.

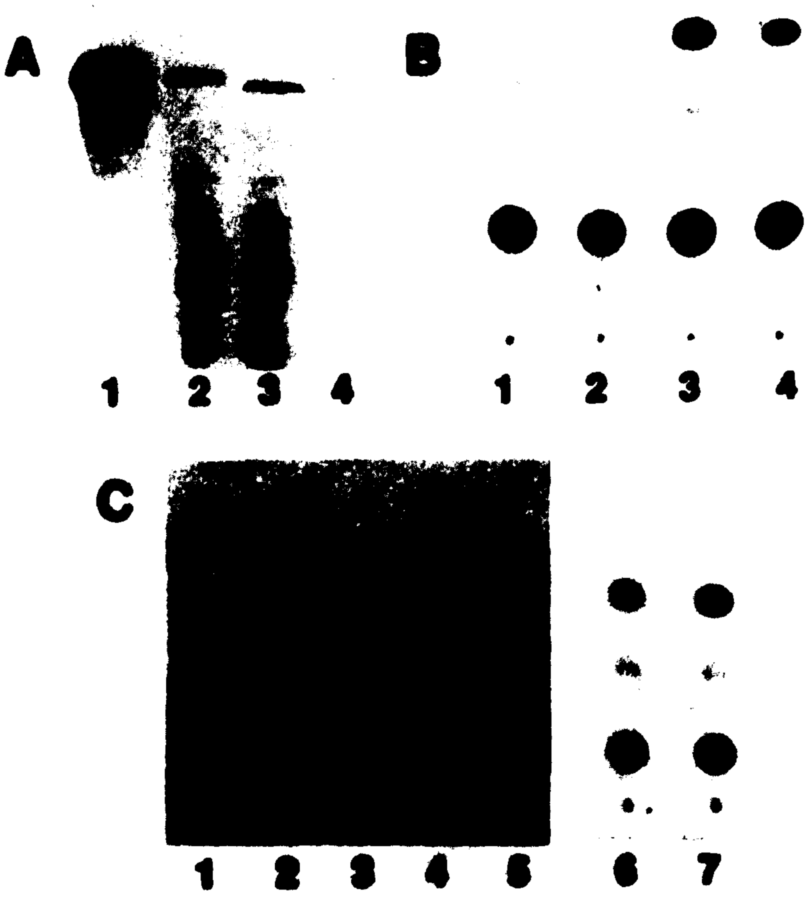
4. SEND-CAT RNA IS REPLICATED BY HELPER VIRUS

In order to determine if the Send-CAT RNA is replicated, the presence of complete positive-sense copies of Send-CAT RNA (cRNA) in the transfected cells was assayed by a ribonuclease (RNase) protection experiment. Cytoplasmic extracts prepared after a typical transfection experiment were first treated with micrococcal nuclease. This treatment removes the nuclease-sensitive mRNAs, but the nucleocapsids remain intact. The resulting nuclease-resistant RNA species were isolated by extensive phenol:chloroform extractions and used for a standard RNase protection experiment. A radiolabelled Send-CAT RNA probe was used to assay for the presence of full-length positive-sense replicative intermediate cRNA. Fig. 8A, lane 1 is the RNase-resistant product after hybridization with a synthetic RNA corresponding to the cRNA of Send-CAT, which was transcribed off a second plasmid construct (Krystal, unpublished data). Fig. 8A, lane 4 is the negative control whereby probe was hybridized to excess non-specific RNA. In this case, all probe sequences are digested. However, if nuclease-resistant RNA from helper virus-infected cells transfected with Send-CAT RNA plus UCE is hybridized to labelled Send-CAT RNA, full-length product is protected (Fig. 8A, lane 2). In addition, the short protected fragments seen in Fig. 8A probably represent Sendai virus sequences present in Send-CAT RNA (the 5' and 3' ends) which are protected by authentic MN-resistant viral RNAs. Therefore, encapsidated replicative intermediate is present, indicating that the synthetic RNA is indeed replicated.

5. SEND-CAT RNA IS PACKAGED INTO INFECTIOUS VIRUS

Since the recombinant Send-CAT RNA can be replicated by the helper Sendai virus, it was tested whether progeny viruses released after a transfection experiment contained the Send-CAT RNA. This would indicate that the Send-CAT RNA contains all signals necessary for virus packaging. To determine this, the medium from the transfected (Send-CAT RNA + UCE)/ infected cells was removed and used for secondary infection of fresh MDBK cells. Cells were harvested at different times after the secondary infection and assayed for CAT activity. Fig. 8B shows that the CAT activity was first detected after 12 hours and increased with time, indicating continued CAT gene expression during this secondary passage. In addition, in this secondary passage, replication of the Send-CAT RNP is still occurring, as nuclease-resistant positive sense RNA is detected in an RNase protection experiment (Fig. 8A, lane 3). Also, to rule out the possibility that either Send-CAT RNP or CAT protein was carried along with the infecting media but not packaged into progeny virions, media was treated with polyclonal antisera prior to infection. Fig. 8C (lanes 2 to 4) shows that antibody to Sendai virus completely abrogates CAT activity whereas antibody to influenza virus has no effect (lane 5). Also, the Send-CAT RNP continues to be retained in the virus population even after multiple passages. This is exemplified by the presence of CAT activity in cells infected with primary (Fig. 8B, lane 1), secondary (lane 6), and tertiary (lane 7) passages of virus from a rescue experiment. It should be noted that rescue of Send-CAT RNA into infectious virus was also achieved with transfection of naked RNA. However, the level of rescued RNA was enhanced upon addition of ICE or UCE. In fact, rescue efficiency of Send-CAT RNA was always proportional to the levels of CAT activity seen in the primary transfected/ infected cells (data not shown).

Figure 8. ANALYSIS OF REPLICATION AND PACKAGING OF SEND-CAT RNA. (*A*) Ribonuclease protection assay was performed using labelled Send-CAT RNA as a probe. The probe was hybridized to: lane 1, 10 ng of synthetic full-length cRNA transcript; lane 2, MN-resistant cytoplasmic RNA (MRCR) isolated after a transfection/infection experiment; lane 3, MRCR isolated after a secondary infection (18 hpi); lane 4, 10 μg tRNA. (*B*) TIME COURSE OF 2^{o} CAT EXPRESSION. Cells were harvested for CAT assays at different times after infection. Lanes 1-4: 1, 4, 12, 24 hours post-infection, respectively. (*C*) CAT activity induced by infection of fresh MDBK cells by media from a transfection/infection experiment in the presence of 0 μl (lane 1), 10 μl (lane 2) and 40 μl (lane 3) of anti-Sendai antibody or 10 μl of anti-influenza antibody (lane 5). Lanes 6 and 7 represent CAT activities observed after second and third passages, respectively, of progeny virus obtained from first passage.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D. DISCUSSION

The ability to manipulate the genomes of negative-sense RNA viruses will have a profound effect on the study of these virus groups. In recent years, artificial genomes have been engineered and used to analyze RNA signals (Luytjes *et al.*, 1989; Parvin *et al.*, 1989) and create new viruses (Enami *et al.*, 1990; Park & Krystal, unpublished data). In this report we modified the technology used to create the influenza virus replication/ rescue system for use with paramyxoviruses. Thus, a cDNA clone was constructed which codes for a marker gene (CAT) bounded by the entire 5' and 3' noncoding termini of Sendai virus. Additional engineering of the cDNA allowed for synthesis of a Sendai genome-like vRNA with exact termini (Fig. 4). When introduced into cells CAT gene is expressed only in the presence of super-infecting Sendai virus (Fig. 5). Thus, the helper virus provides the necessary viral proteins for recognition and transcription of the Send-CAT RNA. That the Send-CAT RNA is also replicated in these cells is evidenced by the presence of full-length positive sense transcripts within cells (Fig. 8A). This synthetic RNA is also packaged into progeny virions as CAT activity is expressed even after multiple passages in tissue culture and the time course of CAT expression approximately follows the kinetics of Sendai virus in tissue culture cells. Therefore, this Send-CAT RNA is analogous to the described fusion DI RNAs of Sendai virus (Amesse *et al.*, 1982; Re *et al.*, 1984). This provides evidence that fusion DI viruses can be transcriptionally active.

A unique finding is that the Send-CAT RNA molecule requires neither *in vitro* encapsidation nor special methods of transfection. This is in contrast to the influenza system, which absolutely requires the addition of purified viral core proteins to reconstitute biologically active RNP. The difference may be due to the fact that influenza virus replicates in the nucleus, whereas paramyxoviruses are exclusively limited to the cytoplasm. It is probable that the nuclear localization signals present in the influenza viral core proteins are required to properly target the transfecting RNA. Nevertheless, in our case, simple DEAE/dextran-mediated transfection of naked Send-CAT RNA transcripts were sufficient for expression and rescue as long as viral proteins were provided via helper virus infection. Perhaps the small size of our fusion DI-like RNA (924 nucleotides) allows for the transfection, subsequent encapsidation and recognition by the Sendai polymerase. How can this recognition occur? The basic model for transcription and replication of paramyxovirus is that during replication, assembly of nucleocapsid by nucleoprotein (NP) is concomitant with RNA synthesis off the intact nucleocapsid (Vidal and Kolakofsky, 1988). Therefore, replicative templates are always encapsidated. Since Send-CAT RNA is not encapsidated *in vitro* (Fig. 6), in order for it to be expressed and replicated it is possible that the helper virus polymerase can recognize and replicate the naked RNA. The subsequent replication products would be biologically active and encapsidated. Alternatively, the full-length naked RNA is first assembled into nucleocapsid *in vivo* before polymerase recognition. In this regard, naked VSV DI RNA was previously shown to be encapsidated *in vitro* through incubation with an infected cell cytoplasmic extract (Mirakhur and Peluso, 1988). However, our similarly derived Sendai virus infected-cell extract does not encapsidate the Send-CAT RNA to any great degree. This may simply reflect different properties of rhabdo- and paramyxoviruses or it may relate to intrinsic differences in the VSV DI RNA and the Send-CAT RNAs, the former of which has been selected *in vivo* by its ability to replicate.

The levels of CAT expression can be markedly enhanced by the addition of cytoplasmic extract from either mock-infected or Sendai-virus infected cells. This enhancement is reflected both in the expression of CAT activity and in the replication of the Send-CAT RNA, as the efficiency of rescue into progeny virions mirrors the levels of CAT activity observed during the initial transfection/ infection (data not shown). This enhancement is not due to increased transfection efficiencies (data not shown) implying that a specific interaction with a cellular protein(s) is involved. The ability of host proteins to stimulate transcription of negative-strand viruses is well documented. It has been reported that β-tubulin not only stimulates the transcription of both VSV and Sendai, but is an absolute requirement for Sendai virus (Moyer *et al.*, 1986). Host factors have also been identified as a requirement for transcription of another paramyxovirus, parainfluenza type IV (De *et al.*, 1990). In addition, a minor microtubule-associated protein (MAP) has been shown to increase VSV transcription *in vitro* (Hill and Summers, 1990). Finally, the replication of other cytoplasmic RNA viruses such as poliovirus and another paramyxovirus, Newcastle disease virus, has also been shown to require host cell cytoplasmic skeleton factors (Lenk and Penman, 1979; Hamaguchi *et al.*, 1985). The stimulatory activity that is detected in our recombinant system may in fact be due to one of the proteins described above, or it may result from the action of an alternative factor which may act to enhance polymerase recognition or packaging of the Send-CAT RNA *in vivo.* In addition, with increasing concentrations, both ICE and UCE completely inhibit replication of Send-CAT RNA. The fact that UCE inhibits at much lower concentrations than ICE may indicate that Sendai virus possesses the ability to modulate the activity of factors inhibitory to its own replication. By adding non-specific RNA such as tRNA during the pre-incubation, the inhibition by UCE or ICE could be partially overcome (data not shown). This suggests that the inhibitory factor(s) may be some non-specific RNA binding protein.

Of premier interest, these data show that the synthetic RNA contains all the signals required for expression, replication and packaging of viral RNA. Thus, the recombinant RNA expression/ rescue system described here will now allow for simple and quantitative studies of the RNA signals present within the paramyxovirus genome. These include the sequences responsible for RNA editing, the intergenic sequences, and the regulatory sequences present at the termini of the paramyxovirus genome.

III.

An *In Vivo* Model for Pseudo-Templated Transcription in Sendai Virus

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A. INTRODUCTION

Sendai virus, a paramyxovirus, is an enveloped virus that has a nonsegmented negative-sense RNA genome of 15.3 kilobases (kb). The genome (vRNA) is transcribed from the 3' end by the viral polymerase, generating an extracistronic leader RNA followed by six transcription units (*NP, P/C, M, F, HN*, and *L*, in 3' to 5' order) (for reviews, see Cattaneo, 1991; Galinski, 1991; Galinski and Wechsler, 1991). The viral polymerase complex, which consists of the L (Large) and P (Phospho) proteins, is thought to terminate and reinitiate at each gene junction, capping and polyadenylylating all mRNAs except the leader RNA. The vRNA also serves as a template for the synthesis of a full-length positive-sense complementary RNA (cRNA), which is the replication intermediate. In contrast to mRNA, both the vRNA and cRNA are unmodified and always found encapsidated by the nucleoprotein (NP) into nuclease-resistant helical nucleocapsids (NCs).

Most paramyxoviruses have the unique property of promoting the transcription of mRNAs containing nontemplated insertions at one locus in the *P/C* gene. Thus, the paramyxovirus *P/C* gene is transcribed into mRNAs that usually differ by one (i.e., measles and Sendai) or two (i.e., simian virus 5 and mumps virus) nontemplated G nucleotide insertions (Cattaneo *et al.*, 1989; Paterson and Lamb, 1990; Thomas *et al.*, 1988; Vidal *et al.*, 1990a). There is a short consensus sequence among these viruses (3' UUYUCCC 5', where Y is either a U or C) where the viral polymerase is suggested to stutter and co-transcriptionally insert one or more G residues (Vidal *et al.*, 1990a). A model for how the consensus and its neighboring sequences may promote one or two G insertions for various paramyxoviruses has been proposed (Vidal *et al.*, 1990b). First, the polymerase pauses at or near the insertion site due to an intrinsic property of the template. When the pause time is sufficiently long, slippage of the nascent mRNA occurs by one (Sendai and measles virus) or two bases (mumps virus and SV5). This way, the polymerase reiteratively adds one or two G's at the slippage site. The insertion(s) creates a frameshift in the ribosomal reading frame, resulting in the synthesis of polypeptides with common N-terminal but alternate C-terminal sequences. For simian virus 5 (SV5) and mumps virus, the edited mRNA is translated into P protein (Paterson and Lamb, 1990; Thomas *et al.*, 1988) whereas for Sendai and measles virus, it usually gives rise to a cystein-rich V protein (Cattaneo *et al.*, 1989; Vidal *et al.*, 1990a). For Sendai virus, and likely for other viruses, one outcome of this type of gene expression appears to be a down-regulation of RNA synthesis by proteins sharing domains for a protein-protein interaction(s) but with alternate functional modules (Curran *et al.*, 1991).

A number of questions remain unanswered over the nature of RNA editing in paramyxoviruses. For example, what is the *cis*-acting sequence element(s) required for pausing and stuttering of the polymerase and slippage of the template? Why isn't it detected at other regions of the genome? What are the proteins involved in initiating and regulating the process of nontemplated transcription that is excluded during replication? To begin to answer these questions via reverse genetics, a model system which accurately mimics the viral RNA editing process and allows mutational analysis both at the level of RNA as well as proteins would be fundamental.

Recently, we have developed an artificial mini-genome that could be expressed, replicated, and packaged efficiently by Sendai virus (Park *et al.*, 1991). The RNA mini-genome consists of the anti-sense chloramphenicol acetyltransferase (CAT) coding region flanked by 145 and 119 5' and 3' terminal sequences derived from the Sendai virus genome. Since the mini-genome is derived from *in vitro* transcription of appropriately constructed recombinant DNA (pSend-CAT), mutations or sequence manipulations introduced at the DNA level would in turn alter the RNA mini-genome. A similar system developed for the influenza virus (Luytjes *et al.*, 1989; Yamanaka *et al.*, 1991) has allowed for new approaches to molecular analyses of negative strand virus replication (Huang *et al.*, 1990; Huang *et al.*, 1992; Luo *et al.*, 1991). An analogous method was also applied to respiratory syncytial virus (Collins *et al.*, 1991), leading to the mutational analysis of the transcription/replication signal(s). In this study, we have developed an *in vivo* model for RNA editing in Sendai virus. By inserting a short nucleotide stretch spanning the editing site of the *P/C* gene into the mini-genome, we have reconstituted the pseudo-templated transcription process using the mini-genome as a template.

B. MATERIALS AND METHODS

1. Virus and Cells

Sendai virus (Sendai/52) was grown in 10-day old embryonated chicken eggs as described (Park *et al.*, 1991). Madin-Darby bovine kidney (MDBK) cells were used for all transfection experiments. Cytoplasmic viral RNAs were prepared from virus infected baby hamster kidney (BHK) cells.

2. Construction of Plasmids

The original plasmid construct was made via PCR mutagenesis using the original pSend-CAT transcription vector (Park *et al.*, 1991). Briefly, two separate PCR fragments were synthesized, and then joined via a common *Bam*HI site. One PCR product was constructed off pSend-CAT via an oligo which contains in 5' → 3' order: a *Bam*HI site, the *P/C* sequence and CAT sequence (5'-GCGCCGGATCCCGACTCAACAAAAAAGGGCATAGGAGAGAAACATCATCTATGAGAAAAAAATCACTGGG-3',p primer-A) and an oligo spanning the *Hind*III site in pSend-CAT (primer-B, Fig. 9A). The second PCR product was constructed off pSend-CAT using an oligo containing a *Bam*HI site followed by 3' untranslated sequences from pSend-CAT (5'-CCCCCGG-

ATCCATCGTGAACTTTGGCAGC-3', primer-C) and an oligo encompassing the *Eco*RI, *Ear*I, and 3' end of the Sendai genome (primer-D). The PCR products were joined at the engineered *Bam*HI site and cloned into the *Eco*RI/*Hind*III sites of pUC19 (pPC-CAT). All subsequent constructs were made via PCR mutagenesis by cloning into the unique *Bam*HI/*Acc*I sites surrounding the *P/C* sequence (Fig. 9A).

3. *In Vitro* Transcription and Transfections

T7 RNA polymerase run-off transcription of *Ear*I-digested plasmids was performed according to the manufacturer's instructions (Gibco-BRL). The transcript was purified by multiple phenol/chloroform extractions, and the yield was quantitated on a 1.2% agarose gel. Approximately 1 μg of RNA was used to transfect a 35-mm dish of MDBK cells (about 10^6 cells) via DEAE-Dextran as described (Luytjes *et al.*, 1989).

4. CAT Assays and Passaging of Transfectant Viruses

CAT assays were performed 18 hours after transfection as detailed (Gorman *et al.*, 1982). Viruses were grown in serum-free minimal essential medium containing 0.2% bovine albumin and trypsin (5 μg/ml) for 48 hours after transfection. 100 μl of undiluted supernatant was injected into 10-day old embryonated eggs for high-titer transfectant virus preparations. Allantoic fluid was harvested after 48 hours and tested for rescued PC-CAT virus through infection of monolayer and CAT assays.

5. Cytoplasmic RNA Preparation

Approximately 10^7 BHK monolayer cells were infected with egg-grown transfectant viruses at an moi $\cong 10$ for 20 hours at 33°C. The infected cells were disrupted in a hypertonic buffer containing 0.6% NP-40, and the viral nucleocapsids and the infected cell cytoplasmic RNA were separated on a CsCl gradient (20-40%) as described (Leppert *et al.*, 1979). The nucleocapsid band was pooled and dialyzed against TE buffer (10mM Tris, 1mM EDTA pH 8.0) for 1 hour, then treated with 500 units of micrococcal nuclease (MN) for 30 minutes at 37°C. The RNA pellet and nucleocapsid band were extracted with phenol/chloroform, precipitated in ethanol, and used for polymerase chain reaction (PCR) and limited primer extension analysis.

6. Polymerase Chain Reaction

Approximately 2 μg of purified cytoplasmic RNA was added in a first-strand cDNA synthesis reaction containing 10 mM Tris-HCl (pH 8.3)/ 50mM KCl/ 1.5 mM $MgCl_2$/ 0.1% (w/v) gelatin/ 1 mM $MgCl_2$/ 2.5 mM each of dNTPs/ 1 unit of RNAsin (Promega, Madison WI)/ 2.5 units of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) and one µg of downstream primer specific for the CAT gene. Either 5'-TGAAAACGTTTCAGTTTGC-TCATGGAAAAC-3' for analyzing mRNA (primer-1) or 5'-GACCCTTTGCTTTGCTGCCAAAGTTCACGATGGATC-3' for analyzing vRNA (primer-2) were used. The mixture was incubated at 42°C for 30 minutes, and the enzyme was inactivated at 99°C for 15 minutes. PCR was performed by diluting the mix to 100µl in the same salt buffer with 0.4 mM $MgCl_2$ and adding one µg of upstream primer specific for the *P/C* gene (primer-2 for analyzing mRNA or primer-1 for vRNA) and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus *AmpliTaq*™, CA). The reaction was carried out at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes, for 30 cycles.

7. Limited Primer Extension Analysis

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by electrophoresis on a 7M urea/12% polyacrylamide. Primers were end-labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Approximately 25 to 50 ng of PCR products were mixed with 5 x 10^6 cpm of labeled primer in 40 mM Tris-HCl (pH 7.5)/ 20 mM $MgCl_2$/ 50mM NaCl, boiled for 3 minutes and allowed to anneal at 37°C for 20 minutes. Either ddA or ddT and appropriate mixes of 3 dNTPs were added to a final concentration of 150 μM and the mixture was incubated at 37°C for 30 minutes after adding 2 μl of 1:8 diluted Sequenase (in 10mM Tris-HCl, pH7.5/ 5mM DTT/ 0.5 mg/ml BSA, US Biochemical). For the PC-CAT PCR product, primer-3 (5'-CTCATAGATGATGTGTTCTCTCCTA-3') was used with dideoxy ATP (Fig. 12B), and for Para 45, 44, and 46 PCR products, primer-4 (5'-CACGATGGATCCCGACT-3') was used with dideoxy TTP (Fig. 10A).

8. Differential Oligonucleotide Hybridization

PCR products were digested with *Bam*HI and *Eco*RI (Fig. 12A) and cloned into an appropriately digested pUC19 vector. Colonies were replica plated to nitrocellulose filters and screened by oligonucleotides as detailed (Vidal *et al.*, 1990a). Primers 5'-CTCCTATGCCCTTTTTTGT-3', 5'-CTCCTATGCCCCTTTTTTG-3' and 5'-TCCTATGCCCCCTTTTTTG-3' were end-labelled and used in screening for 3G, 4G and 5G colonies, respectively. Probes were pre/hybridized in 5X SSC/ 0.5% nonfat dry milk/ 0.05% pyrophosphate for overnight at 37°C and washed in 1X SSC/ 0.1% SDS/ 0.05% pyrophosphate three times at room temperature for 5 minutes each, then at 50°C for 20 minutes. After the filters were air dried, autoradiography was performed as described, and appropriate colonies were picked and grown overnight in 3-ml TB containing ampicillin. Plasmid DNAs were prepared accordingly (Sambrook *et al.*, 1989) and sequenced by dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using Sequenase (US Biochemical).

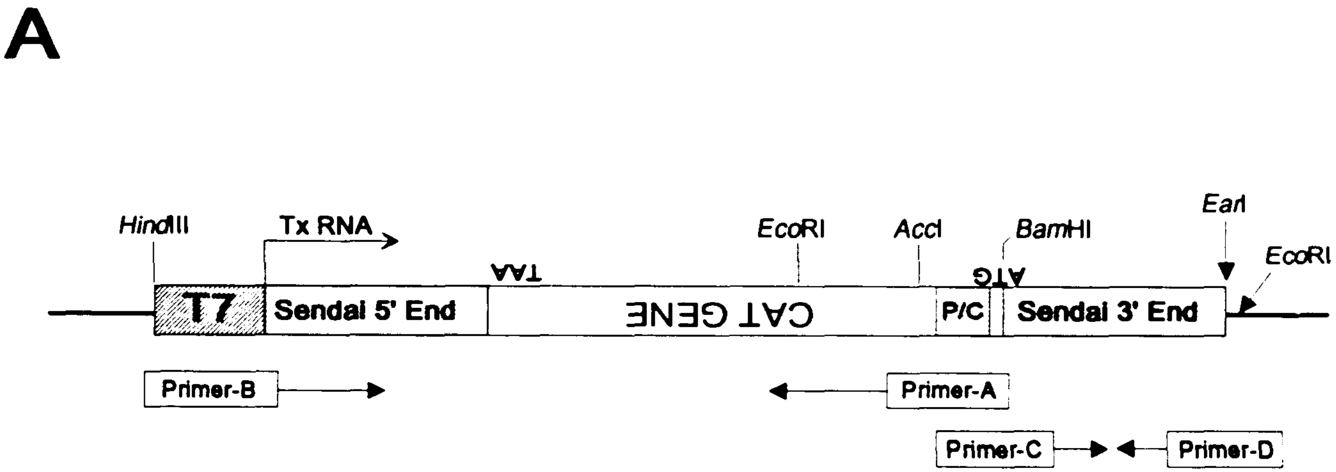
C. RESULTS

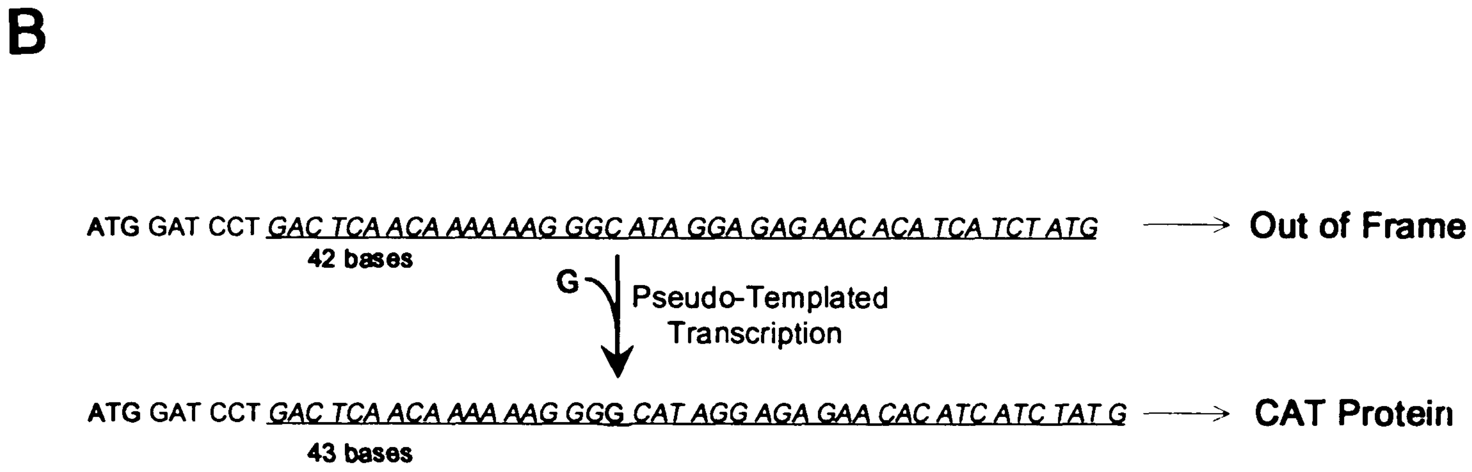
1. RNA Editing Detected by CAT Expression

Previously we have shown that synthetic RNAs containing the 5' and 3' terminal sequences of the Sendai virus genome could be expressed, replicated and packaged very efficiently after transfection in Sendai virus infected cells (Park *et al.*, 1991). In this system, the specific expression of the artificial genome, Send-CAT, was conveniently assayed by expression of the CAT gene inserted between the viral 5' and 3' sequences. Using this system, we attempted to reconstitute the RNA editing event that occurs during transcription of the *P/C* gene of Sendai virus.

Initially, a 42-nucleotide sequence derived from the Sendai virus *P/C* gene spanning the G insertion site was placed two codons downstream of the initiating methionine residue of the CAT gene (Fig. 9A). The resulting construct, PC-CAT, is identical to its parent, Send-CAT, except for the insertion at the initiation codon. The clone was constructed such that the downstream CAT coding region was shifted out of reading frame by one base, so that PC-CAT should not express CAT. If the Sendai virus polymerase recognizes the *P/C* sequence, it may insert one or more G residues. If a single guanine nucleotide were inserted by the viral polymerase, the CAT gene would be returned to the correct reading frame, and CAT activity should be restored, assuming that the 16 amino acid insertion at the amino terminus of the CAT gene is a tolerated insertion (Fig. 9B).

Figure 9. (*A*) DIAGRAMMATIC REPRESENTATION OF RELEVANT REGIONS OF pPC-CAT. The CAT gene is in an antisense orientation (as indicated by upside down letters) relative to the T7-derived RNA transcript. After the initiating methionine codon, the gene is interrupted by a *Bam*HI linker and the insertion of a 42-nucleotide sequence derived from the *P/C* gene. The CAT gene is flanked by 145 and 119 noncoding nucleotides of the Sendai virus genome 5' and 3' termini, adjacent to the T7 phage promoter sequence and the restriction enzyme *Ear*I site, respectively. The primers shown below the gene represent the original PCR oligonucleotides which were used to construct pPC-CAT off the parental pSend-CAT (Park *et al.*, 1991). (*B*) AMINO-TERMINUS SEQUENCE OF THE PC-CAT GENE. The faithful transcript translates into a protein which is out of frame with the subsequent CAT protein. However, if pseudo-templated transcription acts to insert a single G nucleotide, the correct reading frame is restored and CAT protein should be expressed. The *P/C* gene sequences are underlined.


A
HindIII
Tx RNA
EcoRI
AccI
BamHI
EarI
EcoRI
TAA
ATG
T7
Sendai 5' End
CAT GENE
P/C
Sendai 3' End
Primer-B
Primer-A
Primer-C
Primer-D


B
ATG GAT CCT GAC TCA ACA AAA AAG GGC ATA GGA GAG AAC ACA TCA TCT ATG
42 bases
Out of Frame
G
Pseudo-Templated Transcription
ATG GAT CCT GAC TCA ACA AAA AAG GGG CAT AGG AGA GAA CAC ATC ATC TAT G
43 bases
CAT Protein

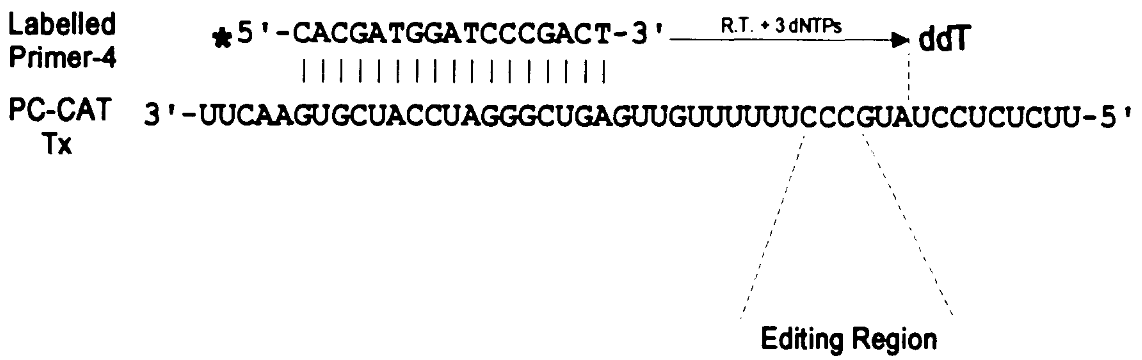
Initially, the PC-CAT RNA was transcribed *in vitro* by T7 bacteriophage RNA polymerase off *EarI* digested pPC-CAT. In order to make sure that this vRNA-like transcript was faithful and did not contain any insertions, this transcript was subjected to a limited primer extension analysis. RNA was hybridized with an end-labelled primer (Fig. 10A) and subjected to extension with reverse transcriptase in the presence of dATP, dGTP, dCTP, and dideoxy TTP (Fig. 10A). The product will extend through the presumptive editing region and terminate to produce a 32-nucleotide product. The resulting 32-nucleotide single band (and a lack of 33-nucleotide product) indicated that the transcript did not incorporate any extra nucleotides during the T7 transcription reaction (Fig. 10B). This is consistent with the previous finding that pseudo-templated transcription is not detected when the *P/C* mRNA was expressed by an alternate RNA polymerase via a vaccinia virus recombinant (Vidal *et al.*, 1990a).

The vRNA-like transcripts are then transfected into cells which are subsequently infected with Sendai virus (Fig. 11A). The helper virus will express, replicate, and package the synthetic RNA transcript. Assays for CAT activity can be used to monitor the transfection. As shown previously and used here as a positive control, the Send-CAT RNA gives rise to a relatively high level of

CAT expression after transfection and Sendai virus infection (Park *et al.*, 1991; Fig. 11B, lane 1). When the PC-CAT RNA was transfected, a relatively small but reproducible amount of CAT expression was detected (Fig. 11B, lane 2). This activity could be the result of pseudo-templated transcription of the PC-CAT mRNA through G insertion by the viral polymerase. Since "edited" version of *P/C* mRNA during a normal virus infection has been estimated to be only about 20-31% of the total *P/C* mRNA produced (Vidal *et al.*, 1990a; Pelet *et al.*, 19), the reduced level of CAT activity would be expected. On the other hand, the CAT activity could also result from unedited mRNA by mechanisms such as an alternate transcription start or a translation frameshift (i.e., pseudoknot). To further address this possibility, the PC-CAT RNA was rescued into progeny viruses and grown to a high titer by passaging undiluted through embryonated chicken eggs (see Fig. 11A). We have previously suggested that the Send-CAT RNA may be analogous to a fusion defective-interfering (DI) RNA and we were able to partially test and confirm one characteristic of a DI RNA by serial passaging of undiluted transfectant viruses (Fig. 11B, lanes 3 and 4).

Figure 10. (*A*) SCHEMATIC OF THE LIMITED PRIMER EXTENSION ANALYSIS. The sequence of the region of the PC-CAT transcript (Tx) where the primer hybridizes is shown. The predicted G insertion site is indicated as the editing region below. The termination site of the extension reaction by reverse transcriptase (RT) after incorporating dideoxythymidine is 15 bases from the start site, thus generating either a 32-nucleotide (no editing) or a 33-nucleotide product (1 G nucleotide inserted). (*B*) Limited primer extension was carried out and analyzed on a PAGE-urea gel. Sequencing reaction of pPC-CAT with the same primer was used to show the proper termination at the first Thymidine residue. A 33-nucleotide extension product was used as a molecular weight marker.

A


Labelled Primer-4
✱5'-CACGATGGATCCCGACT-3'
R.T. + 3 dNTPs
ddT
PC-CAT Tx
3'-UUCAAGUGCUACCUAGGGCUGAGUUGUUUUUUCCCGUAUCCUCUCUU-5'
Editing Region

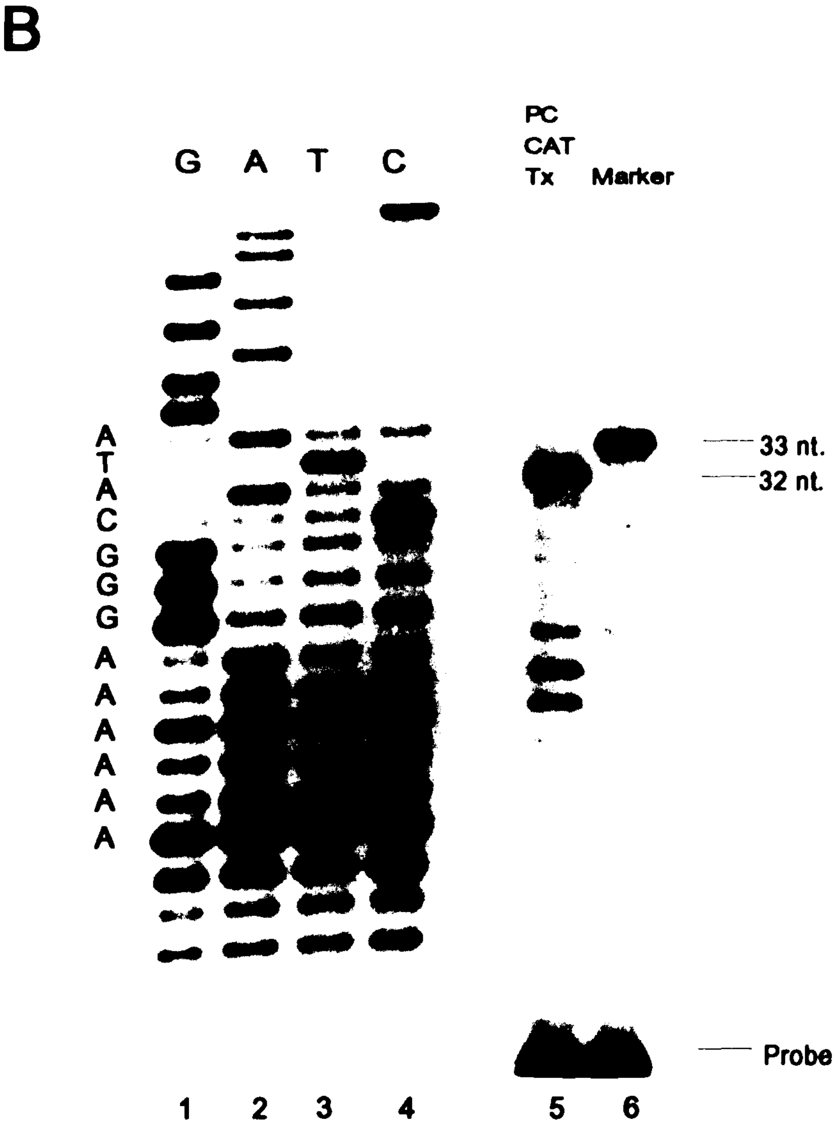
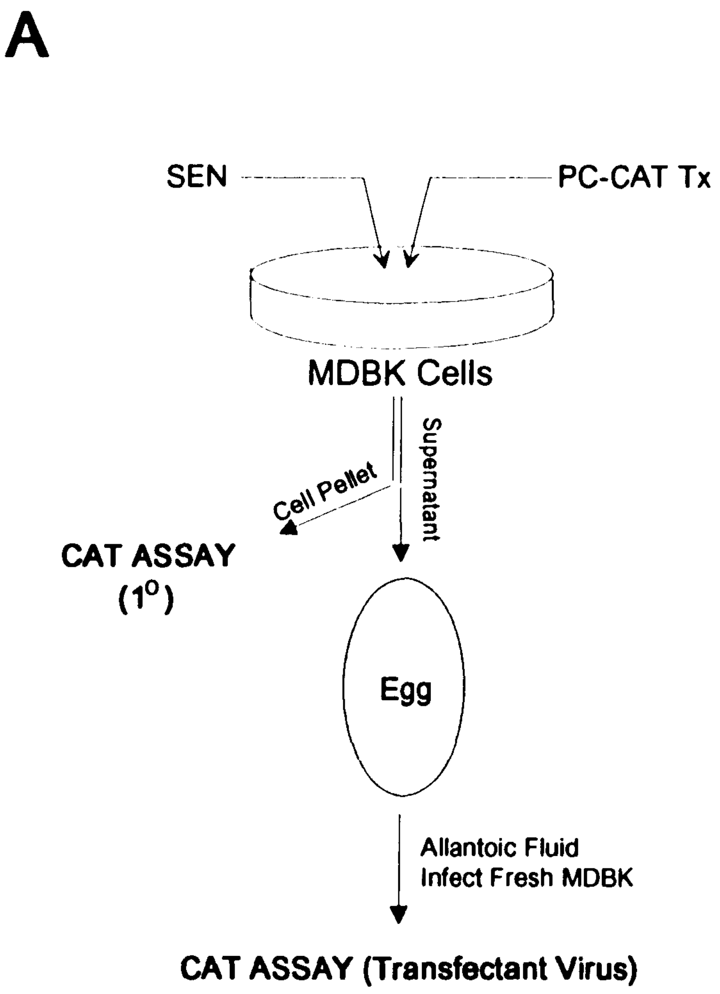
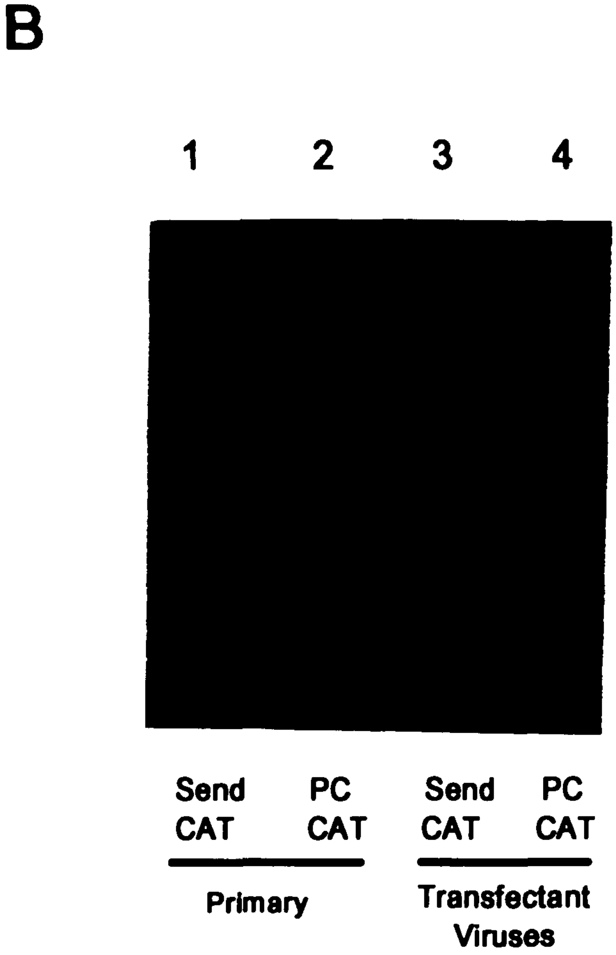

B
G
A
T
C
PC
CAT
Tx
Marker
A
T
A
C
G
G
G
A
A
A
A
A
A
33 nt.
32 nt.
Probe
1
2
3
4
5
6

Figure 11. (*A*) PROTOCOL FOR OBTAINING HIGH-TITER TRANSFECTANT VIRUSES. "SEN" indicates wildtype Sendai virus infection 1 hour after transfection. (*B*) COMPARATIVE CAT ASSAYS AFTER TRANSFECTION OF RNA OR INFECTION WITH THE TRANSFECTANT VIRUSES GROWN IN EGGS. Cells were harvested either 18 hours after transfection (1°) or 12 hours after infection (transfectant viruses). Equivalent amounts of RNA or allantoic fluids were used for both Send-CAT and PC-CAT.


A
SEN
PC-CAT Tx
MDBK Cells
Cell Pellet
Supernatant
CAT ASSAY
(1°)
Egg
Allantoic Fluid
Infect Fresh MDBK
CAT ASSAY (Transfectant Virus)


B
1
2
3
4
Send CAT
PC CAT
Send CAT
PC CAT
Primary
Transfectant Viruses

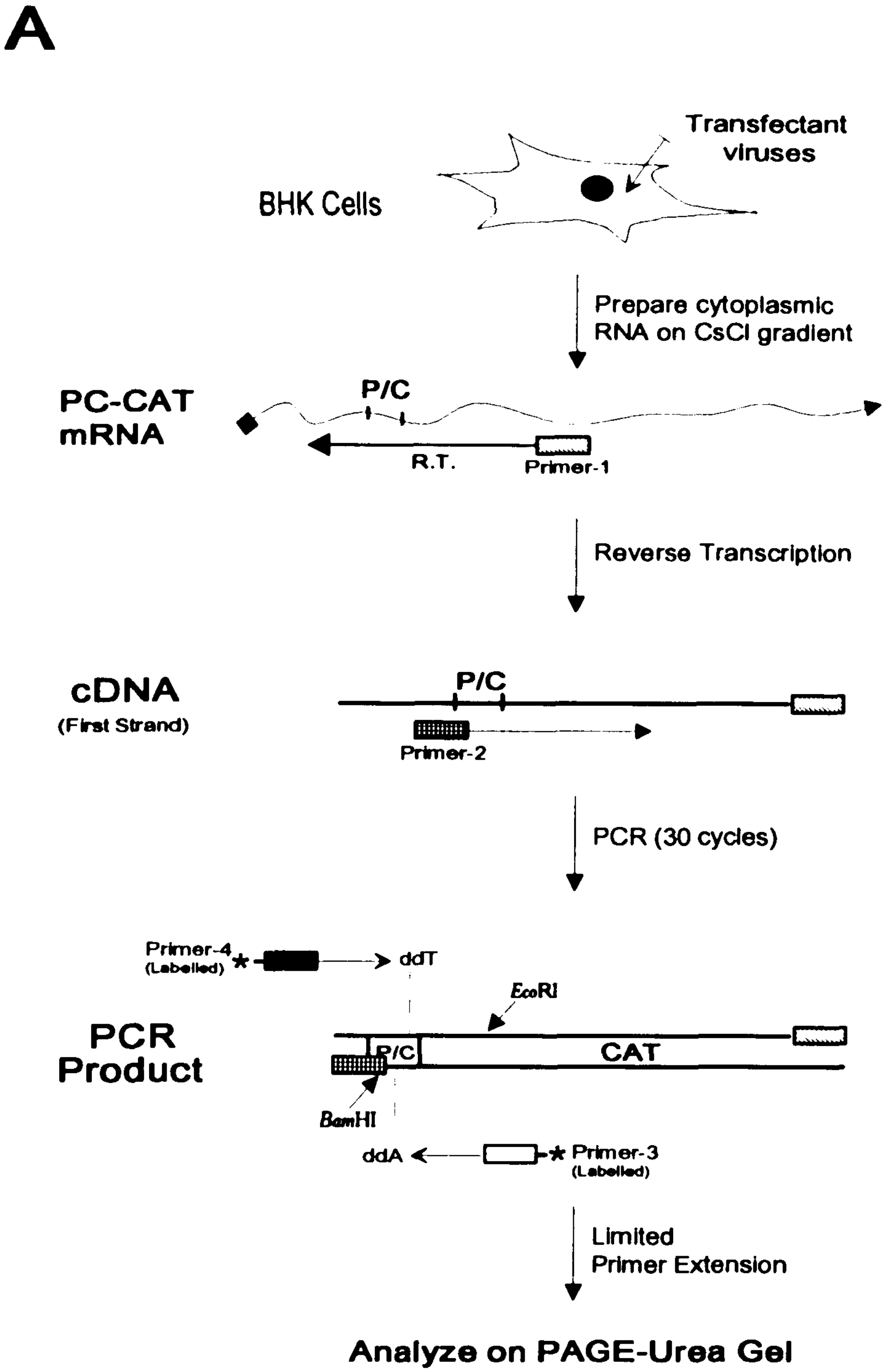
2. Identification of PC-CAT mRNA with G insertions at the correct position.

To determine whether the observed CAT activity was due to PC-CAT mRNA which had undergone pseudo-templated transcription, the rescued transfectant viruses were used to infect large numbers of BHK cells (~10^8 cells). These cells were permeabilized with lysolecithin and cytoplasmic RNA and nucleocapsids were isolated via CsCl gradient centrifugation (Leppert *et al.*, 1979). The PC-CAT infected cytoplasmic RNA was amplified by reverse transcription/ PCR using primers specific for the CAT gene (primer-1) and the inserted *P/C* gene sequence (primer-2) (Fig. 12A). These primers only extend on the synthetic CAT construct and not on viral *P/C* gene mRNA. To control for any possible artifacts which may arise during the reverse transcription and PCR process, the T7-transcribed PC-CAT vRNA was also amplified at the same time. These PCR products were then analyzed by limited primer extension analysis with T7 DNA polymerase (Sequenase, US Biochemical) and *P/C* gene-specific primers in the presence of dGTP, dTTP, dCTP and dideoxy ATP (Fig. 12B). The extension product would terminate at the first Adenosine, which is 16 nucleotides from the end of the primer and would read through the proposed editing site (Fig. 12B).

Therefore, faithful transcripts should align with the first Adenosine in the sequence (Fig. 12C, lane 2) while mRNAs with additional G's inserted would align with bands of increased size. By this analysis, an edited "4G" product was clearly detected when the PCR sample from PC-CAT mRNA was used, whereas with PC-CAT transcript PCR, only the unedited "3G" product was evident (Fig. 12C, lanes 5 and 7). PCR fragments corresponding to mRNA's with more than one insertion could not be definitely identified. However, when the PC-CAT mRNA PCR sample was cloned and screened by a differential oligonucleotide hybridization method that could distinguish 3G's from 4G's or 5G's at the putative editing site (Vidal *et al.*, 1990a), about 10% of the clones contained 4G's while less than 1% had 5G's (Table I). Again, no 4 or 5 G's were identified when the control PC-CAT transcript PCR sample was screened (data not shown). Finally, sequencing analysis of the positive 4G and 5G clones confirmed that the clones contained one and two nontemplated G insertions at the precise position, respectively (Fig. 13). In addition to mRNA, we also examined the PC-CAT vRNA purified from nucleocapsids isolated from a CsCl gradient. In order to further purify the RNP, the banded nucleocapsid was additionally treated with micrococcal nuclease. Since RNP's of Sendai virus are nuclease resistant, this would remove any traces of cytoplasmic RNA which may have co-purified with the nucleocapsid. The limited primer extension analysis showed no evidence of an edited product (Fig. 12B, lane 6), and screening by oligonucleotide hybridization exclusively detected the genomic-like 3 G sequence (data not shown). This strongly suggests that the vRNA in our model system undergoes RNA editing with similar constraints to the intact viral genome, as it occurs during transcription but not replication (Vidal *et al.*, 1990a).

Figure 12. (*A*) PROCEDURE FOR DETECTING EDITED RNA BY PCR AMPLIFICATION AND LIMITED PRIMER EXTENSION ANALYSIS. For analysis of mRNA, primer-1 was used in the first-strand synthesis reaction and primer-2 was added for PCR amplification. For analysis of transcript or rescued vRNA, primer-2 was used in the first-strand synthesis and primer-1 was used for amplication. (*B*) LIMITED PRIMER EXTENSION ANALYSIS ON PCR PRODUCTS. The sequence of the end-labelled primer and template are shown above the figure. The three PCR products indicated CYTO, NC, and Tx are those corresponding to the cytoplasmic RNA, nucleocapsid, and transcript, respectively. A sequence reaction of pPC-CAT using the same labelled primer was used as a size marker and proper termination site indicator. The unedited and edited (+ 1G) products are indicated by 3G and 4G, respectively.

Figure 13. DNA SEQUENCING OF THE cDNA CLONES OF PC-CAT mRNA. Clones positive for the 3G (unedited), 4G (+ 1G), and 5G (+2G) oligoes were sequenced by the dideoxy-NTP method. ● and ➤ indicates unedited 3G's and inserted G's, respectively.


A
Transfectant viruses
BHK Cells
Prepare cytoplasmic RNA on CsCl gradient
PC-CAT mRNA
P/C
R.T.
Primer-1
Reverse Transcription
cDNA
(First Strand)
P/C
Primer-2
PCR (30 cycles)
Primer-4
(Labelled)
ddT
EcoRI
PCR Product
P/C
CAT
BamHI
ddA
Primer-3
(Labelled)
Limited Primer Extension
Analyze on PAGE-Urea Gel

B

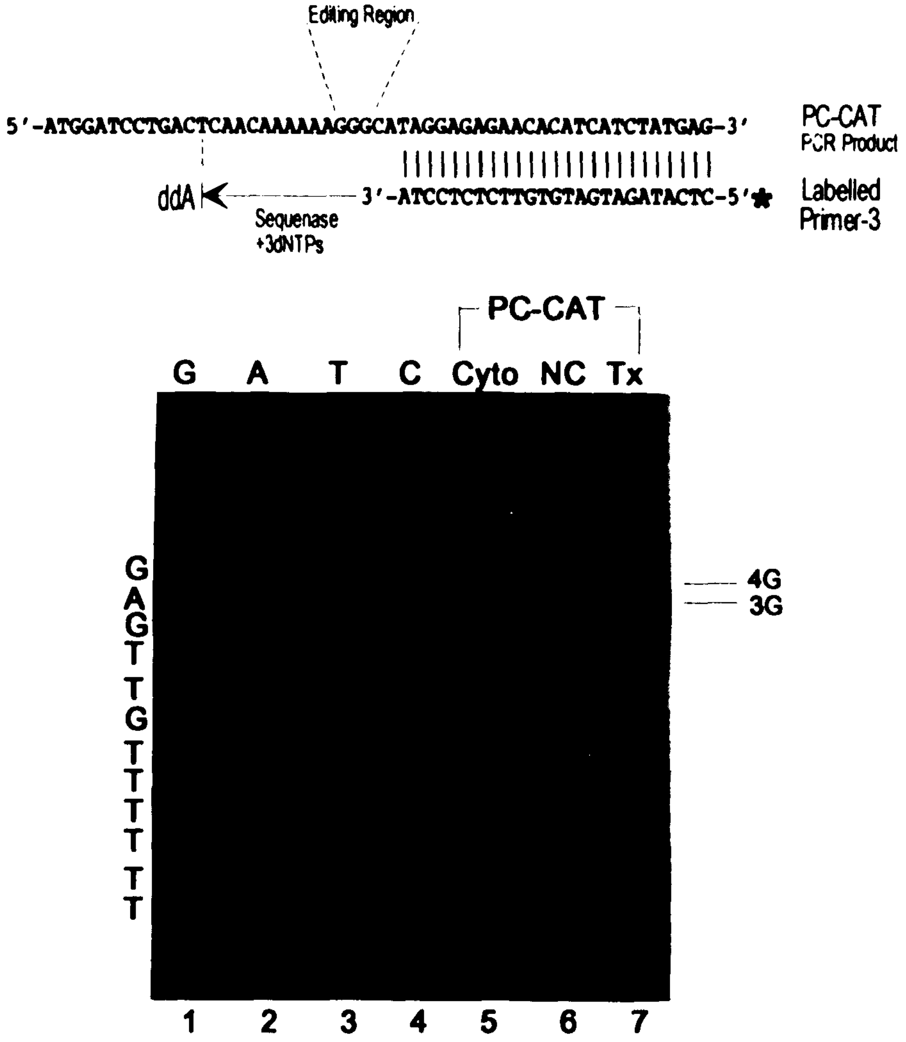
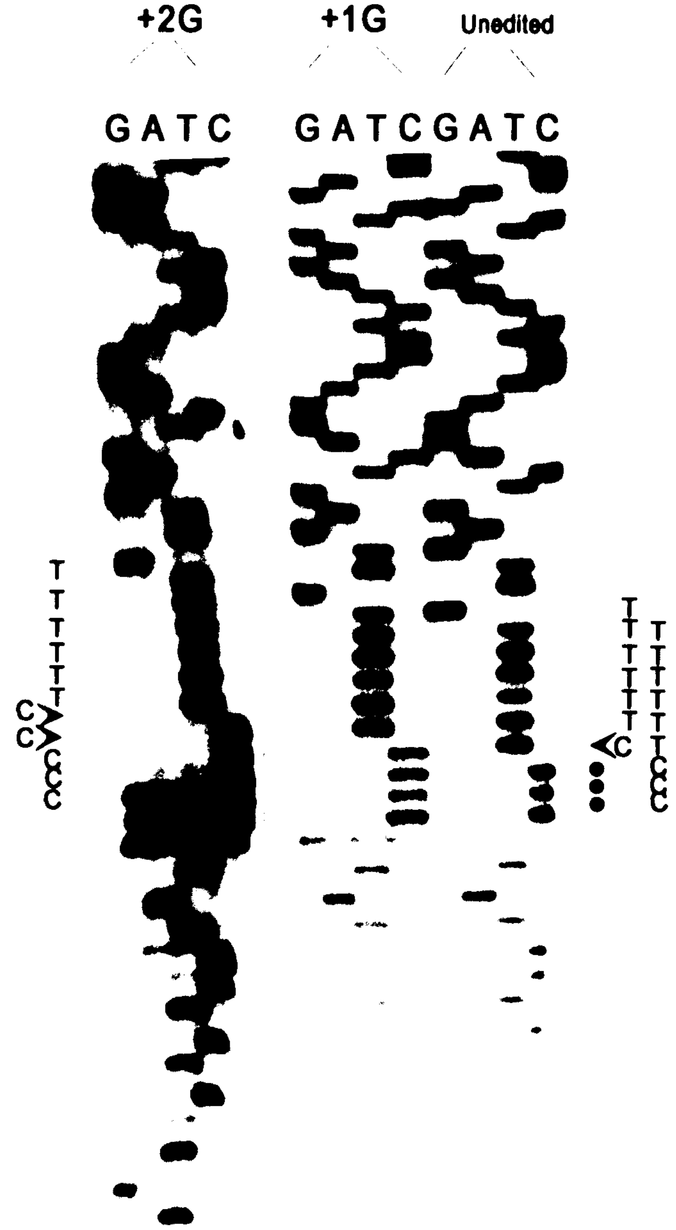

Editing Region
5'-ATGGATCCTGACTCAACAAAAAAAGGGCATAGGAGAGAACACATCATCTATGAG-3'
PC-CAT
PCR Product
ddA
3'-ATCCTCTCTTGTGTAGTAGATACTC-5'
Labelled
Primer-3
Sequenase
+3dNTPs
PC-CAT
G
A
T
C
Cyto
NC
Tx
G
A
G
T
T
G
T
T
T
T
T
T
4G
3G
1
2
3
4
5
6
7

TABLE 1. Frequency of G-nucleotide insertions in PC-CAT mRNA

Experiment #	No. (%) of PC-CAT cDNA clones with the indicated number of G's inserted		
	0	1	2
I	168 (94%)	10 (5.6%)[1]	1 (0.6%)[1]
II	178 (94%)	11 (6%)	0 (0%)
III	125 (91%)	12 (8.7%)	1 (0.7%)
Total (%)	471 (93.1%)	33 (6.5%)	2 (0.4%)

[1] These clones were picked and sequenced. All those sequenced showed the correct number of inserted G nucleotides.


+2G
+1G
Unedited
G A T C
G A T C G A T C
T
T
T
T
T
T
T
C
C
C
C
C
T
T
T
T
T
T
C
T
T
T
T
T
T
T
C
C
C

3. 3' Deletions of the *P/C* Gene Editing Sequence

To determine whether sequences 3' to the G insertion site were essential for pseudo-templated transcription, three new constructs were made which contained progressive deletions of 6 nucleotides (Fig. 14A). Thus, Para 45, 44, and 46 contained 36, 30 and 24 bases of the *P/C* gene, respectively. All of these constructs still retained the conserved 3'-UUUUCCC-5' consensus sequence. The three new derivatives were then transfected, rescued, and used to prepare cytoplasmic RNA. After reverse transcription and PCR, the limited primer extension analysis of the DNA showed that all three constructs underwent editing during transcription by the viral polymerase and now contained an extra nucleotide (Fig. 14B). Cloning, screening, and sequencing of the PCR product of the shortest clone (Para 46) confirmed the presence of pseudo-templated transcription products. Although a smaller sample size was screened, 2 of 35 clones of Para 46 PCR products contained a single G insertion. Thus, a sequence as short as 24 nucleotides could induce editing by the viral polymerase. It should be noted that an additional band corresponding to a one base shorter extension fragment is also evident in Fig. 14B (lanes 5-8). The intensity of this band is highly variable between experiments and is always present in control lanes (Fig. 12B, lane 7). As PCR products shorter by one base have never been isolated we presume that this band is an artifact due to premature termination prior to the dideoxy base addition.

Finally, one other construct was tested for its ability to induce pseudo-templated transcription. Para 54 is identical to PC-CAT except the 3 C's in the consensus sequence were changed to 3 G's (Fig. 14A). When this construct was rescued after transfection and analyzed by limited primer extension, no visible edited product was evident (Fig. 14B). This strongly suggests that the Sendai virus editing mechanism specifically requires the stretch of C's in the vRNA for the insertion to occur, and the substitution by G residues reduces the efficiency of editing to below the detectable level.

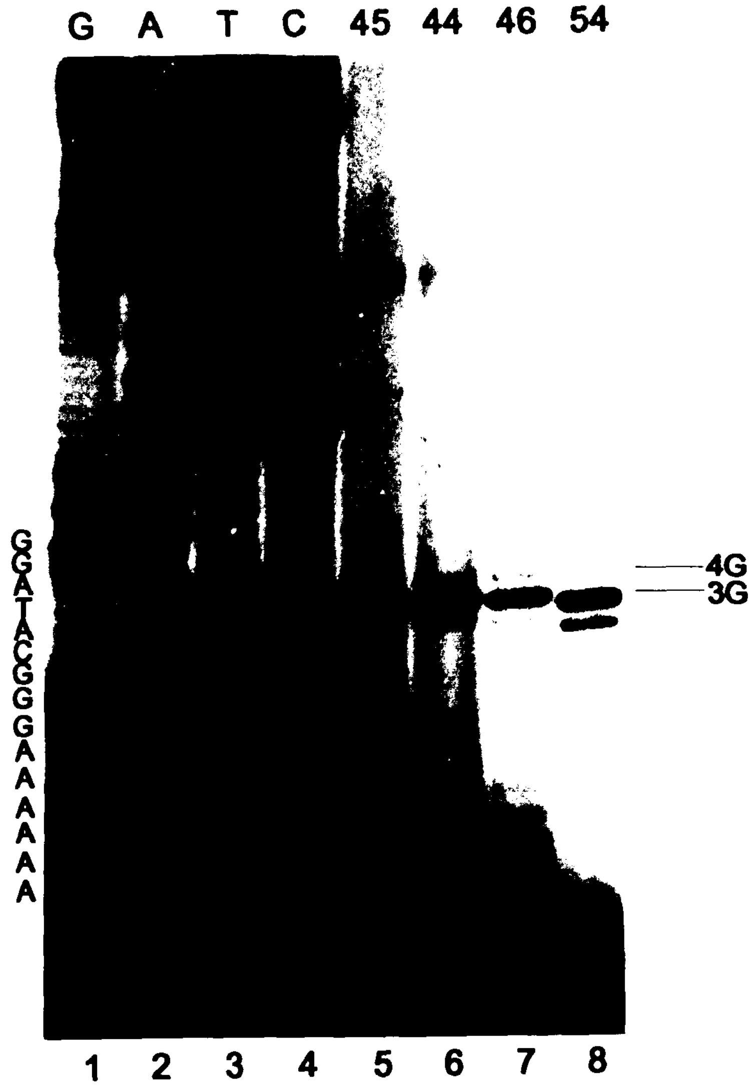
Figure 14. (A) SEQUENCE OF 3' DELETION MUTANTS. Para 45, 44, and 46 contain progressive deletions of 6 nucleotides from the 3' end, whereas Para 54 contains 3C's (shaded) instead of the G's in the consensus sequence. The predicted insertion site is indicated by an arrow. (B) LIMITED PRIMER EXTENSION ANALYSIS ON PCR PRODUCTS OF CYTOPLASMIC RNA DERIVED AFTER INFECTION BY RESCUED VIRUSES OF THE MODIFIED CLONES. The primer for extension is shown in Fig. 10A. Sequence of pPC-CAT using the same primer is shown to indicate the proper termination site at the first Thymidine residue. The unedited and edited products are indicated by 3G and 4G, respectively.

A

G

PC-CAT mRNA	GA CTC AAC AAA AAA GGG CAT AGG AGA GAA CAC ATC ATC TAT G
Para 45 vRNA	CU GAG UUG UUU UUU CCC GUA UCC UCU CUU GUG UAG U
Para 44 vRNA	CU GAG UUG UUU UUU CCC GUA UCC UCU CUU G
Para 46 vRNA	CU GAG UUG UUU UUU CCC GUA UCC UA
Para 54 vRNA	CU GAG UUG UUU UUU $\boxed{\text{GGG}}$ GUA UCC UCU CUU GUG UAG UAG AUA C

B



D. Discussion

The recent success in developing artificial genomes for negative-strand RNA viruses has greatly increased our ability to apply reverse genetics and understand the structure-function requirements of viral elements (Collins *et al.*, 1991; Huang *et al.*, 1990, 1992; Luo *et al.*, 1991; Luytjes *et al.*, 1989; Park *et al.*, 1991; Pattnaik and Wertz, 1990, 1991; Yamanaka *et al.*, 1991). In particular, the analyses of various *cis*-acting signals such as those directing transcription, replication, and packaging of the viral genome have benefited immensely, since mutations are introduced into the model genome with ease and quantitations of the effects of the modifications are accomplished by simple assays of reporter gene expression. Combined with the ability to express viral proteins *in vivo* by T7- or vaccinia-based vectors (Curran *et al.*, 1991; Huang *et al.*, 1990, 1992; Pattnaik and Wertz, 1990, 1991), the artificial genome system provides a powerful tool to dissect and understand the functions of individual *cis*- and *trans*-acting elements as well as their interactions.

In this report, we describe the successful reconstitution of pseudo-templated transcription by the Sendai virus polymerase by placing a 42-base nucleotide sequence derived from the *P/C* gene into the model genome. The initial evidence was provided by CAT expression, which by design, would come from a nontemplated G nucleotide insertion. This was proven by limited primer extension analysis, followed by cloning and sequencing studies. By our assay procedure, which entails rescuing the recombinant genome into transfectant viruses and using these to infect and prepare cytoplasmic RNA samples, we have found that about 10% of the mRNA contained a G insertion at the precise position and less than 1% contained a 2G insertion. During normal paramyxovirus infection, insertion of nontemplated nucleotides occurs only during transcription and not during replication (Cattaneo, 1991). Our model PC-CAT template behaved accordingly, as we did not detect any edited PC-CAT replication products by limited primer extension and oligonucleotide screening. We did, however, find one edited (one G insertion) clone derived from vRNA upon screening of the shorter Para 46 construct (data not shown), but we believe this is probably due to either contamination or an error introduced during our cloning procedure. In this regard, Paterson and Lamb (1990) have also found one mumps virus vRNA cDNA clone after sequencing 70 clones, which was considered an error introduced by reverse transcriptase or *Taq* DNA polymerase. Since no edited PCR product was found when the T7-expressed transcript was used as a starting material for first-strand synthesis and PCR amplification, we feel errors due to our cloning procedure would be introduced at an extremely low rate.

Previous studies have found that during normal Sendai virus infection, 20-31% of viral *P/C* mRNA had a single G insertion while 5-7% had multiple G insertions (Pelet *et al.*, 1991; Vidal *et al.*, 1990a). This variation in frequencies was attributed to be a result of the actual method of quantitation (Pelet *et al.* 1991). In our studies, reverse transcription, PCR amplification and cloning was used and levels of approximately 6% and 0.5% of single and double G insertions were respectively found. Further studies are needed to examine whether this lower value for pseudo-templaed transcription is an inherent property of the multiple steps in our quantitation system or if the PCAT RNA or its derivatives are lacking a regulatory element which may promote more efficient editing. However, it is clear that transcription of a sequence as short as 24 nucleotides is sufficient to promote this polymerase induced event.

From the studies of the known and predicted editing sites among paramyxoviruses, it has been suggested that the linear sequence element plays only a minor role in directing the precise G-nucleotide insertions (Vidal *et al.*, 1990a). This is due to the fact that the consensus site is only weakly preserved among different paramyxoviruses with a strict consensus of only 7 nucleotides present among all paramyxoviruses. In addition, this consensus site is present multiple times throughout the Sendai genome and even in other regions of the *P/C* gene itself without promoting editing. However, the data presented here suggest that the primary sequence does play a major role in editing. In this respect, the 3' sequence deletion analysis strongly suggests that a sequence element as short as 24 nucleotides (Para 46 construct) sequence can by itself confer the ability to undergo RNA editing at the precise position. This property is context-independent, since we have removed these bases from all other *P/C* gene sequences and placed it within a foreign gene. In addition, a substitution of the 3 G residues by C's in the consensus sequence appears to abrogate editing in our system, indicating that there is a very specific sequence requirement. Clearly, a more extensive mutational analysis of the sequence element should allow a better understanding of the sequence requirements.

Although the efficiency of editing appears to be lower than the observed value for the viral genome, the basic rules and mechanisms of RNA editing appears to be preserved in this model. For example, the number of G's inserted heavily favors a single G residue, rather than two or more, and the insertion occurs at the correct predicted position. Furthermore, the editing is only detected as a Sendai virus-encoded activity and is not seen when other polymerases, such as the T7 RNA polymerase, T7 DNA polymerase (Sequenase), M-MLV reverse transcriptase, and *Taq* polymerase are used. Thus, the results obtained with the PC-CAT genome or its derivatives should provide valuable insights into the mechanism of pseudo-templated transcription by the Sendai virus polymerase.

IV. SUMMARY

Our understanding of the molecular properties of paramyxovirus genome thus far has been limited due to a lack of a suitable approach for introducing specific mutations or modifications into the genome. Ideally, one would like to have a cDNA of the entire viral genome, make changes and synthesize vRNA *in vitro*, then efficiently rescue the mutant genome into infectious virions in the absence of any contaminating helper virus genome. The assessment of mutations introduced would then be direct, by simply studying the phenotypic behavior of the rescued mutant. Unfortunately, no such system exists as yet.

The biggest problem associated with studying the paramyxovirus genome is its large size and the negative polarity of the RNA. Having a size of ~15 kb makes it extremely difficult to manipulate at the cDNA level, synthesize vRNA *in vitro*, and transfect into cells. Having a negative polarity, by definition, means that the naked RNA alone is not infectious and thus necessitates one to supply viral proteins *in trans*. In combination, the problems posed mandate a model genome with limited capabilities but nonetheless enables all the appropriate manipulations and assays.

In the first half of this thesis, a suitable model genome was developed for Sendai virus. The model genome, Send-CAT, permits manipulation at the cDNA level, vRNA synthesis *in vitro*, and efficient rescue into virions. The helper virus infection must be provided for replication, expression, and rescue. Consequently, the presence of contaminating wildtype genome is inevitable in this system. One alternative would be to provide the viral proteins through expression vectors, rather than helper viruses. This has been recently achieved for VSV, where DI RNAs were packaged into progeny virions completely free of nondefective genomes (Pattnaik *et al.*, 1992).

In the second half, we utilized the Send-CAT genome to develop an *in vivo* replica of nontemplated transcription by Sendai virus. Here, a sequence element spanning the editing site of the *P/C* gene was taken out of its normal context, put into the model genome, and still retained its fundamental properties. Presumably, the primary and local secondary structure of this element provides the minimally needed regulatory signal for editing. By making deletions from the 3' end of the editing site (in vRNA sense), we showed that the sequence element could be narrowed down to 24 nucleotides.

Undoubtedly, the work done in this thesis represents only a small progress in the field of paramyxovirus studies. Our complete understanding of the molecular properties associated with paramyxovirus genome transcription and replication will require extensive mutational analyses of both the genome and the viral proteins. It is hoped that the studies presented in this thesis will provide some guidance and point to possible pitfalls in future efforts.

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