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SHORT COMMUNICATION

Rescue of Synthetic Measles Virus Minireplicons: Measles Genomic Termini Direct Efficient Expression and Propagation of a Reporter Gene

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Measles virus (MV) mRNA transcription and replication are thought to be controlled by *cis*-acting sequence elements contained within the terminal MV genomic noncoding nucleotides. To validate these promoter and regulatory signal assignments, cDNAs were constructed allowing synthesis of RNAs corresponding to a MV genome in which all coding and intercistronic regions were replaced by the chloramphenicol acetyl transferase (CAT) coding sequence. Transcript production by T7 polymerase starting and ending precisely with the MV genome terminal residues was achieved by fusing the T7 polymerase promoter and the hepatitis delta virus genome ribozyme followed by tandem T7 polymerase termination sequences to the MV genomic 5′ and 3′ ends, respectively. Transfection of these negative polarity transcripts, mimicking natural defective interfering RNAs of the internal deletion type, into MV-infected 293 cells gave rise to CAT activity which could be serially transferred and massively amplified together with progeny helper virus in fresh cells. Transfer was blocked only by antibodies able to neutralize MV infectivity, indicating that the chimeric RNA not only was encapsidated, transcribed, and replicated, but also packaged into virions. Sequence analyses confirmed that both the expected chimeric antigenome and mRNA products were transcribed and replicated with fidelity during serial passage. Minor changes introduced in the transcription promoter markedly compromised function. This system now can be exploited to examine MV genomic *cis*-acting regulatory elements and extended to the development of full-length MV cDNAs.

Measles virus (MV) is a notoriously contagious infectious agent causing sustained epidemic disease associated with severe, even fatal complications, particularly among very young children in developing countries. These features account for MV's continued prominence as a major international health concern (*1*) while pointing to limitations in current vaccine efficacy whose correction ultimately depends on expanded understanding of MV molecular biology and disease pathogenesis.

MV is a *morbillivirus* (*2*). Its genome, like those of its *Paramyxovirus* relatives, is an ~16-kb single-stranded, nonsegmented RNA of negative polarity organized in six contiguous, nonoverlapping, virus protein-encoding genes and additional distinctive nonprotein coding sequence elements thought to direct the transcriptional and replicative pathways. These putative *cis*-acting regulatory domains are localized to the 3′ and 5′ ends of the MV genome and to short regions surrounding each intergenic boundary (*3–5*). The former are thought to contain promoter and/or regulatory sequence elements directing genomic transcription, genome and antigenome encapsidation, and replication. The latter specify transcription termination and polyadenylation of each monocistronic mRNA followed by incrementally less efficient reinitiation of transcription of the next gene.

Identifying and exploring putative MV genome-encoded *cis*-acting regulatory signals has awaited the development of systems by which defined, synthetic MV genome RNA analogs can be generated and then assembled with the virus-specified encapsidating (N) and polymerase (L and P) proteins into functional ribonucleoprotein templates (*6*). This strategy, in fact, has been successfully used recently for the study of several related virus genomes (*7–15*). Artificial RNAs indistinguishable in sequence from naturally occurring influenza virus gene segments (*9*) or well-characterized vesicular stomatitis virus (VSV) (*12*) and Sendai virus (*11*) defective RNA genomes of the copy-back type are efficiently replicated when provided a pool of prerequisite virus-specified *trans*-acting proteins. Genome congeners resembling fusion type defective interfering RNAs (DIs), in which the

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short 3′ and 5′ viral genomic noncoding termini (NCT) are fused to the bacterial chloramphenicol acetyl transferase (CAT) reporter gene open reading frame (ORF), also assume transcriptional and replicative template activity in the presence of proteins specified by homologous helper virus. Such chimeric RNA analogs of a gene segment of influenza virus (*7*) as well as of the genomes of Sendai virus (*10*), respiratory syncytial virus (RSV) (*8*), human parainfluenza virus 3 (HPIV3) (*13, 14*), and rabies virus (*15*) are of particular interest because their expression, amplification, and propagation confirm the contention that the genomic termini of negative strand RNA viruses encompass all *cis*-acting determinants of transcription, replication, and packaging.

This approach now has been extended to the morbillivirus measles. Synthetic MV genome RNA analogs, consisting only of the MV NCTs flanking precisely the CAT ORF, have been generated from corresponding cDNAs. Introducing these MV minigenome RNAs into helper-virus-infected cells leads to the assembly of functional nucleocapsid templates whose MV genomic signals actuate their efficient transcription, replication, amplification, and packaging into viral particles. Thus, the means to simply and quantitatively appraise MV genome-encoded *cis*-acting promoter and regulatory signals has been established and now can be exploited to examine and define their nature and role in detail.

Figure 1 shows the design of plasmid p107MV:CAT, a cDNA from which the synthetic MV genome RNA analog, MV:CAT (−), can be generated. The MV:CAT cDNA insert is placed within a Bluescript plasmid modified so as to produce MV:CAT (−) chimeric RNA with the correct MV genomic NCTs consisting of 107 nt (3′ NCT) and 109 nt (5′ NCT), respectively (Fig. 1A). This was achieved by engineering the T7 bacteriophage RNA polymerase promoter abutting the 5′ terminal MV genomic cDNA sequences to initiate transcription with the MV 5′ end A nucleotide, while the 3′ MV terminal U was generated by hepatitis delta virus (HDV) ribozyme-mediated site-specific endonucleolytic cleavage (*16*) of the run-off transcripts terminated at T7 termination sites (*17*). Other, alternative, cDNA templates for run-off transcription of MV:CAT (−) RNA also have been constructed. These include: (1) plasmids containing a *BsaI* restriction site in place of the HDV ribozyme and T7 terminator sequences; and (2) PCR products generated from appropriate plasmids by using one primer specific for the absolute MV genomic 3′ end and the other for the T7 promoter and contiguous 5′ MV genomic terminal nucleotides. Run-off transcription reactions using these cDNA templates produce identical 876-nt synthetic MV:CAT chimeric RNAs, each having the structural characteristics of a MV internal deletion DI. Less than 1.5% of the nucleotide complement of the standard MV genome is contained within the 3′ and 5′ NCTs of this MV:CAT (−) RNA analog. Figure 1B defines the sequence and location of putative *cis*-acting regulatory elements contained within these NCTs.

Purified synthetic MV:CAT (−) RNA transcripts were transfected into 293 cells previously infected with MV to provide the needed virus-specified *trans*-acting gene products (Fig. 3). The infected/transfected cells then were incubated until extensive, typical viral cytopathic effect (CPE) developed (~24 hr) at which point they were harvested and assayed for CAT activity. As shown in Fig. 2A, lane 2, CAT enzyme was expressed in the above system indicating that a transcriptionally active MV:CAT genome template had been formed. Run-off transcripts generated from each of the above three cDNA templates served equally well as MV genome RNA analogs and, therefore, are not distinguished in the presentation of the results. In addition, the capacity of various tissue culture lines, selected for their ability to support MV reproduction, to serve as helper cells in this MV:CAT expression system was appraised. Reproducible CAT enzyme expression was achieved only in 293, HeLa, and Vero cells; all results described here were obtained using 293 cells.

Successful CAT expression was entirely dependent upon both infection with helper virus and transfection with synthetic MV:CAT (−) RNA (Fig. 2B). Treatment of completed run-off transcription reactions with RNase-free DNase prior to transfection had no adverse effect. In contrast, neither uninfected cells transfected with MV:CAT (−) RNA, nor infected cells transfected with RNase-treated transcripts displayed detectable CAT activity.

The cDNA specifying the synthetic MV:CAT (−) RNA analog was based on the Edmonston MV vaccine strain genomic sequence (*3;* unpublished observations). This sequence, however, differed in its 3′ NCT from one reported previously (*22*) which contained a single additional U residue inserted adjacent to position 30 from the MV genome 3′ terminus. Of note, infected cells transfected with synthetic RNA produced from p108MV:CAT, which contains the corresponding nucleotide insertion (see +T near position 30 in Fig. 1A), consistently failed to give rise to any detectable CAT enzyme activity despite repeated attempts (Fig. 2A, lane 1; 108 MV:CAT). Recently, concurrent transfection/infection was found to markedly increase transfection efficiency allowing demonstration that 3′-108 MV:CAT (−) RNA can indeed specify CAT expression in infected cells, albeit at a level 1000-fold lower than that directed by the authentic 3′-107 MV:CAT (−) RNA (data not shown). Furthermore, it was possible to partially restore the capacity of 3′-108 MV:CAT to direct CAT expression by creating a double mutant in which the U insertion at position 30 is compensated by a deletion at position 40 (Fig. 2A, lane 3; 107* MV:CAT). This level of CAT expression, though readily detectable, was only ~8% (range 7.16 to 9.6%) that mediated by the nonmutated sequence. Nevertheless, it reflects

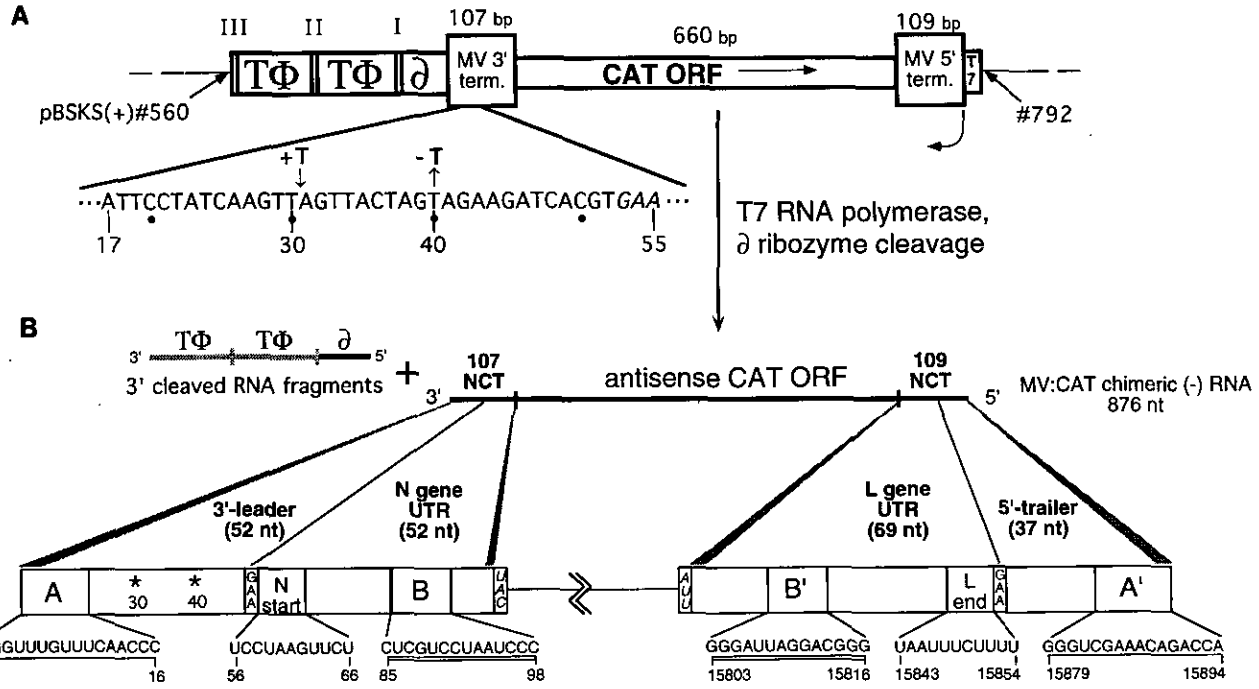

FIG. 1. Schematic representation of the vector p107MV:CAT containing the cDNA insert specifying MV:CAT (−), the MV:CAT genome RNA analog (A), and an expanded view of the predicted MV *cis*-acting transcription/replication regulatory elements (B). (A) p107MV:CAT cDNA consists of the sequence specifying the 3′-107- and 5′-109-nt NCTs of the MV genome, flanking the CAT ORF. The T7 promoter (T7) is designed such that transcription initiates with the precise 5′ terminal nucleotide of the MV genome and proceeds from right to left beyond the hepatitis delta virus (HDV) ribozyme (*δ*) comprising the 84 nt of the HDV genomic sequence (*16*). The T7 RNA polymerase terminators (TΦ) contain nt 24,106 to 24,228 of the bacteriophage T7 genome; transcription stops at 24,209 of the T7 genome (*17*). The vector backbone is based on pBluescript KS(+) (pBSKS(+); EMBL X52331), where nt 792 of the plasmid is linked to a T7 promoter. The small segments I, II, and III between *δ*, the TΦ terminators, and nt 560 of pBSKS(+) are derived from cloning steps. These sequences are (read on the bottom strand) from right to left: I. 5′-GCGGCCGATCCGG-3′; II. 5′-CCGGATCGGCCGATCCGG-3′; III. 5′-CCGGATGGCCGCCACCGGTGG-3′. p108MV:CAT is the plasmid in which an additional T is inserted at position 30; the plasmid p107* MV:CAT contains both the insertion at position 30 and a deletion of the T at position 40. (B) T7 RNA polymerase transcription yields an artificial internal deletion MV:CAT DI RNA beginning with the 5′ terminal nt of the MV genome and ending with the sense HDV ribozyme and sequence(s) of the T7 terminators. Mediated by the site-specific endonucleolytic cleavage activity of the ribozyme, the 876-nt chimeric MV:CAT (−) RNA having the precise 3′ terminal nt of the MV genome, is formed. This RNA analog is denoted 3′-107 MV:CAT to signify that it contains the authentic 3′-107 NCT of the MV genome.

An expanded view of the MV NCTs shows the location and sequence of putative *cis*-acting regulatory signals for transcription and replication. All MV sequences are presented in genome sense, 3′ to 5′ (i.e., as the negative strand; the numbering follows the normal 5′ to 3′ orientation of the antigenome). The 3′ NCT of the MV:CAT (−) chimeric RNA is 107 nt consisting of 52 leader encoding nt [3′-leader] and 52 nt specifying the untranslated region of N message [N gene UTR], separated by the highly conserved trinucleotide punctuation [GAA] which flanks each MV gene. The start codon complement of the N sequence coincides with that of the CAT ORF [broken line] which replaces the MV sequences up to the stop codon complement of the L gene. Thereafter follows the 109-nt-long 5′ NCT composed of 69 nt specifying the 3′ untranslated region of L [L gene 3′ UTR], the intergenic trinucleotide [GAA], and the 37 nt of the 5′ MV trailer.

The putative 3′ extended promoter region of the MV genome spans nt 1 through 98. It is bounded by two highly conserved sequence domains, A of 16 nt and B of 14 nt. Each of these is highly complementary to a sequence element (A′ and B′) present within the 5′-109 NCT of the MV genome. The MV specific signals for MV:CAT mRNA transcription initiation and transcription termination/polyadenylation are probably contained in the 11-nt N gene start sequence (position 56–66) and the 12-nt L gene end (positions 15,843–15,854) and adjacent trinucleotides GAA, respectively. Nucleotide position numbers refer to the full-length, 15,894-nt MV genome RNA. * marks the positions of nucleotide changes in the 3′-107 MV:CAT chimeric RNA defined in the sequence shown in A that alter CAT expression.

a nearly 100-fold increase over the 3′-108 MV:CAT-directed level of CAT expression.

How a single additional U residue, inserted after the 30th nucleotide of the authentic 3′ MV genomic leader sequence, can attenuate CAT expression by three orders of magnitude is as yet undefined. Little insight into this question can be extracted from studies examining the effects of nucleotide changes on CAT gene expression directed by synthetic genome analogs of related viruses, both because such manipulations have not yet been extensively explored and because those examined have focused primarily on nucleotide changes at or near the genomic termini. No significant deleterious effect follows the addition of one or two nucleotides to the absolute 5′ genomic terminus of the HPIV3:CAT genome analog (*13*). In contrast, one nucleotide addition to the 3′ terminus of this same artificial HPIV3 genome is sufficient to abolish CAT gene expression (*13*), indicating that the absolute 3′ end of the genome must be preserved for the polymer-

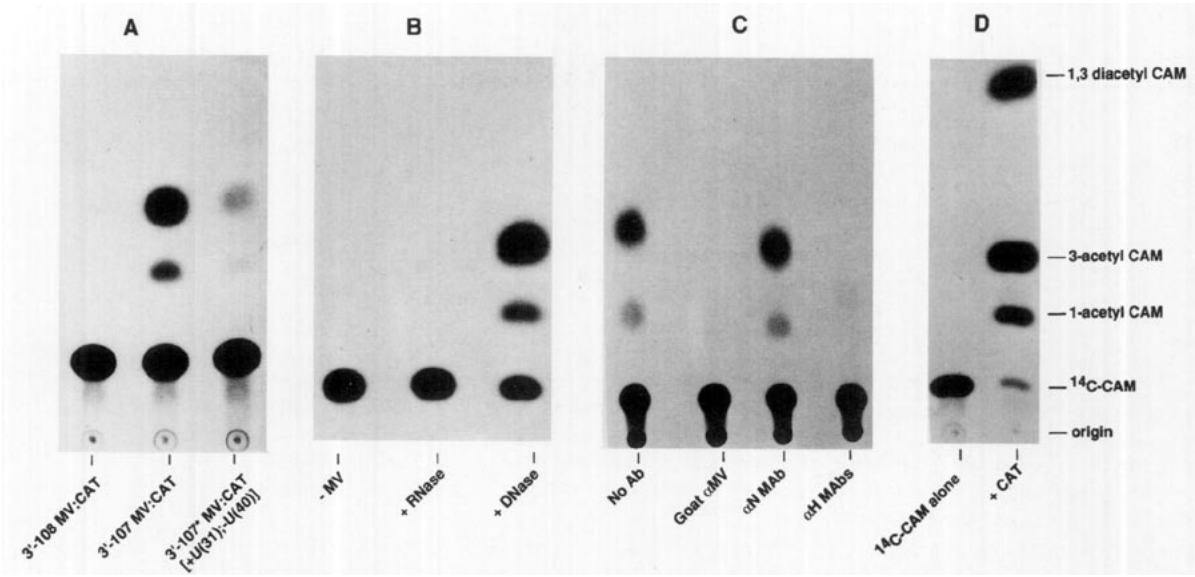

FIG. 2. Synthetic MV:CAT (−) RNA directs expression of CAT enzyme when introduced into MV-infected helper cells and can be further passaged in the form of MV:CAT genome-containing virions. Purified run-off transcripts of MV:CAT (−) were generated from their cognate cDNAs using T7 RNA polymerase as directed by the supplier (Promega). Unless noted otherwise, all completed *in vitro* transcription reactions were treated with RNase-free DNase (RQ 1; Promega) and deproteinized by phenol extraction. Transcript quantity and purity were determined by absorbance at 260 and 280 nm and its integrity appraised by agarose gel electrophoresis. At least 75% of the transcripts generated from p107 MV:CAT were processed by ribozyme-mediated endonucleolytic cleavage to the correct MV:CAT (−) RNA analog prior to being used for transfection.

All transfections were performed with Lipofectace (Gibco/BRL). Each involved transfection of ~2 μg of synthetic MV:CAT (−) RNA into subconfluent monolayers of 293 cells (~2 × 10^5 cells growing in 3.5-cm dishes) infected with a high titer stock (~2 × 10^8 PFU/ml) of the Edmonston B strain of MV free of detectable DI particle activity (*25*). Infections were performed in serum-free medium at a multiplicity of infection of 3–5 PFU/cell for 60 min at 32.5° followed by transfection for 3 hr at 37°. Monolayers then were repeatedly rinsed with fresh serum-containing medium. Infected/transfected cells were incubated with 2 ml of medium at 37° until extensive CPE developed (~24 hr) at which time the entire culture was collected by scraping with a rubber policeman and repeated passage through a pipette. After centrifugation, supernatants provided the inoculum both for further cell passages, whereas the resuspended cells were divided into two equal aliquots, one of which was assayed for CAT enzyme activity (*21, 27*) and the other examined for the presence of authentic MV:CAT mRNA and MV:CAT (+) antigenome replicative intermediates (see Figs. 3 and 4). (A) CAT enzyme expression directed by the authentic MV:CAT (−) RNA analog (denoted 3′-107 MV:CAT) is reduced below detectable levels by a single nucleotide insertion following position 30 in the leader encoding region (denoted 3′-108 MV:CAT). A double mutant in which this addition of a U residue of 3′-108 MV:CAT at position 30 is compensated by removal of a U at position 40 (thus, preserving the length of the authentic 3′-NCT, denoted 3′-107* MV:CAT) partially restores CAT expression. (B) CAT enzyme expression requires a RNA template and virus-specified helper functions: (1) MV:CAT RNA transfected into cells in the absence of prior MV helper virus infection; (2) Transcription reaction mixtures treated with RNase A (10 μg for 15 min at 37°); or (3) with DNase (10 units RQ 1 for 15 min at 37°) prior to transfection into MV-infected cells. (C) Propagation of CAT activity by tissue culture cell passage is selectively inhibited by measles virus neutralizing antibodies. Equal aliquots (0.5 ml) of clarified medium collected from a 10^2-cm dish of MV-infected 293 cells transfected with 3′-107 MV:CAT (−) RNA were incubated for 45 min at 37° with: (1) nonimmune goat serum, 50 μl; (2) goat anti-MV serum, 50 μl; (3) G protein affinity purified anti-MV murine monoclonal antibodies (*26*) to N protein (MAb H-14), 100 μg; or (4) H protein MAbs (H-1 and F/20), 5 μg of each. Fresh 293 cells were incubated with each treated sample for 60 min at 32.5°; the inoculum was removed by aspiration and rinsing with fresh medium and then each monolayer was superinfected with MV (3 to 5 PFU/cell) for 60 min at 32.5° to replace the progeny helper virus neutralized by antibody treatment. Monolayers displayed >90% CPE after ~24 hr at 37° when cells were harvested for CAT assay. (D) Unreacted [^{14}-C]chloramphenicol substrate alone and substrate treated with CAT enzyme under standard assay conditions to provide migration position markers. For all the above, the amount of CAT activity present in ~70 μg of cell lysate protein was determined using a 2-hr reaction and 24-hr autoradiographic exposure of the acetylated chloramphenicol products resolved by ascending thin layer chromatography (*21*).

ase complex to properly engage the nucleocapsid template. The RSV-based analogs are far more tolerant of changes. Even several nucleotides can be removed from the genomic 3′ end without notably compromising CAT expression (*8*). Consequences of nucleotide insertions within the leader region have received less attention still. Only the HPIV3:CAT genome analog has been manipulated in this manner revealing that dinucleotide insertions within this leader region severely compromise transcription (*14*). This effect is consistent with the attenuation associated with nucleotide insertion in the MV:CAT (−) leader, suggesting that paramyxovirus *cis*-acting promoter elements, in general, tolerate little or no sequence, spacing, or alignment alteration.

Perhaps the most compelling possible explanation for the deleterious effect of a single nucleotide insertion follows from the results of recent studies showing that both natural and synthetic Sendai copy-back DI's are efficiently replicated only if their RNA complement is an integral multiple of six nucleotides (*23*). Presumably, this requirement, dubbed the rule of six, reflects an obligatory interaction of six ribonucleotides with

TABLE 1

Propagation and Amplification of CAT on Serial Passage of the MV:CAT Minireplicon

Passage No.	CPE Hr to >80%[a]	Titer $\log_{10}$ PFU[b]	CAT spec. act[c]	CAT amplification[d]
p0[e]	25	5.6	1.6	1
p1[f]	30	6.6	4.5	1.2×10^1
p2	20	6.9	424	4.2×10^3
p3	20	6.4	8,340	3.3×10^5
p4	24	6.2	10,850	1.7×10^6
p5	>52[a1]	<3.0	269	1.7×10^5
p6	>52[a2]	N.D.	12	2.8×10^4

[a] All passages were harvested for assay at a time when the 293 cell monolayer displayed at least 80% CPE, with the following exceptions: [1]CPE developed slowly over 24 hr to affect only ~50% of the cells and failed to progress further; and [2]CPE never progressed beyond a few syncytia.

[b] Plaque assay of total infectious virus units released into 2 ml of medium supporting each passage (*25*).

[c] CAT enzyme activity present in lysates prepared from each passage reported as picomoles acetyl group transferred from acetyl CoA to chloramphenicol/minute/mg cell lysate protein (*27*).

[d] Fold amplification of CAT enzyme activity relative to p0 if all, rather than $\frac{1}{4}$, of the virus progeny in each passage's clarified supernatant had been used as the inoculum for the subsequent passage.

[e] p0 represents the initial transfection of synthetic genome sense MV:CAT (−) RNA into ~2×10^5 MV-infected 293 cells. After >80% of this monolayer developed CPE, cells and the 2 ml of culture fluid were collected, separated by sedimentation, and the former lysed and assayed for CAT enzyme activity while one quarter of the latter was passaged onto fresh 293 cells.

[f] One 293 cell culture was infected with the p0 clarified supernatant (0.5 ml) and the infection allowed to proceed until >80% CPE was displayed. These p1-infected cells and culture fluids then were collected; serial passage was performed in the same fashion through a total of six cycles.

each N protein subunit for template activity to be bestowed. Of note, the nucleotide length of both the standard MV genome (15,894 nt) and the MV:CAT (−) RNA analog containing the precise MV NCTs (876 nt) is a multiple of six. The same is true for the two Sendai virus-based hybrid minigenomes (*10, 24*), whereas neither RSV:CAT (*8*) nor HPIV3:CAT (*14*) are similarly constrained. The 877-nt, almost inactive 3′-108 MV:CAT (−) analog, in contrast, fails to obey this rule. Since encapsidation presumably proceeds vectorially from a nucleation site located at or near the 5′ end of the genome RNA towards the 3′ genomic terminus, a single nucleotide addition to the genome RNA, regardless of its position, would result in the assembly of a nucleocapsid structure in which the 3′ terminal nucleotide is not enwrapped. By leaving this terminal nucleotide outside its prerequisite ribonucleoprotein context, the polymerase complex would fail to recognize the 3′ terminal promoter element. From this perspective, the partial restoration of transcription promoter activity and thus CAT enzyme expression directed by the insertion/deletion mutant (3′-107* MV:CAT) may simply reflect the fact that this MV genome analog conserves an overall nucleotide length obeying the rule of six. A systematic assessment of the applicability of the rule of six to the MV genome currently is underway.

Presence of CAT activity in transfected/infected cells clearly indicates that a functioning transcriptional template was formed. To determine whether these MV:CAT (−) nucleocapsids also contained the signals needed for packaging into viral particles, fresh monolayers of 293 cells were infected with the clarified medium collected from infected/transfected cells. Abundant CAT enzyme activity developed in these cells after ~24 hr of incubation, coincident with the time they displayed extensive CPE. Passage of CAT activity was completely inhibited by pretreatment of the clarified medium with anti-MV antibody preparations, but only if they had virus neutralizing activity (Fig. 2C). Thus, polyclonal, polyspecific anti-MV goat serum containing high titers of antibodies against virion structural proteins and murine monoclonal antibodies with specificity only for the virion envelope hemagglutinin glycoprotein (H) prevented CAT activity passage. In contrast, CAT activity passage was unaffected by both preimmune goat serum (not shown) or murine monoclonal antibody to the N protein of the virion nucleocapsid core.

To ascertain whether the MV NCTs also contain the promoter and regulatory elements directing genome replication, infectious MV:CAT virus particles contained in culture supernatants were serially passaged in 293 cells. Progeny viral particles released during the initial infection/transfection (p0) served as the inoculum for the first cell passage (p1), and so on until a total of six were completed. A parallel set of passages also was performed in which each cycle was supplemented with additional Edmonston MV to guarantee a *trans*-acting helper protein pool sufficient to support multiple rounds of MV:CAT genome replication.

Both sets of serial passages demonstrated that CAT enzyme expression was transmitted through repeated cycles of infection with MV:CAT viral particles. CAT enzyme expression in each passage cycle exceeded

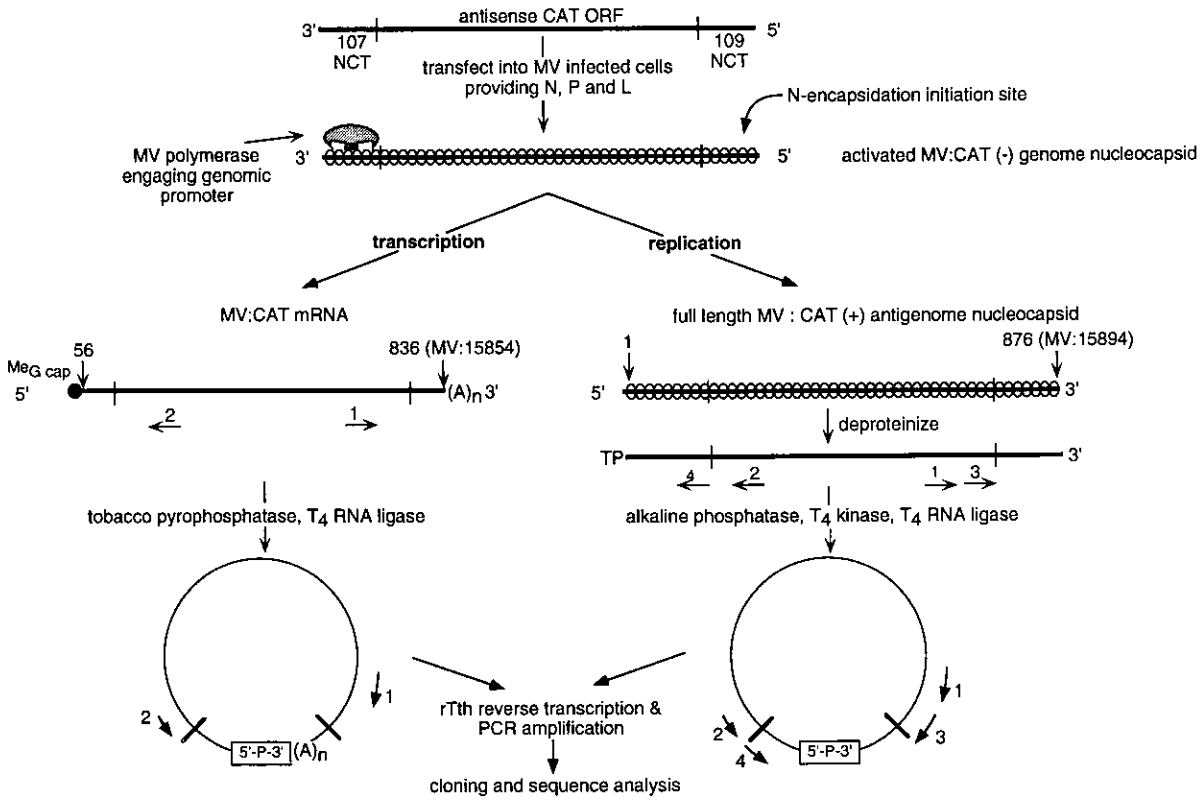

FIG. 3. Proposed scheme of the system by which synthetic MV:CAT (−) RNA analogs are converted into MV:CAT minireplicons and the strategy used to examine their transcription and replication products. The naked MV:CAT (−) RNA synthesized *in vitro* and then introduced into infected cells by transfection presumably is first encapsidated by soluble N protein (or N:P complexes (*18*)), beginning at a nucleation sequence lying at or near the MV genomic 5′ end [5′-109 NCT]. Once assembled into nucleocapsids, the 3′ MV promoter sequences of the synthetic MV:CAT chimeric RNA [3′-107 NCT] are recognized and engaged by the helper virus-specified RNA dependent RNA polymerase (soluble complexes of L and P, perhaps in association with additional MV-specified and/or host-encoded proteins (*6, 18*)). The two predicted products specified by this synthetic template depicted below are: (1) transcription product: 5′ capped and 3′ polyadenylated MV:CAT mRNA; and (2) replication product: full-length MV:CAT (+) antigenome RNA replicative intermediate. This antigenome nucleocapsid template then serves to generate new genomes when its 3′-extended promoter is engaged by the virus-specified polymerase complex (not shown).

The lower part of this figure shows the strategies used to confirm that MV:CAT mRNA and MV:CAT (+) antigenome RNA containing the expected sequences were formed. These rely on analyses of the total cell RNAs prepared from the initial MV:CAT (−) RNA-transfected/infected culture and subsequent passages by sequential guanidinium isothiocyanate disruption and phenol partitioning (*19*); studies focusing on MV:CAT mRNA included an oligo-dT (Dynabeads, Dynal Inc.) preselection step. The monophosphorylated forms of MV:CAT antigenome RNA or of MV:CAT mRNA were prepared by phosphatase/kinase (*5*) or pyrophosphatase treatment (*20*), respectively, after which they were circularized (or concatenated) by T4 RNA ligase. Templates for simultaneous complete sequence analysis of 3′ and 5′ termini of MV:CAT antigenome RNA or of MV:CAT mRNA then were generated by sequence-specific oligodeoxynucleotide-primed reverse transcription followed by polymerase chain reaction amplification (RT-PCR) across the newly created 3′−5′ ligated junction (*5, 20*). The number, sequence, and position of the oligodeoxynucleotide primers used for RT-PCR are: (1) 5′-*TATAAGCTT*AATGCTTAATGAATTACAAC-3′, nt 716−735; (2) 5′-*TATGGATCC*ATTGGGATATATCAACGGTGG-3′, nt 153−133; (3) 5′-*TATAAGCTT*ACTGCGATGAGTGGCAGGGCGGG-3′, nt 739−761; (4) 5′-*TATGGATCC*GATATCCCTAATCCTGCTCTTGTCCC-3′, nt 103−78. Primer positions are numbered relative to the 876-nt MV:CAT genome (see Fig. 1). The 5′ end of primers contain either extended *Bam*HI (primers 2, 4) or *Hind*III sites (primers 1, 3), indicated in italics, to facilitate directional cloning of the amplified cDNA into pGEM 4.

that of the originating transfected cells, regardless of whether additional helper virus was present. The level of increase, however, differed in the two parallel passage series. CAT enzyme specific activity was amplified almost two million-fold during serial passage without additional helper virus (Table 1), but only about seventy-five thousand-fold when supplemental MV was present (data not shown). In both series, CAT activity decreased after the fourth cycle, coinciding with a marked attenuation in the pace with which viral CPE developed and infectious MV progeny were produced when helper virus was omitted. Both of these changes appeared to be masked by the addition of helper virus.

Each of the chimeric synthetic paramyxovirus genome analogs reported previously (Sendai virus (*10*), RSV (*8*), and HPIV 3 (*13, 14*)) and that described here has the structural characteristics of a deletion DI. Since deletion DI RNAs and full-length genomes share the same extended transcription and replication promoter regions, the only obvious replicative advantage of these DI-like RNAs over the standard virus genome is small size. The interference potential of such tran-

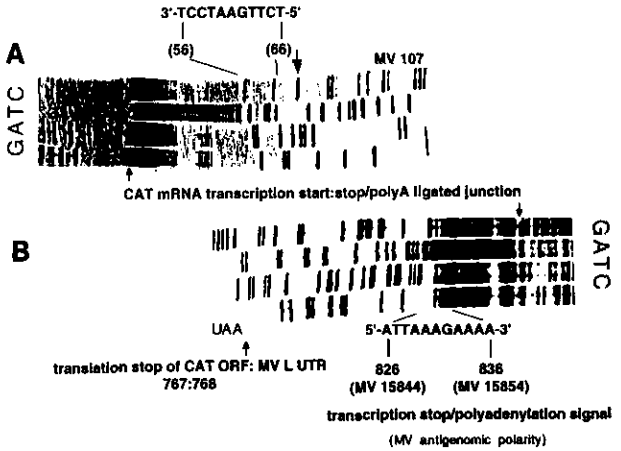

FIG. 4. The MV *cis*-acting mRNA transcription start and transcription stop/polyadenylation signals direct expression of authentic MV:CAT mRNA from the synthetic MV:CAT minigenome. The presence of the N gene start and L gene stop sequences at the MV:CAT mRNA termini was sought by the strategy outlined in the legend of Fig. 3. Each independently isolated cDNA clone was sequenced on both strands using oligodeoxynucleotide primers annealing to the flanking T7 and SP6 bacteriophage promoters of the pGEM4 plasmid. Results from selected regions of a representative pair of dideoxy chain termination sequencing gel analyses are shown. Gel A shows the sequence of the junction created by ligating the 3′ poly(A) tail of the MV:CAT mRNA to the 5′ transcription start (complementary sequence). Gel B shows the sequence analysis performed of the same clone on the opposite strand. Here, the L gene transcription termination/polyadenylation signal is revealed and is followed by a poly(A) stretch ligated to the 5′ end of the MV:CAT mRNA at its transcription start site. Also denoted (▼) is the nucleotide change from T to C at position 71 found in the MV:CAT mRNA clones sequenced, but absent from the synthetic MV:CAT genome template.

scriptionally active deletion DI genomes, therefore, is likely to be limited. Possible interference capacity of MV:CAT RNA was suggested by the serial passage experiments, particularly those from which additional helper virus was omitted. While this interference may reflect competition by the internally deleted MV:CAT chimeric RNA, it seems more likely that Edmonston MV or even MV:CAT copy-back DI's emerged in later passages, accounting for the interference with CAT gene expression and virus production noted. Studies have been initiated to explore this possibility.

Finally, the fidelity with which the MV polymerase complex transcribed and replicated the synthetic MV:CAT genome RNA analog was examined. The general approach currently used by us to create sequencing templates for the entire 3′ and 5′ morbillivirus genomic terminal regions (*5*) was adapted for the current analyses (see Fig. 3). The method entails T4 RNA ligase-mediated circularization (or concatenation) of monophosphorylated RNAs. Circularized RNAs were produced from alkaline phosphatase-treated total cell RNAs isolated from the initial infected/transfected culture or its subsequent passages. To achieve MV:CAT mRNA circularization, the 5′ methyl G cap structure from oligo dT-purified-polyadenylated RNAs was first removed with tobacco acid pyrophosphatase. By designing the primer-pair 1 and 2 oriented such that RT-PCR could proceed only across the newly created 3′–5′ end-ligated junction, the MV terminal nucleotides of each RNA product arising from the MV:CAT genome template was amplified. For the characterization of MV:CAT antigenome, the additional primer pair 3 and 4 was used to perform nested PCR. The resultant cDNAs then were cloned and their sequence determined. It is important to point out that this method, although powerful, is not without technical difficulties. In particular, the phosphatase/kinase steps preceding RNA ligation often lead to the loss of one or more nucleotides from the synthetic genome RNA ends. As a result, several RT-PCR-generated clones must be analyzed to define the terminal nucleotide sequence of the propagated artificial MV genomes with certainty.

Figure 4 shows a sequencing gel analysis defining the MV:CAT mRNA termini. The absolute 5′ end of the CAT mRNA is represented in the upper panel by the predicted N gene UTR beginning with its transcription start signal fused to poly(T); the lower panel shows the CAT ORF translation stop and adjacent L gene UTR with its transcription stop sequence (in antigenome orientation) followed by the 3′ poly(A) tail (see Fig. 1B for localization of the sequences). Similar analyses showed that the 5′ and 3′ ends of MV:CAT antigenomes contained the entire 107 and 109 NCT domains (results not shown). The CAT mRNA clones contained a single unexpected nucleotide change within the N gene-encoded 5′ untranslated region — a C instead of U at genomic position 71. This nucleotide transition was not detected in the MV:CAT antigenome sequence and is currently thought to reflect an error introduced during PCR amplification. The conservation of all predicted MV genomic sequences during the course of synthetic MV:CAT (−) RNA-specified transcription and replication confirms that the *cis*-acting promoter and regulatory signals are fully contained in the 3′ and 5′ NCTs (107 and 109 nucleotides, respectively) and validates the role of MV:CAT (−) as an authentic genome analog.

Our MV minigenome now can be exploited to more precisely define the sequence requirements, limits, and organization of *cis*-acting domains implicated in MV transcription and replication and allow comparative analyses of their relatedness to those of other paramyxovirus genomes. Particular attention currently is focused on recently discovered nucleotide differences distinguishing the genomic regulatory regions of wild-type MVs from those of vaccine strains. Whether these nucleotide changes influence MV virulence or attenuation now can be directly appraised by quantifying their effect on CAT gene transcription and replication. Identifying such candidate virulence/attenuation determinants might ulti-

mately facilitate vaccine design strategies that rely on generating modified infectious MV strains from full-length MV cDNA.

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