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Intracellular amplification and expression of a synthetic analog of rotavirus genomic RNA bearing a foreign marker gene: Mapping cis-acting nucleotides in the 3′-noncoding region

(RNA transfection/viral promoter mapping/mutagenesis/interserotype complementation)

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ABSTRACT **cDNAs were constructed to encode plus- or minus-sense analogs of gene 9 RNA of porcine rotavirus strain OSU in which the bacterial chloramphenicol acetyltransferase (CAT) reporter gene was flanked by the 5′-terminal 44 nucleotides (nt) and 3′-terminal 35 nt of the authentic rotavirus gene. Transfection of plus-sense gene-9-CAT RNA into rotavirus-infected cells resulted in its amplification and in the efficient expression of CAT; this was greatly enhanced by the presence of a 5′ cap structure. Amplification was ablated by omitting the rotavirus superinfection or by removing the 3′-terminal 35-nt rotavirus sequence from the RNA. This result indicated that amplification depended both on rotavirus proteins supplied *in trans* and on cis-acting rotavirus sequences. Minus-sense or double-stranded gene-9-CAT RNA was essentially inactive, indicating that synthetic RNAs can be introduced into the rotavirus replicative cycle *in vivo* only when provided in the plus sense. However, incorporation of the CAT-bearing RNA into infectious rotavirus was not detected. Two heterologous rotaviruses, the simian RRV and chicken Ch2 strains, efficiently complemented the OSU-based gene-9-CAT RNA, even though the Ch2 strain was only 50%–66% related in the noncoding regions. Mutational analysis of the 35-nt 3′-noncoding region showed that the 3′-terminal 12 or 17 nt were sufficient for reduced (12% or 23%, respectively) levels of amplification, whereas inclusion of the 3′-terminal 19 nt fully restored amplification. Thus, the 3′-terminal cis-acting signals required for amplification include the 7-nt-terminal consensus sequence together with 12 nt of adjoining, less-well-conserved sequence.**

Rotaviruses, classified as a genus of the family Reoviridae, are the most important causes of viral gastroenteritis (1). The rotavirus genome consists of 11 segments of double-stranded (ds) RNA, which, for the RNAs sequenced to date, range in length from 667 (segment 11) to 3302 base pairs (bp) (segment 1) (2).

The general features of rotavirus transcription and replication have only begun to be elucidated but appear similar to those described for reovirus (2, 3). Virions contain a viral polymerase packaged with the dsRNA segments in a subviral single-shelled core. The polymerase directs copying of the parental minus strand to yield progeny plus strands (transcription) (4). These plus strands serve as mRNAs and as templates for the synthesis of progeny minus strands to yield dsRNA (replication) (5). Thus, dsRNA synthesis is asynchronous and conservative (6, 7). During the synthesis of the negative strand the nascent dsRNA associates with viral proteins to form subviral cores that eventually are incorporated into virions (4, 5, 8).

The 5′ and 3′ ends of each rotavirus gene segment contain terminal consensus sequences of 7–10 nucleotides (nt) (2). The 5′- and 3′-terminal consensus sequences are unrelated, implying that they have functional differences. The 5′ end of the plus strand has a methylated cap structure, and neither strand is polyadenylylated (9, 10). The terminal consensus sequences are assumed to be important cis-acting signals, which presumably include, at the 3′ ends, the viral promoters. The termini also might contain sequences important in packaging and in the regulation of rotavirus gene expression at the levels of transcription, replication, and translation (2).

Recently, it was shown that authentic plus-sense reovirus RNAs synthesized *in vitro* by subviral cores were infectious when transfected into cells and complemented with a helper reovirus from a different serotype (11). To date, however, similar results have not been reported with synthetic RNAs encoded by cDNA. This latter capability is obligatory for the direct introduction of mutations into rotaviral genes for detailed structure–function studies and engineering attenuated vaccine strains.

As a first step toward this goal, we constructed cDNA clones encoding plus- or minus-sense analogs of rotavirus strain OSU gene 9 [gene 9 encodes the outer capsid VP7 protein (12)] consisting of the bacterial chloramphenicol acetyltransferase (CAT) marker gene flanked by the 5′- and 3′-noncoding regions of gene 9 RNA. Plus-sense synthetic RNAs were replicated and transcribed when transfected into cells and complemented by infection with rotavirus. This procedure provides a sensitive system for characterizing rotavirus gene replication and expression, which was used here to map cis-acting nucleotide sequences at the 3′ end of the plus strand.

MATERIALS AND METHODS

Cells and Viruses. The porcine rotavirus strain OSU (serotype G5), rhesus rotavirus RRV (serotype G3), and chicken rotavirus Ch2 (serotype G7) were grown in MA104 monkey kidney cells as described (13).

cDNA Constructions. A cDNA was constructed by two successive PCRs to encode a plus-sense gene-9-CAT RNA containing a 44-nt 5′-noncoding region and a 19-nt 3′-noncoding region (mutant g in Fig. 4*A*). The length of the 3′ end varied among the different plus-sense cDNAs described below (Fig. 1 and Fig. 4*A*), whereas all had the same 5′-noncoding region. The template for PCR was an *Xba* I–*Pst* I cDNA containing the CAT translational open reading frame (ORF) (Fig. 1). The two primers for the first PCR were: 5′-GAGAATTTCCGACTGGCTATCGGATAGCTCC-

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Abbreviations: nt, nucleotide(s); ds, double stranded; CAT, chloramphenicol acetyltransferase; ORF, open reading frame.

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TCTAGA*ATG*GAGAAAAAAATCAC-3′ (plus sense, with the *Xba* I site underlined, the CAT translational start site italicized, and rotavirus sequences in boldface type) and 5′-**CATACAGTTCTGCAG*TTA*CGCCCCGCCC-3′** (minus sense, with the *Pst* I site underlined, the complement of the translational termination site italicized, and the rotavirus sequences in boldface type). The two primers for the second PCR were: 5′-CCAGGTACC*TTAATACGACTCACTATA*G-**GCTTTAAAAGAGAGAATTTCCGACTGG**-3′ (plus sense, with the *Kpn* I site underlined, the T7 promotor italicized, and the rotavirus sequences in boldface type) and 5′-GGTAAGCTTGACGCTGATAGGTCACATCATACAGT-TCTGCAGTTAC-3′ (minus sense, with the the *Hin*dIII, *Hga* I, and *Pst* I sites underlined and rotavirus sequences in boldface type). The amplified fragment was digested with *Kpn* I and *Hin*dIII and cloned in pUC19.

A second cDNA-encoded RNA, gene-9-CAT(+) RNA (Fig. 1 *A* and *B*), was considered the parental plus-sense RNA because it had the longest (35 nt) 3′-noncoding region. Its cDNA was constructed by digesting the first cDNA (mutant g) described above with *Hin*dIII and *Pst* I and ligating the larger fragment with the following two annealed complementary synthetic oligonucleotides: 5′-GATATATCTTAAGT-TAGAACTGTATGATGTGACCTATCAGCGTCA-3′ (plus sense, with the *Hga* I site and part of the *Hin*dIII and *Pst* I sites underlined and rotavirus sequence in boldface type) and 5′-AGCTTGACGCTGATAGGTCACATCATACAGT-TCTAACTTAAGATATATCTGCA-3′ (minus sense, with the *Hga* I site and parts of the *Hin*dIII and *Pst* I sites underlined and the rotavirus sequence in boldface type).

Additional gene-9-CAT cDNAs (see Fig. 4*A*) containing various other truncations in the 3′-noncoding region were constructed by PCR using the first cDNA described above (mutant g) cut with *Pst* I as template and synthetic oligonucleotides containing the desired mutation as primers. Similarly, gene-9-CAT(−) cDNA (Fig. 1 *A* and *C*), which encodes the minus-sense counterpart of gene-9-CAT(+) RNA, was constructed by PCR with *Pst* I-cut gene-9-CAT(+) cDNA as template and synthetic oligonucleotides as primers.

Transcription and Transfection of Synthetic RNAs. Plasmids were digested with *Hga* I or, as indicated, *Pst* I or *Hin*dIII and used as templates for *in vitro* transcription with T7 RNA polymerase using standard procedures (Promega). MA104 or 293 cells were infected with OSU, RRV, or Ch2 rotavirus at a multiplicity of infection (m.o.i.) of 1–10 plaque-forming units per cell. One hour later the cells were incubated for 3 hr with medium containing synthetic RNA (0.5–1.5 μg per 10 cm^2 per well), and TransfectAce (GIBCO/BRL) used according to the supplier's protocol.

CAT Assays. At 20 hr after infection cell lysates were prepared, protein contents were determined by Bio-Rad protein microassay and normalized, and CAT activities were measured by standard procedures (14) with quantitation of spots by liquid scintillation.

RESULTS

Construction of cDNAs Encoding Synthetic Gene-9-CAT RNAs. Two cDNAs were constructed to contain the CAT ORF flanked by the 5′- and 3′-noncoding sequences of gene 9 RNA of rotavirus strain OSU. Each cDNA would yield, upon digestion with *Hga* I and transcription *in vitro* by T7 RNA polymerase, RNA containing the exact, correct 5′ and 3′ ends.

One cDNA encoded an analog of the gene 9 plus-strand RNA, designated gene-9-CAT(+) (Fig. 1*A*, *Upper*). This RNA contained the CAT ORF in plus sense flanked on the 5′ side by the terminal 44 nt of the 48-nt 5′-noncoding region of the gene 9 plus strand and on the 3′ side by the terminal 35 nt of the authentic plus strand including the 33-nt 3′-terminal noncoding region and 2 nt from the translational stop codon (Fig. 1*B*).

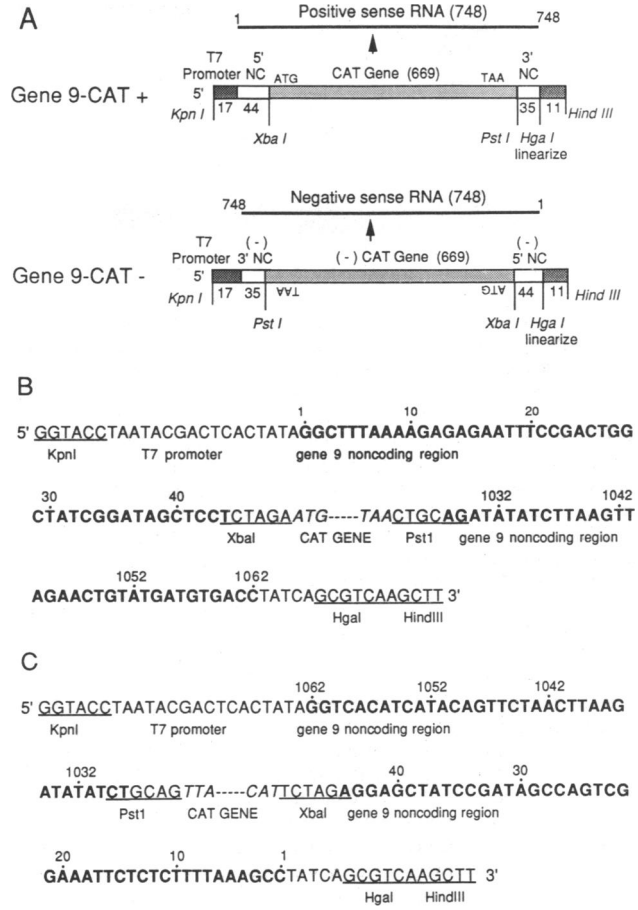

FIG. 1. Structures of cDNAs encoding plus-sense and minus-sense analogs of gene 9 RNA of rotavirus strain OSU. (*A*) Schematic diagrams of cDNAs encoding a plus-sense (*Upper*) or minus-sense (*Lower*) copy of gene-9-CAT RNA. The CAT ORF (light stippling) is flanked by noncoding (NC) regions (open boxes) from the 5′ and 3′ termini of rotavirus gene 9, which, in turn, are flanked by plasmid sequences (dark stippling). Nucleotide lengths are indicated. For each cDNA, cleavage with *Hga* I followed by transcription with T7 RNA polymerase would yield RNA containing the correct 5′ and 3′ ends of gene 9. (*B* and *C*) Nucleotide sequences of the junctions between the plasmid vector and insert cDNAs encoding the plus-sense (*B*) and minus-sense (*C*) gene-9-CAT RNAs. Rotavirus-specific sequences are shown in boldface letters and numbered relative to their positions in the authentic plus strand of gene 9. Restriction sites are underlined and identified. The translational initiation and termination codons of the CAT gene are italicized [shown in mRNA (sense in *B* and antisense in *C*)], and the remainder of the CAT gene is indicated by dashed lines.

A second cDNA encoded an analog of the gene 9 minus-strand RNA, gene-9-CAT(−). This RNA contained the CAT ORF in minus sense flanked by the 5′-terminal 35 nt and 3′-terminal 44 nt of the authentic minus strand (Fig. 1*A*, *Lower*, and Fig. 1*C*).

Amplification of Gene-9-CAT RNA by Complementation with Rotavirus Strain OSU. Plus-sense and minus-sense gene-9-CAT RNAs were synthesized separately *in vitro* with T7 polymerase. RNAs containing a 5′-terminal methylated cap structure were prepared by transcription in the presence of 7-methylguanosine(5′)triphospho(5′)-guanosine (m^7GpppG), which resembles, but is not identical to, the cap structure, 7-methylguanosine(5′)triphospho(5′)-2′-*O*-methylguanosine (m^7GpppGm), found on rotaviral plus-strand RNAs (9). ds RNAs were prepared by heat denaturation of *in vitro*-

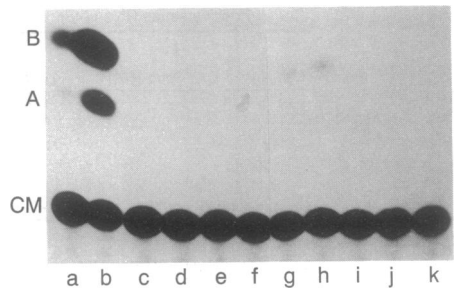

FIG. 2. Expression of CAT in 293 cells infected with rotavirus strain OSU and transfected with gene-9-CAT RNA analogs: significant expression of CAT is specific to the plus strand and depends on complementation by rotavirus coinfection. As indicated below, cells were infected with rotavirus strain OSU or mock-infected, and 1 hr later cells were transfected with *in vitro*-synthesized gene-9-CAT RNA. The RNA was of plus sense or minus sense or was present as dsRNA formed by denaturation and annealing *in vitro*. As indicated, in some cases the RNAs contained a methylated 5′-terminal cap structure. Lanes: a and b, OSU-infected cells, plus-sense RNA without (a) or with (b) a 5′-terminal cap; c, uninfected cells, plus-sense capped RNA; d and e, OSU-infected cells, minus-sense RNA without (d) or with (e) a 5′ cap; f, uninfected cells, minus-sense capped RNA; g, OSU-infected cells, dsRNA in which the plus strand was capped; h, OSU-infected cells, dsRNA in which both strands were capped; i, OSU-infected cells, dsRNA in which neither strand was capped; j, OSU-infected cells, plus-sense uncapped RNA, which was transcribed from cDNA cleaved with *Pst* I; k, same as lane j, except that the cells were uninfected. As described in text and shown in Fig. 1, digestion with *Pst* I (in lieu of *Hga* I) removes the 3′-noncoding region while leaving the CAT ORF and 5′ end intact. The cells were harvested 20 hr after infection and assayed for CAT activity. Autoradiograms of the thin-layer chromatography plates are shown, and the following species are labeled: CM, unreacted chloramphenicol; A, 1-acetylchloramphenicol; B, 3-acetylchloramphenicol.

synthesized plus and minus strands followed by mixing and annealing; the generation of intact dsRNA was confirmed by agarose gel electrophoresis (data not shown). The *in vitro*-synthesized RNAs were tested for biologic activity by transfection into 293 cells 1 hr after the cells had been infected with the homologous rotavirus OSU strain or mock-infected. The cells were then incubated for 20 hr, harvested, and tested for CAT activity.

Minus-sense gene-9-CAT RNA consistently was inactive in directing CAT expression (Fig. 2, lanes d and e). For the plus-sense gene-9-CAT RNA, trace amounts of CAT activity were sometimes detected in uninfected cells after transfection (not evident in the experiment shown in Fig. 2, but see Fig. 4*B*, row b). This sporadic activity was observed only when the RNA was capped and presumably represented direct translation of the input RNA. In contrast, high, reproducible levels of CAT activity were detected when the cells had been infected with rotavirus 1 hr before transfection with the plus-sense strand (Fig. 2, lanes a and b). Interestingly, the expression of CAT was >10-fold greater when the input plus strand contained a 5′ cap (Fig. 2, compare lanes a and b). Expression of CAT was essentially ablated when the cDNA was linearized before transcription with *Pst* I in place of *Hga* I (lane j), which removed the 3′-noncoding region. Efficient complementation was obtained by using MA104 cells (data not shown), a line commonly used for the cultivation of rotaviruses, in place of the 293 cells.

To confirm that the increase in CAT activity was associated with increased levels of plus-sense gene-9-CAT RNA, intracellular RNA was analyzed by PCR. Uninfected or rotavirus-infected cells were transfected with gene-9-CAT RNA, and total RNA was isolated at 24 hr after infection, treated twice with DNase I, reverse-transcribed with a minus-sense CAT gene primer, and amplified by PCR using the same minus-sense primer and a second, plus-sense CAT primer. Aliquots were taken at five-cycle intervals and analyzed by gel electrophoresis, showing that the levels of plus-sense gene-9-CAT RNA were substantially higher in the rotavirus-infected cells (data not shown).

Preparations of gene-9-CAT dsRNA did not direct detectable levels of CAT expression in situations where the two RNA strands were uncapped (Fig. 2, lane i). Very low levels of CAT activity were observed when the plus strand of the dsRNA was capped (lanes g and h). It seems likely that this was due to a low level of residual unannealed plus strand or to plus strand liberated by dissociation *in vivo*.

Complementation with Heterologous Strains of Rotavirus. We tested the abilities of heterologous strains of rotavirus, the rhesus rotavirus RRV and the chicken rotavirus Ch2, to complement gene-9-CAT(+) RNA. From sequence analysis, strain RRV is relatively more closely related to strain OSU than is strain Ch2. For example, RRV is 91% and 80% identical to OSU for the 5′- and 3′-terminal noncoding regions contained in gene-9-CAT(+) (Fig. 3*A* and *B*). In contrast, Ch2 is 50% and 66% identical to OSU for the 5′- and 3′-terminal noncoding regions (Fig. 3*A* and *B*). Because complementation would be expected to involve trans-acting viral proteins in addition to the cis-acting RNA signals, it also would have been of interest to compare these strains for amino acid-sequence relatedness of relevant proteins. However, amino acid sequences were not available for the proteins that would be expected to be involved in replication and transcription, such as VP1-3 and NS34 and -35. Sequences were available for the VP7 proteins of all three viruses: OSU and RRV have

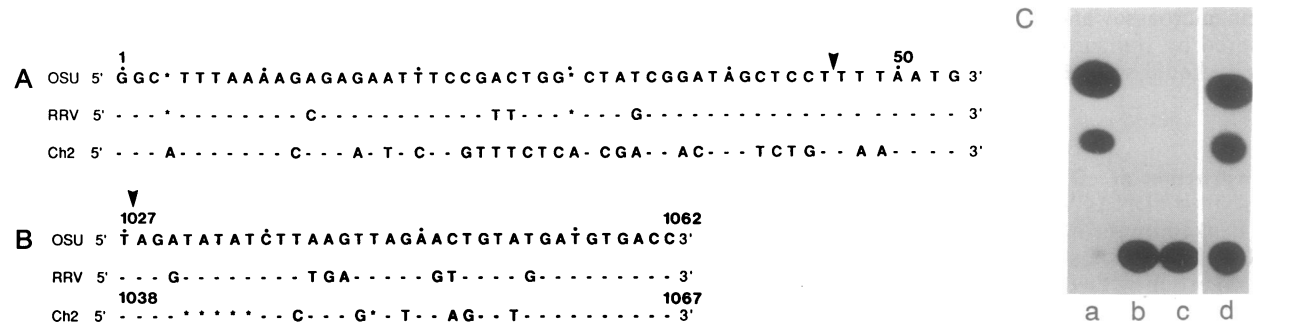

FIG. 3. Complementation of gene-9-CAT RNA by heterologous strains of rotavirus. (*A* and *B*) Comparison of nucleotide sequence of 5′ (*A*) and 3′ (*B*) ends of the plus-sense strand of gene 9 of strains OSU (12), RRV (15), and Ch2 (16). Nucleotide differences between the RRV or the Ch2 strain relative to the OSU strain are given. Numbering refers to positions in the complete RNA sequences, and the arrowhead over each OSU sequence marks the boundary of the noncoding region contained in the gene-9-CAT(+) RNA analog. The last 3 nt (positions 51–53) in the sequences in *A* are the translational initiation codon of the authentic mRNA. Stars indicate gaps. (*C*) 293 cells were infected with Ch2 rotavirus (lanes a and b) or RRV rotavirus (lanes c and d) and subsequently transfected with capped, plus-sense gene-9-CAT RNA (lanes a and d) or mock-transfected (lanes b and c). Cells were harvested 20 hr after infection and assayed for CAT activity.

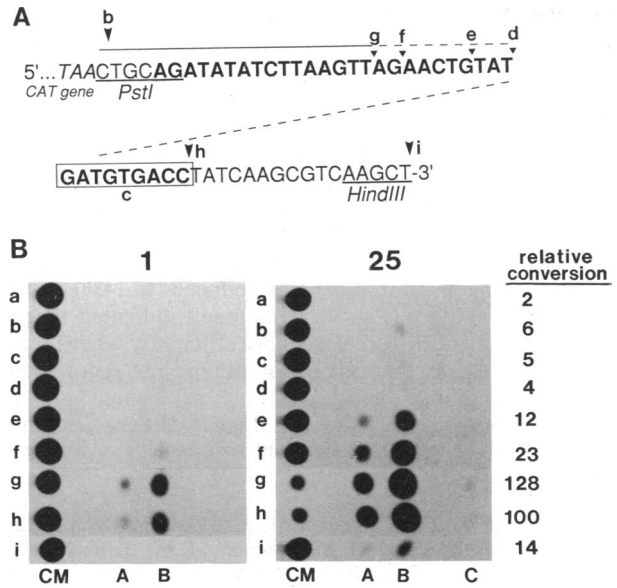

FIG. 4. Mapping cis-acting sequences at the 3' end of plus-sense gene-9-CAT RNA. (*A*) Sequence of the 3' end of plus-sense gene-9-CAT RNA (5' → 3') with the positions of various mutations indicated. Rotavirus-specific sequences of the noncoding region are shown in boldface letters. In the sequence shown, these are flanked on the left by 4 heterologous nt of the *Pst* I site and by the translational stop codon of the CAT ORF (italicized). The rotavirus-specific noncoding sequence is flanked on the right by plasmid-specific sequences. An arrowhead (h) marks the correct, parental 3' terminus, which is generated from cDNA linearized with *Hga* I. Other vertical arrowheads (b and i) mark the 3' ends of RNA synthesized from cDNA linearized with *Pst* I (b), which deletes the entire noncoding region, or *Hin*dIII (i), which results in a 3'-terminal extension of 16 heterologous nt. In another mutant (c) the 3'-terminal 9 nt (boxed) were deleted. Other mutants (d–g) contained internal deletions of increased length spanning from the *Pst* I site on the left (solid overline) to the various positions indicated on the right (dashed overline, small arrowheads). These mutants contained 3'-terminal noncoding regions of the following lengths: 9 nt (d), 12 nt (e), 17 nt (f), and 19 nt (g). (*B*) Assay of the gene-9-CAT RNAs described in *A*. RNAs were transcribed *in vitro* and assayed by transfection into cells infected with the rotavirus strain OSU. Lysates were prepared 20 hr after infection and assayed for CAT activity at two different lysate concentrations (gel sets marked 1 and 25, representing 2% and 50%, respectively, of a 10-cm^2 cell monolayer). Mutants are designated b–i as described in *A*; row a represents a control in which gene-9-CAT RNA containing the correct 3' end was transfected into uninfected cells. All RNAs were synthesized to contain 5'-terminal cap structures.

85% identity with each other and 61% and 60% identity, respectively, with Ch2, a level of relatedness consistent with that described above for the noncoding sequences. Because these viruses had different efficiencies of growth in 293 cells (data not shown), quantitative comparison of their efficiencies of complementation was not attempted. Qualitatively, however, both the more closely related RRV and the more-distantly related Ch2 consistently support efficient complementation of the OSU-based gene-9-CAT RNA (Fig. 3*C*). Indeed, the more-divergent Ch2 virus consistently provided the most efficient complementation, which appeared due, at least in part, to its efficient growth.

Mapping Cis-Acting Signals in the 3'-Terminal Noncoding Region. The 35-nt noncoding region of gene-9-CAT(+) was subjected to mutational analysis to map sequences required for amplification and expression of the CAT gene (Fig. 4). Deletion of the 3'-terminal 9 nt ablated the development of CAT activity (Fig. 4, row c), confirming the expectation that the 7-nt terminal consensus sequence is a requisite cis-acting signal. However, these 9 nt alone were not sufficient; this was demonstrated with another plus-sense RNA analog that contained only the terminal 9 nt as the 3'-noncoding region and was inactive (row d). Length of the noncoding region was then increased incrementally to contain the terminal 12, 17, or 19 nt. This result showed that (*i*) the 3'-terminal 12 nt were sufficient for significant, but low, levels of activity (12%, row e), (*ii*) the 3'-terminal 17 nt provided moderate, but reduced, levels (23%, row f), and (*iii*) the 3'-terminal 19 nt (row g) were equivalent to the complete 35-nt noncoding region (row h). Finally, the addition of 16 heterologous nt to the 3' end, as obtained with cDNA digested with *Hin*dIII in place of *Hga* I, reduced the level of CAT expression to 14% (row i).

DISCUSSION

cDNAs were constructed to encode synthetic analogs of rotavirus gene 9 in which the viral ORF was replaced by that of the CAT marker gene. Gene-9-CAT RNA was synthesized *in vitro* from cDNA and transfected into 293 or MA104 cells. In the absence of rotavirus infection, trace amounts of CAT activity were sometimes detected after transfection of the plus-sense form, which presumably resulted from its direct translation. However, both CAT activity and the amount of intracellular plus-sense gene-9-CAT RNA were greatly increased when the cells were infected with rotavirus immediately before transfection. Thus, the synthetic RNA appeared to be rendered biologically active by viral proteins supplied by the superinfecting rotavirus helper. A role for cis-acting signals was indicated by the finding that deletion of the 3'-terminal rotaviral noncoding sequences (thus leaving the CAT ORF and 5' mRNA end intact) ablated the rotavirus-specific amplification.

The finding that the plus-sense gene-9-CAT RNA was amplified indicated that it was copied into minus-sense RNA, which, in turn, was copied into plus-sense progeny strands. Thus, the synthetic RNA was both replicated and transcribed. It was previously shown, using an *in vitro* transcription-replication system containing subviral cores, that exogenously added naked plus-sense RNA could be converted into dsRNA and packaged into particles (8). That finding indicated that exogenously added RNA could interact with rotavirus proteins *in vitro*. This result was extended in reovirus by the finding that naked plus-sense RNAs were infectious when transfected into reovirus-infected cells (11). Our results further extend this information by demonstrating that biologic activity can be achieved with plus-sense RNA made from cDNA and containing a heterologous reporter gene in place of authentic rotavirus RNA.

Amplification of the synthetic plus-sense RNA was highly, but not completely, dependent on the 5' cap structure. This result would not be from increased translatability of the input RNA because the bulk of CAT synthesis would be from translation of progeny plus strands capped by the viral polymerase. Instead, the 5' cap either stabilized the input-plus strand or was somehow required for its efficient use as template for minus-strand synthesis.

Synthetic, naked minus-sense gene-9-CAT RNA, whether capped or not, was completely inactive after transfection into rotavirus-infected cells. This result is consistent with the available evidence showing that, unlike its plus-sense counterpart, minus-sense RNA is not found intracellularly as a naked single-stranded species (8). Synthetic, naked dsRNA had only a low level of activity when introduced into rotavirus-infected cells, and this activity probably can be attributed to contaminating free plus strands. Thus, neither preformed minus-sense or dsRNA appears able to associate with viral proteins *in vivo* to generate a biologically active form. Perhaps the association between dsRNA and viral proteins that occurs during encapsidation can occur only *in statu nascendi* during minus-strand synthesis.

Roner *et al.* (11) reported that cotransfection of homologous or heterologous dsRNA increased the infectivity of plus-sense reovirus RNAs. However, cotransfection of gene-9-CAT dsRNA did not affect the activity of plus-sense or minus-sense gene-9-CAT RNA (data not shown). The difference between the results of these two studies is unexplained, and we are continuing to explore other parameters, such as to include the full complement of authentic dsRNAs rather than the gene-9-CAT dsRNA alone and to preincubate the synthetic RNA with rotaviral proteins with an *in vitro* translation system.

While the synthetic plus-sense gene-9-CAT RNA was replicated and transcribed in rotavirus-infected cells, its packaging into infectious virions (i.e., its "rescue") was not detected. It might be that a cis-acting structure essential for packaging into virions exists that is not contained in the 5′ and 3′ ends. The possibility that the foreign gene contains a "poison" sequence inimical to packaging can be addressed experimentally by attempting to rescue synthetic RNAs representing natural gene segments with several nt substitutions as markers. One intriguing possibility is that the exogenously supplied rotavirus RNAs are channeled into a separate compartment or factory where they can be expressed and replicated individually but can be efficiently incorporated into virions only when the transfection supplies the full complement of dsRNAs. It seems likely that further characterization of this system will ultimately yield a method for introducing synthetic RNA into particles in an infectious form.

Deletional analysis was performed on the 3′ end of the plus-sense RNA to map requisite cis-acting elements. This analysis showed that the 3′-terminal 12–17 nt were necessary and sufficient for amplification at reduced levels; however, full activity required the 3′-terminal 19 nt. It had been assumed that the 7-nt consensus sequence at the 3′ end would be critical for some aspect of the rotavirus replicative cycle, most likely as a promoter. The identification here that the requisite sequence is 19 nt in length shows that the cis-acting sequences also include a region that is not well-conserved among the different genes as well as among the gene 9 RNAs of different rotaviruses (for example, see Fig. 3*B*). The idea that this region of the requisite domain can accept nucleotide variability was directly shown by the observation that the OSU-based gene-9-CAT RNA was efficiently complemented by the RRV and Ch2 strains (Fig. 3*C*), which have nucleotide differences at positions 10, 11, 14–16, and 18 from the 3′ end (Fig. 3*B*).

Because the synthesis of minus-sense RNA from the plus-sense template probably is concurrent with the encapsidation of the nascent dsRNA to form subviral particles, the 3′-terminal region of the plus strand might encode an encapsidation signal. The 19-nt requisite sequence thus might contain two distinct signals, with the terminal consensus sequence being a promoter and the adjoining less-well-conserved sequence being a signal involved in some other process such as encapsidation or regulation of replication. It is interesting to note that the minimum number of nucleotides found in the 3′-noncoding region of the rotavirus genes sequenced to date is 17 compared with 9 at the 5′ end (17), consistent with the speculation that the former contains two cis-acting signals (the plus-strand promoter and the complement of the encapsidation signal) and the latter one (the complement of minus-strand promoter). Finally, the addition of 16 heterologous nt to the 3′ end reduced (to 14%), but did not abolish, activity (Fig. 4). This result indicated that the cis-acting sequences were used more efficiently when located near the 3′ end but that this is not an absolute requirement.

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