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From the cDNAs of two defective RNAs naturally exhibiting a large difference in replication efficiency, a series of Sendai virus RNA chimeras were constructed by reciprocal exchanges of their 3′ end primary sequences. Using a reverse genetics system, the ability of these RNAs to replicate when expressed from cDNAs in the context of the viral proteins N, P, and L, also expressed from plasmids, was analyzed. First the extent of potential RNA 3′/5′ end complementarity was tested by disrupting and restoring the terminal 110-nucleotide complementarity of a copy-back RNA. Alternatively, this base pairing potential was gradually increased from 12 to 57 or to 98 nucleotides by continuous substitutions. In all cases, the restoration or the creation of more extended base pairing potential had no effect on RNA replication. Reciprocal exchanges were then made in order to identify *cis*-acting sequences that could induce high replication efficiency. It was found that nucleotides 1–31 of the antigenome 3′ end were sufficient to confer a high replication property (more than a 10-fold increase), regardless of the sequence adjacent to these terminal nucleotides. It is concluded that one of the most important features that modulate replication efficiency is contained in the promoter end primary sequence and that this feature is likely to operate independently of the ability to form a potential terminal base pairing.

INTRODUCTION

Negative-stranded RNA viruses initiate the viral multiplication cycle with viral messenger RNA synthesis templated by the infecting genome. This primary transcription leads to *de novo* synthesis of viral proteins, required in turn for viral RNA replication. Genome replication begins with the copying of the incoming viral RNA of negative polarity, i.e., the genome, into its full-length complement of positive polarity, the antigenome, which will serve in turn as template for viral genome amplification. The viral RNA-dependent RNA polymerase is first brought in by the infecting virus, before it is expressed *de novo* following primary transcription and translation. The functional template for transcription and replication is not the naked RNA, but the N:RNA nucleocapsid of helical symmetry.

According to a current model (reviewed in Kingsbury, 1974; Kolakofsky and Blumberg, 1982; Vidal and Kolakofsky, 1989), which applies mainly to viruses of the Rhabdo- and Paramyxoviridae families, the viral RNA synthesis starts with the RNA polymerase entering at the genome or the antigenome 3′ ends. At the genome 3′ end, the RNA polymerase has the apparent choice to synthesize the antigenome or to initiate the sequential synthesis of the messenger RNAs. The RNA polymerase is thought to be in different functional modes, defined, to this day, by the reaction end products. For transcription, the RNA polymerase obeys to start, stop, and restart templated *cis* signals at the borders of each transcription unit. In replication, the RNA polymerase is made insensitive to these *cis* signals and, consequently, synthesizes a full complement of the template. At the antigenome 3′ end, the RNA polymerase will eventually produce only a full copy of its template, i.e., the RNA genome. At the genome and antigenome 3′ ends lie two regions of about 50 to 60 nucleotides called, respectively, the leader plus (le^+) and leader minus (le^-) template regions (Leppert *et al.,* 1979; Vidal and Kolakofsky, 1989; Banerjee and Barik, 1992). These two regions encode respectively the le^+ and le^- RNAs, which are thought to contain the initiation sites for encapsidation (Blumberg *et al.,* 1983). These leader RNAs have further been proposed to be the sites where the decision to replicate is made. Replication would be tied to the leader RNA encapsidation rate, this rate depending itself on the unassembled N protein concentration (Kolakofsky and Blumberg, 1982; Vidal and Kolakofsky, 1989). This model emphasises the importance of the genome and antigenome RNA ends, acting, on one hand, as binding sites for the RNA polymerase (template 3′ ends) and, on the other hand, as check points to confirm replication (nascent strand 5′ ends). For convenience, we refer here to these RNA 3′ ends (still not precisely defined) as promoters. These, for the time being, include indistinctly the template 3′ end nucleotides as well as their templated 5′ end nascent strands. In the case of the Paramyxoviruses, these promoters are likely to extend past the limit of the leader template regions (see text of Results and Discussion). In contrast, for

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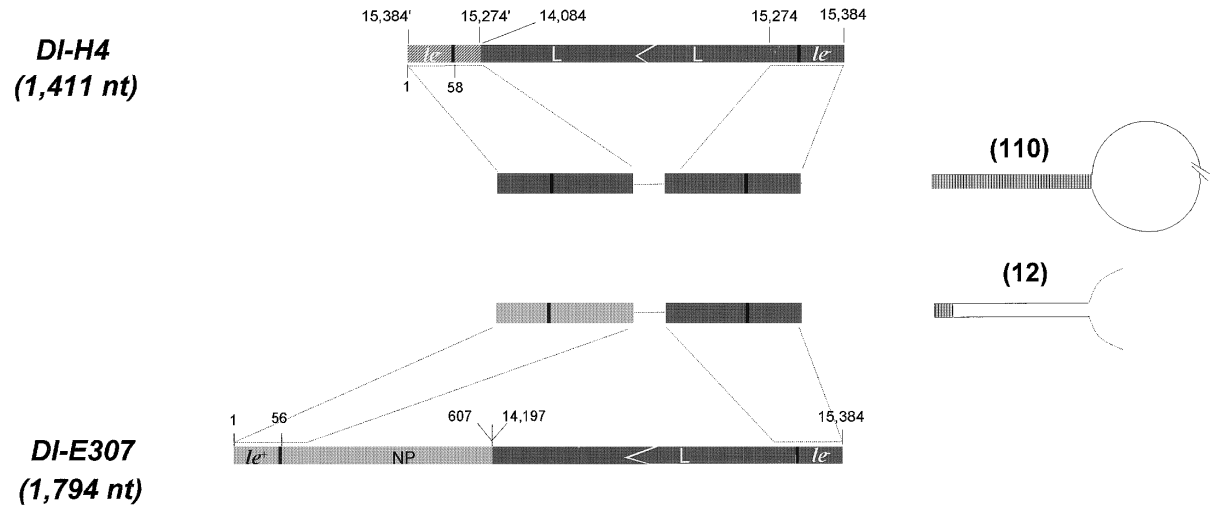

FIG. 1. Schematic representation of the defective RNA templates. The DI-H4 and E307 RNAs are schematically depicted as minus strands oriented 3′ to 5′ from left to right. H4 is a copy-back RNA which contains only a 5′ end portion of the L gene (from nt 14,084), the last SeV RNA transcription unit, followed, at its 5′ end, by the minus-strand leader template region (le^-), present at the 5′ end of the SeV RNA. At its 3′ end, an inverted complementary repeat over 110 5′ end nucleotides (15,384′–15274′) gives the RNA its copy-back feature. This includes the possibility for the extremities of the naked RNA to form an intramolecular hybrid over 110 nucleotides (shown on the second line far right) and implies that both the H4 minus- and plus-strand 3′ ends are identical over 110 nucleotides. This latter feature is pictured on the second line (center), where the two boxes refer to the 3′ end sequences of, respectively, the H4 minus (left)- and plus (right)-strand promoters (of yet undefined length, but likely to be shorter than 110 nucleotides) involved in the control of RNA replication (see Introduction). E307 is an internal deletion RNA, which has conserved the full-length SeV RNA ends. At the 3′ end, the leader plus (le^+) template region is followed by a 3′ end portion of the N gene fused to a 5′ end portion of the L gene. As a naked RNA, E307 can potentially form an intramolecular hybrid over only 12 nucleotides, since the first 12 nucleotides of the plus- and minus-strand 3′ ends are identical (as in the full-length SeV RNA). These are schematized on the line above, along with the two boxes referring to the 3′ end sequences of, respectively, the minus- and the plus-strand promoters, of yet undefined length , involved in the control of RNA replication. Note that E307 and H4 RNAs have the same plus-strand 3′ end, but differ in their minus-strand 3′ ends. The black bar in the middle of the promoter regions refer to the le^+ or le^- template borders at positions respectively, 55 and 57 nucleotides.

the Rhabodviruses, they appear to span only the leader regions (Schubert *et al.,* 1979; Pattnaik *et al.,* 1992, 1995; Conzelmann and Schnell, 1994; Wertz *et al.,* 1994). Early in infection, replication must first produce antigenomes from the incoming genome to build up replication intermediates. Later, an excess of genomes over antigenomes is generally observed (Schincariol and Howatson, 1972; Soria *et al.,* 1974; Kolakofsky and Bruschi, 1975; Simonsen *et al.,* 1979). Therefore, a preferential amplification of the genome over the antigenome has to be orchestrated late in infection. To account for this, the antigenome, template for genome synthesis, has been proposed to contain a stronger replication promoter than the genome, on the rationale that this latter template promoter is weakened by its implication in both replication and transcription.

In a previous work, we have addressed the question of promoter characterization (Calain and Roux, 1995). Using a reverse genetics system, Sendai virus (SeV) defective interfering (DI) RNAs, expressed from cDNA, were replicated in the context of the viral proteins expressed from N, P, and L gene carrying plasmids. Two types of template RNAs, identical to naturally occurring DI RNAs, were used. An internal DI E307-RNA, with both ends of the viral genome conserved (Fig. 1), replicated poorly in the system. This DI genome also produced a functional mRNA. In contrast, a copy-back DI H4-RNA exhibited a 20-fold higher replication efficiency, consistent with the presence of the antigenome promoter on both the minus and plus strands (Fig. 1). Reciprocal sequence exchanges between E307 and H4 promoters generated RNA derivatives whose study led to two main conclusions. First, the ability to transcribe an mRNA did not prevent efficient replication. Thus, in this reconstituted system at least, the replication efficiency of the promoter was found not to be hindered by its participation in both transcription and replication. Second, the efficiency of replication could be then strictly correlated with the promoter primary sequence, since a gradual replacement of H4 promoter (high replication) with the corresponding sequence from the E307 promoter (low replication) resulted in a gradual decrease in RNA replication and vice versa (Calain and Roux, 1995). However, the gradual promoter replacement also diminished the base pairing potential of the H4 RNA 3′/5′ ends which extents for 110 nucleotides, due to the copy-back nature of this RNA (see Fig. 1). As the extent of the RNA 3′/5′ end complementarity has been recently found to affect the replication efficiency of a VSV defective RNA studied in a similar system (Wertz *et al.,* 1994), the question was raised as to whether it was the variations of the extent of the terminal complementarity that was also responsible for the differences of the SeV RNA replication efficiency.

To address this question, new H4 RNA derivatives ex-

hibiting different potential for 3′/5′ end base pairing were constructed and tested for replication. The results obtained do not support the effect of terminal base pairing on replication efficiency. In consequence, a closer examination of the promoter was made, in order to further identify primary sequences controlling SeV RNA replication efficiency. In the end, nucleotides 1 to 31 at the antigenome 3′ end were shown to be sufficient to confer high replication efficiency, regardless of the adjacent downstream sequence.

MATERIAL AND METHODS

Virus and cells

Hela and CV1 cells were grown in regular MEM supplemented with 5% fetal calf serum in a 5% $C0_2$ atmosphere. Vaccinia recombinant virus expressing the T7 RNA polymerase, vTF7-3, a gift from Bernard Moss (National Institutes of Health, Bethesda, MD) has been described by Fuerst and colleagues (1986) and used accordingly. vTF7-3 stocks were grown in HeLa cells with titers ranging from 5×10^8 to 10^9 PFU/ml.

Sequence and plasmids

The complete SeV RNA primary sequence (15,384 nucleotides) was taken from Shioda *et al.* (1983, 1986) with the correction by Neubert *et al.* (1991). The plasmids expressing the SeV NP, P, and L proteins (respectively, pGem-NP, pGem-P/c, and pGem-L) under the control of the T7 RNA polymerase have been described previously (Curran *et al.,* 1991; Kast *et al.,* 1991). The cloning of the natural SeV DI RNAs H4 and E307 in pSP65 under the control of the T7 RNA polymerase promoter has been described previously (Calain *et al.,* 1992; Engelhorn *et al.,* 1993; Calain and Roux, 1995). The nucleotide exchanges performed to generate the DI H4 and E307 RNA derivatives were done by fusion PCR (Ho *et al.,* 1989) with a judicious use of previously existing derivatives and their particular restriction sites. A first round of PCR products were obtained using amplimers composed of about eighteen 3′ end nucleotides complementary to the donor strand and about eighteen 5′ end dangling nucleotides complementary to the recipient strand, along with amplimers common to both DI-H4 and E307 RNA, including a *Sal*1 site to the left of the T7 RNA polymerase promoter. These first PCR products were then combined adequately and amplified with the amplimers common to both RNAs. The recombined final products flanked by the adequate sites were finally subcloned into pSV-H4 or pSV-E307 in replacement of the original fragments. All the sequence modifications were verified by sequencing. The derivatives were identified as having an H4 or E307 backbone. The promoter accepting the substitution was than indicated, as well as the range of nucleotides coming from the donor promoter. For convenience, the 3′ end of the viral genome was called genomic promoter (GP) and the 3′ end of the antigenome, the antigenomic promoter (AGP). In the case of H4 RNA, which contains the same antigenomic sequence (AGP) on both the minus- and the plus-strand RNA, the distinction between the two promoters had to be made [(AGP(−) or (+)]. For example, H4-AGP(−)120 refers to a H4 RNA derivative where the minus-strand 3′ end has been substituted over the first 120 nucleotides with the corresponding nucleotides coming from the E307 RNA minus-strand 3′ end (see also the diagrams presented in the figures).

Replication system

The replication system has been described previously (Calain and Roux, 1995). In brief, CV1 cells (about 10^7) were infected (m.o.i. = 3) with vTF7-3. One hour later, the cells were transfected with 5 μg of pGem4-NP, 5 μg of pGem4-P/c, 1 μg of pGem4-L, and 5 μg of the plasmid carrying the DI RNA sequence. Cytoplasmic extracts were prepared 40 hr later, and CsCl gradient-purified nucleocapsids were analyzed by Northern blotting (Mottet and Roux, 1989). ^{32}P-labeled riboprobes of positive or negative polarity, spanning 1453 nt in the L gene from nucleotides 13,452 to 14,905 (two *Xba*I sites) and stopping at 477 nt from the genome 5′ end, have been described before (5′ex probe, Mottet and Roux, 1989). This probe was shortened by 474 nt by a further internal *Hpa*I cut at position 14,433, to eliminate a cross-reactivity with a nonspecific RNA band appearing unexpectedly in some experiments (as shown in Fig. 5). To prepare the probes, routinely, 1 μg of linearized plasmid was transcribed under standard conditions in the presence of 50 μCi of [α-^{32}P]uridine and 10^6 Cherenkov counts per minute of Sephadex G-50 purified radioactive RNA was added per milliliter of hybridization solution (10 ml total). Calibration of the Northern blot analysis was made using increasing amounts of unlabeled *in vitro*-made transcripts to ensure a linear RNA/signal (not shown), and the 20- to 50-fold signal differences observed reflect this proportionality. The signals were routinely quantitated by PhosphoImager scanning (Molecular Dynamics).

RESULTS

The effect of possible 3′/5′ RNA complementarity

The replication system based on the transfection of the plasmids expressing the viral DI RNAs along with the functions needed for replication (see Materials and Methods) has been found adequate to compare the replication efficiency of various SeV RNAs. This conclusion was reached before, when we examined the effect on RNA replication of reciprocal exchanges of primary sequences present in the viral genome and antigenome promoters (Calain and Roux, 1995): to gradual substitution of sequences corresponded consistent gradual changes in replication efficiency. In this study, the same approach is used to test whether the presence of RNA

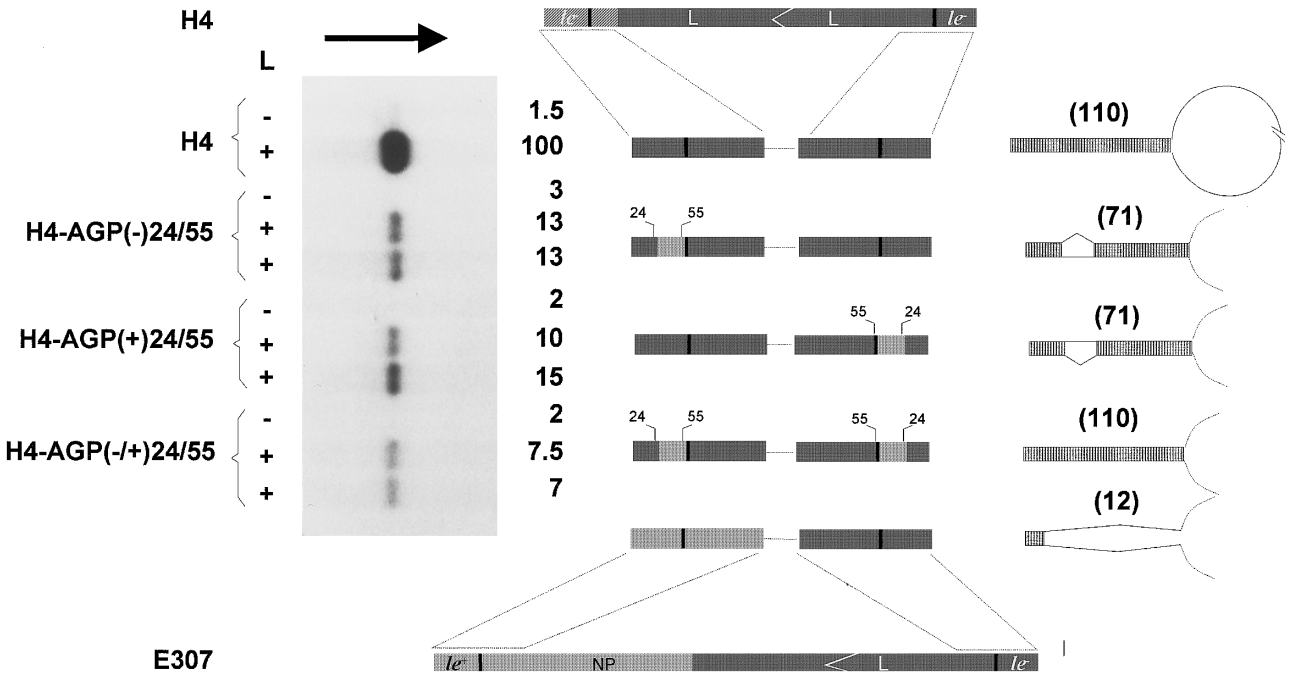

FIG. 2. Disruption and reestablishment of 3′/5′ end complementarity, by intercalation of discontinuous GP sequences into AGP. The H4 RNA plus- or/and minus-strand 3′ ends (dark boxes) were modified by the substitution of nucleotides 24 to 55 with the corresponding nucleotides of the E307 RNA minus-strand 3′ end (GP, light gray box). This substitution was introduced at the 3′ end of the minus or the plus strand as in, respectively, H4-AGP(−)24/55 and H4-AGP(+)24/55, or at both 3′ ends simultaneously, as in H4-AGP(−/+)24/55. The consequence of these substitutions for the 3′/5′ end complementarity is schematically indicated at the right end side of the figure. These RNAs were expressed from plasmids as transcripts of positive polarity by the T7 RNA polymerase in the replication system, as described under Materials and Methods. The replication was monitored by Northern blot analysis of the nucleocapsid RNAs using the probe 5′ ex (see Methods) of positive polarity which reacts with the RNA-strand complementary to the T7 RNA transcript. The −/+ L lanes correspond to replications performed in the absence or in the presence of the viral RNA polymerase L. Duplicate samples are shown for the RNA derivatives. The Northern blot was quantified by PhosphoImager and the numbers refer to the efficiency of replication relative to that obtained for the H4 RNA, arbitrarily set at 100. The arrow indicates the direction of the agarose gel migration.

complementary termini is important for replication efficiency. In a first series of experiments the complementary termini of the H4 RNA are interrupted by the replacement of nucleotides 24 to 55 of its minus strand 3′ end with the corresponding nucleotides coming from the E307 minus strand 3′ end (Fig. 2). This replacement, creating derivative H4-AGP(−)24/55 (for an explanation of the derivative nomenclature see Materials and Methods), decreases the complementary termini from 110 nucleotides down to 71 nucleotides. Figure 2 shows that H4-AGP(−)24/55 replication efficiency decreases correspondingly by about 10-fold relative to H4. Whether the substitution is made in the minus strand 3′ end, as in H4-AGP(−)24/55, or in the plus strand 3′ end, as in H4-AGP(+)24/55, the results are identical. Restoring now the complete H4 RNA end complementarity by introducing the substitution in both 3′ ends [H4-AGP(−/+)24/55] does not restore high replication efficiency. On the contrary, the double substitution has a more pronounced negative effect on replication. Figure 2 presents the results obtained by detecting the minus-strand RNAs. This could, at first, appear inadequate in the cases where modifications are made at the minus-strand 3′ ends and therefore are expected to reflect on the synthesis of the plus-strand RNA. In practice, this possible bias is minimized by the multiple rounds of replication which occur in the system. As a consequence, the probing of the experiment in Fig. 2 with a negative polarity probe gives similar results (not shown).The negative polarity probe, however, can detect, in cases of poor replication, a signal due to the T7 RNA transcript. This is the reason for the preferential use of the positive polarity probe.

At first glance, the results of Fig. 2 do not support the postulate that RNA end complementarity represents a determinant factor of replication efficiency. However, the substitutions may have disrupted the integrity of the H4 RNA promoters, so that the effect of complementarity is now counterbalanced by the creation of weaker promoters. New derivatives are therefore constructed, with substitution of the H4 RNA promoter with noninterrupted sequences coming from the E307 promoter. Since the extent of the template 3′ end RNA sequence necessary to promote replication is yet not defined, nucleotides 1 to 57 or 1 to 98 are substituted at once (Fig. 3). The former region just exceeds the plus leader template region, while the latter extends past the putative BB3 box proposed, on theoretical ground, to be part of the Paramyxovirus promoter (Crowley *et al.,* 1988; Blumberg *et*

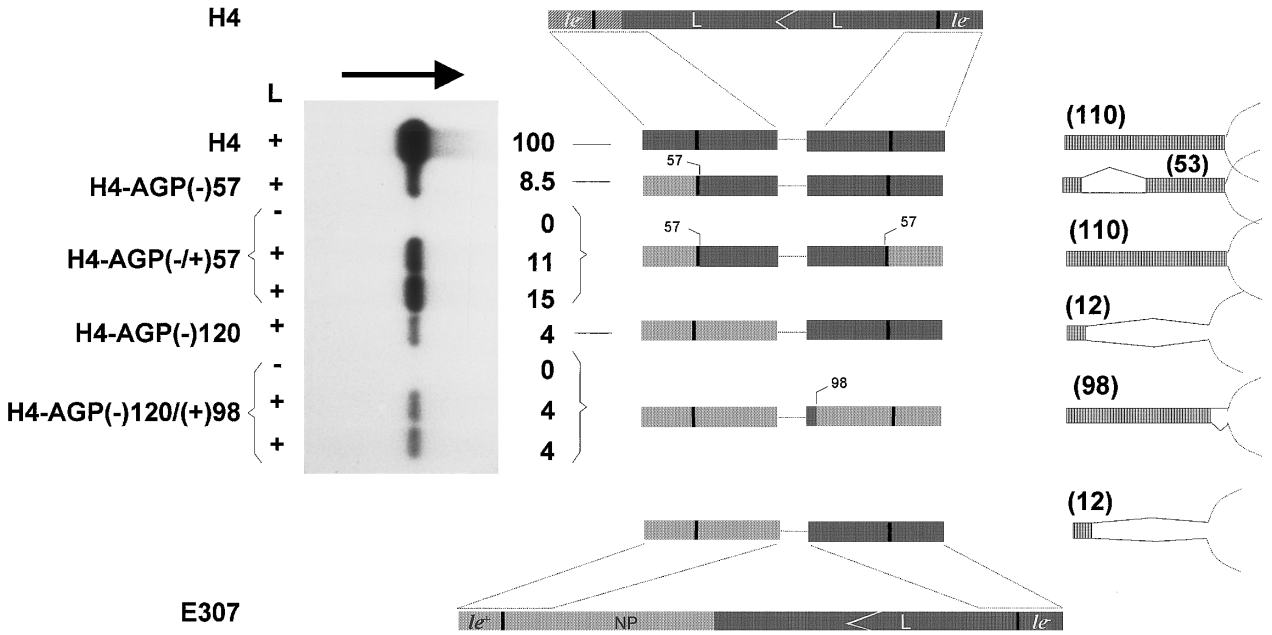

FIG. 3. Generation of further 3′/5′ end complementarity by transfer of continuous GP sequences into AGP in the context of H4 RNA. The experiment was carried out exactly as in Fig. 2, with the 3′ end region substitutions pictured in the middle and the extent of possible 3′/5′ end complementarity drawn on the right end side. The derivatives H4-AGP(−)57 and 120 are taken from Calain and Roux (1995), where they were referred to as, respectively, AGP57 and 120. Arrow as in Fig. 2.

al., 1991). Ninety-eight nucleotides also correspond roughly to the shortest inverted repeat ever described for a Paramyxovirus copy-back RNA (Enami *et al.,* 1989; Calain *et al.,* 1992; Sidhu *et al.,* 1994) and may thus correspond to the minimal Paramyxovirus promoter length. As expected from results obtained previously with similar RNA constructs (in particular H4 AGP65, Calain and Roux, 1995), RNA derivative H4-AGP(−)57, with the minus-strand 3′ end first 57 nucleotides replaced with the corresponding E307 nucleotides, exhibits a decreased replication efficiency (7- to 10-fold compared to the H4 RNA parent, top Fig. 3), which coincides with a decrease in possible base pairing potential (continuous 110 down to discontinuous 12 plus 53). Again, restoring the 110 parental base pairing by introducing the substitution in both the minus and the plus strands [derivatives H4-AGP(−/+)57] does not restore parental replication efficiency. Identical results are obtained with the longer substitutions. When disruption of H4 end complementarity is achieved by a first 120-nucleotide substitution [H4-AGP(−)120], resulting in a more than 10-fold decrease in replication, a second substitution [H4-AGP(−)120/(+)98], restoring continuous possible end complementarity over 98 nucleotides, does not restore high replication. Similar results are obtained with the corresponding derivatives of E307 RNA. In this case it is enough to replace the plus-strand 3′ end with 57 or 98 nucleotides from the minus-strand 3′ end to create a potential base paring over 57 or 98 nucleotides (E307-AGP57, E307-AGP98). E307-AGP98, for instance, with a 98-nucleotide base pairing potential does not replicate better than E307 (not shown). In summary, even when care is taken to create a potential base pairing with an uninterrupted promoter sequence, we are unable, in this system, to find support for the notion that the extent of terminal complementarity affects replication efficiency.

Assessing the high replication property of H4

The observation made in the above experiments rather favors a direct effect of the primary sequence on replication efficiency. Therefore, further substitutions of the H4 RNA minus-strand 3′ end with the corresponding E307 RNA sequence were made in attempts to identify the minimal region carrying the high replication property exhibited by the H4 RNA. In Fig. 2, H4-AGP(−)24/55, with its nucleotides 24 to 55 replaced by the corresponding E307 RNA nucleotides, exhibited a 10-fold decreased replication. This suggested that nucleotides 24 to 55 of the H4 minus-strand 3′ end contains a sequence that confers high replication. In an attempt to more precisely delineate this sequence, the extent of the substitution was first gradually trimmed from nucleotide 55 toward nucleotide 24 to generate derivatives H4-AGP(−)51, H4-AGP(−)48, H4-AGP(−)42 and H4-AGP(−)31 (Fig. 4). Down to the 24 to 31 substitution, all the derivatives exhibit a reduced replication efficiency, showing that it is sufficient to replace the H4 minus-strand 24–31 nucleotides to lose high replication efficiency. Next, the border of the substitution was analyzed from nucleotide 24 toward nucleotide 55 (Fig. 5). The first derivative produced with substitution extending from nucleotide 31 to 55 [H4-AGP(−)31/55] exhibits an almost full parental replication efficiency (Fig. 5). Therefore, if nucleotides 1 to 24 of

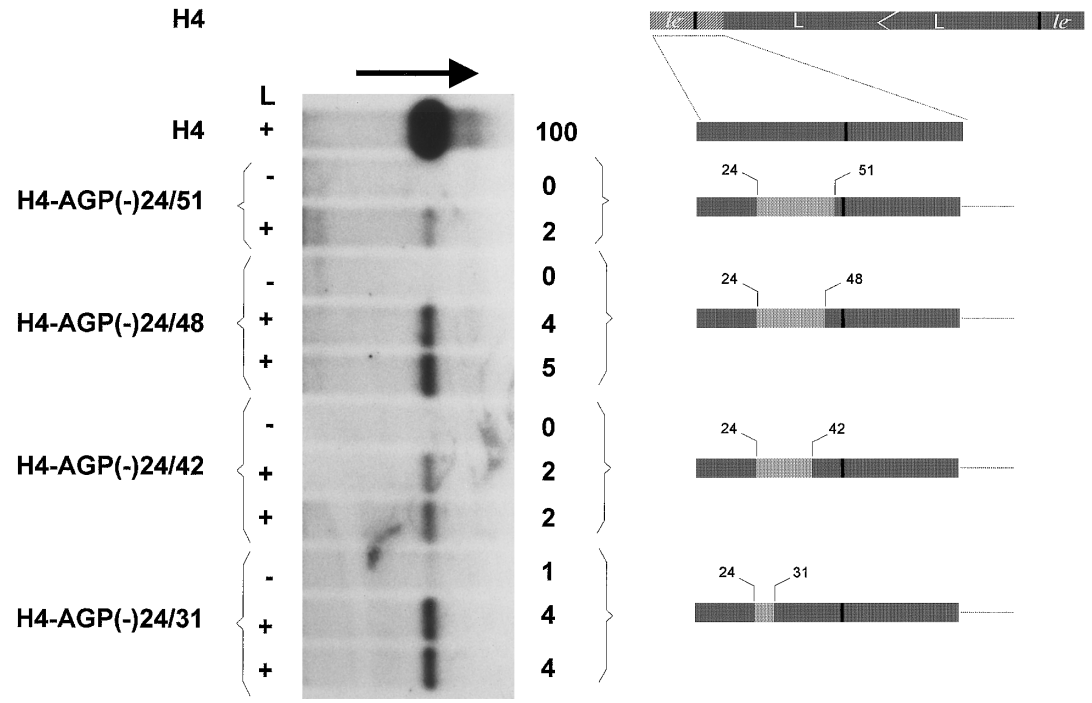

FIG. 4. Analysis of the right end side limit of the AGP sequence required to confer high replication. The experiment was carried out as in Fig. 2, with the H4 derivatives pictured on the right end side. Only the H4 minus-strand 3′ end region has been substituted by the E307 corresponding sequence, and, in consequence, only the H4 minus-strand 3′ end is schematically presented. Arrow as in Fig. 2.

the H4 minus-strand 3′ end region appear insufficient to confer high replication, the extension of these end nucleotides to nucleotide 31 is sufficient. These results agree well with those obtained before, when nucleotides 1 to 33 of the H4 minus-strand 3′ end region, substituting for the corresponding nucleotides in E307 RNA, were found sufficient to increase the E307 RNA replication up to the level of H4-RNA (E307-GP33, Calain and Roux, 1995). In that series of experiments a substitution of 1 to 30 (E307-GP30) had an intermediate effect, close to the

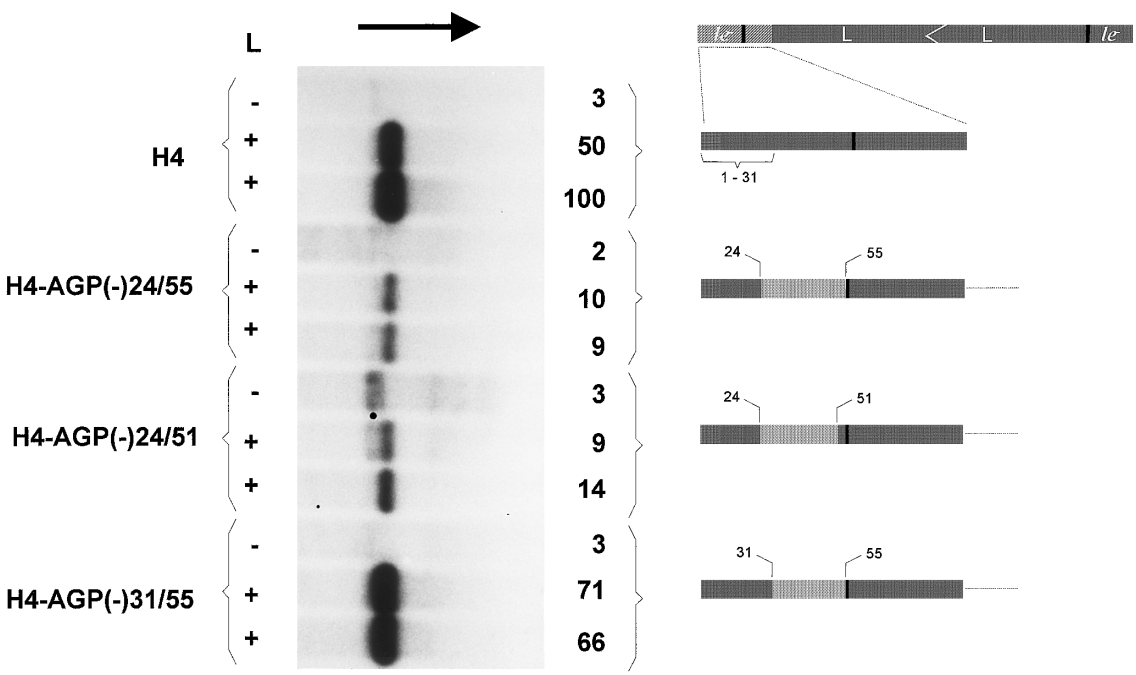

FIG. 5. Left end side limit of the AGP sequence required to confer high replication. As in Fig. 4, but this time H4 derivative AGP(−)31/55 is analyzed in parallel with two derivatives for which the left end border of the substitution of the E307 corresponding sequence is at position 24. The band in lane H4-AGP(−)24/51 L-, marked by a black dot, is explained in the text under Results. Arrow as in Fig. 2.

one exhibited here by H4-AGP(−)31/55. In conclusion, high replication is attached to the H4 minus-strand 3′ end sequence extending from nucleotide 1 to nucleotide 31, regardless of the sequence downstream.

In Fig. 5, an RNA band is visible in lane −L of derivative H4-AGP(−)24/51 (marked by a dot). This band (also visible in the +L lane above the major signal), which migrates at an intermediate position between the H4 and the E307 RNAs, is likely to result from a nonspecific cross-reactivity with the 5′ ex probe. This parasite signal, which appeared unexpectedly, is eliminated by shortening the 5′ ex probe (not shown, see Materials and Methods).

DISCUSSION

In the infected/transfected cell system that we use, the copy-back H4 RNA replication property is compared to that of the internal deletion E307 RNA, and this latter replicates 20-fold less efficiently (Calain and Roux, 1995). As the two RNAs share common plus-strand 3′ ends, but differ in their minus-strand 3′ end, it was reasonable to assume that different replication efficiencies would result from the different minus-strand 3′ end sequences. It is on that basis that the reciprocal replacement approach has been used, assuming, moreover, that there are interchangeable blocks of sequence with different properties. This obviously may have oversimplified the problem. For instance, the displacement of a putative block with a certain property may create a new environment (borders) that can modify the box property. Despite this possible drawback, the reciprocal exchange approach was validated by the results obtained with a series of derivatives for which the replication properties correlated directly to the extent of each other promoter sequence (Calain and Roux, 1995). Moreover, this approach allowed us to test a possible countereffect of transcription over replication by the production of E307 RNA derivatives that exhibited an equally high replication property, independent of their transcription ability. These results failed to support a major effect of transcription on replication in this system (Calain and Roux, 1995).

Encouraged by these previous results, we used this same approach to further define the feature which confers high replication on the H4 RNA. As this RNA contains, on both the minus and the plus strands, the 3′ end sequence of the viral antigenome, the experiments presented here characterize, in essence, the replication promoter present on the antigenome. Moreover, for the H4 RNA, the partial replacement approach has the inherent consequence of partially disrupting the H4 RNA copy-back feature. The study of the RNA derivatives produced to investigate the consequence of this change clearly shows that the extent of the terminal base pairing potential has no consistent effect on replication efficiency. The derivatives presented in Fig. 3 are particularly relevant. The restoration of complete (110 nt) or almost complete (98 nt) H4 base pairing potential in H4-AGP(−/+)57 and H4-AGP(−)120/(+)98 RNAs does not increase their replication above the level of their respective parents, H4-AGP(−)57 and H4-AGP(−)120. These had lost replication efficiency after a putative disruption of their base pairing potential. Also, a disruption of only 7 nucleotides of the base pairing potential has a 20-fold down effect on replication [see H4-AGP(−)24/31, Fig. 4], while a disruption of 24 nucleotides has virtually no effect [see H4-AGP(−)31/55, Fig. 5]. In the case of SeV, therefore, the extent of 3′/5′ end complementarity per se does not represent a factor that can influence replication efficiency.

The data obtained with the various derivatives can be better interpreted as a direct role of the promoter primary sequence. The new derivatives addressing this question (Figs. 4 and 5) identify the first 31 nucleotides present at the antigenome 3′ end as an important determinant for the high replication property. Indeed, the derivatives containing various lengths of genome promoter sequence but containing only the first 24 nucleotides of the antigenome promoter (Figs. 4 and 5) replicate poorly. Only H4-AGP(−)31/55, which has acquired the first 31 nucleotides of the antigenome, recovers high replication (Fig. 5). The question can be raised as to whether the 7 nucleotides (24–31) of the antigenome promoter are sufficient. In fact, when these 7 nucleotides are introduced at the same position in the genome promoter of E307, no increase in replication is observed (E307 GP24/53, Cadd *et al.,* 1996), arguing that the signal for high replication is not confined to these 7 nucleotides, but extends in the 1–24 nucleotides toward the 3′ end. Since high replication depends on the presence of the 31 nucleotides at both the minus- and the plus-strand 3′ ends, this creates the potential for a terminal complementarity over 31 nucleotides. In this respect, it is impossible to formally exclude a participation of this potential complementarity in the high replication property. Were this the case, however, then the effective complementarity could only be created by base pairing of nucleotides 1–31 of the antigenome. Therefore, at the least, terminal complementarity per se would not be enough to confer high replication, since this would have to be generated by a specific sequence.

The identification of the 1–31 nucleotide determinant does not exclude the presence of other features governing replication efficiency. In fact, in the absence of these 3′ end 31 nucleotides, i.e, when they are replaced by nucleotides coming from the viral genome 3′ end, a second region of the antigenome 3′ end, extending from nucleotides 48 to 65, or better 48 to 98, also exerts a positive effect on replication (not shown). This effect, however, is minor (2- to 3-fold) compared to that achieved by the first 31 nucleotides (10- to 50-fold) and is only apparent when the major effect is not present (not shown). All these observations now agree well with those made before with substitutions extending linearly form the 3′ ends for different lengths (Calain and Roux, 1995).

They also support the conclusion that in the case of SeV, and maybe in the case of all the Paramyxoviruses, the promoter regions involved in replication may be longer than the leader template regions (BB3 box, Crowley *et al.,* 1988; Blumberg *et al.,* 1991). From a series of experiments where the genomic promoter, this time, has been under scrutiny through various insertions, a similar conclusion has been drawn: the replication promoter appears to extend past the le^{+} template region and may be discontinuous in nature (Pelet *et al.,* submitted for publication).

The identification of the 1–31 nucleotide determinant does not explain the mechanism controlling replication efficiency. This could apply on the template, if the 31 first nucleotides of the promoter contain a higher-affinity binding site for the RNA polymerase. Alternatively, the encapsidation of the 5′ end nascent strand could be more efficient, allowing the RNA polymerase, which would bind equally to the genome and the antigenome, to proceed more efficiently toward productive replication. Attempts to clarify this mechanism by measuring the ratio of leader RNAs to their final replication products using representative RNA derivatives are under way. The present results can be interpreted by attributing a positive effect on replication efficiency to the identified nucleotides. There is, however, no way to tell whether one deals with a direct positive effect or whether these nucleotides are the sites where a negative control cannot apply. In fact, a recent study by Cadd and co-workers (1996), as well as unpublished experiments, would tend to favor this latter interpretation, since conditions can be found where the replication of most of the poorly replicating RNAs is improved in assays where the C protein is not expressed. This suggests that the replication of these RNAs is under a negative control that can be relieved by the absence of C. Further work is under way to more clearly understand the role of C. In regard to the derivatives presented in this paper, however, we stress that the absence of C does not change the conclusions. For example, if the absence of C results in a significant increase in H4-AGPP(−/+)57 replication, this, however, is paralleled by a similar increase in H4-AGP(−)57 replication under the same conditions, so that the restoration of the end complementarity has still no effect on replication efficiency (not shown).

In the end, our results diverge from those obtained previously with a VSV derivative le47 (analogous to H4-2AGP57) for which the terminal complementarity had a positive effect on replication (Wertz *et al.,* 1994). This result was interpreted as evidence that the nucleocapsid ends communicate to promote efficient replication. Such a control mechanism is believed to apply in the case of the influenza viruses for which partial 3′/5′ end complementarity of the segmented RNAs is described (Robertson, 1979; Desselberger *et al.,* 1980) and proposed to operate in transcription control (Fodor *et al.,* 1994). Partial 3′/5′ RNA end complementarity has been identified as well for the Arenaviruses and the Bunyaviruses over, respectively, about 80 and 20–30 nucleotides. In all these cases, evidence for actual base pairing (Raju and Kolakofsky, 1989) or for the formation of circles has been readily observed in a large proportion of nucleocapsids (Pettersson and von Bonsdorff, 1975; Samso *et al.,* 1975; Obijeski *et al.,* 1976; Palmer *et al.,* 1977; Young and Howard, 1983; Bishop and Auperin, 1987). Although the extent of the H4 RNA base pairing potential is larger than that in any of the cases mentioned above, H4 circular nucleocapsids have been detected with an exceedingly low probability estimated to be 1 in 10,000 (Kolakofsky and Boy de la Tour, unpublished). Whether the results obtained here represent another indication that the nucleocapsid ends do not interact in the cases of the nonsegmented negative-stranded RNA viruses or whether a putative RNA termini interaction takes place via a mechanism that does not involve termini base pairing but rather protein interactions, as suggested more recently by the results of Baudin and colleagues (1994) for influenza virus, is open to question. The answer to this question is likely to come from further studies involving other nonsegmented negative-stranded RNA viruses, such as rabies, measles, and respiratory syncytial viruses, for which reverse genetics now allows this type of analysis (Conzelmann and Schnell, 1994; Garcin *et al.,* 1995; Lawson *et al.,* 1995; Radecke *et al.,* 1995; Whelan *et al.,* 1995).

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