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Internal and terminal *cis*-acting sites are necessary for *in vitro* replication of the L-A double-stranded RNA virus of yeast

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Empty particles of the L-A dsRNA virus of *Saccharomyces cerevisiae* bind to added viral (+) strands and convert them to dsRNA (RNA replication) in an *in vitro* reaction that is dependent on host factors. X dsRNA (530 bp long) is a deletion derivative of L-A dsRNA (4.5 kb). By modifying our cDNA clone of X and testing template activity of T7 RNA polymerase transcripts, we have found that both the 3′ end 30 bases and an internal site on the (+) strand are necessary for optimal replication [*in vitro* (−) strand synthesis]. Changing any one of the 3′ terminal three bases eliminates template activity, but the 3′ terminal five bases of M_1 (a satellite virus of L-A) can replace the 3′ terminal four bases of X. A subterminal stem−loop structure is also important for template activity. The internal site that enhances replication is ~400 bp from the 3′ end and is distinct from the site necessary for binding of (+) strands to the empty viral particles.

Key words: binding sequence/replicase recognition sites/replication enhancer/*Saccharomyces cerevisiae*/stem−loop structure

Introduction

In the replication cycle of positive-strand RNA viruses, dsRNA viruses and retroviruses, the viral plus strands serve as mRNA, as the species packaged to form new virions and as a template for replication. It is essential that the packaging and replication steps have sufficient specificity to prevent propagation of non-viral RNAs at the expense of the virus. Furthermore, packaging and replication signals must be such that splicing or other modification of the (+) strands as mRNA does not produce mutant viruses. The nature of the *cis* sites also gives clues about the interactions of the replication apparatus with its template and knowledge of such signals is important for the development of vectors based on RNA viruses.

The 4.6 kb L-A dsRNA virus of *Saccharomyces cerevisiae* transcribes its genome in a conservative reaction (Fujimura *et al.*, 1986) and extrudes the product (+) ssRNA from the viral particles (Esteban and Wickner, 1986). These plus strands serve as mRNA to synthesize the major coat protein (Hopper *et al.*, 1977) and a minor viral 180-kd protein with single-stranded RNA binding activity (Fujimura and Wickner, 1988b) that is apparently the RNA polymerase (T.Icho and R.B.Wickner, submitted). This 180-kd protein is a fusion protein with an N-terminal major coat protein domain and a C-terminal RNA polymerase/RNA binding domain (Fujimura and Wickner, 1988b; T.Icho and R.B.Wickner, submitted). This gag−pol-like fusion protein is produced by the fusion of two overlapping open reading frames (ORFs), possibly by a ribosomal frameshifting mechanism (T.Icho and R.B.Wickner, submitted). The (+) strands are packaged to form new viral particles (Fujimura *et al.*, 1986) and we have suggested that the 180-kd protein has a crucial role in the packaging process (Fujimura and Wickner, 1988b). These particles then copy the (+) strand [(−) strand synthesis] to make dsRNA, completing the cycle (Fujimura *et al.*, 1986; Fujimura and Wickner, 1987).

M_1 dsRNA, encoding a secreted protein toxin and immunity to that toxin (reviewed by Bussey, 1988), is a satellite virus of L-A, depending on L-A for its own replication (Bostian *et al.*, 1980; Sommer and Wickner, 1982). X dsRNA (530 bp in length) is a deletion mutant of L-A which is, like M_1, packaged, replicated and transcribed in L-A viral particles and so must include all of the *cis* sites necessary for these processes (Esteban and Wickner, 1988).

Template-dependent *in vitro* systems now exist that execute several steps in L-A viral gene expression and replication: (i) specific binding of viral (+) strands to empty L-A viral particles in a gel-retardation assay (Esteban *et al.*, 1988; Fujimura and Wickner, 1988b), (ii) RNA replication [(−) strand synthesis on an added (+) strand template] (Fujimura and Wickner, 1988a) and (iii) RNA transcription [(+) strand synthesis on an added dsRNA template] (T.Fujimura and R.B.Wickner, in preparation). These *in vitro* systems make possible a detailed analysis of the template sites and the enzymes involved in these processes. M_1 and X (+) strands are likewise bound and replicated by these empty particles. Clones of X dsRNA (Esteban *et al.*, 1988) can be used to produce altered viral RNAs which are then tested in the *in vitro* systems. We began such an analysis by defining a 75-bp region of X dsRNA [between bases 117 and 192 of the X (+) strand] that contains a site determining specific binding of viral (+) strands to empty L-A viral particles (Esteban *et al.*, 1988).

Here we report that a 3′ terminal sequence and a nearby stem−loop stucture are, along with a distant site, necessary for the *in vitro* replication reaction. A further definition of the viral particle binding site suggests that an RNA folded structure determines binding. We have suggested that this binding is related to packaging rather than to replication (Fujimura and Wickner, 1988b) and data presented here support this notion.

Results

T7 RNA transcripts from an X cDNA clone are replicated in vitro by empty L-A viral particles

Isolated L-A viral particles, when exposed to low ionic strength, release their dsRNA. These empty L-A viral

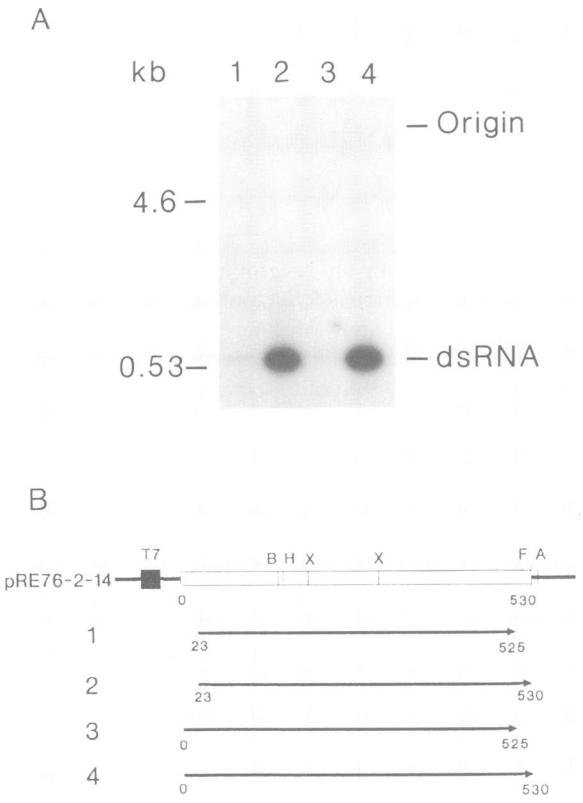

Fig. 1. Replication by empty viral particles of T7 RNA transcripts made from an X cDNA clone. Unlabeled T7 RNA polymerase transcripts (100 ng/reaction) were used as templates for a replication reaction with empty viral particles (15–20 μg of protein) and [^{32}P]UTP. The labeled dsRNA was separated from incorporated nucleotides by agarose gel electrophoresis. An autoradiograph of the gel is shown in the upper part of the figure. In **lanes 1** and **3** T7 transcripts [from pRE76 and pRE76-14 (see Materials and methods) digested with *Bam*HI] lack five bases from the 3′ end of X and have eight extra bases of vector sequence. In **lanes 2** and **4** T7 transcripts (from pRE76-2 and pRE76-2-14 digested wtih *Fsp*I) have the correct 3′ end of X. Sequences of X (+) strands present in each transcript are indicated in the diagram in **part B** of the figure. A schematic representation of plasmid pRE76-2-14 (which contains the entire X cDNA sequence downstream of the T7 RNA polymerase promoter) is also shown. Restriction sites are: B (*Bst*XI), H(*Hae*III), X (*Xmn*I), F (*Fsp*I), A (*Bam*HI). Molecular standards used to determine the size of the dsRNA synthesized *in vitro* were L-A dsRNA (4.6 kb) and X dsRNA (0.53 kb).

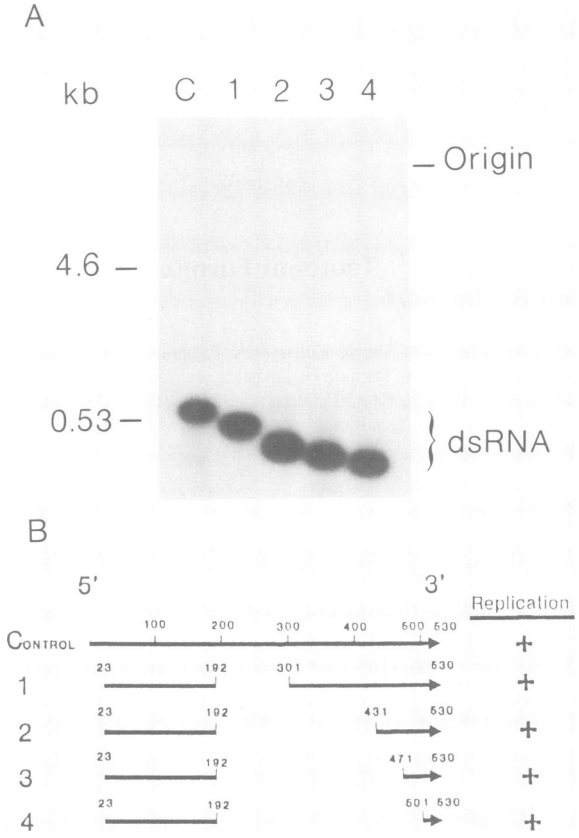

Fig. 2. Effect of internal deletions on X replication. T7 RNA polymerase transcripts were made from plasmids that contained variable portions of the X sequence and the effect of these deletions was tested in a replication reaction. **Lane C**: the T7 transcript of pRE76-2-14 with the complete X cDNA sequence and the correct 3′ end was used. **Lane 1**: pRE89 was used as a template for T7 RNA polymerase transcription. It lacks a 108-bp *Xmn*I fragment. **Lanes 2, 3** and **4** are further deletions of various sizes obtained using pRE89 as the template for oligonucleotide site-directed mutagenesis. The size and location of the remaining X (+) strand sequences in each transcript are indicated in the diagram in part B of the Figure.

particles have a replicase activity (Fujimura and Wickner, 1988a) that can use X (+) ssRNA, produced by the transcriptase of purified X viral particles, as template (Esteban *et al.*, 1988) to produce dsRNA by the synthesis of full-length, complementary X (−) strands. This *in vitro* replication assay provides an excellent tool for the study of the *cis*-acting signals in a template ssRNA molecule that are responsible for the specificity and activity of the viral particle replicase. To modify the X RNA sequence, we used an X cDNA cloned downstream of a T7 RNA polymerase promoter, plasmid pRE76 (see Materials and methods). This plasmid contained all X cDNA sequences except for the first 23 bp of the 5′ end and the last 5 bp at the 3′ end of the X (+) strand.

When T7 RNA transcripts prepared from plasmid pRE76 (digested with *Bam*HI) were used in the replication reaction only a low level of incorporation of [^{32}P]UTP into dsRNA of the size expected from the size of the T7 ssRNA was observed (Figure 1A, lane 1). The ssRNA transcripts used as template lacked the last five nucleotides of the X (+) strand 3′ end, and contained eight extra nucleotides derived from the *Sma*I and *Bam*HI sites of the vector: . . . GGGGGAUC-OH. In contrast, the 3′ end of the X (+) strand is . . . UGC-OH. As will be discussed below, the low, but real, template activity in lane 1 can be explained by the sequence of the last three bases in the T7 RNA transcript which are the same as the last three bases at the 3′ end of M_1 (+) strands.

Lanes 2 and 4 in Figure 1 show the results obtained when T7 transcripts were prepared from plasmids pRE76-2 and pRE76-2-14 which contain the entire 3′ end of the X (+) strand. In each case, following their digestion with *Fsp*I, the expected 3′ end of the run-off transcript is the same as that of the X (+) viral strands. Both RNAs were replicated by the viral particle polymerase and the efficiency of replication was similar to that for X (+) viral strands (produced by viral particles transcribing X dsRNA) (not shown). Plasmids pRE76-2 and pRE76-2-14 differed in their 5′ ends, but that seems to have no effect on replication. Likewise, transcripts of plasmid pRE76-14 (lane 3), that include the

correct 5′ end but the same defective 3′ end as pRE76, showed little template activity.

The results in Figure 1 suggest that the 3′ end of the X (+) viral strand plays an important role in replication [(−) strand synthesis] whereas the 5′ end is not necessary. Furthermore, since the template RNA is made *in vitro* by T7 RNA polymerase from cloned X cDNA, these results indicate that this *in vitro* replication system can initiate and elongate new RNA chains.

In order to study in detail which parts of the X RNA sequence were necessary or sufficient for replication we modified the X cDNA sequences by oligonucleotide site-directed mutagenesis and prepared ssRNA transcripts using T7 RNA polymerase, testing the effect of the changes made on replication. Our previous data (Esteban *et al.*, 1988) delimited a region between bases 117 and 192 necessary for specific binding of X (+) strands to empty viral particles. We therefore next tested what sequences between base 192 and the 3′ end (base 530) were necessary for *in vitro* replication by making a series of internal deletions (Figure 2).

Effect of internal deletions on X replication

The first deletion was made by removing an *Xmn*I fragment of 108 bp from plasmid pRE76-2 to obtain pRE89 (see Materials and methods). This plasmid was used subsequently as a template to eliminate additional downstream regions (Figure 2). All the deletion mutants contained X sequences from base 23 to 192 and variable portions of the 3′ end. There was no effect on replication even when bases 193−500 were eliminated leaving only 30 bases at the 3′ end (Figure 2). This result, together with those summarized in Figure 1, suggests that the information present in the last 30 nt at the 3′ end of the X (+) strand is important for replication and that the >300 nt eliminated between bases 192 and 500 contain no *cis*-acting signals for the replicase associated with the viral particles. In addition, it seems that the distance between the binding region and the 3′ end is not critical for replication.

The sequence at the exact 3′ end of the X (+) strand is required for in vitro replication

The template DNA used for this series of mutants contained the entire X cDNA sequence (pRE76-2-14). When changes were made in the 3′ terminal nucleotides of the X (+) strands (diagrams shown in Figure 3B′), additional changes were also made in adjacent downstream nucleotides in order to produce a restriction site such that after digestion with the appropriate enzyme the template strand for T7 RNA polymerase transcription contained the desired change at the 3′ end. Plasmids modified in that way were also easily detected by the presence of the new restriction site.

Deletion of three, five or nine bases from the 3′ end resulted in complete loss of template activity (data not shown). We also changed each of the last 3 nt to an A, and each of these changes resulted in drastic reduction of template activity, as did deletion of even a single nucleotide (Figure 3B and B′). Addition of a single A residue, however, did not affect template activity as might have been expected since L-A has been shown to have either a paired C or an unpaired A at its 3′ end (Bruenn and Brennan, 1980; Thiele *et al.*, 1984).

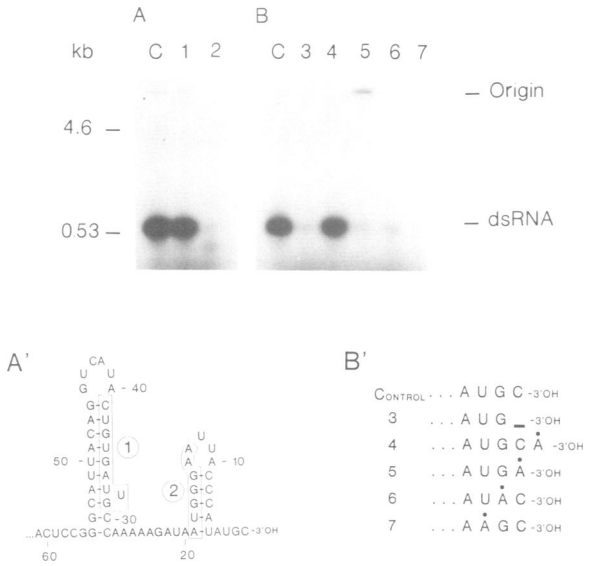

Fig. 3. Effect of changes in the 3′ end region on X replication. The experimental procedure is the same as described in the legend to Figure 1, with non-radioactive ssRNA transcripts used as templates for *in vitro* replication with empty viral particles. Here bases are numbered from the 3′ end. (**A** and **A′**) **Lane C** (control): the T7 RNA transcript was obtained from pRE76-2-14 after digestion with *Fsp*I. **Lane 1**: deletion of nine bases, from base 31 to 39. **Lane 2**: deletion of 7 nt, from base 13 to 19. Part A′ shows where those deletions were located within the 3′ end region of the X (+) strand. Each of these deletions (boxed sequences in A′) eliminates one of the two stem−loop structures described by Thiele *et al.* (1984). (**B** and **B′**) Changes introduced in the last 3 nt of the X (+) strand 3′ end are shown (B′) and the effect on replication is shown in part B. **Lane 3**: the last nucleotide (C) was removed. **Lane 4**: an additional A was added. **Lanes 5, 6** and **7**: the first, second and third bases (from the 3′ end) were changed to an A, indicated by a dot above the base changed.

The stem−loop structure near the 3′ end plays a major role in replication

Deletion of nine bases from base 31 to 39 (from the 3′ end) had no effect on replication while deletion of seven bases from base 13 to 19 (from the 3′ end) reduced template activity to <5% of the control (c.f. lanes 1 and 2 in Figure 3A). The sequences deleted were chosen because of the existence of two stem−loop structures in the L-A (+) strand (and also in X) described by Thiele *et al.* (1984). Our results indicate that the stem−loop structure from bases 29 to 55 is not required for X replication, while some nucleotide sequences upstream of base 12 (from the 3′ end) in the X (+) strand were necessary if not for initiation of replication itself (some replication seems to occur), at least to obtain a high yield of dsRNA synthesis (lane 2). Whether the decreased replication activity resulting from deletion of bases 13−19 was due to the absence of the stem−loop structure or to changes of the nucleotide sequence itself was tested by modifying both sides of the stem such that the sequence of the loop was kept intact and the free energy of the stem−loop structure (−9.0 kcal/mol) was unchanged. Figure 4 shows the effect of changing the nucleotides -15 GGG 17- to CCC (lane 1) or the -7 CCC 9- to GGG (lane 2). In either case, template activity was <5% of the control (similar to the deletion of bases 13−19). However, when both sequences were modified simultaneously (Figure 4, lane 3), replication was similar to the control sample. This result shows that this structure indeed plays a major role in X or

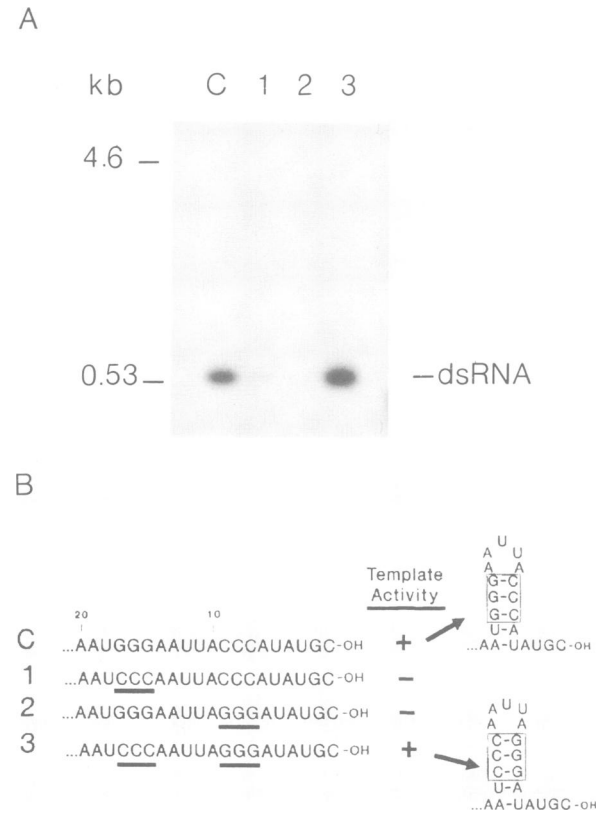

Fig. 4. Importance of the 3′ end stem–loop structure on replication. The experimental procedure is as in Figure 1. **Lane C**: the template RNA used was the same as in Figures 2 and 3 (from pRE76-2-14) and can form the structure indicated in the diagram in part B. **Lanes 1, 2** and **3** represent transcripts with the modifications indicated in part B of the figure. The sequences changed in each template RNA are underlined. The template activity of transcripts C and 3 is similar even though the sequence at the 3′ end is quite different. Both RNAs however, maintain a similar stem–loop structure with the same loop and the same free energy (−9 kcal/mol).

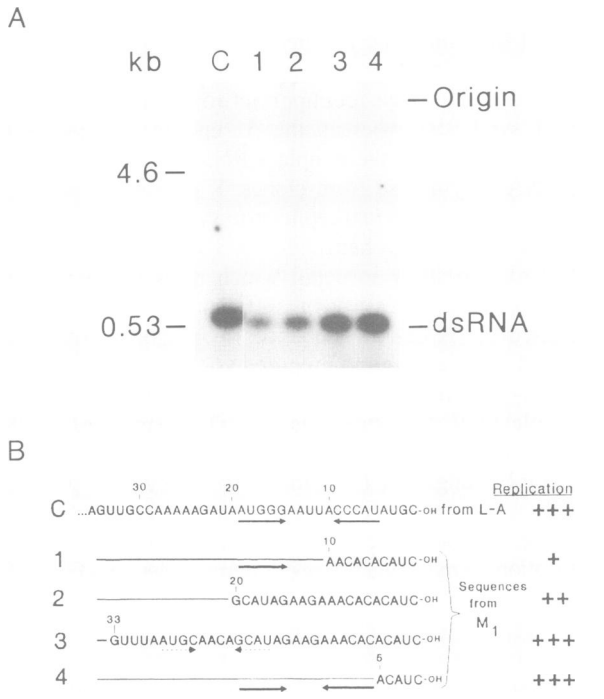

Fig. 5. Effect of M_1 3′ end sequences on replication. The T7 transcripts used as templates for this *in vitro* replication reaction contained variable portions of the M_1 (+) strand 3′ end sequences (indicated in **part B**) and the effect on replication is shown in **part A. Lane C** represents the same control sample containing the entire X sequence shown in previous figures. **Lanes 1, 2** and **3** have 10, 20 and 33 bases of the X 3′ end sequence substituted by M_1 3′ end sequences. In these three samples the stem–loop structure (indicated by arrows underlining the sequences involved) of L-A's 3′ end is eliminated by the changes introduced. Dotted arrows in sequence 3 indicate a possible secondary structure in the M_1 sequence from base 17 to 28, numbering from the 3′ end. **Lane 4**: the transcript has five bases from the 3′ end of M_1, but only four bases of L-A sequence were removed so the L-A stem–loop structure is maintained.

L-A replication. The diagram in Figure 4B shows schematically the effect of changes made in transcripts 1, 2 and 3 on the secondary structure.

M_1 3′ end (+) strand sequences can substitute for the X 3′ end

In addition to L-A and X [both have the same 3′ end (+) strand sequences], M_1 (+) strands can be replicated by empty L-A viral particles (Fujimura and Wickner, 1988a). M_1 and L-A, however, show no nucleotide sequence homology at the 3′ end (Figure 5B, rows C and 3), and as we have shown in Figure 3B, slight changes in the nucleotide sequences near the 3′ end of X cause a drastic decrease in the efficiency of dsRNA synthesis. Our results also show that the stem–loop structure upstream of the 3′ end plays a major role in replication (Figure 4) and no such structure can be found in the M_1 sequence comparably close to the 3′ end: the only possible secondary structure in the M_1 3′ end is 16 nt upstream (indicated by dotted arrows in Figure 5B) and is less stable (free energy = −4.0 kcal/mol) than the one in L-A (free energy = −9.0 kcal/mol).

In order to study whether M_1 sequences were able to support X replication, we substituted the first 10, 20 or 33 nt of the X (+) strand 3′ end by the first 10, 20 or 33 nt of M_1 respectively. pRE76-2-14 was modified using synthetic oligonucleotides based on the M_1 3′ end sequences (Thiele *et al.*, 1982). Introduction of a new *Pst*I site (CAGCTG) [C being the last nucleotide of the M_1 (+) strand 3′ end] and cutting the template with *Pst*I produced a template strand with the correct end.

With 10 and 20 nt of M_1 substituted for X (Figure 5A, lanes 1 and 2), <15 and <25%, respectively, of dsRNA synthesis was observed compared to the original X sequence (lane C). When 33 nt were substituted (lane 3), the amount of dsRNA obtained was similar to that with X. These results suggest that within the first 33 nt of the M_1 (+) strand 3′ end there are *cis*-acting signals that allow a degree of template activity similar to that of the first 30 nt of X, even though both have completely different sequences and structures.

When we substituted the four bases at the 3′ end of X (leaving the stem–loop structure intact) with the five bases of the 3′ end of M_1, the dsRNA synthesis observed was comparable to the control lane C. This shows that the first 5 nt of the M_1 (+) strand 3′ end provide a suitable signal for replication and also that the effect of the secondary structure of L-A is independent of the sequence at the 3′ end of L-A. Thus, there are at least two independent types of signals for replication near the 3′ end: (i) the sequence of the first few nucleotides itself is important for initiation, but appears to exist in two different interchangeable forms

exemplified by the different 3′ ends of the M_1 and L-A (+) strands (Figure 5B) and (ii) some signals upstream that increase considerably the efficiency of replication (the stem−loop structure in L-A and probably some other sequences present in the first 33 nt of M_1). These results may also explain the low, but real, template activity of pRE76 cut with *Bam*HI (Figure 1) which produces T7 transcripts lacking the last five bases of X, but have instead the sequence . . . GGGGGAUC-OH 3′, the last three bases being the same as the last three bases of M_1. The observation that this RNA had very low template activity suggests that either the fourth base from the 3′ end is also important or that the distance from the stem−loop structure is critical.

Structure and location of the viral particle binding region

We have previously shown by a gel-retardation assay that the empty viral particles used in the replication reaction have a binding affinity specific for the template ssRNA (Esteban *et al.*, 1988; Fujimura and Wickner, 1988b). We localized the region responsible for the binding activity to bases 117−192 and suggested that the sequence -151 UUUGGC-CAGG 160-, which is also found in M_1 (+) strands, might be responsible for the binding. This hypothesis was apparently confirmed when we found that neither a subclone with bases 23−156 (ending right in the middle of the putative binding site) nor one with bases 156−523 had binding activity (data not shown). A more detailed analysis of that region of X, however, has allowed us to properly identify the sequence responsible for binding. It includes part of the 10 bases initially proposed but also extends further upstream in the X RNA (+) strand. Using as template ssDNA from pRE89, we created deletions starting at bp 192 and going upstream or starting at bp 95 and going downstream. Deletions of bases 176−192 or of bases 164−170 affected neither binding nor replication (not shown). Figure 6A shows the effect of some of the deletions that abolish binding (transcripts c and e), whereas transcripts that contained only a few more nucleotides upstream (transcript b) or downstream (transcript d) had normal binding affinity. In this way we defined a region of 24 bases between base 131 and base 154 that contains the sequence necessary for binding to the empty viral particles. Figure 7 shows the nucleotide sequence from base 111 to 155 of the X (+) strand. The binding region between base 131 and 154 can be folded forming a stem−loop structure with a free energy of −2.1 kcal/mol. This secondary structure is produced by the FOLD program of the University of Wisconsin Genetics Computer Group package (Zuker and Stiegler, 1981) as the structure for this region when X assumes its optimal conformation. The other sequences shown in Figure 7, upstream of the binding site, have a role in replication that will be described below.

The fact that the binding site was located exclusively upstream of bp 156 was surprising and did not explain why in our previous experiments T7 RNA polymerase transcripts that contained X (+) strand sequences upstream of base 156 (prepared from plasmid pRE97 after digestion with *Sac*I) had no binding affinity. We thought that perhaps the RNA surrounding the binding site (independent of the sequence itself) might stabilize the binding complex or the secondary structure of the binding region. Using the same FOLD program, we found that when the X RNA molecule ends at base 156 other structures for the binding region (bases

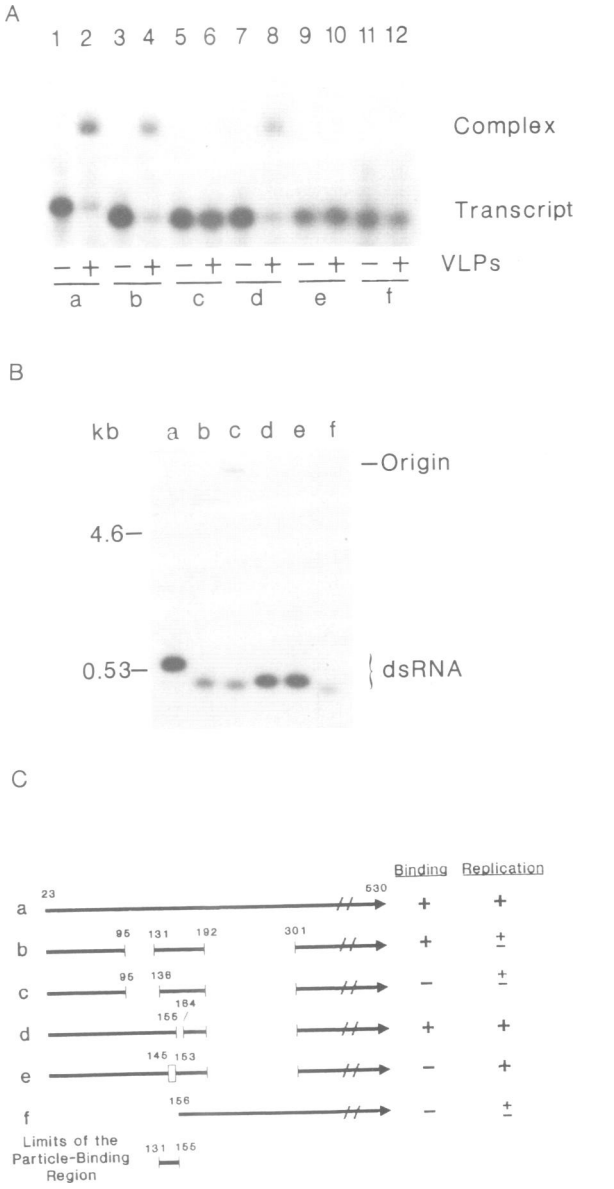

Fig. 6. Viral particle binding site (VBS) and internal replication enhancer (IRE). **(A)** ^{32}P-Labeled T7 transcripts were used in a binding reaction with empty viral particles and the binding complexes formed separated in a gel retardation assay. An autoradiograph of the gel is shown. Transcript a (control) has the complete X sequence. Using pRE89 as a template, internal deletions or substitutions were made within the interval bp 95−bp 192 (see text), and their effects on binding examined. Transcripts b and c define the upstream limit of the particle-binding site as between bases 131 and 136. Transcripts d and e define the downstream limit (between bases 145 and 154). Transcript f was made from pRE91 and lacked binding activity completely. **(B)** Transcripts with the same X sequences as in A, but unlabeled, were used as templates in replication reactions. Transcripts b, c and f have 10−20% template activity for replication compared to transcripts a, d and e. There was no direct relationship between the ability to bind to viral particles and replication efficiency. Transcripts b, c and f lacked some sequence upstream of base 131 that forms part of an IRE, whose upstream limit is defined by results presented in the text. **(C)** Diagram of the deletions (−| |−) or substitutions (−□−) made in each of the transcripts used in A and B.

131−154) appeared to be more stable than that shown in Figure 7. This probably indicates that the binding sequence, to be fully functional, needs to be surrounded by other

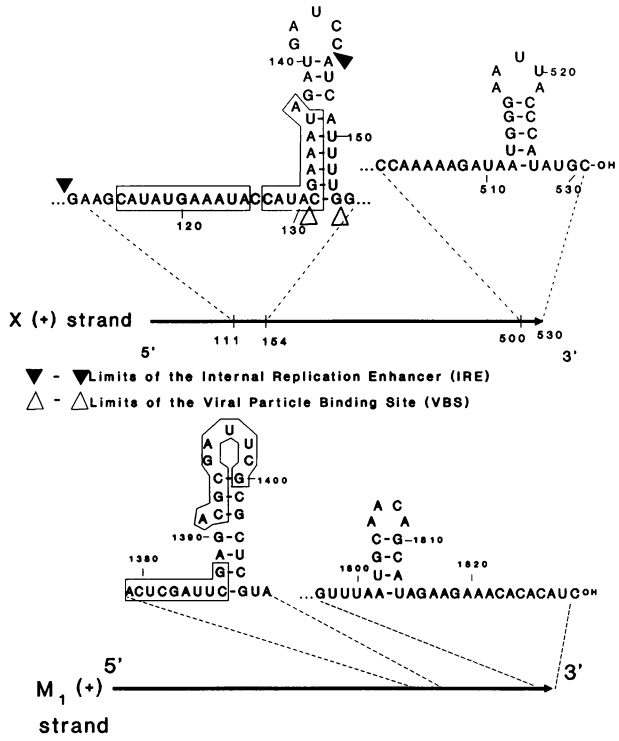

Fig. 7. Summary of sites affecting replication of X (+) strands and their specific binding to empty viral particles. **Upper panel**: X (+) strand. The stem−loop at the 3′ end and the 3′ terminal four bases are necessary for maximal replication. The terminal 30 bases (with the IRE) are sufficient. The VSB could potentially form the stem−loop structure shown. The open and closed triangles show the outer limits of the VBS and IRE respectively. The boxed sequence is a 10 of 11 base direct repeat sequence within the IRE region. **Lower panel**: M_1 (+) strand. The 3′ end of M_1 can substitute for the X 3′ end. the internal region of M_1 shown has a structure like that of the IRE−VBS region of X and we speculate that it may have a similar function for M_1 (+) strand replication and binding.

sequences (to be internal) and not at the 3′ end of the molecule.

The viral particle binding site is not necessary for replication

Transcript f in Figure 6A had no binding activity and its template activity for replication was reduced to <20% that of the control (compare lanes a and f in Figure 6B), suggesting that the viral particle binding site was important for replication. To test this, we examined a series of transcripts for binding activity (Figure 6A) and for replication template activity (Figure 6B). Unexpectedly, we found that a region upstream of the binding site, not the binding site itself, was affecting replication: transcripts b and c that were deleted from base 96 to 130 and base 96 to 135, respectively, had template activity similar to transcript f (<20% of the control sample), whereas transcripts d and e showed full template activity (even though transcript e had no binding affinity). These results show that two distinct sequences exist with different roles in binding and in replication. Transcript b, for example, does bind and shows very low template activity while transcript e does not bind and supports normal replication (Figure 6A and B).

The deletions used to define the binding site (Figure 6C) only established that the region affecting replication includes some of the interval from base 95 to 131 and does not extend downstream beyond base 145. To further define this region, we made several additional deletions using pRE89 as template. Deletions from base 80 to 95, and from 95 to 110 gave template activity similar to the pRE89 control (not shown). Deletion of bases 95−125 did, however, reduce template activity to <20% of the control. These results, together with those shown in Figure 6, define the interval necessary for replication activation as downstream of base 110 and upstream of base 146. We call this the internal replication enhancer (IRE). The sequence affecting replication could partially overlap the binding site (Figure 7), but further work will be necessary to define precisely the sequences involved. A direct repeat of 11 nt with one mismatch and with the repeat copies separated by one base is present within this region.

Discussion

Infectious RNA produced from clones of several (+) ssRNA viruses have been used to study the sites necessary for replication and packaging *in vivo* (Ahlquist *et al.*, 1984; Mizutani and Colonno, 1985; Dasmahapatra *et al.*, 1986; Dawson *et al.*, 1986; Levis *et al.*, 1986; Meshi *et al.*, 1986; Van der Werf *et al.*, 1986; Vos *et al.*, 1988) and in a very few cases, *in vitro* systems are available to define the steps affected by the site mutations. Until now, such studies have not been possible in dsRNA viruses. All the sites needed for brome mosaic virus (−) strand synthesis on the (+) strand template are within the tRNA-like structure present in the terminal 134 bases at the 3′ end (Dreher and Hall, 1988). The poliovirus RNA polymerase copies primed (+) strands and the mechanism of priming appears to involve the viral-encoded VPg peptide and a host-encoded poly(U) polymerase (reviewed by Kuhn and Wimmer, 1987). Since the yeast L-A dsRNA virus has neither a 3′ tRNA-like structure, a poly(A) 3′ end nor a 5′ peptide like VPg, its mechanism of replication probably differs from these cases.

In the replication cycle of L-A dsRNA, the viral plus strands serve as mRNA, as the species to be packaged to form new virions and as a template for replication. It is essential that the packaging and replication steps have sufficient specificity to prevent propagation of non-viral RNAs at the expense of the L-A virus.

We have identified three *cis* sites on L-A (+) strands. Two are necessary for optimal *in vitro* replication—the 3′ end structure and sequence and the internal replication enhancer (IRE). The third is necessary for the specific binding of (+) strands to the viral particles.

The viral binding site (VBS)

We have suggested (Fujimura and Wickner, 1988b) that the specific binding of (+) strands by empty viral particles is a function related to the process of encapsidation rather than to replication. Our finding here that the VBS and the IRE are distinct, though possibly overlapping, sites supports that view. Certainly the particles must bind templates in the course of replicating them, but the VBS is defined by a gel-retardation assay in which nearly quantitative binding is the positive signal scored. This very tight binding is evidently not necessary for (+) strand replication, as was illustrated in Figure 6.

Figure 7 (upper panel) shows the predicted structure of

the viral particle binding site of X (+) ssRNA. We have carried out a similar computer analysis of the likely secondary structure of S14 ssRNA, a deletion mutant of M_1, using the sequence data of Lee *et al.*, (1986). It shows a region of similar secondary structure with an A residue protruding from an analogous point in the stem 5′ to the loop and a loop sequence 5′GAUUC3′ compared to L-A's 5′GAUCC3′. The S14 sequence is from bases 444 to 424 from the 3′ end of S14 or M_1 and has a calculated free energy of −12.8 kcal/mol. (Figure 7, lower panel; Georgopoulos *et al.*, 1986), while the L-A sequence is from bases 399 to 376 from L-A's 3′ end. Experiments are underway to determine whether this M_1 sequence has binding activity.

The 180-kd viral protein encoded by L-A has ssRNA binding activity on Western blots (Fujimura and Wickner, 1988b) and is probably responsible for the viral particle binding activity. This protein is apparently the RNA polymerase (T.Icho and R.B.Wickner, submitted). Examination of the sequence of the ssRNA binding domain of the viral 180-kd protein (Icho and Wickner, submitted) did not reveal any classical RNA-binding protein patterns, although a possible 'leucine-zipper', characteristic of certain DNA-binding proteins, was found.

3′ end replication sites

Template activity requires the 3′ terminal three bases of the X (+) strand. Deletions or single base substitutions eliminate template activity but addition of a single A residue is without effect. This is consistent with the presence of an unpaired A residue on the 3′ end of L-A dsRNA *in vivo* and recalls similar results obtained with Qbeta replicase (Rensing and August, 1969; Bausch *et al.*, 1983).

The stem−loop structure formed by bases 5−19 (from the 3′ end) of the X (+) strand is also necessary for maximum template activity but a low level of replication can be observed even without this structure. Similarly, adenovirus type 2 or 5 has a subterminal sequence that, while not absolutely essential for template activity in the *in vitro* system, stimulates replication 10-fold by allowing the binding of the cellular replication factor NFI (Nagata *et al.*, 1983). The nature of the complex formed between empty L-A particles and viral (+) strands depends on the presence of the host factors necessary for the replication reaction (Fujimura and Wickner, 1988b). Only that formed in the presence of the host factors is replication competent. Further work will be necessary to determine the precise role of the subterminal loop and host factors and whether they are related.

M_1 uses the L-A replication apparatus, but has no sequence homology to L-A in the regions we have shown to be essential for *in vitro* replication. None the less, the sequences at the 3′ end of M_1 can substitute for those at the 3′ end of L-A. One presumes that there exists some structural similarity to the sequences recognized by the L-A particles in the L-A (+) strands and in the M_1 (+) strand. Alternatively, since M_1 and L-A are affected differently by various chromosomal genes (Wickner, 1986) and since M_1, as a satellite virus dependent on L-A, must compete for the replicase, perhaps it is to be expected that its interaction with the viral particle enzyme will differ in detail from that of L-A itself.

Internal replication enhancer

We have shown that a site ~400 bases from the 3′ end of the X (+) strand is required for optimal template activity and have thus named it the IRE. A 10 of 11 base direct repeat sequence is present within the interval to which the IRE has been delimited (Figure 7).

Further work will be needed to clarify the roles of the IRE and the 3′ end in replication. Qbeta replicase first recognizes a site inside the replicase gene of the viral (+) strand, and then binds the 3′ end and begins replication (Meyer *et al.*, 1981). This type of mechanism could explain the requirement of L-A for an internal replication enhancer. The L-A replicase might, for example, bind at the IRE and then slide down the RNA to the 3′ end where initiation would occur. Or it might stay at the IRE and bind the 3′ end from there. Whether the VBS and the IRE are interacting with each other or whether their proximity has any special meaning, either in binding or in replication, remains to be fully investigated. Both processes are affected by the presence of host factors that might interact with these sites. The viral replicase shows binding affinity for the template strand (Fujimura and Wickner, 1988b) and apparently two different complexes may be formed depending on whether the host factor(s) are present. Are these two different complexes related to the two internal regions 380 bases from the 3′ end and are they reflecting binding processes that are part of the encapsidation process rather than the replication process? These questions remain to be answered and more information is required from both the RNA sequence itself and the other factors (the viral replicase and the host factors) involved in these processes.

Materials and methods

Yeast and Escherichia coli strains

Yeast strain RE455 (alpha *arg1 ski2-2* L-A-HN, X) was used to purify X viral particles as described (Esteban and Wickner, 1988). Mature L-A particles were prepared from stationary phase cells of strain TF229 [*a his(3,4) leu2 ski2-2* L-A-HN] grown in YPAD broth for 3 days at 30°C (Fujimura *et al.*, 1986). *Escherichia coli* HB101 and MV1190 (delta(*lac-pro*AB), *thi*, *sup*E, delta (sr1-*rec*A) 306 :: Tn*10*(tet^r)[F′:*tra*delta36, *pro*AB, *lac*I^{q}ZdeltaM15]) were used for the propagation of plasmids. MV1190 was also used as the host for the M13 helper phage R408 (Stratagene) to obtain ssDNA. CJ236 [*dut*-1, *ung*-1, *thi*-1, *rel*A-1/pCJ105 (Cm^r)] was used to prepare uracil-containing ssDNA that served as template for oligonucleotide site-directed mutagenesis (see below). Both *E.coli* strains MV1190 and CJ236 were obtained from BioRad as part of a MUTA-Gene *in vitro* mutagenesis kit.

Oligonucleotide site-directed mutagenesis

In vitro site-directed mutgenesis was carried out according to the method of Kunkel (1985), using uracil-containing DNA as template. The template was prepared by infecting *E.coli* strain CJ236 that carries *ung* and *dut* mutations and the desired plasmid with uracil-containing M13 helper phage R408 (Stratagene) at an m.o.i. of ~20. The infected culture was incubated at 37°C for 7−8 h and the phage were collected and precipitated. An average of 0.2 μg (0.2 pmol) of ssDNA was used in each reaction. Synthetic oligomers were prepared on a Applied Biosystems 381A and 5′ phosphorylated with T4 polynucleotide kinase (BRL), following the recommendations of the supplier, before being used to prime the reaction. Between 5- and 10 pmol of each oligomer (25- to 50-fold molar excess over the template) was annealed to the template ssDNA and second strand synthesis was carried out using T4 DNA polymerase and T4 DNA ligase and buffers from the BioRad Mutagene kit. After completion of second strand synthesis, the DNA was used to transform *E.coli* strain MV1190 (UNG$^+$, DUT$^+$). Single colonies were selected on LB ampicillin plates and the desired changes in sequence were tested by digesting plasmid DNA from minipreparations with appropriate restriction enzymes if pre-existing sites were modified or new sites were introduced. Other changes were detected by direct DNA

sequencing. On average, eight colonies were used to prepare ssDNA using M13 helper phage as described above. The yield of mutant plasmids was generally ≥40%, even in cases of deletions of ≥100 bases. All constructs were checked by sequencing.

Other nucleic acid manipulations

Plasmid purifications, restriction enzyme digestions and transformations were as described by Maniatis *et al.* (1982). ssRNA transcripts were made *in vitro* using T7 RNA polymerase following the recommendations of the supplier (US Biochemical, Cleveland, USA). Before the transcription reaction, plasmid DNA was digested with appropriate restriction enzymes to obtain discrete DNA template fragments for run-off synthesis containing the T7 RNA polymerase promoter and variable portions of the X cDNA sequence. DNA sequencing was done by the dideoxynucleotide chain-termination method of Sanger *et al.* (1977) with [α-^{35}S]dATP, using Sequenase (US Biochemical) (Tabor and Richardson, 1987).

Plasmids and DNA templates for T7 RNA polymerase transcription

pRE76 has been described (Esteban *et al.*, 1988). It contains a 502-bp insert in the unique *Sma*I site of the Bluescript SK$^+$ vector (Stratagene). This 502 bp represents X cDNA sequences from bp 23 to 525 (X dsRNA is 530 bp) numbering from the 5′ end of the X (+) strand. It lacked 23 bp from the 5′ end and 5 bp from the 3′ end of X. Synthetic oligonucleotides were used to construct plasmids with the entire X cDNA sequence as mentioned above. A set of three plasmids was made using ssDNA from pRE76 as a template: (i) pRE76-2, that has the correct 3′ end of X; the last three nucleotides are 5′ . . . TGC 3′. In order to generate a suitable DNA template for T7 RNA polymerase run-off transcription with the same sequence at the 3′ end as the X (+) viral strand, we also inserted into pRE76 the sequence GCA downstream from X. This produced an *Fsp*I site (F in Figure 1), 5′TGCGCA3′, which is cut to give a blunt end. In this way we could make *in vitro* ssRNA transcripts which should have the same 5′ . . . UGC-OH 3′ end as the X (+) viral strands. (ii) pRE76-14 has only been repaired at the 5′ end of the X cDNA sequence. T7 transcripts made from this plasmid contained the entire X (+) strand sequence except for the last 5 nt. Because no restriction site existed at the 3′ end of the X cDNA part of pRE76-14 we used the closest restriction site in the polylinker region, *Bam*HI (A in Figure 1), to digest the template DNA before T7 RNA polymerase transcription. This produces RNA molecules with eight extra bases at the 3′ end (see below). (iii) plasmid pRE76-2-14 (Figure 1B) contains the entire X cDNA sequence. pRE76-2-14 and derivatives obtained by additional *in vitro* site-directed mutagenesis were used for most of the experiments described in this paper.

pRE89 was obtained by removing an *Xmn*I fragment of 108 bp from pRE76-2 (from bp 192 to 300 in the X cDNA sequence). ssDNA from this plasmid was also used to obtain deletions or modifications in the X cDNA sequence.

pRE91 was obtained by removing from pRE76-2 the 160-bp *Hin*dIII–*Hae*III fragment extending from 27 bp upstream from the X sequences in the multiple cloning site through the first 133 bp from the 5′ end of the X cDNA (From bp 23 to 156). pRE91 has the 3′ end corrected to that of the normal X sequence. pRE97 contains sequence from the X cDNA only from bp 23 to 156 and was obtained by removing the *Hae*III–*Xba*I fragment from pRE76.

In vitro replication reaction

The *in vitro* replication reaction was carried out as described in Fujimura and Wickner (1988b) with slight modifications. The standard reaction mixture (25 μl) contained 50 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 0.1 mM Na-EDTA, 20 mM NaCl, 5 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM each of ATP, CTP and GTP, 20 μM [α-^{32}P]UTP (NEN), 3 mg of bentonite per ml, 2.5% polyethylene glycol 6000 (PEG 6000), the host factor fraction (5 μg of protein), unlabeled T7 RNA polymerase transcript (100 ng), and empty particles (10–15 μg of protein) as the source of enzyme. The host factor fraction, a 0–50% saturated ammonium sulfate precipitate of an extract of uninfected cells and empty particles was prepared as described in Fujimura and Wickner (1988a) and Esteban *et al.* (1988) respectively. The reaction mixture was incubated for 90 min at 30°C and the reaction was stopped by addition of 75 μl of 0.3% SDS and 20 mM Na-EDTA. The product was extracted with phenol, then with phenol/chloroform, precipitated with ethanol, and analyzed on a 1.5% agarose gel.

Binding reaction

The binding reaction was carried out as described by Fujimura and Wickner (1988b). The binding mixture (15 μl) consisted of 50 mM Tris–HCl pH 7.6, 0.5% PEG 6000, 5 mM Na-EDTA, 3 mg of bentonite per ml, the host factor faction (5 μg of protein), ^{32}P-labeled T7 RNA polymerase transcript (5–10 ng, 100 000 c.p.m./assay) and empty particles (15 μg of protein). The mixture was incubated for 20 min at 30°C and the binding complex was analyzed on a 1.5% agarose gel and visualized by autoradiography.

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