Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 4411–4415, June 1988
Genetics

Site-specific binding of viral plus single-stranded RNA to replicase-containing open virus-like particles of yeast

(X double-stranded RNA/cDNA sequence/gel retardation)

ROSA ESTEBAN, TSUTOMU FUJIMURA, AND REED B. WICKNER

Section on Genetics of Simple Eukaryotes, Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 8, Room 209, Bethesda, MD 20892

Communicated by Herbert Tabor, February 19, 1988 (received for review January 22, 1988)

ABSTRACT **X double-stranded RNA is a deletion mutant of L-A double-stranded RNA and is encapsidated in viral particles by the L-A-encoded major coat protein. X double-stranded RNA has all the cis sites necessary to be transcribed, encapsidated, and replicated. We have cloned X double-stranded RNA and sequenced it. The complete X double-stranded RNA sequence deduced indicates that the first 25 bases of the X plus-strand 5′ end originated from the 5′ end of the L-A plus strand and that most, if not all, of the rest comes from the 3′ end of the L-A plus strand. The X plus strand made by X double-stranded RNA-containing virus-like particles binds specifically to empty open virus-like particles and is converted by these particles to X double-stranded RNA. RNA transcripts of the X complementary DNA clones and deletion derivatives thereof were made *in vitro* by T7 and T3 RNA polymerases and tested for specific binding to the virus-like particles. The results suggest that the binding is due to the sequence UUUGGCCAGG, 370 bases upstream from the X plus-strand 3′ end. This sequence is also present in the M_1 plus strand 140 bases from its 3′ end.**

Most strains of the yeast *Saccharomyces cerevisiae* harbor intracellular virus-like particles (VLPs) that contain linear double-stranded RNA (dsRNA) molecules of various sizes. The most widely distributed is L-A dsRNA [4.8 kilobases (kb)], which encodes the major coat protein of the VLPs in which itself and another dsRNA, M_1 (1.8 kb), are separately encapsidated. M_1 encodes a polypeptide of ≈31 kDa that carries the toxin and immunity determinants characteristic of killer strains (for recent reviews, see refs. 1 and 2). Other minor species of different dsRNAs are also present in most yeast strains (3, 4). L-A and M_1 are transcribed and replicated *in vivo* in a conservative, sequential process (5–7). Messenger polarity single-stranded RNAs (ssRNAs) or plus strands are synthesized by the VLP-associated RNA polymerase with the dsRNA inside the VLPs used as template (8–11). At least in the case of L-A VLPs, the transcripts are extruded or released into the medium (12). Some of the plus ssRNAs become encapsidated in VLPs that have the same size and protein composition as the mature dsRNA-containing particles. Then minus-strand synthesis occurs on a plus-strand ssRNA template inside the VLPs to produce dsRNA (13).

L-A VLPs disrupted *in vitro* by low ionic strength treatment lose most of their L-A dsRNA but maintain the overall structure of the particle and the replicase activity. These "empty" VLPs replicate exogenous plus L-A and M_1 ssRNAs (14). Empty VLPs thus constitute an excellent tool to study how modifying an RNA template may affect replication.

Recently, we isolated and characterized a deletion mutant of L-A dsRNA, X dsRNA (24). X is 0.53 kb and originated as a result of a large internal deletion of the L-A parental molecule. X is encapsidated in L-A-encoded VLPs, and *in vivo* and *in vitro* experiments have shown that X is transcribed and replicated. Direct RNA sequencing showed that only the first 25 base pairs (bp) from the 5′ end of the L-A plus strand were present at the 5′ end of X but that at least the 110 bp sequenced from the X plus-strand 3′ end were exactly the same as the 3′ end of L-A, raising the possibility that the 3′ region of L-A may contain some internal signals necessary for replicating or encapsidating the dsRNA or for stabilizing the particles (24). Other deletion mutants, such as S (*suppressive*) mutants of M_1 dsRNA, have also arisen by large internal deletions of the parental molecule, and the bulk of the material retained in these deleted molecules also derives from the 3′ end of the parental genome (15–17).

We decided to clone X dsRNA to study in detail the replication of X and, in turn, of L-A. We describe here the isolation and sequence of a cDNA clone of X.* This clone contains all but the first 23 bp from the 5′ end of the X plus strand and 5 bp from the 3′ end. Using RNA transcripts made from that X cDNA clone, we have established that the X plus strand binds specifically to the empty L-A VLPs, and we have identified a binding site in the X plus strand that is also present in M_1. The importance of this binding site in the replication process is discussed.

MATERIALS AND METHODS

Strains. Yeast strain RE455 (*α argl ski2-2*, L-A-HN, X) was used to purify X VLPs as described (24). These particles served as a source of X dsRNA (obtained by disrupting the VLPs with phenol) or were used for *in vitro* synthesis of X plus ssRNA transcripts by using the VLP-associated RNA polymerase. Mature L-A VLPs were prepared from stationary cells of strain TF229 [*a his(3,4) leu2 ski2-2*, L-A-HN] grown in YPAD broth for 3 days at 30°C as described in ref. 12. *Escherichia coli* HB101 was used for the propagation of plasmids, and JM109 was used as the host for the M13 helper phage R408 to obtain ssDNA.

Empty Particle Preparation. Mature L-A VLPs (37 mg of protein per ml; 200 μl), which contain one L-A dsRNA per particle, were dialyzed against 300 ml of 2 mM Tris·HCl, pH 7.6/1 mM EDTA/1 mM dithiothreitol for 3 hr at 4°C. These ruptured particles have replicase activity able to synthesize a minus strand on exogenous plus-strand RNA template (14). Then, empty particles were separated from L-A dsRNA by CsCl density-gradient centrifugation for 20 hr at 130,000 × *g* and at 4°C. (The initial density of the solution was adjusted to 1.31 g/ml by adding CsCl.) The empty particle fraction (ρ

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: VLP, virus-like particle; ds, double stranded; ss, single stranded.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03234).

= 1.31 g/ml) was dialyzed against a buffer of 50 mM Tris·HCl, pH 7.6/150 mM NaCl/5 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol, and kept at −70°C before use.

Binding and Replication Reactions. The standard binding reaction mixture (15 μl) consists of 50 mM Tris·HCl, pH 7.6/2.5 mM EDTA/9 μg of yeast tRNA (Sigma)/empty particles (15–25 μg of protein)/20–60 ng of [^{32}P]RNA. The mixture was incubated for 20 min at 30°C, and the binding complex was then separated by electrophoresis on a 1.5% agarose gel and detected by autoradiography. The *in vitro* replication reaction was carried out as described in ref. 14.

X cDNA Synthesis and Cloning. X cDNA synthesis was carried out with a cDNA synthesis kit purchased from Amersham. X dsRNA was obtained from purified X VLPs by phenol extraction and was separated from contaminating L-A dsRNA by a LMP agarose gel. One microgram of pure X dsRNA in 4 μl of TE buffer (10 mM Tris·HCl, pH 8/1 mM EDTA) was treated with 1 μl of 50 mM methylmercuric hydroxide for 10 min at room temperature to denature the dsRNA. Excess mercuric ions were sequestered by adding 1 μl of 700 mM 2-mercaptoethanol, and the mixture was kept at room temperature for 5 min more. The denatured X dsRNA was used in a reverse transcriptase reaction primed with 1 μl (2 μg/ml) of the 18-mer synthetic oligonucleotide 5′ TGCATATGGGTAATTCCC 3′ (complementary to the first 18 nucleotides of the 3′ end X plus strand) in a 20-μl total reaction volume. The experimental conditions for first- and second-strand synthesis were as recommended by Amersham. After synthesis of both strands, larger fragments of X cDNA were purified in a LMP agarose gel and cloned into the unique *Sma* I site of a Bluescript-SK$^+$ vector obtained from Stratagene (San Diego, CA).

Other Nucleic Acid Manipulations. Plasmid purification (minipreparations or large-scale purification), restriction enzyme treatments, and transformation procedures were as described by Maniatis *et al.* (18). ssRNA probes were made *in vitro* by T7 and T3 RNA polymerases following the recommendations of the suppliers [Bethesda Research Laboratories for T3 RNA polymerase and United States Biochemical (Cleveland) for T7 RNA polymerase]. 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 or T3 RNA polymerase promoters and variable portions of the X cDNA sequence. DNA sequencing was done by the dideoxynucleotide chain-termination technique of Sanger *et al.* (19) with [α-^{32}P]dATP, using a Sequenase kit provided by United States Biochemical. For all regions of the X molecule, both strands of at least two independent cDNA clones were sequenced.

The plus or minus polarity of T7 or T3 RNA polymerase transcripts from X cDNA clones was confirmed by RNA·RNA hybridization (20) with cold X plus ssRNA transcribed by X VLPs used as a probe.

Plasmids. Plasmid pRE76 contained a 502-bp insert (X cDNA clone) in the unique *Sma* I site of the Bluescript-SK$^+$ vector. Plasmid pRE51 was obtained by removing an *Eco*RI fragment of 291 bp from pRE76. This 291-bp fragment included 14 bp from the multiple cloning site upstream of the *Sma* I site in the vector and 277 bp from the X cDNA sequence (from base pair 23 to 300). pRE57 was an independently derived clone that contained an insert of only 183 bp (from base pair 117 to 300 of the X cDNA sequence) in the *Sma* I site of the same vector. The orientation of this 183 bp in pRE57 was opposite that of pRE76. pRE90 was obtained by removing the 160-bp *Hin*dIII/*Hae* III fragment from pRE76, which contained 27 bp from the multiple cloning site, upstream of the *Sma* I site, and the first 133 bp from the 5′ end of the X cDNA sequence (from base pair 23 to 156).

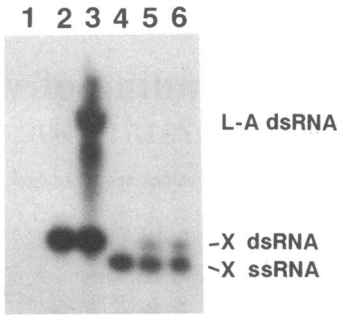

FIG. 1. Replication of X plus ssRNA by L-A empty VLPs. Lanes 1–3, nonlabeled RNA and [^{32}P]NTP were used. Lanes 4–6, the template was ^{32}P-labeled, and unlabeled NTP was used. The *in vitro*-synthesized labeled dsRNA was separated from the unused template by agarose gel electrophoresis and visualized by autoradiography. In lanes 2 and 4, VLPs were purified by a CsCl gradient equilibrium sedimentation after being disrupted by low ionic strength treatments. Lanes 3 and 5, replicase activity before the CsCl purification. The L-A dsRNA in lane 3 represents the replication product of some L-A plus ssRNA made *in vitro* by a small portion of nondisrupted particles; these unopened particles were removed during the CsCl sedimentation, and so L-A dsRNA is not produced in lane 2. Lanes 1 and 4, control reactions with no VLPs added. CsCl-purified VLPs were used in all subsequent binding reactions (see Figs. 4 and 5).

RESULTS

X Plus ssRNA Can Be Replicated by Empty L-A VLPs. X is a mutant of L-A resulting from a very large deletion of the parent molecule. Although it is only 0.53 kb, X contains all the information necessary to be packaged, transcribed, and replicated (24). Dialysis of L-A VLPs against very low ionic strength results in release of the L-A dsRNA. The empty particles produced can now convert added L-A plus ssRNA or M_1 plus ssRNA to the respective dsRNAs *in vitro* (14). When X plus ssRNA is added to empty L-A VLPs, it is converted to X dsRNA with net RNA synthesis (Fig. 1). The reaction depends on the presence of both the X plus-strand template and the disrupted L-A VLPs. The newly synthesized strand was the X minus strand (data not shown).

Cloning and Sequence of X. The cDNA synthesis and cloning of X was carried out as described. Among six cDNA clones of X, pRE76 had the largest insert (Fig. 2).

The complete sequence of both strands of the insert in pRE76 was determined. The sequence of the first 60 nucleotides from the 5′ end and >100 nucleotides from the 3′ end of the X plus strand had already been established by direct RNA sequencing (24). All but the 5′ terminal 23 bp and the

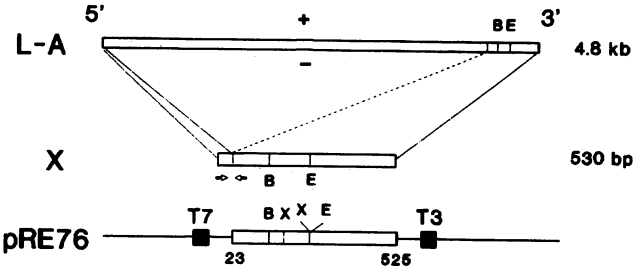

FIG. 2. Schematic representation of L-A and X dsRNAs and the plasmid pRE76 that contains X cDNA sequences from base pair 23 to 525, numbering from the 5′ end of the X plus strand. Solid boxes, T7 and T3 RNA polymerase promoters flanking the X cDNA clone. Between the promoters and the X sequence, there are two stretches of 73 bp (T7) and 57 bp (T3) that correspond to sequences of the multiple cloning site from the Bluescript-SK$^+$ vector used to clone X. The locations of the *Bst*XI (B) and *Eco*RI (E) restriction sites in X and in the parental L-A molecule are indicated. X, *Xmn* I.

3′ terminal 5 bp of the X sequence were found in the cDNA clone pRE76. The complete sequence of the X plus strand is shown in Fig. 3.

The restriction sites for *Eco*RI (E) and *Bst*XI (B) in the X plus-strand sequence are also present, at the same distances from the 3′ end, in L-A (T. Icho and R.B.W., unpublished data) (Fig. 2). This suggests that only the first 25 bp from the 5′ end of X (where the breakpoint between X and L-A occurs) were directly derived from the 5′ end of L-A. Apparently, the rest of X comes from the 3′ end of L-A, with no internal portions of L-A present in X.

Fig. 3 also shows that X encodes an uninterrupted 156-amino acid sequence from base pair 27 to 494. This truncated open reading frame might represent the carboxyl terminus of a larger protein encoded in L-A. Two perfect inverted repeats of 18 bp each, from base pair 1 to 18 and from base pair 24 to 42, are indicated, the first of which is present in the L-A 5′ end sequence. The second inverted repeat probably was generated in the course of formation of X, although until the complete sequence of L-A is known, this will be uncertain.

X Plus ssRNA Shows Binding Affinity for L-A VLPs. In the course of the replication assays performed with empty VLPs (Fig. 1), we observed that the template ssRNA used had affinity for the VLPs and that the VLP–RNA complex (binding complex) formed was easily detected by gel retardation experiments (Fig. 4). The formation of the complex was dependent on the type of ssRNA template used. Fig. 4*A* shows that the plus strand of X dsRNA has binding affinity, whereas the minus strand does not.

To determine which part of the X plus strand was responsible for the binding activity, we used T7 or T3 RNA polymerases to make *in vitro* RNA transcripts from X cDNA clones that contained only part of the X sequence and used them in the binding reaction. Fig. 4*B* shows the binding activity of four such RNA transcripts. Only transcript b, made from plasmid pRE57 by T3 RNA polymerase and which represents X plus-strand sequences from base pair 117 to 300, formed a binding complex with the empty VLPs. Neither the two RNA species that contained X minus-strand sequences

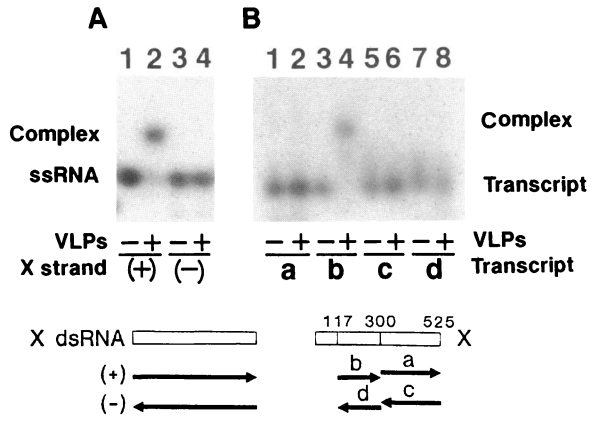

FIG. 4. (*A*) Identification of the binding complex formed between empty VLPs and X plus ssRNA by agarose gel electrophoresis (gel retardation assay). X dsRNA was 3′-end-labeled with [^{32}P]pCp by T4 RNA ligase, and the plus and minus strands were separated and purified from a polyacrylamide strand-separating gel as described (22). The binding reaction was carried out as described, and the complex was separated from the template RNA on an agarose gel. An autoradiogram of the gel is shown. (*B*) Binding complexes formed between empty VLPs and RNA transcripts made *in vitro* by T7 and T3 RNA polymerases from plasmids pRE51 (transcripts a and c) and pRE57 (transcripts b and d). Transcripts a and b have sequences of the X plus strand, and transcripts c and d represent parts of the X minus strand. The parts of the X sequence present in the *in vitro* transcripts are indicated by the numbers above the X molecule. Binding reactions in *B* contained 0.7 pmol of labeled RNA strands and 2.3 pmol of empty VLPs (≈3-fold excess over RNA strands) in a total volume of 15 μl.

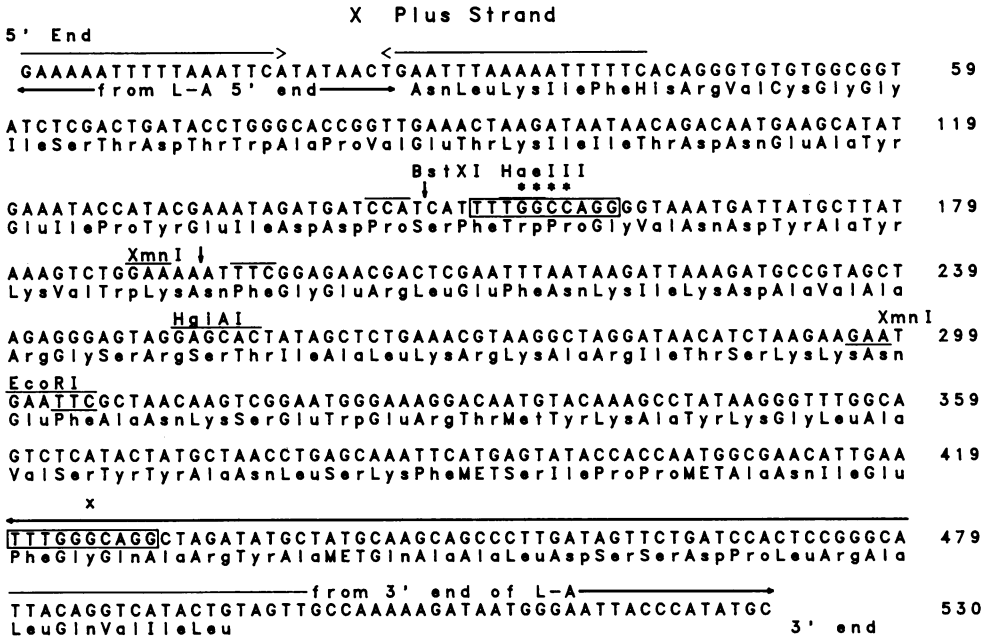

FIG. 3. Nucleotide sequence of the X plus strand. Since most of the sequence analysis was done on a cDNA clone, thymine (T) was used instead of uridine (U). The amino acid sequence of an uninterrupted reading frame from base pair 27 to 494 is shown. Two perfect inverted repeats of 18 bp, from base pair 1 to 18 and from base pair 24 to 42, are indicated by horizontal arrows above the nucleotide sequence. At both ends of X, the sequences known to be present also in L-A are indicated. [L-A sequences were determined by direct RNA sequencing by Thiele *et al.* (21).] The restriction sites shown represent some of the enzymes used to cut plasmid DNA before synthesizing *in vitro* transcripts with T7 or T3 RNA polymerases; vertical arrows, position of the last nucleotide in the transcript, assumed to be the place where the enzyme cuts the template strand. Asterisks, restriction site used to disrupt the putative binding site (*Hae* III). The boxed 10-bp sequence from base pair 151 to 160 is the putative VLP binding sequence determined by experiments such as those in Figs. 4 and 5. The sequence at positions 420–429 is identical except for 1 base (indicated by the X) and is also boxed.

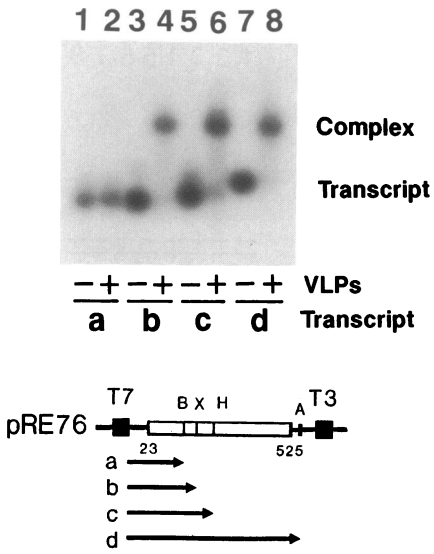

FIG. 5. Binding reaction with empty VLPs and T7 RNA polymerase transcripts made from plasmid pRE76 digested with the enzymes indicated. B, *Bst*XI; X, *Xmn* I; H, *Hgi*AI; A, *Bam*HI. RNA transcripts include X sequences upstream of base pair 148 (transcript a), 192 (transcript b), 251 (transcript c), and 525 (transcript d). Lanes 1, 3, 5, and 7, no VLPs added. Lanes 2, 4, 6, and 8, empty VLPs were present. The mobility of complexes is similar, independent of the size of the template RNA.

nor X plus-strand sequences from base pair 300 to 525 (transcript a) showed binding activity.

We next studied in more detail the region from base pair 117 to 300 of the X plus strand with some of the restriction enzymes indicated in Fig. 3 (*Bst*XI, *Xmn* I, and *Hig*AI) to digest DNA from plasmid pRE76 prior to transcription with T7 RNA polymerase. Fig. 5 shows the binding activity of some of the *in vitro* RNA transcripts. The minimum size RNA that has binding activity is transcript b containing X sequences from the 5′ end (base pair 23) to base pair 192. Thus, the region from base pair 117 to 192 is sufficient for binding. RNA transcript a containing information only upstream of base pair 148 has no binding activity. This result would suggest that the binding site was located somewhere between base pairs 148 and 192; however, it is also possible that the *Bst*XI site is within the binding site itself or disrupts a binding site located only a few nucleotides upstream of the *Bst*XI site at base pair 148. Thus, results shown in Figs. 4 and 5 only allow us to affirm that the binding site is located either between base pairs 148 and 192 or close to base pair 148.

We next prepared an RNA transcript from plasmid pRE90 (that lacks X sequences upstream of base pair 156) and found that <10% of the binding activity remained associated with this RNA species as compared to RNA prepared from plasmid pRE76 (data not shown). This last result, together with those described in Figs. 4 and 5, suggests that the binding site is located in an interval of the X plus-strand sequence that includes the *Bst*XI site (base pair 148) and the *Hae* III site (base pair 156). As we discuss below, this region of X contains a 10-bp sequence (from base pair 151 to 160) (Fig. 3) that is also present in the M_1 plus-strand sequence and is thus a good candidate for being the RNA sequence that is recognized and to which some L-A VLP-associated protein binds.

X and M_1 Contain the Same Binding Site. Increasing amounts of transcript b (the same as in Fig. 5 but without being radiolabeled) were added to a binding reaction with ^{32}P-labeled X (plus strand) or M_1 (plus strand) viral ssRNAs (synthesized *in vitro* by the X or M_1 VLP-associated RNA polymerase). We found that the presence of transcript b interfered with the binding reaction, competing actively not only with the X plus ssRNA but also with M_1, for binding to the empty VLPs (data not shown). In each case, a 10-fold excess of transcript b produced a decrease by a factor of ≈5 of X or M_1 plus ssRNA bound. In the same experiment, transcript a, which showed no binding activity (Fig. 5), did not affect the formation of the complex between X or M_1 and the VLPs. This result indicates that X and M_1 bind to the same site on the VLPs and suggests that they might bind by a similar RNA sequence. We searched for nucleotide similarity between X sequences between base pairs 117 to 192 and the 3′ part of the M_1 sequence (23) and found that there was a 10-bp sequence, UUUGGCCAGG, from base pair 151 to 160 in X (numbered 5′ to 3′) and present between base pairs 144 and 153 from the 3′ end of M_1. The 10-bp sequence (boxed in the X sequence in Fig. 3) is aligned with the same sequence in M_1 in Fig. 6. Adjacent regions are also indicated. The distance between the binding site and the 3′ end is different in the two molecules: 370 bp in X and only 143 bp in the case of M_1. If we consider, however, the total length of L-A (parental molecule of X) and M_1, this binding site is located about the same relative distance from the 3′ end. Downstream of the X sequence, there is another 10-bp sequence almost identical to the first binding site, with only a single nucleotide change (C→G) (Figs. 3 and 6). This second putative binding site may explain the residual binding activity (≈10%) of transcripts made from plasmid pRE90 that lacked the first binding site.

DISCUSSION

The molecular characterization of X dsRNA described here, combined with that reported earlier (24), indicates that only 25 bp from the L-A plus- strand 5′ end are present in X; the remainder (at least the 390 bp from the *Bst*XI site to the 3′ end) (Fig. 2) is the same as the 3′ end of L-A (T. Icho and R.B.W., unpublished results).

Even though X dsRNA carries only 530 bp of L-A's original 4.8 kb, X is packaged, transcribed, and replicated by the L-A VLP system. This means that X carries all the sites necessary for these processes. The studies reported here have delimited a site around residue 155 (from the 5′ end of X plus strands) that is necessary for the specific binding of X to the opened empty VLP particles capable of converting X plus strands to X dsRNA. While further work will be necessary to exactly define the necessary and sufficient sequence, a good candidate is the 10-bp sequence 5′ UUUGGCCAGG 3′ present from residues 151 to 160 in X and also near the 3′ end of M_1 dsRNA (23) and its deletion derivatives, S3 and S14 (16, 17). This sequence is present on the plus strands of X, L-A, and M_1, and only the plus strands

		5′ Plus Strands	3′
X dsRNA	390	GAUCCAUCAU**UUUGGCCAGG**GGUAAAUGAU	361
X dsRNA	121	GAACAUUGAA**UUUGGGCAGG**CUAGAUAUGC	92
M1 dsRNA	163	AAUACGACUA**UUUGGCCAGG**UCUGGACGGG	134

FIG. 6. Comparison of RNA sequences from X and M_1 plus strands that contain the 10-nucleotide putative binding site boxed in the middle of the sequence. Numbers indicate distance of the binding site from the 3′ end in each molecule. The second (alternative) binding site in the X plus-strand sequence has only 1 nucleotide change (C → G) in the first cytosine, counting from the 5′ end. We present direct evidence here that the region of X shown in the top line is responsible for binding to VLPs.

of these molecules are replicated. Furthermore, a detailed analysis indicates that this binding site is necessary for the *in vitro* replicase reaction that converts plus strands of L-A, M_1, or X to the respective dsRNAs (unpublished data).

The generation of the X deletion molecule, a process in which >90% of L-A was lost, has resulted in a molecule with an inverted repeat of the first 18 bases of the 5′ end beginning at base 24. Whether this inverted repeat was generated during the deletion process or was already present in the original L-A sequence is as yet unclear. It will be interesting to determine whether the inverted repeat found near the 5′ end of X is an essential feature to allow replication of this molecule. The S mutants (replicating deletion mutants of M_1), S3 and S14, have no such inverted repeat. VLPs containing a wide range of deletion derivatives of L-A have been demonstrated in wild-type strains (12, 13). Our evidence suggests that, unlike X, most of these molecules are not replicating.

The uninterrupted reading frame from base pair 27 to 494 potentially encodes 156 amino acids that could constitute the carboxyl terminus of a larger protein encoded by L-A. It is already known that L-A encodes an 81-kDa protein, the major component of the VLPs in which L-A, M_1, or X are encapsidated; the coding capacity of L-A (4.8 kb), however, is at least twice that required for this protein. Near the 5′ end of the L-A plus strand there is an initiation codon at base pair 30, and this reading frame extends through the 110 bp so far sequenced (21). The fact that the 156-amino acid sequence (carboxyl terminus) is located at the very 3′ end of X (and thus of L-A) suggests that in addition to the 81-kDa protein, a much larger polypeptide might be also encoded by L-A. No coding capacity seems to be associated with the X minus strand (similar to M_1). This result is expected because only the plus strands are synthesized and released from the VLPs into the cytoplasm, where they can be translated into proteins.

We thank Dr. Gerald Zon (Division of Blood and Blood Products, Center for Drugs and Biologics) for synthesis of the oligonucleotide primer.

1. Wickner, R. B. (1986) *Annu. Rev. Biochem.* **55**, 373–395.
2. Wickner, R. B., Hinnebusch, A., Lambowitz, A. M., Gunsalus, I. C. & Hollaender, A., eds. (1986) *Extrachromosomal Genetic Elements in Lower Eukaryotes* (Plenum, New York).
3. Sommer, S. S. & Wickner, R. B. (1982) *Cell* **31**, 429–441.
4. Wesolowski, M. & Wickner, R. B. (1984) *Mol. Cell. Biol.* **4**, 181–187.
5. Newman, A. M., Elliott, S. G., McLaughlin, C. S., Sutherland, P. A. & Warner, R. C. (1981) *J. Virol.* **38**, 263–271.
6. Sclafani, R. A. & Fangman, W. L. (1984) *Mol. Cell. Biol.* **4**, 1618–1626.
7. Newman, A. M. & McLaughlin, C. S. (1986) in *Extrachromosomal Genetic Elements in Lower Eukaryotes*, eds. Wickner, R. B., Hinnebusch, A., Lambowitz, A. M., Gunsalus, I. C. & Hollaender, A. (Plenum, New York), pp. 173–187.
8. Herring, A. J. & Bevan, E. A. (1977) *Nature (London)* **268**, 464–466.
9. Welsh, J. D., Leibowitz, M. J. & Wickner, R. B. (1980) *Nucleic Acids Res.* **8**, 2349–2363.
10. Welsh, J. D. & Leibowitz, M. J. (1980) *Nucleic Acids Res.* **8**, 2365–2375.
11. Bruenn, J., Bobek, L., Brennan, V. & Held, W. (1980) *Nucleic Acids Res.* **8**, 2985–2997.
12. Esteban, R. & Wickner, R. B. (1986) *Mol. Cell. Biol.* **6**, 1552–1561.
13. Fujimura, T., Esteban, R. & Wickner, R. B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4433–4437.
14. Fujimura, T. & Wickner, R. B. (1988) *J. Biol. Chem.* **263**, 454–460.
15. Fried, H. M. & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4224–4228.
16. Thiele, D. J., Hannig, E. M. & Leibowitz, M. J. (1984) *Virology* **137**, 20–31.
17. Lee, M., Pietras, D. F., Nemeroff, M. E., Corstanje, B. J., Field, L. J. & Bruenn, J. A. (1986) *J. Virol.* **58**, 402–407.
18. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
20. Fujimura, T. & Wickner, R. B. (1986) *Mol. Cell. Biol.* **6**, 404–410.
21. Thiele, D. J., Hanning, E. M. & Leibowitz, M. J. (1982) *Mol. Cell. Biol.* **4**, 92–100.
22. Thiele, D. J. & Leibowitz, M. J. (1982) *Nucleic Acids Res.* **10**, 6903–6918.
23. Georgopoulos, D. E., Hannig, E. M. & Liebowitz, M. J. (1986) in *Extrachromosomal Genetic Elements in Lower Eukaryotes*, eds. Wickner, R. B., Hinnebusch, A., Lambowitz, A. M., Gunsalus, I. C. & Hollaender, A. (Plenum, New York), pp. 203–213.
24. Esteban, R. & Wickner, R. B. (1988) *J. Virol.* **62**, 1278–1285.