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Long internal inverted repeat in a yeast viral double-stranded RNA

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ABSTRACT

The Saccharomyces cerevisiae viruses are non-infectious double-stranded (ds) RNA viruses present in most laboratory strains of yeast. Their genome consists of one or more dsRNAs separately encapsidated in particles composed mainly of one polypeptide, which has a M_r of 88 kdaltons in the best-studied viral subtype. A large viral dsRNA (L_1, of 4.7 kb) encodes the capsid polypeptide. We have determined the sequences of a number of cDNA clones homologous to portions of L_1 and mapped them by a novel heteroduplex technique. Several of these clones originate from a region of L_1 2.3 - 2.5 kb from the 5' end of the plus strand that contains stop codons in all three reading frames in the plus strand. We therefore suspect that the capsid polypeptide gene lies in the 5' 2.3 - 2.6 kb of the plus strand. One of the cloned cDNAs has an inverted repeat of 170 bp that appears to be present in its parental RNA. The inverted repeat in L_1 is the longest known inverted repeat in a viral dsRNA and the only known non-terminal inverted repeat. It might serve the function of creating two mRNAs from one viral dsRNA.

INTRODUCTION

The Saccharomyces cerevisiae viruses (ScV) are small double-stranded (ds) RNA viruses similar to other fungal viruses. In viral subtype 1, a large viral dsRNA (L_1) of about 4.7 kb encodes the major viral capsid polypeptide (P1) of 88 kdaltons in ScV-L_1 (1). Other, smaller viral dsRNAs, if present, are separately encapsidated in particles composed mainly of P1 (2). ScV-L particles, like all dsRNA virus particles, have a RNA-dependent RNA polymerase activity (3, 4) that synthesizes the full-length plus strand (the coding strand) in vitro (5). By random-primed reverse transcription, we have synthesized cDNA on the L_1 plus strand and constructed cDNA clones homologous to portions of L_1 (6, 7, 8).

Since present techniques do not readily generate full-sized cDNA clones of the large viral dsRNAs (9), we have sought methods to

rapidly map partial cDNA clones on their parental dsRNAs. By a method analogous to R-looping, in which a single-stranded RNA is insinuated into a dsDNA duplex, we have made heteroduplexes between a linear viral dsRNA and restriction fragments from its cDNA clones. In most cases, the resultant heteroduplex results in a loop, in which one branch is single-stranded and the other is an RNA-DNA duplex. The position of the loop accurately reflects the positon of the cDNA sequence within the viral dsRNA, as independently measured by primer extension, and in the case of an inverted repeat in L_1, by mapping stem and loop structures in the single-stranded RNA. We have completed sequence analysis of four independent cDNA clones mapping about 2500 bp from the 5' end of the plus strand. This anaylsis demonstrates an inverted repeat of at least 170 bp in this region of L_1.

Several viral dsRNAs have been entirely sequenced by a combination of direct RNA sequencing and sequencing of cDNA clones (9, 10, 11, 12). Only one of these has any extensive repeating structure, and that is rotavirus segment 11, in which a 53 bp inverted repeat exists at residues 29-82 and 609-661 in a dsRNA of 681 bp. This includes both the initiation and termination codons of the segment 11 gene (9). The inverted repeat in L_1 occurs close to the middle of the dsRNA and may include the termination codon of the P1 gene. All the rotavirus and reovirus dsRNAs sequenced have only short non-coding regions 5' and 3' to the single genes located on each segment. The longest of the 3' non-coding regions is in simian rotavirus gene 10 and constitutes 24% of the dsRNA (12). If the cloned region of L_1 constituted the end of the P1 gene, then L_1 would have about 2100 bp, or 44% of the dsRNA, 3' to the P1 gene. An in vivo mRNA coding for P1 is about 2300 b long, consistent with this model for L_1 (13).

MATERIALS AND METHODS

Heteroduplex analysis. Restriction enzyme fragments from cDNA inserts of clones pL1-21 or pL1-26 were combined with L_1 dsRNA, denatured and renatured as for dsRNA-dsRNA heteroduplexes (14), except that the temperature of renaturation was 50°C. Spreading and electron microscopy with pBR322 open circular DNA internal standard was as previously described (15).

Primer extension. Primer extension using restriction fragments of the clone pL1-21 cDNA insert were performed essentially as described (16), except that in some experiments, one dNTP was reduced to 50 micromolar and an alpha-^{32}P-dNTP included at a final specific activity of 35 Ci/mM. Analysis of products was by alkaline agarose gel electrophorsis (17). The substrate for primer extension was the L plus strand, produced in vitro by the ScV-L transcriptase and purified by CF-11 chromatography (5). No detectable ds L_1 was present, and hybridization of denatured restriction fragments (1-2 micrograms) to L_1 plus strand (25-100 micrograms) was confined to 30 min. at 50°C. Reverse transcriptase was from Life Sciences Inc.

DNA sequence analysis. Sequence analysis used the Maxam-Gilbert technique on cDNAs cloned in pBR322 (18) or the Sanger technique on subcloned fragments in M13 mp8, 9, 10, or 11 (19). Computer programs for data analysis were from J. Pustell (20). Restriction enzymes were from New England Biolabs and Bethesda Research Labs, T4 polynucleotide kinase and deoxynucleotidyl terminal transferase from PL Biochem., bacterial alkaline phosphatase from Boeheringer-Mannheim and isotopes from ICN.

Filter hybridizations. Southerns were performed as described (17). For hybridization to unlabeled RNA, methylmercuric hydroxide denatured RNA was bound to nitrocellulose filters by spotting and then baking as usual (17). Probes of the M13 cDNA clones were made by in vitro synthesis primed by the M13 probe primer (21). The alpha-^{32}P-UTP labeled L transcript probe was made as previously described (5).

RESULTS

Sequence analysis of cDNA clones. The cDNA inserts of pL1-26, pL1-21, and pL1-53 have previously been shown to overlap by Southern analysis (7). We have sequenced these four cDNA inserts, as well as an additional cloned cDNA from this same region (pL1-17) by Maxam-Gilbert and Sanger method sequence analyses. This scheme of sequencing is outlined in Fig. 1. Repeat experiments through the same region on different clones are not shown. Sequences have been obtained across each restriction site used for sequencing, and all of the predicted restriction sites that have been checked (32 of 41 predicted) have been confirmed. A composite DNA sequence of the

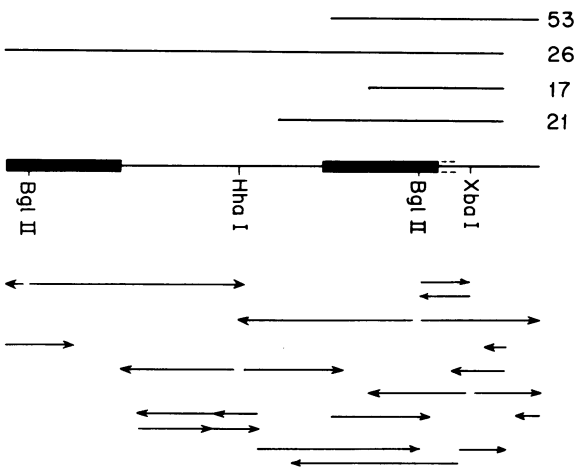


Figure 1. Sequence analysis of the inverted repeat region of L_1. The extent of clones pL1-21, 17, 26, and 53 is shown above a restriction map of the region. The inverted repeat is indicated by heavy lines. Dashed lines indicate the undefined extent of the inverted repeat at one end: the inverted repeat probably extends past the XbaI site (see text). The extent of individual sequencing experiments is shown by arrows (repeat experiments are not indicated).

strand with the same sequence as the L_1 plus strand (see below), beginning at the first base of clone pL1-26 and extending to the last base of clone pL1-53, is shown in Fig. 2.

There are a number of remarkable things about this sequence. The sequences of all the cDNA clones agree exactly, even at the termini of the inserts, despite the fact that they all originated by random-primed reverse transcription and were prepared for tailing by S1 digestion. Three of the clones terminate on the right within five bases of each other and the remaining clone (pL1-53) some 50 bases farther. The most obvious structural feature is the inverted repeat from 1-170 and 499-668. These 170 bp are a perfect inverted repeat, except for bases 81 and 588, which are both G in this strand. The cloned region establishes only one end of the inverted repeat, since the left end of pL1-26 is within the inverted repeat. Clone pL1-21 confirms the other end of the inverted repeat (Fig. 2).

This region may punctuate a gene, since at least one branch of the inverted repeat and the intervening region are closed in all three reading frames. The orientation of the sequenced region on

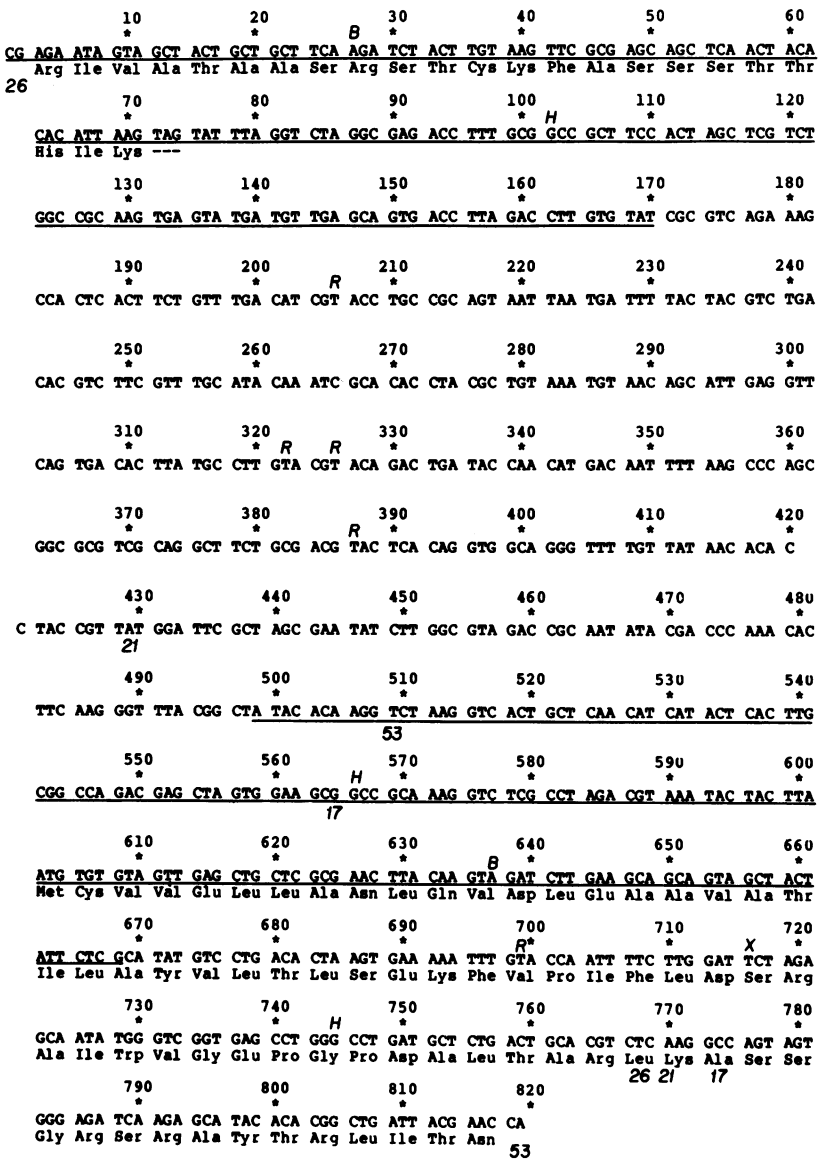


Figure 2. Sequence of the inverted repeat region of L_1. The DNA strand with the sequence of the L_1 plus strand is shown. The longest open reading frames are indicated. The ends of the cDNA clones are indicated by italicized numbers below the sequence. Key restriction sites are noted by italicized letters above the sequence: X is XbaI, B is BglII, H is HaeIII, R is RsaI. The inverted repeat is underlined.

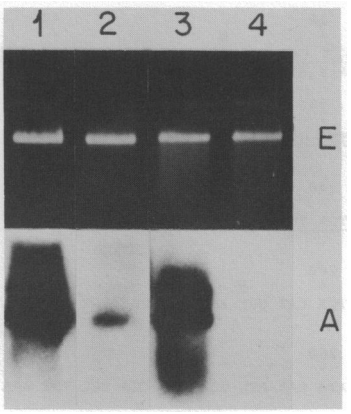


Figure 3. Hybridization of phage DNAs with L_1 transcript. Lane 1 has the XbaI-BglII fragment (in that orientation) in M13 mp11. Lane 2 has the BglII-XbaI fragment (in that orientation) in M13 mp10. Lane 3 has the RsaI fragment from residue 322 to 206 (minus strand in phage DNA) and lane 4 has the same fragment from 206 to 322 (plus strand in phage DNA) in the SmaI site of M13 mp9. The upper portion of the figure is the ethidium bromide stained gel (E) and the lower portion an autoradiograph (A) of a Southern of that gel hybridized to the alpha-^{32}P-UTP labeled in vitro transcript of L. All lanes received the same photographic and autoradiographic exposures.

its parental dsRNA was established by use of fragments subcloned in M13. The RsaI fragments from 206 to 322 and from 326 to 387 were cloned in both orientations in the SmaI site of mp9 or mp11 and sequenced by the Sanger method. In Southen hybridization experiments, only the DNAs of those clones carrying the strand complementary to that shown in Fig. 2 hybridized to the alpha-^{32}P-UTP labeled viral transcript made in vitro by viral particles (Fig. 3). Since the transcript is the plus (messenger) strand of the viral dsRNA (5), this experiment establishes that the orientation of the sequenced region is as shown in Fig. 2, and that the region between the inverted repeats occurs only in one orientation in the dsRNA.

The existence of the inverted repeat in the viral dsRNA was verified by subcloning the XbaI-BglII fragment from pL1-21 into BamHI, XbaI digested M13 mp10 and mp11. Since the two phages have these sites in opposite order, the fragment is cloned in opposite orientation in the two phages. The phage DNA of each hybridizes to the L_1 viral transcript, verifying that at least part of the

Table 1. Hybridization of phage DNAs with L transcript.

(DNA probe hybridized to L)/(DNA probe hybridized to 1)				
DNA probe	1	2	3	4
Measured value	2.0	2.1	1.2	32.3
Expected value	2.0	2.0	1.0	infinite

Filters had about 2 micrograms of L or about 0.8 micrograms of 1. The four DNAs are as in Fig. 3.

XbaI-BglII fragment is present in an inverted repeat in L_1 (Fig. 3). The first (left) branch of the inverted repeat hybridizes less well to the in vitro transcript than does the second (right) branch. We do not understand this result, but it might be explained by initiation of in vitro transcription between the inverted repeats.

We repeated this experiment using the cloned DNAs as probes and the RNA on nitrocellulose (Table 1). Approximately equimolar quantities of L and its plus strand transcript (1) were bound to separate sets of filters. The M13 probe primer was used to make labeled probes using the four DNAs of Fig. 3. As expected, all probes hybridized to L, but of the two clones carrying the RsaI fragment from between the inverted repeats, only the clone with DNA equivalent to the minus strand (DNA 3) hybridized to 1. Both of the probes with the XbaI-BglII fragment in opposite orientation (1 and 2) hybridized to 1, as expected. The ratio of counts hybridized to L to counts hybridized to 1 was twice as great for each of the probes carrying the inverted repeat as for the probe carrying the RsaI fragment from between the inverted repeats. Again, this was as expected, since the inverted repeat is present twice in each orientation in L but only once in each orientation in 1. The DNA with the plus strand of the RsaI fragment did not hybridize significantly to 1 (1510 cpm hybridized to L, 227 cpm hybridized to 1 and 186 cpm background).

The longest open reading frames are shown in Fig. 2. There are no open reading frames that traverse the entire region, even if the left branch of the inverted repeat is ignored. Since L is known to code for a polypeptide of about 88 kdaltons (2), which would require a coding region of about 2.4 - 2.6 kb, we would expect this sequenced region to be at least that distance from one end of L_1 if

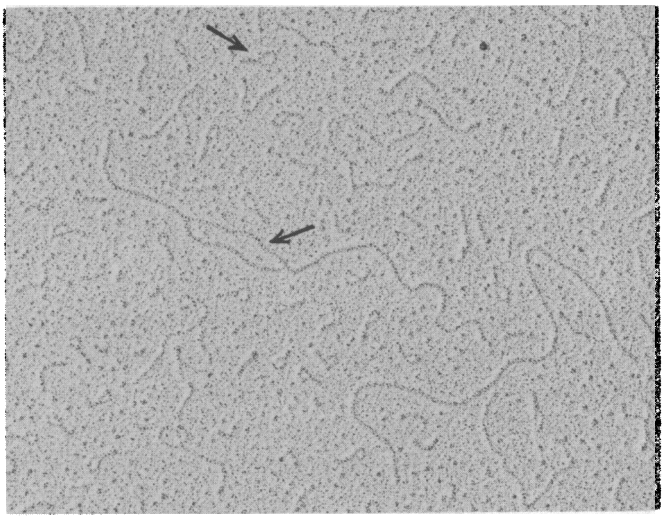
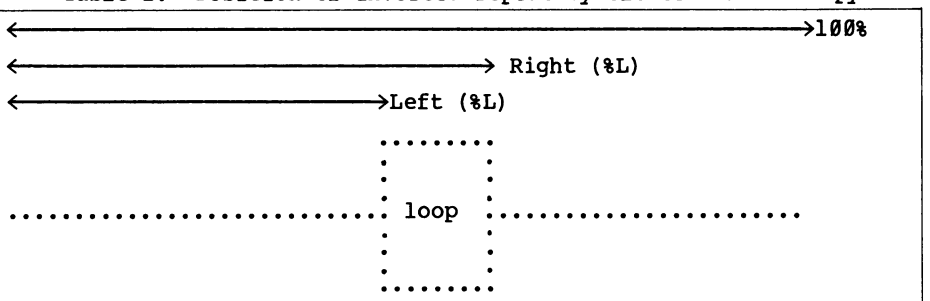


Figure 4. Heteroduplex of L_1 with the BglII-BglII fragment. The BglII-BglII 611 bp fragment (residues 27-637) was denatured and renatured with L. One of the resultant heteroduplexes, with two double-stranded branches, is shown. Arrows indicate the heteroduplex region and a BglII-BglII stem and loop. Magnification is 60,000X.

it coded for the C-terminus of P1. Direct RNA sequencing results show that the first long open reading frame (beginning with a methionine) starts at base 30 from the 5' end of the plus strand (22; J. D. Reilly and J. Bruenn, unpub.). We have mapped the region of these four clones in L_1 in several independent ways.

Heteroduplex analysis. In experiments analogous to R-looping, we denatured and renatured L_1 in the presence of restriction fragments from the cloned region. Heteroduplexes, consisting of ds L with inserted DNA restriction fragments, were identified by the presence of a displacement loop. No such loops were observed in the control experiment in which L was denatured and renatured by itself. Most of the resultant heteroduplexes have only one DNA strand in an RNA-DNA duplex, so that the looped region consists of one single-stranded RNA branch and one RNA-DNA duplex. On occasion, the loop consists of two RNA-DNA duplexes. One of these, with the BglII-BglII fragment of 611 bp (residues 27-637) is shown in Fig. 4. As expected, the two RNA-DNA branches of the displacement loop are identical in length. It is clear, from the formation of such

Table 2. Position of inverted repeat by electron microscopy



Experiment	Postion (%L)		N[1]	Length	
	Left	Right		Meas.	Calc.
stem and loop ss L	45.4 ± 5.4	68.2 ± 5.0	11	982 ± 258	-
BglII heteroduplex	50.2 ± 8.5	64.9 ± 7.5	14	691 ± 211	611
XbaI heteroduplex	50.7 ± 2.6	73.8 ± 5.5	28	1084 ± 286	769

The BglII heteroduplexes were formed between L and the BglII-BglII 611 bp fragment. The XbaI heteroduplexes were formed between L and the XbaI-HaeIII 151 bp fragment. The calculated lengths for the regions of the heteroduplexes are 611 for the BglII-BglII heteroduplex and 769 for the XbaI-HaeIII heteroduplex (assuming that the inverted repeat extends at least to the XbaI site, 52 bp further than documented by sequence analysis). For ss L, the positions of the beginning and end of the stem and loop are given; for the heteroduplexes, the beginning and end of the displacement loop.
[1] N is the number of molecules measured. The measured lengths assumed 3.4 angstroms/bp for dsDNA, 3.0 angstroms/bp for dsRNA, and 3.0 angstroms/bp for RNA-DNA duplexes. This gave a value of 4700 bp for L in comparison with the pBR322 internal standard.

heteroduplexes without DNA tails, that the inverted repeat must exist in the parental RNA. Measurement of these heteroduplexes places the 5' proximal BglII site at about 50% from the 5' end of L_1 (Table 2). In the heteroduplex shown (Fig. 4), the loop begins at 55.4% of L_1 and ends at 73.7% of L_1. In some molecules, such as this one, regions of L_1 beyond the heteroduplex region are melted out, and the resultant single-stranded regions are difficult to distinguish from the adjacent duplex regions. Hence the standard deviations of the positions of the end points are large, and the length of the hybrid region is overestimated (691 ± 211 bp vs. an actual length of 611 bp).

This experiment was repeated with the XbaI-HaeIII 151 bp

fragment from residues 566-716. In this case, the result is more complicated, since the fragment may form a duplex with L_1 in either of two regions. Again, the formation of the heteroduplex also tends to melt out regions of L_1 beyond the duplex region, so that these two sets of heteroduplexes give overlapping ends on the inside of the inverted repeat. Nevertheless, the outside extremities of the duplexes map to the same places as those of the BglII-BglII fragment (Table 2). The standard deviations are considerably larger than the distance between the BglII and XbaI sites (79 bp). The duplexes with the XbaI-HaeIII fragment suggest that the inverted repeat extends at least to the XbaI site (52 bp further than documented by sequence analysis). The BglII-BglII fragment usually forms a hairpin and loop structure (Fig. 3) rather than a heteroduplex: consequently the proportion of molecules of L_1 with an inserted BglII fragment was small (about 1%). The proportion of molecules of L_1 heteroduplexed with the XbaI-HaeIII fragment was considerably higher (5-10%). Both experiments were performed with the DNA fragment in molar excess.

Electron microscopy of single-stranded L. Although the electron microscopy of single-stranded L was more difficult, we were able to identify a number of stem and loop structures in denatured L. One of these single-stranded forms is shown in Fig. 5. Single-stranded RNA (ssRNA) was distinguished from dsRNA by its more kinky appearance and reduced amount of platinum shadowing. We previously determined that ssRNA is about 2/3 as extended as dsRNA under our spreading conditions, by measuring the ss regions of heteroduplexes between the yeast viral dsRNA M and one of its defective interfering RNAs (S) derived by internal deletion (L. Field and J. Bruenn, unpub.). Measurements of ss L and ds L in the current experiments agree with this previous determination: for instance, the ss L of Fig. 5 is 64% of the length of ds L in the same plate. The map position of stem and loop structures corresponded to the position of the inverted repeat mapped by heteroduplex analysis (Table 2). The extent of the ds regions (the stems) of the stem and loop structures varied considerably, but averaged 325 bp; in the molecule of Fig. 5 it is about 100 bp. Although there are two species of L present in that used for electron microscopy (L_1 and L_a), that homologous to the cloned cDNAs

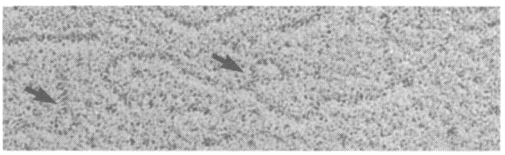


Figure 5. Stem and loop structure in denatured L. Magnification is 104,000X. Arrows indicate the stem and loop in ss L and a BglII-BglII fragment in a hairpin and loop structure, as in Fig. 4.

(L_1) consitutes 95% of the preparation (8), so the stem and loop structures observed are probably in L_1.

Another heteroduplex form that could be unambiguously identified as of L_1 was also observed. On occasion, two identical strands of the BglII-BglII fragment will renature with each other: the result is a fragment with a single-stranded loop (the middle 330 bp). Analogous forms were also observed in which a BglII fragment renatured with the identical strand of a denatured molecule of L_1, creating a single-stranded RNA with two short double-stranded regions bracketing a loop (not shown). These loops mapped to the same position as the stem and loop structures.

The extent of the inverted repeat estimated from the stem and loop structures in single-stranded L_1 is about 325 bp. This agrees well with the S1 resistant fraction of denatured L_1 or L_1 plus strand (6-10%) and is larger than the minimum of 170 bp from the sequenced region (data not shown). Control S1 treatments of tRNA gave less than 0.5% resistant to digestion.

From the three sets of electron microscopic measurements, the mean distance from the 5' end of the plus strand of L_1 to the vicinity of the BglII or XbaI sites in the cloned region is 49.5% of L_1 (Table 2). If our best estimate of the size of L_1 is 4700 bp (Table 2), this places the left BglII site at about 2325 bp from the end of L_1.

Primer extension. Our primer extension experiments had a twofold purpose: to map the position of the cloned region on L_1 and to verify the presence of the inverted repeat in L_1. To verify the existence of the inverted repeat in L_1, we did the following experiments. Plasmid DNA from pL1-21 was cut at the unique XbaI site (bp 716 of Fig. 2), dephosphorylated, and labeled at its 5'

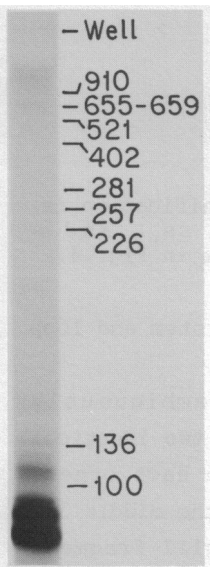


Figure 6. Primer extension on L_1. The denatured HinfI-Sau3A fragment (residues 713-785), labeled at the 5' side of the HinfI site, was used to prime synthesis by reverse transcriptase on the L_1 plus strand. The resultant products were electrophoresed on a 8% sequencing gel. The positions of markers made by 5' end labeling an AluI digest of pBR322 and run in an adjacent lane are shown at the side.

termini. A secondary restriction cut was made with HaeIII and the XbaI-HaeIII 151 fragment (from residues 566-716) was isolated by polyacrylamide gel electrophoresis and used for primer extension by reverse transcriptase on the L_1 plus strand. Another fragment, from the HinfI site at 713 to the Sau3A site at 785, labeled at the 5' end of the HinfI site, was similarly isolated and also used for primer extension. The L_1 plus strand was the product of in vitro transcription by the ScV-L_1 particles and was separated from ds L_1 by CF11 chromatography. If the inverted repeat does not exist in L_1, or does not extend as far as bp 785, only the fragment from 566-716 will give labeled primer extension products. If the inverted repeat does extend to 785, then both fragments should give labeled extension products. Both denatured fragments did produce labeled extension products, confirming the existence of the inverted repeat (Figs. 6 and 7). The inverted repeat therefore probably extends at least to the Sau3A site, 120 bp farther than the left end of clone pL1-26. Most of the extension products primed by the 566-716 fragment are smaller than 1500 b (Fig. 7), but the 713-785 fragment primes the synthesis of a large product of about 2500 b, whose size cannot be accurately estimated from the gel shown (Fig. 6).

Figure 7. Primer extension on L_1. The denatured XbaI-HaeIII 151 fragment (residues 566-716), labeled at the 5' side of the XbaI site, was used to prime synthesis by reverse transcriptase on the L_1 plus strand. The products were electrophoresed on a 1.4% alkaline agarose gel, and the gel was dried and autoradiographed. Size markers were the fragments resulting from EcoRI digestion of pL1-17 DNA previously cut with BglII and labeled at the 5' termini (3766 and 848 bp); from SalI digestion of the same DNA (3116 and 1498 bp); and the same DNA uncut (4614 bp, not shown).

In order to accurately determine the position of the cloned region in L_1, we therefore performed a primer extension experiment with the XbaI-HaeIII (566-716) fragment in the presence of alpha-^{32}P-dATP. The result is shown in Fig. 8. The same smaller products appear as in the experiment with the 5' end labeled primer

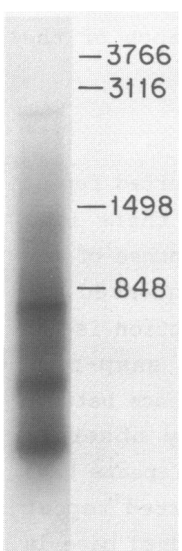


Figure 8. Primer extension on L_1. As in Fig. 6, but synthesis occured in the presence of alpha-^{32}P-dATP.

(Fig. 7), but now the largest primer extension product of 2650 b is clearly visible. Since this product is primed on the left hand branch of the repeat (see below), this places the left BglII site 2578 bp from the 5' end of the plus strand of L_1, within one standard deviation of the 2325 bp predicted from the heteroduplex mapping and from the mapping of stem and loop structures in denatured L_1.

The major products of primer extension with the XbaI-HaeIII 151 fragment are considerably smaller. The largest of these is estimated from the alkaline agarose gel as being about 770 b long. If the XbaI-HaeIII fragment were to prime synthesis by hybridization to the right hand branch of the inverted repeat (Fig. 2), then a primer extension product of this length would end within the inverted repeat. This primer extension product does arise from hybridization to the right hand branch of the inverted repeat because it is also a major product in the primed synthesis with the same fragment labeled at the 5' end of the XbaI site and extended in the absence of labeled nucleotides (Fig. 7). The smaller primer extension products, also present in the absence of labeled nucleotides (Fig. 7), have not been sized accurately, but probably end within the region between the inverted repeats. The existence of only one full-length primer extension product in the experiment with labeled nucleotides may be due to the fact that almost all the primer extension products begun on the right hand branch of the inverted repeat end within or before the inverted repeat.

DISCUSSION

We have several reasons for believing that the inverted repeat present in pL1-26 is not a cloning artifact. First, there is a single base difference between the left and right branches of the repeat, while all overlapping regions of four clones sequenced are identical. The observed accuracy of reverse transcription is not consistent with generation of the inverted repeat by snap-back synthesis, since there should then be no sequence difference between the branches of the inverted repeat (23). The only observed differences in sequence between artifactual inverted repeats have been in the first few base pairs, where an imperfect inverted repeat in the RNA causes snap-back synthesis (23). Our mismatched base is

88 bp into the repeat. Second, the inverted repeat region hybridizes to the L_1 plus strand in both orientations, while the region between the inverted repeats hybridizes in only one orientation. Third, the primer extension experiments imply that a region around the XbaI site exists in both orientations in the L_1 plus strand. Fourth, the entire BglII-BglII fragment, including a large portion of both branches of the inverted repeat, forms a continuous heteroduplex with L_1. Fifth, stem and loop structures at the appropriate position are present in single-stranded L. The sequenced region of this inverted repeat is 170 bp long, with one mismatched base. Our heteroduplex analysis suggests that the repeat is at least 220 bp long, while measurement of stem and loop structures in single-stranded L and S1 digestion experiments indicate that the inverted repeat is about 300 bp long. Primer extension experiments imply an inverted repeat of at least 289 bp. This is the longest inverted repeat presently known in dsRNA viral genomes, and the only internal inverted repeat. Final confirmation of the existence of the inverted repeat and measurement of its extent await the complete sequence determination of L_1.

All experiments are consistent with the mapping of the first branch of the inverted repeat to 2.3 - 2.5 kb from the 5' end of the plus strand of L_1. Both the left branch of the inverted repeat and the region between the inverted repeats have termination codons in all three reading frames, so that the cloned region may punctuate a gene. A subgenomic in vivo mRNA that codes for P1 in in vitro translation experiments has been estimated to be 2.3 kb on the basis of comparison to migration of yeast rRNAs in a Northern gel (13). The gene for P1 may therefore occupy the 5' 2.3-2.6 kb of the L_1 plus strand.

Previous mapping by hybridization to ScV transcriptase pause products placed this sequenced region much closer to the 5' end of the L_1 plus strand (6). This mapping was erroneous because the longer pause products do not originate, as previously thought, at the 5' end of the L_1 plus strand (J. D. Reilly and J. Bruenn, unpub.).

There is also an open reading frame in L_1 whose first methionine codon is within the second inverted repeat. Since most eucaryotic mRNAs are translated from the first AUG downstream from

their 5' ends (24), this open reading frame could serve for the synthesis of a second polypeptide on L_1 plus strands if the message were cleaved between the inverted repeats and prior to its AUG at position 602 or if termination and reinitiation of transcription occurred in this region. This would be the first example of a dsRNA coding for two polypeptides by cleavage or reinitiation of transcription. A reovirus dsRNA may encode two polypeptides in overlapping reading frames (A. Shatkin, personal communication). If the 3' region of the L_1 plus strand does not encode any polypeptide, it would constitute a much larger untranslated region than demonstrated for any other dsRNA.

The heteroduplex mapping technique we have used should be useful for the rapid mapping of partial cDNA clones on their parental dsRNAs. It does not require the preparation of separated strands of the dsRNAs for primer extension, nor the preparation of separated strands of cDNA restriction fragments, and only very small quantities of restriction fragments and dsRNA are necessary.

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