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Biochemistry

In vivo mapping of a sequence required for interference with the yeast killer virus

(cDNA/expression vector/defective interference/double-stranded RNA virus/*Saccharomyces cerevisiae*)

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ABSTRACT **The *Saccharomyces cerevisiae* viruses are noninfectious double-stranded RNA viruses whose segments are separately encapsidated. A large viral double-stranded RNA (L1; 4580 base pairs) encodes all required viral functions. M1, a double-stranded RNA of 1.9 kilobases, encodes an extracellular toxin (killer toxin) and cellular immunity to that toxin. Some strains contain smaller, S, double-stranded RNAs, derived from M1 by internal deletion. Particles containing these defective interfering RNAs can displace M1 particles by faster replication and thus convert the host strain to a nonkiller phenotype. In this work, we report the development of an assay in which the expression of S plus-strand from an inducible plasmid causes the loss of M1 particles. This assay provides a convenient method for identifying *in vivo* cis-acting sequences important in viral replication and packaging. We have mapped the sequence involved in interference to a region of 132 base pairs that includes two sequences similar to the viral binding site sequence previously identified in L1 by *in vitro* experiments.**

Most laboratory *Saccharomyces cerevisiae* (yeast) strains contain virus particles in the cytoplasm in which segmented double-stranded RNAs (dsRNAs) are separately encapsidated. The large dsRNA of one viral family, L1, is 4580 base pairs (bp) (1) and encodes on the plus-strand in reading frames that overlap by 130 bases the major capsid protein (cap; ref. 2) and an RNA-dependent RNA polymerase (pol; refs. 1, 3, and 4) that are used by all dsRNAs of this family. The pol protein is translated as a cap–pol fusion product by a frameshift event (1). Replication and transcription in this system may be of general interest, since the pol protein shares conserved regions with the RNA-dependent RNA polymerases of the plus-strand RNA viruses (1, 3).

In k1 killer strains, there is a second dsRNA, M1, of about 1.9 kilobases (kb), which encodes a secreted toxin that kills sensitive cells (5–8). The M1 preprotoxin functions as an immunity protein (9, 10). Suppressive-sensitive mutants (11) of the *S. cerevisiae* virus (ScV) contain L1 and smaller dsRNAs, S, which vary from 0.6 to 1.6 kb (12, 13). S dsRNAs are derived from M1 by internal deletion, sometimes followed by tandem duplication (14–19), and are thus defective in synthesis of toxin. In a cross between a k1 killer strain (containing ScV-L1 and ScV-M1) and suppressive-sensitive mutants (containing ScV-L1 and ScV-S), ScV-S particles can displace ScV-M1 particles by faster replication (20). Thus, the S dsRNAs are analogous to defective interfering (DI) genomes of viruses of more complex eukaryotes.

Defective genomes are one way to approach the definition of cis-acting sites necessary for replication and packaging. Only a small portion of the 5′ region of the plus-strand is common to all three S dsRNAs so far characterized, while a very large region (>500 bp) from the 3′ end of the plus-strand is common (19). There are chromosomal genes (*MAK*) required for M1 and S maintenance but not for L1 maintenance (21). At least some of these are also necessary for an L1 DI mutant (L1X) lacking all but 25 bp from the 5′ end and 491 bp from the 3′ end of the L1 plus-strand (22). There is a 10-bp sequence present in the 3′ region of the L1 plus-strand (bases 4200–4209) and in the 3′ region of the M1 and S plus-strand (bases 642–651 of S14) that was thought (23) to play a part in recognition by proteins that recognize both L1 and M1 (e.g., the major viral capsid polypeptide and the viral RNA polymerase). More recent *in vitro* experiments implicate overlapping sequences acting as a viral binding site (VBS) and an internal replication enhancer (IRE) (24) that do not include this 10-bp sequence but are located within L1X (bases 4160–4203 in L1).

The dsRNAs of ScV replicate conservatively. As in reovirus, transcription within viral particles is followed by extrusion of the newly synthesized plus-strand, which then (presumably) interacts with cellular and viral proteins. The plus-strand is then packaged in viral particles and replicated by synthesis of minus-strand, resulting in duplex formation (25–27). Although some viral particles may contain more than one copy of a viral dsRNA when transcription is not followed by extrusion of the new plus-strand (22, 28), new viral particles are only formed as described. Viral replication might be most vulnerable to interference during the period in which the viral plus-strand is not yet packaged and must interact with cellular and viral proteins. We would predict that excess S plus-strand expressed in a cell containing ScV-L1 and ScV-M1 might interfere with replication and/or packaging of M1. We envision that the cis-acting sites of RNA transcribed from a cDNA could titrate out one or more proteins recognizing sites in M1 or S. In this work, we show that the expression of an S (S14) plus-strand by an inducible yeast expression vector in k1 killer cells can indeed cure ScV-M1. This is a unique *in vivo* assay for cis-acting functions of the plus-strands of dsRNA viruses. These experiments show that the 10-bp sequence in common between L1 and M1 is not required for interference with M1 replication or packaging. The sequences necessary for interference (INS) are located within a 132-bp region that includes two sequences homologous to the IRE–VBS in L1. These sequences are two of the five repeats of a consensus 11-bp repeat that we previously postulated was important for M1 and S replication or packaging (19).

MATERIALS AND METHODS

Strains and Media. Bacterial transformations were done with HB101. Yeast transformations were done with the

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Abbreviations: ScV, *Saccharomyces cerevisiae* virus; dsRNA, double-stranded RNA; DI, defective interfering; IRE, internal replication enhancer; VBS, viral binding site; INS, interference sequence.
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recipient BFH2 (*ura3 Gal*$^+$ *L1 M1*), a sporulation product of a diploid formed between strain 18 (*MATa lys1 L1 M1*; ref. 29) and CGY339 (*MATα ura3 his4 pep4 gal*$^+$ *L1*; ref. 30). The rate of displacement of ScV-M1 by ScV-S14 was measured in a cross of LO14 (*MATa ade2-1 lys1-1 SUP4-2 S14*; ref. 13) and LF822 (*MATα cyc1 rad1 can1 ura3 L1 M1*). Sensitive cells for killer tests were S7 (31).

All bacterial transformants were selected on LB ampicillin medium by growth at 37°C overnight. BFH2 was grown in yeast extract/peptone/glucose medium (1% yeast extract/2% peptone/2% dextrose) at 30°C. Yeast transformants were selected and maintained on either selective glucose plates (0.67% yeast nitrogen base/2% dextrose/2% Casamino acids) or on selective galactose plates (0.67% yeast nitrogen base/2% galactose/2% Casamino acids) at room temperature. All diploids were grown on a minimal glucose medium (0.67% yeast nitrogen base without amino acids/2% dextrose). Medium for killer tests was buffered yeast extract/peptone/glucose with methylene blue (29).

Expression Vector Constructions. The *Xba* I fragment containing a full-length S14 cDNA clone (32) was inserted into one of the *Xba* I sites in pCGS152 or the *Bam*HI site in pCGS110 (30) after making both fragments flush-ended and positive clones were identified by colony hybridization (33). Plasmids with the desired structure were identified and orientations of S14 cDNA inserts were determined by restriction enzyme mapping and sequencing. Insertion of smaller restriction fragments of the S14 cDNA was performed by making the ends of the fragments flush-ended with DNA polymerase I (EC 2.7.7.7) Klenow fragment and ligating them to the similarly treated vector (33). All plasmid constructions were verified by sequencing.

Yeast Transformations. All yeast transformations were performed by the lithium acetate procedure (34).

Yeast Killer Tests. A loop-full of S7 sensitive cells was suspended in 7.5 ml of buffered yeast extract/peptone/glucose with methylene blue and with 1% agar (liquefied) and poured in a Petri plate (29). Cells to be tested were streaked on the surface of the solidified medium with S7 cells embedded and the plates were incubated at room temperature until killer rings were visible.

Generation Determination and Subcloning of Transformants. A number of independent, original transformants were streaked on selective plates with galactose and incubated until individual colonies were formed (≈4 days). From two colonies of about the same size, one colony was used to do a killer test and streaked again to get subclones; the other colony was suspended in water and a series of dilutions were plated to determine the number of cells in the colony and thus calculate the number of generations elapsed since the last plating. In each experiment, 10–45 original (independent) transformants were streaked for subclones on minimal galactose medium. From each of these transformants, one subclone was tested for killer phenotype after each subcloning (≈20 generations). Once a clone became a nonkiller, all its progeny remained nonkillers.

Total RNA Preparations. Stationary phase cells (50 ml) were harvested and washed with 10 ml of cycloheximide (0.1 mg/ml) and suspended in 3 ml of lysis buffer (25 mM Tris·HCl, pH 7.5/25 mM NaCl/5 mM $MgCl_2$). Cells were then broken by adding an equal volume of autoclaved glass beads and Vortex mixing at top speed for 2 min. The supernatant was removed from the glass beads and cell debris by centrifugation and was extracted by water-saturated phenol with 0.1% SDS. Total RNAs were ethanol precipitated and dissolved in 0.4 ml of TE (Tris·HCl, pH 7.5/1 mM EDTA).

Northern Blot Transfers. Ten microliters of each total RNA preparation was loaded on a 1.4% agarose gel. After electrophoresis, the gel was treated with a solution of TAE (33), 50% formamide, and 17% formaldehyde for 60 min at 65°C. The gel was then incubated with 50 mM NaOH/100 mM NaCl for 45 min at room temperature followed by two 30-min incubations in 18× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate). Transfer to nitrocellulose, prehybridization, and hybridization were performed as described (33).

RESULTS

Curing of ScV-M1 by DI Particles. Since we have completed the sequence of S14 (792 bp) (19, 32) and have constructed full-sized cDNA clones within one *Xba* I fragment (32), we chose to use S14 for these experiments. Control experiments were performed by mating a ScV-S14 strain (LO14) with a killer haploid (with ScV-M1) and selecting diploids. Individual colonies were followed for many generations by repeated subcloning followed by killer tests. Curing of ScV-M1, monitored by loss of killer toxin production by all progeny of a single cell, is not very efficient when mediated by introduction of ScV-S14 particles to a diploid containing ScV-M1. This is shown in Fig. 1, which plots the percentage of nonkillers arising as a function of the number of generations elapsed since formation of the diploid. After 130 generations, nonkiller diploids reached a plateau of 16% (3/19) of colonies tested. Comparison with previously published results (20) indicates that the rate of curing is dependent on the kind of S particles introduced and perhaps on the recipient strain as well. Other crosses with this same ScV-S14 haploid (LO14) gave similar results (data not shown).

Curing of ScV-M1 by DI cDNAs. We performed similar experiments to test for curing of ScV-M1 by the expression of S14 plus- and minus-strands in transformants carrying an inducible plasmid. S14 cDNAs were inserted into the *Xba* I site of pCGS152 that immediately follows a *GAL1* promoter, so that S14 plus-strand (in all clones except pS14-4) or minus-strand (pS14-4) would be expressed. Killer strain BFH2 was transformed by either pCGS152 (vector alone), pS14-1 (plus-strand expression vector with the entire S14 cDNA), or pS14-4 (minus-strand expression vector with the entire S14 cDNA). Transcription is under the control of the *GAL1* promoter. All transformants were isolated on minimal glucose medium and subclones were grown on galactose minimal medium. In every case, individual clones were tested for killer phenotype and their progeny were followed for ≈200 generations.

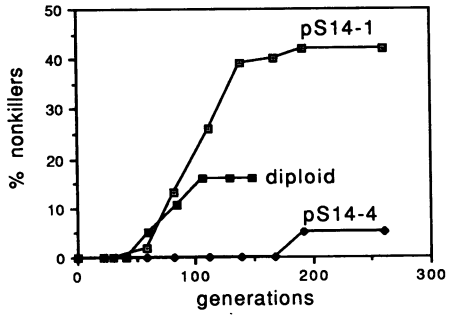

FIG. 1. Segregation of nonkiller progeny from crosses with defective interfering mutants and from defective interfering cDNA transformants. A minimum of 19 individual clones of each type was followed by repeated subcloning for many generations. The percentage of clones tested that were nonkillers is plotted as a function of generations elapsed since growth on galactose medium (or since diploidization) commenced. The pS14-1 transformants express the S14 plus-strand and the pS14-4 transformants express the S14 minus-strand. The diploid initially had both ScV-S14 and ScV-M1 particles. The negative control (the vector pCGS152 transformants) gave no nonkillers at any time in this experiment.

All of the transformants carrying the vector pCGS152 alone remained killers, whether grown on galactose medium or glucose medium. Some 42% (19/45) of the transformants expressing the S14 plus-strand (pS14-1) in galactose medium became nonkillers. This is quite repeatable and has varied from 25% (6/24) to 90% (15/16) in four independent experiments. All but 1 of the 20 transformants expressing the S14 minus-strand in galactose medium remained killers. All nonkillers remain so after being subcloned on glucose medium, so that the loss of killer function is permanent. Control pS14-1 transformants never plated on galactose medium retain killer function and ScV-M1 (see below). A comparison of the percentage of clones becoming nonkillers as a function of generations elapsed after expression of S14 cDNAs by pS14-1 and pS14-4, and after introduction of ScV-S14 particles by mating is shown in Fig. 1. Curing by expression of cDNA is more efficient than the natural process of competition between ScV-S14 and ScV-M1 in this experiment. Expression of the S14 minus-strand does not efficiently cure ScV-M1: the plateau level of cured clones was never more than 5% in each of four experiments. This may, however, reflect the steady-state level of transcript in pS14-4 transformants (see below). In some control experiments with transformants carrying the vector alone, spontaneous loss of ScV-M1 also reached 5%.

Expression of Viral RNAs. There may be numerous points between synthesis of viral plus-strand and completion of dsRNA viral particles at which viral interference might take place. We can arbitrarily divide these into processes that take place on the free viral plus-strand, on the packaged viral plus-strand, and on the packaged viral dsRNA. Since our hypothesis is that S14 competes with M1 by titration of factors required for replication or packaging, we sought to roughly quantify the amounts of S14 single-stranded RNAs and dsRNA present in our transformants. We isolated the total RNAs from transformants and performed Northern transfers to measure S14 minus-strand, S14 plus-strand, and M1 dsRNA (Fig. 2). Note that these are nondenaturing gels in which the RNAs are denatured after electrophoresis but prior to transfer to nitrocellulose filters, so that dsRNA, plus-strands, and minus-strands can all be distinguished from each other. After growth in galactose, all of the nonkiller pS14-1 transformants tested had lost M1 dsRNA and expressed an RNA homologous to the S14 plus-strand, with a size of ≈1500 bases as judged by its mobility compared to those of the yeast ribosomal RNAs (Fig. 2). Southern blots and curing experiments demonstrate that all transformants grown on selective medium retain the unintegrated plasmid (data not shown). These nonkiller transformants had not acquired any new dsRNAs, including S14. A pS14-1 transformant grown in glucose medium and never exposed to galactose remained a killer, retained ScV-M1, and did not express S14 RNA, as expected. None of the transformants that should express minus-strand (pS14-4), nor those with vector alone (pCGS152), expressed S14 plus-strand RNA, whether grown in glucose or galactose. The controls with vector alone retained M1 and remained killers. Nonkiller diploids resulting from a cross between a ScV-S14 strain and a k1 killer strain have lost M1 and have S14 dsRNAs (data not shown). All strains retain L1, as judged both by ethidium bromide staining (Fig. 2) and by Northern blot analysis (data not shown).

The expression of M1 plus-strand in ScV-M1 strains (Fig. 2) or S14 plus-strand in ScV-S14 strains (data not shown) is barely detectable. The pS14-1 transformants express much higher levels of the S14 transcript than are expressed from the dsRNA viral genome. The simplest interpretation of these results is that the rate of production of nonkillers is a function of the amount of interfering single-stranded RNA present. Interference does not take place during a process that operates on mature viral particles, since there are no ScV-S particles in our nonkiller transformants (Fig. 2); nor does it take place during a process operating on packaged single-stranded RNA, since the S14 transcripts made in pS14-1 transformants are not present in viral particles (data not shown). We conclude that interference takes place during a process that operates on viral single-stranded RNAs prior to or during packaging. The pS14-4 transformants express the S14 minus-strand at a low level (Fig. 2), so it is difficult to say whether the minus-strand is capable of curing ScV-M1.

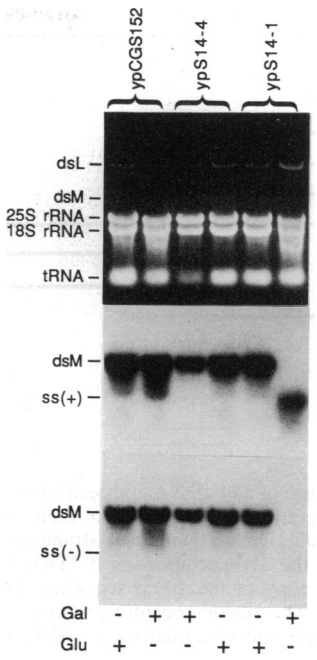

FIG. 2. Characterization of ScV RNAs present in transformant strains. Total RNA was electrophoresed on 1.4% agarose nondenaturing gels, denatured, and transferred to nitrocellulose. Hybridization was to "probe-primer"-extended viral DNA from M13 cDNA clones. (*Upper*) Ethidium bromide-stained gel. (*Middle*) Hybridization to minus-strand cDNA. (*Lower*) Hybridization to plus-strand cDNA. Results are shown for two representatives of each transformant type, one grown in glucose (Glu) and one in galactose (Gal) minimal medium. The ypS14-1 clone, expressing the S14 plus-strand, was a nonkiller; the ypS14-4 clone, expressing the S14 minus-strand, was a killer; and the ypCGS152 clone, with vector alone, was a killer. Results were similar for all clones of each type tested. dsL, L dsRNA; dsM, M dsRNA; ss(+), plus-strand homologous to S14; ss(−), minus-strand homologous to S14.

Although our Northern blots are of whole cell RNA, the S14 cDNA transcripts are probably in the cytoplasm, since the entire ScV life cycle takes place in the cytoplasm. The transport of S14 cDNA transcripts to the cytoplasm is as expected, since most yeast mRNAs are unspliced and since transcripts of M1 cDNA are known to be transported to the cytoplasm (9, 10).

Region of S14 Required for Interference. Fig. 3 shows an abbreviated map of the S14 full-length cDNA, including the five occurrences of the 11-bp repeat previously noted (19) and the single occurrence of the 10-bp site present both in L1 and M1, as well as the region with some homology to the IRE–VBS region of L1 (24). Subclones of the S14 cDNA from the 5′ end, the 3′ end, and the region suspected to be involved in interference were placed in the pCGS152 vector in the proper orientation for expression of the plus-strand. Expression did occur in each case (see below). Curing experiments identical to those of Fig. 1 were performed. The results are summarized in Fig. 3, which gives the plateau values of percentages of clones becoming nonkillers. Plateau values were reached after 120–200 generations. All plasmids expressing the region of S14 from bases 336–468 were capable

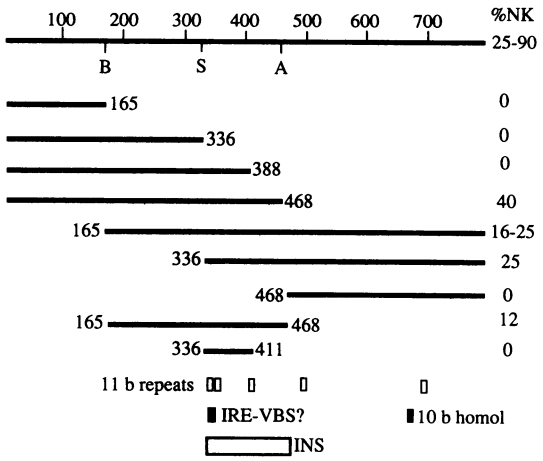

FIG. 3. Structure of S14 cDNA and restriction fragments cloned into expression vectors and the results of their expression as plus-strand in killer strains. Bases on the plus-strand are numbered. Open boxes, copies of the 11-bp consensus (C/T)GCGATTCGC(G/A); solid box, single copy of the 10-bp sequence also present in L1, TTTGGCCAGG. The region recently proposed to be an internal replication enhancer and viral binding site (IRE–VBS) is indicated. Solid bars indicate regions present in the indicated plasmids. Fragments for subcloning were generated by using the *Bgl* II site at 165 (B), the *Sph* I site at 336 (S), the *Acc* I site at 468 (A), or by other methods. The last column indicates the plateau percentage of clones cured of ScV-M1 by expression of the indicated cDNA. A minimum of 16 clones of each transformant type was followed for 130–200 generations. All deletion constructs were tested for curing in at least two independent experiments. NK, nonkiller.

of curing ScV-M1. No plasmids expressing any portion of the S14 plus-strand not including the region from 411 to 468 could cure ScV-M1. The region from 336 to 411 is insufficient by itself. We conclude that the region from 336 to 468 includes sequences necessary and sufficient for viral interference, and we have designated this region INS, for interference sequence (Fig. 3). Included within this region is the postulated IRE–VBS (24), but it falls within bases 336–411, which are not sufficient by themselves. Neither the 5′ nor the 3′ end of the S14 plus-strand is necessary for interference, although inclusion of the 5′ end increases the efficiency of interference. Lack of the 5′ end in the construction including bases 336–411 is not the explanation for the failure of its expression to cure, however, since expression of the construction including bases 1–388 also fails to cure (Fig. 3). The INS lies entirely within the region of S14 derived from the 3′ end of the M1 plus-strand (the last 540 bases) and outside the region derived from the 5′ end of the M1 plus-strand (the first 252 bases).

An additional control plasmid expressing bases 4349–4580 of the L1 plus-strand, lacking the IRE–VBS (bases 4160–4203 of L1) failed to interfere with either ScV-M1 or ScV-L1 (data not shown). All of the transformants expressing portions of the S14 plus-strand gave detectable levels of transcript in Northern blots, including those transformants failing to show any curing. There was no correlation between the level of curing and the amount of transcript detectable in transformants, but all transformants gave a molar quantity of transcript within a factor of 2 of that present in pS14-1 transformants (data not shown).

DISCUSSION

The likely stages at which interference occurs in our cDNA expression system are packaging and replication. Since packaging precedes replication and no S14 single-stranded RNA or dsRNA occurs in the viral particles in the pS14-1 transformants, we can rule out replication as the stage at which interference occurs. Replication is also eliminated because the 3′ end of the plus-strand, known to be required for replication (24), is absent from two constructions whose expression eliminates ScV-M1 (Fig. 3). Authentic M1 transcripts and the transcripts of the S14 cDNA therefore appear to compete during packaging.

The region of S14 responsible for interference in our cDNA expression system (INS) has striking similarity to the L1 IRE–VBS sequence (Fig. 4). The secondary structures predicted by FOLD (35, 36) for L1X and S14 within the interesting regions are shown in Fig. 4. Note that these same structures (excluding that predicted for bases 450–468 of the S14 plus-strand) are predicted to occur in the complete L1X (531 bases) and S14 (792 bases) plus-strand sequences (data not shown). There is a dramatic similarity between the L1X VBS sequence (bases 131–154) and the two stem–loop structures predicted for the S14 plus-strand centered about bases 360 and 415. In both DI plus-strands, a long stem is topped with a loop with the sequence GAU(U/C)C. Both the GAUUC sequences of S14 present in such predicted loops are sequences within the 11-bp repeats previously noted (19). A third 11-bp repeat is present in the stem of the first stem–loop in S14. Sequences within the stems of the L1X and S14 RNAs in this region have little sequence homology. We propose that the sequence GAU(U/C)C within a loop at the top of a stem of 16–20 bp serves as the recognition sequence for packaging. The somewhat less efficient interference manifested by plasmids carrying the INS but lacking adjacent regions, especially the 5′ region, may be the result of stabilization of the INS secondary structure by the secondary structure in adjacent regions. The mapping is not a trivial effect due to inadequate expression of some cDNAs, however, since Northern blots demonstrate approximately equimolar levels of transcripts in all the plus-strand expressing constructions (data not shown). In all cases in which the INS plus-strand is expressed, curing is at least as efficient as it is by ScV-S14 particles. A minimal sequence from bases 336–468 is necessary and sufficient for interference as deduced from overlapping deletions, although the minimal sequence tested as a single fragment whose expression did cure ScV-M1 was from bases 165–468. Our data show that one stem–loop structure, previously identified (24) as similar to the L1 IRE–VBS (bases 354–365), is insufficient for interference; both stem–loop structures may be necessary for interference, or the second structure, by itself, may be sufficient.

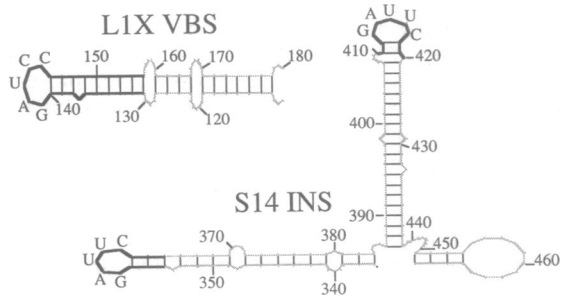

FIG. 4. Secondary structure predictions using FOLD (35, 36) for the VBS region of L1X and the INS region of S14. The highlighted region in L1X is the VBS. The highlighted regions in S14 are the two 11-base repeats that fall in the loops of predicted stem–loop structures. A third repeat is also present in this region, at bases 342–352. Neither of the two remaining 11-base repeats falls in such predicted structures. The stability of the predicted L1X structure is −13.6 kcal/mol (1 cal = 4.184 J) and that of the S14 structure is −47 kcal/mol.

The curing of only a portion of the cells in every case is difficult to explain but occurs both with expression of cDNA clones and with interference by DI particles (Fig. 1).

Either cellular or viral proteins might interact with viral plus-strands during packaging, but the protein responsible for viral interference is probably the cap–pol fusion protein (1), which is thought to initiate packaging (24). The homology with the L1X IRE–VBS sequence (Fig. 4) implicates the viral cap–pol fusion protein, which has been shown to bind to this sequence (4, 24). The absence of S14 transcripts in viral particles in transformants expressing large quantities of S14 transcript from cDNAs may be due to abortive packaging. This might be due to incorrect 5′ and 3′ end structures, which are not formed in our cDNA expression vectors, since these initiate and terminate outside the cDNA sequence. The 3′ end structure of the L1 plus-strand is known to be essential for its replication (24). Neither the 5′ nor the 3′ end of S14 is necessary for interference. Our results do not demonstrate that the process of interference by expression of cDNAs is the same as that mediated by ScV-S14 particles, but they are suggestive, since the INS includes two sequences similar to the L1X VBS (24) and since the kinetics and specificity (ScV-M1) of curing are the same in both cases. All of the known S dsRNAs include the INS region (ref. 19; M. Lee, H. Kang, and J.A.B., unpublished data).

The similarity between the region of S14 near base 360 and L1X has been noted (24), although only a small portion of the predicted secondary structure was shown, and the second stem–loop centered about base 415 was not detected. This second region is necessary for interference. Our results rule out the participation of the 10-bp region in S14 also present in L1 (bases 640–649 of the S14 plus-strand) in interference.

The S14 cDNA transcripts fail to interfere with L1 packaging, even though our assay could have detected such interference, granted that production of S14 transcript in our plasmid-carrying strains is no longer dependent (as it is in LO14) on the maintenance of ScV-L1. One possible explanation is that packaging of the two viral plus-strands takes place in different cellular compartments, so that S14 transcripts can only titrate out the cap–pol protein in one compartment. This would be consistent with the requirement for numerous cellular genes (e.g., *MAK* genes) by ScV-M1 (or ScV-S) but not by ScV-L1 (21). On the other hand, L1X, although dependent on the same *MAK* gene products as ScV-M1, does lower the copy number of ScV-L1 (22). If L1X plus-strands were present in both the L1 and M1 compartments, these results might be explained. Alternatively, lack of interference with ScV-L1 by S14 transcripts may simply reflect the much higher copy number of ScV-L1 particles (37) or the higher affinity of the cap–pol protein for L1.

The mechanism of packaging of viral dsRNAs is still mysterious. The assay we have described should be useful for further elucidating this process *in vivo*.

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