JOURNAL OF VIROLOGY, Mar. 1997, p. 2157–2162 Vol. 71, No. 3
0022-538X/97/$04.00+0

In Vitro Selection of Packaging Sites in a Double-Stranded RNA Virus

WENSHENG YAO, KAREN ADELMAN,† AND JEREMY A. BRUENN*

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260

Received 15 October 1996/Accepted 9 December 1996

The *Saccharomyces cerevisiae* double-stranded RNA virus ScVL1 recognizes a small sequence in the viral plus strand for both packaging and replication. Viral particles will bind to this viral binding sequence (VBS) with high affinity in vitro. An in vitro selection procedure has been used to optimize binding, and the sequences isolated have been analyzed for packaging and replication in vivo. The selected sequence consists of a stem with a bulged A residue topped by a loop of several bases. Four residues of the 18 bases are absolutely conserved for tight binding. These all fall in regions that appear to be single stranded. Eight more residues have preferred identities, and six of these are in the stem. The VBS is similar to the R17 bacteriophage coat protein binding site. Packaging and replication require tight binding to viral particles.

Of the many examples of specific RNA-protein interactions, there are several in which binding is as tight as it is in DNA-protein interactions. In these cases, the dissociation constant of the complex (K_d) is in the nanomolar range (1, 3, 23). One of these interactions is necessary for the packaging of the viral plus strand in the *Saccharomyces cerevisiae* virus (ScV). ScV is a persistent double-stranded RNA (dsRNA) virus in yeast which is passed from cell to cell solely by mitosis and meiosis. It belongs to the *Totiviridae*: that is, it has a single essential dsRNA (L). This genomic RNA encodes two proteins: a capsid polypeptide (Cap) and a Cap-Pol fusion protein synthesized by a translational frameshift between the *cap* and *pol* genes (7, 27). There appear to be two copies of the Cap-Pol protein per viral particle (6, 15, 23), which has 120 copies of the Cap domain (5). A satellite dsRNA (M1) is present in some strains and is separately packaged in ScV particles of the L1 virus (ScVL1). L1 is also known as L-A (4). A second dsRNA virus, ScVLa, with a similarly organized genome (17), is also present in some strains. La is also known as L-BC (4, 30). ScVL1 and ScVLa package only their own RNAs (8, 18, 25).

As in other dsRNA viruses, ScV replication proceeds by the synthesis within the viral particle of the viral plus strand, extrusion of the plus strand, packaging of the plus strand in nascent particles, and synthesis of the minus strand within completed viral particles. The whole cycle depends on the specificity of packaging of the plus strand, and this is accomplished in ScV by a domain within the Pol region of the Cap-Pol protein (12, 32). The region of the RNA recognized (the viral binding site [VBS]) is a minimum of 20 bases long and consists of a stem interrupted by a bulged A residue and with a central loop of several bases (11, 23), although a second type of binding site with three bases in place of the bulged A residue also functions well (23). L1 has a single VBS, while M1 has two sites, SL1 and SL2. The minimal binding site functions both for packaging and for viral interference in vivo (12, 14, 23). In vitro mutagenesis has defined the stems of the VBS as necessary but their sequence as nonspecific, while at least one base in the loop appeared sequence specific and the bulged A residue appeared conserved as well (11, 23). In this work, we have determined the secondary and primary structure requirements for the VBS by in vitro selection (2, 26) and shown that the selected VBSs are competent as packaging and replication signals. The ScV VBS is similar to the coat protein binding site for the single-stranded RNA bacteriophage R17 (19, 22, 28).

MATERIALS AND METHODS

In vitro selection. In vitro selection of RNAs by gel shift (see below) followed schemes previously described (2, 26). First, the five oligonucleotides for the in vitro selection experiment (see Fig. 1) were purified by 8% polyacrylamide gel electrophoresis (PAGE) in 7 M urea. Oligonucleotides 2 and 3 were phosphorylated with T4 polynucleotide kinase, and oligonucleotides 2, 3, and 5 were ligated together while hybridized to oligonucleotides 1 and 4. The full-length oligonucleotide 2+3+5 was purified by 8% PAGE in 7 M urea. Oligonucleotide 1 was used as a primer to verify the sequence of oligonucleotide 2+3+5. Oligonucleotide 1 was annealed to oligonucleotide 2+3+5, and double-stranded DNA was synthesized with DNAPI Klenow fragment. This double-stranded DNA was used as a template for in vitro RNA synthesis by T7 RNA polymerase. The RNA was subjected to in vitro binding with ScVL1 viral particles. The binding mixture was separated by 1.5% agarose gel electrophoresis, and the gel-shifted band was excised and had its RNA extracted with the RNaid kit (Bio 101). The purified RNA was reverse transcribed into cDNA with oligonucleotide 5 as a primer. Oligonucleotide 1 was added to amplify cDNA by PCR. The PCR products were used for in vitro transcription, and the RNA was subjected to the next round of selection. Cloning of PCR products (20) was by cleavage with *Apa*I and *Xba*I and insertion into a similarly cut pGEM7Zf+. Sequencing was by the Sanger technique (21).

Viral binding. Gel shift experiments were performed as described elsewhere (9, 23). The *Acc*I-*Sph*I (AS) fragment of S14 cDNA of 134 bp, containing both SL1 and SL2, or cloned, selected cDNAs, in pGEM7Zf+ was used to make labeled viral plus strand containing the VBS. The standard binding mixture contained 50 mM Tris-Cl (pH 7.5), 0.5% polyethylene glycol 8000, 5 mM EDTA, 3 mg of bentonite per ml, 1 to 5 ng of [α-^{32}P]UTP-labeled RNA (10^5 cpm per assay), and 2 μg of viral particles in a total volume of 15 μl.

***K_d* determinations.** Determination of K_d was done by competition with the AS fragment, assuming a K_d of 8.1×10^{-10} M for AS, as described elsewhere (23), or directly, by fitting a binding curve to data points as in Fig. 4, with DELTAGRAPH.

Viral packaging and replication. Packaging of viral plus strands was determined by expression of a viral plus strand containing the binding site to be tested (11, 23) embedded in several hundred bases of vector sequences from a hybrid *GAL/ENO* promoter (16, 24) in a strain with ScVL1 particles but no ScVLa particles. This was strain 2818H$^+$ (R. B. Wickner) cured of ScVLa as described elsewhere (31, 32). Packaging was determined by nondenaturing Northern blots (14) of fractions from CsCl gradients with probes derived from the expressed vector sequences (23). The packaging efficiency was calculated as the ratio of radioactivity in the CsCl fraction with the greatest amount of packaged vector sequences to the total amount of vector RNA expressed in the cell divided by the total amount of ScVL1 particles in the peak fraction estimated by sodium

* Corresponding author. Mailing address: Department of Biological Sciences, SUNY/Buffalo, Buffalo, NY 14260. Phone: (716) 645-2868. Fax: (716) 645-2975. E-mail: cambruen@ubvms.cc.buffalo.edu.

† Present address: Centre de Genetique Moleculaire, CNRS, Gif-Sur-Yvette 91190, France.

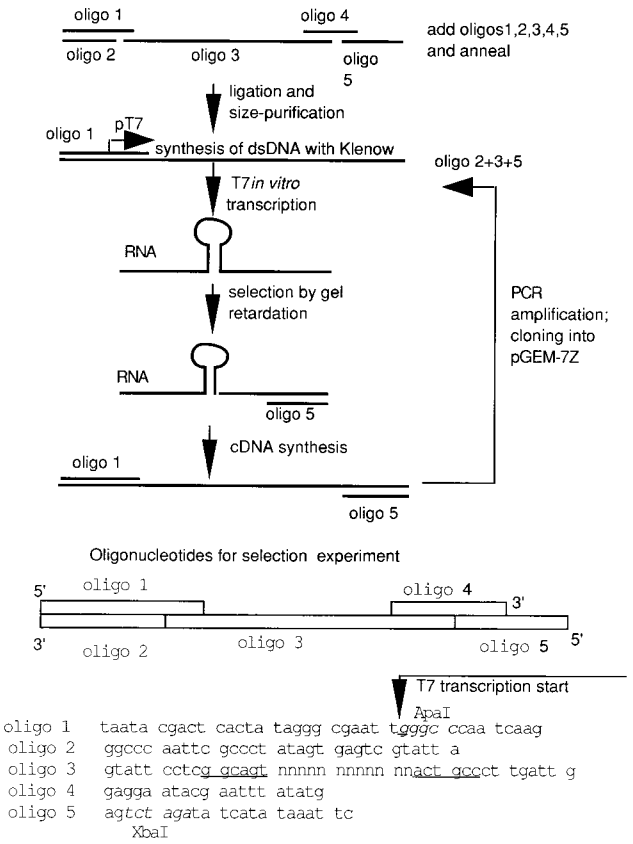

FIG. 1. In vitro selection procedure for high-affinity VBS. See Materials and Methods for details.

dodecyl sulfate (SDS)-PAGE (in arbitrary units). Phosphorimager scans of fraction 6 (e.g., Fig. 6) from each isolate were normalized by dividing by the amount of total RNA and by the amount of Cap protein in fraction 6 (from optical scans of Coomassie brilliant blue-stained SDS-PAGE gels). The packaging efficiency is the last column of Table 1. Given the accuracy of this determination (see below), only a single significant figure is given.

RESULTS

In vitro selection. The overall strategy in the in vitro selection is outlined in Fig. 1. The selection itself was by isolation of bound RNA in viral particle-RNA complexes separated from unbound RNA by agarose gel electrophoresis (11, 13, 23). The bound RNA was purified, reverse transcribed, amplified by PCR, and used for the next cycle of selection. Samples were taken by cloning after each round of selection.

There are three known VBSs in the wild-type ScV RNAs: two sites, SL1 and SL2, in M1 and its defective interfering derivatives, and one binding site in L1. We chose to model our selectable VBS partially on the stem of SL2 and partially on the upper stem and loop sequence of SL1. Hence the lower portion of the stem is defined by the unpaired bases at the beginning and end of the unpaired region of oligonucleotide 3 (Fig. 1). This gives a 6-bp stem with 12 ambiguous nucleotides between the branches of the stem (see Fig. 5). We chose this design, since previous results had indicated that the precise sequence of the stem was not important for VBS function (11, 23) and we wanted to maximize the proportion of RNAs that might bind to viral particles.

The K_d of the mixture of RNAs recovered after each round of selection was determined by competition with plus-strand

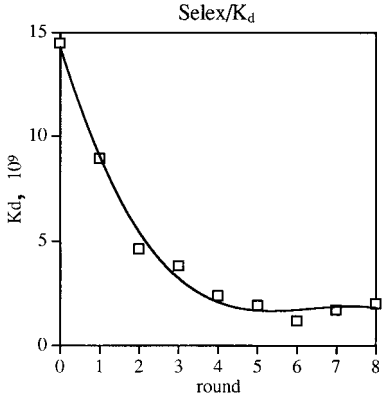

FIG. 2. Dissociation constant of VBS-viral particle complex versus round of selection. A crude K_d was determined for each pool of VBS sequences by competition with the AS fragment, as described elsewhere (23).

RNA transcripts of the AS fragment; this is the *Acc*I-*Sph*I fragment of S14 cDNA, of 134 bp, containing the two binding sites, SL1 and SL2 (23). This is not a true average K_d, but it is an indicator of how the selection is proceeding. After a decrease to the nanomolar range, the K_d ceased to decrease after about the fifth round (Fig. 2). Enrichment in K_d was about 12-fold (14.5/1.2).

Analysis of a number of clones from every round (some 50 clones were analyzed) showed that many of the cDNA clones represented RNA sequences with a structure similar to that of SL1 but that many had no similarity to SL1. Those RNAs that did not have similarity to SL1 did not bind to viral particles in the gel shift assay. The binding to viral particles of T7 plus-strand RNAs from a selection of clones is shown in Fig. 3. All of the clones which failed to show any binding (e.g., 8-7, 8-13, 8-14, and 8-19) lacked the consensus secondary and primary structure (see below). Only those clones that had the consensus structure showed significant binding. Even at the eighth round of selection, nearly 50% of the clones isolated did not represent binding sequences. We have no ready explanation for this result. It may be that the gel electrophoretic separation of bound and unbound RNA was inadequate. Adding excess competitor AS transcript or excess noncompeting RNA to the binding reaction during selection had little effect on the clones isolated. A summary of the sequences of binding RNAs is shown in Table 1.

***K_d* determination of individual RNAs.** The K_d's of selected RNAs were determined by competition with the AS transcript, as previously described (23, 29). This method is considerably faster than doing individual binding curves for each isolate and

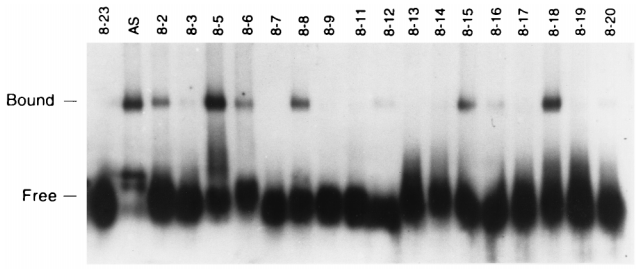

FIG. 3. Binding of some selected sequences by mobility shift analysis. Each sequence, cloned in pGEM7Zf+, was transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP, and the radioactive plus strand was used for a binding experiment with ScVL1 particles, as described elsewhere (23).

TABLE 1. Consensus of tight binding sequences

Bulged residue	Second portion stem, 5′ branch	Loop	Second portion stem, 3′ branch	Isolate no.	Binding	K_d (10^{-9} M)	Packaging[a]
A	UGC	CUCCG	GUA	8-9	+/−	2.95	0
A	GGC	GAUUC	GUC	8-12	+	1.58	30
A	GU	CUCCGUC	AC	8-17	+/−	ND[b]	ND
A	GUU	GUUUC	AAC	8-18 (6)	++	0.68	8
A	GUU	GUCUC	AAC	8-20	+	3.23	0
A	GGU	GACAC	ACU	8-15	+	1.13	20
A	GGU	GCUAC	AUC	8-29	+	0.79	4
A	GUU	GCUUC	GAC	8-32	+	1.29	1
A	GGU	GCUUC	AUC	8-5	++	0.21	30
A	GAU	GAUUC	AUU	8-2	+	1.73	1
A	GU	GCUUCC	AC	6-9	+	0.65	1
A	GGU	GAUUC	AUC	6-21	+	2.48	8
A	GAU	GCUUC	GUC	6-37	+	1.3	10
A	GGU	GAUUC	AUC	6-38	+	0.9	40
A	GUC	GAUUU	GAC	7-11	+	1.28	0
A	GGU	GCUUC	AUC	7-12	++	0.32	8
A	GGU	GUUGC	GCC	7-14	+	0.66	10
A	GAU	GAUCC	AUC	L1	+	1.6	ND
A	CGC	GAUUC	GCG	SL1	+	1.4	8
A	UGC	GAUUC	GCG	SL2	+	0.8	20
A	RRY	GHUUC	RYY[c]				

[a] Relative packaging efficiency; see Materials and Methods.
[b] ND, not done.
[c] R, purine; Y, pyrimidine; H, A, C, or U.

is just as accurate. For instance, binding curves for the AS and 8-18 transcripts are shown in Fig. 4. From this data, the calculated K_d for AS is 0.86 nM and that for 8-18 is 0.74 nM. A previous determination of the K_d for the AS transcript gave 0.81 nM (23). The K_d ratio of 8-18 to AS was determined by competition to be 0.84. This corresponds to 0.86, calculated by dividing the two independently determined K_d's. The K_d's in Table 1 are given with respect to a K_d for the AS transcript of 0.81 nM. All the sequences that fit the consensus (see below) had K_d's in the nanomolar range, and several had K_d's several times lower than that of any wild-type VBS.

Consensus binding site of selected sequences. The consensus structure is summarized in Fig. 5. The minimum number of base pairs in the bottom portion of the stem (4 bp) has been assigned by analysis of RNAs such as isolate 6-9 (see below). Of the three wild-type VBSs, one (SL1) has 4 bp prior to the bulged A. Tight binding (a K_d in the nanomolar range) re-

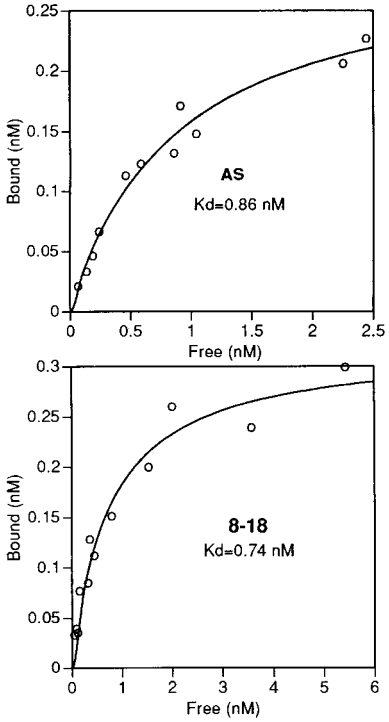

FIG. 4. Direct K_d determination for two RNAs: the AS transcript and the 8-18 transcript. The amounts of bound and unbound RNA as a function of concentration of RNA were measured by phosphorimager scans of 1.5% agarose gels prepared as described elsewhere (23). Standard binding curves were fitted to the data by DELTAGRAPH.

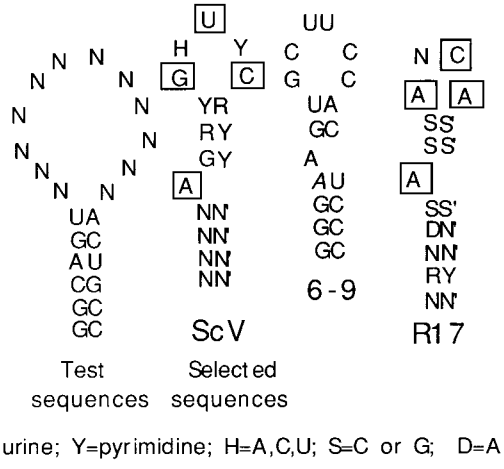

FIG. 5. Consensus ScV VBS compared to the R17 coat protein binding site. Complete conservation of a given nucleotide is indicated by a square box. The proposed structure of isolate 6-9 is also shown. This isolate has a mutation in the "fixed" portion of the test sequence (a C-to-A change, in italics), apparently creating a new bulged A, a loop with an extra U, and a shorter stem.

quires a stem with a bulged A in the middle and a loop with the sequence GHUYC, where H is A, C, or U and Y is a pyrimidine. There are preferences for two purines and a pyrimidine in the 5′ branch of the stem following the bulged A. The conserved G and C in the loop might be drawn as an eighth base pair in the stem (reducing the loop to three bases), a conjecture made more likely by the existence of isolates with the G and C reversed (e.g., 8-9), but this G appears to be unambiguously in a single-stranded region by nuclease sensitivity (23). The conserved U in the loop contributes a large proportion of the stability of the complex, since alteration of this single base reduces affinity for the sequence by more than fivefold (compare 8-18 to 8-20). Packaging with isolate 8-20 is undetectable, while packaging with isolate 8-18 is essentially identical to that of wild-type SL1 (see below and Table 1).

Alterations outside the 12 selected bases. There were several examples in which a consensus sequence was created by mutations outside the region intended to be variable. We presume that these unintended (but informative) mutations occurred during PCR. For instance, isolate 6-9, with a very respectable K_d of 0.65 nM, has an A in the first branch of the stem of the wild-type sequence rather than a C, so that bases within the variable region now form the second branch of this portion of the stem. This A now becomes the fourth base of the stem. The sequence GGGCAGUNNNNNNNNNNNNACUGCCGAGG AAUAC has been altered to GGG*A*AGUGCUUCCACUCC CACUGCCGAGGAAUAC, where the change in the "invariable" portion of the oligonucleotide sequence is in boldface italics and the 12-base variable region is underlined. This creates a loop with an extra base and shortens the second portion of the stem. The proposed secondary structure for this isolate is shown in Fig. 5.

The two isolates with the lowest K_d's are 8-5 and 7-12, which are identical in the second portion of the stem, the bulged A, and the loop sequence (Table 1) and have very similar K_d's but differ in the first portion of the stem. The first base pair of the stem is GU in 8-5 and GC in 7-12, due to a mutation in 8-5 in the invariable portion of the sequence.

Packaging and replication. Most of the selected VBS sequences were tested for incorporation into viral particles and for replication within viral particles (synthesis of minus strand). Cells lacking any ScVLa viral particles (but with ScVL1) were transformed with a plasmid expressing a VBS sequence as a 5′ portion of a transcript with several hundred vector-derived bases. The endogenous wild-type ScVL1 particles provided both Cap and Cap-Pol in *trans* for particle assembly. Viral particles were isolated and purified by CsCl equilibrium gradient centrifugation, fractions had RNA extracted, and Northern blots were performed. An example of a Northern blot using the vector minus strand as probe is shown as Fig. 6A. Here the 8-18 and 6-38 transcripts are efficiently packaged into viral particles that peak at fraction 6 of the gradient. The negative control, in which pm (expression vector without insert) is present, makes no particles containing vector sequences, although the pm transcript is readily detected in the total RNA, in the same quantities present in the other strains. The 8-18 plus strand is readily packaged, but the 8-20 RNA is not. This corresponds to the difference in their K_d's (a fivefold difference) and is the result of a single base difference (the conserved U in the loop).

The efficiency of packaging of the selected RNAs was quantified by phosphorimager scans of the peak fraction (fraction 6) in Fig. 6A and normalized by dividing by the amount of the selected RNA present in the total RNA lane and by the amount of Cap protein present in fraction 6 (as determined by optical scanning of Coomassie brilliant blue-stained SDS-PAGE gels). The efficiency of packaging (viral plus strand normalized to RNA and Cap protein) is summarized in the last column of Table 1. Since all the isolates had a maximum in packaged RNA in fraction 6 of the CsCl gradient, only the amount of Cap protein in this fraction was quantified, to simplify the measurements. The relative packaging efficiency so measured is only crudely reproducible (by a factor of two). However, there is no simple relationship between efficiency of packaging and K_d, although several isolates, especially 6-38, are packaged more efficiently than the wild type. Isolate 6-38 reproducibly gives the best packaging efficiency.

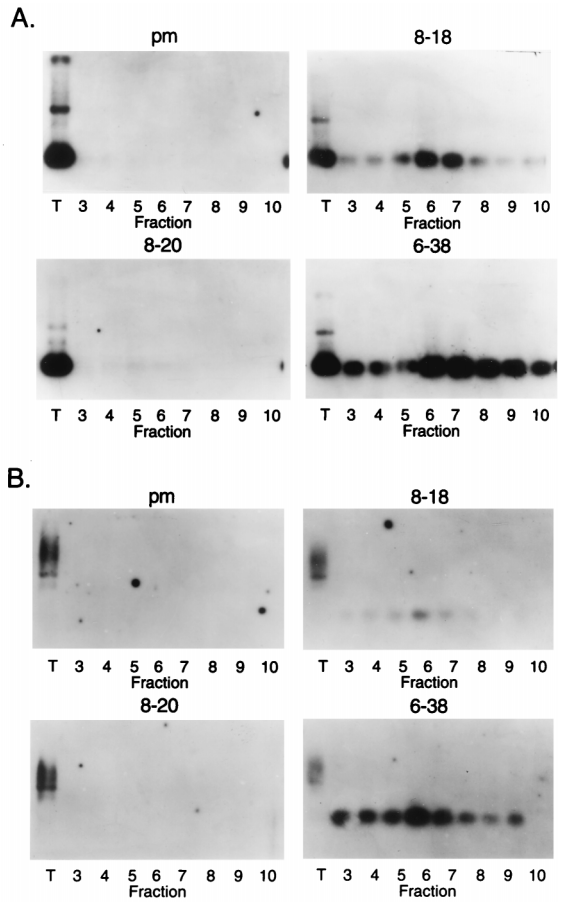

FIG. 6. Packaging of VBS sequences: detection of viral plus strand (A) and minus strand (B). The viral plus strand, with several hundred bases from the vector included, is produced from an expression vector with various VBS inserts or without insert (pm), and the viral proteins are produced by endogenous ScVL1 particles. CsCl fractions of purified viral particles (fractions 3 to 10) had RNA extracted, were run on a nondenaturing 1.5% agarose gel, were denatured, were transferred to nitrocellulose, and were probed with labeled RNA transcripts of the vector sequence included in the VBS transcripts, as described elsewhere (23). Each set of fractions had a positive control, which is total RNA from the same cells from which viral particles were purified. The probe in panel A is the minus strand of the vector sequence, and the probe in panel B is the plus strand. The autoradiograph of panel B was exposed about five times as long as that of panel A and had four times as much RNA from each of the CsCl fractions.

Similar experiments in which fractions from CsCl gradients were probed in Northern blots with the plus-strand probe (Fig. 6B) demonstrate that all the packaged RNAs are replicated, as previously noted (23), although very poorly, since they lack the 3′ end normally required for replication (10). The limited hybridization to RNA in the total cellular RNA is nonspecific. The single band of minus strand present in particles is not detectable in the total RNA and is clearly present only in

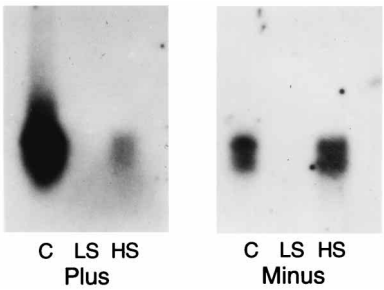

FIG. 7. Replication of VBS sequences. The peak particle fraction (fraction 6) from the 6-38 particle preparations had RNA extracted and was treated with RNase A (20 μg/ml) with high salt (HS) (0.6 M NaCl) or low salt (LS) (no added salt) or was untreated (C), and Northern blots were performed as for Fig. 6. The plus strand was detected on the left, and the minus strand was detected on the right, as indicated.

particles in which the plus strand has been packaged (compare Fig. 6A and B).

All the viral minus strand is in dsRNA, as shown in Fig. 7. Clearly, there is much less minus strand than plus strand present in viral particles. Phosphorimager scans of Fig. 7 show about 80 times as much plus strand in single-stranded RNA as in dsRNA, implying that only about 1.2% of the plus strand is replicated. As expected, the amount of minus strand present is the same with and without RNase treatment at high salt, implying that all the minus strand is present as dsRNA. Note that, in these gels, the dsRNA and single-stranded RNA are not well separated.

DISCUSSION

In vitro selection has made possible the derivation of a consensus VBS for the *S. cerevisiae* dsRNA virus ScVL1. The ScV VBS requires conserved bases in the loop as well as a stem with a bulged A residue. There are three conserved bases in the ScV VBS loop: a G, a C, and a U. The other two positions are preferably a pyrimidine and a residue other than G. Because the G and C residues are at the top of the stem, it is possible that they form another base pair in the stem, rather than being single stranded in the loop, so that the loop would consist of three instead of five nucleotides. There are VBS isolates that bind in which the G and C are reversed (e.g., 8-9), so that a CG pair rather than a GC pair would exist at this position. However, these bind rather poorly to viral particles. The nuclease sensitivity mapping of secondary structure of the VBS of the unbound RNA (23) shows the G residue in question as in a single-stranded region. It is possible that the tertiary structure of the VBS is altered during binding so that a GC pair and a three-base loop result.

The ScV VBS is quite similar to the coat protein binding site in R17 (Fig. 5), also determined by in vitro selection (22), as well as by site-directed in vitro mutagenesis (19). However, the affinity of the wild-type R17 coat protein binding site for the coat protein is 1 to 2 orders of magnitude less than that of the wild-type VBS for the Cap-Pol fusion protein (19). The conserved bases in the R17 coat protein binding site are two A residues in the loop and the bulged A residue, as well as a pyrimidine in the loop (19, 22).

The three-dimensional structure of the MS2 coat protein-R17 operator site complex (28) has been determined. All of the protein-RNA contacts are to nucleotides outside the stem regions in this system. In MS2, two coat protein monomers form a dimer with a 10-stranded antiparallel β-sheet, in which residues from both monomers form contacts with the single RNA operator. This situation may be different with ScV, in which there is some evidence that two Cap-Pol monomers form two independent binding domains (23). The evidence is that binding to two nearby sites in one viral RNA (such as AS RNA) is independent but that both sites are recognized, while most viral particles appear to have only two Cap-Pol monomers. This cannot be due to binding of two separate particles, since the mobility shift remains the same as with an RNA with a single site (e.g., SL1).

Since most of the sequences selected in vitro as binding to viral particles are packaged and replicated in vivo, binding and packaging appear to be synonymous. However, the fact that there appears to be no direct correlation between the equilibrium dissociation constant (K_d) and packaging efficiency may mean that the rate of association, rather than the equilibrium constant for binding, is important in packaging. Other cellular or viral factors may also affect the process of packaging in vivo.

ACKNOWLEDGMENTS

We thank Angelo Pulgiano and Scott Zimmer for technical assistance, Jing Zhang for phosphorimager scans, and R. B. Wickner for strains.

We also thank the National Science Foundation (grant MCB9505829) for support.

REFERENCES

1. **Antson, A. A., J. Otridge, A. M. Brzozowski, E. J. Dodson, G. G. Dodson, K. S. Wilson, T. M. Smith, M. Yang, T. Kurecki, and P. Gollnick.** 1995. The structure of the TRP RNA-binding attenuation protein. Nature **374:**693–700.
2. **Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. Cell **67:**529–536.
3. **Baumann, C., J. Otridge, and P. Gollnick.** 1996. Kinetic and thermodynamic analysis of the interaction between TRAP (*trp* RNA binding attenuation protein) of *Bacillus subtilis* and *trp* leader RNA. J. Biol. Chem. **271:**12269–12274.
4. **Bruenn, J. A.** 1988. Replication of dsRNA mycoviruses, p. 195–209. *In* R. D. J. Holland and P. Ahlquist (ed.), RNA genetics. CRC Press, Inc., Boca Raton, Fla.
5. **Cheng, R. H., J. R. Caston, G.-J. Wang, R. Gu, T. J. Smith, T. S. Baker, R. F. Bozarth, B. L. Trus, N. Cheng, R. B. Wickner, and A. C. Steven.** 1994. Fungal virus capsids: cytoplasmic compartments for the replication of double-stranded RNA formed as icosahedral shells of asymmetric Gag dimers. J. Mol. Biol. **244:**255–258.
6. **Diamond, M. E., J. J. Dowhanick, M. E. Nemeroff, D. F. Pietras, C.-L. Tu, and J. A. Bruenn.** 1989. Overlapping genes in a yeast double-stranded RNA virus. J. Virol. **63:**3983–3990.
7. **Dinman, J. D., T. Icho, and R. B. Wickner.** 1991. A −1 ribosomal frameshift in a double-stranded RNA virus of yeast forms a gag-pol fusion protein. Proc. Natl. Acad. Sci. USA **88:**174–178.
8. **El-Sherbeini, M., D. J. Tipper, D. J. Mitchell, and K. A. Bostian.** 1984. Virus-like particle capsid proteins encoded by different L double-stranded RNAs of *Saccharomyces cerevisiae*: their roles in maintenance of M double-stranded killer plasmids. Mol. Cell. Biol. **4:**2818–2827.
9. **Esteban, R., T. Fujimura, and R. B. Wickner.** 1988. Site-specific binding of viral plus single-stranded RNA to replicase-containing open virus-like particles. Proc. Natl. Acad. Sci. USA **85:**4411–4415.
10. **Esteban, R., T. Fujimura, and R. B. Wickner.** 1989. Internal and terminal cis-acting sites are necessary for in vitro replication of the L-A double-stranded RNA virus of yeast. EMBO J. **8:**947–954.
11. **Fujimura, T., R. Esteban, L. M. Esteban, and R. B. Wickner.** 1990. Portable encapsidation signal of the L-A double-stranded RNA virus of S. cerevisiae. Cell **62:**819–828.
12. **Fujimura, T., J. C. Ribas, A. M. Makhov, and R. B. Wickner.** 1992. Pol of *gag-pol* fusion protein required for encapsidation of viral RNA of yeast L-A virus. Nature **359:**746–749.
13. **Fujimura, T., and R. B. Wickner.** 1988. Gene overlap results in a viral protein having an RNA binding domain and a major coat protein domain. Cell **55:**663–671.
14. **Huan, B.-F., Y. Shen, and J. A. Bruenn.** 1991. *In vivo* mapping of a sequence required for interference with the yeast killer virus. Proc. Natl. Acad. Sci. USA **88:**1271–1275.
15. **Icho, T., and R. B. Wickner.** 1989. The double-stranded RNA genome of yeast virus L-A encodes its own putative RNA polymerase by fusing two

open reading frames. J. Biol. Chem. **264:**6716–6723.
16. **Innis, M. A., M. J. Holland, P. C. McCabe, G. E. Cole, V. P. Wittman, R. Tal, K. W. K. Watt, D. H. Gelfand, J. P. Holland, and J. H. Meade.** 1985. Expression, glycosylation, and secretion of an Aspergillus glucoamylase by Saccharomyces cerevisiae. Science **228:**21–26.
17. **Park, C.-M., J. Lopinski, J. Masuda, T.-H. Tzeng, and J. A. Bruenn.** 1996. A second double-stranded RNA virus from yeast. Virology **216:**451–454.
18. **Reilly, J. D., J. Bruenn, and W. Held.** 1984. The capsid polypeptides of the yeast viruses. Biochem. Biophys. Res. Commun. **121:**619–625.
19. **Romaniuk, P. J., P. Lowary, H. N. Wu, G. Stormo, and O. C. Uhlenbeck.** 1987. RNA binding site of R17 coat protein. Biochemistry **26:**1563–1568.
20. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, A. Higuchi, G. T. Horn, F. B. Mullis, and H. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. Sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
22. **Schneider, D., C. Tuerk, and L. Gold.** 1992. Selection of high affinity RNA ligands to the bacteriophage R17 coat protein. J. Mol. Biol. **228:**862–869.
23. **Shen, Y., and J. A. Bruenn.** 1993. RNA structural requirements for RNA binding in a double-stranded RNA virus. Virology **195:**481–491.
24. **Tao, J., I. Ginsberg, N. Banerjee, Y. Koltin, W. Held, and J. A. Bruenn.** 1990. *Ustilago maydis* KP6 killer toxin: structure, expression in *Saccharomyces cerevisiae*, and relationship to other cellular toxins. Mol. Cell. Biol. **10:**1373–1381.
25. **Thiele, D. J., E. M. Hannig, and M. J. Leibowitz.** 1984. Multiple L double-stranded RNA species of *Saccharomyces cerevisiae*: evidence for separate encapsidation. Mol. Cell. Biol. **4:**92–100.
26. **Tuerk, C., and L. Gold.** 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science **249:**505–510.
27. **Tzeng, T.-H., C.-L. Tu, and J. A. Bruenn.** 1992. Ribosomal frameshifting requires a pseudoknot in the yeast double-stranded RNA virus. J. Virol. **66:**999–1006.
28. **Valegard, K., J. B. Murray, P. G. Stockley, N. J. Stonehouse, and L. Liljas.** 1994. Crystal structure of an RNA bacteriophage coat protein-operator complex. Nature **371:**623–626.
29. **Weeks, K. M., and D. M. Crothers.** 1991. RNA recognition by Tat-derived peptides: interaction in the major groove? Cell **66:**577–588.
30. **Wickner, R. B.** 1996. Double-stranded RNA viruses of *Saccharomyces cerevisiae*. Microbiol. Rev. **60:**250–265.
31. **Yao, W., and J. A. Bruenn.** 1995. Interference with replication of two double-stranded RNA viruses by production of N-terminal fragments of capsid polypeptides. Virology **214:**215–221.
32. **Yao, W., K. Muqtadir, and J. A. Bruenn.** 1995. Packaging in a yeast double-stranded RNA virus. J. Virol. **69:**1917–1919.