Characterization of the TMV encapsidation initiation site on 18S rRNA

Jaya P.Gaddipati, C.D.Atreyia, D'Ann Rochon* and Albert Siegel⁺

Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

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ABSTRACT

Tobacco Mosaic Virus capsid protein oligomers react with and encapsidate 18S rRNA from both plant and mammalian sources in vitro. The site (ei) in 18S rRNA which reacts with capsid protein to initiate the packaging reaction has been localized and partially characterized by testing the ability of transcripts from different regions of a cloned Cucurbita pepo rDNA repeat unit to become encapsidated. The 18S rRNA ei is found to react more slowly with capsid protein than does the functional virion ei and to lie within a 43 nucleotide region which starts at position 157 from the 5' terminus of 18S rRNA. When 6 nucleotides are removed from the 5' end, the remaining 37 nucleotide segment is still reactive, but with reduced efficiency. The primary structure of the reactive segment has limited similarity to the virion ei and can be folded into a stem-loop. The first 18 nucleotides of the ei region is highly conserved from an evolutionary standpoint and this may account for the ability of 18S rRNAs from both plant and mammalian sources to be encapsidated.

INTRODUCTION

Assembly of the tobacco mosaic virus (TMV) particle is initiated by a specific reaction of a capsid protein oligomer with an internal region of the virion RNA known as the encapsidation initiation site (ei) [1, 2] and this is followed by subsequent rod elongation toward both the RNA 5' and 3' termini. Although this mechanism appears to be one that ensures that only virion RNA would become encapsidated, the reaction specificity proves not to be absolute; capsid protein oligomer is found to react with and encapsidate selected non-virion RNA species in addition to virion RNA. Some of the reactive non-virion RNA species are encapsidated in vivo, during the infection process to form the pseudovirions observed in preparations of all strains of TMV thus far examined [3]. These are, for the most part, chloroplast DNA transcripts [4] which can also be packaged by an in vitro assembly reaction. Other RNA species, however, are not found to be packaged in vivo but can be encapsidated in vitro. Predominant in this class is 18S ribosomal RNA (rRNA) where, because of sequence conservation, both plant and animal species are reactive.

The present study was undertaken to locate and characterize a presumed site on 18S rRNA that reacts with capsid protein oligomer to initiate encapsidation in an attempt to determine the critical features that imbue it with this property.

MATERIALS AND METHODS

Plasmid Construction

The plasmid pKH was constructed by inserting the 3.6 kb KpnI-HindIII restriction fragment from a *Cucurbita pepo* rDNA repeat unit (Fig. 1) [5] into KpnI and HindIII cleaved transcription vector pBS(+) (Stratagene). This insert contains the coding sequences for 18S RNA and a portion of the preceding intergenic region and is oriented so that sense transcript can be obtained with the aid of T7 RNA polymerase when pKH is cleaved at any one of several unique restriction sites (Fig. 1A). The plasmids pXH and pSH (Fig. 1B, C) were constructed by subcloning XbaI-HindIII and SalI-HindIII restriction fragments, respectively, into appropriately cleaved pBS(+). The plasmid pXS was constructed in a similar manner by inserting the XbaI-SalI fragment into pBS(+) but in this case, an additional fragment (a 1.6 kb SacI-SacI fragment from the 25S RNA coding region of the *C. pepo* rDNA repeat unit) was inserted between the T7 promoter and the insert of interest in order to generate a larger RNA molecule upon transcription of appropriately cleaved plasmid (Fig. 2A). The plasmid pX'S was obtained by cleaving pXS with XbaI, treating with mung bean nuclease and recircularizing with the aid of T4 DNA ligase. It proved to have all six nucleotides of the XbaI site missing as determined by dideoxynucleotide sequencing (Fig. 2B). The plasmid pTMV was constructed by subcloning the EcoRI-SalI fragment from pSP64TMV [6] (a gift from Dr. T.M.A. Wilson, Department of Virus Research, The John Innes Institute, Norwich, UK) which contains the origin of assembly of the TMV common strain into EcoRI-SalI double digested pBS(+). The insert is oriented so that sense transcript can be obtained from EcoRI cleaved plasmid with the aid of T3 RNA polymerase.

Transcript Preparation

The *in vitro* transcription reaction was at 37° for 1 hr in a 50 µl volume containing 0.4 mM of each of the 4 nucleoside triphosphates, 75 mM dithiothreitol, 10 µCi α[³²P]ATP, 0.2 µg of appropriately restricted plasmid template, 20 U T7 RNA polymerase and 10 µl 5x transcription buffer (200 mM Tris pH 8.0, 40 mM MgCl₂, 10 mM Spermidine, 250 mM NaCl). Linearized plasmids with 3' overhangs were blunted by treatment with *E. coli* DNA polymerase I, large (Klenow) fragment prior to use as transcriptional template. Labelled

transcripts were purified by phenol/chloroform extraction and ethanol precipitation.

Preparation of Capsid Protein

Virus was purified from infected Nicotiana tabacum L. cv. Samsun plants as described [7]. Capsid protein was prepared by the acetic acid method [8]. Protein oligomers were prepared by incubating capsid protein for 18 hr at 24° in 0.1 M sodium phosphate buffer, pH 7.0.

In Vitro Reconstitution Assays

Freshly diluted coat protein oligomer was mixed with RNA transcript in 0.1 M sodium phosphate buffer, pH 7 to give a final concentration of 1 mg/ml protein and 33 µg/ml RNA. Incubation was at 24° for 8-12 hr when time is unspecified or for shorter specific periods, as indicated in the experimental section. To test for protection from RNase, samples were diluted with 10 mM Tris buffer, pH 8.0, at the end of the incubation period, pancreatic ribonuclease A was added to a final concentration of 0.1 µg/ml and incubation was allowed to proceed for 20 min at room temperature following which proteinase K was added to a final concentration of 0.5 mg/ml and incubation was for an additional 30 min. Five µg of carrier tRNA were added and the preparation extracted with phenol/chloroform/SDS. The RNA, collected by ethanol precipitation and resuspension in H₂O, was analyzed by radioautography after electrophoresis through a methyl mercuric hydroxide containing 1% agarose gel [9].

Measurement of reaction kinetics

The kinetics of RNA protein interactions were followed by a nitrocellulose filter binding assay [10]. Samples removed from a reaction mixture at specified times were diluted two fold with 0.1 M phosphate buffer, pH 7 and filtered through a phosphate buffer washed nitrocellulose filter (Schleicher and Schuell BA 85). The filters were washed further with 1 ml phosphate buffer and retained radioactivity determined with the aid of a scintillation spectrometer.

RESULTS

Localization of the 18S rRNA ei site

Transcripts, generated from different regions of a cloned C. pepo 18S rRNA coding region, were tested for reactivity with TMV capsid protein as a means of localizing the 18S rRNA ei site. The test consisted of a determination of whether reaction with capsid protein oligomers would confer ribonuclease resistance.

Runoff transcripts were generated with T7 polymerase from pKH cleaved at

Figure 1. Transcripts from portions of a cloned *Cucurbita pepo* rDNA repeat unit tested for reactivity with TMV capsid protein. The top long line is a limited restriction map of the rDNA repeat unit with the 18S and 25S coding regions overlined. The letters above the map indicate restriction sites (B-BglI, H-HindIII, Ha-HaeII, K-KpnI, S-SalI, Sa-SacI, X-XbaI, Xm-XmnI). The numbers below the line are kilobases. The lines labeled pKH, pXH, and pSH indicate sections of the repeat unit inserted between the T7 and T3 promoters of pBS(+). A 161 nucleotide region of interest between the broken lines has been expanded sixteen fold in relation to the rest of the insert. Lines terminating with an arrow indicate tested runoff transcripts generated with T7 RNA polymerase from appropriately cleaved pKH, pXH or pSH. ● indicates that the transcript became protected from ribonuclease upon reaction with capsid protein; ○ indicates that it did not.

its HindIII and other upstream sites in order to obtain a nested set of different length transcripts with a common 5' terminus. The results shown in Figure 1 were obtained upon testing these for reactivity with capsid protein.

Figure 2. Analysis of the XbaI-SalI region. A. Plasmid pXS was constructed by inserting the XbaI-SalI portion of the 18S coding region into pBS(+). The SacI-SacI region between kb 1.7 and 3.3 (see fig. 1) was also inserted, as indicated, for experimental convenience. The region to the right of the broken line is expanded ca. sixteen fold with respect to that on the left. N=NsiI; other abbreviations and symbols are as in figure 1. The KpnI site is in a DNA segment that is part of the plasmid multi-cloning site. B. Plasmid pX'S is the same as pXS except that the 6 nucleotides constituting the XbaI site have been deleted.

All transcripts from plasmid cleaved at SalI or downstream sites become protected from ribonuclease, whereas those from plasmid cleaved at XbaI or other upstream sites do not. Likewise, transcripts from pXH, linearized with HindIII, become protected but transcripts from pSH, also linearized with HindIII do not, even after extensive incubation with capsid protein. Thus, it would appear that the 18S rRNA ei site lies between the XbaI and SalI sites. Those transcripts which contain this region are reactive with capsid protein, those which lack it, are not. This is so even when XbaI-SalI fragment appears out of context (Fig. 2), as it does in pXS which has an exogenous segment inserted between the T7 promoter and the XbaI site. In conformity with the previous results, linearization of pXS at SalI yields a transcript that becomes protected, whereas linearization at XbaI does not. The 18S rRNA ei was further localized by observing that the transcript from NsiI linearized pXS reacted with capsid protein to become ribonuclease resistant and this also proved true for transcripts of SalI and NsiI linearized pX'S which lack the 6 XbaI site nucleotides (Fig. 3).

Relative reactivity of the 18S rRNA ei site

The foregoing experiments clearly confirm the suggestion that 18S rRNA

Figure 3. Example of ribonuclease protection assay. Radioactive transcripts were reacted with TMV capsid protein oligomers for 8 hr at 24°C and then subjected to ribonuclease treatment. The RNA was reextracted and analyzed by radioautography after electrophoresis through a 1% denaturing agarose gel. a and a' - Transcript from NsiI linearized pX'S before and after reaction; b and b' - Transcript from KpnI linearized pX'S before and after reaction.

contains a region which reacts with TMV capsid protein to initiate encapsidation [3] and, in addition, serve to localize the site. They do not, however, indicate the relative affinity of the site in comparison with the functional viral ei site. Thus, the reaction time course of capsid protein oligomers with transcripts containing the 18S rRNA ei site was determined by a nitrocellulose filter binding assay and compared with that of the virion site. The results shown in figure 4, illustrate that a transcript generated from pTMV, which contains the viral origin of assembly, is retained completely on a membrane after 30 minutes of reaction with capsid protein, the shortest time period tested in these experiments. In contrast, only 15% of a transcript from PvuI cleaved pBS(+), not known to have an ei site, is retained after 4 hours, presumably as a consequence of non-specific reaction. Transcripts from plasmids which contain at least the XbaI to NsiI segment

Figure 4. Comparative reaction rate analysis between transcripts and capsid protein oligomers. Transcripts were reacted with capsid protein oligomers for different times and then subjected to nitrocellulose filter binding assay. The letters indicate transcripts from the following plasmids : a - d, pKH cleaved at XmnI, XbaI, SalI and HindIII, respectively; e, pXH linearized at HindIII; f, pSH linearized at HindIII; g - i, pXS cleaved at XbaI, NsiI and SalI, respectively; j, pX'S linearized at NsiI; k, pBS(+) linearized at PvuI; and l, pTMV linearized at EcoRI.

behave in a similar manner; 35-38% is retained after a 4 hour reaction period. Thus, although the 18S rRNA ei reacts specifically with virus protein to engender encapsidation, it has a considerably lower affinity for capsid protein than does the virion site. Shortening of the 5' end of the tran-

Cucurbita	UCUAGAGCUAAUACGUGCAxACAACCCCGACxxxxxUUCxxxUGGAAGGGA	[Ref. 11]
Soybean	-----x-----xxxxx---xxx-----	[Ref. 12]
Maize	-----x-----xxxxx---xxxC--G----G	[Ref. 12]
Rat	-----A---CG--GGG-G-U---CCCC--CCG--GG--A-	[Ref. 13]
Mouse	-----A---CG--GGG-G-U---CCCC--CCGx--GG----	[Ref. 14]
Rabbit	-----A---CG--GGx-G-U---UCCCx--UGxx--Uxx----	[Ref. 15]
Human	-----A---CG--GGG-G-U---CCCCx---GCxx--GG----	[Ref. 16]
Xenopus	-----A---CG--G-G-G-U---CCCCxxxxxxxxxxxxx----	[Ref. 17]
Shrimp	-----A---C-AU-G---A-xxxxx---ACxx-----x	[Ref. 18]
Yeast	-----A---UU-A-U-U-x---CCxxx--xxxx-----xx--	[Ref. 19]

Figure 5. Nucleotide sequence of the XbaI-NsiI segment of *Cucurbita pepo* 18S rRNA and comparison with a similar region in the 18S rRNAs of other species.

script by removing the 6 nucleotide XbaI site, reduces the affinity still further but activity is not completely lost. The transcript from pX'S linearized at NsiI is retained to 27% after 4 hours, consistently greater than the 15% for control reaction but lower than the 38% for the other reactive 18S rRNA based transcripts.

Primary and possible secondary structure of the 18S rRNA ei

The nucleotide sequence of the *C. pepo* XbaI-NsiI rRNA segment is shown in figure 5. It consists of 43 nucleotides which can possibly assume the least energy secondary structure configuration shown (Fig. 6A) derived from the Zucker and Stiegler computer program [20]. This is compared with that of the

Figure 6. Possible secondary structure of A) the 43 nucleotide XbaI-NsiI segment (-6.5 kcal) and B) the same segment lacking the 6 nucleotides XbaI site (-6.0 kcal).

U1	UgUuGAugxAgUuCaUGgAAGauguCCCuAugUCgaucAGGcu	[Ref. 21]
<u>C. pepo</u> ei	UCUAGAGCUAAUACGUGCAACAAACCCGACUUCUGGAAGGGA	[Ref. 11]
Cc	UCUAcAGacAxcACGUaaUAgAguCauCGAggUugaGAAcucu	[Ref. 22]

Figure 7. Comparison of the 18S rRNA ei with regions in virus RNA assembly sites; (nucleotides 915-874 and 440-399 from the 3' termini of U1 and Cc, respectively).

lower affinity 37 nucleotide segment which lacks the XbaI site (Fig. 6B). Both can possibly assume a well defined stem-loop structure.

DISCUSSION

Previous experiments have shown that the 5' three quarters of native and all of in vitro transcribed 18S rRNA becomes encapsidated when incubated with TMV protein under reconstitution conditions, suggesting the presence of a region(s) on 18S rRNA capable of serving as an encapsidation initiation site [4]. The data presented here indicate that this function lies within a 43 nucleotide region located between the XbaI and NsiI sites of C. pepo 18S rDNA which, by comparison with other known plant rRNA sequences puts it at nucleotides 157-199 from the 5' terminus. The failure of capsid protein to package runoff transcripts which lack this segment rules out the presence of other possible downstream sites.

A mammalian 18S rRNA (bovine) has also been shown to be encapsidated by TMV protein [3] and it would seem likely that this is a consequence of the presence of an evolutionarily conserved reactive segment comparable to the one found in the plant 18S rRNAs. A comparison of the Cucurbita 43 nucleotide segment with that in several other species where the sequence of a comparable segment is known is shown in figure 5. The Cucurbita segment is identical to that of soybean and differs from that of maize in only 3 positions, all of which are transitions. A mammalian sequence, rat, differs from that of Cucurbita by ten nucleotides, only one of which, however, is a transversion and also contains 9 additional interspersed nucleotides in this region. This leads to the speculation that either a shorter region within the segment imbues it with the ei property or that particular positions require either a purine or a pyrimidine for activity but that it makes little difference which purine or pyrimidine it is. It is of interest to note in this regard that the region of greatest homology comprises the first 18 nucleotides where there is identity between the three plant sequences and from which vertebrates, invertebrates and yeast are different in only one

position, a transition. An indication that this region has a functional property is provided by the observation that removal of the 6 nucleotides at its 5' terminus markedly reduces ei activity.

The 18S rRNA ei site is clearly not as efficient in initiating encapsidation as is the site responsible for inducing viral RNA packaging and, thus, one might not expect close homology between the two. Indeed, comparison reveals only limited regions of sequence homology such as that shown in figure 7, the significance of which remains to be determined. It may be that rather than a precise primary sequence, a structural feature is of functional significance. The minimum energy configuration of the XbaI-NsiI segment, as determined by the Zucker and Stiegler algorithm [20], indicates a stem-loop structure and, possibly, this is responsible for the ei activity. However, this loop lacks the AAGAAGUCG sequence postulated to be responsible for ei activity in, at least, the common strain of TMV [10]. At the present time it is not at all clear what attributes of the 43 nucleotide sequence are responsible for its ei activity. It does appear, however, that initiation, and not elongation, is limiting because complete transcripts are seen to be encapsidated at all incubation times tested, rather than a distribution of shorter RNAs [3].

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* Present Address : Agriculture Canada, 6660 NW Marine Drive, Vancouver, BC, Canada V6T 1X2.

+ To whom correspondence should be addressed.

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