A Dynamic in Vivo View of the HIV-I Rev-RRE Interaction

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The export of pre-mRNAs coding for the structural genes of the human immunodeficiency virus type I depends on the interaction of the Rev protein with a highly structured viral RNA sequence, the Rev-responsive element (RRE). To gain information about the structure of the RRE and the determinants of the in vivo RRE-Rev interaction, we have analyzed the structure of the 351 nt RRE RNA within living yeast (Saccharomyces cerevisiae) by dimethyl sulfate probing with or without Rev. The in vivo structure in the absence of Rev is generally similar to the previously established solution structure. In addition, we observe a single hypermethylated guanine residue (G128), located within the Rev high-affinity binding site, in vitro as well as in vivo. The important homopurine interaction between residues 129 and 106 is required for the hyperreactivity, confirming its biological relevance. Expression of wild-type Rev leads to a protection of this region and to modifications of the RRE structure: the high-affinity site becomes further structured, and Stem IIA is destabilized. High-level expression of the oligomerization-defective mutant M4 protein leads to the same protections without destabilization of Stem IIA. Taken together with other observations, the data suggest that Rev captures the unusual conformation of the high-affinity site, followed by additional changes in the structure of the RRE.

Abbreviations used: HIV-I, human immunodeficiency virus type I; DMS, dimethyl sulfate; RRE, Rev-responsive elements; RBE, Rev binding element.

Introduction

Lentiviruses, e.g. human immunodeficiency virus type 1 (HIV-I), encode transcriptional (Tat) and post-transcriptional (Rev) regulatory proteins in addition to the genes encoding structural proteins (Env, Gag and Pol) and enzymes required for virus replication (for a review, see Cullen, 1992). Tat and Rev identify their viral RNA targets by binding to cis-acting regulatory elements. Tat interacts with the trans-activation-responsive region (TAR), a stem–loop structure found at the 5′ end of all HIV-I transcripts (for a review, see Gait & Karn, 1993). Rev, a 116 amino acid residue nuclear phosphoprotein, promotes the cytoplasmic accumulation and expression of singly spliced (~4 kb) and unspliced (~9 kb) viral mRNAs coding for the structural proteins (Cullen, 1992). All these Rev-dependent RNAs contain a highly structured sequence within the intron of the env gene, called the Rev-responsive element (RRE: Rosen et al., 1988; Dayton et al., 1989; Hadzopoulou-Cladaras et al., 1989; Malim et al., 1989a,b). Direct binding of Rev to the RRE in vivo is critical for Rev function (Emerman et al., 1989; Hammarskjöld et al., 1989; Heaphy et al., 1990; Malim et al., 1990; Mann et al., 1994), and purified Rev binds to the RRE RNA in vitro with some specificity (Daly et al., 1989; Zapp & Green, 1989; Cochrane et al., 1990; Heaphy et al., 1990). A current view of the Rev-RRE interaction comes from mutagenesis analyses coupled with non-denaturing gel electrophoresis of in vitro-formed complexes (Bartel et al., 1991; Malim & Cullen, 1991; Holland et al., 1992; Mann et al., 1994; Zemmel et al., 1996): a Rev monomer first binds to a high-affinity binding site (Heaphy et al., 1991; Bartel et al., 1991; Kjems et al., 1992; Tiley et al., 1992; Iwai et al., 1992), followed by the cooperative addition of more Rev molecules via protein-protein and protein-RNA interactions all along the RRE (Heaphy et al., 1991; Malim & Cullen, 1991; Zapp et al., 1991; Mann et al., 1994; Zemmel et al., 1996).

What kind of mechanism explains the role of Rev in relieving the nuclear retention of incompletely
sulfate (DMS) within living yeast. In vivo probing of pre-RNAs can be achieved with dimethylsulfate (DMS) within living yeast (Charpentier & Rosbash, 1996). In addition, some reports have shown viral sequences that reduce the appearance of chimeric reporter genes in the cytoplasm in the absence of Rev expression (Rosen et al., 1988; Maldarelli et al., 1991; Cochrane et al., 1990). One of these cis-acting repressive sequences (CRS) overlaps the RRE (Brighty & Rosenberg, 1994) and is proposed to retain viral RNAs within the nucleus.

Everything that is known about the complete RRE structure comes from structure probing in solution (Kjems et al., 1991; Mann et al., 1994). NMR was employed to analyze the structure of a small RRE subregion, called the RRE (rev binding element), that represents the minimal Rev binding sequence (Peterson et al., 1994; Battiste, 1994, 1996). The RRE structure consists of three stem–loops (Stems IIA-ID-IIC-IIIB, III-IV and V) protruding from a periphery of duplex RNA (Heaphy et al., 1991; Bartel et al., 1994; Figure 2(b)). The high-affinity site consists of an unusual bubble structure between Stems IIA and IIB, where non-Watson-Crick interactions, i.e. G:A and G:G, produce a distortion of the major groove of duplex RNA (Heaphy et al., 1991; Bartel et al., 1994; Dayton et al., 1989; Mann et al., 1994; see Figure 2(b)). The high-affinity site consists of a 22 amino acid residue Rev peptide was established by NMR (Battiste et al., 1996).

Despite these advances, there has been no in vivo assay of the Rev-RRE interaction. Structure probing of pre-RNAs can be achieved with dimethyl sulfate (DMS) within living yeast Saccharomyces cerevisiae (Charpentier & Rosbash, 1996) but not yet in mammalian cells, and important aspects of Rev function can be recapitulated in yeast (Stutz & Rosbash, 1994; Stutz et al., 1995). Therefore, we have analyzed the structure of the RRE with and without Rev in this organism; the results have led to a dynamic model of the Rev-RRE interaction.

Results
In vivo probing of the RRE

Pre-mRNAs produced from the plasmid PC-CUP-RRE carry the CUP1 gene interrupted by a 65 nt artificial intron, followed by the RRE sequence (Figure 1). The nuclear export of these pre-mRNAs is stimulated by Rev expression in yeast cells in a Rev and RRE dependent manner (Stutz & Rosbash, 1994). In order to analyze the in vitro RRE structure, cells were incubated with DMS (see details in Materials and Methods) and methylation at Watson-Crick positions of adenine (N1-A) and cytosine (N3-C) residues were analyzed by primer extension. Four different oligonucleotide primers were used to analyze the whole 351 nt sequence (Figure 1).

Examples of gels obtained with three primers are shown in Figure 2(a). Reproducible methylation hits were assigned on the latest published structure of the RRE (Mann et al., 1994; Figure 2(b)). The majority of the reactive adenine and cytosine residues are located in loops or bulges outside stems, confirming that these residues are not engaged in Watson-Crick interactions in vivo. Nevertheless, some bases reported to be in stem structures were found to be methylated. Examples include A172, C196, C204 and A205 in Stems III and IV; A251 in Stem V; and A102, C132, A133, A142 and A143 in Stems IIA, IIB and IIC. Most of these positions are at the ends of stems or in short stems. Therefore, a possible explanation is that they may reflect breathing, or the secondary structures may be locally modified by RNA binding proteins during exposure to DMS. Also, a few bases, particularly in Stem I, reported to be single-stranded did not appear to be reactive here, e.g. A18, A19, A40 and A41. One obvious explanation is that these positions could be masked by proteins in vivo or simply inaccessible to DMS in the context of a tertiary structure. This was observed previously with another pre-mRNA structure we analyzed with this method (Charpentier & Rosbash, 1996).

Hyperreactivity of a guanine residue in the high-affinity binding site

Some of the guanine residues of the high-affinity binding site are contacted, since their N7-carboxymethylation with diethylpyrocarbonate interferes with in vitro Rev binding (Kjems et al., 1992; Tiley et al., 1992). Although the core of the high-affinity binding site (residues 102 to 135) shows some N1-A and N3-C methylations that would allow the de-
tection of a Rev footprint, we also analyzed N7-methylated guanine residues: after treatment with aniline (see Materials and Methods), in vivo methylated N7-G residues were analyzed by primer extension (Figure 3). Analysis of the 90-170 region with primer BC3 shows that some guanine residues are methylated, whereas others are not. Strikingly, the guanine residue at position 128 within the core is highly reactive. As there is no report of in vitro DMS reactivity at N7-G, we modified a phage T7-RRE transcript RNA with DMS in vitro and analyzed the aniline-cleaved products by primer extension. G128 was indeed highly methylated (data not shown), indicating that methylation at this N7-G is not an idiosyncracy of the in vivo assay. Positions N1-A and N3-C were also analyzed and confirmed the proper in vitro folding of the RRE (data not shown).

To see whether this N7-G128 reactivity was dependent on the whole RRE sequence or was a consequence of a peculiar conformation of the high-affinity binding site itself, we analyzed the in vitro methylation of short RNA oligonucleotides (Figure 4(a)). As a high level of reactivity of G128 (G24 in the RNA oligonucleotide sequence) is detected on rBC-6 (Figure 4(b)), we conclude that the second possibility is correct.

Several reports have shown the role of the non-Watson-Crick interactions G107:G129 and G106:A131 (also called G48:G71 and G47:A73 using another numbering system) for in vitro Rev binding (Bartel et al., 1991; Heaphy et al., 1991; Kjems et al., 1992; Tiley et al., 1992; Iwai et al., 1992; Werstuck et al., 1996). Their role is to widen the major groove of the helix, allowing binding of Rev into it (Bartel et al., 1991; Iwai et al., 1992; Leclerc et al., 1994; Battiste et al., 1994, 1996). To check whether the high level of reactivity of G128 within the oligonucleotide was also sensitive to these non-canonical interactions, we analyzed the N7-G

Figure 2(a) legend on p. 953
Figure 2. In vivo probing of the RRE structure. (a) Autoradiograms of gels obtained by primer extension with 10 mg of total RNA extracted from DMS-treated cells carrying the PC-CUP-RRE plasmid (lanes 1 to 3). Oligonucleotides BC3, BC10 and BC11 were used as the primers, as indicated at the top of each gel. A control elongation reaction was done with 10 mg of total RNA extracted from non-treated cells (lanes EL). The numbering starts at position +1 of the 351 nt of the RRE sequence and is indicated along each gel. Positions of methylated bases are indicated by arrows. The A, C, G and U lanes refer to the RNA sequence obtained by dideoxynucleotide sequencing of total RNA from non-treated cells with each oligonucleotide. Stars indicate bands that were not reproducibly detected. (b) In vivo accessibility of DMS to positions N1 of adenine residues and N3 of cytosine residues of the RRE sequence are symbolized on the structure of the RRE established in vitro by Mann et al. (1994). The relative intensities of the reactivities, indicated by different numbers of dots, are based on visual estimation from several experiments.
methylation pattern of two mutant RNA oligonucleotides, rBC-5 and rBC-7 (Figure 4(b)). rBC-5, in which G107 and G129 (G7 and G25 in the RNA oligonucleotide sequence) were each changed to A, showed a highly reactive G128. This mutant sequence was found to be bound by Rev in vitro with a greater efficiency than the wild-type sequence (Bartel et al., 1991). When only G107 was changed to A (G7 in rBC-5), no binding was found in vitro (Bartel et al., 1991) and no G128 (G24 in the RNA oligonucleotide sequence) reactivity was observed. We conclude that the presence of a homopurineinteraction is required for exposing position N7 of G128 to DMS methylation. The hyperreactivity is thus a manifestation of the unusual RNA conformation important for Rev binding.

In vivo footprinting of Rev on the RRE

Cells transformed with PC-CUP-RRE and the Rev-expressing plasmid pG1-Rev (TRP1/ARS1-CEN4) or pG1-MB3 (TRP1/ARS1-CEN4), expressing a mutant of the Rev RNA binding domain that cannot
bind RNA; Hope et al., 1991), were treated with DMS. Positions N1-A, N3-C and N7-G were analyzed by primer extension (Figure 5). The pattern obtained when MB3 is expressed was identical with that obtained in the absence of protein and confirmed that this mutant Rev protein cannot bind RNA. Two of the reactive adenine residues and one cytosine residue (A131, A133 and C132) were reproducibly protected in the presence of wild-type Rev (Figure 5(a) and (c)). This could result from either a direct interaction or an indirect modification of RNA structure (see Discussion). In the latter case, C132 and A133, which were apparently single-stranded in the absence of protein, could interact more strongly with G104 and U103 to form Stem IID in the presence of Rev (Figure 5(c)). Three guanine residues (G104, G105 and G128) were also protected (Figure 5(b) and (c)). Surprisingly, two adenine residues became hypermethylated (A102 and A158; Figure 5(a)), a result suggesting that these two nucleotides are not just protected by a single Rev monomer. The same results were obtained whether Rev was expressed from a high or a low copy number plasmid (data not shown), and no other protection or enhancement was detected elsewhere in the 351 nt sequence (data not shown). This is consistent with the fact that Rev interacts tightly only with the high-affinity site (Kjems et al., 1991; Tiley et al., 1992). We also analyzed the footprint of the transdominant M10 mutant, which carries a mutation in the effector domain and does not shuttle between the nucleus and the cytoplasm (Malim et al., 1989a, b; Kalland et al., 1994; Meyer & Malim, 1994). The wild-type patterns for A, C and G residues were obtained with M10 Rev (data not shown). Although A131, C132 and A133 appeared slightly less protected with M10, we conclude that: (1) the in vivo probing technique detects the majority of Rev/RRE complexes within the nucleus; and (2) in vitro as well as in vivo, Rev protects G residues at the junction of the high-affinity binding site bubble (Kjems et al., 1992; Iwai et al., 1992; Zapp et al., 1993; Figure 5(c)). The hypermethylation suggests that a change in the RRE conformation, particularly in Stem IIA, occurs as a result of Rev in vivo binding (Figure 5(c)).

M4 mutant can bind RNA in vivo

We used this in vivo footprinting approach to address the relationship between Rev multimerization and RNA binding. In vitro data indicate that a biologically inactive oligomerization mutant, M4 (Malim et al., 1989a,b), has much less specificity for the RRE high-affinity site (Zapp et al., 1991); others reported no effect on binding to the RRE (Berger et al., 1991; Daly et al., 1995; Malim & Cullen, 1991; Tiley et al., 1992; Madore et al., 1994). In yeast, we previously showed that M4 has no dominant negative effect, even when over-expressed in the presence of wild-type Rev (Stutz et al., 1995). To extend this observation, we directly assayed M4

Figure 5. In vivo footprinting of Rev on the RRE. Cells transformed with plasmid PC-CUP-RRE (LEU2/2 μm) and either the wild-type Rev-expressing vector pG1-Rev (TRP1/ARS1-CEN4) (+) or an RNA binding domain mutant pG1-MB3 (TRP1/ARS1-CEN4) (−) were treated with DMS and methylated (a) adenine, cytosine and (b) guanine residues were analyzed with primer BC3, as described for Figures 2(a) and 3, respectively. Lanes EL show primer extension reactions done on RNA extracted from non-treated cells. Protected adenine and cytosine residues are indicated with open circles whereas more reactive adenine residues are indicated with arrows. Protected guanine residues are indicated by open boxes. (c) Summary of the data obtained from the in vivo probing and footprinting experiments. Reactive adenine and cytosine residues are indicated with black dots and guanine residues with black squares, with the number of symbols representing the degree of reactivity. Adenine and cytosine residues protected when Rev is expressed are circled, and protected guanine residues are boxed. The adenine residues that are more reactive when Rev is expressed are indicated with arrows.
function in yeast with the original Rev copper growth assay (Stutz & Rosbash, 1994). The data show that M4 has only a low level of export activity (Figure 6(a); compare lane M4/2 µm with the control lane MB3), and only when expressed from a high copy number plasmid (Figure 6(a); compare lanes M4/CEN and M4/2 µm at 0.9 and 1 mM). This is consistent with an effect of the M4 mutation on affinity, specificity or oligomerization.

Mutant M4 was expressed in yeast from a low or a high copy number plasmid (TRP1/ARS1-CEN4 and TRP1/2 µm, respectively), and the footprint was analyzed (Figure 6(b)). The protection pattern of M4 was quite different from that of wild-type: A131, C132 and A133 are protected only when M4 is expressed at a high level (Figure 6(b)). Even more surprising was the absence of the reactivity enhancements at A102 and A158. The results suggest that M4 binds in vivo with a lower affinity or probably a lower specificity (Daly et al., 1995). In addition and as M4 binds to RNA as a monomer (Daly et al., 1995), the absence of the two reactivity enhancements indicates that one Rev molecule is not sufficient to produce the reorganization in Stem IIA.

**RRE structure modification in Stem IIA occurs upon oligomerization**

To check further whether the number of interacting Rev molecules affects the RNA structure, a short RRE was constructed: the RRE sequence was reduced to 176 nt (87 to 262 in Figure 2(b)). Binding experiments had shown that two Rev molecules could bind this truncated RRE sequence in vitro at low Rev to RRE ratios (Mann et al., 1994). Yeast cells transformed with the Rev-expressing plasmid pG1-Rev (TRP1/ARS1-CEN4) and the PC-CUP-Δ5 reporter constructs were treated with DMS. No difference in pattern was observed with the Δ5 construct as compared to the full-length RRE, including the two methylation enhancements (Figure 7). We conclude that the binding of at least two molecules produces the structural change in Stem IIA of the RRE and that this is a consequence of Rev oligomerization on the RRE.

**Discussion**

The aim of this work was to analyze the in vivo structure of the HIV-I RRE sequence and to obtain information about structural features that might occur upon Rev binding. Although several enzymes and chemical reagents can be used to examine RNA structure and RNA-protein complexes in vitro, few are suitable for in vivo studies. DMS is currently the best compromise in terms of its membrane permeability, high level of reactivity, and small size (Charpentier & Rosbash, 1996). It has been successfully used for RNA studies in yeast (Charpentier & Rosbash, 1996), Trypanosoma (Harris et al., 1995) and Tetrahymena (Zaug & Cech, 1995), but not yet in mammalian

![Figure 6. In vivo footprinting of Rev oligomerization-deficient M4 mutant. (a) Effect of the level of expression of wild-type or mutant Rev on the export of PC-CUP-RRE pre-mRNA. Cells containing plasmid PC-CUP-RRE (LEU2/2 µm) in the presence of wild-type or the M4 mutant Rev, expressed from a high or a low copy number plasmid (TRP1/2 µm or ARS1-CEN4), respectively, were analyzed by growth on copper-containing plates. Growth on five different copper concentrations is shown. Cells expressing MB3, a mutant of the RNA binding domain, serves as a negative control for Rev function. (b) Methylation and analysis of the methylated bases were done as described for Figure 5. Experiments were done with cells expressing low levels of MB3 (lane 1), Rev (lane 2) and M4 (lane 3) or high levels of M4 (lane 4). A control (lane EL) was loaded as in Figure 5. The band in lane 3 indicated by a star was not detected reproducibly.](image-url)
cells. Primer extension identifies non-base-paired adenine and cytosine residues, methylated by DMS at positions N1 and N3, respectively, as well as methylated guanine residues at position N7 after treatment with aniline (Inoue & Cech, 1985). Position N1 of adenine and N3 of cytosine become protected upon base-pairing, tertiary structure formation or protein binding, whereas position N7 of guanine becomes protected due to tertiary structure formation or protein binding (Ehresmann et al., 1987). Our first attempt to analyze the RRE structure and the RRE-Rev complex was therefore with DMS within living yeast cells, where Rev is able to target and promote the export of RRE-containing transcripts (Stutz & Rosbash, 1994; Stutz et al., 1995).

In vivo RRE structure

The in vivo methylation pattern we obtained fits well with the in vitro structures established with a wider set of chemical and enzymatic probes (Kjems et al., 1991; Zapp et al., 1993; Mann et al., 1994), and it confirms that the majority of RRE molecules are folded properly in vivo.

NMR studies have been performed on a small 34 nt RNA sequence representing a minimal Rev binding sequence (Battiste et al., 1994, 1996; Peterson et al., 1994). This RNA, which appeared poorly structured in the absence of protein (Battiste et al., 1994), is similar to the rBC-6 oligoribonucleotide used in our in vitro experiments.

Notably in the study by Battiste et al. (1994), neither C107 nor G108 was found in a Watson-Crick configuration with G128 and C127, respectively. In contrast, we have not detected any methylation at the N1 of C107 or C127, suggesting that those nucleotides are base-paired at the end of Stem IIB in vivo. We also found the N7 of G128 to be highly reactive in vivo, in vitro and in small RNA oligonucleotides in the absence of protein. This hyperreactivity was obvious compared to all other G residues and appeared dependent on the presence of two purines (a homopurine pair) at neighboring positions 106 and 129 (Figures 4(a) and (b), and 5(c)). This suggests that a non-Watson-Crick interaction (G106:G129 or A106:A129) is indeed formed and the region structured in all of the tested substrates. An explanation for this difference between NMR and our direct probing is that structural breathing causes a fast exchange of imino protons at the end of stem IIB despite base-pair formation (Peterson et al., 1994). Alternatively, only a fraction of the protein-free RNA is in a proper partially folded conformation at any moment. The structure probing technique as well as Rev, captures the partially folded conformation (Figure 8, structure A). We prefer this second possibility because of the stringent single conformation requirement for NMR structure determination: the high-affinity site will be scored as unstructured if a substantial fraction of the RNA explores two or more conformations (Figure 8, conformations A’ and A).

Also in contrast to the NMR data, C132 and A133 of Stem IID appear highly reactive and are therefore probably not in a stable stem with G104 and U103 in the large RRE sequence. In vitro probing of a 66 nt RNA sequence carrying Stems IIA, B, C and D also showed that the right side of the high-affinity site was not well structured (Zapp et al., 1993). One can imagine that Stem IIC, present in the wild-type RRE but absent from the NMR substrates, plays a role in preventing a Watson-Crick interaction between the two nucleotides of Stem IID. Thus in the absence of protein, the left side of the bubble and Stem IIB appear well formed in vivo whereas the other side, including Stem IID, is less defined.

In vivo footprinting of the first Rev molecule

It is intriguing that, as Rev oligomerizes on the RRE (Malim & Cullen, 1991; Zapp et al., 1991; Mann et al., 1994), we still observed protection only of the high-affinity binding site. Possible explanations for this result are that (1) some points of RNA-protein interaction are still modified by DMS because of its high reactivity, and (2) weak or predominantly backbone binding could go undetected because of the small size of the reagent. Indeed, very faint protections from chemical probes were observed by Kjems et al. (1991) in Stem I when Rev was present at a level that allowed formation of
multiple complexes. In general, only RNases have been successfully used to detect the presence of Rev molecules on Stem I (Mann et al., 1994).

We interpret the Rev protection pattern as due to RNA structure changes as well as direct Rev binding. The simplest explanation for the protection of positions A133 and C132 is a stabilization of Stem IID (C132A133:G104U103 stem; Figure 5(c)). This agrees with prior in vitro data (Zapp et al., 1993; Battiste et al., 1994, 1996; Peterson et al., 1994; Kjems et al., 1991). The protection of A131 is probably due to the formation of an adjacent non-canonical A131:G105 interaction, which could be considered part of Stem IID (Bartel et al., 1991; Iwai et al., 1992; Peterson et al., 1994; Battiste et al., 1994, 1996). For G 104 and G105, the decrease in methylation is probably also due to a direct protection by Rev: modification of these N7 positions interferes with Rev binding, and a direct contact of Rev with these nucleotides was proposed from the NMR data (Kjems et al., 1992; Tiley et al., 1992; Iwai et al., 1992; Battiste et al., 1996). The same arguments explain protection of the prominent G128.

In conclusion, we propose that, in addition to direct Rev-RNA contacts, a structural reorganization occurs in the right part of the high-affinity site (Figure 8, Stem IID in structure B).

**Figure 8.** A model for the structural rearrangements that occur during Rev binding on the RRE. Four different RNA structures formed by Stems IIA, IIB, IIC and IID of the RRE in the absence of Rev (structures A and A') or upon Rev binding (structures B and C) are represented. A Rev monomer interacts first with the partially structured high-affinity binding site (structure A), thereby inducing a stabilization of both the right side of the high-affinity site bubble and Stem IID (structure B). Addition of a second Rev molecule results in a melting of the first two base-pairs of Stem IIA (structure C). Broken lines represent the non-Watson-Crick G:G and A:G interactions.

**Rev oligomerization**

Although a Rev-Stem I interaction was not detected with DMS, the structural modifications in Stem IIA and use of the M4 mutant gave us a way to visualize an effect of oligomerization on the RRE structure in vivo. M4 contains mutations at the N terminus of the protein that abolish oligomerization (Mermer et al., 1990; Malim et al., 1989a,b, 1991; Zapp et al., 1991; Madore et al., 1994; Daly et al., 1995). Although we observed M4 protection of the high-affinity site, it required higher protein expression than the wild-type protein; by Western blotting, we measured a tenfold to 20-fold difference between the M4 level from a high copy number plasmid and the Rev level from a low copy number plasmid (data not shown). Moreover, this observation agrees well with recent findings by Daly et al. (1995) concerning the effect of this N-terminal region of the protein on in vitro binding. M4 displays a slightly reduced binding affinity to the RRE and a tenfold decreased ability to discriminate the target RRE from non-specific RNA in vitro (Daly et al., 1995). A higher level of expression is then required to allow an equivalent extent of M4 binding. The absence of robust M4 biological activity even when M4 is expressed from a high copy number plasmid (Figure 6) is probably due to the fact that a single monomer bound to the
high-affinity binding site is not sufficient for wild-type activity (Huang et al., 1991).

Stem IIA is stabilized by M4 binding, confirming that this effect results from a single monomer binding to the high-affinity site. The absence of the Stem IIA enhancements suggests that this requires the binding of a second Rev molecule. We confirmed this interpretation with the truncated RRE D5, which binds two Rev molecules in vitro (Mann et al., 1994).

We interpret the unusual enhancements in Stem IIA as a melting in this region due to Rev oligomerization. Recently, Zemmel et al. (1996) showed that bulged regions near the bubble of the high-affinity site are required both for the binding of a second Rev molecule on the RRE and for a proper directionality of oligomerization. These sequences need to be close to the bubble, i.e. at the junction of Stems I/IIA/IIID. This suggests that bulged regions on synthetic RNAs initiate oligomerization like the melted sequences in the complete RRE. In the absence of an appropriate RNA sequence, inefficient melting could be rate-limiting for Rev oligomerization and reduce Rev activity. This predicts that mutagenesis of the two relevant base-pair interactions U101:A158 and A102:U157 should affect Rev function.

Despite the expected oligomerization of additional Rev molecules along with Stem I, no other structural change was detected. Different RNA-protein and protein-protein interactions could take place after addition of the first two molecules. Also, the presence of multiple bulges in Stem I may allow the development of the multi-protein complex without dramatic changes in RNA structure.

In conclusion, we propose that dynamic RRE structural changes occur before as well as after Rev binding (Figure 8): (1) the first molecule captures the peculiar structure at the left of the high-affinity site, dependent on the G:G interaction (Figure 8, structure A); (2) Rev binding stabilizes the right side of the high-affinity bubble (A131:G105 interaction) as well as Stem IID (Figure 8, structure B); and (3) binding of a second Rev molecule produces a melting of Stem IIA and initiates oligomerization into Stem I (Figure 8, structure C).

Materials and Methods

Plasmid construction

The PC-CUP-RR (LEU2/2 µm) plasmid carries the CUP1 reporter gene with the whole RRE sequence (Stutz & Rosbash, 1994). Wild-type Rev and mutant Rev proteins MB3, M10 and M4 were expressed from TRP1/ARS1-CEN4 or TRP1/2 µm versions of Rev plasmids pG1-Rev, pG1-MB3, pG1-M10 and pG1-M4 (Stutz & Rosbash, 1994; Stutz et al., 1995). A fragment carrying 176 nt of the RRE (positions 87 to 262 in the 351 nt sequence; Mann et al., 1994) was amplified by PCR with oligonucleotides BC33 and BC34 to introduce a XhoI site at the 5'end and a SacI site at the 3'end, respectively. The PC-CUP-Δ5 plasmid (LEU2/2 µm) was obtained by cloning the XhoI-SalI fragment into the SalI site of the PC-CUP plasmid.

Yeast strain and copper growth assay

All the in vivo tests were done with the copper-sensitive strain Y59ΔCUP1 (MATa, leu2-3, leu2-112, ura3-52, trp1-289, arg4, ade2, ΔCUP1; Stutz & Rosbash, 1994). The cells were transformed with the CUP1 reporter constructs (PC-CUP-RR) and the Rev-expressing plasmids according to standard procedures (Ito et al., 1983) and selected on Leu-Trp plates. Saturated liquid culture (6 µl) was spotted onto Leu-Trp plates containing a defined concentration of copper and incubated for four days at 30°C.

In vivo DMS probing

Transformed yeast cells were treated with DMS as described (Charpentier & Rosbash, 1996). Briefly, cells were grown overnight in Leu-Trp medium and diluted in YM-1 medium to an A600 of 0.3. After two hours at 30°C, 15 ml of cells was treated for five minutes with 200 µl of a 1:4 (v/v) dilution of DMS in 95% ethanol. The reaction was stopped with 5 ml of 0.6 M 2-mercaptoethanol and 5 ml of water-saturated isoaamylic alcohol. Cells were washed once with 5 ml of 0.6 M 2-mercaptoethanol. Total RNA was extracted as described (Pikielny & Rosbash, 1985).

In vitro DMS probing

Phage T7 transcripts and chemically synthesized RNA oligonucleotides were exposed to DMS methylation in vitro. Primer BC5 (5'-TAATTACGACCTCATAATTAGGAGGAGATATGAGGGACAAT 3') carrying a T7 promoter (underlined) and DT2163 (5'-GGGGGC-TGGAGCTCTGTCCCACTCCACGGTGCG 3') were used to amplify (by PCR) a 487 nt region of the env gene, carrying the 351 nt of the RRE (Mann et al., 1994). Cold RRE RNA was synthesized by in vitro T7 transcription in conditions described by Séraphin & Rosbash (1991). RNA oligonucleotides were labeled at their 5' ends with [γ-32P]ATP and phage T4 polynucleotide kinase following standard procedures (Sambrook et al., 1989). T7 RNA transcript (100 ng) or 50,000 cpm of labeled RNA oligonucleotides were pre-incubated for ten minutes at 30°C in 100 µl of 80 mM Hepes (pH 7.9), 100 mM KCl, 0.1 mM EDTA. RNAs were methylated for ten minutes with 1 µl of a 1:10 (v/v) DMS dilution in 95% ethanol. Reactions were stopped with 100 µl of 1 M 2-mercaptoethanol, 0.6 M sodium acetate, 2 mM EDTA. RNAs were precipitated twice with ethanol before further analysis.

Guanine reaction

In vivo and in vitro DMS-treated RNAs were treated with aniline according to D’Alesio (1982) with some modifications. Total in vitro methylated RNA or 5 µg of total extracted RNA was treated in 20 µl of 0.1 M sodium borohydride on ice in the dark for 30 minutes. After two precipitations in ethanol, dried pellets were dissolved in 20 µl of 1 M aniline acetate (pH 4.5) and incubated at 60°C in the dark for 20 minutes. RNAs were precipitated with ethanol. Products from reactions done on unlabeled RNAs were analyzed by primer extension.
Analysis of DMS modifications

A portion (5 ng) of (5'-32P)-end-labeled oligonucleotides (BC3, BC10, BC11 or BC-13) was annealed to 5 μg of total RNA extracted from DMS-treated or untreated cells or to total in vitro treated RNAs, and extended by primer extension (Pikielny & Rosbash, 1985). The cDNAs were fractionated on denaturing 6% polyacrylamide gels.

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References


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