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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.

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Keywords: HIV-1; RRE; Rev; *in vivo* probing; oligomerization

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

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

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spliced RNA? The most likely mechanism is that the Rev effector domain interacts with components of a nuclear transport machinery (Malim *et al.*, 1991; Fischer *et al.*, 1994; Meyer & Malim, 1994; Stutz *et al.*, 1995; Wen *et al.*, 1995; Fridell *et al.*, 1996; Wolff *et al.*, 1995). 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 RBE (rev binding element), that represents the minimal Rev binding sequence (Peterson *et al.*, 1994; Battiste *et al.*, 1994, 1996). The RRE structure consists of three stem–loops (Stems IIA-IID-IIC-IIB, III-IV and V) protruding from a long central stem (Stem I; Malim *et al.*, 1989a,b; Kjems *et al.*, 1991; Dayton *et al.*, 1989; Mann *et al.*, 1994; see 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.*, 1991; Iwai *et al.*, 1992). The opened major groove allows the Rev basic arginine-rich α-helix domain to interact with nucleotides at the borders of Stems IIA and IIB (Kjems *et al.*, 1992; Tan *et al.*, 1993; Iwai *et al.*, 1992; Leclerc *et al.*, 1994; Jain & Belasco, 1996). Recently, a more refined structure of the high-affinity binding site associated with a 22 amino aacid 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 *Saccharmoyces 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 vivo* 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).

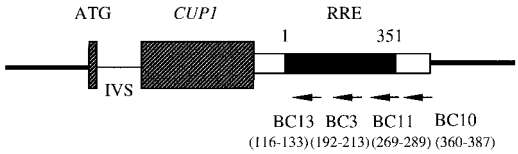

Figure 1. A representation of the PC-CUP-RRE reporter construct (Stutz & Rosbash, 1994). The *CUP1* gene is represented by the striped box, interrupted by the 65 nt intron of the pLGNdeAcc construct (Legrain & Rosbash, 1989). The RRE sequence cloned in the reporter construct is represented by the white box. The 351 nt of the RRE are represented by a black box. Positions of oligonucleotides BC3, BC10, BC11 and BC13 used in probing experiments are indicated.

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-carboxyethylation 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

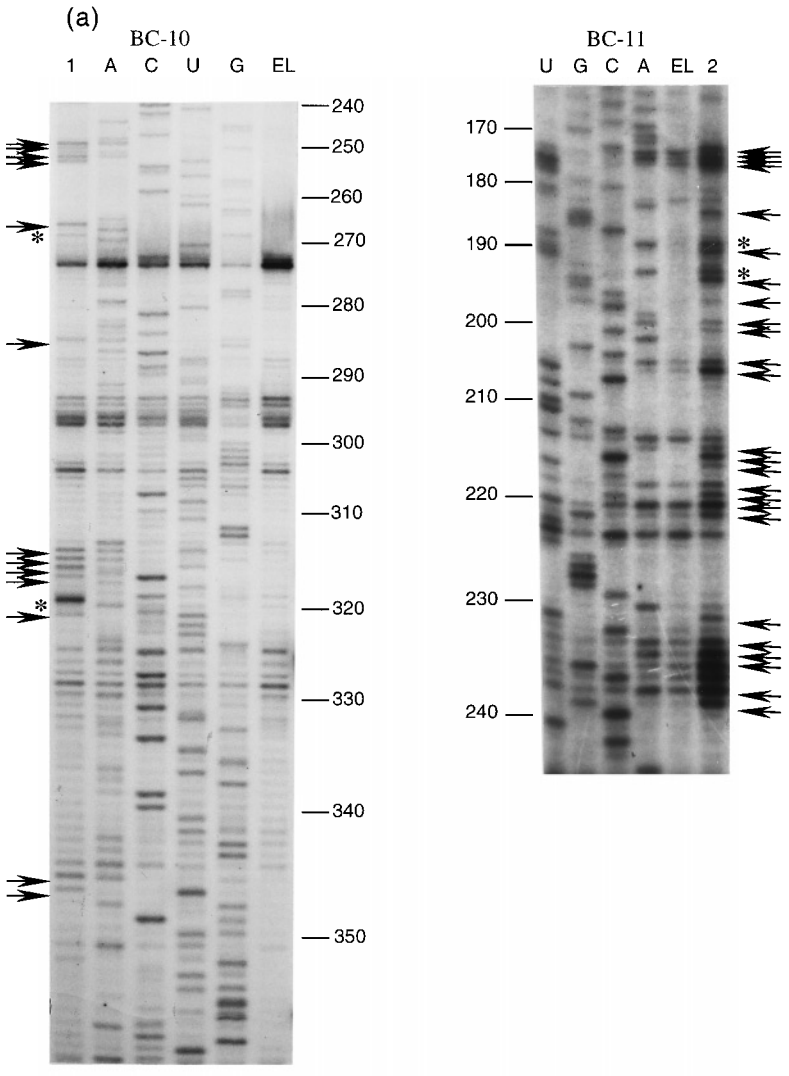

Figure 2(a) *legend on p. 953*

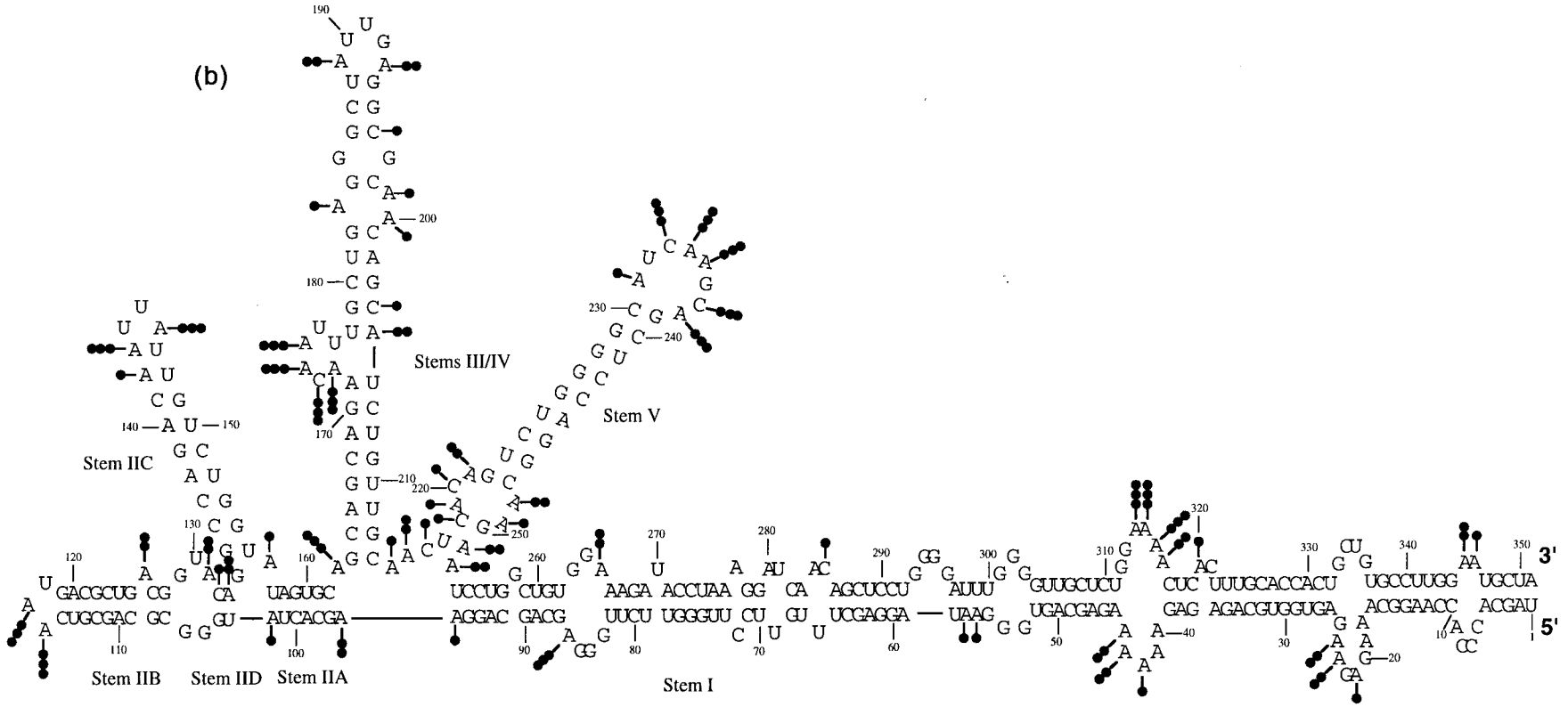

Figure 2. *In vivo* probing of the RRE structure. (a) Autoradiograms of gels obtained by primer extension with 10 mg of total RNA extracted from DMS-treated cells carrying the PC-CUP-RRE plasmid (lanes 1 to 3). Oligonucleotides BC3, BC10 and BC11 were used as the primers, as indicated at the top of each gel. A control elongation reaction was done with 10 μg 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.

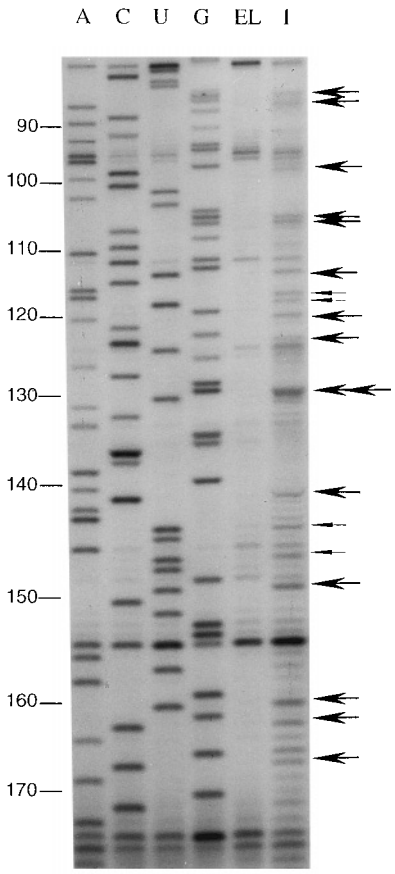

Figure 3. *In vivo* DMS probing of guanines. Total RNA (5 μg) extracted from DMS-treated (lane 1) or non-treated cells (lane EL) was analyzed by primer extension after treatment with aniline (see details in Materials and Methods). Oligonucleotide BC3 was used as the primer to analyze the sequence from nucleotides 90 to 170. Methylated guanine residues are indicated by large arrows along the gel. Highly reactive G128 is indicated by the two-headed arrow. Methylated N1 ademome resodies that are detected by primer extension are indicated by small arrows. Lanes A, C, U and G refer to RNA sequencing with the same primer.

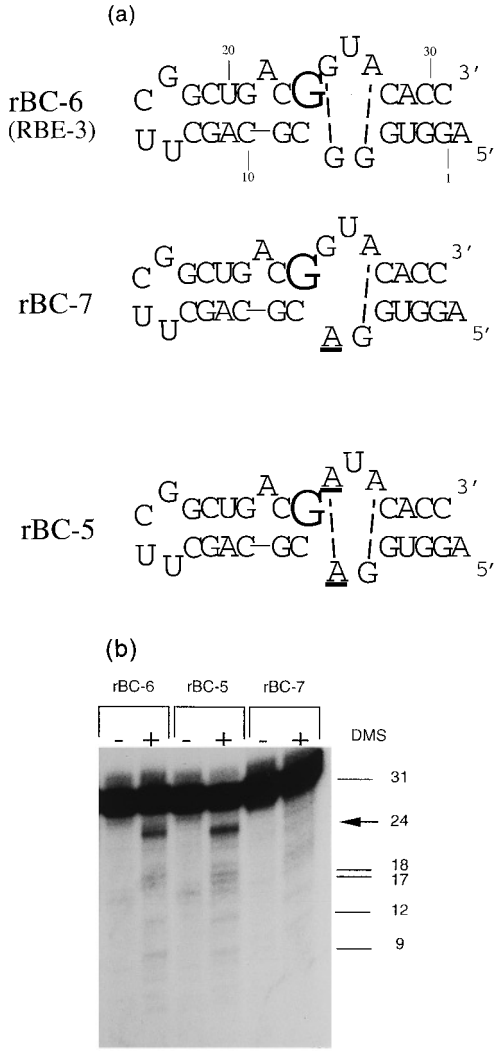

Figure 4. Reactivity of guanine residues in RNA oligonucleotides. (a) Sequences and proposed secondary structure of 31 nt RNA oligonucleotides used in probing experiments *in vitro*. The rBC-6 sequence is the same as RBE-3 (Bartel *et al.*, 1991), except that an extra A was added at the 5′ end to facilitate the ^{32}P labeling. Mutations carried by rBC-5 and rBC-7 are underlined. Broken lines represent the non-Watson-Crick interactions G:A and G:G. The guanine residue that is highly reactive to DMS is indicated by a larger letter. (b) *In vitro* DMS reactivity of guanine residues in the RNA oligonucleotides. The 5′ end-labeled RNAs were (+) or were not (−) treated with DMS before the treatment with aniline. Products of the reaction were fractionated on a denaturing 15% polyacrylamide gel. The position of the highly methylated guanine residue is indicated by an arrow.

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 homopurine interaction 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

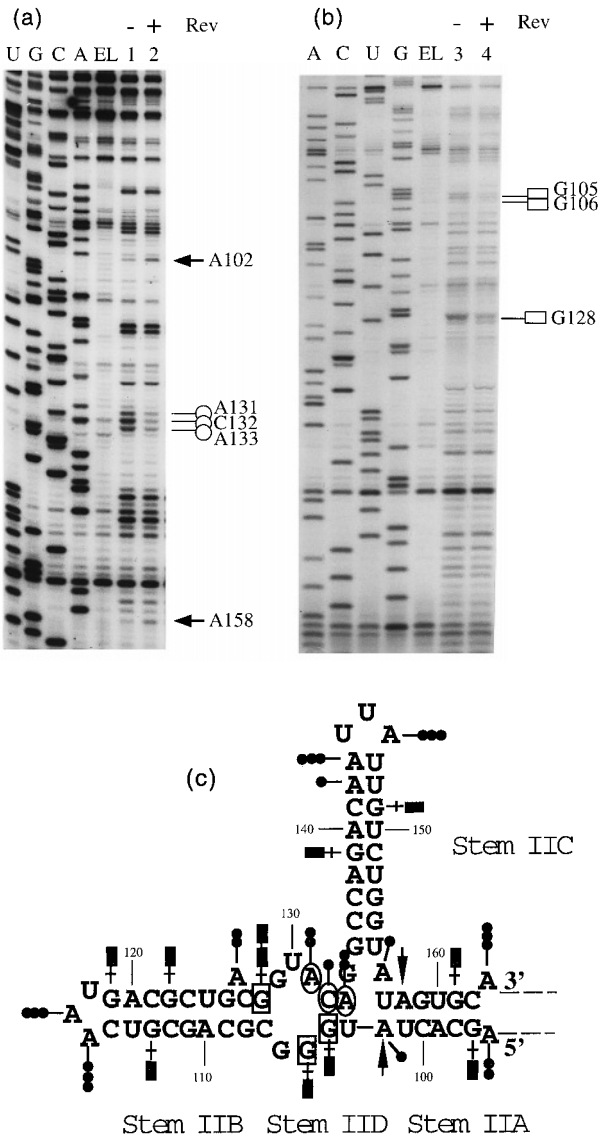

Figure 5. *In vivo* footprinting of Rev on the RRE. Cells transformed with plasmid PC-CUP-RRE (*LEU2*/2 μm) and either the wild-type Rev-expressing vector pG1-Rev (*TRP1*/*ARS1*-*CEN4*) (+) or an RNA binding domain mutant pG1-MB3 (*TRP1*/*ARS1*-*CEN4*) (−) were treated with DMS and methylated (a) adenine, 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.

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 hypermethylations suggest 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

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

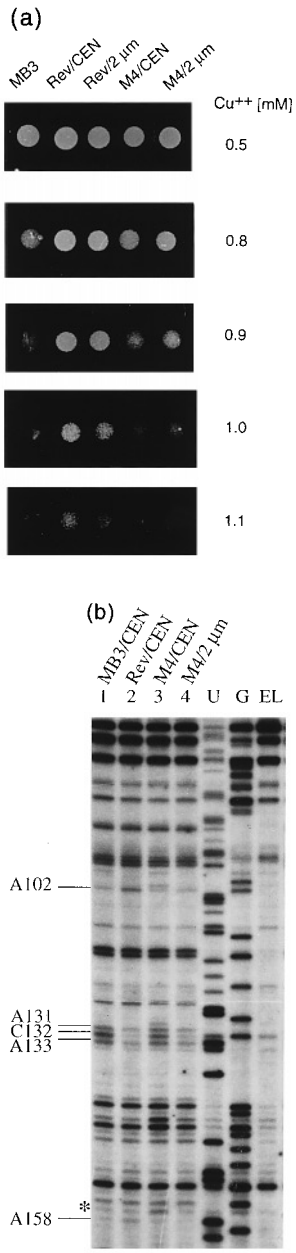

Figure 6. *In vivo* footprinting of Rev oligomerization-deficient M4 mutant. (a) Effect of the level of expression of wild-type or mutant Rev on the export of PC-CUP-RRE pre-mRNA. Cells containing plasmid PC-CUP-RRE (*LEU2*/2 μm) in the presence of wild-type or the M4 mutant Rev, expressed from a high 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.

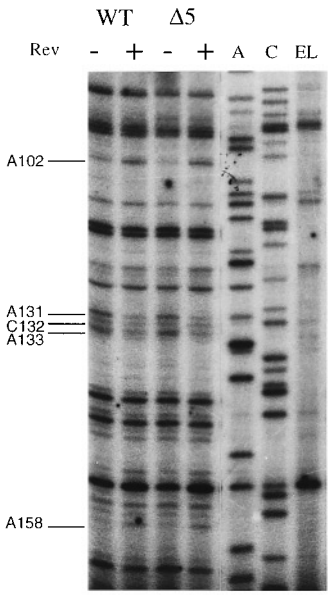

Figure 7. *In vivo* footprinting on a truncated RRE. Cells expressing wild-type Rev (+) or the MB3 mutant (−), as well as a PC-CUP-RRE construct containing a truncated version of the RRE (Δ5 construct), were treated with DMS and methylations were analyzed by primer extension with primer BC3 as described for Figure 5(a).

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

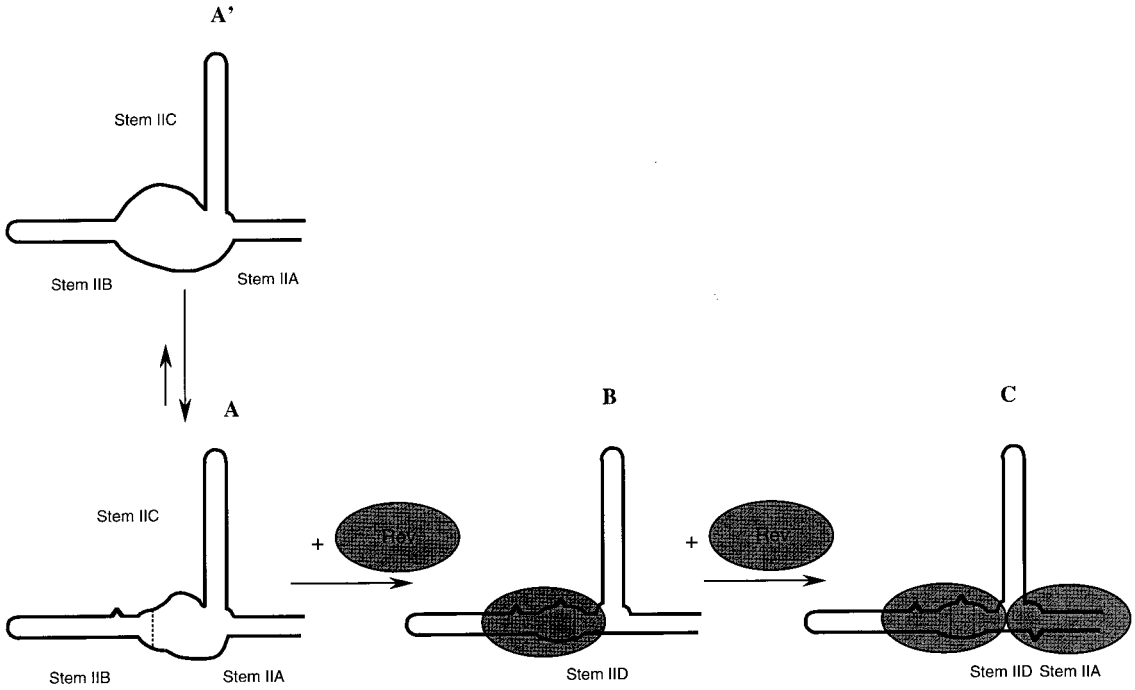

Figure 8. A model for the structural rearrangements that occur during Rev binding on the RRE. Four different RNA structures formed by Stems IIA, IIB, IIC and IID of the RRE in the absence of Rev (structures A′ and A) or upon Rev binding (structures B and C) are represented. A Rev monomer interacts first 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.

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).

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 IID 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 IIC/IIA/IID. 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 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-RRE (*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 BC53 and BC54 to introduce a *Xho*I site at the 5′end and a *Sal*I site at the 3′ end, respectively. The PC-CUP-Δ5 plasmid (*LEU2*/2 μm) was obtained by cloning the *Xho*I-*Sal*I fragment into the *Sal*I 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*, Δ*CUP1*; Stutz & Rosbash, 1994). The cells were transformed with the CUP1 reporter constructs (PC-CUP-RRE) 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 A_{600} 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 isoamyl 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′ TAATACGACTCACTATAGG-GAGGAGATATGAGGGACAAT 3′) carrying a T7 promoter (underlined) and DT2163 (5′ GGGGGC-TCGAGCTCTGTCCCACTCCATCCACGTCG 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-^{32}P]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 with RNA oligonucleotides were analyzed on denaturing 15% polyacrylamide gels. Pellets from reactions done on unlabeled RNAs were analyzed by primer extension.

Analysis of DMS modifications

A portion (5 ng) of (5′-^{32}P)-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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