Sensitive in vitro analysis of HIV-1 Rev multimerization
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ABSTRACT
Oligomerization of the Rev protein of human immunodeficiency virus type 1 on its cognate response element is essential for export of the late viral mRNAs from the nucleus. Two regions of the protein, flanking the RNA binding site, have been defined as oligomerization sites after mutants (M4 and M7) had been reported to bind specifically to the response element but not to oligomerize in vivo or in vitro. These mutants are often used as paradigms for studies of Rev multimerization. We have re-examined the in vitro binding of these mutants to model Rev response elements, using improved gel mobility assays. We find that both mutants will form oligomers on the Rev response element, but have somewhat lower affinities for RNA than the wild-type protein. M7 has lower specific affinity, but shows little deficiency in oligomerization once binding starts. In contrast, M4 is multimerization deficient, as previously reported. Therefore, while the sites are correctly defined, it is inappropriate to employ the original M7 deletion mutant to study Rev oligomerization.

INTRODUCTION
The Rev protein of human immunodeficiency virus type 1 (HIV-1) is essential for the late phase of infection of a cell, leading to the production of infectious virions (1,2); it acts post-transcriptionally to permit cytoplasmic expression of incompletely spliced HIV-1 mRNAs (reviewed in 3). There are two classes of HIV-1 mRNAs; the ‘early’, fully spliced transcripts encode regulatory proteins, including Rev and the trans-activator protein Tat, and are constitutively exported from the nucleus. The incompletely spliced and unspliced ‘late’ mRNAs, encoding the viral enzymes and structural proteins, require the direct interaction of the Rev protein with its RNA target element, the Rev response element (RRE), to allow their export from the nucleus and hence their expression.

The RRE is a 351 nt stem–loop structure located in the env coding region, present in all incompletely spliced HIV-1 mRNAs (4–8). Initially, a single Rev monomer binds to a high affinity site consisting of a purine-rich ‘bubble’ structure with bulged GG and GU residues, located near the apex of Stem I of the RRE, with a reported Kd of 1–3 nM (9–13). This permits the subsequent binding of further Rev monomers in an ordered and partially cooperative assembly process, mediated by protein–protein interactions, along Stem I of the RRE. It has been suggested that binding of a single Rev monomer stabilizes the high affinity site bubble, while binding of a second Rev initiates the process of oligomerization by melting part of Stem IA of the RRE (14). This multimerization is proposed to be the mechanism by which the threshold Rev concentration for the RRE response is determined—the RRE is thought to act as a ‘molecular rheostat’ (15) such that the degree of multimerization of Rev along its target RRE corresponds to the degree of the resulting Rev response. A later in vitro study provided evidence that the multimerization process was only partially cooperative; the wild-type RRE is not optimal for Rev oligomerization, allowing a graded response to increasing Rev concentrations (16). Multimerization is essential for the generation of a Rev response in vivo (13,15,17–21).

Rev is a 13 kDa protein which localizes predominantly to the nucleolus of HIV-1 infected cells (22–25) and is able to shuttle between the nucleus and the cytoplasm (26,27). The functional regions within the protein have been defined by mutagenesis studies combined with in vitro and in vivo functional assays. Within the N-terminal half of the protein, an arginine-rich motif spanning residues 35–50 mediates the sequence-specific binding of Rev to the high affinity bubble within the RRE target RNA element (11,28–32). This motif also acts as a nuclear localization signal (7,25,32,33). Between residues 75 and 84 in the C-terminal portion of Rev lies a leucine-rich sequence termed the activation domain (34–36); this sequence is a nuclear export signal which is able to bind the conserved nuclear export factor Crm1 (37–40). This interaction with Crm1 and other nucleoporins, probably including RIP/Rab (41,42), allows the activation domain to direct the nuclear export of Rev proteins and the mRNAs to which they are bound.

The multimerization, which is essential for in vivo Rev activity, involves two loci flanking the arginine-rich motif; these multimerization domains, between residues 18–26 and 54–56, were originally defined by two mutants, M4 (Y23D; S25D; N26L) and M7 (ΔS54; ΔS56) (7,18). When expressed as Glutathione-S-transferase (GST)-fusions and assayed by in vitro gel retardation, these mutants were reported to bind to RRE RNA efficiently, giving rise to an initial complex corresponding to the RRE with a single Rev monomer bound. However, neither mutant formed any larger complexes, with more than one Rev bound, suggesting that they were unable to multimerize on the RRE. Other studies have suggested that these mutants retain a degree of in vivo activity, perhaps due to partial ‘rescue’ of multimerization by bridging cellular cofactors (21). The mutations have been shown to map to an exposed hydrophobic surface patch on the Rev protein (43), and it was proposed that the mutant phenotype arises.

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from localized structural changes in this surface region which disrupt the multimerization interfaces.

The in vitro gel retardation assay remains the best method for detailed analysis of the multimerization ability of wild-type and mutant Rev proteins, by showing the formation of specific complexes between the RRE with one or more Rev monomers (11–13,15–18,31,43,44). Here we report an improved protocol for such assays which, by permitting the sensitive resolution of specific Rev–RRE complexes, has allowed the re-evaluation of the M4 and M7 Rev mutants. These mutants are of particular importance since they are widely accepted as the classic, reference examples of Rev variants which are unable to multimerize. Rev M4 is confirmed by these results to be substantially deficient, whereas Rev M7 appears to be defective in multimerization. This result has important implications for any interpretation of the behaviour of Rev M7 in in vivo studies.

MATERIALS AND METHODS

Preparation of Rev mutants

Wild-type Rev protein was produced from a synthetic gene inserted in a pT7-Sc plasmid vector (United States Biochemicals) as previously described by Zemmel et al. (16). An EcoRI site was introduced to the Syn-Rev gene at codon 58 by SalI/BstII insertion, producing a pT7-EcoRev vector. Mutations in the M4 region were created by BstII/SalI insertion of double-stranded DNA oligonucleotides (Rev M4 = Y23D; S25D; N26L); mutations in the M7 region were created similarly by SalI/EcoRI insertion (Rev:S54G:S56G). The M7 mutant itself (∆S54; ∆S56) was prepared by single-stranded mutagenesis employing a dut′ ung′ strain of Escherichia coli (45) by R. J. Marshall. To confirm the sequences of the Rev mutants, DNA sequencing was performed using a Sequenase version 2.0 kit (Amersham), based on di-deoxy chain termination (46).

Oligonucleotides used for mutagenesis (mutations shown in bold, deletions shown by dashes):

(i) Rev M4 (double-stranded mutagenesis)

5′-gatcaagatcccgagaggactcgctccgcgaaccccagaggggtct-3′
3′-tccaaagagtgtagcctgctgaggagcgctttggttgtcctccatgagcgcagggcgcgtcgacagggcg-3′

(ii) Rev:S54G:S56G (double-stranded mutagenesis)

5′-tcgacgtgctgccggaacgctcgctcaaatcccagcggatctgtgacggc-3′
3′-gccacccgcctgtgcacgtggatctgtgacggc-3′

(iii) Rev M7 (single-stranded mutagenesis)

5′-cgtcaaatccacatt-----agaggcgcctctg-3′

Rev protein purification

Rev protein was expressed as described by Zemmel et al. (16) and purified in three stages: cell lysis, ion-exchange chromatography (Q-Sepharose column) and affinity chromatography (Heparin Sepharose column), as described by Karn et al. (47). Expression of the correct protein was confirmed by comparison of the predicted masses with those of the expressed proteins, as determined by electrophoresis mass spectrometry.

RNA transcription

The sequences and suggested secondary structures of all RNAs used are shown in Figure 1. RNA transcripts of the full-length RRE (RRE-WT; 16) were produced from pUC19 plasmids into which the RRE sequence has been cloned under a T7 promoter. The plasmids were linearized by SmaI restriction digestes before use in run-off transcription reactions. Transcription reactions of 50 µl were set up, each containing 40 nM Tris–HCl (pH 7.4), 25 mM NaCl, 16 mM MgCl2, 10 mM DTT, 1 mM ATP, 1 mM GTP, 1 µM CTP and 100 µM UTP; 40 µCi of [α-35S]UTP and 5 µg of linearized plasmid were used for each reaction, along with 40 U of RNasin (Promega) and 0.2 µg T7 RNA polymerase. Shorter RNA transcripts RWZ2A, RWZ3 and RRE-T5 were transcribed from partially double-stranded synthetic oligodeoxyribonucleotides as described by Zemmel et al. (16). Reactions were incubated for 1.5 h (RRE-WT) or 1 h (shorter transcripts) at 37°C then run on a denaturing gel containing 8 M urea, 6% polyacrylamide (12% for RWZ2A and RWZ3 transcripts; 20% for RRE-T5) and 1x TBE (23 mM Tris base, 23 mM boric acid, 5 mM EDTA pH 8.3) at 37 W. The major RNA band was visualized by autoradiography, excised and purified as described by Zemmel et al. (16) except that shorter transcripts were eluted in RNA elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA) containing 0.1% SDS.

Transcription yields were calculated by scintillation counting for all reactions. The RNA was annealed by heating to 90°C for 2 min and slow cooling before being stored at 4°C for use in gel retardation assays at a final concentration of 2 nM (full-length RRE) or 1 nM (shorter RNA transcripts).

Gel retardation assays

Binding reactions (total volume 20 µl) were set up in siliconized Eppendorf tubes on ice. RNA mixtures were prepared, containing 84 µl 2.5% Triton, 58 µl 50 mM NaCl buffer (50 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM DTT, 2 mg/ml BSA), 5 µl RNasin and 10.5 µl refolded RNA transcript. Aliquots of 15 µl of this mixture were placed in 10 tubes. Rev protein was diluted in 0.5 M NaCl buffer (as 50 mM NaCl buffer, but containing 0.5 M NaCl) to an initial concentration of 0.2 mg/ml and then diluted further 50 or 100-fold for use in binding reactions. A variable volume of diluted protein was added to each tube, from 0 to 5 µl; a corresponding volume of 50 mM NaCl buffer was added to each tube beforehand to adjust the total volume of each reaction to 20 µl. The contents of each tube were mixed carefully and the reactions incubated on ice for 15 min before the addition of 5 µl loading buffer (30% glycerol, 0.2% bromophenol blue, 0.25% xylene cyanol FF) per tube. Samples (5 µl) from each tube were loaded on polyacrylamide gels (4% for full-length RRE-WT transcripts, 6% for RWZ2A and RWZ3 transcripts, 7.5% for RRE-T5 transcripts) containing 0.5x TBE buffer at 4°C and run at 20 mA for 2 h (RRE-WT bandshifts) or 1 h (shorter RNA bandshifts).

Gels were dried and bands containing RNA visualized by autoradiography with film or with an image plate. Phosphoimages were visualized using a Molecular Dynamics Densiometer and the program ImageQuant. Densitometry was carried out using the Geltrack program (48) and data analysis was carried out as described by Zemmel et al. (16), with an estimated error of 10% of the data value (or 0.02 nM if greater).
RESULTS

We investigated the regions of the Rev protein previously reported to be involved in multimerization (18) with an in vitro RNA binding assay, initially concentrating on the area implicated by the M7 mutation (7), between residues 50 and 60. A series of Rev variants (produced by R. J. Marshall) containing double and triple mutations in the M7 region, including the M7 mutant itself, were assayed for in vitro binding to the full-length RRE (Fig. 1a).

The gels with wild-type Rev show the expected pattern of up to 13 discrete shifted bands, corresponding to the successive addition of single Rev monomers from initial binding at the high affinity site (15,16). At higher protein concentrations, smeared bands, which we refer to as ‘super-shift’, are observed near the top of the gel; these arise from non-specific Rev aggregation (10). The results for the mutants were surprising; it was found that all the mutants tested produced higher-order complexes of five or more Rev monomers bound to the RRE at Rev concentrations between 7.5 and 15 nM (with 2 nM RRE RNA) (Fig. 2). The M4 mutant was also assayed and produced a similar result, suggesting that none of the mutants was wholly defective in multimerization (Fig. 2). With all the Rev mutants tested, up to 13 shifted bands could be seen by 40 nM Rev However, below 10 nM Rev some mutants showed a tendency to stall at lower-order complexes corresponding to one to three Rev bound to the RRE, when compared to the wild-type Rev (Fig. 2). Strikingly, no mutant gave only a single Rev complex.

The resolution of these lower-order complexes being relatively poor with the full-length RRE and, therefore, difficult to quantitate, the multimerization behaviour of the Rev mutants was investigated further using a shorter RNA transcript, RWZ2A (Fig. 1b). This RNA molecule is a truncated form of the RRE, comprising the high affinity site and sufficient flanking duplex RNA to bind a total of three Rev monomers; these complexes are easier to resolve than those with the much longer, full-length RRE. It was observed that the M4 and M7 mutants bound RWZ2A less readily than wild-type Rev, and produced much less trimer complex (Fig. 3a–c). While the mutants appeared qualitatively similar in their behaviour, quantitative analysis of the binding data to calculate the dissociation constants (K_d) for the binding of the first, second and third Rev monomers (K_1, K_2 and K_3, respectively) revealed significant differences (Table 1).

Table 1. Quantitative analysis of binding of wild-type and mutant Rev proteins to a shortened RRE, RWZ2A

<table>
<thead>
<tr>
<th>Mutant</th>
<th>K_1 ± K_1</th>
<th>K_2 ± K_2</th>
<th>K_3 ± K_3</th>
</tr>
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<tbody>
<tr>
<td>Wild-type Rev</td>
<td>1.2 ± 0.12</td>
<td>6.5 ± 0.2</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>Rev M4</td>
<td>5.4 ± 0.3</td>
<td>36.5 ± 1.5</td>
<td>157 ± 46</td>
</tr>
<tr>
<td>Rev M7</td>
<td>13 ± 0.6</td>
<td>6.3 ± 0.2</td>
<td>263 ± 57</td>
</tr>
<tr>
<td>Rev:S54G;S56G</td>
<td>6.0 ± 0.4</td>
<td>25 ± 1</td>
<td>57 ± 7</td>
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</table>

K_d values are shown in nM in each case.

The first molecule of either mutant M4 or M7 bound with a lower affinity than wild-type (i.e. K_1 is larger), M7 even less well than M4. However, a major difference in behavior occurs with
binding of the second Rev. M7 binds with the same $K_d$ as wild-type, while M4 shows the same lowering of affinity as for the first molecule to bind. Both M4 and M7 show a lowered affinity for the third molecule, which in the case of RWZ2A is binding onto an uninterrupted double-helical region of RNA. (Note that the accuracy of fitting the data for the binding of the third Rev molecules is poor, as shown by the high standard deviations of the fitted values, due entirely to the very small amounts of complex forming with the mutants.) These affinities are fully compatible with the appearance of the gels (Fig. 3a–c).

Overall binding of the first M4 or M7 Rev is lower than wild-type at low concentrations and there is very little Rev–trimer complex up to 100 nM added Rev. The most striking feature, however, is the abrupt formation of Rev–dimer complex with M7, even when very little monomer complex has formed, due to the low Rev concentration. This is due to a greater cooperativity than wild-type, shown by the $K_d$ of only $0.5$ (compared with 5.4 for wild-type).

To investigate the dimer complex formation further, an additional mutant was produced containing a double substitution of glycines, rather than simple deletion of the serines (as in Rev M7), at positions 54 and 56. This Rev:S54G;S56G mutant displayed oligomerization to monomer and dimer complexes similar to that of Rev M4 (Fig. 3d; Table 1) and bound the high affinity site more strongly than Rev M7, supporting the hypothesis that the deletions in M7 are responsible for disruption of specific RNA binding and Rev/Rev interaction. These results are surprising, as they contradict previous reports that M4 and M7 are multimerization deficient Rev mutants (18,21,43). To confirm the initial binding of the mutants to the high affinity site, without any possibility of a second Rev binding to perturb the measured $K_d$, the M4 and M7 mutants were tested on a shorter RNA transcript, RRE-T5 (Fig. 1c). This contains the high affinity site, but is very short and will bind only a single Rev protein. These results supported those obtained with the RWZ2A transcript; the $K_d$ value for M4 monomer binding to the high affinity site is about twice that for wild-type Rev, but that for M7 is a further two times higher (Fig. 4; Table 2). Although it was suspected that these results arose from a defect in sequence-specific RNA binding of M7 at the high affinity site, it is possible that M7

![Figure 3. Gel retardation assay of Rev binding to shortened RRE, RWZ2A (1 nM). (a) Wild-type Rev; (b) mutant Rev M4; (c) mutant Rev M7; (d) mutant Rev:SS4G;SS6G.](Image 65x615 to 277x771)

![Figure 4. Gel retardation assay of Rev binding to high affinity RRE site, RRE-T5 (1 nM). Wild-type Rev run on each gel with (a) mutant Rev M4 and (b) mutant Rev M7. Note that the origin of the gels is not shown; neither the origin nor additional bands were visible on the original autoradiographs.](Image 323x643 to 552x771)

is also defective in non-specific RNA binding. In order to test this, we used wild-type Rev and both the M4 and M7 mutants in a binding assay with the RWZ3 transcript (Fig. 1d). RWZ3 is, like RWZ2A, derived from an original RWZ2 transcript (16) but residues within the high affinity site bubble structure have been transposed, preventing site-specific recognition by Rev. The transcript therefore gives a measure of Rev binding to a non-specific sequence (although one which still contains a double-stranded bulge). Quantitative analysis of the resulting band-shift gels (Table 2; gels not shown) revealed that the non-specific RNA binding activity of the M4 and M7 mutants is essentially the same, but roughly an order of magnitude worse than that of wild-type Rev.

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<tr>
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<th>RRE-T5</th>
<th>RWZ3</th>
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<tbody>
<tr>
<td>Mutant</td>
<td>$K_f$</td>
<td>Mutant</td>
</tr>
<tr>
<td>Wild-type Rev</td>
<td>0.8 ± 0.2</td>
<td>Wild-type Rev</td>
</tr>
<tr>
<td>Rev M4</td>
<td>1.2 ± 0.4</td>
<td>Rev M4</td>
</tr>
<tr>
<td>Rev M7</td>
<td>3 ± 1</td>
<td>Rev M7</td>
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$K_f$ values are shown in nM in each case.

**DISCUSSION**

This study shows that of the two Rev mutants which originally defined the regions of the protein involved in the multimerization process, neither is simply multimerization deficient. Rev M4 binds the high affinity site less well than wild-type Rev and the binding of subsequent molecules is similarly impaired. It therefore multimerizes along the RRE only at higher Rev concentrations than wild-type, although it will form a normal pattern of bands on a native RRE construct. Most notably, Rev M4 forms trimer complexes with target RNA significantly less well than wild-type Rev, which is indicative of a multimerization defect. Rev M7 is substantially defective in sequence-specific recognition and binding of the high affinity site, rather than in its ability to multimerize; indeed, the second M7 monomer binds with unusually high affinity. It is probable that the deletion of two

Quantitative analysis of binding of wild-type and mutant Rev proteins to the RRE high affinity site, RRE-T5, and to a shortened RRE with a defective high affinity site, RWZ3

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<th>RRE-T5</th>
<th>RWZ3</th>
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<tbody>
<tr>
<td>Mutant</td>
<td>$K_f$</td>
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<td>Wild-type Rev</td>
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</tr>
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<td>Rev M4</td>
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<td>Rev M4</td>
</tr>
<tr>
<td>Rev M7</td>
<td>3 ± 1</td>
<td>Rev M7</td>
</tr>
</tbody>
</table>

$K_f$ values are shown in nM in each case.
amino acids at positions 54 and 56, being so close to the RNA binding domain (residues 35–50), distorts the folding of the protein and hence the presentation of the arginine residues necessary for sequence-specific RNA recognition.

Is the M7 region of the protein therefore excluded from involvement in the multimerization process? The Rev:S54G:S56G mutant, which contains a double substitution at positions 54 and 56 (as opposed to the double deletion in M7), gave first and mutant, which contains a double substitution at positions 54 and 56 (as opposed to the double deletion in M7), gave first and second dissociation constants very similar to those for M4 when assayed with our in vitro system (Fig. 3; Table 1). This supports the hypothesis that the deletions in M7 are responsible for misfolding and the disruption of specific RNA binding ability. The M4 mutations are also substitutions, which are likely to have a less major effect on the folding of the protein (they are in any case further from the RNA binding region). The decreased overall affinity for the high affinity site on the RNA, when compared with wild-type Rev, shown by both the M4 and Rev:S54G:S56G mutants may result from a poorer presentation of the RNA binding motif. This would also be compatible with the lower affinity for non-specific RNA of both Rev M4 and M7 compared to wild-type Rev, as shown with RW3Z. Daly et al. (49) have previously reported that Rev M4 shows a lower affinity for binding to the RRE than wild-type Rev. However, they also reported a lower specificity whereas our results suggest that the specificity is actually greater than that of the wild-type, albeit starting from a lower non-specific affinity for RNA.

Thomas et al. (43) proposed that neither of the residues deleted in Rev M7 were functionally important for multimerization of the protein, but rather were of structural significance, correctly positioning the key isoleucine residue at position 55. The deletion of the serines at positions 54 and 56 was therefore suggested to cause highly localized disruption of the proposed oligomerization surface, and hence to prevent multimerization. Our results suggest that the disruption is more severe, reducing the overall RNA binding. This, of course, does not argue against a direct functional role for Ile55 in multimerization, but would suggest that the effect of the structural alterations on the multimerization ability of the M7 mutant protein cannot be analyzed, given that RNA binding is disrupted. Zapp et al. (50) reported that M7 was defective in both multimerization and sequence-specific RNA binding. Similarly, Olsen et al. (51) noted the importance of distinguishing between mutations which caused a defect in multimerization ability, and those which affected RNA binding, as RNA binding assays alone could not necessarily distinguish between them. It seems likely that the region of the Rev protein defined by the M7 mutation is still likely to be involved in multimerization, given preliminary studies of our own and other published data (18,21,43,51–53). Whether the M7 and M4 regions are of equal importance is another question, although there is evidence to suggest that they may be within two α-helices which contact each other via hydrophobic residues (52).

The effects of both the M4 and M7 mutations in vitro have been examined in a number of previous studies. The mutants were originally described by Malim et al. (7) as failing to give the export of unspliced Tat mRNA in a co-transfection experiment. This absence of Rev activity was subsequently attributed to a multimerization defect (18). Madore et al. (21) employed a transactivation assay using Tat/Rev fusions to confirm that both Rev M4 and Rev M7 bound the RRE as well as wild-type. They went on to employ an indirect activation assay and showed that binding of Rev M4, and to a lesser extent M7, could occur to wild-type Rev, but found that self-oligomerization was much less efficient. The same assay was employed by Thomas et al. (43), who confirmed the result for Rev M4. The multimerization of Rev M4 in vivo was also studied by Charpentier et al. (14), who demonstrated that it was showing a lower affinity for RRE than the wild-type Rev, only binding to the high affinity site when present at high concentrations.

Clearly, a more sensitive in vitro binding assay is of value in the study of Rev multimerization since, as shown here, it can distinguish between apparent and actual multimerization mutants. The results of this study may, for instance, explain the greater degree of in vivo Rev activity observed for M4 than M7 (21), despite both mutants failing to show any multimerization in earlier in vitro studies (18). The original in vitro assays, being less sensitive than our system, did not show any multimerization, whereas we observe it for both mutants above a threshold concentration. Furthermore, we have established that M7 is significantly defective in RNA binding, the essential initial event in the generation of a Rev response. This may well account for the almost complete absence of in vivo Rev activity compared with the partial activity of M4, especially given the proposed explanation of partial ‘rescue’ of multimerization by cellular cofactors (21)—no such compensatory effects could aid multimerization of M7 when the defect is in overall RNA binding. Given the ample evidence that the Rev effect requires multimerization, it is our belief that an in-depth understanding of how the M4 and M7 mutants affect the in vivo response will only come from knowledge of the detailed binding steps as the Rev molecules oligomerize from the high affinity site on the RRE. We have therefore been concerned with improving the in vitro assay for Rev oligomerization.

The gel retardation assay is widely used to study the in vitro multimerization behavior of Rev. The major difficulty with this method is the high propensity of Rev to self-aggregate in vitro, producing a high molecular-weight smear on gels (supershift). To avoid this, Rev-GST fusion proteins (54) have often been used for in vitro multimerization assays [e.g. the original characterization of M4 and M7 by Malim and Cullen (18)], being easy to purify and less inclined to aggregate. A maximum of two complexes of Rev-GST proteins with RRE RNA can be resolved, doubtless due to steric hindrance from the GST protein which at 26 kDa is around twice as large as the Rev monomer (13 kDa). Such experiments can identify Rev variants which fail to produce a dimer complex with the RRE, and this is taken to be indicative of a multimerization defect. However, it is a fairly crude assay, obviously far from the true in vivo situation, and reveals nothing about the binding of multiple Rev monomers along the RRE. Moreover, GST proteins, including the specific GST usually used for the hybrid proteins (SjGST), are themselves capable of dimerization (55), and hence may contribute to the apparent dimerization of the hybrid proteins. It is therefore desirable to use purified Rev protein, rather than a hybrid, in reactions with RRE RNA.

We have refined the protocol for RNA binding assays in order to minimize non-specific Rev aggregation. With these improved conditions we have been able to resolve up to 13 clearly defined bands on gels, corresponding to the RRE with from 1 to 13 Rev monomers bound. We have also been able to demonstrate the highest affinity, i.e. lowest Kd, for Rev binding. A comprehensive study of Rev multimerization behaviour in vitro should, we suggest, employ the most sensitive system possible. Such a
system provides more exhaustive data which, as demonstrated here, are of relevance, and must be a closer re-creation of the true in vivo situation. The additional use of shorter, model RRE transcripts is also of value; not only does it allow quantitation, but it can also elucidate the precise nature of binding behaviour of Rev mutants.

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