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A rev/β-galactosidase fusion protein binds in vitro transcripts spanning the rev-responsive element of human immunodeficiency virus type 1 (HIV-1)

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The rev protein of human immunodeficiency virus type 1 (HIV-1), a phosphoprotein of 20 kDa apparent molecular mass, is essential to target the mRNA for virion polypeptides into the cytoplasm. This effect is mediated by a specific RNA stretch (rev-responsive element = RRE) localized within a 3'-terminal segment of the mRNA encoding virion proteins. We present evidence that rev expressed as a β-galactosidase fusion protein in *E. coli* forms a complex with in vitro transcripts containing the RRE; it can be precipitated by monoclonal antibodies with rev or β-galactosidase specificity. In addition, specific binding of rev protein to RNA could be demonstrated by Northwestern blotting.

Rev-protein; Human immunodeficiency virus type 1; Sequence-specific RNA-binding; Rev responsive element; Immunoprecipitation; Northwestern blot

1. INTRODUCTION

Gene expression of human immunodeficiency virus (HIV) is regulated by a number of viral and cellular factors [1,2]. The rev protein, one of the important viral regulatory polypeptides, is encoded by a double-spliced mRNA [3,4]; it is a phosphoprotein of approximately 20 kDa that has been shown to be essential for the expression of viral structural proteins [4,5]. If expression plasmids are transfected into cell cultures, the rev polypeptide appears to be accumulated in the nucleus, particularly in the nucleoli [6–10]. There is evidence that rev functions by supporting transport to cytoplasm of single-spliced and unspliced (i.e. genomic) HIV-1 transcripts [4,5,7,11–14]. Furthermore, a role in mRNA stabilization and translation of these messages has been demonstrated [7,14]. Phosphorylation that is performed by an intranuclear kinase at serine residues appears not to be required for rev function, at least not for cytoplasmic RNA targeting [10,15–17].

A *cis*-acting element that is essential and sufficient to mediate the rev effect has been mapped in the 3'-half of the env mRNA [14,18]. More precisely, it corresponds to a *Sty*I–*Sau*3A fragment of 210 bp coding for the N-terminus of the transmembrane glycoprotein gp 41 [13]; the target has been termed *cis*-acting rev-responsive sequence (CAR) [18] or rev-responsive element (RRE) [13,14]. The element has a stable stem-loop structure; it is conserved among all HIV isolates investigated so far [13]. The effects of rev were independent of the presence of splice donor/acceptor sites [7,13]. To be active, the *cis*-acting element needs to be in sense orientation of the message; it is functional, if CAR/RRE is localized in an intron or exon structure [13]. *Trans*-dominant negative repressors of the rev function were created by site-directed mutagenesis of the rev reading frame, suggesting that, akin to transcription factors [19], the rev polypeptide has two domains. One may be required for RNA binding and one for another function related to RNA targeting. Recently, specific interaction of prokaryotically expressed unfused rev peptide with the RRE has been shown by RNase-protection [20] and by filter-binding and gel-shift assays [21]. This study provides evidence for a direct and specific in vitro binding of a rev/β-galactosidase fusion protein to the RRE. These rapid procedures should help to facilitate further investigations of rev and RRE interaction.

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2. MATERIALS AND METHODS

2.1. *Cloning procedures and prokaryotic expression*

The reading frames for HIV-1 regulatory proteins rev, tat, and nef were inserted into the prokaryotic expression vector pROS [22]. Restriction fragments coding for HIV-1 regulatory proteins were taken from the cDNA plasmid pCV-1 [3] that was kindly provided by F. Wong-Staal. Ligation was performed with blunt-ended fragments (*Dpn*I) or after filling in recessed ends (*Xho*I, *Hin*dIII, *Hin*fI, *Sfa*nI) [23]. The expression plasmid pRVA was constructed with a 266 bp

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*Hin*fI fragment, the plasmid pRVB by inserting a 599 bp *Sfa*nI fragment into pROS (Fig. 1). The resulting fusion proteins are composed of 375 N-terminal amino acids of *E. coli* β-galactosidase, a cleavage site for the protease factor Xa (FXa), 12–17 amino acids from the polylinker of pROS, followed by HIV-1 specific sequences. The proteins encoded by the vector pTAT contain the amino acids 4–86 of the tat polypeptide. PRVA specifies amino acids 17–104 of rev, pRVB the amino acids 2–116 of rev, and pNEF the amino acid residues 36–206 of the nef-polypeptide. Fusion proteins were purified from transformed *E. coli* BMH 71–18 [24] as published by Nagai et al. [25]. All expression plasmids were verified by DNA sequencing [26]. The induced fusion proteins and their FXa cleavage products were checked by SDS-polyacrylamide gel electrophoresis.

2.2. *In vitro transcription*

The blunt-ended *Sty*I fragment spanning the RRE from position 7358 to 7628 of HIV-1 clone BH10 [27] was inserted into the *Sma*I site of the vector pBS (Stratagene, Heidelberg). The resulting clone p14STM-4 was used for in vitro transcription as published by Krieg and Melton [28]. The T_7 polymerase transcripts from *Sal*I-cut plasmid p14STM-4 consist of 312 nucleotides and correspond to the sense-orientated RRE. Bacteriophage T_3 RNA polymerase transcripts from the *Eco*RI-cleaved clone pST14M-4 had 330 nucleotides and antisense orientation. The transcripts were labeled by the addition of 50 μCi [α-^{32}P]UTP (400 μCi/mmol; Amersham, Braunschweig). Radioactivity was quantified by Cerenkov counting for each transcript; the quality of RNA was analysed in denaturing 6% polyacrylamide gels.

2.3. *Monoclonal antibodies and immunoprecipitation*

The monoclonal antibodies directed against rev (mab 111, 348) [9] and β-galactosidase (mab 87-55/60) [27] were purified through a protein A Sepharose column (Pharmacia, Uppsala) [30]. RNA/protein complexes were precipitated with the monoclonal antibodies as described [31]. In vitro transcripts from clone p14STM-4 (10^6 cpm) were incubated in a volume of 10 μl with approximately 10 μg of the fusion proteins for 30 min at 0°C prior to reaction with monoclonal antibodies and subsequent precipitation with Pansorbin cells (Calbiochem, Frankfurt). Protein/RNA incubation was performed in NET-2 buffer (100 mM NaCl/50 mM Tris, pH 7.4; 250 μM/ml RNasin and 0.3% vanadyl ribonucleotide complexes (VRC)) in 4 M urea and 0.05% NP40. Pansorbin cells were suspended in NET-2 buffer in the presence of 200 μg/ml *E. coli* tRNA (≈3 × 10^{-5} mol/ml) as nonspecific competitor. Washing was done in NET-2 for 5 times.

2.4. *Northwestern blotting*

Demonstration of RNA/protein binding was performed by Northwestern blotting essentially as published [32,33]. The fusion

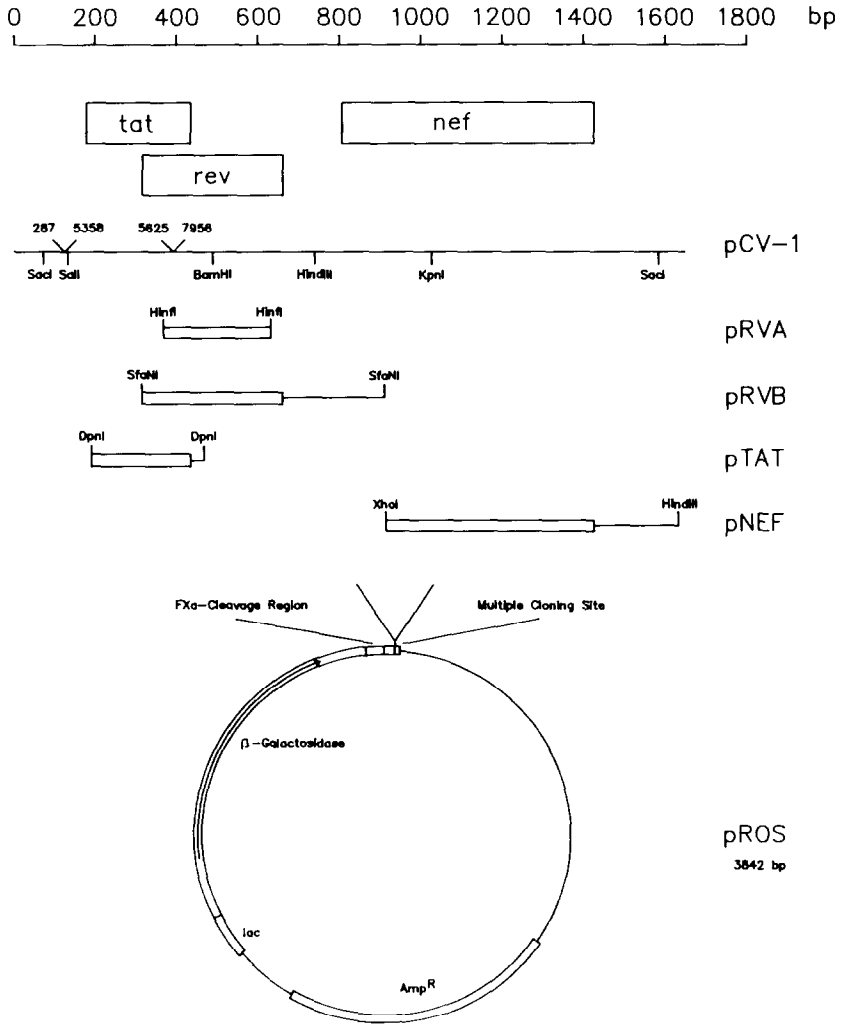

Fig. 1. Structure of the prokaryotic expression clones pRVA (for rev fragment), pRVB (for rev), pTAT, and pNEF. The plasmids are derived from the cDNA clone pCV-1 [3]. The numbering of the base pairs and relevant restriction enzymes is according to [3]. Open segments, expressed HIV-specific cDNA sequences; thin lines adjacent to open segments, nontranslated HIV-specific cDNA sequences.

proteins from *E. coli* were purified [25], separated by 12.5% (w/v) polyacrylamide gels, blotted to nitrocellulose (NC) filters, and were allowed to react with ^{32}P-labeled in vitro transcripts of sense or antisense orientation. NC-strips after blot procedures were preincubated for 1 h in probing buffer (50 mM NaCl; 10 mM Tris, pH 7.5; 1 mM EDTA; 0.02% bovine serum albumin (BSA); 0.02% Ficoll; 0.02% polyvinylpyrrolidone). Incubation was performed in the same buffer for 1 h and 1×10^6 cpm of either transcript added. To lower nonspecific background *E. coli* tRNA in a concentration of 20 μg/ml ($\approx 3 \times 10^{-6}$ mol/ml) was present in the incubation reaction. Washing was done 3 times in probing buffer without BSA. Immunostaining of NC-strips post-exposure by rev-mab 348 has been described [9].

3. RESULTS

3.1. *Binding of HIV-1 rev protein to in vitro transcripts can be demonstrated by an immunoprecipitation assay*

To substantiate the hypothesis of direct RNA binding by the rev polypeptide, we tried to precipitate an RNA/protein complex with monoclonal antibodies. Labeled in vitro transcripts from clone p14STM-4 spanning the RRE (Fig. 2) were incubated with the fusion protein derived from the expression vector pRVB and immunoprecipitated by monoclonal antibodies. Fig. 3, lane 2, shows that the expected RNA/protein complex is formed in vitro, since the radioactive transcript is precipitated by the rev specific monoclonal antibody (rev-mab 348). The same complex could also be detected using the monoclonal antibody (mab 87-55/60) that is directed against the N-terminal part of β-galactosidase within the fusion protein (Fig. 3, lane 5). No significant amounts of radioactive RNA were precipitated by either monoclonal antibody, if antisense-transcripts were incubated with the rev-specific fusion protein (Fig. 3, lanes 4 and 6) or if prokaryotic protein was missing (Fig. 3, lanes 1 and 3). Reaction of the monoclonal antibody rev-mab 348 with the fusion protein prior to the incubation with in vitro transcripts prevented appreciable precipitation of sense or antisense RNA (Fig. 3, lanes 7 and 8, respectively). This suggests that the binding site of the monoclonal antibody rev-mab 348 is in close vicinity to the RNA binding domain of the rev polypeptide, or that interaction of the fusion protein with rev-mab 348 results in changes of the secondary/tertiary structure, thus inhibiting complex formation with RNA. The fusion proteins from the expression plasmids pTAT and pNEF did not bind to either type of in vitro transcribed RNA (data not shown). Fusion protein from expression plasmid pRVA, lacking the 17 N-terminal amino acids of rev, showed diminished binding (data not shown).

3.2. *Interaction between a prokaryotic rev-β-galactosidase fusion protein and in vitro transcribed RNA is visualized by Northwestern blotting*

The partially purified fusion proteins from the prokaryotic expression clones (Fig. 1) were separated by

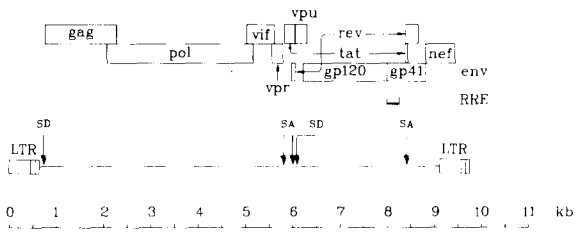

Fig. 2. The reading frames of HIV-1 and map position of a *Sty*I-*Sau*3A fragment containing the rev responsive element (RRE). Major splice donor (SD) and splice acceptor (SA) sites for tat and rev mRNA are indicated.

polyacrylamide gels, transferred to nitrocellulose, and incubated with the radioactive in vitro transcripts from plasmid p14STM-4. Fig. 4, lane b, shows significant complexing of labeled sense RNA to the fusion polypeptide from clone pRVB. The signal obtained with the fusion protein pRVA was equal to pRVB in repeated experiments (Fig. 4, lane c). Equal amounts of radioactivity in antisense orientated transcripts did not result in visible binding. Neither sense nor antisense

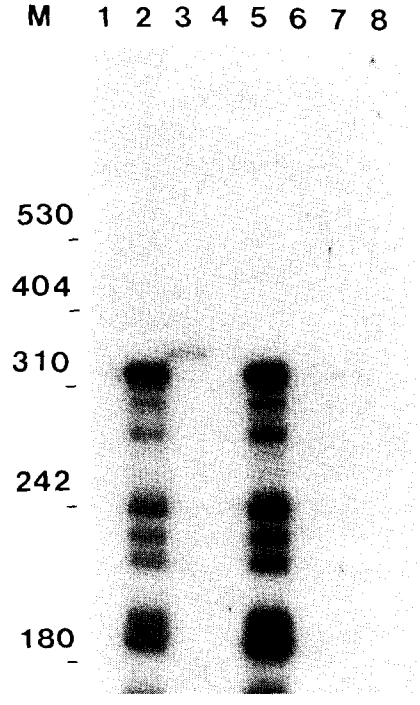

Fig. 3. Demonstration of RNA/protein binding by immunoprecipitation of rev protein/RNA complexes with monoclonal antibodies. In vitro transcripts in sense orientation (lanes 1, 2, 5, 7) and in antisense direction (lanes 3, 4, 6, 8) were incubated with fusion protein from clone pRVB. Precipitation with mab 348 against rev (lanes 1, 2, 3, 4, 7, 8) and with mab 87-55/60 directed against β-galactosidase (lanes 5, 6). For lanes 7 and 8, the monoclonal antibody was allowed to react with pRVB protein prior to RNA incubation. Fusion protein was omitted in lanes 1 and 3. The size of full length transcripts corresponds to 312nt (sense) and 330nt (antisense), respectively. The smaller RNAs apparently resulted from degradation and radiolysis during experimental procedures. M, DNA size markers.

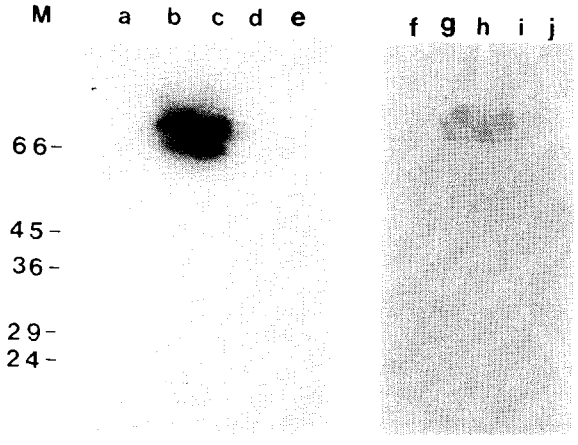

Fig. 4. Northwestern blot to demonstrate specific binding of RRE sense transcripts with prokaryotic rev fusion protein. Partially purified proteins from expression plasmids pNEF (lanes a, f), pRVB (lanes b, g), pRVA (lanes c, h), pTAT (lanes d, i) and marker proteins (lanes e, j) blotted to NC. Lanes a–e are an autoradiography post-incubation with RRE sense transcripts (antisense RRE incubated strips showed no real hybridization signal, therefore data not shown). Lanes f–j are the same strip after immunostaining with rev-mab 348. In addition, efficiency of protein transfer by blotting was checked after exposure by Ponceau S staining. M are marker proteins, numbers are molecular mass of marker proteins in kDa.

transcripts reacted with the fusion proteins from pTAT and pNEF, where the β-galactosidase component is identical to the pRVB-derived polypeptide (Fig. 4).

4. DISCUSSION

This study has shown by two independent techniques that a rev-specific polypeptide can specifically bind to RNA containing the rev-responsive element (RRE) of human immunodeficiency virus type 1. The two procedures, Northwestern blot and immunoprecipitation by monoclonal antibodies against rev, are based on in vitro assays that are conducted under quite distinct buffer conditions. Sense-transcripts are reacting with rev-specific proteins expressed in *E. coli*, but antisense transcripts do not. It indicates that a rev polypeptide is able to bind RNA in a sequence-specific fashion in the absence of other viral or cellular proteins. It may be concluded that the binding of rev protein to the RRE is a functionally important step in cytoplasmic targeting of mRNA for the structural proteins of immunodeficiency viruses.

In vitro mutagenesis of eukaryotic rev expression vectors revealed some mutants that were unable to mediate the effects of rev. One of these mutants, pM_{1-3}, produced a truncated rev polypeptide in which the first 17 amino acids were lacking [10]. We found that the fusion protein from pRVA with a similar N-terminal deletion shows diminished binding of RNA in the precipitation assay, and it reacts equally to pRBV in Northwestern blots. This may indicate that the loss of rev activity in the eukaryotic deletion variant could have a correlation with diminished RNA binding in vitro. The polypeptide from pRVA does not contain the 12 amino acids of the rev protein carboxy-terminus. Two studies have shown that this part of the protein is not required for the function of rev [8,10]. The nuclear targeting sequence of rev, a basophilic region between amino acids 38 and 45 [8,10], appears not to be sufficient for RNA binding. The recent study by Daly et al. demonstrated 50% binding inhibition of rev and RRE in sense-orientation by 2.5×10^{-4} mol/ml *E. coli* tRNA, while 7×10^{-7} mol/ml were required to achieve an equal inhibition by the antisense transcript. We found the optimal concentration for Northwestern blot to be 3×10^{-6} mol/ml *E. coli* RNA, for the immunoprecipitation procedure at 3×10^{-5} mol/ml of the unspecific competitor. The use of *E. coli* fusion proteins, purified by a rapid method, should be advantageous in defining more precisely structural requirements for binding at the level of RNA and protein.

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