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# Specific Complex of Human Immunodeficiency Virus Type 1 Rev and Nucleolar B23 Proteins: Dissociation by the Rev Response Element

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**The human immunodeficiency virus type 1 (HIV) Rev protein is thought to be involved in the export of unspliced or singly spliced viral mRNAs from the nucleus to the cytoplasm. This function is mediated by a sequence-specific interaction with a *cis*-acting RNA element, the Rev response element (RRE), present in these intron-containing RNAs. To identify possible host proteins involved in Rev function, we fractionated nuclear cell extracts with a Rev affinity column. A single, tightly associated Rev-binding protein was identified; this protein is the mammalian nucleolar protein B23. The interaction between HIV Rev and B23 is very specific, as it was observed in complex cell extracts. The complex is also very stable toward dissociation by high salt concentrations. Despite the stability of the Rev-B23 protein complex, the addition of RRE, but not control RNA, led to the displacement of B23 and the formation of a specific Rev-RRE complex. The mammalian nucleolar protein B23 or its amphibian counterpart No38 is believed to function as a shuttle receptor for the nuclear import of ribosomal proteins. B23 may also serve as a shuttle for the import of HIV Rev from the cytoplasm into the nucleus or nucleolus to allow further rounds of export of RRE-containing viral RNAs.**

The genome of the human immunodeficiency virus (HIV) encodes at least five regulatory proteins in addition to the three polyproteins common to all retroviruses (Gag, Pol, and Env). Among these regulatory proteins, Tat and Rev, expressed from partially overlapping reading frames, are essential for viral replication in human cells (3, 14, 19, 38, 41).

Transcription from the 5′ long terminal repeat of the provirus generates a full-length genomic RNA harboring at least two introns. This primary transcript undergoes differential splicing pathways, resulting in the production of three distinct classes of mRNAs (16, 36, 40): unspliced, singly spliced, and fully spliced mRNAs. The unspliced genomic RNA of 9 kb encodes the polyproteins Gag and Pol. Removal of the first intron results in the production of singly spliced RNAs of about 4 kb that encode, among other proteins, the Env polyprotein. Extensive processing of the primary transcript generates a family of doubly spliced mRNAs of about 2 kb that encode the viral regulatory proteins Tat, Rev, and possibly Nef.

Gene expression of HIV-1 is controlled by temporal regulation (reviewed by Cullen and Greene [11]). During the early phase, translation of the intronless class of transcripts takes place so that Tat, Rev, and Nef are the first viral proteins produced in the permissive infected cells. The biological role of Nef protein remains undefined. Tat protein acts on the 5′ long terminal repeat promoter to ensure high levels of transcription from the viral genome (25), whereas Rev protein acts postranscriptionally to selectively increase the cytoplasmic concentration of the *gag-pol* and *env* mRNAs (16, 40). Tat and Rev are therefore required to induce transition to the late phase of viral gene expression, during which the structural proteins Gag, Pol, and Env are synthesized.

The action of Rev depends on the presence of a specific RNA sequence in *cis* referred to as the Rev response element (RRE) (15, 22, 32, 37). The RRE is about 234 bases long and is predicted to have extensive secondary structure of five stem-loops (32). This sequence forms part of the most highly conserved region of the *env* gene. The RRE is present within the intron-containing mRNAs but is spliced out of the mRNAs encoding the regulatory proteins, whose expression is then independent of Rev function. Evidence for the specific binding of Rev protein to RRE sequences in vitro has been reported (13, 44), and the minimal region necessary for the specific interaction is about 70 bases (26, 33).

The mechanism by which Rev protein increases the cytoplasmic levels of the unspliced or singly spliced viral mRNAs is unclear. Available data support the hypothesis that Rev binding somehow prevents splicing of these intron-containing RNAs and then channels them toward the exporting machinery (9, 17, 23, 32).

To study the mechanism of Rev function, we took the biochemical approach of searching for Rev-binding host proteins. Using affinity chromatography, we identified the mammalian nucleolar protein B23 (6) or its amphibian homolog No38 (39) as the major HIV Rev-binding protein. This abundant nucleolar protein is thought to play a role in the translocation of ribosomal components across the nuclear envelope by shuttling constantly between nucleus and cytoplasm (4). Several authors have suggested a possible involvement of the nucleolus or some nucleolar factor in Rev function on the basis of the nucleolar localization of this protein in expressing cells (12, 18, 37). Our results suggest that B23 may serve as a shuttle protein for the import of Rev, thus permitting additional cycles of export of RRE-containing viral RNAs.

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## MATERIALS AND METHODS

**Cells.** HeLa S3 cells were grown in suspension cultures at about $2 \times 10^5$ cells per ml at 37°C in RPMI medium supplemented with 5% newborn calf serum.

**Nuclei.** HeLa nuclei were isolated as described by Mirkovitch et al. (35). Rat liver nuclei were isolated as described by Lichtsteiner et al. (30) and stored at −70°C in homogenization buffer containing 50% glycerol.

**Nuclear extract.** All manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 0°C. Phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were added to the buffers just prior to use. Nuclei (1,000 $A_{260}$ units) isolated as described above were pelleted and resuspended at 40 $A_{260}$ units per ml in extraction buffer (15 mM Tris-HCl [pH 7.4], 100 mM KCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mM benzamidine, 10% glycerol, 0.1 mM PMSF, leupeptin [0.5 μg/ml], antipain [0.5 μg/ml], pepstatin A [50 ng/ml]. The KCl concentration was then adjusted to 0.55 M by adding an appropriate amount of a 3 M stock solution. The extract was incubated for 30 min on ice with occasional mixing. The viscous lysate was then centrifuged at 35,000 rpm for 60 min in a Ti50 rotor. The supernatant was dialyzed twice for 2 h against 25 volumes of extraction buffer. During dialysis, a white precipitate formed which was removed at the end of dialysis by 15 min of centrifugation at 35,000 rpm in a Ti50 rotor. The extracts were used immediately or stored at −70°C. Final protein concentration was between 0.6 and 1 mg/ml.

**Affinity chromatography.** Proteins (2 mg) were coupled to 1 ml of an Affi-Gel 10 support (Bio-Rad) according to the recommendations of the manufacturer. Coupling efficiency was determined by comparing the input protein solution with the remaining free protein in the supernatant on sodium dodecyl sulfate (SDS)-polyacrylamide gels. For Rev protein, coupling efficiency was about 95%. The resin was then poured in 4-ml Bio-Rad columns and stored at 4°C in extraction buffer containing 0.02% $NaN_3$. For longer storage periods, columns were kept at −20°C in the same buffer containing 50% glycerol. Columns were prewashed with 3 ml of extraction buffer, loaded with 1 to 3 ml of the nuclear extract, and washed in a stepwise manner with 8 ml of extraction buffer, followed by three washes with 3 ml of the same buffer containing 0.3, 0.6, and 1.0 M KCl, respectively. Columns were run at 4°C at 3 ml/h. In the experiment described in Fig. 2, sonicated salmon sperm DNA (Sigma type III; mean size, 2 kb) was added to the extract at 1.5 mg/ml (final concentration) prior to loading, and washes were carried out in the presence of the same amount of DNA. DNA (5 μg/ml) was included in the elution steps. In a typical experiment, proteins in column fractions were precipitated with 20% trichloroacetic acid, washed with acetone-ethanol (7:2), resuspended in protein gel sample buffer containing 4 M urea, and loaded onto a 7.5 to 15% linear gradient acrylamide gel.

**Immunoaffinity chromatography.** The anti-Rev serum used was obtained by immunization of rabbits as described by Harlow and Lane (24). The serum shows high affinity for Rev in Western immunoblotting experiments, and no immunoreactivity against total nuclear proteins was observed. Rabbit polyclonal anti-Rev serum (0.2 ml) was coupled to a 1-ml Affi-Gel 10 (Bio-Rad) column as described above. Two columns were prepared by pouring 0.5 ml of the resin in two sterile 1-ml syringes. To each syringe, 1 ml of rat liver nuclear extract (about 0.6 mg of nuclear proteins) was applied. Rev protein (10 μg) was added to one of the samples and incubated for 15 min at 4°C prior to loading. Columns were washed as described above except that the last elution step was 100 mM glycine (pH 2.5) instead of 1.0 M KCl.

**Sucrose gradients.** Rev protein and affinity-purified B23 protein were labeled with the Bolton and Hunter reagent (Amersham) according to the recommendations of the manufacturer. In the experiment shown in Fig. 4, 0.1 μg of $^{125}$I-labeled Rev protein ($10^6$ cpm/μg) and 0.4 μg of unlabeled Rev were incubated in the absence or presence of unlabeled B23 protein (1 μg) for 30 min at room temperature in 10 mM sodium phosphate (pH 7.4)–140 mM NaCl–200 mM KCl–0.05% (wt/vol) digitonin (RBB buffer). Digitonin does not seem to interfere with the assay and prevented loss of Rev protein by adsorption to the walls of the plastic tubes. Final sample volumes were 100 μl. In parallel, B23 protein was incubated under the same conditions in the absence of Rev. In this case, $3 \times 10^4$ cpm of $^{125}$I-labeled B23 (at $6 \times 10^4$ cpm/μg) was added to the unlabeled protein. Samples were then layered on top of 5 to 30% linear sucrose gradients made up in the same buffer supplemented with 1% (vol/vol) Trasylol (Bayer). Gradients were centrifugated at 35,000 rpm in a Beckman SW50.1 rotor for 22 h at 4°C. For the estimation of S values, a standard mixture of proteins (see legend to Fig. 4) was used. The gradients were collected into 23 fractions of 200 μl and counted in a gamma scintillation spectrometer. Fractions were diluted three times with water and extracted with 200 μl of equilibrated phenol. Aqueous phases were discarded, and phenol phases were extracted with 2 volumes of ether saturated in water. The organic phases were discarded, and the residual aqueous phases were reextracted with ether and concentrated on a Speed Vac concentrator (Savant Instruments). Samples were analyzed on SDS-containing 7 to 15% linear gradient polyacrylamide gels. Gels were silver stained, dried, and exposed. In the experiment described in Fig. 7, 0.5 μg of labeled Rev protein ($5 \times 10^4$ cpm) and 2.5 μg of B23 protein were used per sample analyzed on an individual gradient. Samples (3 μg) of sense or antisense RRE RNA were added to the protein complexes in the presence of 2.5 μg of yeast tRNA. Centrifugation tubes used to pour sucrose gradients were incubated overnight in 0.5% gelatin prior use.

**Protein sequencing.** Samples (20 μg) of affinity-purified B23 protein were loaded onto a 12% SDS-polyacrylamide gel and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore). Transfer buffer was 15 mM Tris base–192 mM glycine–20% methanol. Internal sequences were obtained by tryptic digestion of the blotted protein essentially as described by Aebersold et al. (2). Peptides were fractionated by reverse-phase high-performance liquid chromatography on a C18 Vydac column (4.6 by 250 mm), with a 0 to 70% acetonitrile gradient in 0.1% trifluoroacetic acid. The purified peptides were sequenced with a model 470A gas-phase sequenator (Applied Biosystems) with an on-line PTH amino acid analyzer and 03R PTH program.

**Gel electrophoresis and Western blotting.** Protein gels were run according to Laemmli (29). Gels were stained with Coomassie brilliant blue R or with silver nitrate. Molecular size standards used were the Sigma MW-SDS-200 kit composed of rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (BSA; 66 kDa), ovalbumin (45 kDa), and bovine erythrocyte carbonic anhydrase (29 kDa). Western blots were carried out according to Harlow and Lane (24). The quality of electrotransfer was controlled by transiently staining the membrane with Ponceau S. Nitrocellulose filters were blocked by incubations in phos-

phate-buffered saline containing 5% fat-free milk powder and 0.1% Tween 20. The monoclonal anti-chicken B23 antibody was diluted 1:1,000 in phosphate-buffered saline supplemented with 0.1% Tween 20 and 0.1% BSA. Detection was with a secondary antibody (goat anti-mouse immunoglobulin G; Sigma) coupled to alkaline phosphatase diluted 1:1,000 in the same buffer and developed with bromochloroindolyl phosphate (BCIP) at 0.2 mg/ml in 100 mM $Na_2CO_3$ (pH 10.2). Incubation was for 4 h with the primary antibody and for 2 h with the secondary antibody.

**Recombinant plasmids and generation of in vitro-synthesized RNA transcripts.** HIV DNA clone pGEM4z-1430 was provided by J. Delamarter. This plasmid was obtained by insertion of a *Bgl*II RRE-containing fragment of 1,430 bp (from HIV strain B10) to the 3′ side of the SP6 promoter present in the pGEM4z vector (Promega Biotec). For gel shift assays, a 550-nucleotide sense RNA fragment containing the RRE was used. Plasmid pGEM4z-1430 was digested with *Hin*dIII, and in vitro transcription using SP6 RNA polymerase was performed according to the instructions of the manufacturer (Promega Biotec). The two similar-size RNA fragments used as controls were in vitro synthesized by transcription using SP6 polymerase. The 255-nucleotide-long RNA fragment was obtained by transcription of plasmid pSP65 (Promega Biotec) digested with *Hae*III. The 906-nucleotide-long fragment was transcribed from plasmid pSP64-34 (27) digested with *Hae*III. The specific activity of the synthesized RNAs was about $10^7$ cpm/μg. For sucrose gradients shown in Fig. 7, 1,430-nucleotide sense or antisense RNA fragments were used. Plasmid pGEM4z-1430 was digested with *Sal*I or *Eco*RI and in vitro transcribed by using SP6 or T7 RNA polymerase, respectively. Specific activity was about $4 \times 10^4$ cpm/μg. All RNA products were phenol extracted, purified by gel filtration on Sephadex G-50 spin columns, and concentrated by ethanol precipitation. The integrity of the transcribed products was examined by analysis on denaturing 6% polyacrylamide gels. Yeast tRNA (Sigma type III; purified by phenol extraction) was used as an unlabeled competitor.

**Gel shift assay.** Reactions were carried out in RBB buffer. All samples contained 2.5 μg of unlabeled yeast tRNA, 1 U of RNasin (RNase inhibitor; Promega) per μl, and a mixture of the three radiolabeled RNA fragments described above (approximately $2 \times 10^4$ cpm per fragment). Final sample volumes were 10 μl. Rev and B23 proteins were added to the reaction mixtures from a diluted stock solution in the same buffer. Protein concentrations are indicated in the legend to Fig. 6. After the incubation period, 5 μl of final sample buffer (30% glycerol and 0.12% bromophenol blue in RBB buffer) was added to each sample. Samples were applied to a 1.3% agarose gel. Electrophoresis was carried out at a constant voltage of 100 V (75 to 80 mA) at room temperature in TBE buffer (34).

## RESULTS

**Rev protein specifically interacts with a 38-kDa nuclear protein.** To identify possible Rev-host protein interactions by affinity chromatography, we coupled Rev protein to an Affi-Gel 10 support. For this purpose, a full-length Rev protein, purified from an *E. coli* expressing strain, was used (42). This protein was able to bind specifically to an RNA containing the RRE as shown below. As a potential source for Rev-binding proteins, we extracted HeLa cell nuclei with 0.55 M KCl. The extract was applied to the affinity column, and after an initial wash, elution was carried out in a stepwise manner with 0.3, 0.6, and 1.0 M KCl.

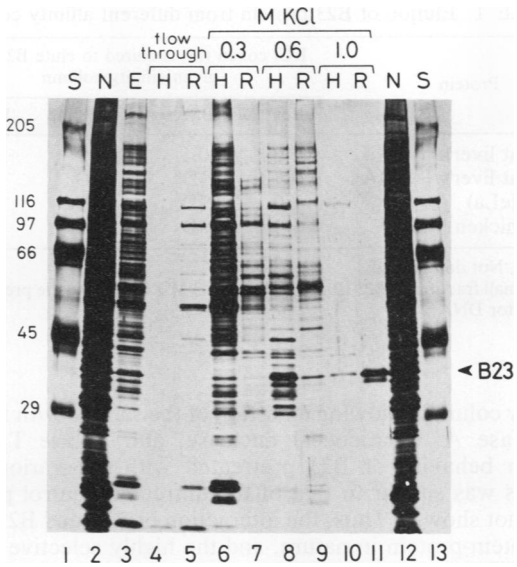


FIG. 1. Evidence that a 38-kDa nuclear protein is specifically retained by a HIV Rev affinity column. A HeLa nuclear extract was fractionated on both a Rev and a histone affinity column, and fractions were analyzed by SDS-gel electrophoresis. Elution steps (0.3, 0.6, and 1.0 M KCl) and the flowthrough are indicated above the lanes. Salt washes were five times overloaded compared with the other samples. Eluents from the Rev column (R) alternated with those from the histone column (H) to facilitate comparison. The position of B23 protein is indicated by an arrowhead. The gel was stained with silver nitrate. Lanes: 2 and 12, HeLa nuclei (N); 3, input nuclear extract (E); 4 and 5, flowthrough of the histone and Rev columns, respectively; 6 and 7, 0.3 M KCl eluates; 8 and 9, 0.6 M KCl eluents; 10 and 11, 1.0 M KCl eluents. Positions of molecular size standards (S; lanes 1 and 13) are indicated in kilodaltons on the left.

The results shown in Fig. 1 reveal a protein gel pattern of reduced complexity at increasing salt concentrations (lanes 7, 9, and 11). The protein pattern of the 1.0 M KCl eluent is striking and composed primarily of a 38-kDa protein (indicated by an arrowhead; lane 11). This protein will be identified as the nucleolar protein B23 and is referred to as such in the text that follows. B23 amounts to about 80% of the total protein in this eluent, and only a minor portion of B23 is observed in the 0.6 M KCl eluent (lane 9). Although B23 was the major protein in the 1 M KCl eluent, another minor satellite band was occasionally observed (lane 11). This band may have been derived from one of the three forms (α, β, and δ) of HeLa B23 protein (7). This question was not further studied. B23 is known to be an evolutionarily conserved protein (39). Thus, we have observed a tight interaction of both the rat and chicken B23 proteins with immobilized Rev protein. Several of the experiments described below were carried out with the rat B23 protein, which was more easily purified with good yields from rat liver nuclei (see below).

We prepared a variety of control experiments to assess the specificity of the interaction of Rev with B23. The first addressed the question of the nature of the interaction, which could be a protein-protein interaction or possibly one mediated by a nucleic acid component. For this purpose, we reapplied the B23-containing fraction on a second Rev

TABLE 1. Elution of B23 protein from different affinity columns

| Protein | KCl concn (M) required to elute B23 from given affinity column | | | |
|---|---|---|---|---|
| | Rev | BSA | Lysozyme | Histones |
| B23 (rat liver) | 1.0 | 0.1 | 0.1 | 0.6 |
| B23 (rat liver) + DNA | 0.6–1.0 | ND[a] | ND | 0.1[b] |
| B23 (HeLa) | 1.0 | ND | ND | 0.6 |
| B23 (chicken liver) | 0.6–1.0 | ND | ND | ND |

[a] ND, Not determined.
[b] A small fraction of B23 is also eluted at 0.3 M KCl despite the presence of competitor DNA.

affinity column following digestion of the sample with DNase I, RNase A, micrococcal nuclease, and RNase $T_1$. The elution behavior of B23 pretreated with the various nucleases was similar to that of the untreated control protein (data not shown). Thus, the interaction of Rev and B23 must be protein-protein in nature, and the highly selective retention of B23 by the Rev column from a crude nuclear extract suggests specificity for this interaction.

We also prepared a number of control affinity columns. Rev is a basic protein with a pI of 9.46 (unpublished observations). For this reason, we prepared an affinity column with egg white lysozyme, which is also a basic protein (pI ~ 11) and is similar in size to Rev. No specific retention of B23 by the lysozyme affinity column was observed (Table 1). Similarly, B23 did not bind to an affinity column composed of BSA (Table 1). Curiously, we did observe some retention of B23 on a histone affinity column, albeit with a lower affinity, as judged from the sensitivity to elution by KCl. The gel pattern of the histone column is also shown in Fig. 1 (lanes 6, 8, and 10). The B23 protein (positively identified as such below) was eluted from the histone column, together with a number of other proteins, predominantly in the 0.6 M KCl eluent (lane 8). Only a trace of this protein was observed in the 1.0 M KCl eluent (lane 10).

The histone affinity column used above was prepared with a mixture of rat liver core and H1 histones. However, our analysis indicated a preferential coupling of histone H1 to this control column. Histone H1 is a very sticky protein which interacts nonspecifically with many different proteins, and the retention of B23 on the histone (histone H1) column is unlikely to be specific. Thus, histone H1 is one of the major proteins retained when nuclear extracts are applied to several affinity columns. These affinity columns were prepared with BSA, topoisomerase II (1), total rabbit serum (see Fig. 3), and Rev (see Fig. 5). Consistent with the general stickiness of histone H1 for many different proteins is a comparison of the gel patterns of the 0.3 M KCl eluent derived from the histone and Rev affinity columns (Fig. 1, lanes 6 and 7). Whereas numerous proteins are eluted from the histone column at this salt concentration (lane 6), the pattern derived from the Rev column is much less complex (lane 7).

To establish further the nonspecific nature of the H1-B23 interaction, we applied a rat liver nuclear extract to both Rev and histone (histone H1) affinity columns in the presence of salmon sperm DNA as a competitor. Figure 2 shows the protein gel patterns of the different salt eluents derived from these columns. In the presence of DNA, B23 was no longer significantly retained on the histone column and was observed mainly in the flowthrough fraction (Fig. 2, lane 4).

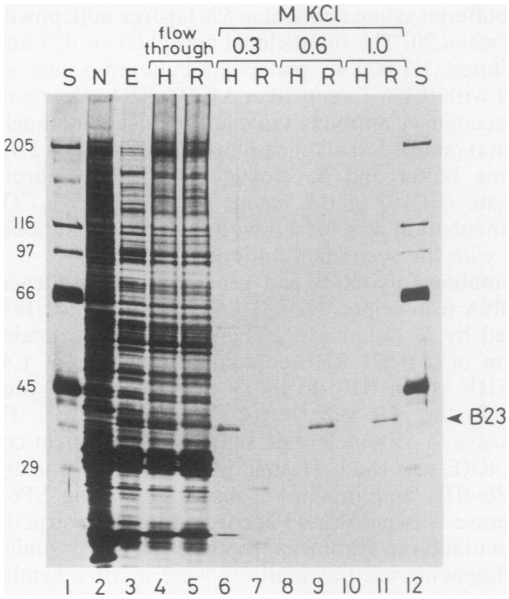


FIG. 2. Stability of Rev-B23 complexes in the presence of DNA. A rat liver nuclear extract was fractionated in parallel on Rev and histone affinity columns as described in the legend to Fig. 1 in the presence of competitor DNA. Loading was done in the presence of 1.5 mg of sonicated salmon sperm DNA per ml. Elution steps (indicated above the lanes) were carried out at 0.3, 0.6, and 1.0 M KCl containing 5 μg of salmon sperm DNA per ml. The position of B23 protein is indicated by the arrowhead. Four times more sample was applied to lanes 6 to 11 than to the other lanes. Other details are as for Fig. 1.

The B23 band observed in the 0.3 M KCl eluent (lane 6) represents only a minor fraction since we overloaded this sample fourfold. This is also obvious from a comparison of the B23 staining intensities in the flowthrough and input samples, which are visibly similar (lanes 3 and 4, respectively). The interaction of B23 with Rev, however, was maintained in the presence of competitor DNA. About half of the B23 protein was eluted in the 1.0 M KCl eluent (lane 11), and the remainder was located in the 0.6 M KCl eluent (lane 9). It is noteworthy that B23 was quantitatively bound to the Rev column, since it was absent in the gel pattern of the flowthrough fraction (lane 5). Moreover, closer inspection of the gel shows that the addition of DNA increased the relative enrichment of B23 in the 0.6 and 1.0 M salt elutions of the Rev column, as many of the minor protein bands observed without DNA competitor are no longer found in the high-salt elutions (compare Fig. 1 and 2, lanes 9).

**Immunoaffinity chromatography.** The interaction between Rev and B23 was also tested by immunoaffinity chromatography. For that purpose, we coupled a specific antiserum raised against Rev to an Affi-Gel 10 support. To two separate columns we applied equal amounts of rat liver nuclear extract, but to one extract we added Rev protein prior to loading to allow formation of the expected Rev-B23 complex. Rev would then be expected to mediate the binding of B23 to the anti-Rev serum column, the second column serving as a control to detect nonspecific binding. Elution with KCl was performed as described above for the Rev affinity column except that the last elution step was 100 mM glycine (pH 2.5) instead of 1.0 M KCl. Most nuclear proteins did not exhibit any affinity for the columns and were recovered in the flowthrough fractions (Fig. 3, lanes 4 and 5).

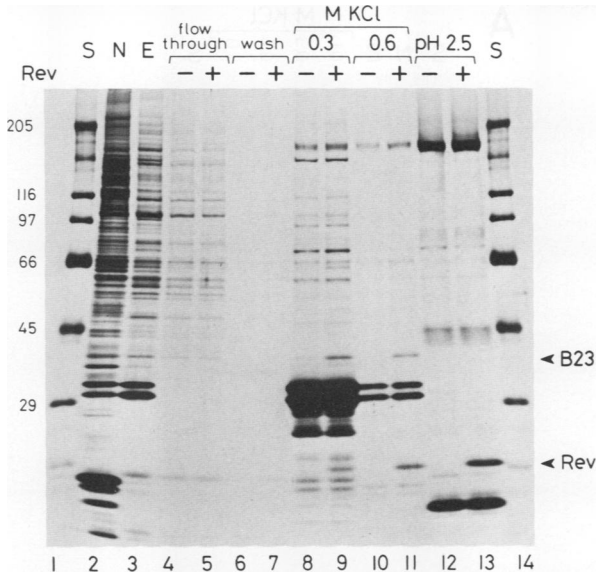


FIG. 3. Immunoadsorption of Rev-B23 complexes. Rabbit polyclonal antibody against Rev protein was coupled to Affi-Gel 10, and two identical columns were prepared. Equal amounts of rat liver nuclear extract were applied to each of two columns. Rev protein was added to one of the extracts prior to loading (+) but not to the other (−). Elution steps are indicated above the lanes. Arrowheads mark the positions of B23 and Rev proteins. Equal amounts of nuclei (N), nuclear extract (E), and flowthrough but 50 times more of the KCl and glycine eluates were applied to the SDS-gel. Other details are as for Fig. 1.

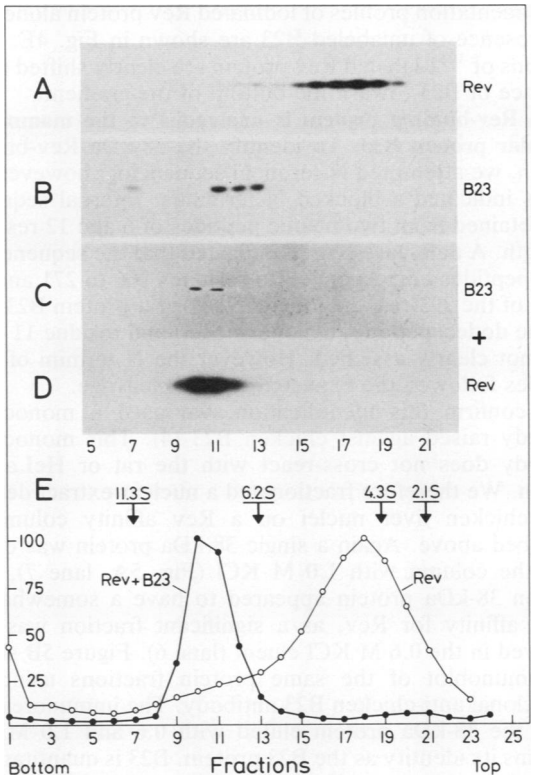


FIG. 4. Sedimentation analysis of Rev-B23 protein complexes. Rev and B23 proteins or their complex were subjected to sedimentation on 5 to 30% linear sucrose gradients. Sedimentation was from right to left. Fractions numbers are indicated below the lanes. (A and B) Sedimentation positions of iodinated Rev and B23 proteins applied individually to separate gradients. (C and D) Sedimentation position of the Rev-B23 complex. In this case, the unlabeled B23 was revealed by silver staining (C) and the cosedimenting Rev protein was revealed by radioautography (D). (E) Sedimentation profiles for Rev protein in the absence (○) or presence (●) of B23 protein. The sedimentation positions of several markers are indicated by arrows and correspond to horse heart cytochrome *c* (2.1S), beef blood hemoglobin (4.3S), calf intestine alkaline phosphatase (6.2S), and beef liver catalase (11.3S).

Histone H1 was eluted with 0.3 and 0.6 M KCl from either column, demonstrating again the general stickiness of H1, in this case for a complex mixture of serum proteins (lanes 8 to 11). The gel patterns of the two columns were identical with one exception: the B23 protein (indicated by an arrowhead) was observed in the 0.3 and 0.6 M KCl elution steps derived from the Rev-containing extract. Nonspecific binding of B23 on the control column was not observed (lanes 8 and 10). Thus, the anti-Rev serum column specifically retained the Rev-B23 complex. Besides B23, we occasionally observed other minor protein differences in this experiment which were not further analyzed. Rev protein was primarily observed in the elution step containing 100 mM glycine (pH 2.5), which dissociated the antigen-antibody interaction (lane 13). A small amount of Rev also coeluted with B23 in the 0.3 and 0.6 M KCl eluents, probably because of a weakening of the immunological interaction of Rev complexed with B23.

From the results of presented above, we conclude that the Rev-B23 interaction occurs despite the compositional complexity of the total nuclear extract, adding further support for the specificity of the interaction.

**Rev-B23 interaction in solution.** Finally, the specific interaction between Rev and B23 was studied by sedimentation analysis. Iodinated Rev protein was prepared and applied to linear sucrose gradients, either alone or together with unlabeled B23. The sedimentation behavior of B23 alone was examined in a parallel gradient. Proteins separated in sucrose fractions were resolved on SDS-polyacrylamide gels and visualized by silver staining followed by radioautography (Fig. 4A to D). An S value of about 5S was estimated for Rev protein, suggesting that the protein exists as a multimer (Fig. 4A). B23 has a sedimentation coefficient of about 7S, but a small amount of this protein cosediments with the 11.3S marker (Fig. 4B). Interestingly, our S value estimation for B23 correlates with that of Schmidt-Zachmann et al. (39), who obtained a sedimentation coefficient of 7S for the *Xenopus* No38 protein. These authors suggested that this protein is either pentameric or hexameric in solution. Yung and Chan (43) have also observed homo-oligomers of 10S for HeLa B23 and proposed that the protein exists as an hexamer of 230 kDa composed of four α and two β monomers.

Mixtures of B23 and Rev cosedimented, indicating complex formation (Fig. 4C and D). In the presence of B23, Rev no longer sedimented near the top of the gradient but cofractionated with B23 protein in a 9S peak (Fig. 4, fractions 10 and 11). Figure 4C shows the silver-stained gel to reveal B23; in Fig. 4D, Rev is identified in the radioautograph. It is difficult to calculate the stoichiometric ratio of B23 and Rev since we have not carried out titration experiments. But assuming no free B23 in the leading edge of the B23-Rev peak, we estimate a B23-to-Rev ratio of about 1.

Sedimentation profiles of iodinated Rev protein alone or in the presence of unlabeled B23 are shown in Fig. 4E. Peak fractions of $^{125}$I-labeled Rev protein are clearly shifted in the presence of B23 toward the bottom of the gradient.

**The Rev-binding protein is analogous to the mammalian nucleolar protein B23.** To identify the 38-kDa Rev-binding protein, we attempted N-terminal sequencing; however, the results indicated a blocked N terminus. Internal sequence was obtained from two tryptic peptides of 6 and 12 residues in length. A data base search indicated that the sequences of these peptides corresponded to residues 266 to 271 and 276 to 287 of the 293-residue-long nucleolar rat protein B23 (10). For the dodecapeptide, residue 1 (Met) and residue 11 (Trp) were not clearly assigned. However the N termini of both peptides followed the expected tryptic cleavage.

To confirm this identification, we used a monoclonal antibody raised against chicken B23 (4). This monoclonal antibody does not cross-react with the rat or HeLa B23 protein. We therefore fractionated a nuclear extract derived from chicken liver nuclei on a Rev affinity column as described above. Again a single 38-kDa protein was eluted from the column with 1.0 M KCl (Fig. 5A, lane 7). This chicken 38-kDa protein appeared to have a somewhat reduced affinity for Rev, as a significant fraction was also observed in the 0.6 M KCl eluent (lane 6). Figure 5B shows an immunoblot of the same protein fractions using the monoclonal anti-chicken B23 antibody. The immunoreactivity of the 38-kDa protein eluted with 0.6 and 1.0 M KCl confirms its identity as the B23 protein. B23 is quantitatively retained on the Rev affinity column, as is evident from a comparison of the input extract with the flowthrough fraction and the 0.3 M KCl eluent (Fig. 5B, lanes 3, 4, and 5, respectively). We also loaded as a positive control total cellular proteins from chicken DU249 hepatoma cells; B23 was the only protein detected by this highly specific antiserum in this total cell extract (lane 1).

These results clearly identify the major Rev-binding protein as the nucleolar protein B23 (6, 8). As B23 is homologous to the amphibian protein No38 (39), we also applied a total cytoplasmic extract derived from *Xenopus* eggs to a Rev affinity column. The immunoreactivity of the proteins eluted from this column, using a monoclonal antibody specific for No38 (39), demonstrated the quantitative partitioning of this protein into the 1.0 M KCl eluent. This result further demonstrates the high degree of specificity of the Rev and No38 or B23 interaction. We should mention, however, that in the affinity chromatography of the *Xenopus* extract, a considerable number of other proteins also coeluted with No38 (data not shown).

**B23-Rev complex is dissociated by the RRE.** Rev protein binds in a sequence-specific manner to the RRE (13, 33, 44). Does B23 interfere with or facilitate the specific interaction of Rev with its RNA target element? Gel retardation assays and sucrose sedimentation analysis were carried out to elucidate these questions. We used two control RNA fragments of 906 and 255 bases and an RRE-containing RNA of 550 bases (Fig. 6, lanes 1, 5, and 9). Yeast tRNA was chosen as an unlabeled nonspecific competitor.

Upon addition of Rev, a discreet shift of the RRE-containing fragment was observed (Fig. 6; indicated by an arrowhead; lanes 2, 6, and 10). No mobility shift was observed following addition of B23 alone at increasing concentrations to the RNA probes, indicating no RNA-binding capacity for this protein (lanes 4, 8, and 12). Interestingly, the mobility shift of the RRE-Rev complex remained unaltered when increasing amounts of B23 were added to a fixed amount of Rev protein (lanes 3, 7, and 11). This result showed that B23 does not facilitate or interfere with the specific interaction of Rev with the RRE RNA. Moreover, if B23 and Rev were able to bind to the RNA as a complex, we would expect a mobility shift in addition to that caused by Rev alone, and this was not observed.

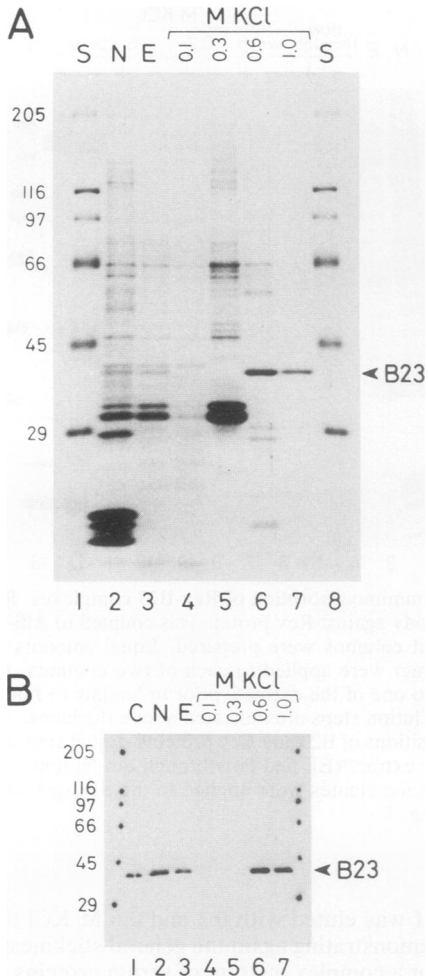


FIG. 5. Interaction of anti-chicken B23 monoclonal antibody with chicken major Rev-binding protein. A chicken liver nuclear extract was applied to a Rev affinity column and eluted as described in the legend to Fig. 1. Sample preparation and gel conditions were as described for HeLa and rat liver nuclear extracts. (A) Coomassie blue-stained gel of eluted fractions. (B) The same samples shown in panel A plus a total chicken cell extract (lane 1). The extracts were loaded onto an SDS-polyacrylamide minigel, transferred to a nitrocellulose filter, and probed by immunoblotting using anti-chicken B23 monoclonal antibody as described in Materials and Methods. Lanes: S, marker proteins; 1, total cell extract from chicken DU249 hepatoma cells (C); 2, chicken liver nuclei (N); 3, input chicken liver nuclear extract (E); 4, flowthrough. The elution steps are indicated above the lanes. Chicken B23 protein is indicated by an arrowhead. Positions of size standards (in kilodaltons) are shown on the left.

In the aforementioned experiment, Rev and the RNA probes were mixed prior to addition of B23 protein. We also added the components in all possible combinations, but we noted an unchanged mobility of the RRE-Rev protein complex (not shown). This finding suggests the following: Rev is likely to bind to the RRE RNA without the assistance of B23, and by inference, the Rev-B23 complex, which is very

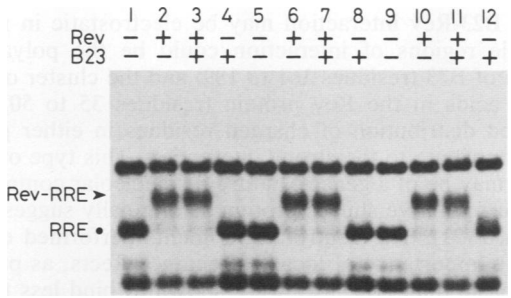


FIG. 6. Evidence that B23 does not facilitate or interfere with the binding of Rev to the RRE RNA. A gel retardation assay was carried out using an RNA probe composed of three internally labeled RNA fragments of 906, 550, and 255 nucleotides. The 550-base RNA fragment contains the RRE. Rev protein was added to the RNA mixtures and incubated for 15 min at 30°C. After the incubation period, B23 protein was added to the indicated samples and incubation was carried out for an additional 15 min. Samples were loaded onto a 1.3% agarose gel in TBE buffer. Rev concentrations were 5 ng/μl on lanes 2, 3, 6, 7, 10, and 11. The B23 concentration was 0 for the samples analyzed on lanes 1, 2, 5, 6, 9, and 10, 1.5 ng/μl for lanes 3 and 4, 3 ng/μl for lanes 7 and 8, and 6 ng/μl for lanes 11 and 12. The circle indicates the RRE-containing RNA fragment; the arrowhead indicates the altered mobility of the RRE-containing RNA in the presence of Rev.

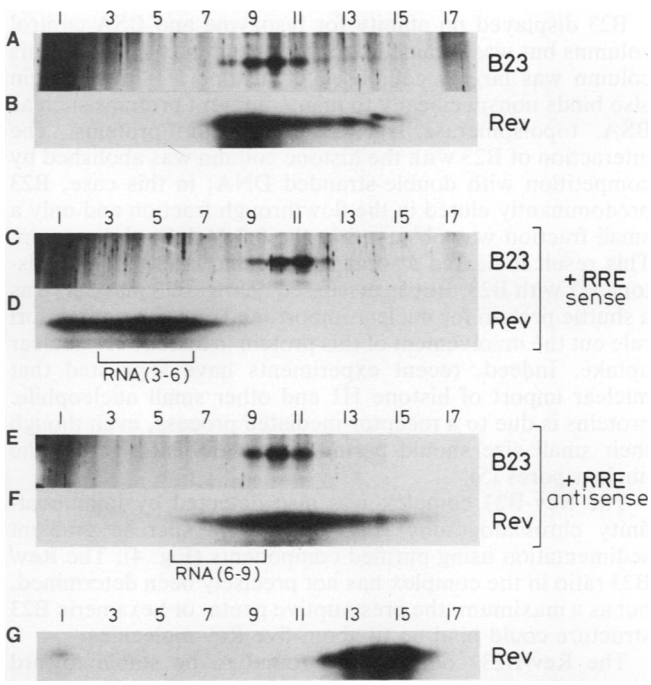


FIG. 7. Dissociation of Rev-B23 protein complexes by the RRE RNA. Rev-B23 complex was prepared by mixing iodinated Rev protein with unlabeled B23 protein. The complex was challenged with RNA fragments containing sense or antisense RRE in the presence of yeast tRNA. Samples were layered on top of a 5 to 30% linear sucrose gradient. Sedimentation was from right to left. Panels A to G represent SDS-gels of the individual fractions from the separate gradients. Fractions numbers are indicated above the lanes. B23 protein was detected by silver staining (A, C, and E), and Rev protein was detected by radioautography (B, D, F, and G). Positions of the labeled RNA were monitored by Cerenkov counting and are indicated by brackets below the corresponding panels. (A and B) Rev-B23 complex as observed without competitor RNA; (C and D) dissociation of the Rev-B23 complexes in the presence of sense RRE RNA; (E and F) stability of the Rev-B23 complexes in the presence of the antisense RNA fragment; (G) sedimentation position of Rev protein run alone.

stable in the absence of RNA, appears to be dissociated upon addition of RNA (tRNA and the radioactive probes).

Is the Rev-B23 complex dissociated specifically by the RRE-containing RNA or nonspecifically by any RNA? We used sucrose gradient analysis to study this question. Iodinated Rev and nonradioactive B23 proteins were mixed to allow complex formation. This sample was subsequently split and incubated in the presence of competitor tRNA with either sense or antisense $^{32}$P-labeled RNA fragments containing the RRE. The samples were centrifuged on linear 5 to 30% sucrose gradients, and the individual gradient fractions were subjected to SDS electrophoresis. The results indicated that B23 and Rev cosedimented, forming a complex in the absence of added RNA, as revealed by silver staining (Fig. 7A) and radioautography (Fig. 7B). Both proteins were recovered in fractions 8 to 11. (The heavy staining in lane 1 of Fig. 7A was due to the gelatin used to coat the tubes; see Materials and Methods.) If Rev was applied to the gradient alone, it sedimented near the top of the gradient (Fig. 7G, fractions 12 to 16).

Preincubation of the Rev-B23 complex with RRE RNA prior to loading onto the gradient resulted in dissociation of the protein complex. Rev protein was bound to the RNA, as it cosedimented with the RNA fragment to near the bottom of the gradient (Fig. 7D, fractions 1 to 6). The dissociated B23, however, was observed in fractions 9 to 12 (Fig. 7C), which corresponds to its usual sedimentation position without the addition of RNA.

The dissociation of the Rev-B23 complex required an RRE-containing RNA. This was demonstrated in a control experiment using antisense RNA. In the presence of the antisense RNA, Rev-B23 complexes were stable, since the bulk of both proteins, B23 and Rev, cosedimented in fractions 9 to 11 (Fig. 7E and F, respectively). In addition, the antisense RNA sedimented higher up in the gradient (Fig. 7F; indicated with a bracket) as expected, since it was not complexed with Rev.

These results confirm and extend those obtained for the gel retardation assay: the results demonstrate that RRE-containing RNAs can specifically dissociate Rev-B23 protein complexes.

## DISCUSSION

The nucleolar protein B23 is the major protein in a crude nuclear extract which specifically and tightly interacted with the HIV-1 Rev protein. Rev is one of the key proteins in viral propagation and is thought to mediate, via the RRE, the export of singly spliced and full-length viral RNAs from the nucleus to the cytoplasm. In the absence of the Rev function, transition from the early to the late phase of viral gene expression cannot occur, and as a consequence the provirus becomes replication defective (16, 40).

A Rev affinity column selectively retrieves B23 from a complex nuclear extract derived from HeLa, rat liver, or chicken liver nuclei. The elution of B23 from a Rev affinity column required between 0.6 and 1.0 M KCl, which was indicative of a tight association. B23 was the major component in the high-salt eluates and ran similarly when reapplied to a second affinity column following digestion with various nucleases, supporting the view for a protein-protein interaction.

B23 displayed no affinity for lysozyme and BSA control columns but was retained by a histone affinity column. This column was largely composed of histone H1; this protein also binds nonspecifically to many different proteins such as BSA, topoisomerase II, Rev, and serum proteins. The interaction of B23 with the histone column was abolished by competition with double-stranded DNA; in this case, B23 predominantly eluted in the flowthrough fraction and only a small fraction was observed in the 0.3 M KCl elution step. This result indicated a weak nonspecific interaction of histone H1 with B23. But as discussed below, B23 may serve as a shuttle protein for nuclear import, and one can not a priori rule out the involvement of this protein in histone H1 nuclear uptake. Indeed, recent experiments have suggested that nuclear import of histone H1 and other small nucleophilic proteins is due to a receptor-mediated process, even though their small size should permit simple diffusion across the nuclear pores (5).

The Rev-B23 complex was also detected by immunoaffinity chromatography (Fig. 3) and by sucrose gradient sedimentation using purified components (Fig. 4). The Rev/B23 ratio in the complex has not precisely been determined, but as a maximum, the presumptive penta- or hexameric B23 structure could bind up to about five Rev molecules.

The Rev-B23 complex was found to be stable toward dissociation by double-stranded DNA but was dissociated by an RRE-containing RNA. The RRE RNA having a higher affinity for Rev than B23 displaced the latter, as determined by sedimentation analysis. As a control, we used the antisense RNA, which did not affect the stability of the Rev-B23 complex. The specific dissociation of the Rev-B23 complex by RRE RNA is of interest in view of a possible in vivo function of this interaction, as discussed below.

B23 migrates constantly between nucleus and cytoplasm, acting as a shuttle protein for the nucleocytoplasmic transport of ribosomal components (4). It is possible that Rev takes advantage of this shuttle service, being imported into the nucleus as a Rev-B23 complex. Given that the Rev-B23 complex can be specifically dissociated in vitro by the RRE RNA, we might also assume a similar exchange reaction taking place in the nucleus. The RRE-containing RNA complexed with Rev would then be ready for nuclear export, thus closing the cycle.

Despite the proposed role of Rev in nuclear export of unspliced viral RNAs, this protein localizes into the nucleolus, as observed by immunofluorescence studies (12, 18, 31, 37). Several authors have discussed for this reason the possible involvement of the nucleolus or some nucleolar factor in Rev function. B23 is a major nucleolar component (6), and the fact that it forms a tight complex with HIV Rev, as established in this report, provides a rationale for the localization of Rev in the nucleolus. The Rev-B23 complex in the nucleolus may also serve as a storage device, from which Rev is recruited, as RRE-containing RNAs become available.

Rev protein contains a nucleolar localization signal (NOS) which may directly target this protein to the nucleolus via a receptor-mediated process (28). Whether such a receptor is cytoplasmic or form parts of the nuclear pore complex is not known. Evidence for cytosolic or nuclear receptors which bind proteins harboring nuclear localization signals has been reported (for a review, see Goldfarb [21]). Similarly, such soluble receptors may exist for proteins containing nucleolar localization signals. Thus, despite the NOS sequence in Rev, a mediator might be necessary and B23 could carry out this role.

The B23-Rev interaction may be electrostatic in nature. Possible regions of interaction could be the polyanionic stretch of B23 (residues 161 to 189) and the cluster of basic amino acids in the Rev protein (residues 35 to 50). This sectored distribution of charged residues in either protein may contribute to the strong interaction. This type of interaction may be of a general nature for nucleolar components and their putative shuttle protein as originally suggested by Goldfarb (21). The control experiments performed emphasize the importance of localized charge effects, as proteins more basic than HIV Rev were shown to bind less tightly, such as lysozyme and histone H1, as shown in this study.

Affinity column chromatography has often been successfully used to discover biologically important protein-protein interactions such as between the proteins of the bacteriophage T4 replication complex (20). The biological importance for the Rev-B23 interaction reported here has not been established, but an experimental study of the in vivo role of B23 is feasible and of importance, given the key role played by Rev in HIV replication.

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