In Vitro Binding of Human T-Cell Leukemia Virus *rex* Proteins to the *rex*-Response Element of Viral Transcripts

RALPH GRASSMANN,^{1*} SUSANNE BERCHTOLD,¹ CHRISTIAN AEPINUS,¹ CLAUDIA BALLAUN,²
ERNST BOEHNLEIN,² AND BERNHARD FLECKENSTEIN¹

Institut für Klinische und Molekulare Virologie der Universität Erlangen-Nürnberg, Loschgestrasse 7, D-8520 Erlangen, Federal Republic of Germany,¹ and Sandoz Research Institute, A-1235 Vienna, Austria²

Received 30 November 1990/Accepted 26 March 1991

Human T-cell leukemia virus (HTLV-I, HTLV-II) *rex* protein function is required for the cytoplasmic expression of incompletely spliced viral transcripts encoding structural proteins. The effect is mediated by a *cis*-acting *rex*-response element (RRX) which is located near the 3' end of all viral mRNAs. We show that *rex* polypeptides of HTLV-I and HTLV-II expressed in *Escherichia coli* are capable of specifically binding RRX-containing transcripts of both viruses in cell-free assays. Binding analyses with deletion variants of *rex* proteins revealed a domain with RNA-binding activity in the first 77 N-terminal amino acids. Removal of a basic peptide of 19 amino acids from the N terminus abrogated RNA binding, whereas a β -galactosidase fusion protein containing this peptide bound to the RRX. These results suggest that direct binding of *rex* protein to the RRX is important for *rex*-mediated regulation of viral gene expression and that a short stretch of positively charged amino acids contributes to the specific binding of *rex* to its target RNA.

Human T-cell leukemia virus (HTLV) genomes contain, in addition to the genes common to all retroviruses, a segment of about 1.6 kb termed the X region (21, 27). The X region is located near the 3' terminus of the viral genome and is related to the T-lymphocyte-immortalizing phenotype of HTLV-I (8). The X genes are translated in two different reading frames from a doubly spliced mRNA (15, 22, 34). The HTLV-I transcript codes for at least two *trans*-acting regulatory proteins, the 40-kDa *tax* protein, a transcriptional activator (4, 24, 30), and the 27-kDa phosphoprotein, *rex* (11). The equivalent HTLV-II mRNA is translated into proteins of 38 and 26 kDa (25, 26). The *rex* protein is localized mainly within the nucleoli of infected cells; this is mediated by a highly basic sequence motif (nucleolar localization signal [NLS]) at the amino terminus (29). The HTLV-I *rex* function is required for the expression of the structural proteins *gag* and *env* (10), facilitating the transport of unspliced or singly spliced mRNA from the nucleus into the cytoplasm (9). Viral mRNA contains a sequence near the 3' terminus that mediates *rex* responsiveness (9, 23). The *rex*-response element of HTLV-I (RRX1) resides within a sequence of about 280 nucleotides (nt) that is homologous to the U3 and R region of the viral long terminal repeat (LTR) and can form a stable secondary structure (32). To get some insight into the mechanism of selective *rex*-regulated HTLV mRNA export, we expressed the *rex* gene in *Escherichia coli* and analyzed the RNA-binding properties of *rex* protein by using Northwestern (RNA-protein) blot and filter-binding analyses. Our data indicate that *rex* polypeptides bind specifically to RRX-containing in vitro transcripts and suggest that the RNA-binding domain, at least in part, is localized at the amino terminus of the protein.

MATERIALS AND METHODS

Construction of *rex* expression plasmids. HTLV *rex* polypeptides were expressed as fusion proteins by using the plasmid pROS comprising the 375 N-terminal amino acids of

β -galactosidase and the cleavage site of blood-clotting factor Xa, a sequence-specific protease (7). To obtain pROS-Rex1, the *rex* open reading frame (ORF) of HTLV-I (Fig. 1a), including its genuine start and stop codons, was taken from pLHcRex, a plasmid containing a 1.1-kb cDNA segment (Fig. 1b) (3). A 2.0-kb *Sph*I fragment was inserted into the *Sph*I site of pROSS, a variant of pROS having a *Sph*I octamer linker (New England BioLabs, Beverly, Mass.) inserted in the unique *Eco*RV site. The HTLV-II *rex* gene was obtained from pcXIIILTR, a plasmid containing a cDNA copy of the doubly spliced mRNA and a complete LTR (kindly provided by K. Shimotohno, National Cancer Research Institute, Tokyo, Japan). To construct the prokaryotic expression plasmid pROS-Rex2, a 1.8-kb *Sph*I-*Hind*III fragment was isolated and, after selective removal of the 3' protruding *Sph*I end, inserted between the *Eco*RV and *Hind*III sites of pROS. Proper insertion of the open reading frame was verified by DNA sequencing. Plasmids expressing carboxy-terminal-deleted *rex* fusion proteins were generated by removing *Hind*III-*Fsp*I (pRexN-126), *Hind*III-*Cla*I (pRexN-77), and *Hind*III-*Nco*I (pRexN-19) fragments from pROS-Rex1. Protruding ends were filled in with Klenow polymerase and religated. In analogy, plasmid pRex2N-19 was constructed by using pROS-Rex2 as progenitor. Mutants containing N-terminal-truncated *rex* open reading frames were obtained by deleting a *Sal*I-*Nco*I or a *Sal*I-*Acc*I fragment from pROS-Rex1 by Klenow polymerase treatment and by religation, resulting in the expression plasmids pRexC-170 and pRexC-154, respectively.

Expression and purification of *rex* proteins. Fusion proteins were isolated from transformed *E. coli* BMH 71-18 and W3110 after inducing the *lac* promoter (2, 7). The polypeptides could be visualized in Coomassie-stained sodium dodecyl sulfate (SDS) gels and were enriched as described previously (1, 7). Complete fusion proteins were isolated from preparative SDS-10% polyacrylamide gels by electroelution. The fusion proteins were cleaved with factor Xa (Boehringer, Mannheim, Federal Republic of Germany) into *rex* polypeptide and the β -galactosidase portion. Fusion proteins and eukaryotic cleavage products were identified by

* Corresponding author.

FIG. 1. Construction of prokaryotic *rex* expression plasmid and RRX in vitro transcription cassettes. (A) Genetic map of HTLV-I provirus, the open reading frame for p27^{rex}, and the doubly spliced mRNA. (B) Expression cloning of a *rex* β -galactosidase fusion protein (▨). A 2.0-kb *Sph*I fragment was isolated from the eukaryotic expression plasmid pLHcRex. It consists of 1.0 kb of cDNA derived from the doubly spliced mRNA (nt 253 to 1316) and vector sequences. It was cloned in the unique *Sph*I site of pROSS. (C) Origin of RRX-containing RNA probes obtained by in vitro transcription. RRXs, RRX3/8, and LTRs are sense transcripts; RRXa and LTRa are in the antisense orientation.

Western immunoblot analyses with anti-peptide antisera directed against the carboxyl termini of *rex* proteins. The fusion polypeptides isolated from bacteria transformed with pROS-Rex1 had a molecular mass of about 70 kDa. Factor Xa treatment yielded a cleavage product of about 27 kDa, which reacted with the anti-*rex1* rabbit serum (Fig. 2A). Cleavage of the 70-kDa *rex2* fusion protein yielded a *rex2*-specific polypeptide of about 26 kDa that stained specifically in Western blots. A *rex2*-specific antiserum recognizing the 30 C-terminal amino acids was kindly provided by K. Shimotohno.

Subcloning and in vitro transcription of RRXs. RRX DNA of HTLV-I was obtained by polymerase chain reaction amplification. The primers used contained recognition sequences for *Bgl*III (5' end) and *Hind*III (3' end). Amplified fragments were cloned into the *Bam*HI-*Hind*III sites of pBluescriptII KS- (Stratagene, La Jolla, Calif.), a plasmid providing bacteriophage T3 and T7 promoters. The resulting plasmid, pKSRRX25/26, contained a 286-bp (nt 302 to 574) fragment of HTLV-I DNA representing a complete RRX; plasmid pKSRRX3/8 contains a nonfunctional RRX subfragment of 137 bp (nt 334 to 471) (2b). Runoff transcripts were synthesized after linearization with *Hind*III (sense) or *Xba*I (antisense) by using T3 or T7 polymerase. For the generation of RRX2-containing transcripts, a 0.9-kb *Hind*III LTR fragment was isolated from pILTRCAT (28) and ligated into the

*Hind*III site of pBS- (Stratagene). Two resulting plasmids, pBSL3s and pBSL3a, containing the inserts in opposite orientations were used to generate sense and antisense transcripts initiating at the T3 promoter. To generate runoff transcripts, pBSL3s was cleaved with *Eco*RI and pBSL3a was cleaved with *Asp*718. In vitro transcripts were labeled by addition of 50 μ Ci of [α -³²P]UTP (400 μ Ci/mmol) and purified by phenol extraction, repeated ethanol precipitations, and, in some cases, additional Sephadex G-50 chromatography. Transcripts were monitored by Cerenkov counting and electrophoresis in 5% denaturing polyacrylamide gels. The method used results in RNA with specific activity of about 60 nCi/ng for all transcripts.

RNA-binding analysis. Northwestern blots were performed as described previously (1) with minor modifications. Briefly, protein preparations containing 2×10^{-11} to 6×10^{-11} mol of *rex* polypeptide were separated on SDS-polyacrylamide gels (10 to 17.5% acrylamide), electroblotted onto nitrocellulose (NC) membranes, and allowed to renature for at least 12 h in NW buffer (50 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone). Incubation was carried out in NW buffer which also contained ³²P-labeled in vitro transcripts (1×10^6 cpm), *E. coli* tRNA (200 μ g/ml), 0.02% bovine serum albumin (BSA), and 0.5 mM dithiothreitol. Blots were washed three

FIG. 2. *rex* protein binding with the *rex* RRR. (A) Western blot analysis of the *rex* protein expressed in *E. coli* to be used in binding studies. Factor Xa-treated (lane R) and untreated (lane F) *rexI* fusion protein were separated on an SDS-12.5% polyacrylamide gel and electroblotted. The NC filter was probed with an anti-peptide rabbit serum directed against amino acids 173 to 189 of *rexI*. (B) Northwestern blot with RRR1 in vitro transcripts. Factor Xa-digested *rexI* fusion protein (lanes R), partially purified β-galactosidase (lanes G) derived from vector pROSS, and protease factor Xa (1 μg) (lanes P) were electrophoresed and blotted. Filters derived from the same gel were probed with riboprobes HPV, RRRs, and RRRa. (C) Northwestern blot with HTLV-II specific transcripts. The NC-filter prepared as described for panel B was incubated with riboprobe LTRs. (D) Competition of *rex* binding to ³²P-labeled specific and nonspecific RNA fragments by unlabeled *E. coli* tRNA. Factor Xa-treated fusion protein purified from pROSS-*rexI*-infected cells was incubated with specific ³²P-labeled RRRs transcripts (○) and nonspecific ³²P-labeled human papillomavirus RNA (●) and in the presence of various amounts of unlabeled competing *E. coli* tRNA. Protein-RNA binding was measured by a filter-binding assay. The values represent an average of three independent experiments. The radioactivity bound in the absence of competing tRNA was taken as 100%.

times with NW buffer prior to drying and exposed to X-ray films.

To measure RNA-protein interaction in solution, we used NC filter-binding assays. Radioactively labeled in vitro transcripts (0.5×10^{-11} to 2×10^{-11} M) were incubated with factor Xa-cleaved *rexI* fusion protein (1.2×10^{-8} to 2.5×10^{-8} M) in the presence of various amounts of *E. coli* tRNA. Binding reactions were performed by using 1 ml of filter-binding buffer (50 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 0.2 mM dithiothreitol, 0.01% BSA, 20 U of RNasin per ml) for 15 min on ice. Protein-RNA complexes were recovered by filtration through 25-mm NC round filters (pore size, 45 μm; Schleicher und Schüll, Dassel, Germany) at a constant low flow rate. Filters were dried for 20 min at 37°C,

and bound radioactivity was evaluated by liquid scintillation counting.

RESULTS

Specific binding of *rex* to the RRR. To analyze the binding capacity of *rex* protein to radiolabeled RRR transcripts, we used the Northwestern blotting assay, which was previously used successfully to demonstrate specific binding of the human immunodeficiency virus proteins *rev* and *tat* to their corresponding RNA targets, the *rev*-response element and the *tat*-response element, respectively (1, 14, 35). The factor Xa-cleaved polypeptides were separated by SDS-gel electrophoresis, transferred to NC filters, and incubated with

32 P-labeled in vitro transcripts. Several independent experiments demonstrated that recombinant *rex1* protein strongly binds sense transcripts of RRX1 (Fig. 2B, lane R), whereas neither factor Xa (Fig. 2B, lane P) nor β -galactosidase (Fig. 2B, lane G) shows any reaction. In agreement with previous results (9), this RRX1 sequence displayed biological activity (2a). A significantly weaker interaction was observed with the antisense transcript, RRXa (Fig. 2B). To estimate the difference in the RNA-binding capacity, the bound radioactivity from three independent Northwestern blot experiments was determined by liquid scintillation counting. Sense transcripts bound much more efficiently than antisense RRX RNA (9.8 times; standard deviation, 3.7). To analyze whether nonspecific binding to double-stranded RNA or complex RNA secondary structures could account for the affinity to antisense transcripts, we used a deleted version of RRX1 (RRX3/8) as the probe. This transcript of 137 nt (-0.4 kcal per base [-1.67 kJ per base]) essentially contains stem1 as well as stem-loops A, B, and C of RRX1 (32) and was shown to be biologically inactive (2b). Binding of RRX3/8 was far weaker and could be visualized only after extended exposure. Again, the radioactivity bound was estimated by liquid scintillation counting. There was no significant difference from background values, but the activity was 12- to 17-fold lower than with complete sense transcripts. In vitro RNA was also generated from plasmid pEBF33, a pBS derivative containing a 554-bp insert from the human papillomavirus type 33 E2 region (nt 2796 to 3356) (5). Computer predictions suggest that the corresponding RNA probably does not form a highly stable secondary structure (-0.18 kcal per base [-0.75 kJ per base]). As expected, this in vitro transcript did not yield any detectable signals (Fig. 2B), even after long exposure.

To demonstrate specific binding of dissolved *rex* protein to RRX1, we also analyzed complex formation by protein-mediated retention of radioactive RNA on NC filters (filter-binding assay). An indirect competition assay was used to determine the relative affinities of *rex* to its natural target RNA and to an unspecific transcript. *rex* protein in excess was incubated with labeled RRXs and human papillomavirus transcripts in the presence of various concentrations of unlabeled competing tRNA (Fig. 2D). Competitor concentrations required to reduce radioactive complex formation to 50% were taken as a measure of the relative binding affinities. To obtain 50% reduction of complex formation, 40 to 50 ng of tRNA per ml was required when the unrelated human papillomavirus transcript was used as the labeled probe. In contrast, however, about 20 μ g/ml was necessary to achieve 50% inhibition of *rex*-RRXs formation, which demonstrates that the affinity of *rex* to RRXs is about 400-fold higher than its affinity to an unrelated RNA.

Binding properties of *rex2* protein and RRX2. *rex* polypeptides from HTLV-I and HTLV-II can functionally substitute for each other (9). We found specific binding in Northwestern blots when HTLV-I *rex* protein was probed with HTLV-II LTR transcripts (Fig. 2C). It was comparable in strength to the binding with RRX1 sense transcript, whereas antisense RNA (LTRa) (Fig. 1C) resulted in a significantly weaker signal. A very similar pattern of physical interaction was detected when *rex2* proteins were probed with HTLV-I sense (RRXs) and antisense (RRXa) transcripts (Fig. 3). Again, the papillomavirus transcripts did not lead to any detectable interactions with *rex2* protein.

Localization of RNA-binding activity within the *rex* protein. To map domains of *rex1* required for RNA binding, we generated a series of deletion mutants of the procaryotic

FIG. 3. Binding of RRX to *rex* protein of HTLV-II. Factor Xa-digested fusion proteins derived from pROS-Rex2 (lane 1) and pROS-Rex1 (lane 2) were separated on an SDS-17.5% polyacrylamide gel and transferred to NC filters. The corresponding blot was cut into two equal filter strips and probed with RRXs and RRXa. Lane 3 contains a β -galactosidase fragment expressed from pROS; lane 4 contains factor Xa.

expression plasmid pROS-Rex1. Fusion proteins derived from plasmids pRexN-126, pRexN-77, and pRexN-19 are truncated at the carboxy terminus; fusion proteins from pRexC-154 and pRexC-170 suffer N-terminal deletions in the *rex* portion (Fig. 4). The plasmids were named according to the number of *rex*-corresponding amino acids retained in the peptides. Factor Xa-cleaved fusion proteins with C-terminal deletions (N-126, N-77) bound as efficiently as full-length protein to RRXs (Fig. 5A, left panel, lanes 1 to 3). Interaction with RRXa and RRX3/8 transcripts was significantly lower, confirming the specificity of the reaction (Fig. 5A, middle and right panels, lanes 1 to 3). The deleted peptides, however, exhibited less-specific binding than did full-length protein. These experiments indicate the presence of an RNA-binding domain within the first 77 amino acids of *rex1*. To map its location more precisely, two fusion proteins containing N-terminally deleted *rex* polypeptides were treated with factor Xa and tested in Northwestern blots. The *rex* polypeptides with deletions of 35 (C-154) or 19 (C-170) amino acids did not show any binding activity even after

FIG. 4. Diagram of truncated *rex* fusion proteins. The *rex* portion is depicted as a black bar; deletions are indicated as thin lines.

FIG. 5. Binding of C-terminal-truncated *rex* protein to RRX RNA. (A) Northwestern blot with factor Xa-cleaved fusion proteins. Equal filter strips derived from a single SDS-17.5% polyacrylamide gel were incubated with ³²P-labeled transcripts RRXs, RRXa, and RRX3/8. Proteins were purified from *E. coli* transfected with pROS-Rex1 (lane 1), pRex-N126 (lane 2), pRex-N77 (lane 3), and pROSS (lane 4). Lane 5 contains factor Xa. (B) Northwestern blots of fusion proteins N-19. Equal filter strips were obtained from a single SDS-17.5% polyacrylamide gel loaded with the fusion proteins N-19 (lane 1) and C-170 (lane 2) and with β -galactosidase (lane 3). The riboprobes used were the same as in panel A.

very long exposure times (Fig. 6). This suggested that the first 19 amino acids, a highly basic region which also contains the NLS (29), is required for RRX binding in vitro. A β -galactosidase fusion protein (N-19) containing the NLS was tested in Northwestern blots. This polypeptide bound RNA with a clear preference for RRXs (Fig. 5B, left panel). The control transcripts, RRXa and RRX3/8, bound much more weakly, resulting in signals with 5- and 15-fold less intensity, respectively (Fig. 5B, middle and right panels). Analogous results were obtained with protein expressed from pRex2-N19 (N-19₂), a fusion protein including the homologous sequence motif of HTLV-II. The polypeptide N-19₂ bound equally strongly to the sense transcripts RRXs and LTRs (Fig. 1B), whereas antisense or RRX3/8 tran-

scripts bound significantly more weakly (data not shown). In summary, these experiments suggest that the known NLSs (29) are able to confer RRX-binding specificity to a β -galactosidase fusion protein.

DISCUSSION

The *rex*-regulated nuclear export of incompletely spliced HTLV mRNA is mediated by the RRX sequence (9, 23, 32). Using Northwestern blot and filter-binding analyses, we demonstrated that *rex* polypeptides bind specifically to in vitro-generated RRX transcripts and less specifically to a biologically inactive deletion variant or to unrelated RNA. This strongly suggests that direct complexing of *rex* protein

FIG. 6. Absence of RNA-binding capacity in N-terminal-truncated *rex* proteins. (A) Western blot of fusion proteins to confirm correct translation of the C terminus of *rex*. Proteins were separated on an SDS-10% polyacrylamide gel and electroblotted. The NC filter was probed with an antipeptide antiserum directed against amino acids 173 to 189 of *rex1*. (B) Northwestern blot analysis of factor Xa-treated fusion proteins. Filters derived from an SDS-17.5% polyacrylamide gel were probed with the RRXs (sense) transcript. Lanes: 1, N-77; 2, C-154; 3, C-170.

to RRX RNA is a functionally important step in the nuclear export of incompletely spliced retroviral mRNA. The sense transcripts from both HTLV-I and HTLV-II interacted equally well with *rex* polypeptides of both viruses. This is consistent with the observation that *rex2* can fully substitute for *rex1* function in vivo (9). Our studies showed a weaker, although significant, in vitro binding of HTLV RRX antisense transcripts. Computer predictions indicate that RRX antisense RNA forms a mirror-image secondary structure including identical double-stranded sequences. Our results agree with results of previous studies suggesting that the secondary structure of HTLV RRX is important for *rex* function (32). Preliminary experiments have also shown that in vitro-transcribed RNA from the HIV-1 *rev*-response element, which also has the potential to form a stable stem-loop structure (13), weakly interacts with *rex* polypeptides of both HTLV types. This is consistent with the observation that *rex* can functionally substitute for *rev* (12, 17) with lower efficiency (20, 31), although neither polypeptides nor RNA targets share recognizable sequence homologies. In conclusion, these observations suggest a model of specific *rex* binding to the RRX and less-specific binding to complex secondary structures. Although this work suggests that *rex* binding to RRX is a prerequisite, it is not sufficient for mediating the *rex* effect. Host proteins have been shown to be important for the biological activities of the RNA-binding HIV proteins *rev* and *tat* (6, 33). Cellular factors may directly interact with HTLV mRNA or also with the *rex* protein, as suggested by the existence of *trans*-dominant *rex* mutants (3, 18). The requirement for *rex* function of a 15-bp sequence element at map position 5171 to 5185 (23) could indicate a binding site for such a factor. An appropriate topological arrangement of *rex* and cellular factors on the viral RNA may be relevant for biological activity. This could explain the failure to detect in vivo function of reverse-positioned RRX (9).

RNA-binding analysis of the truncated *rex* proteins N-126 and N-77 resulted in specific recognition of the RRX by these peptides. The deleted peptides however, exhibited less specific binding than the wild-type protein. This loss in speci-

ficity could be due to the deletion of protein domains involved in the recognition of the RNA target. Alternatively, an altered secondary structure or partially nonspecific binding caused by the increasing isoelectric points of the shortened proteins could be responsible for the less-specific binding observed. These experiments indicate the presence of an RNA-binding domain within the first 77 N-terminal amino acids. Deletion of 35 or 19 amino acids from the N terminus of the *rex* protein completely abolished the RNA-binding capacity in Northwestern blot analyses. This could mean either that the first N-terminal amino acids are important for the generation of secondary or tertiary structures necessary for RNA binding or that this short stretch of amino acids directly interacts with RNA. The first 19 N-terminal amino acids (N-19 peptide) were attached to a β -galactosidase fragment that had no RNA-binding capacity by itself. The resulting chimeric polypeptide bound complete RRXs with significantly higher affinity than it bound biologically inactive RNA. Although it cannot be ruled out that β -galactosidase influences RNA binding by some nonspecific interaction, our experiments suggest that the N-19 peptide is involved in the RNA binding by HTLV *rex* protein. The N-19 peptide could either represent a part of a larger RNA-binding structure or represent the complete binding domain. This highly positively charged peptide sequence was previously described as an NLS (29), which was shown to be essential for the biological function of *rex* (16). Coincidence of RNA-binding domains consisting of short stretches of basic amino acids with NLSs were recently described for the HIV regulatory proteins *tat* and *rev* (17, 19, 36). A *tat* peptide of 14 amino acids containing the corresponding region was shown to specifically bind to a target structure within the *tat*-response element (19, 36). This indicates that a small basic oligopeptide is sufficient to produce a functional RNA-binding domain capable of recognizing a specific target structure. Bifunctional domains that bind to complex RNA structures and mediate nucleolar targeting may define a class of small regulatory proteins including the lentivirus proteins *tat* and *rev* as well as the HTLV *rex* proteins.

ACKNOWLEDGMENTS

The excellent technical assistance of Ingrid Buckreis is greatly appreciated. We thank E. Beyer-Finkler for providing pEBF33 and J. Hauber for providing plasmid pLHcRex.

This work was supported by Deutsche Forschungsgemeinschaft (Forschergruppe "DNA-Viren des hämatopoetischen Systems") and Projektträger AIDS des Bundesministeriums für Forschung und Technologie (FKZ II-100-89).

REFERENCES

1. Aepinus, C., R. Voll, M. Bröker, and B. Fleckenstein. 1990. A *rev*/ β -galactosidase fusion protein binds *in vitro* transcripts spanning the *rev*-responsive element of human immunodeficiency virus type 1 (HIV-1). *FEBS Lett.* 263:217-221.
2. Amann, E., and J. Brosius. 1985. 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* 40:183-190.
- 2a. Ballaun, C. Unpublished data.
- 2b. Ballaun, C., and E. Böhnlein. Unpublished data.
3. Böhnlein, S., F. P. Pirker, L. Hofer, K. Zimmermann, H. Bachmayer, E. Böhnlein, and J. Hauber. 1991. Transdominant repressors for human T-cell leukemia virus type I Rex and human immunodeficiency virus type 1 Rev function. *J. Virol.* 65:81-88.
4. Cann, A. J., J. D. Rosenblatt, W. Wachsman, N. P. Shah, and I. S. Y. Chen. 1985. Identification of the gene responsible for human T-cell leukaemia virus transcriptional regulation. *Nature*

- (London) 318:571-574.
5. Cole, S. T., and R. E. Streek. 1986. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. *J. Virol.* 58:991-995.
 6. Cullen, B. R. 1990. The HIV-1 *tat* protein: an RNA sequence-specific processivity factor? *Cell* 63:655-657.
 7. Ellinger, S., R. Glockshuber, G. Jahn, and A. Plückthun. 1989. Cleavage of procaryotically expressed human immunodeficiency virus fusion proteins by factor X_a and application in Western blot (immunoblot) assays. *J. Clin. Microbiol.* 27:971-976.
 8. Grassmann, R., C. Dengler, I. Müller-Fleckenstein, B. Fleckenstein, K. McGuire, M.-C. Dokhelar, J. G. Sodroski, and W. A. Haseltine. 1989. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a herpesvirus saimiri vector. *Proc. Natl. Acad. Sci. USA* 86:3351-3355.
 9. Hanly, S. M., L. T. Rimsky, M. H. Malim, J. H. Kim, J. Hauber, M. Duc Dodon, S.-Y. Le, J. V. Maizel, B. R. Cullen, and W. C. Greene. 1989. Comparative analysis of the HTLV-I Rex and HIV-1 Rev trans-regulatory proteins and their RNA response elements. *Genes Dev.* 3:1534-1544.
 10. Inoue, J.-I., M. Yoshida, and M. Seiki. 1987. Transcriptional ($p40^{tax}$) and post-transcriptional ($p27^{ex}$) regulators are required for the expression and replication of human T cell leukemia virus type I genes. *Proc. Natl. Acad. Sci. USA* 84:3653-3657.
 11. Kiyokawa, T., M. Seiki, S. Iwashita, K. Imagawa, F. Shimizu, and M. Yoshida. 1985. $p27^{x-III}$ and $p21^{x-III}$, proteins encoded by the pX sequence of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* 82:8359-8363.
 12. Lewis, N., J. Williams, D. Rekosh, and M.-L. Hammar-skjöld. 1990. Identification of a *cis*-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 *rev* and human T-cell leukemia virus types I and II *rex* proteins. *J. Virol.* 64:1690-1697.
 13. Malim, M. H., L. S. Tiley, D. F. McCarn, J. R. Rusche, J. Hauber, and B. R. Cullen. 1990. HIV-1 structural gene expression requires binding of the *rev* trans-activator to its RNA target sequence. *Cell* 60:675-683.
 14. Müller, W. E., T. Okamoto, P. Reuter, D. Ugarkovic, and H. C. Schröder. 1990. Functional characterization of *tat* protein from human immunodeficiency virus. Evidence that *tat* links viral RNAs to nuclear matrix. *J. Biol. Chem.* 265:3803-3808.
 15. Nagashima, K., M. Yoshida, and M. Seiki. 1986. A single species of pX mRNA of human T-cell leukemia virus type I encodes *trans*-activator $p40^x$ and two other phosphoproteins. *J. Virol.* 60:394-399.
 16. Nosaka, T., H. Siomi, Y. Adachi, M. Ishibashi, S. Kubota, M. Maki, and M. Hatanaka. 1989. Nucleolar targeting signal of human T-cell leukemia virus type I *rex*-encoded protein is essential for cytoplasmic accumulation of unspliced viral mRNA. *Proc. Natl. Acad. Sci. USA* 86:9798-9802.
 17. Olsen, H. S., A. W. Cochrane, P. J. Dillon, C. M. Nalin, and C. R. Rosen. 1990. Interaction of human immunodeficiency virus type 1 *rev* with a structured region in *env* mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. *Genes Dev.* 4:1357-1364.
 18. Rimsky, L., M. Duc Dodon, E. P. Dixon, and W. C. Greene. 1989. Trans-dominant inactivation of HTLV-I and HIV-1 gene expression by mutation of the HTLV-I Rex transactivator. *Nature (London)* 341:453-456.
 19. Roy, S., U. Delling, C.-H. Chen, C. A. Rosen, and N. Sonenberg. 1990. A bulge structure in HIV-1 TAR RNA is required for *tat* binding and *tat* mediated *trans*-activation. *Genes Dev.* 4:1365-1373.
 20. Sakai, H., H. Siomi, H. Shida, R. Shibata, T. Kiyomasu, and A. Adachi. 1990. Functional comparison of transactivation by human retrovirus *rev* and *rex* genes. *J. Virol.* 64:5833-5839.
 21. Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida. 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc. Natl. Acad. Sci. USA* 80:3618-3622.
 22. Seiki, M., A. Hikikoshi, T. Taniguchi, and M. Yoshida. 1985. Expression of the pX gene of HTLV-I: general splicing mechanism in the HTLV-family. *Science* 228:1532-1534.
 23. Seiki, M., J. Inoue, M. Hidaka, and M. Yoshida. 1988. Two *cis*-acting elements responsible for posttranscriptional trans-regulation of gene expression of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* 85:7124-7128.
 24. Seiki, M., J. Inoue, T. Takeda, and M. Yoshida. 1986. Direct evidence that $p40^x$ of human T cell leukemia virus type I is a *trans*-acting transcriptional activator. *EMBO J.* 5:561-565.
 25. Shima, H., M. Takano, K. Shimotohno, and M. Miwa. 1986. Identification of $p26^{xb}$ and $p24^{xb}$ of human T-cell leukemia virus type II. *FEBS Lett.* 209:289-294.
 26. Shimotohno, K., M. Miwa, D. J. Slamon, I. Chen, H. Hoshino, M. Takano, M. Fujino, and T. Sugimura. 1985. Identification of new gene products coded from X regions of human T-cell leukemia viruses. *Proc. Natl. Acad. Sci. USA* 82:302-306.
 27. Shimotohno, K., Y. Takahashi, N. Shimizu, T. Gojobori, D. W. Golde, I. S. Y. Chen, M. Miwa, and T. Sugimura. 1985. Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: an open reading frame for the protease gene. *Proc. Natl. Acad. Sci. USA* 82:3101-3105.
 28. Shimotohno, K., M. Takano, T. Teruuchi, and M. Miwa. 1986. Requirement of multiple copies of a 21-nucleotide sequence in the U3 regions of human T-cell leukemia virus type I and type II long terminal repeats for *trans*-acting activation of transcription. *Proc. Natl. Acad. Sci. USA* 83:8112-8116.
 29. Siomi, H., H. Shida, S. H. Nam, T. Nosaka, M. Maki, and M. Hatanaka. 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. *Cell* 55:197-209.
 30. Sodroski, J. G., W. C. Goh, C. A. Rosen, Z. Salahuddin, A. Aldovini, G. Franchini, F. Wong-Staal, R. C. Gallo, K. Sugamura, Y. Hinuma, and W. A. Haseltine. 1985. *trans*-activation of the human T-cell leukemia virus large terminal repeat correlates with the expression of the *x-lor* protein. *J. Virol.* 55:831-835.
 31. Solomin, L., B. K. Felber, and G. N. Pavlakis. 1990. Different sites of interaction for *rev*, *tev*, and *rex* proteins within the *rev*-responsive element of human immunodeficiency virus type 1. *J. Virol.* 64:6010-6017.
 32. Toyoshima, H., M. Itoh, J. Inoue, M. Seiki, F. Takaku, and M. Yoshida. 1990. Secondary structure of the human T-cell leukemia virus type I *rex*-responsive element is essential for *rex* regulation of RNA processing and transport of unspliced RNAs. *J. Virol.* 64:2825-2832.
 33. Trono, D., and D. Baltimore. 1990. A human cell factor is essential for HIV-1 *rev* action. *EMBO J.* 9:4155-4160.
 34. Wachsman, W., K. Shimotohno, S. C. Clark, D. W. Golde, and I. S. Y. Chen. 1984. Expression of the 3' terminal region of human T-cell leukemia viruses. *Science* 226:177-179.
 35. Walley, A. J., S. Heathy, and I. M. Jones. 1989. Direct RNA-binding by the *rev* product of HIV-1. *AIDS* 3:859-861.
 36. Weeks, K. M., C. Ampe, S. C. Schultz, T. A. Steiz, and D. M. Crothers. 1990. Fragments of the HIV-1 *tat* protein specifically bind TAR RNA. *Science* 249:1281-1285.