Functional mapping of the rev-responsive element of human immunodeficiency virus type 2 (HIV-2): influence of HIV-2 envelope-encoding sequences on HIV-1 gp120 expression in the presence or absence of Rev

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Introduction

Replication of the human immunodeficiency virus type 1 (HIV-1) regulatory protein Rev stimulates expression of structural viral proteins via a target response element (RRE) located within gag-pol and env mRNAs. To analyse the HIV-2 Rev trans-activation effect on the expression of the envelope protein, we cloned a functionally active HIV-2 rev cDNA and showed that it contained four exons. Using transient expression assays, we mapped a 353 bp RRE fragment within the env gene of HIV-2 on which both HIV-1 and HIV-2 Rev could act. Interestingly, smaller fragments suppressed the use of additional splice sites within the env gene and caused envelope protein expression independent of Rev.

Methods

Plasmids. Nucleotide (nt) numbers are based either on HIV-1 LAI (formerly BRU; Wain-Hobson et al., 1985, 1991) or on HIV-2 ROD proviral clones (Guyader et al., 1987).

HIV-2 rev and env gene expression vectors were constructed by using cDNA clones of a λgt10 library (Gubler & Hoffman, 1983) prepared from HUT cells infected with a proviral clone of HIV-2 ROD provided by the laboratory of R. Desrosiers (New England Regional Primate Research Center, Harvard Medical School, Southborough, Mass., U.S.A.). The HIV-2 rev gene expression vector pSP264, a pBR322-derived plasmid, contains the entire cDNA fragment coding for the Rev protein inserted in a pSV2-derived vector (Gorman et al., 1987). The HIV-2 env gene expression vector pSP275 contains a cDNA fragment from nt 6090 (56 bases upstream of the ATG start codon from...
the HIV-2 envelope gene) to the poly(A) tail. This fragment was cloned into the restriction sites for SmaI and SpeI in Bluescript vectors (Stratagene), downstream of the simian virus 40 (SV40) origin (ori) (between 5171 and 294 nt of the SV40 sequence) and HIV-2 long terminal repeat (LTR) sequences (275 bp of U3 and all of R from HIV-2 ROD; Gyuyader et al., 1987). The hepatitis B virus sequence used at the 3' end for the poly(A) signal is from positions 1465 to 1985. pSP275 contains the 5' and 3' splice sites for tat and rev but cannot express these genes since their ATG start codons are upstream of nt 6090.

The HIV-1 rev gene expression vector, the plasmid pSVar, provided by Craig Rosen expresses a cDNA copy of the rev gene from the SV40 early promoter (Rosen et al., 1988). The HIV-1 env gene expression vectors pME252 and pME246 have been described previously (Emerman et al., 1989).

Transfection. COS-1 cells (Gluzman, 1981) were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Twenty-four hours prior to transfection, the cells were plated at 1 × 10⁶ cells per 100 mm dish. Transfections were done as described using DEAE-dextran and chloroquine (Cullen, 1987) with the following modifications: 6 μg of a plasmid containing the env gene was transfected with either 2 μg of a plasmid encoding HIV-1 Rev (pSVar), HIV-2 Rev or 2 μg of a carrier plasmid. When indicated, 2 μg of a plasmid encoding HIV-2 Tat (pEM221; Emerman et al., 1987) or HIV-1 Tat (pEM241; Emerman et al., 1989) was included in the transfection. Sixteen hours after transfection, 5 mM-sodium butyrate was added to the transfected cells and incubated for 24 h.

Labelling and immunoprecipitations. Forty hours after transfection, cells were labelled with 100 μCi each of [35S]cysteine and [35S]methionine for 6 to 8 h. After labelling, cells were centrifuged at 2500g for 5 min and resuspended in 50 μl per 1 × 10⁶ cells of NTE buffer (20 mM-Tris-HCl pH 8.3, 75 mM-KCl, 1-5 mM-MgCl2, 10 mM-EDTA, 0.05% aprotinin (Intripl), 0.2 mM-PMDS, (Sigma), 7 mM-2-mercaptoethanol). Cells were boiled for 3 min and resuspended in 50 μl of lysis buffer (2% Triton X-100 in NTE) was added per 1 × 10⁶ cells. A second 5 min incubation on ice was followed by a centrifugation for 5 min at 12000 g. The soluble fraction was used for immunoprecipitation. The supernatant of the cell culture was centrifuged at 2000 g for 5 min, and 0-1 volume of NTE–Triton X-100 was added and left for 10 min on ice. After centrifugation at 12000 g for 5 min, the soluble fraction was used for immunoprecipitation.

For each reaction, 25 μl of the cell extract or 250 μl of supernatant was preincubated with Protein A–Sepharose (50 μl) for 1 h at 4 °C and then incubated overnight with either HIV-2 patient serum or with the monoclonal antibody (MAB) 110-4 (anti-HIV-1 gpl20 amino acids 303 to 323; Linsley et al., 1988). Fifty μl of a Protein A–Sepharose suspension was added and incubated for 1 h at 4 °C, pelletted and washed four times with 1 ml of NP40–DOC buffer (1% NP40, 0.2% sodium deoxycholate, 120 mM-NaCl, 20 mM-Tris–HCl pH 8.0). 25 μl of loading buffer (20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 125 mM-Tris–HCl pH 6-8) was added, samples were boiled for 3 min and loaded onto a 7-5% polyacrylamide–SDS gel.

RNA analysis. RNA was extracted 48 h after transfection as described (Favoloro et al., 1980). Owing to the purification protocol, the nuclear RNA fraction is contaminated by cytoplasmic RNA, but the cytoplasmic fraction does not contain nuclear RNA. This was checked by hybridization with a ribosomal probe. Five μg of RNA was used for Northern blot analysis using a formaldehyde–agarose gel. Hybridization was done as previously described (Fourney et al., 1988).

Amplification of RNA. cDNA synthesis was done synthetized for 90 min at 37 °C in 20 μl containing 1 μg of RNA, 50 mM-Tris–HCl pH 8.3, 75 mM-KCl, 3 mM-MgCl₂, 10 mM-DTT, a 1 mM concentration of each dNTP, 100 pmol of random hexamers (Pharmacia), and 200 units of Moloney murine leukemia virus reverse transcriptase. Two μl of the cDNA was used for PCR amplification in 50 μl containing 10 mM-Tris–HCl pH 8.3, 50 mM-KCl, 1-5 mM-MgCl₂, 0.01% gelatin, 0.2 mM of each dNTP, 0.2 μM of each primer, and 0-25 units of Cetus AmpliTaq polymerase. After an initial 5 min step at 94 °C, amplification was done for 30 cycles (2 min at 60 °C, 2 min at 72 °C, 1 min at 94 °C). Amplified fragments were purified on agarose gels using glass milk, filled with the Klenow enzyme and cloned into the phosphatase-treated SmaI site of the Bluescript M13 vector (Stratagene). DNA sequencing was performed on double-stranded plasmid DNA, by the deoxyribonucleotide chain termination method (Sanger et al., 1977) using T3 or T7 primers (Stratagene).

Oligonucleotide sequences from 5' to 3' are as follows: ME43 in the sense orientation at the beginning of the HIV-1 env gene (positions 5853 to 5870) GGGCACCATGCTCCTTGGG; ME35 in the antisense orientation downstream from the splice acceptor (SA) site of SV40 poly(A) (positions 4546 to 4564) CATTTCATGGTCATAGG.

Results

Molecular cloning of a functional rev gene of HIV-2

In order to analyse the specificity of action of the rev gene of HIV-2, functional complementary (cDNA) clones were obtained. HIV-2 viral stock was used to infect HUT78 cells. Poly(A)+ RNA was extracted 4 days after infection and used to construct an oligo(dT)-primed cDNA library in the λgt10 vector. Clones were identified and classified based on their hybridization patterns. By analogy with HIV-1, the rev mRNA is predicted to be a spliced message. The cDNA clones selected for analysis were those which hybridized to probes derived from the 5' part (U5 probe A) of the virus as well as the end of the env gene (probe C) but not to sequences corresponding to the intron within the env gene (probe B). Fig. 1 shows a schematic representation of one of the selected clones (clone 106) which was further characterized by restriction mapping and complete DNA sequencing. The clone contains four exons, of which exon 1 [from R to the major splice donor (SD) at position 470] and exon 2 (72 bp within the pol gene) are non-coding. The two coding exons contain an open reading frame (ORF) starting with the predicted initiator codon for the rev gene (at position 6071) up to the 3' region of the env gene, U3 and R. The viral insert in clone 114 hybridized with both probes B and C and appeared to be a partial transcript colinear with unspliced viral genomic RNA. Sequence analysis of this clone showed that it extended from nt 6090 to the end. The first ATG triplet proximal to the 5' end of the sense strand corresponds to the start codon of the env gene.

To ascertain that clone 106 contained a functional rev gene able to trans-activate the expression of HIV-2 env, we used the fragments corresponding to the two cDNA clones 106 and 114 for the construction of HIV-2 rev (pSP264) and HIV-2 env (pSP275) expression vectors respectively. The latter construct cannot express tat or rev genes because it does not contain the start codon for
HIV-2 rev-responsive element

Fig. 1. Schematic representation of HIV-2 cDNA clones. Top: genomic organization of the HIV-2 proviral DNA showing the position of the major ORFs and LTRs. Middle: restriction fragments from HIV-2 ROD proviral clone used as probes for the identification of cDNA clones; probe A, nt 184 to 395, probe B, nt 6156 to 7803 and probe C, nt 7968 to 8569. Bottom: schematic representation of clones 106 and 114. Exons and introns are depicted by solid and broken lines, respectively. Positions of the donor and acceptor splice sites are indicated. Residues from nt 8957 to 9314 are absent from all cDNA clones sequenced and presumably from the proviral clone.

Fig. 2. Immunoprecipitation of HIV-2 Env protein synthesized in COS-1 transfected cells. COS-1 cells were transfected with no env plasmid (lane 1), 6 μg pSP275 (lanes 2 to 5), 2 μg pSP264 (lanes 3 and 5) or 2 μg pME221 which expresses HIV-2 Tat (lanes 4 and 5). A schematic diagram of plasmid DNAs pSP275 and pSP264 are shown in Fig. 3.

These genes. We cotransfected COS-1 cells with pSP275 (HIV-2 env) in the presence or absence of pSP264 (HIV-2 rev). Cells were metabolically labelled with [35S]cysteine and [35S]methionine after transfection and protein extracts were immunoprecipitated with an HIV-2 patient serum (Fig. 2). When HIV-2 rev was included in the transfection, we detected one major high Mr protein of 140K. An additional minor protein of 125K was occasionally detected (see Fig. 3). The sizes of these proteins correspond to those of the HIV-2 envelope precursor and extracellular glycoproteins respectively (Rey et al., 1989). Furthermore, their expression was amplified by the presence of Tat in trans (Fig. 2, lane 5), which was expected because the env sequence was linked to HIV-2 LTR in the expression vector pSP275. These results indicate that pSP264 does indeed encode a functional Rev protein and that the expression of the env-encoded product directed by pSP275 is dependent on the presence of Rev in trans.
Non-reciprocal cross-trans-activation of HIV-1 and HIV-2

The HIV-2 rev-encoded protein shares only 45% amino acid identity with HIV-1 Rev and contains 100 amino acids compared to 116 for HIV-1. We cotransfected COS-1 cells with HIV-2 or HIV-1 expression vectors for Env and Rev proteins. HIV-1 env expression directed by plasmid pME252 has been shown to be rev-dependent (Emerman et al., 1989). Immunoprecipitation of transfected cell extracts showed that HIV-1 Rev can trans-activate the expression of HIV-2 Env whereas HIV-2 Rev did not trans-activate the expression of HIV-1 Env (Fig. 3). This result indicates that the specificity of the HIV-2 Rev protein is different from that of HIV-1 and confirms the published data of Malim et al. (1989b). HIV-2 appears to require sequences that are not found in the HIV-1 RRE whereas the HIV-2 RRE contains all the sequences necessary for the full activity of HIV-1 Rev. Alternatively, the non-reciprocal cross-trans-activation of HIV-1 and HIV-2 might be due to different structures within the HIV-1 RRE which impair HIV-2 Rev binding or activity.

Mapping of the HIV-2 RRE

The region of the env gene required in cis for trans-activation by Rev in HIV-1 lies within the region that encodes the N terminus of the transmembrane protein (Malim et al., 1989a). Comparison of this region with the corresponding domain of HIV-2 ROD showed that they are closely related in primary sequence and that both can form a highly stable RNA secondary structure (Malim et al., 1989b; Sakai et al., 1990). We constructed a series of plasmids in which HIV-2 fragments from this region were inserted after a stop codon introduced at the end of HIV-1 gp120 coding sequence. The test sequence which serves as a marker for HIV-2 RRE-directed expression would therefore not affect the stability of the final protein. A schematic representation of these vectors is shown in Fig. 4.

The plasmid pME236 (Emerman et al., 1989) contains the sequence for the HIV-1 extracellular protein gp120, flanked by the SV40 ori and HIV-2 LTR on one side and signals for 3' RNA processing on the other side. Transfection of this vector did not lead to the production of the Env protein even when the HIV-1 or HIV-2 rev gene encoded on a separate plasmid was provided (Fig. 5). When an HIV-1 fragment (nt 7179 to nt 7735) containing the HIV-1 RRE was inserted after the gp120 sequence (pME246), production of the HIV-1 Env protein occurred only in the presence of the HIV-1 rev gene (Emerman et al., 1989). HIV-2 Rev was not able to trans-activate gp120 production directed by pME246 (Fig. 5). In contrast, addition of HIV-2 env fragments allowed the expression of HIV-1 gp120 when either HIV-1 or HIV-2 rev plasmids were cotransfected. We observed that the full Rev response was obtained for the largest fragments (ABCDE or BCDE), indicating that sequences outside the minimal RRE influenced Rev action. We observed also that HIV-1 Rev gave rise to...
greater expression of Env than HIV-2 Rev, possibly because it accumulates in the cell at higher concentrations than does HIV-2 Rev. Finally, we mapped a 353 bp fragment (fragment BCD) within the HIV-2 env gene that could function as HIV-2 and HIV-1 RRE. This fragment, lying between nt 7615 and nt 7968 (pSP289, Fig. 4 and 5) is larger than the putative RRE deduced by sequence comparison of HIV-1 and HIV-2 env sequences (Malim et al., 1989b; Sakai et al., 1990). Thus, additional sequences located on both sides of the central stem and not found in HIV-1 might be required for the effective presentation of the HIV-2 RRE to Rev.

Production of HIV-1 Env protein in the absence of Rev

A significant amount of HIV-1 gp120 was produced upon transfection of plasmids containing smaller fragments of the RRE in the absence of Rev (Fig. 6). Cotransfection with either HIV-1 or HIV-2 rev did not increase gp120 production from these plasmids (except from pSP289), indicating that none of the sequences added after the stop codon in pME236 functions as an RRE. Expression of the Env protein in the absence of Rev was not sequence-dependent since it was observed with different subfragments of the RRE in sense or antisense orientations. However this effect was not observed after transfection of plasmids containing no RRE (pME236) or the entire RRE (pSP278 and 279).

To understand the molecular basis of this constitutive expression, we performed Northern blot analysis of mRNA from cells fractionated into nuclear and cytoplasmic compartments. There was no expression of gp120 from pME236 in the presence or absence of Rev because pME236 does not contain the RRE (Fig. 5 and 7a). Although we found full-length RNA in the nucleus of transfected cells, as expected from previous studies
(Malim et al., 1989a; Felber et al., 1989) there was no detectable full-length RNA in the cytoplasm. On the other hand, there was a smaller mRNA in both the nuclear and cytoplasmic fractions (Fig. 7b). pSP294 is identical to pME236 except that it contains a 165 nt fragment of the HIV-2 env gene (fragment D; Fig. 3). This fragment is sufficient to allow expression of gpl20 independent of Rev expression (Fig. 7a) and we found the presence of full-length RNA in the cytoplasm in the absence of Rev (Fig. 7b).

Both nuclear and cytoplasmic fractions of cells transfected with pSP294 expressed a small mRNA identical in size to that of pME236 (Fig. 7b). To characterize this small RNA, we performed RNA amplification using one primer (ME43) at the beginning of the env gene, and one antisense primer (ME35) downstream from the intron of SV40 poly(A) (Fig. 8). We obtained an unique fragment of 490 bp for both constructs, pME236 and pSP294. Sequence analysis of this fragment revealed that the small RNA species was generated by splicing between a 5' splice site at position 6316 [already described as the SD for Tev by Benko et al. (1990)] in the env gene and the 3' splice site in SV40 sequences.

Although the total amount of nuclear RNA produced by transfection of pSP294 and pME236 is the same, the ratio of full-length to spliced mRNA produced by pSP294 was increased when compared with the amount produced by pME236. This suggests that the presence of fragment D in pSP294 influences intron recognition and affects splicing efficiency. Changes in splicing were correlated with an increase in the cytoplasmic expression of envelope mRNAs which are subsequently translated (Fig. 7).

**Discussion**

We have isolated an HIV-2 cDNA clone that encodes a functionally active Rev protein. Cloning and sequencing of this multiply spliced mRNA allowed us to define the position of the HIV-2 major splice site and a non-coding exon within the pol gene. We have also identified an SA site between the tat and rev AUG codons (16 nt upstream of rev AUG) allowing expression of Rev but not of Tat. This SA is conserved among HIV-1 isolates, HIV-2 and SIV. These findings are in good agreement with those of Schwartz et al. (1990) showing that HIV-1 produces distinct mRNAs for each regulatory protein (Tat, Rev and Nef), unlike human T lymphotropic virus which uses a bicistronic mRNA for the production of both Tax and Rex proteins (Nagashima et al., 1986).

In an attempt to define precisely the HIV-2 RRE and to determine the basis of non-reciprocal cross-transcriptional activation, we tested different HIV-2 env fragments for their ability to permit HIV-1 Env expression after transfection with either HIV-1 or HIV-2 Rev. We localized the HIV-2 RRE to a 353 bp fragment, which is smaller than the RRE of 1042 bp first described by Lewis et al. (1990) in a similar study using the HIV-2 SBL/ISY clone. This study also showed that a subfragment corresponding to fragment CD in Fig. 4 could function as RRE only for HIV-1 Rev. In a recent study, Dillon et al. (1990) demonstrated that the same portion of the RRE allowed binding of both HIV-1 and HIV-2 Rev. RRE function may be influenced by flanking regions that differ in the constructs. In our assay, we failed to detect Rev induction since fragments smaller than BCD were used. Rev responsiveness may be masked by the constitutive expression that we observed in these cases.

Surprisingly, we found that several fragments of the HIV-2 env gene could cause expression of HIV-1 gpl20 independent of rev. These fragments must act in cis because they are placed after a stop codon and do not contain an initiator methionine. Northern blot analysis showed that these fragments are not enhancers because they did not increase the total amount of RNA produced. Rather, insertion of fragment D (nt 7803 to 7968) 3' to the HIV-1 env sequences encoding gp120 increased the ratio of full-length to aberrantly spliced messages (Fig. 7).

Recent work has shown that the env gene contains a family of 5' splice sites that are activated when the major 5' splice site for tat and rev is deleted (C. Deminie & M. Emerman, unpublished results). The effect of fragment D in suppressing these splice sites is to increase the pool of unspliced nuclear RNA. Therefore fragments of HIV-2 env may allow the env mRNA to leave the nucleus independent of Rev because a subfraction of the unspliced nuclear mRNA is no longer recognized by the splicing machinery. This effect was not observed when the fragment inserted after the stop codon corresponded to the entire RRE (pSP278 and pSP279). It is possible that the formation of a stable secondary structure brings the required cis-acting elements into appropriate proximity and splicing can occur as efficiently as observed for pME236, which contains no RRE. In the same way, folding of the Rex-responsive element mediates the juxtaposition of two sequences essential for polyadenylation (Ahmed et al., 1991).

Previous work has shown that mutation of both 5' and 3' splice sites in a β-globin-RRE construct caused cytoplasmic expression of the RNA independent of Rev (Chang & Sharp, 1989). We previously showed that changes at the 3' end of the mRNA can have a significant effect on the expression of the HIV-1 envelope protein (Emerman et al., 1989). Therefore, it is likely that
expression of env in HIV is controlled by a complex interaction of secondary and tertiary RNA structures with the cellular splicing apparatus and with the Rev protein to regulate the amount of splicing and type of spliced messages produced.

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References


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