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Functional Comparison of Transactivation by Human Retrovirus *rev* and *rex* Genes

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The effect of *rev*-responsive element deletion on human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) gene expression was examined. The phenotypes of HIV-1 and HIV-2 provirus DNAs lacking the *rev*-responsive element, as determined by transfection experiments, were indistinguishable from those of virus DNAs carrying *rev* gene mutations. By using *rev*-responsive elements derived from these two viruses, we developed two monitoring systems to evaluate the functionality of HIV-1 *rev*, HIV-2 *rev*, and human T-lymphotropic virus type I *rex*. In both systems, HIV-1 *rev* and human T-lymphotropic virus type I *rex* transactivated HIV-2 very efficiently. On the contrary, HIV-2 *rev* and human T-lymphotropic virus type I *rex* were poor activators of HIV-1. No functional replacement of *rex* by HIV-2 *rev* was observed.

Primate lentiviruses carry a unique transactivator gene called *rev* in their genomes (8). The *rev* gene product regulates virus replication by selective enhancement of structural gene expression (5, 6, 12, 13, 36). Proviruses lacking a functional *rev* gene cannot produce *gag*, *pol*, and *env* and are therefore replication defective (33, 34, 36, 37). Extensive molecular biological studies on human immunodeficiency virus type 1 (HIV-1) have revealed that this effect of *rev* is primarily on the cytoplasmic mRNA (5, 7, 12, 13, 20–23, 28). In the absence of *rev*, unspliced and singly spliced viral mRNA species cannot be detected in the cytoplasm. The *trans* regulation of viral mRNA distribution in the cells by *rev* requires a *cis*-acting *rev*-responsive element (RRE) within the *env* region of HIV-1 (4, 7, 12, 13, 21, 22, 29). The RRE can form a highly ordered and stable RNA secondary structure, and the highly structured nature of RRE is maintained among all known primate lentiviruses (18). Evidence now exists that HIV-1 *rev* (*rev1*) actually binds the RRE (RRE1) to exert its function (3, 16, 23, 26, 40).

Recently, the functional replacement of *rev1* and *rev2* by *rex* of human T-lymphotropic virus type I (HTLV-I), belonging to a group of viruses that are distinct from the lentiviruses, has been described (14, 17, 19, 20, 28). *trans* regulation by *rex* also requires a *cis*-acting *rex*-responsive element (RXE) that is similar to RRE1 for *rev1* (14, 32). However, this genetic complementation of *rex* for *rev1* was nonreciprocal, since *rev1* was unable to substitute for *rex* (14, 17). Interestingly, this nonreciprocal complementation was also observed within the primate lentivirus subgroup. The *rev* proteins of HIV-2 (*rev2*) and simian immunodeficiency virus from a rhesus monkey were unable to activate the cytoplasmic expression of unspliced HIV-1 mRNA (20). Proviral *gag* gene mutants of HIV-2 and simian immunodeficiency virus from an African green monkey could not complement an HIV-1 *rev* mutant for reverse transcriptase (RT) production (30). In both reports, HIV-1 highly activated other viruses. These findings have prompted us to investigate the functional compatibility of human retrovirus *rev* and *rex* in more detail.

In this communication, we have constructed various reporter plasmids bearing bacterial chloramphenicol acetyltransferase (CAT) genes and a series of proviral clones to assess the responsiveness to *rev1*, *rev2*, and *rex*. We demonstrate here that *rev1* and *rex* augment the expression of HIV-2 very efficiently, whereas HIV-1 is activated very poorly by *rev2* and *rex*. We also demonstrate the nonfunctionality of *rev2* in the HTLV-I system in addition to *rev1*.

MATERIALS AND METHODS

Cell culture and DNA transfection. A human colon carcinoma cell line, SW480 (ATCC CCL228), was maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. For transfection, uncleaved plasmid DNA was introduced into SW480 cells by the calcium phosphate coprecipitation method (11, 38).

RT assays. RT activity was measured as described previously (39). For quantitation, spots on DE81 paper were cut out, and RT activity was determined by scintillation counting.

CAT assays. CAT assays have been previously described (10). CAT levels were assayed in equivalent amounts of cell lysates from transfected SW480 cells.

Northern (RNA) blotting analysis. Total cellular RNA was prepared from transfected SW480 cells by the guanidinium isothiocyanate-cesium chloride method, electrophoresed (5 μg of RNA) through 1% agarose gel containing 2.2 M formaldehyde, and analyzed by Northern blot hybridization as previously described (27). As probes, DNA fragments containing complete long terminal repeats (LTRs) were cut out from HIV-1 and HIV-2 DNA clones and labeled with ^{32}P by the multiprime DNA labeling system (Amersham International plc, Little Chalfont, Buckinghamshire, England).

Western immunoblotting analysis. Lysates of transfected SW480 cells were prepared as described previously (39), and proteins were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated overnight at room temperature with sera from individuals infected with viruses and with ^{125}I-labeled protein A for 3 h, washed, and visualized by autoradiography (39).

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Proviral DNA constructs. The proviral DNA clones pNL432 (HIV-1 wild type), pNL-Ba (HIV-1 *rev* mutant), pGH123 (HIV-2 wild type), and pGH-Ps (HIV-2 *rev* mutant) were described previously (1, 30, 33). RRE deletion mutants (pNLΔR and pGHΔR) and chimeric clones with respect to RRE (pNLR2 and pGHR1) were constructed by blunt end ligation with the restriction sites indicated in Fig. 1. Mutations were introduced into the *rev* coding sequence of these chimeric clones as described previously (30, 33) to generate pNLR2-Ba and pGHR1-Ps.

CAT reporter and effector constructs. An expression vector pRVSV was constructed by transferring an *Nde*I-*Hin*dIII fragment of pRSVCAT (9) containing the Rous sarcoma virus LTR into the *Nde*I and *Hin*dIII sites of pUC18 (pRSVLTR) and by inserting a *Bgl*II-*Bam*HI fragment of pSV2-βG (25) containing the simian virus 40 (SV40) small t intron and polyadenylation signal at the unique *Bam*HI site of pRSVLTR in a sense orientation. All reporter and effector clones except pCATLTRs were constructed by insertion of the necessary DNA fragments into pRVSV (see Fig. 1 and 3). To insert the fragments, restriction sites were modified by T4 DNA polymerase or linker insertion if needed. Plasmid designations and DNA fragments used for construction of the plasmids are as follows: pRSpCAT-RRE1 (*rev1* reporter), pRSpCAT-RRE2 (*rev2* reporter), pRSpCAT-RXE (*rex* reporter), prev1 (*rev1* effector), prev2 (*rev2* effector), sd (*Hin*dIII-*Ssp*I fragment of pNL432 [Fig. 1 top] containing the HIV-1 splice donor site), CAT (*Hin*dIII-*Sau*3A-I fragment of pSV2 cat [10] containing the CAT coding sequence), RRE1, (*Ssp*I-*Hin*dIII fragment of pNL432 [Fig. 1 top] encompassing HIV-1 RRE), RRE2 (*Ssp*I-*Nco*I fragment of pGH123 [Fig. 1 bottom] encompassing HIV-2 RRE), sa (*Hin*dIII-*Bam*HI fragment of pNL432 [Fig. 1 top] containing the HIV-1 splice acceptor site), HTLV LTR (*Sma*I-*Rsa*I fragment of HTLV LTR [31]), *rev1* (*Axy*I-*Ssp*I fragment and *Hin*dIII-*Hpa*I fragment of pNL432 [Fig. 1 top] carrying HIV-1 *rev*), and *rev2* (*Ava*I-*Ssp*I fragment and *Nco*I-*Bam*HI fragment of pGH123 [Fig. 1 bottom] carrying HIV-2 *rev*). Plasmids prev1m and prev2m contained mutations within the *rev* coding sequence as described for proviral clones. Another reporter construct, pCATLTRs (for *rex*), has been described elsewhere (H. Shida and H. Siomi, submitted for publication) (see Fig. 3). Briefly, an *Sma*I-*Rsa*I fragment of HTLV LTR (31) was cloned into the *Hpa*I site of pRSVCAT (9) after the insertion of a *Hin*dIII linker. The HTLV *rex*-expressing plasmid prex and its mutant clone prexm were constructed from pCDMRex (Shida and Siomi, submitted) and pCDMD1 (Siomi and Shida, unpublished data), respectively. To obtain prex, a *Hin*dIII-*Sma*I fragment of pCDMRex, which contains the intron-free HTLV-I *rex* gene (35), was cloned into pRVSV and 240 bp (between *Apa*I and *Stu*I [31]) were deleted by blunt-end ligation to remove the *tax* coding sequence. The same strategy was used to generate prexm from pCDMD1. pCDMD1 contains a small deletion within the *rex* coding sequence (amino acid residues 8 to 18).

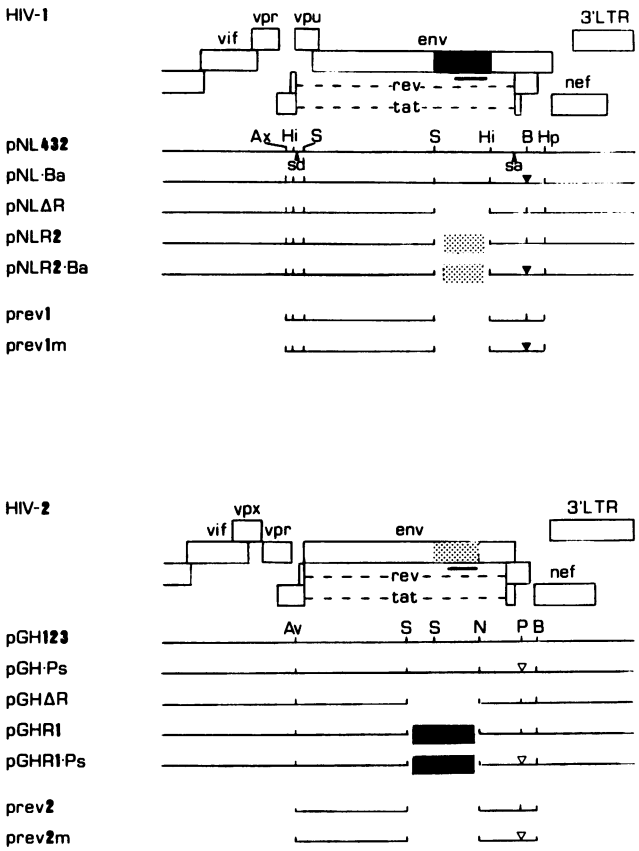

FIG. 1. Structures of proviral clones used in this study. The upper and lower parts show HIV-1 and HIV-1 clones, respectively. DNA fragments used for the construction of effector plasmids (prev1, prev1m, prev2, and prev2m) are also shown (see Fig. 3 for the structures of effectors). DNA fragments used to exchange RREs are indicated by closed boxes (between S and Hi in pNL432) and dotted boxes (between S and N in pGH123). The sequences that represent RREs (30) are underlined. The sites of mutations in the *rev* coding regions are shown by closed triangles (4-bp insertion) and open triangles (4-bp deletion) as reported previously (30). Abbreviations: Ax, *Axy*I site (nucleotide 5954 in pNL432); Hi, *Hin*dIII sites (nucleotides 6026 and 8131); S, *Ssp*I site (nucleotides 6153 and 7556); B, *Bam*HI site (nucleotide 8465); Hp, *Hpa*I site (nucleotide 8648). Av, *Ava*I site (nucleotide 6167 in pGH123); S, *Ssp*I sites (nucleotides 7338 and 7629); N, *Nco*I site (nucleotide 8053); P, *Pst*I site (nucleotide 8551); B, *Bam*HI site (nucleotide 8663). Nucleotide sequence data were from the Los Alamos data bank (Los Alamos, N.M.) for pNL432 and from a published article (15) for pGH123.

RESULTS

Phenotypical characterization of RRE deletion mutants. The effect of RRE deletion on HIV-2 provirus has not yet been described. To confirm the reported observation that in HIV-1 the phenotypes of proviruses carrying either a defective *rev* gene or a defective RRE are identical (23) is valid for HIV-2, a deletion mutant of HIV-2 lacking the putative RRE (30) was constructed and analyzed comparatively and phenotypically (Fig. 1). The viral gene expression of mutant clones was monitored by transfection into SW480 cells (1), followed by Northern blot analysis of viral RNAs (Fig. 2A), Western blot analysis of viral proteins (Fig. 2B), and a functional RT assay (Fig. 2C). As previously reported (6, 24, 33), the intact HIV-1 and HIV-2 proviruses expressed three distinct size classes of viral RNA (Fig. 2A). The major difference in the RNA expression patterns of *rev* mutants (pNL-Ba and pGH-Ps) from those of wild types (pNL432 and pGH123) observed in our system (Northern analysis of total RNA prepared from transfected SW480 cells) was the reduced levels of 9.2- and 4.3-kb RNA species. RRE deletion mutants (pNLΔR and pGHΔR) exhibited essentially the same pattern of viral RNA expression as *rev* mutants (Fig. 2A). Examination of viral *gag* and *pol* expression indicated

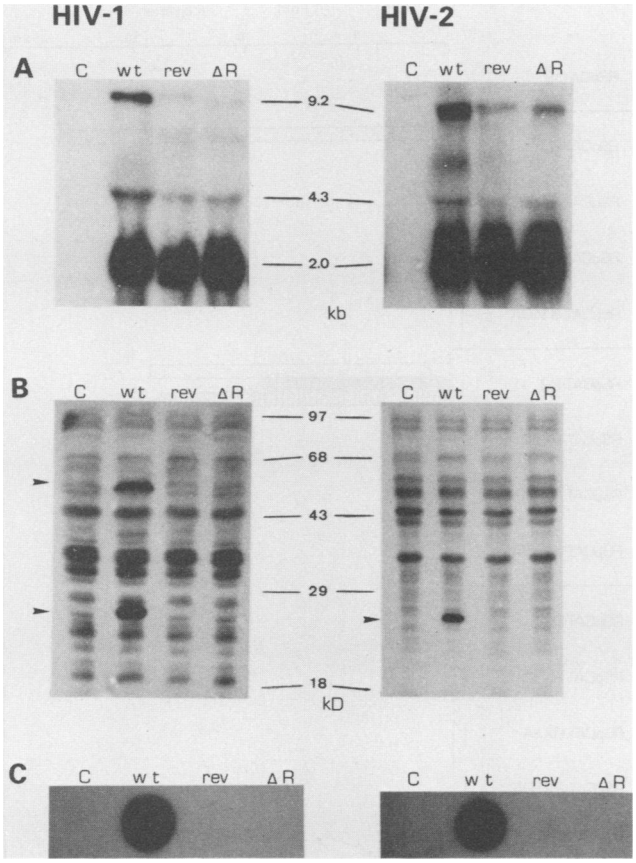

FIG. 2. Viral gene expression of various proviral clones. SW480 cells were transfected with the clones, and viral RNA expression (A), *gag* gene expression (B), and RT production (C) 48 h later were monitored. C, pUC19 (control); wt, wild type (pNL432 for HIV-1 and pGH123 for HIV-2); rev, *rev* mutant (pNL-Ba for HIV-1 and pGH-Ps for HIV-2); ΔR, RRE deletion mutant (pNLΔR for HIV-1 and pGHΔR for HIV-2). Arrowheads in panel B show the position of the *gag* protein (precursor p55 and mature product p24 for HIV-1, mature product p24 for HIV-2).

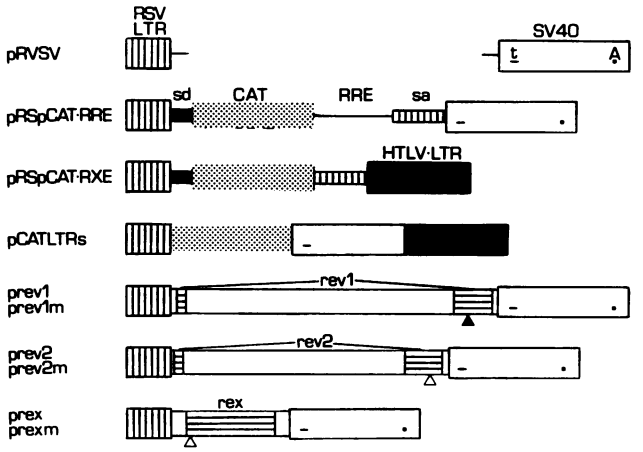

FIG. 3. Schematic representation of the expression vectors used in this study. Symbols for each signal and coding sequence are indicated. For sd, sa, RRE, and coding sequences of *rev*, see Fig. 1. Sequences of Rous sarcoma virus (RSV), SV40, and HTLV are described in Materials and Methods. t and A indicate the SV40 small t intron and polyadenylation signal, respectively. The sites of mutations in the coding regions of activators are indicated by a closed triangle (prev1m, see Fig. 1) and open triangles (prev2m, see Fig. 1; prexm, see Materials and Methods).

that *rev* and RRE deletion mutants shared the same phenotype (Fig. 2B and C). Neither mutant produced *gag* proteins or functional RT. Our previous experience showed that deletions in *env* (not affecting RRE, *tat*, and *rev*) had no effects on *gag* and RT expression (30; Adachi and Sakai, unpublished observations). The results presented in this section demonstrate that *rev* and RRE deletion mutants are indistinguishable phenotypically.

Responsiveness of various reporter CAT constructs to *rev1*, *rev2*, and *rex*. For quantitative determination of the activation potentials of *rev1*, *rev2*, and *rex*, a series of CAT reporters was constructed (Fig. 1 and 3). Two representative constructs, designated pRSpCAT-RRE and pRSpCAT-RXE, consisted of several signal elements derived from HIVs and HTLV-I and the CAT-coding sequence. The structure of constructs was designed to mimic the viral genome. As effector plasmids, coding sequences of *rev1*, *rev2*, and *rex* were cloned into the expression vector pRVSV (Fig. 1 and 3). All reporters and effectors contained the same Rous sarcoma virus LTR as a promoter.

We first estimated the basal CAT activity of reporter constructs to ascertain the effect of signal element insertions into the plasmids. The CAT activities detected in SW480 cells transfected with 16 reporter clones (four groups classified by the activator-responsive element in Fig. 4). Generally, similar CAT expression patterns in each group were observed (particularly pRSpCAT-R1 and pRSpCAT-R2 in Fig. 4), although the introduction of RRE or RXE into the constructs had some effects. The constructs without splice donor (sd) and acceptor (sa) regions had the highest CAT activity (ΔDA in Fig. 4), whereas reporters bearing both regions generated the lowest activity (full in Fig. 4). The insertion of splice signals appeared to affect the CAT expression of reporters similarly, regardless of responsive elements. However, the responsiveness of each construct to activators differed drastically (Fig. 5). Various CAT constructs and *rev*- or *rex*-expressing DNAs were cotransfected into SW480 cells, and CAT production was monitored. The construct lacking RRE or RXE (RSpCATΔR in Fig. 5), as predicted, did not respond to *rev1*, *rev2*, or *rex*. In contrast, all effectors highly augmented the expression of reporters carrying self-activator-responsive elements (*rev1* for RSpCAT-R1, *rev2* for RSpCAT-R2, and *rex* for RSp CAT-RX in Fig. 5). The absence of sd (ΔD), sa (ΔA), or both (ΔDA) resulted in a reduced level of activation. The reactivity of the reporters to non-self-activators diverged greatly depending on the pair used. The HTLV-I reporters pRSp CAT-RX and pCATLTRs were activated solely by *rex*. Quite interestingly, even *rex* did not significantly enhance the expression of pRSpCAT-RXΔDA, the structure of which is very similar to that of pCATLTRs except for the absence of the SV40 sequence (Fig. 3). HIV-2 reporter pRSpCAT-R2 expression was equally well enhanced by all activators. *rev1*, *rev2*, and *rex* stimulated all HIV-2 constructs irrespective of the presence or absence of sd and sa, although the magnitude of activation was much higher in the case of pRSpCAT-R2. In contrast to pRSpCAT-R2, pRSpCAT-R1 (HIV-1) responded well only to *rev1*. The activation of pRSpCAT-R1 by *rev2* and *rex* was extremely low, but it was reproducibly observed in repeated experiments. No significant activation by *rev2* and *rex* was detected when constructs without splice signals were used.

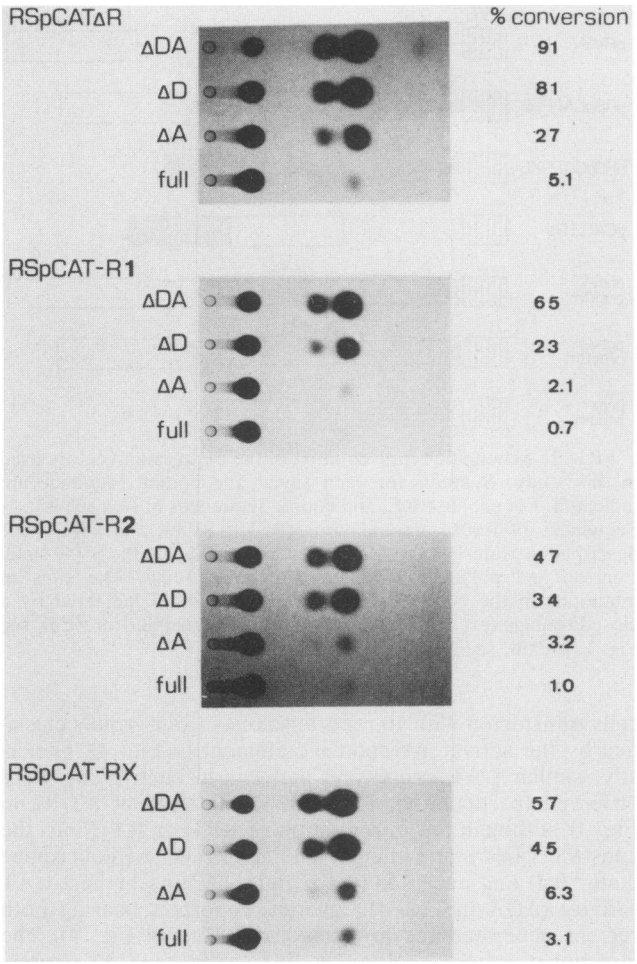

FIG. 4. Basal CAT activities of the reporter constructs. SW480 cells were transfected with each construct (1 μg), and CAT activity in the cell lysates (30-min reaction) 48 h later was determined. The reporters used are pRSpCAT lacking RRE (RSpCATΔR), pRSpCAT-RRE1 (RSpCAT-R1), pRSpCAT-RRE2 (RSpCAT-R2), and pRSpCAT-RXE (RSpCAT-RX). The CAT activity directed by the constructs with a full set of splice signals (full), without sd and sa (ΔDA), without sd (ΔD), and without sa (ΔA) is shown. The percent conversion of chloramphenicol to its acetylated forms is indicated on the right.

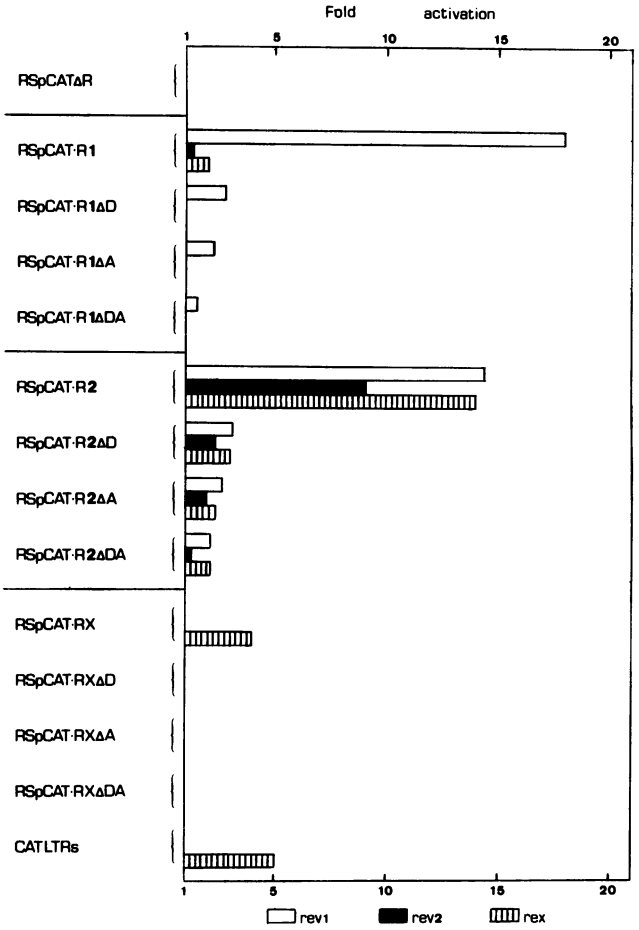

FIG. 5. Relative CAT production in SW480 cells cotransfected with the reporter (1 μg) and the effector (19 μg). CAT activities in cell lysates were determined, and relative activation rates of the reporters (CAT activity in the presence of activator/CAT activity in the control [pRVSV as a control]) are presented. The amount of cell lysates and reaction time were adjusted to obtain linearity. Three to five independent experiments were performed, and the average is presented. R1, RRE1; R2, RRE2; RX, RXE. ΔR, ΔD, ΔA, and ΔDA indicate the lack of RRE, sd, sa, and both sd and sa in the reporter constructs, respectively. pCATLTRs is a *rex* reporter (Fig. 3).

Responsiveness of various proviral clones to *rev1*, *rev2*, and *rex*. To confirm the validity of the observations obtained in the CAT assay system, we generated several proviral clones for phenotypic analysis (RT as the marker) within the context of the proviral genome (Fig. 1). The RRE was exchanged between HIV-1 and HIV-2, mutations were introduced into the *rev* coding sequence of chimeric clones, and their responsiveness to *rev1*, *rev2*, and *rex* was determined by transfection analysis. The RT activity produced in SW480 cells cotransfected with proviral clones and effector plasmids is presented in Fig. 6. Single transfection of the two constructs designated pNL-R2 (HIV-1 bearing RRE2) and pGH-R1 (HIV-2 bearing RRE1) gave the expected results from CAT experiments described above. pNL-R2 yielded RT activity similar to that of the wild type, whereas pGH-R1 generated a very low level of RT activity. The *rev* mutants of various proviral clones had distinct RT expression patterns in response to *rev1*, *rev2*, and *rex*. Proviral *rev* mutant clones carrying RRE1 (pNL-Ba and pGHR1-Ps) were rescued by *rev1* to produce high titers of RT. The ability of *rev2* and *rex* to complement the two clones with regard to RT production was very limited. The results for *rev* mutants carrying RRE2 (pNLR2-Ba and pGH-Ps) were very different. All activators could rescue the mutants to express RT equally well. Mutations within the *rev* coding sequence of all effectors completely abolished the activation potentials described above. As predicted, cotransfection of the viral clones lacking RRE (pNLΔR and pGHΔR) and prex produced no RT activity (data not shown). In total, the data presented in Fig. 6 were consistent with those in Fig. 5.

DISCUSSION

We quantitatively determined the functionality of three viral transactivators (*rev1*, *rev2*, and *rex*) within the context of HIV-1, HIV-2, and HTLV-I in this communication. Several reports, including ours (30), concerning genetic

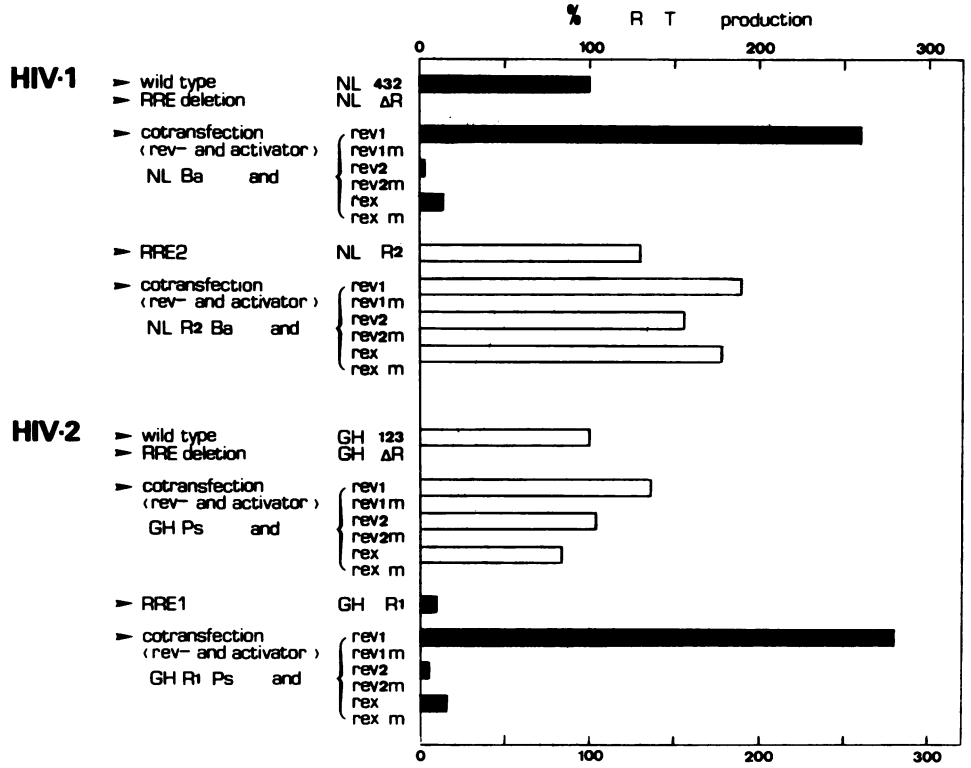

FIG. 6. RT production relative to the wild-type proviral clone. SW480 cells were cotransfected with various proviral clones (20 μg) and effectors (20 μg), and RT production 48 h later was determined. pUC19 was used as cotransfected DNA for pNL432, pNLΔR, pNLR2, pGH123, pGHΔR, and pGHR1. The data presented are percent RT production relative to that by the wild-type clone as indicated. Closed and open bars show the result of RT production by proviruses containing RRE1 and RRE2, respectively. The prefix p is omitted from the plasmid designations. The actual RT counts (1.5 μl of culture fluid) were 3,175 cpm for pNL432, 2,250 cpm for pGH123, and 25 cpm for pUC19 (negative control). The experiments were repeated three times with essentially the same results.

complementation among various *rev* and *rex* have already been published (14, 17, 19, 20, 28). The common conclusion drawn from those works is the inability of *rev* gene products derived from HIV-2 and simian immunodeficiency viruses to substitute for *rev1* and the one-way functional replacement of *rev1* by *rex*. Our results presented here indicate that *rev2* can recognize RRE1 and function as a *rev* transactivator in the HIV-1 system. We also show that all DNA clones bearing RRE2 are equally highly activated by any of the activators, unlike those carrying RRE1. Finally, nonfunctionality of *rev2* in the HTLV-I system is proven.

The apparent disparity regarding the low functionality of *rev2* in the HIV-1 system between this study and previous reports most likely reflects the assay methods used. The complementation analysis between the two proviral mutant clones (30) and the pgTAT assay (20) may be less sensitive than the methods used here, as mentioned before (23). The reason for the observed differences in reactivity of RRE1-containing constructs to *rex* is presently not clear if the above consideration is correct. Our results and others (17, 19) show that *rex* is not an efficient activator of HIV-1, whereas the HIV-1 reporter pgTAT is fully responsive to *rex* (20) and *rex* can rescue the replication of a defective HIV-1 provirus mutated in the *rev* gene quite efficiently (28). In our two systems, *rex* was consistently a poor activator of HIV-1. We considered the possible contribution of the RRE sequence and the *rex* protein itself to the apparent disagreement regarding the activation of HIV-1 by *rex*. Comparison of RRE1 sequences in reporter constructs has revealed no significant differences (17, 20; this report). There is only one amino acid substitution between predicted *rex* sequences (17, 20, 35). It is possible that various constructs used for the studies and experimental designs may affect the results. Detailed mutational analyses of RRE1 and *rex*, coupled with studies on the RRE2 reporter (high responder to *rex*), would help elucidate the mechanism(s) by which *rex* activates the heterologous virus HIV-1. The *rex* reporters did not respond to *rev2*, as reported for *rev1* (14, 17), within the limit of our CAT assay system. Neither *rev1* nor *rev2* augmented the expression of the reporter containing the full set of signal sequences (pRSpCAT-RXE).

The importance of the sd and sa regions for *rev1* responsiveness has been recently reported (2). We also observed a drastic reduction in the reactivity of pRSpCAT-R1 (HIV-1 reporter) to *rev1* in the absence of sd or sa. This effect was not confined to *rev1* responsiveness. The various CAT constructs lost high reactivity with *rev1*, *rev2*, and *rex* by deletions of sd and sa. The *rex* reporter pCATLTRs harboring SV40 small-t splice signals still retained responsiveness to *rex* without the sd and sa from HIV-1.

On the basis of our findings described here and previous works (14, 17, 19, 20, 30), we can develop a system for differentiating the primate lentiviruses. Taking advantage of the differential responsiveness of RRE1 and RRE2 to virus-coded activators, an assay system for this purpose may be constructed. It may be useful for the phylogenic classification of viruses and in the characterization of and search for unknown viruses.

ACKNOWLEDGMENT

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