Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus

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ABSTRACT The art gene product of the human immunodeficiency virus is required for the expression of virion capsid and envelope gene proteins. The experiments presented here show that sequences located within the coding region of the envelope gene exert a negative effect on the expression of heterologous genes and that the negative effect of these sequences can be relieved by the art gene product. This region in the env gene contains negative regulatory sequences that inhibit gene expression, as well as a sequence necessary for the art gene product-dependent relief of repression. The experiments define the cis- and trans-acting components of a regulatory system that permits differential expression of human immunodeficiency virus virion structural and regulatory proteins.

The human immunodeficiency virus (HIV), the etiological agent of acquired immune deficiency syndrome (1–4), contains two essential regulatory genes, tat (5, 6) and art (7–11). The tat gene encodes a 14-kDa protein (12, 13), and the art gene encodes a 20-kDa protein from overlapping reading frames (7–11). No viral proteins are detected in cells transfected with provirus defective for the tat gene. By contrast, although a provirus defective in the art gene fails to express structural proteins, regulatory proteins, including the products of the tat and the 3′-orf genes, are synthesized (7, 9, 14). This observation suggests that the art gene product regulates gene expression posttranscriptionally, as all viral mRNAs are derived from the same primary transcript (Fig. 1a). Consideration of the mRNA species that encode the structural proteins and the tat gene leads to the hypothesis that the structural gene mRNAs contain negative regulatory sequences that repress their expression in the absence of the art gene product (7). A likely location for such a sequence is in a region present in the gag and env gene mRNAs that is removed by splicing from the tat, art, and 3′-orf gene mRNAs (Fig. 1a).

To test this hypothesis, we mimicked selective regulation of viral structural genes by using a heterologous gene as an indicator. This method was adopted as it was considered possible that the cis-acting negative regulatory sequences as well as the art gene product-responsive sequences might lie within coding regions of the structural genes themselves, in the regions removed by splicing from the tat and art gene mRNA.

MATERIALS AND METHODS

Cell Lines, Transfections, and Transient Assays. Jurkat-tat111 cells have been described (19). Lymphoid cells were transfected with 3–5 μg of the individual plasmid DNAs by using DEAE-dextran (20). Adherent cells were transfected by the method of Lopata et al. (21). Chloramphenicol acetyltransferase (CAT) assays were performed as described (17). For human growth hormone (hGH) assays, 100 μl of medium obtained 48 hr after transfection was used to measure the level of secreted hGH by using an hGH radiimmunoassay kit (Nichols Institute, San Juan Capistrano, CA).

Construction of Recombinant Plasmids. Recombinant plasmids were prepared according to established techniques (22). The art gene expression vector pH3-art was prepared by treating the tat–art gene cDNA (6) with BAL–31 exonuclease to remove the tat gene ATG codon. The resultant fragment, which contains 3 nucleotides (nt) on the 5′ side of the art gene ATG codon and extends to the Bgl II site within the 3′ long terminal repeat (LTR), was cloned into the Sal I and BamHI sites present in the multiple restriction site linker present in plasmid pH3. Plasmid pH3 contains an SP64 multiple restriction site linker (Promega Biotec, Madison, WI) sandwiched between HIV LTR sequences from nt −167 to nt +80 and simian virus 40 (SV40) polyadenylation sequences (Bgl II–EcoRI fragment of plasmid pSV2 NEO [17]). Deletion mutants were generated from plasmid PA-6376 (see Fig. 3) by using BAL–31 (23). The 5′ border of each deletion was confirmed by DNA sequence analysis (24). Other deletion mutants were created by using internal restriction enzyme sites. The activity of pH3-art is shown in Fig. 2.

RESULTS

Regulation of Heterologous Gene Expression by Using HIV Intragenic Sequences. For the initial experiments, the 3′ half of an HIV provirus was placed distal to either the CAT gene or the hGH gene (25) (plasmids pIIIAR and pIIGHAR; Fig. 1b). In this context the HIV env sequences will be incorporated within the 3′-noncoding region of the CAT and hGH transcripts. The segment of the HIV genome present on the plasmid contains a mutation in the art gene coding sequence that inactivates the potential art gene function (7).

The level of CAT activity directed by plasmid pIIIAR in HeLa, Jurkat, and CHO cells that express that tat gene product was markedly reduced as compared to the activity of the plasmid pU3R–III (26), which contains the HIV LTR but which lacks the 3′ HIV sequences (Fig. 2b and Table 1, experiment 1). However, cotransfection of pIIAR with plasmid pH3-art, a plasmid that produces a functional art gene (Fig. 2), resulted in an increase in the level of CAT activity by >20-fold in all three cell lines (Fig. 2b and Table

Abbreviations: HIV, human immunodeficiency virus; CAT, chloramphenicol acetyltransferase; hGH, human growth hormone; LTR, long terminal repeat; SV40, simian virus 40; HTLV–I, human T-cell leukemia virus type I; nt, nucleotide(s).

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1, experiment 1). Similarly, the level of the hGH directed by plasmid pIIGHAR (Fig. 1b) in the absence of the art gene product was very low relative to the parental plasmid (pIIGHGSV) that lacks the 3' HIV sequences (Table 1, experiment 6). However, cotransfection with pH3-art resulted in a marked increase in hGH activity directed by pIIGHAR. Evidently, the 3' half of the HIV genome contains sequences that negatively regulate heterologous gene expression. Moreover, the negative regulatory effect of these sequences can be overcome by the product of the art gene. In this respect, the expression of CAT and hGH genes linked to the HIV provirus sequences mimics the regulation observed with the gag and env genes themselves (7, 14).

Env Gene Sequences Are Required for art Gene-Mediated Regulation of Gene Expression. To determine whether sequences within the HIV LTR were required for art gene regulation, plasmids were constructed in which either the 5' LTR was replaced with a human T-cell leukemia virus type I (HTLV-I) LTR or the 3' LTR was replaced with SV40 poly(A) sequences (Fig. 1b). The level of CAT activity directed by the 5' LTR replacement (pI-AAR) was much lower than that of the control plasmid pU3R-I (28) that lacks the 3' HIV sequences (Fig. 2b and Table 1, experiment 2). However, cotransfection with pH3-art resulted in a substantial increase in the level of CAT activity.

In other experiments the 3' LTR was replaced by SV40 poly(A) sequences (plasmid PA-6376; Fig. 3). In this construct, the suppression of CAT activity by HIV env gene sequences was relieved upon cotransfection with pH3-art (Table 1, experiment 5). These experiments show that neither the 5' nor 3' LTR of HIV is required for the negative and art gene product-responsive regulatory effects.

To determine if the regulatory effects were due to env gene sequences, the majority of these sequences were removed from plasmids pIIAAR1 and pIAAR (Fig. 1b). Deletion of these sequences resulted in a significant increase in CAT activity relative to the parental plasmids pIIAAR3 and pIAR (Table 1, experiments 1 and 2). Moreover, cotransfection with pH3-art did not increase CAT activity directed by these plasmids. Therefore, sequences located entirely within the env gene are necessary for the negative and art gene responses.

To examine the potential role of the splice donor and acceptor sites that are located within the env gene (15), these sequences were deleted from plasmids pIIAAR3 and pIIAR4, respectively (Fig. 1b). The low level of CAT activity directed by these plasmids was substantially increased in the presence of the art gene product (Table 1, experiment 3). We conclude that the splice acceptor or donor sites are not required for art gene regulation. This conclusion is strengthened by the observation that expression of CAT by plasmid PA-6376 (Fig. 3) that contains only that region of the env gene located between the splice donor and acceptor sites is subject to regulation by art gene product (Table 1, experiment 5).

The 3' HIV sequences were placed on the 5' side of a heterologous promoter to determine if they exhibit an enhancer (29) or silencer (30) function. The promoter used was derived from the LTR of HTLV-I. Plasmids used for this experiment, pC-55/III1470 and pC-55/III2479, are described in Table 1. The HTLV-I LTR sequence of plasmid pC-55 contains a promoter activity but lacks a functional enhancer (31). This promoter can be stimulated by heterologous enhancers (31). The CAT activity directed by plasmids pC-55/III1470 and pC-55/III2479 was similar to the
product was substantially greater than that directed by pA-6376. This result indicates that a negative regulatory sequence was removed by deletion of the env gene region between these nucleotides. However, the remaining portion of the env gene contains an additional negative regulatory sequence that is responsive to the art gene product, as the level of CAT activity directed by pA-6725 is increased 5-fold by cotransfection with pH3-art. A response to the art gene product was also retained by plasmids pA-6725 to pA-7283. Removal of 42 nt from pA-7283 to produce pA-7325 eliminated the art response, pinpointing an element important for suppression and/or art gene response to this small region. The sequence from nt 7283 to nt 7325 is rich in purines. This region is highly conserved among diverse HIV isolates (refs. 28 and 32 and M. Alizon, personal communication).

A small deletion of part of this region was introduced into the pIIAR plasmid to yield pIIAR7315-7374 (Table 1). The CAT activity directed by this plasmid was much lower than that directed by pU3R-III and was not increased in the presence of the art gene product (Table 1, experiment 4). This result supports the conclusion drawn from the nested set of deletions that a sequence necessary for the art gene product response is located within this sequence.

**DISCUSSION**

The experiments presented here show that sequences in the env gene that are spliced from the tat, art, and 3'-orf gene mRNAs can negatively regulate the expression of heterologous genes. Moreover, the negative regulatory effect exerted by this region is relieved by the art gene product. In this respect, regulation of the CAT and hGH genes mimics that observed for the viral structural genes gag and env, which are synthesized in detectable amounts only in the presence of a functional art gene product.

Within the env gene are cis-acting sequences that negatively regulate gene expression as well as cis-acting sequences that relieve the negative effect of the env gene sequences in the presence of the art gene product. The regulatory effects of this region are independent of the LTR and the source of polyadenylation sequences and seem not to depend on tissue-specific or species-specific factors, as art gene function is observed in lymphoid, fibroblast, and epithelial cell lines of human and hamster origin. These experiments leave open the possibility that additional negative and art gene product-responsive regulatory sequences exist in other regions of the HIV genome, particularly within the gag and pol genes.

The experiments also show that at least some of the sequences that negatively regulate heterologous gene expression do not entirely overlap with those sequences required for the response to the art gene product. For example, deletion of the sequence from nt 7315 to nt 7374 eliminates the response to the art gene product without eliminating the negative regulatory effect of HIV env gene sequences. In the absence of the art gene product, the effect of the HIV env sequences is to inhibit gene expression.

The mechanism of inhibition of gene expression by HIV env gene sequences is as yet unclear. It has been reported that levels of gag and env gene mRNAs are notably reduced relative to tat–art and the 3'-orf gene mRNAs in cells transfected with art-defective provirus (9). Others have reported that substantial levels of gag and env gene mRNAs are made in the absence of the art gene but that the corresponding proteins are not made in detectable amounts (7, 14). Our attempts to measure RNA levels in these experiments were not successful, possibly due to the short half-life of these mRNA species. Whatever the mechanism, repression of expression of proteins encoded for by mRNAs that carry HIV env gene sequences is independent of virus-
encoded proteins, as such repression is observed upon transfection of uninfected cells.

The existence of repressive and *art* gene responsive sequences within the *env* gene provides an explanation of the dependence on the *art* gene for *gag* and *env* gene expression. Differential expression of virion structural and regulatory proteins may play an important role in the establishment of HIV latency by permitting expression of regulatory genes in the absence of gene products such as the *env* gene protein that are lethal to T4 lymphocytes (33, 34). Differential expression of regulatory and structural genes may also play a role in the lytic cycle permitting gene products such as the *tat* gene product to accumulate prior to the synthesis of *gag* and *env* gene proteins. Similar regulation by intragenic sequences may exist for the human T-cell leukemia viruses types I and II (35, 36). A regulatory role for nontranslated or internal sequences of other mRNAs has also been described (37, 38). The concept of a regulatory role of intragenic sequences should be explored for other viral and cellular genes.

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