Functional Similarities between Human Immunodeficiency Virus Type 1 and Simian Virus 40 κ B Proto-Enhancers

WILLIAM PHARES AND WINSHIP HERR*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

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To search for broadly active enhancer elements within the human immunodeficiency virus type 1 (HIV-1) long terminal repeat, we have used a proto-enhancer amplification assay. In this assay, the enhancer region of simian virus 40 (SV40) is replaced by heterologous regulatory sequences. Upon passage in African green monkey kidney cells, SV40 growth revertants can arise by amplification (usually duplication) of active proto-enhancers within the heterologous sequences. Most of the HIV-1 U3 regulatory sequences were assayed; only amplification of one or both of the HIV-1 enhancer core κ B motifs consistently resulted in viable SV40 virus. Examination of the cell-specific enhancer activity of the individual HIV-1 κ B proto-enhancers showed that, like the broadly active SV40 κ B proto-enhancer (C proto-enhancer), they are all active in noninduced cell lines of either lymphoid (H9 and Jurkat) or nonlymphoid (HeLa and CV-1) origin. Unexpectedly, one of three κ B point mutants that exhibit little or no activity in unstimulated cells is as highly induced in stimulated Jurkat cells as are the wild-type κ B proto-enhancers. This point mutation shows that κ B-related proto-enhancers can display markedly different activation properties in unstimulated cells yet still activate transcription to similar levels in stimulated cells.

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus associated with human AIDS. The virus was originally isolated in association with lymphadenopathy (5) and has been recovered from blood cells of individuals who are seropositive against HIV antigens by cocultivation with CD4⁺ T-cell lines in tissue culture (18, 38). While HIV-1 predominantly infects CD4⁺ helper-inducer T lymphocytes in vivo and in vitro (32), the ability to replicate is not limited to lymphoid cells because virus can be recovered following transfection of an infectious clone of HIV-1 into lymphoid or nonlymphoid cell lines (1). These data indicate that viral tropism is restricted by infectivity, mediated by the CD4 receptor molecule (11, 33). Consistent with the ability of the virus to replicate in a broad range of cell lines, the HIV-1 promoter within the long terminal repeat (LTR) directs transcription in transient assays in both lymphoid and nonlymphoid cell lines (53, 59).

The cell-specific activity of promoters, either restricted to one or a few cell types or relatively unrestricted, usually results from interactions between multiple individual promoter modules; seldom is a single element responsible for the full activity of the promoter (reviewed in reference 13). An extensively studied promoter that is broadly active in tissue culture cells is the simian virus 40 (SV40) early promoter. This promoter is typical of a complex promoter and shares several features with the HIV-1 promoter (Fig. 1). Both promoters contain multiple binding sites for the ubiquitous mammalian transcription factor Sp1 (14, 28) and enhancer sequences which can activate transcription from a heterologous transcriptional start site over large distances (4, 40, 53). Functional dissection of the SV40 enhancer has revealed that enhancers consist of individual elements that can be categorized into two different types of organizational units called enhansons and proto-enhancers (17, 46). Enhansons are the fundamental structural units of enhancers and correlate with protein binding sites (12). Proto-enhancers,

which can be composed of one or two enhansons, are functional units that possess the ability to create an effective enhancer (i.e., can activate at a distance) when present in multiple copies, without a requirement for precise spacing between proto-enhancers.

The three SV40 proto-enhancers, A, B, and C (Fig. 1), were originally identified by genetic selection (10, 25, 26). Phenotypic revertants of SV40 viruses carrying point mutations that debilitated one or two of these three proto-enhancers invariably contained duplications of the remaining wild-type proto-enhancer(s). The boundaries of each proto-enhancer were originally defined as the region of overlap among the many revertant duplications. Subsequent analysis of the A, B, and C elements by multimerization of synthetic oligonucleotides showed that each of these elements displays a unique pattern of cell-specific proto-enhancer activity; this activity is generally more restricted than the activity of the entire SV40 enhancer (47, 55). Thus, the combination of different cell-specific proto-enhancers can explain the broad activity of the wild-type SV40 enhancer.

The HIV-1 U3 regulatory region has not been characterized in as much detail as the SV40 enhancer, but deletion analyses reveal both positive and negative regulatory elements (see references 15 and 22 for reviews). Furthermore, several protein binding sites and regions of sequence similarity with other promoters have been identified (Fig. 1). The most active region of the enhancer has been called the enhancer core (EC) and contains two copies of a 10-bp motif called κ B which was originally identified as a binding site for the nuclear factor NF- κ B within the κ immunoglobulin light-chain enhancer (56). This motif has since been found in a large number of viral and cellular enhancers (reviewed in reference 35); indeed, it is the functional motif within the SV40 C proto-enhancer (26, 29).

Although the 10-bp κ B motif is identical between the κ light-chain, HIV-1, and SV40 enhancers, these elements display different cell-specific activities when assayed in vivo. For example, the κ B motif within the SV40 C proto-en-

* Corresponding author.

FIG. 1. Diagram of the HIV-1 U3-R region and the SV40 early promoter, showing the homologous κ B sites and other regulatory sites. Shown from right to left in the HIV-1 LTR are the TAR region (responsive to the viral transactivator Tat); the transcriptional start site (wavy line with arrow); the TATA box (A/T); three binding sites for the Sp1 transcription factor (I, II, and III; hatched boxes); the enhancer core sites (κ B sites I and II; black boxes); a site homologous to the binding site for the upstream stimulatory factor (USF) in the major late promoter of adenovirus; the binding site for a factor found in nuclei of stimulated T cells (NFAT-1); a small region homologous to sequences upstream of the interleukin-2 (IL-2), interleukin-2 receptor- α (IL-2R), and gamma interferon (IFN γ) promoters; binding sites for the AP-1 transcriptional activator complex; and a negative regulatory region (NRE). Positions of the four *Hae*III restriction sites used to clone HIV U3 segments into SV40 are indicated by vertical lines above the diagram. The *Kpn*I and *Bam*HI sites in the SV40 early region used for enhancer replacement are indicated in the diagram of the SV40 early promoter. Here are shown from right to left the SV40 early start sites, A/T-rich TATA element, six (I to VI) Sp1 binding sites, 72-bp element, and major late start sites. Positions of the A, B, and C proto-enhancers are shown by the boxes. The tandem HIV-1 κ B sites and the SV40 C proto-enhancer κ B site are indicated by the black boxes, with the relative orientation shown by the arrows beneath. The sequences contained in each of the three wild-type and three mutant synthetic multimerized proto-enhancer constructs are listed below the HIV-1 κ B sites. The three boxed nucleotides at each end represent the *Xho*I linker sequences that separate each repeat. When these nucleotides match the wild-type sequence flanking the κ B sequences in HIV-1 or SV40, they are shown in capital letters. The 3' T residue of ECII is the same position in the HIV-1 LTR sequence as the 5' T residue of ECI. Mutations in the SV40 C16 construct (*spm*5, *dpm*10, and *tpm*1) are indicated by arrows at specific bases.

hancer is very broadly active in different uninduced cell types (29, 47, 55), whereas the original κ enhancer κ B motif is active primarily in mature B cells or cells stimulated with the tumor promoter phorbol 12-myristate 13-acetate (PMA) (43, 51, 67). The activity of the HIV-1 κ B motifs generally has been observed in a broad array of cell types (19, 24, 30, 41, 62), although in one report activity could not be detected unless the cells (Jurkat T cells) were stimulated with T-cell activators (42). To explain the differences between the SV40 and κ enhancer κ B motifs, Pierce et al. (51) suggested that the broad activity of the SV40 C proto-enhancer might result from the activity of overlapping motifs defined by sequences flanking the κ B motif.

In this study, we have adapted the SV40 genetic selection strategy, which successfully identified the three SV40 proto-enhancers, to assay and to identify broadly active proto-enhancers within the HIV-1 U3 region. For this proto-enhancer amplification assay, the SV40 enhancer region was replaced by the heterologous HIV sequences. The parental SV40-HIV recombinants are not capable of lytic growth in the permissive African green monkey kidney cell line CV-1, but certain recombinants yield phenotypic revertants which invariably contain rearrangements, usually simple tandem duplications, that amplify the number of heterologous proto-enhancers. Of the HIV-1 sequences tested (the three *Hae*III fragments shown in Fig. 1), only amplification of the HIV-1 κ B motifs consistently resulted in viable SV40 virus. Thus, the HIV-1 and SV40 κ B motifs both possess the ability to restore growth of SV40 when present in multiple copies. The

functional similarities among the one SV40 and two HIV-1 κ B proto-enhancers were further established by assay of their enhancer activity as multimerized synthetic enhancers in both lymphoid and nonlymphoid cells.

MATERIALS AND METHODS

Tissue culture and cell lines. CV-1 and HeLa cells were grown in Dulbecco's modified Eagle minimum essential medium supplemented with 5% fetal calf serum, penicillin, and streptomycin. The human T-lymphoid H9 and Jurkat cell lines (18) were grown in RPMI 1640 medium supplemented with 10% (H9) or 5% (Jurkat) fetal calf serum, 2 mM glutamine, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.3), and antibiotics.

Construction of replication-defective SV40/HIV-1 recombinants. SV40 enhancer substitutions were created in the SV40 enhancer replacement vector pSVER. pSVER was constructed in two steps. First, the SV40 early promoter region (*Eco*RI-*Hind*III fragment from the recombinant pAO plasmid [68]) was inserted into pUC119, thus creating pUC119AO; second, a unique *Sac*I restriction site was created in the SV40 sequences of this plasmid by successive steps of digestion with *Asp*718, treatment with the large fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates, digestion with *Pvu*II, and insertion of a *Sac*I linker (CGAGCTCG) by ligation. This ligation recreated the SV40 *Kpn*I-*Asp*718 restriction site. Recombinant pSVER/HIV-1 plasmids were constructed by ligation of the

three HIV-1 LTR *Hae*III fragments, indicated in Fig. 1 and isolated from pCD12 (45), into flush-ended *Sac*I-*Bam*HI-digested pSVER; the structure and orientation of each insert were confirmed by DNA sequence analysis. The following point mutations relative to the HIV-1 sequence reported by Ratner et al. (52) were found: in the 91-bp *Hae*III fragment (positions -69 to -159), C→T at positions -125 and -110; in the 117-bp fragment (-160 to -276), G→T at position -215; and in the 76-bp fragment (-277 to -352), A→C at -308 and G→A at -347. All sequence positions are given relative to the RNA start site as +1. Recombinant SV40/HIV-1 fragments were subsequently transferred into the remainder of the SV40 genome (strain 776) by ligation of pSVER/HIV-1-derived *Kpn*I-*Bgl*II fragments into the SV40-containing pK1K1 plasmid (20), resulting in the SV40/HIV-1 recombinant plasmids used for transfection.

Plaque assay and revertant isolations. The pK1K1 derivatives contain a terminal duplication of 0.27 copy of the SV40 late region which permits excision of unit-length SV40 genomes by homologous recombination upon transfection of the DNA into permissive cells. For higher transfection efficiencies for the revertant isolations, closed circular SV40/HIV-1 DNA (obtained by linearization of the SV40/HIV-1 plasmid DNAs with *Eco*RI, followed by ligation at a dilute DNA concentration) was transfected into CV-1 cells by DEAE-dextran- and chloroquine-mediated transfection, as described previously (25). For each recombinant, 14 plates of cells were transfected. To obtain growth revertants, a virus lysate was prepared by twice freeze-thawing the cell cultures 3 days posttransfection and used to infect freshly confluent CV-1 cells. Subsequent transfers were 12 to 14 days postinfection. Once a visibly active virus stock was obtained, revertant viruses were purified by two rounds of plaque purification. Two plaque isolates were purified from each initial transfection. These isolates were frequently identical or related (see Results). Plaque purification and analysis of Hirt DNA extracts were performed as described previously (25). For DNA sequence analysis, the small SV40 *Bgl*II-*Eco*RI fragment of each revertant was cloned into the large *Sfi*I-*Eco*RI fragment of pUC119AO. Single-stranded DNA templates were sequenced with a primer complementary to SV40 at sequence positions 455 to 473 (63).

Activity assays of multimerized synthetic proto-enhancers. Human β -globin expression vectors, containing six tandem copies (6X) of synthetic proto-enhancers cloned into the *Sph*I site downstream of the β -globin sequences, were constructed from p β e⁻ as described previously (46, 47). The sequences of the two oligonucleotides used to prepare the 6XECI construct were AGTGGGGACTTTCCAGGCTCG and GCCTGGAAAGTCCCCACTCGA. When annealed, the underlined nucleotides form 3' overhangs; ligation of double-stranded oligomers forms an *Xho*I recognition site, CTC GAG, between adjacent subunits. The other constructs were made with identical oligonucleotides except for the changes between the *Xho*I linker sequences as shown in Fig. 1. The orientation of 6XECII, 6XC16, 6XC16*spm*5, 6XC16*dpm*10, and 6XC16*tpm*1 is (-) as defined by Ondek et al. (47); i.e., the GGAAAGTCCC-containing strand reads counterclockwise in the p β vector as shown by Ondek et al. (47). 6XECI was constructed in the (+) orientation; the (-) orientation was obtained by inverting the orientation of the *Hind*III-*Pst*I fragment of p β 6XECI⁺ by religation into the blunt-ended *Sph*I site of p β e⁻.

Purified plasmid DNAs were transfected along with a human α -globin internal control plasmid into CV-1 and HeLa cells by calcium phosphate coprecipitation (47) or into Jurkat

or H9 cells by electroporation. Either π SVHP α 2 (64) or pBS α 2 (kindly provided by M. Gilman) was used as an internal control. Jurkat and H9 cells were electroporated (8) as follows: 1.5×10^7 cells in 0.25 ml of complete growth medium, containing 10 μ g each of the p β test plasmid and α -globin control plasmid per ml and sufficient pUC119 carrier DNA for a final DNA concentration of 80 μ g/ml, were subjected to a pulse of 200 V at a capacitance of 960 μ F in a Gene Pulser apparatus (Bio-Rad). These conditions result in α - and β -globin expression levels that are proportional to the amount of transfected test plasmid. For stimulation of Jurkat cells by mitogenic lectins and tumor promoters, 1 μ g of phytohemagglutinin (PHA) per ml and 10 nM PMA were added 14 to 16 h after transfection, and incubation was continued for 8 h prior to isolation of RNA; RNA was isolated from unstimulated cells 22 to 24 h after transfection of parallel cultures. Isolation of RNA and RNase protection of α - and β -globin antisense probes were performed as previously described (26). The bands corresponding to correctly initiated α - and β -globin RNAs were quantitated by liquid scintillation spectrometry.

RESULTS

Proto-enhancer amplification assay of HIV-1 U3 region sequences. To establish the proto-enhancer amplification assay of a heterologous enhancer, an SV40 enhancer replacement vector, pSVER, was created that allows substitution of the SV40 sequences between the unique *Kpn*I recognition site and an engineered *Bam*HI recognition site (68) (Fig. 1) with heterologous sequences. Such a substitution removes the majority of the SV40 enhancer sequences (49, 66, 68) while maintaining the *Kpn*I site sequences which can influence usage of the SV40 major late initiation site (7). The 76-, 91-, and 117-bp HIV-1 *Hae*III fragments indicated in Fig. 1 were individually cloned into the pSVER vector. The 91-bp *Hae*III fragment, which spans the two κ B motifs, was cloned in both orientations to create pSV/HIV91⁺ and pSV/HIV91⁻. The (+) orientation indicates that the HIV sequences are in the same orientation with respect to the SV40 early promoter as they are normally with respect to the HIV-1 promoter. Because the κ B motifs are positioned in opposite orientations in the HIV-1 and SV40 early promoters (Fig. 1), it is the pSV/HIV91⁻ construct which contains the HIV-1 κ B motifs in the same orientation as the SV40 C proto-enhancer κ B motif. The 76- and 117-bp HIV-1 fragments were assayed only in the (-) orientation.

To assay virus viability, the recombinant SV40/HIV-1 enhancer regions were transferred to the SV40-containing vector pK1K1 (20). Under conditions that yielded 10^5 plaques per μ g of wild-type (2X72) SV40 pK1K1 plasmid DNA, no plaques were observed upon transfection of 400 ng of the pK1K1 SV40/HIV-1 recombinants. These results indicate that the SV40/HIV-1 recombinants are growth defective. We were never able to obtain virus from the SV/HIV76⁻ and SV/HIV117⁻ recombinants, but virus could be isolated from the SV/HIV91⁺ and SV/HIV91⁻ recombinants after repeated serial passage of infected cell lysates onto fresh CV-1 cell cultures. Transfer of lysates from independently transfected SV/HIV91⁺ and SV/HIV91⁻ cell cultures exhibited cytopathic effects by the third transfer, and the fourth transfer resulted in confluent lysis of the cultures. The inability to detect virus growth from the SV/HIV76⁻ and SV/HIV117⁻ recombinants even after four serial transfers suggests that these HIV-1 sequences do not contain en-

hancer elements that can function effectively in CV-1 cells and in the context of the SV40 vector (see Discussion).

Replication-competent viruses arising from multiple independent transfections of SV/HIV91⁺ and SV/HIV91⁻ DNAs were purified twice by plaque assay, and the structures of independent isolates were examined. Electrophoretic analysis of revertant SV40 genomic DNAs digested with *Nco*I indicated that rearrangements had occurred in the recombinant enhancer regions but not elsewhere in the genome (data not shown). DNA sequence analysis of the altered regions revealed duplications of the heterologous HIV sequences. Figure 2 illustrates the structures of the 12 revertants isolated from each of the parental SV/HIV91⁺ (Fig. 2A) and SV/HIV91⁻ (Fig. 2B) recombinants. Each rectangle, or set of rectangles when multiple duplications are present, represents the sequences from the parental SV/HIV91 recombinant shown at the top of each panel that are tandemly duplicated in the revertants. The revertants are identified by the size of the revertant duplication in base pairs (i.e., SV/HIV91⁺rd26 contains a 26-bp duplication). Two SV/HIV91⁺ revertants, rd26/dl97 and rd45/dl77, contain deletions of sequences flanking the duplication (Fig. 2A). These two revertants probably arose in two steps, a duplication followed by a deletion, because they each appeared from the same transfected samples as the related rd26 and rd45 revertants, which contain only the duplications. (See the legend to Fig. 2 for the origin of each revertant and the coordinates of each duplication.) We do not know whether the deletions contribute to improved growth. Finally, one pair of revertants, rd45 and rd45/dl77, contain an extra A residue at the duplication junction that was probably inserted at the time the duplication arose.

The duplication patterns generated by the two sets of SV/HIV91 revertants exhibit subtle differences from one another. The SV/HIV91⁺ revertants generally contain smaller (26- to 57-bp) duplications, whereas the SV/HIV91⁻ revertant duplications frequently include flanking SV40 late region sequences and the rearrangements are sometimes quite complicated, since they can contain multiple tandem duplications (i.e., rd70/rd75 and rd14/rd50/rd28). We do not know the reasons for these differences, but they may include the orientation or position of critical elements within the HIV sequences with respect to the SV40 early and late promoters. Even though differences exist, in each case there is a single 24- or 38-bp region that is included in all of the duplications of the SV/HIV91⁺ or SV/HIV91⁻ revertants, respectively. It is precisely such consistently duplicated regions that in SV40 enhancer revertants allowed the identification of the three SV40 A, B, and C proto-enhancers (10, 25, 26). In both sets of revertants, the commonly duplicated regions encompass the κ B motif, either both motifs, as in the SV/HIV91⁺ revertants, or just one of the two κ B motifs along with the neighboring Sp1 binding site, as in SV/HIV91⁻ revertants (Fig. 2). These results indicate that duplication of the HIV-1 κ B motifs can replace the SV40 enhancer for virus growth in CV-1 cells and that this property is independent of the exact position and orientation of the κ B motifs. Thus, within the three HIV-1 regions tested in the proto-enhancer amplification assay, the κ B motifs may be the only broadly active enhancer elements.

HIV-1 κ B proto-enhancers are broadly active. Previous studies showed that the revertant duplications that arose in SV40 enhancer mutants are directly responsible for both the revertant phenotype and improved enhancer function (10, 25, 26). Here, we have not assayed the activities of the SV/HIV91 revertant duplications. Instead, we chose to

assay directly the proto-enhancer activity of the two HIV κ B motifs by constructing synthetic multimerized enhancers and assaying activation of the human β -globin promoter in different cell types. This strategy permits a direct comparison of the activities of the SV40 C proto-enhancer, which is known to be active in a broad array of noninduced cell types (29, 44, 47, 55), and the two HIV κ B motifs ECI and ECII, which were originally shown to be active only in induced cells (42).

Separate multimerized ECI and ECII enhancers were constructed that matched a set of wild-type and mutant SV40 C proto-enhancer constructs previously assayed in CV-1 cells (the C16 series [61]) (Fig. 1). To align correctly the ECI and ECII κ B motifs with the SV40 counterpart, they are each flanked by 2 bp of 5' and 3 bp of 3' wild-type HIV sequence (Fig. 1) and separated by an *Xho*I recognition site. The three SV40 C16 point mutants shown in Fig. 1 contain either single (*spm*5), double (*dpm*10), or triple (*tpm*1) point mutations. The *spm*5 (25) and *dpm*10 (2) mutations, in the context of an SV40 virus with a single 72-bp element (1X72), are each independently deleterious for SV40 growth; these mutations mutate the first and last residues of the C proto-enhancer κ B motif. The *tpm*1 mutation is identical to a mutation used previously to study HIV-1 κ B motif-binding proteins (16).

Six tandem copies of the test sequences were inserted 2.2 kb downstream of the human β -globin gene transcriptional initiation site in the plasmid p β e⁻ (46), and enhancer activity was assayed by transient expression in different cell types; the same vector with a single copy of the 72-bp element (p β 1X72) served as a positive control and as the reference for normalization. The p β e⁻ derivatives were transfected into cells along with an internal reference plasmid containing the human α -globin gene either by calcium phosphate coprecipitation (CV-1 and HeLa) or electroporation (H9 and Jurkat). Cytoplasmic RNAs isolated from transfected cells were probed for α - and β -globin transcripts by an RNase protection assay.

The results of assays performed in three different cell types, simian kidney cells (CV-1), human cervical carcinoma cells (HeLa), and human CD4⁺ T cells permissive for HIV-1 replication (H9), are shown in Fig. 3; quantitation of the results is presented in Table 1. Between the bands that represent correctly initiated α - and β -globin RNAs (labeled α and β in Fig. 3) are two bands labeled it1 and it2 which represent incorrectly initiated β -globin transcripts that are aberrantly spliced (see reference 57 for a full description of the "it" transcripts). The assays in Fig. 3 revealed significant activity of the three wild-type κ B motifs (ECI, ECII, and C16; Fig. 3, lanes 2 to 4 in all panels) in all three cell lines. These results are consistent with the broad activity previously reported with use of different synthetic multimers of the SV40 C proto-enhancer (29, 47, 55); the activity of the multimerized HIV ECI and ECII proto-enhancers in uninduced H9 cells is consistent with results reported by Kaufman et al. (30).

In general, the ECI, ECII, and SV40 C16 sequences activated transcription to similar levels, and the *spm*5 and *dpm*10 mutations (Fig. 3, lanes 5 and 6) resulted in similar activation in all three cell types; in each case, however, the *dpm*10 mutant was slightly more active than the *spm*5 mutant. The wild-type κ B activities in uninduced cells are significant, since they compare favorably to activation by the wild-type SV40 enhancer control (lanes 7). Some of the differences in the relative activation potential of ECI, ECII, and C16 may reflect relatively subtle effects of the different

FIG. 2. Tandemly duplicated sequences in 24 revertants of the SV/HIV91 recombinant viruses in CV-1 cells. The structures of the parental growth-defective recombinants SV/HIV91⁺ (A) and SV/H91⁻ (B) are shown at the top of each panel. The sequences that are duplicated in each of the 12 plaque-forming revertants of the SV/HIV91⁺ and SV/HIV91⁻ viruses are indicated below each diagram by the horizontal bars. The revertant duplications are referred to as rd (revertant duplication) followed by the length of the duplication in base pairs. The region common to all revertant duplications from each recombinant is indicated by the stippled region, and the sequence is given at the bottom of each diagram; where included, the positions of the ECI, ECII, and Sp1 binding site GCIII is indicated below the commonly duplicated sequence. Isolates of two SV/HIV91⁺ revertants, rd26/dl97 and rd45/dl77, contained deletions (dl) of upstream sequences, indicated by the dashed lines. Two related revertants (rd45 and rd45/dl77) contained a base insertion at the duplication junction, indicated by the A residue next to the arrowhead above the duplication. Two SV/HIV91⁻ revertants, rd70/rd75 and rd14/rd50/rd28, contained multiple duplications in tandem; in these revertants, the sequence from left to right through the duplicated segments (horizontal boxes) begins with the upper box and then reads through the lower boxes. Each revertant arose from an independent transfection of CV-1 cells except for the following pairs of revertants, which arose from common transfections: SV/HIV91⁺ revertants rd26 and rd26/dl97, rd45 and rd45/dl77, and rd34 and rd53, and SV/HIV91⁻ revertants rd183 and rd70/rd75, and rd55 and rd58. Not all transfections resulted in purified and sequenced revertants. Below are listed the exact recombination points for each revertant rearrangement. The left and right (as shown in the figure) recombination points in each revertant are given. Recombination points within SV40 sequences are identified by the suffix S, and those within the HIV-1 sequences are given the suffix H. In the parental constructs, SV40 positions 106 to 292 inclusive were deleted. The 91-bp HIV-1 *Hae*III fragment contains HIV-1 positions -69 relative to the transcription initiation site to -159 inclusive. The size of the recombination point ambiguity caused by homology at the recombination endpoints is indicated in parentheses. The rearrangement coordinates for SV/HIV91⁺ are as follows: rd26, -104H/-79H (2); rd26/dl97, -104H/-79H (2) and Δ 348S/-118H (2); rd51, -111H/95S (0); rd45, -106H/96S (0); rd45/dl77, -106H/96S (0) and Δ 320S/-111H (0); rd52, -121H/105S (0); rd33, -104H/-70H (2); rd57, -124H/103S (0); rd38, -115H/-78H (1); rd34, -114H/-81H (0); rd53, -122H/105S (1); and rd32, -104H/-71H (4). The rearrangement coordinates for SV/HIV91⁻ are as follows: rd183, 404S/-138H (0); rd70/rd75, 323S/-107H (0) followed by 325S/-110H (4); rd134, 393S/-101H (1); rd118, 378S/-100H (0); rd112, 366S/-107H (1); rd97, 338S/-121H (0); rd60, 306S/-114H (0); rd54, 300S/-114H (0); rd14/rd50/rd28, -91H/-104H (0) followed by 298S/-114H (0) and then -87H/-114H (0); rd114, 374S/-102H (4); rd58, 309S/-111H (0); and rd55, 309S/-108H (3).

sequences flanking each 10-bp κ B motif (see Fig. 1). Inactivation of the κ B proto-enhancer by both the *spm5* and *dpm10* mutations is consistent with the results of Kanno et al. (29), who, using a similar assay, tested the effect of individually mutating every position of a 13-bp sequence spanning the 10-bp SV40 κ B motif. Comparison with their

results suggests that the deleterious effect of the *dpm10* mutation is entirely due to the single mutation within the κ B motif. Both sets of results argue that in these cell lines the κ B motif is entirely responsible for the activity of the SV40 C proto-enhancer.

The *dpm10* κ B mutation is active in stimulated Jurkat cells.

FIG. 3. Evidence that enhancer activity of multimerized κ B sites, as assayed by transient human β -globin gene expression, is broadly distributed in different cell lines. The autoradiographs show the levels of α - and β -globin probe RNAs protected from RNase digestion by cytoplasmic RNA isolated from transfected CV-1 cells (A), HeLa cells (B), and H9 cells (C). Transfection of the 6X series of β -globin reporter constructs indicated at the top of each lane was done as described in Materials and Methods. Protected fragments corresponding to correctly initiated α -globin (α) and β -globin (β) transcripts are indicated; incorrect β -globin transcripts are labeled it1 and it2. Enhancer activity was assayed relative to activities of the enhancerless β -globin reporter p β e⁻ (e⁻; lanes 1) and p β 1X72, which carries a single SV40 72-bp enhancer element (lanes 7). Results of transfections with α -globin alone or mock transfections are shown for HeLa cells (panel B, lanes 8 and 9, respectively); similar results were obtained with CV-1 and H9 cells (not shown). Quantitative results are summarized in Table 1.

TABLE 1. Summary of transient activity assays

Cell line	Relative activity ^a							
	e ⁻	ECI	ECII	C16	<i>spm5</i>	<i>dpm10</i>	<i>tpm1</i>	1X72
CV-1	0.02	0.4	0.9	0.2	0.01	0.03	ND	1
HeLa	<0.05	0.4	0.5	0.2	<0.05	<0.05	ND	1
H9	0.02	0.6	0.9	0.8	0.04	0.09	ND	1
Jurkat	0.11	1.0	0.8	0.4	0.16	0.14	0.05	1
Jurkat + PMA-PHA	0.07	16	16	13	0.12	5.8	0.05	5.5
Fold PMA-PHA activation ^b	0.6	16	20	32	0.8	41	1	5.5

^a Levels of β -globin RNA were determined directly from the experiments shown in Fig. 3 and 4 as described in Materials and Methods. The levels of β -globin RNA were normalized to the α -globin RNA reference and are shown relative to the value for the SV40 enhancer (1X72) control. Induced activity in Jurkat cells treated with PMA and PHA was normalized to uninduced levels of 1X72 expression; in these experiments, PMA-PHA treatment reproducibly induced α -globin expression by 2- to 4-fold (standardized against total cytoplasmic RNA) and was 2.4-fold in this experiment. After normalization to the induced α -globin RNA signal, the values were multiplied by 2.4 to take into account the α -globin induction. Results shown are from the single series of transfections with each cell type shown in Fig. 3 and 4, in which the activity with ECI, ECII, and C16 was determined in duplicate and did not vary by more than 20%; in most cases, variability was less than 10%. Other experiments for each cell line were consistent with the results shown here. The orientation of the multimerized oligonucleotides was (-) as described in Materials and Methods, except for 6XECI in CV-1, HeLa, and H9 cells, in which the (+) orientation was used. e⁻, Enhancerless; ND, not determined.

^b Ratio of the levels of β -globin RNA in the presence of PMA and PHA versus in their absence.

Figure 4 shows a β -globin activation assay of the multimerized κ B elements in uninduced (lanes 1 to 9) and induced (lanes 10 to 17) human Jurkat T cells. Jurkat cells serve as a model to study activation of latent HIV provirus by inducers of T-cell proliferation such as the phorbol ester PMA and the lectin PHA (23, 39). The HIV-1 ECI and ECII elements respond to PMA and PHA activation of Jurkat cells (42, 62). We therefore used Jurkat cells to compare the responses of

the multimerized wild-type and mutant SV40 C proto-enhancer constructs with the HIV-1 ECI and ECII proto-enhancers.

In uninduced Jurkat cells, the wild-type HIV-1 and SV40 κ B multimerized proto-enhancers displayed three- to nine-fold higher levels of activity than the enhancerless control (compare lanes 3 to 5 with lane 2 in Fig. 4; Table 1). As in the other three cell lines tested, these activities were similar to the activity of the wild-type SV40 enhancer, and the *spm5* and *dpm10* mutants were weakly active, if at all. In Jurkat cells, we also assayed the *tpm1* mutation, which, as expected from the results with a similar mutation (42), was not active. Upon stimulation for 8 h with a mixture of PMA and PHA, the ECI, ECII, and C16 enhancers were all induced 10- to 30-fold while the SV40 enhancer was activated 5- to 6-fold (Table 1). Activation of the SV40 enhancer by PMA is consistent with previous results (27); indeed, expression from the SV40 enhancer-containing α -globin internal reference plasmid was also consistently induced two- to fourfold by the stimulation with PMA and PHA.

As expected, the *spm5* and *tpm1* κ B mutants were not activated by PMA and PHA induction, but surprisingly the *dpm10* mutant, which in all the other assays exhibited only a small amount of activity, if any, was highly induced. Indeed, the 40-fold induction of this sample was greater than the fold induction of any of the other samples (Table 1) because of the low uninduced levels of expression. Because activation by the SV40 κ B proto-enhancer is still sensitive to the *spm5* and *tpm1* mutations yet relatively insensitive to changes in the sequences flanking the κ B motif, as evidenced by the similar activities of the ECI, ECII, and C16 constructs, it is probable that induction of the *dpm10* mutant is not due to an overlapping motif but instead is still due to κ B proto-enhancer-binding proteins. The robust activity of the C16 *dpm10* mutant in stimulated Jurkat cells shows that κ B proto-enhancers that can display marked differences in activation properties in uninduced cells can respond similarly to Jurkat cell activation.

DISCUSSION

Proto-enhancer amplification to identify generally active proto-enhancers. To identify proto-enhancers (enhancer subelements that upon multimerization can create an enhancer), we have used an in vivo selection assay which we term

FIG. 4. Induction of κ B-directed enhancer activity in the human lymphoid Jurkat T-cell line. The autoradiograph shows the β -globin expression with multimerized κ B proto-enhancers in unstimulated Jurkat cells and Jurkat cells stimulated by combined treatment with PMA and PHA as described in Materials and Methods; 30 μ g of total RNA from unstimulated cells and 20 μ g from PMA-PHA-stimulated cells was used in each hybridization reaction with probe. Quantitative results are summarized in Table 1.

proto-enhancer amplification. In this assay, the SV40 enhancer can be replaced by a heterologous sequence; upon passage of the recombinant virus, any proto-enhancer that is active in cells permissive for SV40 replication can be duplicated or further amplified to produce better-replicating virus. This assay is related to the SV40 enhancer trap assay developed by Weber et al. (66) in which already functional enhancers can be identified by selection of viable SV40 virus carrying random fragments of DNA in place of the enhancer. But the enhancer trap assay differs from the proto-enhancer amplification assay because it generally selects enhancers that are already functional, not rearrangements that create an enhancer.

The proto-enhancer amplification assay is designed to elucidate the substructure of a known enhancer by selection for viral rearrangements that can identify proto-enhancers. This assay led to the identification of the three SV40 proto-enhancers, A, B, and C. An advantage of this strategy in comparison with a deletion or point mutagenesis analysis is that enhancer elements are identified functionally by positive selection rather than by loss of function; therefore, individual elements that display little activity on their own can be identified by the increased potency resulting from proto-enhancer duplication. For example, mutations in any one of the three SV40 proto-enhancers do not have a very large effect on SV40 enhancer function (68), because these proto-enhancers are functionally redundant (25). A disadvantage of this SV40 selection assay is that it is limited to proto-enhancers that are active in the context of SV40 and in cells permissive for SV40 replication. To overcome the cell type limitation, we have developed polyomavirus, which can grow in many different murine cell lines, as a vector for proto-enhancer amplification; this polyomavirus vector system has allowed proto-enhancer amplification in polyomavirus/SV40 enhancer recombinants in murine F9 embryonal carcinoma cells (61).

Of the three HIV-1 *Hae*III fragments assayed here in the SV40 vector, only the 91-bp *Hae*III fragment yielded viable virus. This result suggests that the sequences contained within the two upstream *Hae*III fragments do not contain any proto-enhancers that are active in the CV-1 cells used to propagate the SV40/HIV recombinants. There are two caveats to this conclusion. First, a proto-enhancer active in CV-1 cells could have been disrupted by digestion of the HIV-1 LTR by *Hae*III endonuclease. For example, the putative HIV LTR USF/MLTF binding site (54) is cleaved by *Hae*III (Fig. 1). Second, a negative regulatory element (53) could mask the activity of a positive element. With these caveats in mind, the SV40 proto-enhancer amplification assay should be generally applicable for the identification of broadly active proto-enhancers. To identify cell-specific HIV-1 proto-enhancers, the polyomavirus proto-enhancer amplification assay may prove useful. Other, probably cell-type-specific, HIV-1 proto-enhancers are likely to exist because deletion of the HIV-1 κ B motifs does not abolish virus replication in T cells (37).

The SV40 and HIV-1 κ B proto-enhancers are functionally similar. The two different assays described here, proto-enhancer amplification and transient expression assay of multimerized enhancers, both indicate that the HIV-1 κ B motifs are broadly active proto-enhancers in uninduced cells in culture and are functionally similar to the SV40 κ B proto-enhancer. The activity observed here is consistent with the uninduced activities observed previously for the SV40 κ B proto-enhancer (29, 47, 55) and the HIV-1 κ B proto-enhancers (24, 30, 41, 62). The reported inactivity of

the HIV-1 κ B proto-enhancers in Jurkat cells (42) may have resulted from the use of a less sensitive assay because these motifs display little activity in these cells (Fig. 4). The similar activities of the HIV-1 and SV40 κ B proto-enhancers are in stark contrast to the lymphoid-specific or activation-specific activity observed for the κ enhancer region spanning the κ B motif (43, 51, 67). These latter results suggested that the broad activity of the SV40 κ B proto-enhancer (C element; 47, 55) was due to overlapping elements (43, 51). The results described here, however, in which a matched set of three active κ B proto-enhancers, two of which (ECI and ECII; Fig. 1) do not share any κ B flanking sequences but display similar activation potentials, argue against an overlapping element. Furthermore, the inactivity of the *spm5* and *dpm10* κ B mutants, in which opposite extremes of the κ B motif are mutated, argues that the full κ B motif is responsible for the broad activities observed here in uninduced cells.

Perhaps it is the κ enhancer κ B motif, instead of its SV40 counterpart, that contains an overlapping element, in this case lymphoid specific, which overshadows the activity of the κ enhancer κ B motif in lymphoid cells. By comparison, then, any activity of the κ enhancer κ B motif that could be observed in uninduced nonlymphoid cells (as can be observed in the results of Nelsen et al. [43] in HeLa cells) would appear relatively weak and for this reason may have been ignored. The SV40 B proto-enhancer is an extensively studied example in which overlapping proto-enhancers display different cell-type-specific activities: the octamer motif is lymphoid specific, and the sph motifs are active in many non-B-cell lines (12, 17, 60). One way to unravel such complex proto-enhancers is to make many individual point mutations both within and flanking the sequence motif suspected to be responsible for a particular activity. Previously described κ enhancer κ B mutations in which three (51) or six (67) base pairs of the κ B motif were mutated are unlikely to uncover overlapping elements, because such mutations are likely to inactivate both elements simultaneously. Indeed, it is the individual mutagenesis of every position within and surrounding the SV40 κ B proto-enhancer by Kanno et al. (29) that convincingly shows that there are no overlapping elements that lie entirely within the SV40 C proto-enhancer.

The importance of the κ B motif for transcriptional activation in nonlymphoid cells is emphasized by the ability of duplications of the SV40 κ B proto-enhancer to substitute effectively for loss of A and B proto-enhancer function during SV40 growth in CV-1 cells (26). The κ B motif is also found in the very strong enhancer of the human cytomegalovirus (CMV) early promoter (6). Because treatment of cells with agents that mimic viral infection (e.g., double-stranded RNA) induces the activity of the κ B motif-binding protein NF- κ B (35, 65), DNA viruses such as SV40 and CMV may stimulate their own expression indirectly by incorporating the NF- κ B responsive element within their enhancers. In HIV-1-infected individuals, the progression of AIDS symptoms is associated with other viral infections that may act as cofactors. One prevalent viral infection in AIDS patients is by CMV, which grows well in immunocompromised individuals. The presence of shared transcriptional regulatory elements between a DNA virus like CMV and a retrovirus like HIV-1 may play an important role in cross-talk between coinfecting viruses.

The broad activity observed with multimerized κ B elements in transient assays in cell lines, which mimics a similar broad expression pattern observed with large HIV-1 LTR fragments linked to expression vectors, may be particular to

cells that can grow continuously in culture. In transgenic mice, the HIV-1 LTR can display restricted, albeit variable, cell-type-specific patterns of expression (36, 58), and a similar in vivo restriction of an otherwise broad activity in cell culture has been seen for the SV40 72-bp enhancer element in transgenic mice (48). These results suggest that the potent ubiquitous activity of the κ B elements in tissue culture cells may be the result of adaptation to continuous growth in vitro. In vivo the difference between HIV expression in nonstimulated versus stimulated lymphoid cells could be much greater than seen in transient assays in tissue culture because of lower basal levels of activity in nonstimulated cells.

Differential response of κ B point mutations to stimulation of Jurkat cells with PMA and PHA. The activation of κ B function by PMA and PHA shown here in Jurkat cells (Fig. 4) has been observed previously in various cell types for the SV40 C proto-enhancer (9, 29) and the HIV-1 κ B motifs (24, 30, 42, 62). To our surprise, however, one of the κ B mutations, *dpm10*, although generally inactive in uninduced cells, was very active in induced Jurkat cells. We envisage two possible mechanisms to explain the robust activity of the *dpm10* mutant in activated Jurkat cells. There could be one or a family of κ B-specific transcription factors that interact weakly with the *dpm10* mutant; in unstimulated cells, this weak affinity could result in a low activity in comparison with the wild-type κ B element. In stimulated cells, however, the active form of the κ B-specific transcription factor(s) may no longer be limiting. Then under such saturating conditions, the activity of the mutant κ B motif could approach that of the wild-type element. Alternatively, among a family of κ B-specific transcription factors there may exist members that are more sensitive and others that are less sensitive to subtle alterations in the κ B binding site. This second model proposes that one or more inducible κ B-specific transcription factors are insensitive to the *dpm10* mutations whereas another (or others), constituting the basal activity, is sensitive.

Consistent with the second model, the two κ B-binding factors NF- κ B and KBF-1/H2TF-1, which share overlapping but different binding specificities (3), share a DNA-binding subunit that is related to the proto-oncogene *c-rel* (21, 31). Furthermore, the human κ B-binding protein HIVEN86A (16) is structurally related or identical to the product of the human *c-rel* gene (34). Thus, there may exist a family of *rel*-related factors that display different affinities for the *dpm10* mutant or different activation potentials when bound to the *dpm10* mutant κ B motif. An analysis of nuclear κ B-binding proteins from PMA- and PHA-treated Jurkat cells by gel retardation has indeed revealed a complicated pattern of at least six distinct protein-DNA complexes. Some of these complexes still form with the *dpm10* mutant, albeit weakly, but we have been unable as yet to establish a clear correspondence between the activity of the *dpm10* mutant in stimulated Jurkat cells and one or more of these κ B-specific complexes (50).

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