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TAR independent activation of the human immunodeficiency virus in phorbol ester stimulated T lymphocytes

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Multiple regulatory elements in the human immunodeficiency virus long terminal repeat (HIV LTR) are required for activation of HIV gene expression. Previous transfection studies of HIV LTR constructs linked to the chloramphenicol acetyltransferase gene indicated that multiple regulatory regions including the enhancer, SP1, TATA and TAR regions were important for HIV gene expression. To characterize these regulatory elements further, mutations in these regions were inserted into both the 5′ and 3′ HIV LTRs and infectious proviral constructs were assembled. These constructs were transfected into either HeLa cells, Jurkat cells or U937 cells in both the presence and absence of phorbol esters which have previously been demonstrated to activate HIV gene expression. Viral gene expression was assayed by the level of p24 *gag* protein released from cultures transfected with the proviral constructs. Results in all cell lines indicated that mutations of the SP1, TATA and the TAR loop and stem secondary structure resulted in marked decreases in gene expression while mutations of the enhancer motif or TAR primary sequence resulted in only slight decreases. However, viruses containing mutations in either the TAR loop sequences or stem secondary structure which were very defective for gene expression in untreated Jurkat cells, gave nearly wild-type levels of gene expression in phorbol ester-treated Jurkat cells but not in phorbol ester-treated HeLa or U937 cells. High level gene expression of these TAR mutant constructs in phorbol ester-treated Jurkat cells was eliminated by second site mutations in the enhancer region or by disruption of the *tat* gene. These results suggest that in stimulated T lymphocytes *tat* is likely capable of interacting with cellular factors which bind to both upstream and downstream regulatory elements resulting in activation of HIV gene expression.

Key words: enhancer/human immunodeficiency virus/phorbol esters/*tat*/transcriptional regulation

Introduction

The regulation of the human immunodeficiency virus (HIV) (Barre-Sinousi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984; Arya *et al.*, 1985) is dependent on specific regulatory regions in the long terminal repeat (LTR) (Rosen *et al.*, 1985; Jones *et al.*, 1986; Peterlin *et al.*, 1986; Garcia *et al.*, 1987; Muesing *et al.*, 1987; Siekevitz *et al.*, 1987; Feng and Holland, 1988; Harrich *et al.*, 1989). These elements can be divided into core promoter elements required for activation of HIV gene expression in most cell lines (SP1, TATA and TAR) (Rosen *et al.*, 1985; Peterlin *et al.*, 1986; Garcia *et al.*, 1987; Muesing *et al.*, 1987; Feng and Holland, 1988; Hauber and Cullen, 1988; Jakobovits *et al.*, 1988; Jones *et al.*, 1988; Garcia *et al.*, 1989; Selby *et al.*, 1989) and elements which are important in activated T cells (enhancer and IL-2 like elements) (Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1987; Shaw *et al.*, 1988; Crabtree, 1989). DNase I footprinting and gel retardation have indicated that these regulatory regions serve as binding sites for cellular proteins. At least two of these factors, NF-κB, which binds to the enhancer (Nabel and Baltimore, 1987; Kawakami *et al.*, 1988) and NF-AT (Durand *et al.*, 1988; Shaw *et al.*, 1988; Crabtree *et al.*, 1989), which binds to the IL-2 like sequences, are expressed in activated but not resting T cells. SP1 (Jones *et al.*, 1986; Harrich *et al.*, 1989), TF-IID (Nakajima *et al.*, 1988; Garcia *et al.*, 1989) and the TAR DNA (Jones *et al.*, 1988; Wu *et al.*, 1988b; Garcia *et al.*, 1989) and RNA (Gatignol *et al.*, 1989; Gaynor *et al.*, 1989) binding proteins are expressed constitutively in most cells.

SP1 (Jones *et al.*, 1986; Harrich *et al.*, 1989), TATA (Garcia *et al.*, 1987, 1989; Jones *et al.*, 1988) and TAR (Rosen *et al.*, 1985; Garcia *et al.*, 1987, 1989; Feng and Holland, 1988; Hauber and Cullen, 1988; Jakobovits *et al.*, 1988; Berkhout *et al.*, 1989; Braddock *et al.*, 1989; Laspia *et al.*, 1989; Selby *et al.*, 1989) regions comprise elements required for high levels of HIV gene expression. The SP1 and TATA elements are important in both basal and *tat*-induced gene expression (Rosen *et al.*, 1985; Garcia *et al.*, 1987, 1989; Jones *et al.*, 1988; Harrich *et al.*, 1989). The TAR region has been demonstrated to be essential for *tat* activation (Rosen *et al.*, 1985; Garcia *et al.*, 1987, 1989; Feng and Holland, 1988; Hauber and Cullen, 1988; Jakobovitz *et al.*, 1988; Berkhout *et al.*, 1989; Braddock *et al.*, 1989; Laspia *et al.*, 1989; Selby *et al.*, 1989). This region has been mapped by mutagenesis between -17 and $+44$ in the HIV LTR and it is capable of forming a stable RNA stem$-$loop structure (Muesing *et al.*, 1987; Feng and Holland, 1988; Hauber and Cullen, 1988; Jakobovits *et al.*, 1988; Garcia *et al.*, 1989; Hauber *et al.*, 1989; Selby *et al.*, 1989). Both stem base pairing and the primary sequence of the loop are required for high levels of *tat* activation (Feng and Holland, 1988; Garcia *et al.*, 1989; Selby *et al.*, 1989). The TAR region serves as the binding site at the RNA level for both *tat* (Dingwall *et al.*, 1989) and a group of cellular proteins known as URBP (Gaynor *et al.*, 1989; Gatignol *et al.*, 1989), and at the DNA level for cellular proteins UBP1 and UBP2 (Jones *et al.*, 1988; Wu *et al.*, 1988b; Garcia *et al.*, 1989).

Several studies have examined the role of mutated viral regulatory regions in the HIV LTR in the context of infectious virus (Lu *et al.*, 1989; Leonard *et al.*, 1989). One study using constructs containing deletions in the HIV LTR

demonstrated an effect on removal of potential negative regulatory elements on viral growth (Lu *et al.*, 1989). Another study showed that mutations of the enhancer motif did not result in marked defects in viral growth unless coupled to additional mutations of the SP1 binding site (Leonard *et al.*, 1989). The focus of both of these studies was to determine the effect of these HIV LTR regulatory region mutations on viral growth.

To determine the role of viral regulatory regions in the context of proviral constructs, 5′ and 3′ LTRs containing mutations of either the enhancer, SP1, TATA or TAR regions or multiple regulatory domains were inserted into infectious virus constructs. These constructs were transfected into either HeLa, U937 or Jurkat cells in both the presence and absence of phorbol esters. Previous studies have indicated that the presence of phorbol esters can stimulate HIV gene expression (Harada *et al.*, 1986). The quantity of p24 *gag* protein released from cultures transfected with these proviral constructs was determined from 48 to 96 h post-transfection to determine the roles of these mutations on HIV gene expression. Alterations in transfection efficiency were monitored by the inclusion of Rous sarcoma virus LTR chloramphenicol acetyltransferase (RSV CAT) constructs into each set of transfections. The role of mutations of the *tat* gene in these constructs was also determined. These studies define the patterns of early gene expression in proviral constructs containing promoter element mutations.

Results

Construction of HIV LTR mutated constructs

Previously we constructed oligonucleotide-directed mutations in the enhancer, SP1, TATA and TAR regions in the HIV LTR (Garcia *et al.*, 1989; Gaynor *et al.*, 1989; Harrich *et al.*, 1989). These mutant constructs were previously assayed in both DNase I footprinting assays and transient expression assays using truncated LTR constructs linked to the chloramphenicol acetyltransferase gene. The mutation of the enhancer altered cellular protein binding to both NF-κB motifs in this region (NENH1 and NENH2), the mutation of their SP1 region eliminated SP1 binding to all three SP1 sites (NSP1, NSP2, NSP3), and the TATA mutant (NTATA) altered binding of proteins to this region (Figure 1). The mutations in the TAR region included a single point mutation of the loop (+34), a 4 bp substitution in the loop (+31/+34), interruption of much of the primary sequence of the stem with preservation of stem base pairing and stem energy (ΔTAR-sense), and interruption of stem base pairing (+11/+14, +40/+43) (Figure 1).

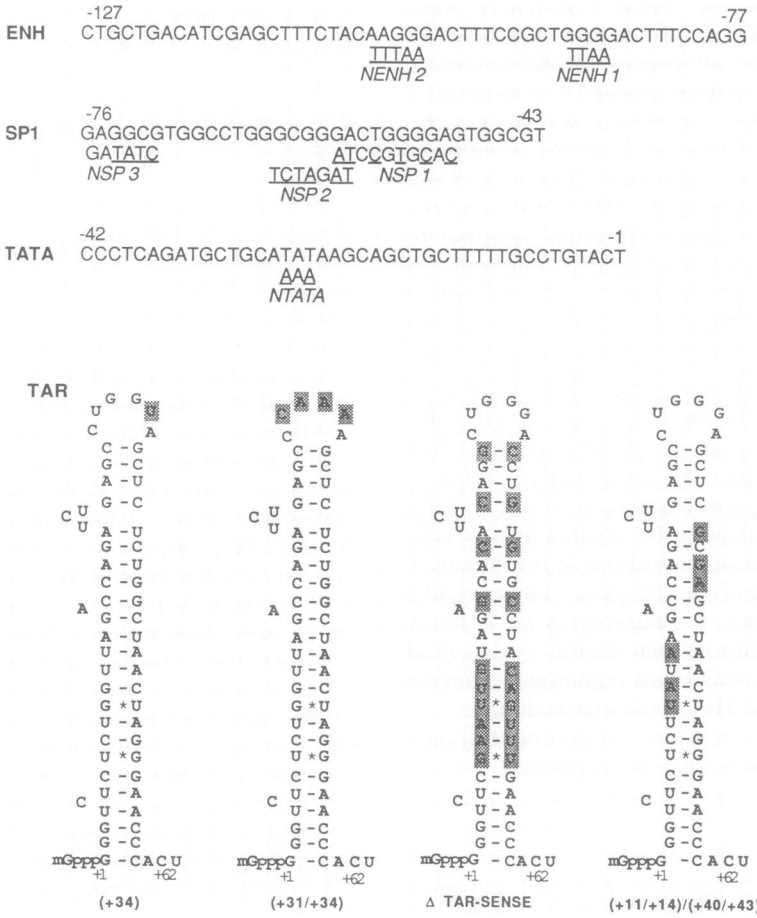

Fig. 1. Schematic of HIV LTR mutations. A portion of the HIV LTR extending from −127 to +1 is indicated. The position and nucleotide changes for mutations of both NF−κB motifs in the enhancer (ENH 1 and ENH 2), the three SP1 sites (NSP1, NSP2 and NSP3) and the TATA element (NTATA) are indicated. The TAR mutations in the context of the RNA secondary structure are indicated. The TAR mutations in the context of the RNA secondary structure are indicated. The dark boxes indicate mutated nucleotides for a point mutation of the loop (+34), a 4 bp loop substitution (+31/+34), a change in the primary sequence of TAR (ΔTAR-sense), and a stem disruption of the stem structure (+11/+14, +40/+43).

Each of these mutations was placed into both the 5′ and 3′ LTRs and inserted into a plasmid containing a derivative of ARV-2B (Levy *et al.*, 1984; Luciw *et al.*, 1984). This proviral construct was able to replicate in a variety of lymphoid and monocyte cell lines (U937,H9) as determined by the presence of both high levels of secreted p24 *gag* protein and reverse transcriptase in tissue culture media samples (data not shown). p24 antigen (Ag) assays in this study were performed at 48 h post-transfection with Jurkat and U937 cells in both the presence and absence of phorbol esters. This is prior to the onset of viral replication. p24 Ag assays on HeLa cells were performed at 96 h post-transfection due to the inability to detect substantial levels of this protein at earlier times. A diagram of the mutations introduced into the LTRs in these constructs is shown in Figure 1.

TAR independent activation of HIV in PMA-treated Jurkat cells

Each of the proviral constructs was introduced into Jurkat cells by electroporation. An RSV CAT plasmid was included in each transfection to normalize for differences in transfection efficiency. At 24 h post-transfection, the transfections were divided and one set was untreated and the other treated with either phorbol ester (PMA) or PMA and phytohemagglutinin (PHA). Supernatants were harvested at 48 h post-transfection and p24 Ag levels were quantitated (Figure 2).

In untreated Jurkat cells, the wild-type construct yielded ~8000 pg/ml of p24 Ag antigen (Ag) (Figure 2). Mutations of both κB motifs in the enhancer resulted in a <2-fold decrease in p24 Ag levels. Mutations of either the three SP1 binding sites or the TATA element resulted in levels of p24 only slightly above detection limits (Figure 2). Mutations of both the TAR loop and stem structure were also tested. A single base pair mutation of the loop sequences (+34) resulted in an ~10-fold decrease in p24 Ag levels compared with the wild-type construct. A 4 bp substitution in the loop sequences (+31/+34) resulted in a 35-fold decrease in p24 Ag levels compared with the wild-type construct. Mutations in the primary sequence of the TAR region (ΔTAR-sense) which left stem base pairing and stem energy intact exhibited a 2.5-fold decrease in gene expression. A mutation that disrupted stem base pairing in the TAR region (+11/+14/+40+43) resulted in a 40-fold decrease in gene expression. Thus multiple upstream and downstream regulatory regions alter early viral gene expression.

Each of these constructs was also assayed in the presence of PMA. Minimal differences were noted in the level of induction of these constructs by PMA alone or PMA in combination with PHA (data not shown). In the following experiments, transfections containing only PMA treatment alone are shown. The wild-type HIV construct was only slightly induced in the presence of PMA as compared with its absence. Similar low levels of PMA induction were seen with the enhancer mutation. The levels of expression of the SP1 and the TATA mutants were extremely low in the presence of PMA as they were in its absence. However, the results with several of the TAR region mutants were unexpected (Figure 2). p24 Ag levels for both mutations in the loop region, either a single base pair mutation (+34) or a 4 bp substitution (+31/+34) were strongly induced by the addition of PMA. This induction ranged from 8-fold (+34) to 20-fold (+31/+34). Similarly, a mutation that disrupted the stem structure (+11/+14/+40/+43) resulted in a 15-fold level of induction in p24 Ag levels. This level of p24 Ag induction was much greater than that seen with the wild-type or enhancer mutant constructs. A mutation of the TAR region primary sequence (ΔTAR-sense) was only induced 2 to 3-fold.

These results suggest that in activated Jurkat cells, two critical determinants of TAR function, the loop sequences and the stem secondary structure, were much less critical for gene expression than in unstimulated Jurkat cells. This was demonstrated by the fact that in the absence of PMA, loop and stem mutants were 10 to 35-fold less active than wild-type while in the presence of PMA these same mutants are only 2 to 3-fold decreased compared with wild-type. The wild-type construct was only minimally induced by PMA as was the enhancer mutant. Thus, mechanisms exist in PMA-treated Jurkay cells to induce strongly the gene expression of proviral constructs containing mutations in the HIV TAR region.

The enhancer region is required for TAR independent activation of HIV gene expression in PMA-treated Jurkat cells

Previous studies have demonstrated that the enhancer element composed of two NF-κB motifs was critical for PMA activation of the HIV LTR (Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1987). These studies were performed using transient expression assays with the HIV LTR fused to the CAT gene. However, our studies (Figure 2) and previous studies using infectious proviral constructs did not indicate that the enhancer element had marked effects on HIV gene expression (Leonard *et al.*, 1989). To determine whether the enhancer region was involved in PMA activation of TAR mutants, we constructed double regulatory region mutants in which a mutated enhancer region was fused to TAR mutations in either the loop, stem or primary sequence. These viral constructs containing either double of single mutations were transfected into untreated or PMA-treated Jurkat cells and p24 Ag levels determined.

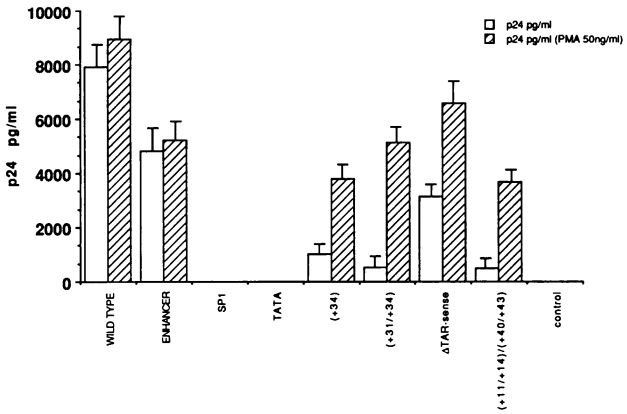

Fig. 2. Expression of HIV mutants in untreated and PMA-treated Jurkat cells. The amount of p24 Ag (pg/ml) detected in the tissue culture media at 48 h post-transfection for the wild-type construct and mutations of the enhancer, SP1, TATA, point mutations of the loop (+34), 4 bp substitutions of the loop (+31/+34), primary sequences of the stem (ΔTAR-sense), and stem secondary structure (+11/+14,+40/+43) are indicated. All transfections were normalized for the expression of RSV CAT. The clear boxes indicate expression in untreated Jurkat cells and the hatched boxes indicate expression in PMA-treated Jurkat cells. The results are the average of four independent experiments with the standard deviation indicated.

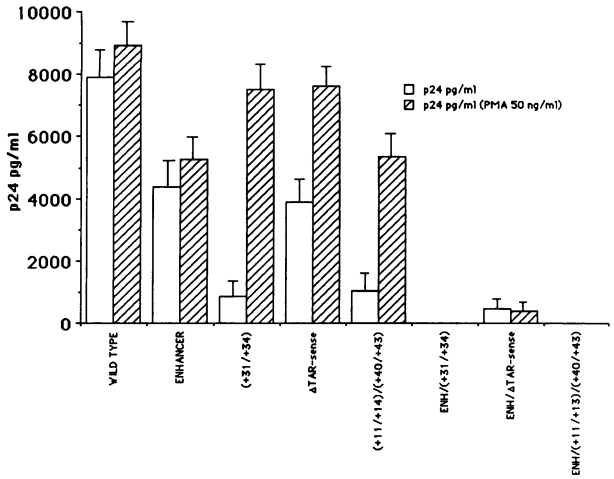

Fig. 3. Expression of HIV enhancer−TAR mutants in untreated and PMA-treated Jurkat cells. The amount of p24 Ag (pg/ml) detected in the tissue culture media at 48 h post-transfection for the wild-type construct and mutations of the enhancer, 4 bp substitutions of the loop (+31/+34), primary sequence of the stem (ΔTAR-sense), and stem secondary structure (+11/+14,+40/+43) are indicated. The enhancer mutation was also coupled to each of the following TAR mutants; ENH/(+31/+34), ENH/ΔTAR-sense, and ENH/(+11/+14,+40/+43). All transfections were normalized for the expression of RSV CAT. The clear boxes indicate expression in untreated Jurkat cells and the hatched boxes indicate expression in PMA-treated Jurkat cells. The results are the average of four independent experiments with the standard deviation indicated.

As shown in Figure 3, mutations of the enhancer alone had minimal effects on HIV gene expression both in the presence and absence of PMA. Mutations of the loop (+31/+34) or the stem structure (+11/+14/+40/+43) were very defective for gene expression in untreated Jurkat cells but each was induced 15 to 20-fold in PMA-treated Jurkat cells (Figure 3). The ΔTAR-sense construct was somewhat defective relative to wild-type (decreased ~2-fold) in untreated Jurkat cells but its gene expression was induced in PMA-treated Jurkat cells (Figure 3). Coupling the enhancer mutation to each of these TAR mutations resulted in marked changes in gene expression. The mutated enhancer coupled to either the loop (+31/+34) or the stem disruption (+11/+14/+40/+43) constructs resulted in no detectable gene expression in either untreated or PMA-treated Jurkat cells (Figure 3). Coupling the ΔTAR-sense and the enhancer mutations resulted in decreased gene expression in untreated Jurkat cells with no further increase in PMA-treated Jurkat cells (Figure 3). The severe decreases in gene expression obtained by coupling mutations in TAR and the enhancer suggest a synergy between these two elements in regulating HIV gene expression. Furthermore, it points to a requirement for the enhancer element in regulating PMA induced expression of HIV in Jurkat cells. However, we cannot rule out that other upstream regulatory elements may also influence PMA induction of HIV in Jurkat cells.

tat is required for TAR independent activation of HIV in PMA-treated Jurkat cells

It was important to determine whether the *tat* protein (Arya *et al.*, 1985; Sodroski *et al.*, 1985; Cullen, 1986; Dayton *et al.*, 1986; Feinberg *et al.*, 1986; Fisher *et al.*, 1986; Rosen *et al.*, 1986) was required for high levels of gene expression with HIV TAR mutants in PMA-treated Jurkat cells. A deletion of the cysteine domains in the *tat* gene was

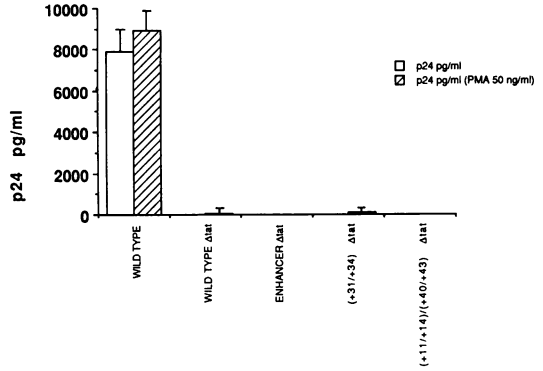

Fig. 4. Expression of *tat* mutants in unstimulated and PMA-stimulated Jurkat cells. The amount of p24 Ag (pg/ml) detected in the tissue culture media from either untreated or PMA-stimulated Jurkat cells at 48 h post-transfection for the wild-type construct or the wild-type, enhancer, 4 bp substitutions of the loop (+31/+34), and stem secondary structure (+11/+14,+40/+43) mutants containing an additional mutation of the *tat* gene are indicated. All transfections were normalized for expression of the RSV CAT. The clear boxes indicate expression in untreated Jurkat cells and the hatched boxes indicate expression in PMA-treated Jurkat cells. The results are the average of four independent experiments with the standard deviation indicated.

constructed which resulted in a protein defective for *trans*-activation (Garcia *et al.*, 1988; Sadaie *et al.*, 1988; Ruben *et al.*, 1989). Constructs containing this *tat* mutation included the wild-type construct, the enhancer mutant, the loop substitution mutant (+31/+34), and the stem disruption mutant (+11/+14/+40+43). An RSV CAT construct confirmed that equal transfection efficiencies were maintained in these experiments. As shown in Figure 4, the wild-type construct gave high levels of expression in Jurkat cells in both the presence and absence of PMA. However, constructs containing *tat* deletions were extremely defective in both the presence and the absence of PMA as compared with the wild-type construct. This indicated that *tat* was required for high levels of gene expression with both the wild-type construct and with TAR mutants in activated Jurkat cells.

Expression of HIV constructs in unstimulated and PMA-stimulated HeLa cells and U937 cells

To determine whether the effects of TAR region mutants seen in activated Jurkat cells was seen on other cell types, each of the HIV constructs was transfected onto either untreated or PMA-treated HeLa and U937 cells. As shown in Figure 5, the level of expression with the wild-type HIV construct transfected by calcium phosphate precipitation onto HeLa cells was markedly decreased as compared with that on Jurkat cells. Similar levels of gene expression were seen when these constructs were introduced by electroporation. However, there was still a ~2-fold induction in p24 Ag levels of the wild-type construct by PMA. Gene expression of the enhancer mutant and the ΔTAR-sense construct were each decreased by ~2-fold compared with the wild-type and each construct was induced ~2-fold by PMA treatment. Mutations of the SP1, TATA or TAR region mutations (+34,+31/+34 and +11/+14/+40/+43) resulted in extremely low levels of p24 Ag with minimal activation by PMA (Figure 5).

These constructs were also introduced into U937 cells by electroporation, the cells divided at 24 h, and one portion of the cells was untreated and the other half treated with

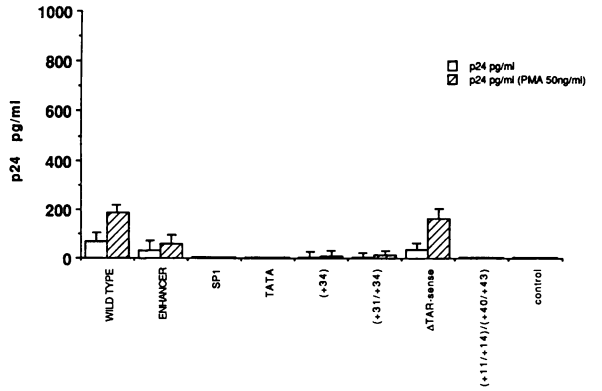

Fig. 5. Expression of HIV mutants in untreated and PMA-treated HeLa cells. The amount of p24 Ag (pg/ml) detected in the tissue culture media at 96 h post-transfection for the wild-type construct and mutations of the enhancer, SP1, TATA, point mutations of the loop (+34), 4 bp substitutions of the loop (+31/+34), primary sequence of the stem (ΔTAR-sense) and disruption of the stem secondary structure (+11/+14,+40/+43) are indicated. All transfections were normalized for expression of RSV CAT. The clear boxes indicate expression in untreated HeLa cells and the hatched boxes indicate expression in PMA-treated HeLa cells. The results are the average of four independent experiments with the standard deviation indicated.

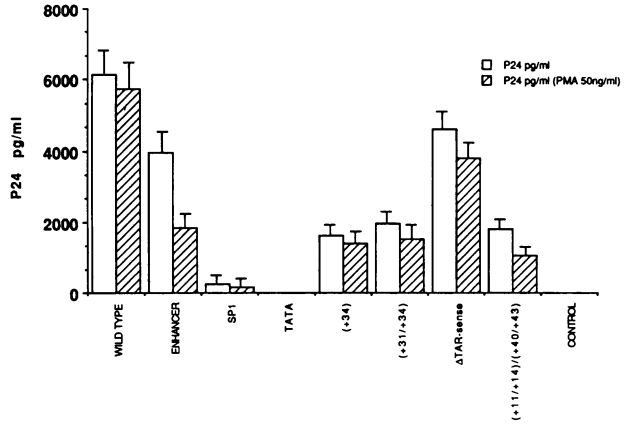

Fig. 6. Expression of HIV mutants in untreated and PMA-treated U937 cells. The amount of p24 Ag (pg/ml) detected in the tissue culture media at 48 h post-transfection for the wild-type construct and mutations of the enhancer, SP1, TATA, point mutations of the loop (+34), 4 bp substitutions of the loop (+31/+34), primary sequence of the stem (ΔTAR-sense) and disruption of the stem secondary structure (+11/+14,+40/+43) are indicated. All transfections were normalized to expression of RSV CAT. The clear boxes indicate expression in untreated U937 cells and the hatched boxes indicate expression in PMA-treated U937 cells. The results are the average of four independent experiments with the standard deviation indicated.

PMA. p24 Ag levels were determined at 48 h post-transfection (Figure 6). In contrast to results seen with PMA on HeLa and Jurkat cells, each of the constructs had slightly lower levels in the presence of PMA than in its absence. This decreased gene expression in the presence of PMA was not due to cell death as determined by equivalent cell viabilities in both the presence and absence of PMA. The wild-type construct both in the presence and absence of PMA yielded ~6000 pg/ml of p24. The enhancer mutant was decreased 2- to 3-fold as compared with the wild-type construct both in the presence and absence of PMA. Mutations of the SP1 and TATA regions were decreased >50-fold compared with the wild-type construct (Figure 6).

The effects of various TAR region mutations was much less pronounced as compared with the wild-type construct in untreated U937 cells than Jurkat cells. Mutation of the ΔTAR-sense construct was decreased ~2-fold (Figure 6). Mutations of either the loop (+34, and +31/+34) or the stem structure (+11/+14/+40/+43) were decreased ~3-fold as compared with the wild-type construct in both the presence and absence of PMA (Figure 6). Thus, all the TAR mutants exhibited slightly decreased expression in the presence of PMA as compared with its absence.

Discussion

A number of regulatory regions in the HIV LTR are critical for the regulation of HIV gene expression. Previous mutagenesis and transient expression assays have shown that the enhancer (Kaufman *et al.*, 1987; Muesing *et al.*, 1987; Nabel and Baltimore, 1987; Tong-Starksen *et al.*, 1987; Gaynor *et al.*, 1988) SP1 (Jones *et al.*, 1986; Harrich *et al.*, 1989), TATA (Garcia *et al.*, 1987, 1989; Muesing *et al.*, 1987; Jones *et al.*, 1988) and TAR (Rosen *et al.*, 1985; Peterlin *et al.*, 1986; Garcia *et al.*, 1987; Feng and Holland, 1988; Hauber and Cullen, 1988; Jakobovits *et al.*, 1988; Berkhout *et al.*, 1989; Braddock *et al.*, 1989; Garcia *et al.*, 1989; Laspia *et al.*, 1989; Selby *et al.*, 1989) regions were critical for high level basal and *tat*-induced activity. In this study, mutations within these regions were placed into infectious proviral constructs. These constructs were assayed before the onset of significant viral replication in Jurkat, U937 and HeLa cells in both the presence and absence of PMA.

Previous data has indicated that several upstream regulatory domains in the HIV LTR are required for high level gene expression in activated T cells (Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1987; Durand *et al.*, 1988; Shaw *et al.*, 1988; Crabtree, 1989). These studies demonstrated that the enhancer region containing two NF-κB motifs was one element required for efficient expression in T cells (Nabel and Baltimore, 1987; Kaufman *et al.*, 1987; Tong-Starksen *et al.*, 1987). Activation of Jurkat or other cells by PMA and PHA resulted in increased binding to the NF-κB motifs (Nabel and Baltimore, 1987; Wu *et al.*, 1988a). This increased binding was correlated with increased expression from the HIV LTR and this expression was augmented by *tat* (Kaufman *et al.*, 1987; Nabel and Baltimore, 1987). Our results demonstrated that isolated mutations of the enhancer were not required for high level gene activation in any of the cell lines tested. Neither did mutations of the enhancer region alone markedly alter HIV gene expression in Jurkat cells in the presence or absence of PMA. Similar lack of dependence on the enhancer element using assays of viral growth have recently been obtained with infectious viral constructs (Leonard *et al.*, 1989). In contrast to studies with HIV LTR CAT constructs which are strongly induced in PMA-treated Jurkat cells (Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1987; Kaufman *et al.*, 1987), our results suggest that either viral regulatory proteins and/or *cis* acting control elements in the wild-type proviral construct may inhibit high levels of PMA induction.

Mutations of the SP1 and TATA motifs were previously found to result in severe defects in basal and *tat*-induced gene expression by transient gene-expression assays (Jones *et al.*, 1986; Garcia *et al.*, 1987; Muesing *et al.*, 1987; Jones *et*

al., 1988; Garcia et al., 1989; Harrich et al., 1989). These results were confirmed when these mutations were introduced into proviral constructs. Both of these mutations resulted in severe defects in viral gene expression on all cell lines tested. These mutants appeared to have a very low induction by PMA.

The TAR region has been shown to be required for activation by *tat*. This region is the site for DNA binding by cellular proteins UBP1 and UBP2 (Wu et al., 1988a; Garcia et al., 1989). In addition, this region is the site for a stable stem−loop structure at the RNA level (Muesing et al., 1987; Feng and Holland, 1988; Garcia et al., 1989; Selby et al., 1989). The maintenance of both the stem structure and the primary sequence of the loop were required for *tat* activation (Feng and Holland, 1988; Hauber and Cullen, 1988; Jakobovits et al., 1988; Garcia et al., 1989; Selby et al., 1989). Mutations in the TAR region were tested which eliminated stem base pairing (+11/+14/+40/+43), that introduced either a single base pair mutation (+34) or a 4 bp substitution (+31/+34) in the loop, or that extensively changed the primary sequence of TAR while preserving stem base pairing and stem energy (ΔTAR-sense).

These TAR mutations exhibited very different effects on different cell lines. On untreated Jurkat cells, the mutations of the primary sequence of TAR resulted in only 2-fold decreases in gene expression compared with the wild-type. Mutation of the loop sequences or disruption of the stem secondary structure resulted in 10 to 35-fold decreases in gene expression compared with wild-type. However, expression of these TAR mutants was much less defective in PMA-treated Jurkat cells being reduced only 2 to 3-fold compared with the wild-type. This marked induction of gene expression with these TAR mutants was not seen in either PMA-treated U937 or HeLa cells. This indicates that alternative mechanisms of activation may occur in PMA stimulated Jurkat cells to circumvent TAR region mutations.

By coupling TAR mutants to mutants in the enhancer region, increased expression from these proviral constructs was eliminated in PMA-treated Jurkat cells. Thus, the enhancer is an essential regulatory element required for PMA inducton of HIV gene expression in the presence of TAR mutations. Synergistic effects of the enhancer and TAR mutations in regulating HIV gene expression were seen in both untreated and PMA-treated Jurkat cells, suggesting potential interactions of cellular factors binding to these sites (Garcia et al., 1987). In addition to cellular factors, *tat* was also required for high level gene expression in PMA-treated Jurkat cells. Due to the deleterious effects of mutations of the SP1 and TATA elements on HIV gene expression, it was not possible to determine whether these elements were also involved in increasing HIV gene expression in PMA-treated Jurkat cells. The NFAT binding sites (Durand et al., 1988; Shaw et al., 1988; Crabtree, 1989) in the HIV LTR did not appear to play a critical role in regulating HIV gene expression in PMA-stimulated Jurkat cells. The high level of gene expression in PMA-treated lymphocytes in the presence of TAR mutations may be due to suboptimal activation by *tat* due to decreased affinity to *tat* binding to mutated TAR RNAs (Dingwall et al., 1989). Our results are consistent with a model in which *tat* may interact with both upstream and downstream cellular factors to increase HIV gene expression.

Similar to results in Jurkat cells, TAR region mutations of the loop or stem structure resulted in large defects in gene expression on HeLa cells. However, the induction of expression with these mutants by PMA was minimal. The effect of TAR region mutants introduced into U937 cells was much less pronounced than seen on HeLa or untreated Jurkat cells. Mutations of the loop and stem disruption were only 3 to 4-fold reduced compared with the wild-type construct on U937 cells. Furthermore, all constructs were reduced in expression in the presence of PMA. This was not due to cell death as determined by studies of cell viability, but it may reflect the effect of PMA on monocyte differentiation (Rovera et al., 1979). Thus, modulation in either the level or activity of cellular regulatory proteins in different cells may alter the phenotype of constructs containing TAR mutations.

The studies outlined herein define the activity of HIV LTR mutants in proviral constructs. Due to the fact that PMA was toxic to cells with prolonged incubation, assays were performed before the onset of significant viral replication. This was reflected in the absence of reverse transcriptase at the time of the assays. However, with prolonged infection, significant levels of reverse transcriptase were detected with a number of these constructs. Further studies using high titered virus stocks will be required to determine the kinetics of viral growth for these various mutant viruses. In addition, viruses containing TAR region mutations coupled to different upstream regulatory regions will be required to determine other potential regions required for efficient viral growth in activated Jurkat cells. These studies will be important in defining potential sites for *tat* action in activated T lymphocytes.

Materials and methods

Plasmid constructs

Two HIV-1 LTR shuttle vectors were constructed. For the 5′ LTR shuttle, a *Bam*HI restriction site was inserted just upstream of −454 from the start of transcription by site-directed mutagenesis of ARV 2B (Luciw et al., 1984). A *Bam*HI−*Nar*I(+186) fragment of this HIV-1 LTR was cloned into the corresponding unique sites of pUC18 (*Bgl*II$^+$/SmaI$^-$ polylinker by linker addition). For the 3′ LTR shuttle, an *Asp*718(+8581)−*Nar*I(+186) fragment, which includes the LTR from ARV 2B, was cloned into pUC18(*Bgl*II$^+$/SmaI$^-$). *Ava*I(−160)−*Hin*dIII(+83) fragments from previously made LTR mutants were cloned into the two shuttle vectors. The *Bam*HI−*Nar*I(+186) fragments from these 5′ LTR shuttles were subcloned into an ARV 2B *Bam*HI−*Eco*RI(+5299) pUC19(*Sph*I$^-$) construct (designated as the 5′ LTR arm) and the *Asp*718 (+8581)−*Nar*$^+$ (+186) pUC 18 construct (designated as the 3′LTR arm). To assemble the proviral construct, a *Bgl*I−*Eco*RI(+5299) fragment from each of the LTR arms was gel isolated, ligated and positive clones were confirmed by dideoxy sequencing. Finally, a *Sph*I(+999)−*Nco*I(+5111) fragment from PBH 10 was cloned into each of the proviral constructs (Shaw et al., 1984). The Δ*tat* proviral constructs contain an internal deletion from +5409 to +5492 which deletes the putative zinc finger. The expression plasmid RSV CAT is described elsewhere (Gorman et al., 1982).

Transfections

HeLa plates were maintained on complete Iscove's medium containing penicillin and streptomycin. Plates were split the day prior to transfection so that each 100 mm plate was 50−70% confluent at the time of transfection. Using the calcium phosphate precipitation technique, 10 μg of each proviral DNA and 5 μg RSV CAT DNA were co-transfected onto identically prepared plates. The cells were glycerol shocked at 4 h post-transfection. The media from one set of duplicate plates was removed and replaced with complete Iscove's containing phorbol 12-myristate 13-acetate at 50 ng/ml (PMA, Sigma) at 24 h post-transfection.

Jurkat cells and U937 cells were maintained on Iscove's medium containing 10% fetal calf serum plus penicillin and streptomycin. Cells were grown to a density of 1×10^6 cells/ml and supplemented with fresh media 16−20 h

prior to electroporation. Cells (2×10^7) were electroporated using the BRL cell porator at 250 V, 1180 μF and low resistance, in growth media containing the proviral DNA (10 μg/ml) plus RSV CAT (5 μg/ml). The cells were split into identical flasks at 24 h post-transfection and one set was supplemented with PMA (50 ng/ml).

p24 ELISA

Culture supernatants were collected 48 h post-transfection (for U937 and Jurkat cells) and at 96 h for HeLa cells. HIV-1 p24 antigen in culture supernatants was detected using the Abbott Laboratories' HIVAG-1 enzyme immunoassay. To normalize for transfection efficiencies, the cells for each transfection were harvested and assayed for the presence of the CAT gene product.

Reverse transcriptase assay

Culture supernatants were assayed for reverse transcriptase activity as described (Melish *et al.*, 1989). Briefly, cleared cell supernatants were precipitated with PEG (30% PEG 6000, 0.4 M NaCl), spun at 2000 *g* for 30 min, and the resulting pellet was resuspended in TNE (10 mM Tris pH 8.0, 1.0 mM EDTA, 100 mM NaCl). Samples were frozen and thawed twice and added to a reaction containing appropriate buffers plus [^{3}H]thymidine. The templates used were poly(rA)/oligo(dT), and separately poly(dA)/oligo(dT) (Pharmacia).

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