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Functional domains required for *tat*-induced transcriptional activation of the HIV-1 long terminal repeat

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The transcriptional regulation of the human immunodeficiency virus (HIV) type I involves the interaction of both viral and cellular proteins. The viral protein *tat* is important in increasing the amount of viral steady-state mRNA and may also play a role in regulating the translational efficiency of viral mRNA. To identify distinct functional domains of *tat*, oligonucleotide-directed mutagenesis of the *tat* gene was performed. Point mutations of cysteine residues in three of the four Cys-X-X-Cys sequences in the *tat* protein resulted in a marked decrease in transcriptional activation of the HIV long terminal repeat. Point mutations which altered the basic C-domain of the protein also resulted in decreases in transcriptional activity, as did a series of mutations that repositioned either the N or C termini of the protein. Conservative mutations of other amino acids in the cysteine-rich or basic regions and in a series of proline residues in the N terminus of the molecule resulted in minimal changes in *tat* activation. These results suggest that several domains of *tat* protein are involved in transcriptional activation with the cysteine-rich domain being required for complete activity of the *tat* protein.
Key words: human immunodeficiency virus/viral protein *tat*/mRNA

Introduction

The human immunodeficiency virus (HIV) type I is an etiologic agent of the acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984). Transcriptional regulation of HIV involves both cellular and viral proteins. DNase I footprinting of the HIV long terminal repeat (LTR), using HeLa extracts, revealed five regions of the LTR that served as binding sites for cellular proteins including the negative regulatory, enhancer, SP1, TATA and TAR regions (Dinter *et al.*, 1987; Garcia *et al.*, 1987; Jones *et al.*, 1987). Mutagenesis studies have indicated the importance of these sites in HIV transcriptional regulation (Peterlin *et al.*, 1986; Garcia *et al.*, 1987; Kaufman *et al.*, 1987; Muesing *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1987; Hauber and Cullen, 1988).

In addition to the HIV LTR regulatory regions and the cellular factors that bind to these regions, at least two viral proteins, *tat* and *rev*, are important in viral gene regulation. The *rev* protein (Feinberg *et al.*, 1986; Sodroski *et al.*, 1986) functions in increasing the level of *gag* and *env* mRNA levels by affecting mRNA splicing or stability, while the *tat* protein (Arya *et al.*, 1985; Sodroski *et al.*, 1985a,b; Cullen, 1986; Dayton, 1986; Feinberg *et al.*, 1986; Fisher *et al.*, 1986; Peterlin *et al.*, 1986; Hauber *et al.*, 1987; Kao *et al.*, 1987; Muesing *et al.*, 1987) functions to increase the level of HIV LTR mRNA (Cullen *et al.*, 1986; Gendelman *et al.*, 1986; Peterlin *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.*, 1987) and may also regulate the translation of viral mRNA (Cullen, 1986; Feinberg *et al.*, 1986; Rosen *et al.*, 1986). Deletion mutagenesis of the *tat* gene in HIV viral constructs resulted in marked defects in viral gene expression (Dayton, 1986). The *tat* protein requires both the enhancer and the TAR regions for complete activation of HIV LTR (Rosen *et al.*, 1985; Peterlin *et al.*, 1986) though potential RNA stem-loop structures in the TAR region have also been postulated to be important for *tat* activation (Okamoto *et al.*, 1986; Muesing *et al.*, 1987).

The *tat* protein is a nuclear protein (Hauber *et al.*, 1987) consisting of 86 amino acids although only the first 58 are required for transactivating activity (Siegel *et al.*, 1986). Examination of the amino acid sequence of the *tat* reveals the presence in the N terminus of *tat* of four potential zinc-binding sites each encoded by the amino acid sequence Cys-X-X-Cys (Miller *et al.*, 1985; Berg, 1986; Evans and Hollenberg, 1988). Variations of this sequence have been found in several DNA-binding proteins, e.g. the *Drosophila* proteins Kruppl (Rosenberg *et al.*, 1986), Serendipity (Vincent *et al.*, 1985) and hunchback (Tautz *et al.*, 1987), the yeast Gal4 (Johnston and Dover, 1987; Johnston, 1987) and ADR proteins (Blumberg *et al.*, 1987), SP1 (Kadonaga *et al.*, 1987), TFIII-A (Miller *et al.*, 1985) and the glucocorticoid (Hollenberg *et al.*, 1985) and estrogen receptors (Green *et al.*, 1986). In addition, similar motifs are found in other proteins that have not been shown to bind DNA including the adenovirus E1A protein (Ferguson *et al.*, 1985; Green *et al.*, 1988), a positive activator of the early adenovirus transcription units. Recent studies with a bacterial-produced *tat* protein have indicated that the *tat* binds to such divalent ions as cadmium and zinc as a dimer (Frankel *et al.*, 1988). The cysteine motif in the *tat* protein differs significantly from the classic description of a zinc finger binding domain, in which each repeat unit contains 30 amino acids which bind a zinc atom using two cysteines and two histidines as ligands (Miller *et al.*, 1985).

There are several other notable regions in the *tat* protein. In the N terminus of the *tat* gene there are three repeats of the sequence Pro-X-X-X-Pro which may also have functional significance. In addition, there is a basic region of nine amino acids in the C terminus of *tat* consisting of mainly lysine and arginine residues. Similar basic domains have been noted in the DNA binding domains of other proteins such as GCN-4, AP1/c-*jun* and c-*fos* (Hope and Struhl, 1985; Vogt *et al.*, 1987). They have also been found in the nuclear transport signals of the simian virus 40 (SV40) and

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polyomavirus T antigens, SV40 VP1, and the *Xenopus laevis* N_1/N_2 and nucleoplasmin proteins (Kalderon *et al.*, 1984; Kleinschmidt *et al.*, 1986; Richardson *et al.*, 1986; Wychowski *et al.*, 1986; Burglin and De Robertis, 1987).

To determine the importance of each of these regions in *tat* activation, oligonucleotide-directed mutagenesis of the *tat* gene was performed. Point mutations were made which changed the first cysteine of each of the four Cys-X-X-Cys motifs to serine residues. Additional mutations were constructed which replaced several of the lysine or arginine residues in the basic region of the gene with the acidic amino acid glutamic acid. Mutations which altered both the proline residues in the N terminus of *tat*, and truncated *tat* proteins with alterations in the positions of the N and C termini were also constructed. These results indicate that the cysteine-rich and basic domains were both required for full transcriptional induction by the *tat* protein. In addition, truncated forms of *tat* did not function well if either the N terminus was displaced or the C terminus was moved upstream into the basic domain suggesting that those regions contain information required for optimal function of the *tat* protein.

Results

Oligonucleotide-directed mutagenesis of the tatI protein

Figure 1 shows the structure of the first 72 amino acids in the second exon of the *tat* gene and the amino acid substitutions introduced by oligonucleotide mutagenesis. This exon was cloned into the M13 vector, mp19, and subjected to oligonucleotide-directed mutagenesis. Mutants which replaced both the second and third prolines or the third and fourth prolines in the N terminus of the protein were constructed as were mutants in which the first cysteine of each of the four potential zinc fingers was replaced with serine residues (Figure 1). A conservative change was also made in this latter region whereby an asparagine residue was changed to a threonine residue. In the basic region at the carboxy end of this exon, mutations were made which substituted glutamic acid residues for lysine or arginine residues (Figure 1). A conservative substitution in this domain was also made by changing an asparagine to a threonine. An additional construct was made which destroyed the initiating methionine for *art*, but retained the *tat*-protein-coding sequence. Finally, truncations of the N terminus of the *tat* protein were constructed in which the initiating methionine in *tat* was moved downstream while another series of mutations introduced stop codons at various locations in the C terminus of the protein. These mutants were then cloned downstream from the Rous sarcoma virus promoter in an expression vector containing the SV40 splice acceptor and polyadenylation signals and tested for their ability to transactivate the HIV LTR (Garcia *et al.*, 1987).

Single amino acid substitutions alter tat activation of the HIV LTR

Each of the mutant *tat* constructs or a control expression plasmid (RSV-β-globin) were transfected into HeLa cells together with a construct containing a portion of the HIV LTR from −177 to +83 fused to the chloramphenicol acetyltransferase (CAT) gene. Each set of transfections was repeated five times with similar results in each experiment.

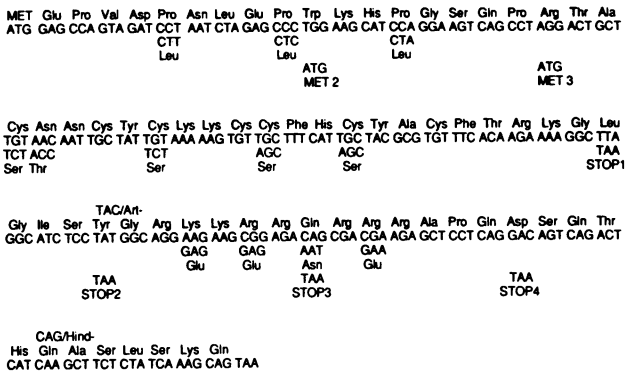

Fig. 1. HIV *tat* amino acid sequence and oligonucleotide-directed mutants. The first 72 amino acids in the HIV *tat* protein and the substitutions introduced by oligonucleotide-directed mutagenesis are indicated.

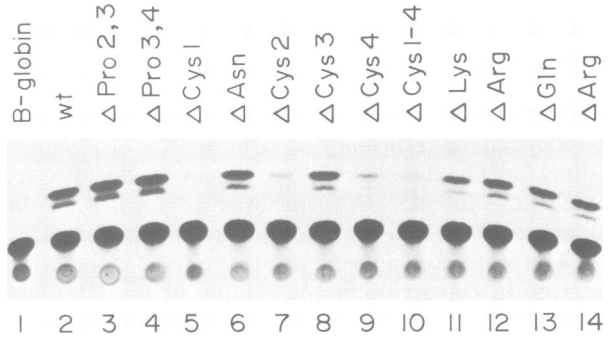

Fig. 2. CAT assays of HIV *tat* point mutations. HIV *tat* constructs β globin (1), wt (2), Δ Pro 2, 3 (3), Δ Pro 3, 4 (4), Δ Cys 1 (5), Δ Asn (6), Δ Cys 2 (7), Δ Cys 3 (8), Δ Cys 4 (9), Δ Cys 1−4 (10), Δ Lys (11), Δ Arg 1 (12), Δ Gln (13) and Δ Arg 2 (14) were transfected into HeLa cells with the HIV LTR CAT construct, harvested at 48 h post-transfection, and CAT activity determined.

CAT assays were performed to assay for the effects of these mutations on *tat*-induced transcriptional activation of the HIV LTR (Gorman *et al.*, 1982). Mutations of either the second and third or third and fourth proline residues in the N terminus of the *tat* protein had minimal effects on *tat* activation of the HIV LTR (Figure 2, lanes 3 and 4). Mutations of three of the four Cys-X-X-Cys motifs (Figure 2, lanes 5, 7 and 9) resulted in a marked decrease in *tat*-induced transactivation as compared with wild-type *tat* (Figure 2, lane 2). Mutagenesis of the third cysteine motif resulted in a minimal change in *tat*-induced CAT activity (Figure 2, lane 8). A mutation of all four cysteine motifs also resulted in a marked decrease in *tat*-induced activity (Figure 2, lane 10). A conservative mutation of the amino acid asparagine in this same region did not affect the transactivating ability of the *tat* protein (Figure 2, lane 6). Mutations which substituted the acidic amino acid glutamic acid for several of the lysine or arginine residues in the basic domain of the *tat* protein resulted in a decrease in *tat*-induced activity, but not as severe as seen with the mutations of the cysteine-rich domain (Figure 2, lanes 11, 12 and 14). A conservative mutation in this basic domain that replaced an asparagine with a glutamine residue had minimal effects on *tat* transactivation (Figure 2, lane 13). Thus, at least two domains of the *tat* protein appear important for *tat*-mediated transactivation of the HIV LTR.

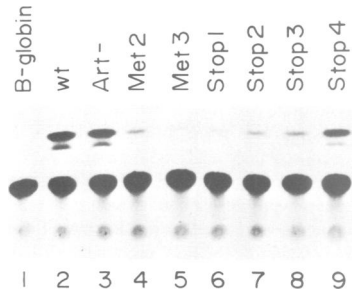

Fig. 3. CAT assays of HIV truncated *tat* genes. HIV *tat* constructs β globin (1), wt (2), Art(-) (3), Met 2 (4), Met 3 (5), Stop 1 (6), Stop 2 (7), Stop 3 (8) and Stop 4 (9) were transfected into HeLa cells with the HIV LTR CAT construct, harvested at 48 h post-transfection, and CAT activity determined.

Table I. CAT activity of HIV *tat* point mutants

Construct	Percent CAT conversion	Relative CAT activity
(1) β globin	0.1	0.01
(2) Wt	13.0	1.00
(3) Δ Pro 2,3	19.5	1.50
(4) Δ Pro 3,4	24.7	1.90
(5) Δ Cys 1	0.2	0.02
(6) Δ Asn	11.8	0.86
(7) Δ Cys 2	0.6	0.05
(8) Δ Cys 3	7.8	0.60
(9) Δ Cys 4	0.9	0.07
(10) Δ Cys 1–4	0.4	0.03
(11) Δ Lys	3.5	0.27
(12) Δ Arg 1	7.1	0.55
(13) Δ Gln	6.1	0.47
(14) Δ Arg 2	4.9	0.38

Both unacetylated and acetylated chloramphenicol (C^{14}) were determined by scintillation counting of CAT assays to determine the percent CAT conversions. A 13% conversion for the Wt construct was assigned a CAT activity of 1.00.

Table II. CAT activity of HIV truncated *tat* proteins

Construct	Percent CAT conversion	Relative CAT activity
(1) β globin	0.1	0.01
(2) Wt	13.0	1.00
(3) Art(-)	8.5	0.65
(4) Met 2	0.9	0.07
(5) Met 3	0.4	0.03
(6) Stop 1	0.9	0.07
(7) Stop 2	1.0	0.08
(8) Stop 3	1.2	0.09
(9) Stop 4	4.5	0.35

Both unacetylated and acetylated chloramphenicol (C^{14}) were determined by scintillation counting of CAT assays to determine the percent CAT conversion. A 13% conversion for the Wt construct was assigned a CAT activity of 1.00.

Truncated tat proteins function in transcriptional activation

A series of mutations which resulted in truncations at the N or C terminus of the *tat* protein were also constructed (Figure 3). Mutations which interrupted the first methionine in the *tat* gene and introduced one of two downstream methionines shown in Figure 1, exhibited a decrease in *tat*-induced activity of the HIV LTR (Figure 3, lanes 4 and 5). Introduction of a series of stop codons in the C terminus of the *tat* protein before the basic domain gave low levels of *tat* induction (Figure 3, lanes 6, 7 and 8), whereas a stop codon introduced after this domain retained partial activity (Figure 3, lane 9). These results support point-mutation studies of the *tat* gene which indicate that the cysteine-rich domain is a critical domain for *tat* activation, although both the amino terminus and the basic domains also contribute significantly to full *tat* activity. The construct which destroys the initiating methionine for *art* (ART-) retained near-wild-type activity (Figure 3, lane 3). Tables I and II show CAT conversion for both the point mutations and the truncated *tat* constructs. S1 analysis of *tat* mRNA from the transfected HeLa cells was performed for these constructs and gave similar levels of *tat*-specific mRNA indicating that these mutations did not alter the steady-state level of *tat* mRNA (data not shown). These results suggest that the changes in the amino acid composition of important domains of the *tat* protein directly altered its transactivating ability rather than effects on *tat* mRNA stability due to the introduction of mutations.

Discussion

The HIV viral protein *tat* is an activator of transcription of the HIV LTR (Arya *et al.*, 1985; Sodroski *et al.*, 1985a,b; Cullen, 1986; Dayton, 1986; Feinberg *et al.*, 1986; Fisher *et al.*, 1986; Gendelman *et al.*, 1986; Peterlin *et al.*, 1986; Rosen *et al.*, 1986; Siegel *et al.*, 1986; Wright *et al.*, 1986; Hauber *et al.*, 1987; Kao *et al.*, 1987). The target sequence for *tat*-induced transactivation was previously shown to reside in the 5′ untranslated end of the HIV LTR message (Rosen *et al.*, 1985; Peterlin *et al.*, 1986; Garcia *et al.*, 1987; Muesing *et al.*, 1987; Tong-Starksen *et al.*, 1987; Hauber and Cullen, 1988) with a 3′ boundary between +37 and +52 from the start of transcription in a region containing a direct repeat CTCTCTGG which is important for *tat* activation (J.Garcia *et al.*, unpublished). The activation of this region is orientation dependent, unlike enhancer elements found in the SV40 promoter, and requires upstream promoter elements for full activation (Rosen *et al.*, 1985; Peterlin *et al.*, 1986).

An examination of the sequence composing this region in the HIV LTR has indicated a potential stem and loop structure for messages containing this sequence. Using SP6-produced RNA, it has been demonstrated that this leader sequence, referred to as the TAR element, is capable of forming stable stem structures under certain experimental conditions (Muesing *et al.*, 1987). Several studies have suggested that one function of *tat* may be to relieve translational repression of TAR-containing mRNA (Feinberg *et al.*, 1986; Rosen *et al.*, 1986) or to function as an anti-terminator allowing the synthesis of long mRNAs (Kao *et al.*, 1987). However, there are no data available to date which indicate that *tat* or any cellular factor binds directly to the TAR sequence of mRNA, although a cellular protein, UBP-1, which binds to DNA sequences in the TAR region, has been purified (Wu *et al.*, 1988b).

Several studies have demonstrated that *tat* increases the level of message produced from the HIV LTR (Gendelman *et al.*, 1986; Peterlin *et al.*, 1986; Wright *et al.*, 1986; Garcia *et al.*, 1987; Muesing *et al.*, 1987). The *tat* protein may not bind directly to the HIV LTR. Instead, *tat* may activate

transcription indirectly in a similar manner to other viral transactivators such as the E1A protein of adenovirus. Recent studies of a bacterially synthesized *tat* protein have indicated that this protein binds divalent ions such as zinc and cadmium and the protein forms a metal-linked dimer (Frankel *et al.*, 1988). The metal-binding domain has been suggested to be localized to the cysteine-rich region, although this region does not conform to previously described zinc fingers (Frankel *et al.*, 1988). Our studies indicate the importance of this domain for *tat*-induced transcriptional activation. The 13S E1A protein of adenovirus also contains a domain with a potential zinc finger (Green *et al.*, 1988) which may be required for transcriptional activation and which is absent in the 12S E1A protein, a poor activator of early adenovirus genes (Montell *et al.*, 1984). The TAR region itself functions as a domain to which cellular factors can bind as demonstrated by DNase I footprint analysis (Garcia *et al.*, 1987). Neither the levels nor the characteristics of the cellular proteins which bind to the LTR appear to be altered in the course of an HIV infection (Wu *et al.*, 1988a). This would suggest that *tat* may act indirectly on viral gene expression of the HIV LTR as suggested for the E1A protein which does not directly bind to DNA (Ferguson *et al.*, 1985).

Mutations which alter this basic region in *tat*, such as the substitution of glutamic acid residues for lysine or arginine residues, resulted in decreased *tat* transactivation and stop codons which eliminate this domain resulted in even further decreases in *tat* transactivation. The basic domain present in the *tat* protein is analogous to a basic region important for DNA binding of the yeast DNA-binding protein GCN4. Homology to this portion of the GCN4 protein is also found in both the c-*fos* and the c-*jun*/AP-1 protein (Vogt *et al.*, 1987). Similar basic domains have also been shown to be important as nuclear transport signals for a number of other nuclear localized proteins including SV40 and polyomavirus T antigens, SV40 VPI, and the *X.laevis* N_1/N_2 and nucleoplasmin proteins (Kalderon *et al.*, 1986; Kleinschmidt *et al.*, 1986; Richardson *et al.*, 1986; Wychowski *et al.*, 1986; Burglin and De Robertis, 1987). Further studies will be required to determine if the basic domain in *tat* is required for its nuclear localization and/or nucleic acid binding. The function of the N terminus of the *tat* protein is not known but it may play a role in either transcriptional activation, protein transport and localization, or nucleic acid binding.

One potential mechanism of action for *tat* may be a direct interaction with a cellular transcription factor. In transcriptional-activator proteins such as the yeast GAL4 protein, it has been found that DNA binding and transcriptional activation were separable and activation was dependent on an acidic activating region of the protein (Ma and Ptashne, 1987a,b). The *tat* protein may establish a domain-specific contact via an opposite charge attraction to a cellular transcription factor containing an acidic activating domain. Such a mechanism has been proposed for the interaction of certain bacteriophage repressors with RNA polymerase (Hothschild *et al.*, 1983). By displacing some factor from, or altering the structure of, a transcription complex, *tat* may mediate its effect with further specificity defined by the zinc finger domain.

Materials and methods

Mutagenesis

A *Hin*cII–*Ssp*I fragment (nt 5791–6061 of the Arv 2 genome) containing the second exon for the *tat* message (first 72 amino acids) was cloned into the *Hin*cII site of pUC19 (Sanchez-Pescador *et al.*, 1984). A partial *Hin*dIII–*Bam*HI fragment containing the *tat* gene was then cloned into the M13 vector mp18 for subsequent mutagenesis. Oligonucleotides (21mers) made to the coding strand of *tat* and containing the mutations indicated in Figure 1 were synthesized for use in site-directed mutagenesis of the *tat* gene. Mutagenized plasmids were transformed into JM103 and plaques picked for subsequent sequence analysis. All clones were dideoxy sequenced to verify the appropriate changes. RF DNA was prepared from positive clones and fragments were gel-isolated which contained the mutated *tat*I genes. These fragments were used to replace the wild-type *tat* gene in an expression plasmid containing the RSV promoter, SV40 splice acceptor and SV40 polyadenylation sequences (Garcia *et al.*, 1987).

Transfections and CAT assays

Using the calcium phosphate transfection protocol, 5 μg of the RSV-*tat* expression plasmids or a control plasmid RSV-β-globin were transfected onto each 100-mm HeLa plate (50–70% confluent at the time of transfection) along with 5 μg of an HIV LTR CAT construct containing nt 287–476 of the Arv 2 genome (−177 to +83 from the start of transcription). This HIV LTR construct contains transcriptional control elements in the HIV LTR required for expression in HeLa cells (Garcia *et al.*, 1987). Transfections were glycerol shocked 4 h post-transfection and harvested 44 h later and CAT activity determined as described (Gorman *et al.*, 1982).

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