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Multiple functional domains of Tat, the *trans*-activator of HIV-1, defined by mutational analysis

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ABSTRACT

The *tat* gene of HIV-1 is a potent *trans*-activator of gene expression from the HIV long terminal repeat (LTR). To define the functionally important regions of the product of the *tat* gene (Tat) of HIV-1, deletion, linker insertion and single amino acid substitution mutants within the Tat coding region of strain SF2 were constructed. The effect of these mutations on *trans*-activation was assessed by measuring the expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene linked to the HIV-LTR. These studies have revealed that four different domains of the protein that map within the N-terminal 56 amino acid region are essential for Tat function. In addition to the essential domains, an auxiliary domain that enhances the activity of the essential region has also been mapped between amino acid residues 58 and 66. One of the essential domains maps in the N-terminal 20 amino acid region. The other three essential domains are highly conserved among the various strains of HIV-1 and HIV-2 as well as simian immunodeficiency virus (SIV). Of the conserved domains, one contains seven Cys residues and single amino acid substitutions for several Cys residues indicate that they are essential for Tat function. The second conserved domain contains a Lys X Leu Gly Ile X Tyr motif in which the Lys residue is essential for *trans*-activation and the other residues are partially essential. The third conserved domain is strongly basic and appears to play a dual role. Mutants lacking this domain are deficient in *trans*-activation and in efficient targeting of Tat to the nucleus and nucleolus. The combination of the four essential domains and the auxiliary domain contribute to the near full activity observed with the 101 amino acid Tat protein.

INTRODUCTION

The human immunodeficiency viruses (HIV) have been etiologically linked with the acquired immunodeficiency syndrome (AIDS). HIV undergoes remarkably controlled states of replication during its pathogenesis. Initial infections appear to be silent with no or low levels of virus replication. Disease progression leads to a state of prolific viral replication and cell death (1-3). The differential state of HIV replication appears to be controlled by an elaborate network of regulatory circuits that control gene expression. In addition to the three common retroviral genes (*gag*, *pol* and *env*), the HIV genome includes at least six other genes. Three of these genes (*tat*, *rev* and *nef*) have been shown to play important regulatory roles in increasing or decreasing viral gene expression (reviewed in Ref. 4 and 5). The *tat* gene of HIV-1 codes for a protein (Tat) of 86 to 102 amino acids, depending on the strain. Tat is a strong *trans*-activator of genes that are expressed from the viral LTR (6) and is essential for virus replication and virus mediated cytopathicity (7-9).

The mechanism of Tat-mediated *trans*-activation is not clear. It is generally believed that the regulatory role of Tat is bimodal since it has been shown to increase the level of HIV mRNA (10-14) and may also regulate the translation of viral mRNA

(10,15,16). It has also been proposed that the effect of Tat at the level of transcription may involve anti-termination of transcription (13,17). Tat-mediated *trans*-activation depends on a *cis*-acting sequence termed *tar* (11,18) which has the potential to form an extensive stem-loop structure (12,19,20). Although recent mutagenesis studies (13,20) have revealed that the loop sequence within the *tar* element is the primary site of Tat-mediated *trans*-activation, the mechanism of interaction between the loop sequence and Tat is not known.

Elucidation of the essential structural features of Tat may facilitate understanding of the mechanism of *trans*-activation and suggest strategies to suppress this viral function. Comparison of the amino acid sequences of Tat protein coded by all known strains of HIV-1, HIV-2 and SIV (21) has revealed regions of Tat that are conserved among these related viruses. One striking feature is the presence of seven Cys residues at identical spacing in all the Tat proteins. Recent biochemical studies have indicated that the Cys residues are involved in the metal-linked dimerization of Tat (22,23). In addition to the Cys-rich domain, a highly basic domain and a Lys X Leu Gly Ile X Tyr motif are also conserved. Our mutational analysis of the *tat* gene has demarked the essential and non-essential regions of Tat involved in *trans*-activation and indicates that the essential region may consist of four different domains and further identifies an auxiliary domain.

MATERIALS AND METHODS

Plasmids and Mutants:

Plasmids pTAT expressing the *tat* gene of HIV-1, strain SF2, and pLTR-CAT expressing the bacterial chloramphenicol acetyl transferase (CAT) gene under the control of HIV-1 (SF2) LTR have been described (11,24). Mutants *tat*-82, *tat*-67, and *tat*-56 were constructed by ligating SpeI linkers (New England Biolabs, #1061) to pTAT DNA linearized with either ApaI or HindIII or SacI (see Figure 1 for restriction map of these enzymes), respectively, and blunt-ended with T4 DNA polymerase. Mutant *tat*Δ58-66 was generated by circularization of pTAT cut with SacI and HindIII after blunt-ending with T4 DNA polymerase. Mutants *tat*Δ3-19 and *tat*Δ2-35 were constructed by insertion of a double stranded oligonucleotide 5'-GATCTGGAGAGCAAGAAGAAATGGA-3'/ 3'-ACCTCTCGTTCTTCTTTACCT-5' between the BglII and AvrII (blunt ended with mung bean nuclease) or between the BglII and MluI (blunt ended with T4 DNA polymerase) sites. Mutant *tat*Δ2-6 was derived from *tat*Δ2-35 by inserting a 149 bp Tat coding sequence from pTAT located between the XbaI (blunt-ended with T4 DNA polymerase) and SacI sites between the MluI (blunt) and the SacI sites of *tat*Δ2-35. Mutant *tat*Δ19-35 was constructed by removal of the Tat coding region between the AvrII and MluI sites followed by blunt (T4 DNA polymerase) end circularization. Mutant *tat*Δ40-49 was constructed from *tat*-49 (which contains an NheI site introduced at the end of codon 48 by oligonucleotide-directed mutagenesis) by replacing the sequences between the BglII and NheI (blunt-ended with mung bean nuclease) of *tat*-49 with a 136 bp BglII to SnaBI fragment from another mutant, *tat*-39 that contains a SnaBI site after the 39th codon. Mutant *tat*Δ48-59 was constructed by inframe insertion of a 10 bp SalI linker between the NheI and SacI sites of *tat*-49. All linker insertions were constructed by the method of linker tailing (25). Mutants *tat*-22, -30, -31, -34, -37, -41, -44, -47H, -47A and -49 were carried out by oligonucleotide-directed mutagenesis using a template cloned in M13mp18. Mutagenesis and mutant enrichment was carried out by the procedure of Taylor *et al* (26). DNA sequence determinations were carried out by the dideoxy method using Sequenase (United States Biochemical Corporation).

DNA transfections, CAT assays and RNA analysis:

HeLa cells (about $2x10^6$ cells/ 100 mm dish) were transfected with 1 μg of LTR-CAT and 1 μg of various TAT plasmids along with 18 μg of carrier pUC13 or 18

DNA by the calcium phosphate method. Cells were harvested 48 hour after transfection and the expression of the CAT gene was determined by measuring the CAT enzyme activity (27) or the level of CAT-specific cytoplasmic RNA. Total cytoplasmic RNA was treated with 50 μg/ml DNAse (RQ1, Promega) and immobilized on nitrocellulose sheets using a Schleicher and Schuell slot-blot manifold. The blots were hybridized with a 551 bp nick translated CAT-specific probe (HindIII-NcoI fragment from pRSV-CAT) and autoradiographed.

Immunoflorescence:

COS1 cells were grown on 12mm glass coverslips and transfected with *tat*-67 or various mutant plasmids. Forty-eight hours after transfection, cells were fixed with 3.5 % formaldehyde in PBS at room temperature for 10 min, with methanol at -20° C for 6 min and then rehydrated with PBS. Coverslips were incubated with Tat-specific antibodies (1:1000 dilution) directed against the N-terminal 61 amino acids (28) for 30 min at 37° C in a humidified chamber, then washed three times with PBS at 22° C and finally incubated with goat anti-rabbit immunoglobulin (1:50 dilution) conjugated with rhodamine (Cappel Immunological Reagents) for 30 min. The cover slips were washed three times with PBS, once with water and mounted with glycerol-gelatin (Sigma Chemical Co). Photographs were taken on a Nikon fluorescence-phase microscope using Kodak Tri-X film.

RESULTS

In order to delineate the functional domains of Tat, a combination of deletion, linker insertion and single amino acid substitution mutants were used as it may not have been possible to define a functional domain with single amino acid substitution mutants alone. In these studies, we used the *tat* gene of HIV-1, strain SF2 which codes for a protein of 101 amino acids (see Figure 1; also Ref. 24). The SF2 Tat differs from the Tat of the prototype strain HXB2 (86 amino acid-long) only by five conservative amino acid substitutions.

C-terminal boundary.

To define the C-terminal boundary of Tat, chain termination mutations were introduced after amino acid residues 82, 67, and 56 taking advantage of convenient restriction sites for ApaI, HindIII and SacI (Figure 1). These mutations were generated by ligation of an oligonucleotide linker that introduces a chain termination mutation in all three reading frames in addition to creating a diagnostic SpeI restriction site. The effect of these mutations on *trans*-activation was determined by the CAT activity expressed in Hela cells cotransfected with pLTR-CAT reporter plasmid and various mutant *tat* plasmids. These results (Figure 2A) revealed that mutants coding for 82 (*tat*-82) or 67 (*tat*-67) residues from the N-terminus induced *trans*-activation of LTR-CAT to near *wt* levels. However, the activity of a mutant coding for the N-terminal 56 amino acids (*tat*-56) was only about one fifth of the activity of *tat-wt* or tat-82 or *tat*-67 mutants suggesting that the sequence between residues 57 and 66 enhances the activity of the N-terminal 56 amino acids. In order to determine whether this enhancement is uniquely contributed by this sequence or other C-terminal sequences of Tat could substitute for this region, an inframe deletion mutant lacking residues 58 to 66 (*tat*Δ58-66) was constructed and its activity was determined. The activity of this mutant was more or less similar to that of mutant *tat*-56 suggesting that the protein region between residues 58 to 66 plays an auxiliary role.

N-terminal boundary.

To define the N-terminal boundary essential for Tat activity, three different deletion mutants lacking the various N-terminal regions were constructed by ligation of a synthetic double stranded oligonucleotide containing sequences 20 bp upstream and 2 bp down stream of the ATG codon to various N-terminal deletions generated by digestion with a combination of BglII and XbaI or BglII and AvrII or BglII and MluI (Figure 2B). All the three deletion mutants (*tat*Δ2-6, *tat*Δ3-9, *tat*Δ2-35) lacking the various N-terminal sequences between residues 2 to 6, 3 to 19 or 2 to 35 were almost

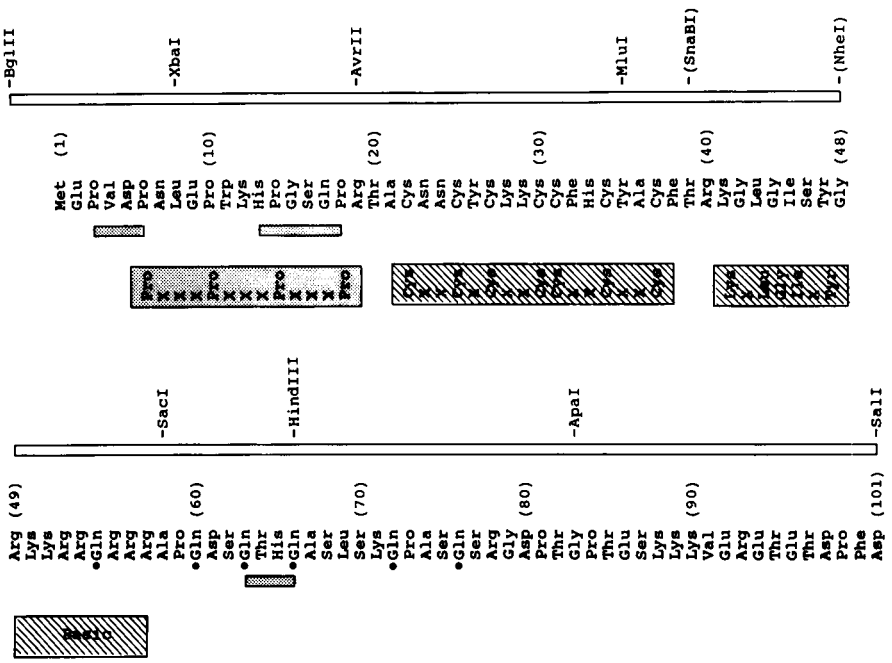

Figure 1: *Structural features of HIV (SF2) Tat protein.* The amino acid sequence of the entire protein is shown. The broader boxes indicate the conserved structural features and the thinner boxes indicate conserved sequences. Boxes with dots indicate sequences and features conserved among all HIV-1 strains and boxes with slashed lines indicate features conserved among all HIV-1, HIV-2 and SIV strains. Some of the restriction sites present in pTAT are indicated above the double lines. The restriction sites introduced into pTAT by site-directed mutagenesis and used in constructing other mutants are shown in parenthesis. The Gln residues of the Gln-rich region are indicated by dots.

totally defective for *trans*-activation (Figure 2B). In addition to these mutants, an additional deletion mutant (*tat*Δ9-18) defective in coding residues 9 to 18 (XbaI-AvrII) was also found to be defective for Tat activity. These results indicate that the integrity of most of the N-terminal 46 amino acids is essential for Tat activity.

Conserved domains:

The conserved region of Tat could be divided into two obvious domains (Cys-rich and basic) based on their distinguishing characteristics. However, a highly conserved motif of Lys X Leu Gly Ile X Tyr, present between the Cys-rich and the basic domains, may also represent a different functional domain. Therefore, mutagenization experiments were designed under the assumption that the conserved sequences constitute three different domains.

a. Cys-rich domain.

The effect of the Cys-rich domain was assessed by using a deletion mutant (*tat*Δ19-35) lacking six of the seven conserved Cys residues as well as mutants with single amino acid substitutions where five of the seven Cys residues were replaced with various

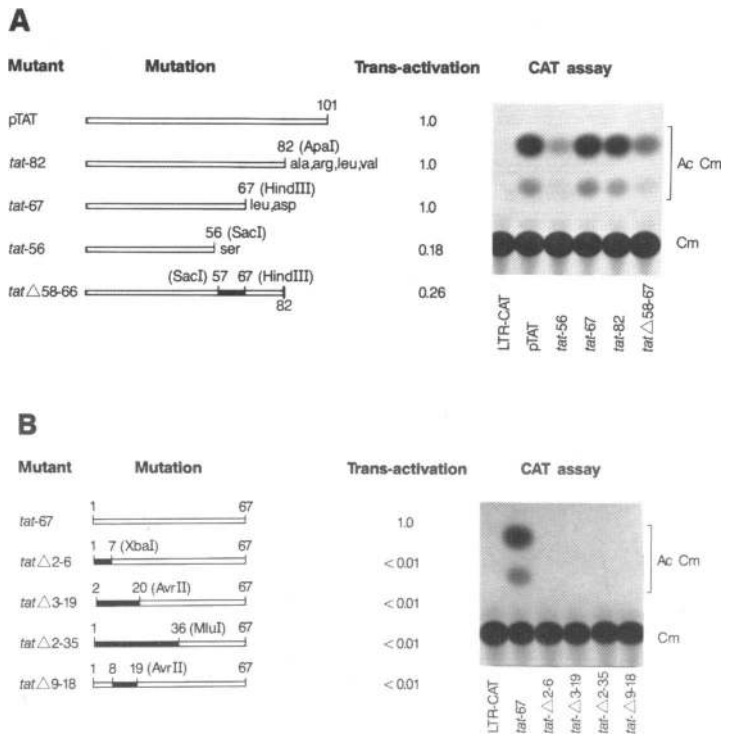

Figure 2: *C-terminal (A) and N-terminal (B) mutants of tat gene.* The figures shown under the column *trans*-activation represent relative CAT-activity determined in four to six different transfection experiments. Representative CAT-assays are shown on the right hand side. The missense amino acids generated by the insertion are indicated in all-lower cases. Amino acid sequences not expressed or deleted are indicated by filled bars. Target restriction sites where the mutations were introduced are shown in parentheses. The *trans*-activation potentials of the N-terminal mutants and most of the mutants described in other figures were determined relative to that of mutant *tat*-67. However, many of the mutations were also assayed under parental pTAT background and were found to function similarly as in the *tat*-67 background.

amino acids (Figure 3A). Functional analysis revealed that mutant *tat*Δ19-35 was almost totally defective in *trans*-activation. Similarly, all the five single amino acid substitution mutants were also functionally defective indicating that the Cys residues are essential for Tat activity.

b. Lys X Leu Gly Ile X Tyr motif.

In order to determine whether this motif is essential for Tat activity, single amino acid substitutions in three of the five conserved amino acids were introduced (Figure 3B). Analysis of these mutants revealed that the *tat*-41 (Lys→Thr) mutant was severely defective in *trans*-activation whereas mutations *tat*-44 (Gly→Ser), *tat*-47H (Tyr→His) and *tat*-47A (Tyr→Ala) reduced Tat activity by about 60 to 70 per cent. These results

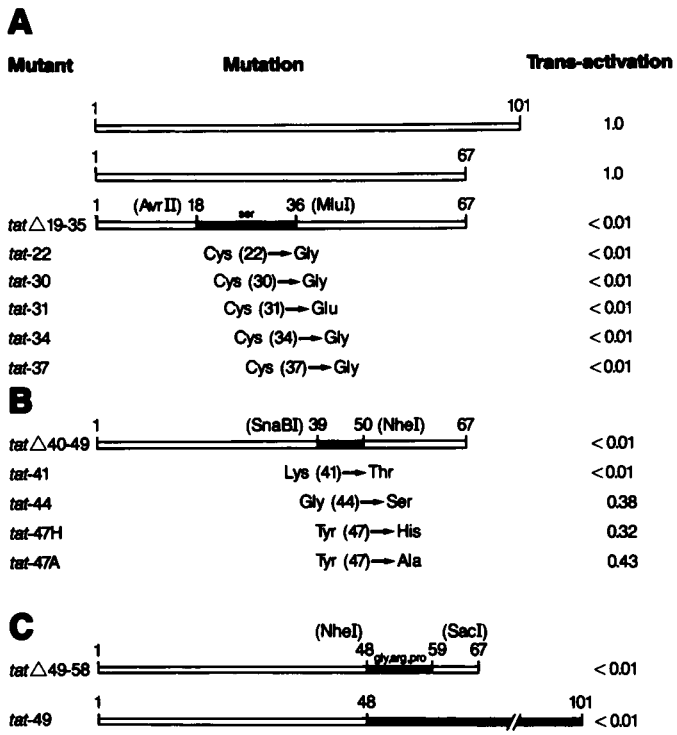

Figure 3: *Mutants of tat gene in the conserved sequences.* A. Cys-rich region. B. Lys X Leu Gly Ile X Tyr motif. C. Basic domain. Activities of the various mutants were determined as described under Figure 2.

indicate that the Lys residue within this motif is essential for *trans*-activation while the other residues (Gly#44 and Tyr#47)are partially essential. The importance of this motif is also evident from the analysis of a deletion mutant *tat*Δ40-49 lacking residues 40 to 49 that was found to be severely *trans*-activation defective as expected. Although mutant *tat*Δ40-49 has a deletion of one of the basic residues (Arg#49), it is unlikely that this may have a significant effect on Tat activity (see Discussion).

c. Basic domain.

The role of the conserved basic domain was determined using an inframe substitution mutant (*tat*Δ49-58) lacking the entire basic domain (residues 49 to 58). As in the case of mutants in the Cys-rich domain, the Lys (#41) mutant and the mutants in the N-terminal domain, mutant *tat*Δ49-58 was severely defective in Tat activity (Figure 3C). Similarly, mutant *tat*-49 which has a TAG codon after the 48th codon was also defective for *trans*-activation, indicating that the basic domain is essential for the *trans*-activation function.

CAT-RNA accumulation.

In order to determine whether the effects of the various mutations observed with the CAT assays were also reflected at the level of RNA accumulation, total cytoplasmic RNA from cells transfected with deletion mutants in each of four essential domains and the auxiliary domain and the CAT-specific RNA was analyzed by RNA slot blots (Figure 4). In accordance with the CAT assays, mutants in each of the four

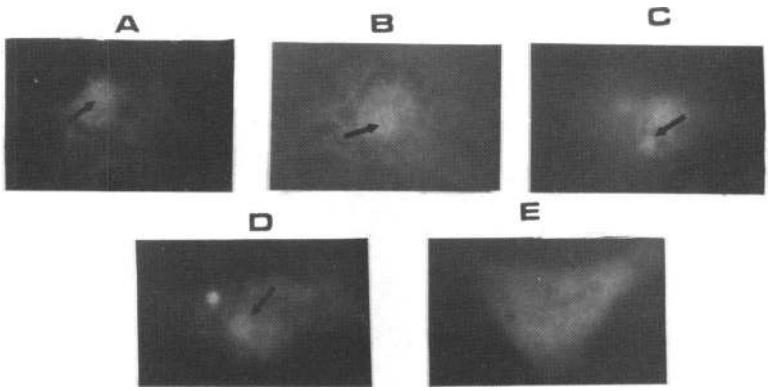

Figure 4: *Slot-blot analysis of CAT RNA.* In the top panel each slot contained 1 μg samples and in the lower panel each slot contained 10 μg samples.

essential domains induced CAT-RNA at less than one hundredth of the level induced by *tat*-67 whereas cells transfected with the mutant (*tat*Δ58-66) in the auxiliary domain induced low levels of RNA. These results indicate that the observed effects of the various mutations may primarily be at the level of RNA synthesis and accumulation.

Subcellular localization of Tat.

It is known that Tat is a nuclear protein (28). The basic domain of Tat resembles the region essential for nuclear transport of several well characterized proteins such as SV40 and Polyoma T antigens (29-32) and the nucleolar targeting signal of HTLV-I Rex protein (33). In order to determine whether the basic domain of Tat is essential for transport to the nuclear/nucleolar locations and to determine whether the Tat protein coded by the mutants is stable and localizes to appropriate cellular compartment, COS1 cells were transfected with parental *tat*-67 plasmid or mutants in various essential domains and the expression of Tat was examined by indirect immunoflorescence using Tat peptide antiserum directed against the N-terminal 61 amino acids. These experiments revealed that in cells transfected with *tat*-67 (Figure 5A), the fluorescence was primarily localized in the nucleus and in the nucleolus. Similarly the proteins coded by mutants *tat*Δ2-6 (Figure 5B) or *tat*-31 (Figure 5C) or *tat*-47A (Figure 5D) were also localized in the nuclear/nucleolar locations. However, in cells transfected with mutant *tat*Δ49-58 (Figure 5E) the fluorescence was weaker and was distributed throughout the cell. These results indicate that the basic domain of Tat is essential for efficient transport to the nucleus and nucleolus. However, in the absence of this domain still a significant amount of the protein appears to be translocated to the nucleus.

DISCUSSION

The mutational analysis of the *tat* gene of HIV-1 (SF2) presented here reveals that the amino acid sequences of Tat essential for its activity are located within the N-terminal 56 amino acid region and this region could be divided into four essential domains (Figure 6). Although the immunoflorescence analysis of Tat expression in cells transfected with various mutants indicates that they code for stable proteins, in the absence of a quantitative analysis of mutant Tat protein levels, the effect of differential levels of protein expression on *trans*-activation cannot be ruled out at present. In addition to the essential domains, an auxiliary domain also appears to be important for its full activity. The evidence for an auxiliary domain comes from our results with an

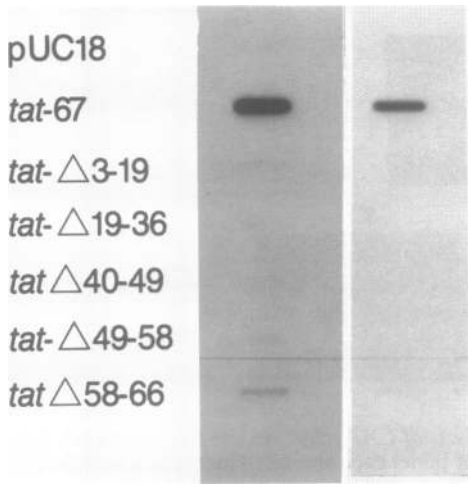

Figure 5: *Subcellular localization of Tat protein.* Immunoflorescence photographs of COS cells transfected with *tat*-67 (A) or *tat*Δ2-6 (B) or *tat*-31 (C) or *tat*-47A (D) or *tat*Δ49-58 (E). The nucleolar regions are indicated by arrows.

inframe deletion mutant (*tat*Δ58-66) that deletes nine amino acids immediately adjacent to the essential basic domain. It should be noted that a previous mutational analysis of the *tat* gene of HIV-1 (HXB2) also revealed that a mutant with N-terminal 58 codons had reduced *trans*-activation potential (34) indicating the requirement for additional sequences for full activity. Although the auxiliary domain identified here is not conserved among HIV-1, HIV-2 and SIV, sequence comparisons among different HIV-1 strains reveal some conserved sequences in this region(21; also see Figure 1). The auxiliary domain may contribute to enhanced Tat activity by structural stabilization or by direct functional contribution. This domain contains three Gln residues out of nine residues in all HIV-1 strains (Figure 1). It has recently been shown with the transcription factor Sp1 that a Gln-rich domain is involved in transcriptional activation (35). It is possible that the Gln-rich auxiliary domain that we have identified may contribute to enhanced Tat activity. It should be noted that HIV-2 Tat also contains a Gln-rich (four out of eighteen) motif but near the N-terminal region(21).

Our results indicate that the N-terminal 20 amino acid region is crucial for Tat activity. However, the identity of the essential sequence element is not clear. All HIV-1 strains contain three repeats of a Pro X X X Pro motif (Figure 1). The deletion mutants that we have used lack one (*tat*Δ2-6) or all three (*tat*Δ3-19; *tat*Δ9-18) of these motifs and are almost totally defective in Tat activity, apparently suggesting that it may be functionally important. However, a recent mutagenesis study (36) showed that when some of the Pro residues were substituted with Leu residues in such a way that two or all three of these motifs were altered, Tat activity was not affected thereby casting a doubt on the requirement of the Pro X X X Pro motif. The deletion mutant *tat*Δ2-6 localizes one of the critical region within the N-terminal five amino acid region between residues 2 and 6. This region contains a Pro Val Asp Pro motif conserved in all HIV-1 strains. Mutant *tat*Δ9-18 retains this motif but is still functionally inactive. It however, lacks another conserved (HIV-1) sequence, His Pro Gly Ser Glu Pro. This raises the possibility that the overall integrity of the N-terminal 20 amino acid region is essential and indicates that further mutagenization is required to identify the exact N-terminal sequence element essential for Tat activity.

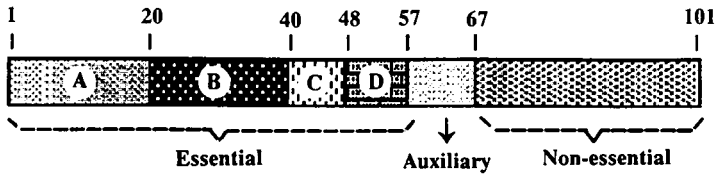

Figure 6: *Organization of Tat functional domains.* A, N-terminal domain; B, Cys-rich domain; C, Lys X Leu Gly Ile X Tyr motif; D, Basic domain. The amino acid positions are given on the top.

The results presented here and those of others (36) indicate that a Cys-rich domain is essential for Tat-activity. The use of the deletion mutant *tat*Δ19-35 which deletes six of the seven Cys residues appears to emphasize the importance of this domain as it could be argued that single amino acid substitution mutants could lead to 'prohibitive' interactions by other Cys residues. This domain contains seven Cys residues that are highly conserved and has been suggested to be important for metal-linked dimerization of Tat (22,23). Other suggestions include that this domain may be a potential zinc finger (37). It is not known whether this domain plays any functional role other than the suggested structural role in protein dimerization. Mutations resulting in the substitutions of other amino acid residues within this domain should be valuable to unravel other potential roles of this domain in Tat function.

We have identified that the conserved Lys X Leu Gly Ile X Tyr motif located between the Cys-rich domain and the basic domain is important for Tat activity. Although, among these conserved residues, only the Lys(#41) residue appears to be most critical, other amino acid residues (Gly#44; Tyr#47) are also at least partially important. The possibility that this motif is not a separate functional domain and that the essential Lys (#41) residue may well be a part of the neighboring Cys-rich domain cannot be ruled out.

It has previously been reported that Tat is a nuclear protein (28). Our immunoflorescence studies indicate that in addition to the nuclear localization, Tat also preferentially localizes in the nucleolus. As expected, deletion of the entire basic domain (residues 49 through 58) had a severe effect on Tat activity. Mutagenization experiments by other investigators where the individual Lys or Arg residues were substituted with acidic or polar amino acids did not have a severe effect on the *trans*-activation potential (9,36). In contrast, mutant (*tat*Δ49-58) exhibited near total loss of activity. These results suggest that the overall basic nature of this domain is essential for Tat function. We have shown that the basic domain is essential for efficient transport of Tat to the nucleus and nucleolus. However, it should be noted that deletion of the basic domain does not result in total loss of transport to the nucleus although nucleolar localization appears to be excluded. This observation is similar to certain other nuclear proteins such as adenovirus E1a where deletion of the signal sequence for nuclear transport did not impair nuclear transport totally and also did not seem to significantly affect the function (38,39). We have recently observed that a fusion protein consisting of E. coli β-galactosidase and the basic domain of Tat efficiently localizes in the nucleus and nucleolus (in preparation). It remains to be determined whether the nuclear/nulceolar localization targeting is the only function of the basic domain. It has been proposed by a number of investigators that the basic domain of Tat may be involved in nucleic acid binding. However, the observation (22) that bacterially expressed Tat does not bind to specific RNA or DNA suggests that it may be involved in *trans*-activation by an alternate mechanism such as interaction with other protein factors.

Since *tat* gene is essential for viral replication and cytopathicity, understanding of the mechanism of Tat-mediated *trans*-activation should be useful in designing strategies

to interfere with this function. Demarcation of various functional domains and the availability of defined mutants should be useful in this endeavor.

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