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Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus

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The human immunodeficiency virus (HIV) is a human retrovirus which is the etiologic agent of the acquired immunodeficiency syndrome. To study the cellular factors involved in the transcriptional regulation of this virus, we performed DNase I footprinting of the viral LTR using partially purified HeLa cell extracts. Five regions of the viral LTR appear critical for DNA binding of cellular proteins. These include the negative regulatory, enhancer, SP1, TATA and untranslated regions. Deletion mutagenesis of these binding domains has significant effects on the basal level of transcription and the ability to be induced by the viral *tat* protein. Mutations of either the negative regulatory or untranslated regions affect factor binding to the enhancer region. In addition, oligonucleotides complementary to several of the binding domains specifically compete for factor binding. These results suggest that interactions between several distinct cellular proteins are required for HIV transcriptional regulation.

Key words: AIDS/HIV/transcription factors

Introduction

The human immunodeficiency virus (HIV, LAV, HTLV-III, ARV-2) is a human retrovirus which is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984). This virus has been shown to infect OKT4 positive lymphoid (Dalgleish *et al.*, 1984; Klatzman *et al.*, 1984a,b; McDougal *et al.*, 1985) and non-lymphoid cells (Levy *et al.*, 1985; Maddon *et al.*, 1986). In addition, the virus has been grown in a number of different cell lines, including HeLa cells, when introduced into the cells by calcium phosphate transfection (Levy *et al.*, 1986). This ability to produce viral mRNA and proteins in diverse cell lines indicates that the virus is able to use a number of general cellular transcription factors to regulate its gene expression.

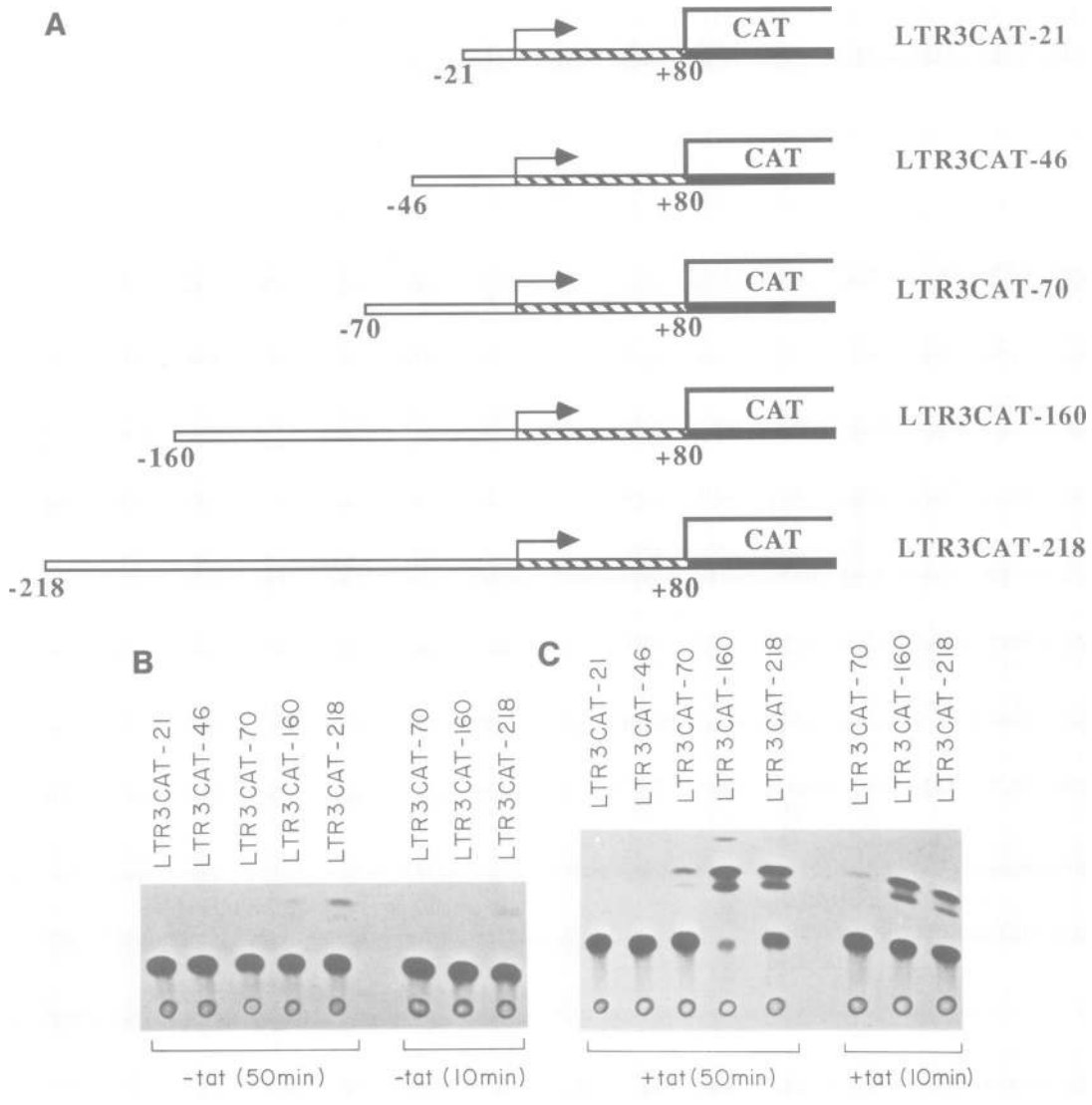
A number of genetic studies have defined seven unique gene products involved in the viral life cycle (reviewed by Rabson and Martin, 1985; Chen, 1986). One of these genes, *tat*, appears to play a central role in viral gene expression and replication (Arya *et al.*, 1985; Sodroski *et al.*, 1985a,b; Cullen, 1986; Dayton *et al.*, 1986; Feinberg *et al.*, 1986; Fisher *et al.*, 1986; Gendelman *et al.*, 1986; Peterlin *et al.*, 1986; Rosen *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.*, 1987). This 86 amino acid protein is encoded by two exons (Arya *et al.*, 1985; Sodroski *et al.*, 1985b). Only the first 58 amino acids (58AA) have been found necessary for transactivation (Siegel *et al.*, 1986). Transient expression assays with the HIV LTR fused to the bacterial chloramphenicol acetyltransferase (CAT) gene have shown that the *tat* gene is capable of inducing CAT activity from 40- to 300-fold in a variety of cell lines (Arya *et al.*, 1985; Sodroski *et al.*, 1985a,b; Cullen, 1986; Dayton *et al.*, 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). The mechanism of transactivation by the *tat* protein has been reported by several investigators to be an increase in the translation of viral mRNA (Feinberg *et al.*, 1986; Rosen *et al.*, 1986) while others have suggested the primary effect to be an increase in the steady-state level of viral mRNA (Gendelman *et al.*, 1986; Peterlin *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.*, 1987). This protein may have dual functions (Cullen, 1986; Wright *et al.*, 1986). Genetic data indicate that the target sequences for *tat* activity are located in the untranslated region of the viral LTR (Rosen *et al.*, 1985). The untranslated region is not sufficient for complete transactivation, since the level of transactivation when the untranslated region is fused to heterologous promoters is 5% or less of that seen when it is present on the complete HIV LTR (Rosen *et al.*, 1985; Peterlin *et al.*, 1986). These data suggest that the untranslated region may interact with upstream control elements.

To understand the role of cellular factors in the transcriptional regulation of the viral LTR, we performed a combined *in vitro* and *in vivo* analysis of the HIV LTR. This involved the use of DNase I footprinting assays (Galas and Schmitz, 1978) to identify the sites of binding of cellular transcription factors, the subsequent mutagenesis of these regulatory regions, and transfection assays of these constructs fused to the CAT gene both in the presence or absence of the *tat* gene. We found five distinct regulatory regions that serve as binding sites for cellular proteins. These include the negative regulatory, enhancer (Nabel and Baltimore, 1987), SP1 (Jones *et al.*, 1986), TATA and untranslated region binding proteins. Our results indicate that the regulation of the HIV LTR involves the interaction between several distinct cellular transcription factors.

Results

Mutational analysis of the viral LTR

Previous genetic analysis has defined several regions of the HIV LTR which are important in the gene expression of the virus (Rosen *et al.*, 1985; Peterlin *et al.*, 1986; Wright *et al.*, 1986). These include a region between −17 and +80, the TAR region, which is required for *tat*-induced activation, a region between −17 and −137 which functions as an enhancer element for heterologous promoters, and a putative negative regulatory element located in a region from −167 to −423 (Rosen *et al.*, 1985). Two copies of a sequence GGGACTTTCC are present in the enhancer region and these sequences are important for both basal and *tat*-induced gene expression (Nabel and Baltimore, 1987). Three SP1 binding sites have been demonstrated by DNase I footprinting from −43 to −83 using highly purified SP1 (Jones *et al.*, 1986). The addition of highly purified SP1 to *in vitro* transcription systems containing the HIV LTR results in a 10-fold stimulation of transcription (Jones *et al.*, 1986).



D

LTR Construct	% CAT Conversion (-)tat	(+)tat	Fold Induction
LTR3CAT-21	0.1	0.1	-
LTR3CAT-46	0.1	0.1	-
LTR3CAT-70	0.4	1.6	4.0
LTR3CAT-160	1.0	61.3	61.3
LTR3CAT-218	2.6	19.0	7.3

Fig. 1. Transfection assays of HIV deletion mutants. (**A**) Diagram of HIV LTR constructs. The HIV LTR was first cut in the upstream region at either −21, −46, −70, −160 and −218 from the start of transcription and then at +80 and these fragments were placed upstream of the CAT gene as described. Arrows indicate start (+1) and direction of transcription. (**B,C**) CAT assays using HIV LTR constructs. Each of the constructs was used in transfection assays with either RSV beta globin as a control (**B**) or with a viral expression plasmid for RSV-*tat* (**C**). CAT assays were performed as described, and each reaction was harvested at 10 min and 50 min to ensure linear conversion of the chloramphenicol with each extract. (**D**) Per cent of chloramphenicol acetylated with each HIV LTR construct. The per cent conversion and the fold induction in response to *tat* relative to LTR3CAT −160 are also shown.

Deletion mutagenesis of the HIV LTR was performed to further define the regions important for gene expression and to study the interaction of specific DNA binding proteins. We used convenient restriction sites in the HIV LTR (Sanchez-Pescador *et al.*, 1984) to create a series of deletion mutants and then fused these to plasmids containing the CAT gene (Gorman *et al.*, 1982). A group of five mutants demonstrated distinct patterns of transcriptional regulation. These mutants extend from −21, −46, −70, −160 and −218 relative to the start of transcription to +80 in the untranslated region of the virus (Figure 1A). The deletion mutants were then co-transfected into HeLa cells with expression plasmids containing the Rous sarcoma virus (RSV) LTR (Gorman *et al.*, 1983) fused to either the beta globin gene (RSV-beta globin, Figure 1B) or a restriction fragment encoding the first 58AA of the *tat* protein (RSV-*tat*, Figure 1C). This fragment encoding the first 58AA of the *tat* protein has been shown to be sufficient for transactivation of the HIV LTR (Siegel *et al.*, 1986). Transfections were harvested at 48 h post-transfection and

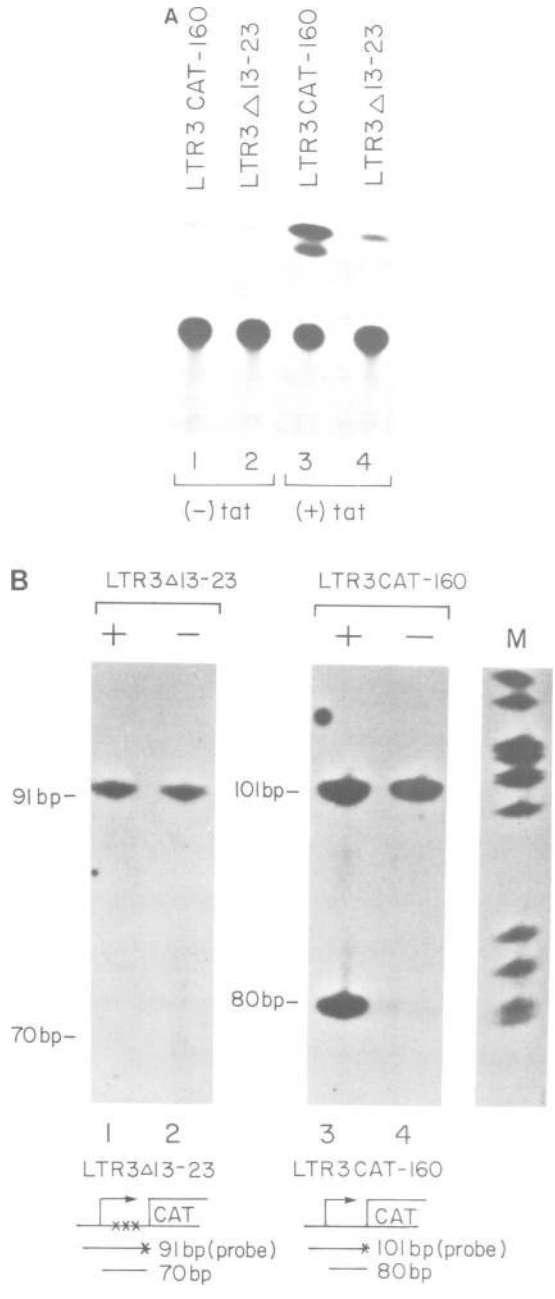


Fig. 2. Mutations in the untranslated region decrease the level of HIV LTR CAT activity and steady state mRNA. (**A**) Transfections both in the presence and absence of the *tat* gene were performed for both LTR3CAT −160 and LTR3CAT −160Δ13−23. CAT activity was determined 48 h post-transfection as described. (**B**) 50 μg of cytoplasmic RNA from each transfection was used in S1 nuclease mapping as described. DNA probes used in the S1 analysis for both the −160 and +80 and the −160 to +80 Δ13−23 LTR3 fragments were end-labeled at the *Hin*dIII site.

CAT activity was determined (Gorman *et al.*, 1982). All transfections and CAT assays were repeated five times and similar results were obtained in each experiment. S1 analysis of both the RSV-beta globin and the RSV-*tat* constructs revealed no significant difference in the level of the mRNA starting from the RSV promoter in each experiment (data not shown). Thus, there were minimal differences in transfection efficiency.

As shown in Figure 1B, C and D, constructs which contain a portion of the untranslated region either without (LTR3CAT −21) or with (LTR3CAT −46) the TATA sequence had very low basal and induced levels of CAT activity. LTR3CAT −70, which in addition contains the first two of the three SP1 recognition sequences in the viral LTR, gave approximately a 4- to 5-fold stimulation of CAT activity in the presence of the *tat* gene (Figure 1B and C). LTR3CAT −160, which contains the untranslated, TATA, SP1 and enhancer sequences, gave a 60-fold stimulation in response to the *tat* protein (Figure 1B and C). LTR3CAT −218, which contains a putative negative regulatory element in addition to the other elements, gave only a 7-fold induction in response to the *tat* protein (Figure 1B and C). This was due to a 2- to 3-fold increase in the basal level as compared to LTR3CAT −160 and a 3-fold decrease in *tat*-induction. Further upstream portions of the viral LTR had no significant effect on either basal or *tat*-induced CAT activity of the viral LTR (data not shown).

To study the untranslated region in more detail, a series of deletion mutants in this region were constructed. One deletion, LTR3CAT −160Δ13−23, created a 9-bp deletion between +13 and +23, thereby removing the palindrome TAGACCAGAT found in the untranslated region. This fragment was then joined to the CAT gene and tested by transfection into HeLa cells both in the presence and in the absence of the *tat* gene. The gene expression of this mutant was compared to the level of activity of the parental plasmid LTR3CAT −160. Transfection experiments with this mutant resulted in a marked decrease in *tat*-induced CAT activity (Figure 2A). S1 analysis from this transfection revealed extremely low levels of specific mRNA when this plasmid was transfected alone or in the presence of the *tat* gene (Figure 2B). In contrast, the LTR3CAT −160 construct showed a marked increase in the level of CAT activity with *tat* (Figure 2A) and a corresponding increase in the steady-state level of HIV LTR mRNA (Figure 2B). Nuclear run-off experiments will be required to determine whether the mutation in LTR3CAT −160Δ13−23 affects transcription initiation or RNA stability.

Several regions of the viral LTR appear to be critical for both basal and *tat*-induced gene expression of the viral LTR (Figure 1D). The level of CAT activity correlated with the level of HIV LTR steady-state mRNA (Figure 2A and B). Thus the gene expression determined by CAT assays reflects the transcriptional activation of the HIV LTR constructs.

DNase I footprint assays of HIV deletion mutants

Experiments with a number of different promoters including SV40 (Wildeman *et al.*, 1986; Zenke *et al.*, 1986) and c-*fos* (Gilman *et al.*, 1986) have shown that mutagenesis of factor binding sites identified *in vitro* can result in severe defects in the transcriptional activity of these promoters when tested *in vivo*. Thus it appears that the binding sites demonstrated *in vitro* by DNase I footprinting are likely to be important sites for factor binding required for *in vivo* transcriptional regulation. DNase I footprinting analysis (Galas and Schmitz, 1978) was performed on both the coding and non-coding strands of the HIV LTR deletion mutants using partially purified HeLa cell nuclear extracts (Dignam *et al.*, 1983). To standardize all cellular extracts used in this study, extracts were first tested with the SV40 promoter to ensure no variation in the level of total protein required for complete protection of the SP1 binding sites (Gidoni *et al.*, 1984, 1985; Kadonaga and Tjian, 1986). All extracts subsequently used in these DNase I footprinting experiments resulted in complete protection of five of six SP1 binding sites in the SV40 promoter between 50 and 100 μg of added extract (data not shown). The fragments that were used for footprinting were the same fragments that were cloned into the plasmid containing the CAT

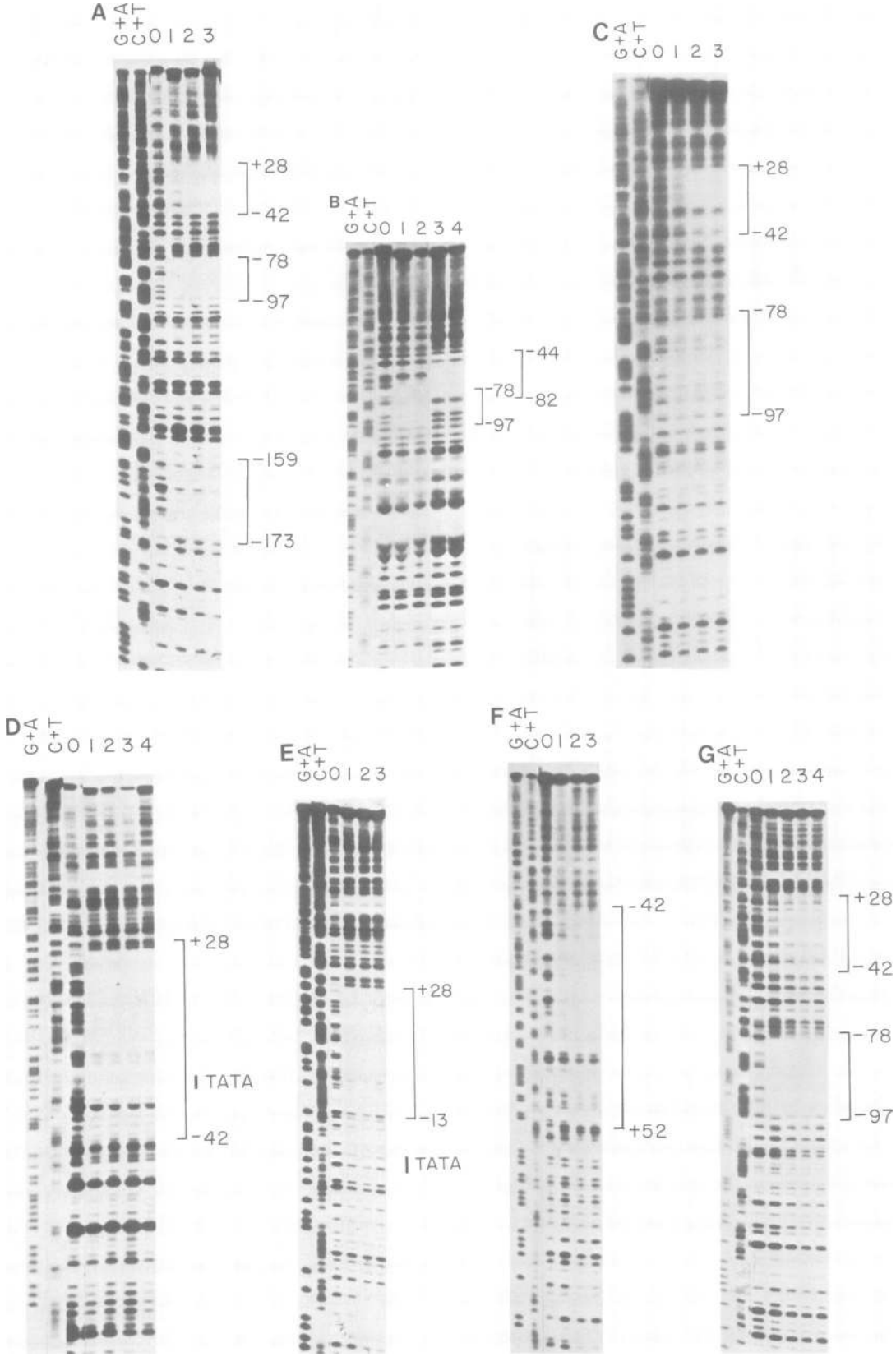


Fig. 3. DNase I protection assays using HIV LTR fragments. The order of HIV fragments used for DNase I footprinting assay is (**A**) −218 to +80 (LTR3 −218), (**B**) −218 to +80 (LTR3 −218) using partially purified HeLa cell extract 100 μg (**lane 1**) and 150 μg (**lane 2**) or oligonucleotide affinity column purified SP1 20 μl (**lane 3**) and 40 μl (**lane 4**), (**C**) −160 to +80 (LTR3 −160), (**D**) −70 to +80 (LTR3 −70), (**E**) −46 to +80 (LTR3 −46), (**F**) −160 to +80 (LTR3 −160) and (**G**) LTR3 −160Δ13−23. **A**−**E** show the coding strand; **F** shows the non-coding strand. G + A and C + T are Maxam−Gilbert sequencing lanes of each probe. **Lane 0** is the probe with DNase I and no added protein, and **lanes 1**−**4** contain increasing amounts of partially purified HeLa extract (**lane 1**, 50 μg; **lane 2**, 100 μg; **lane 3**, 150 μg; **lane 4** 200 μg).

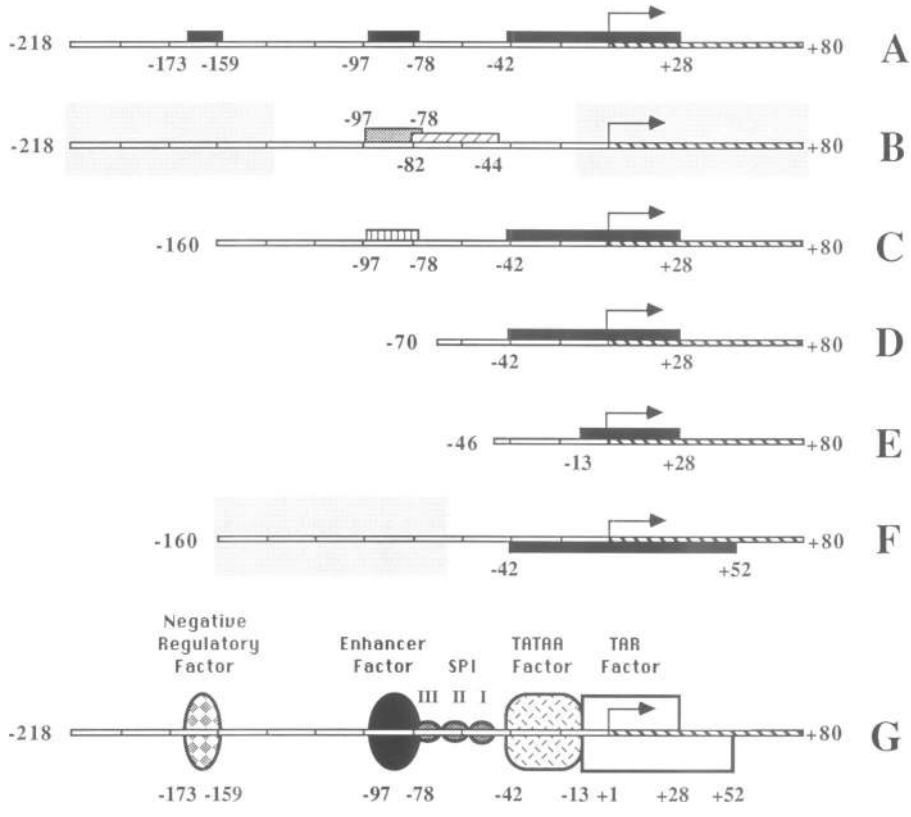


Fig. 4. Diagram of binding sites of HIV LTR. The protected regions (black boxes) in the HIV LTR corresponding to Figure 3A–F are shown. The numbers below the binding sites indicate the extent of protection relative to the start of transcription (arrow). The vertically lined box (**C**) indicates partial protection. Grey areas indicate areas of gel not resolvable (**F**) or not shown for better resolution of highlighted regions (**B**). Gray box (**B**) corresponds to extent of protection over the enhancer region with HeLa extracts (−97 through −78) while slanted line box (**B**) indicates extent of protection for partially purified SP1 (−82 through −44). **G** summarizes binding domains of HIV LTR seen with HeLa cell extracts.

gene for the transfection experiments. They include LTR3 −218, LTR3 −160, LTR3 −70, LTR3 −46 and LTR3 −21. The end point of all of these constructs is +80 in the untranslated region. DNase I footprinting experiments were performed with probes labeled to approximately the same specific activity and with the same HeLa extract preparation.

The coding strand of the LTR3 −218 fragment gave three protected regions (Figure 3A). The first region, which included a portion of the untranslated region, was between +28 and −42; the second region was between −78 and −97 in a domain containing both a portion of the third SP1 binding site (Jones *et al.*, 1986) and the repeated enhancer sequences (Nabel and Baltimore, 1987); and the third region was between −159 and −173. No other protected regions were found upstream of −218 (data not shown). The region between +28 and −42 will be referred to as the TATA-untranslated region, the region between −78 and −97 as the enhancer region, and the region between −159 and −173 as the negative regulatory region.

Previous data using highly purified preparations of SP1 indicated that three SP1 binding sites between −43 and −83 in the HIV LTR were protected in DNase I footprinting studies (Jones *et al.*, 1986). These data indicated that the third SP1 binding site had a higher affinity for purified SP1 than the other two sites. We could not determine from the DNase I footprint (Figure 3A) whether the entire region from −78 to −97 was protected by enhancer binding factor alone or together with SP1. However, competition studies using oligonucleotides to a high-affinity SP1 binding domain suggested that the third SP1 binding site was protected by SP1 (see below).

To address this point further, partially purified HeLa cell extract was passed twice over an SP1 oligonucleotide affinity column as described by Kadonaga and Tjian (1986). This partially purified SP1, in the absence of other HIV LTR binding proteins, resulted in protection over the three SP1 binding sites from −44 to −82 (Figure 3B, lanes 3 and 4). The third SP1 binding site overlaps the enhancer binding domain (Figure 3B, lanes 1 and 2). When the same amount of oligonucleotide affinity column purified SP1 from HeLa cells which gave protection over the HIV LTR binding sites was added to the partially purified HeLa cell extracts, there was still no protection over the first two SP1 binding sites (data not shown). This result suggested that other proteins present in these HeLa cell extracts may inhibit SP1 binding to the two low-affinity SP1 binding sites or that SP1 may bind transiently to these sites and possibly interact with other HIV LTR binding proteins. A role for these two lower affinity SP1 sites in the transcriptional regulation of the HIV LTR was seen in the higher level of CAT activity of LTR3CAT −70 (containing SP1 binding sites I and II) as compared to LTR3CAT −46 (lacking all three SP1 binding sites).

The LTR3 −160 fragment which gave maximal *tat*-induced transactivation (Figure 1B and C) contains the TATA-untranslated, SP1 and enhancer regions. On the coding strand this fragment gave complete protection over only one region at the protein concentrations tested. There was protection over the TATA-untranslated region, but minimal protection was seen over the enhancer or SP1 regions (Figure 3C). However, at protein concentrations >200 μg per reaction, partial protection was seen over the enhancer region (data not shown). There was a marked

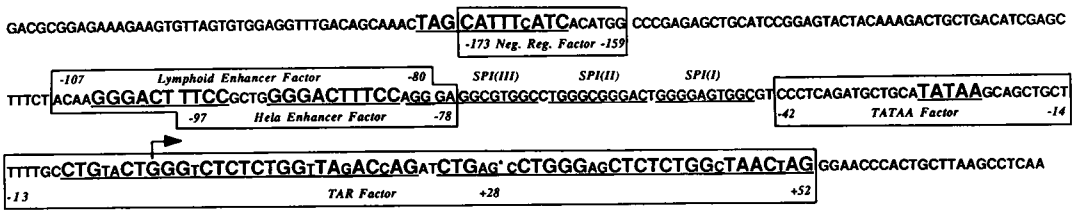


Fig. 5. Nucleotide sequence of HIV LTR with DNase I protected regions. Boxed areas indicate extent of protection for regions shown. Protection for the coding strand of the TAR factor extends to +28 (*) while non-coding strand protection ends at +52. Underlined sequence in the negative regulatory region indicates homology to sequences found in the arabinose operon with large letters indicating exact match (Ogden *et al.*, 1980). The underlined sequence in lymphoid/HeLa enhancer region is the sequence found in other viral and cellular enhancer sequences. The underlined sequences in the TAR factor region indicate homologous regions in tandem direct repeat orientation. The palindrome (TAGACCAGAT) separating the core sequence of these regions (CTCTCTGG) is not indicated.

difference in the amount of protection over the enhancer region at equivalent protein concentrations in the presence of the negative regulatory element (Figure 3A, lanes 2 and 3) as compared to its absence (Figure 3C, lanes 2 and 3). Thus, deletion of the negative regulatory region decreases the amount of protection over the enhancer region.

The LTR3 −70 fragment which contains the TATA-untranslated and first two SP1 binding sites gave protection on the coding strand over only the TATA-untranslated region (Figure 3D). This construct had a much lower level of transactivation than the LTR3 −160 fragment (Figure 1B and C).

The LTR3 −46 fragment which contains the TATA-untranslated region gave only one major protected region on the coding strand, but it extended from +28 to −13 (Figure 3E) in contrast to the result with the LTR3 −70 fragment where the region of protection extended from +28 to −42 (Figure 3D). It appears that the protection observed between −14 and −42 is diminished in the absence of the flanking sequences between −46 and −70. The results of this deletion mutant suggest the existence of at least two protein binding sites between −42 and +28, one of which binds over the TATA sequence, in addition to a further downstream factor which extends into the untranslated region. The level of transactivation of LTR3CAT −46 was significantly lower than seen for LTR3CAT −70 (Figure 1B and C). The LTR3 −21 fragment on the coding strand also gave protection from +28 to −13 on the coding strand (data not shown).

These HIV LTR fragments were also footprinted on the non-coding strand. There were minimal differences in the protection patterns seen between the coding and non-coding strands in the negative regulatory and enhancer regions. However, the non-coding strand gave extended downstream protection over the untranslated region with all fragments tested. The non-coding strand protection pattern using the LTR3 −160 fragment is shown in Figure 3F. A protected region from −42 to +52 is seen. Thus we conclude that at least two, but perhaps more, cellular proteins bind between −42 and +52 with binding seen only on the non-coding strand between +28 and +52.

Finally, to investigate the binding over the untranslated region in more detail, we performed DNase I footprinting on the LTR3 −160Δ13−23 fragment which contains a 10-bp deletion in the untranslated region. DNase I footprinting studies on the LTR3 −160Δ13−23 fragment, which contains all of the regulatory regions except the negative regulatory region, demonstrated a moderate decrease in the amount of protection on the coding strand from +28 to −42 (Figure 3G) and on the non-coding strand from +52 to −42 (data not shown) compared to the parental plasmid. This suggested that the palindrome was not the major recognition sequence for proteins binding to the untranslated region. Surprisingly, with this mutant we noted the appearance of complete protection over the region from −78 to −97 which contains the enhancer binding domain (Figure 3G). This differed markedly from the result seen with the parental plasmid LTR3 −160 (Figure 3C). Thus, mutations in the untranslated region have marked effects on both transactivation of the LTR (Figure 2A and B) and DNase I protection patterns over the enhancer region (Figure 3G).

Deletions of several upstream binding domains appear to alter the binding to domains located downstream (Figure 3A−G). An illustration of the fragments used in DNase I footprinting is shown in Figure 4A−G. The deletion of the negative regulatory element results in decreased protection over the enhancer region and deletion of the SP1 binding sites results in decreased protection over the TATA sequence. Furthermore, deletions in the untranslated region appear to affect binding over the enhancer region. The deletion of these binding sites also has marked effects on the ability to transactivate the HIV LTR (Figure 1B and C). These results suggest that factor interactions are important both for *in vitro* and *in vivo* transcriptional regulation.

Sequences in the HIV LTR binding domain

Figure 5 illustrates the DNase I protected sequences in the HIV LTR. The sequences protected in the negative regulatory region have strong homology to sequences found in the binding site of the araC protein in the arabinose operon (Hendrickson and Schlief, 1985). This procaryotic protein is also capable of functioning in repression of gene expression (Dunn *et al.*, 1984). Purification of the HIV negative regulatory protein from HeLa cell extracts and immunoprecipitation with antibody to the araC protein are underway to determine if the DNA binding domains of these proteins may be conserved.

The enhancer region (Nabel and Baltimore, 1987) contains two direct repeats (GGGACTTTCC) which are also found in other viral and cellular enhancer sequences including SV40 (Herr and Gluzman, 1985; Davidson *et al.*, 1986; Wildeman *et al.*, 1986; Zenke *et al.*, 1986), the immunoglobulin kappa gene (Picard and Schaffner, 1984; Sen and Baltimore, 1986) and cytomegalovirus (Boshart *et al.*, 1985). Differences in binding to the enhancer domain found with HeLa cell extracts compared to lymphoid extracts is also indicated (F.Wu, J.Garcia, R.Mitsuyasu and R.Gaynor, submitted).

The presence of direct repeated sequences (CAGNTGCT) are found flanking the TATA sequence and also in the untranslated region (CTCTCTGG) where they are separated by a palindrome TAGACCAGAT. Thus, the binding domains of several regions of the HIV LTR contain 8−10 nucleotide direct repeats. Muta-

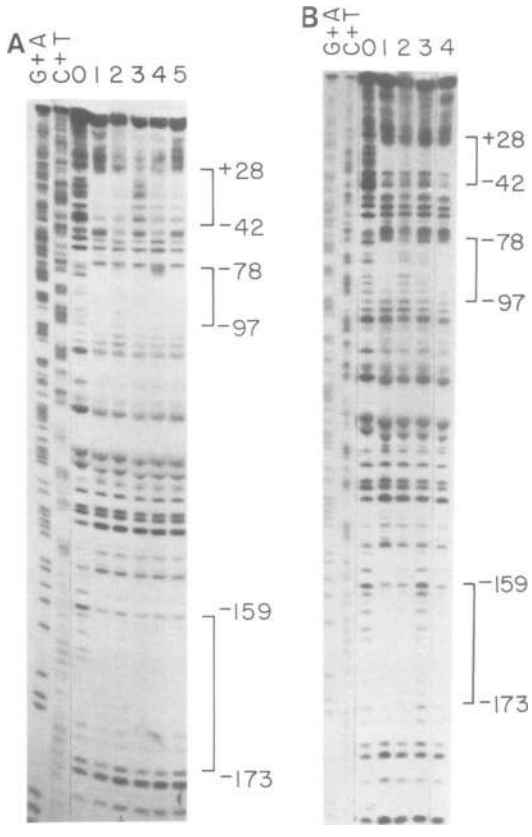


Fig. 6. Oligonucleotide competition of the HIV LTR binding domains. **(A)** The LTR3 −218 fragment was incubated with 100 μg of partially purified HeLa cell extracts following a 20-min preincubation in the absence of added oligonucleotides (**lane 1**) or with ligated oligonucleotides containing sequences complementary to either the enhancer region (**lane 2**), the untranslated region (**lane 3**), a high-affinity SP1 binding site (**lane 4**) or a portion of the SV40 enhancer AP1 binding site (**lane 5**). **Lane 0** is the probe without added protein and G + A and C + T are Maxam−Gilbert sequencing lanes. **(B)** the LTR3 −218 fragment was incubated with 100 μg of partially purified HeLa cell extract following a 20-min preincubation with either no added oligonucleotide (**lane 1**) or ligated oligonucleotides containing sequences complementary to the enhancer (**lane 2**), the negative regulatory region (**lane 3**) or to a portion of the AP1 binding site of the SV40 enhancer (**lane 4**). **Lane 0** is no added protein and G + A and C + T are Maxam−Gilbert sequencing lanes for the probe.

genesis of each of these repeated sequences will be required to definitively show their role in DNA binding.

Oligonucleotide competition of the HIV LTR binding domains

To demonstrate the specificity of binding using these extracts, specific oligonucleotides to several of these binding domains were synthesized for use in competition experiments. Double-stranded oligonucleotides complementary to the negative regulatory, enhancer, untranslated and to SV40 high-affinity SP1 binding site (Kadonaga and Tjian, 1986) were synthesized, annealed and ligated. These oligonucleotides were then used in preincubation experiments with partially purified HeLa cell extracts prior to DNase I footprinting of the LTR3 −218 fragment which contains all five HIV LTR binding domains (Figure 6A and B).

As shown in Figure 6A, preincubation with either the enhancer region oligonucleotides (Figure 6A, lane 2) or the untranslated region oligonucleotides (Figure 6A, lane 3) resulted in the loss of DNase I protection of these respective domains. Preincubation with the high-affinity SP1 oligonucleotides resulted in a loss of protection over the region of the third SP1 binding domain such that protection over the region containing the enhancer sequences extended from −83 to −97 rather than from −78 to −97. The difference in protection pattern seen with the SP1 oligonucleotide competition experiment (Figure 6A, lane 4) was the appearance of four bands of equal intensity between −78 and −83. With extract alone, the band located at −78 was the most intense (Figure 6A, lane 1). A longer exposure of this lane showed the appearance of the three other bands with decreased intensity relative to the band at −78 (data not shown). Thus, it is likely that a portion of the protection over the region from −78 to −97 is due to protection by SP1 of the third SP1 binding site. There was no change in the protection patterns over the first two SP1 binding sites. An oligonucleotide made to a portion of the AP1 binding site of the SV40 enhancer (Lee *et al.*, 1987) did not compete for binding with the HIV LTR binding domains (Figure 6A, lane 5).

Oligonucleotides complementary to the negative regulatory region competed for binding to the negative regulatory region (−159 to −173) and appeared to decrease binding to the enhancer region (−78 to −97) using the LTR3 −218 fragment (Figure 6B, lane 3) while oligonucleotides complementary to the enhancer region only competed for binding to the enhancer region (Figure 6B, lane 2). Oligonucleotides complementary to a portion of the AP1 binding site of the SV40 enhancer (Figure 6B, lane 4) did not compete for binding to either the negative regulatory or enhancer regions. These results show the specificity of binding to the HIV LTR regulatory regions and suggest that the proteins binding to the negative regulatory region likely interact with the proteins binding to the enhancer region.

Discussion

At least five regions of the HIV LTR sites serve as binding sites for cellular proteins. DNase I footprinting of a number of HIV LTR deletion mutants show that deletion of certain binding regions results in alterations in binding to other regions. The most likely explanation for these results would be that cellular proteins bound to the HIV LTR interact to stabilize factor binding. The deletion of binding sites for the untranslated TATA, SP1, enhancer and negative regulatory regions have marked effects on both *in vitro* binding and on gene expression assayed *in vivo*. Thus these binding regions demonstrated *in vitro* are likely to correlate with regions required for factor binding *in vivo*.

The untranslated region has been shown to serve as a binding domain for the B factor in the *Drosophila* histone and actin genes (Parker and Topol, 1984). In addition, untranslated regions have been shown to be important for transcriptional regulation of other cellular and viral genes (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Mansour *et al.*, 1986) and for translational control of the heat shock gene (Hultmark *et al.*, 1986). We have demonstrated binding of factors to the untranslated region of the HIV LTR. The protection seen over the untranslated region is more extensive on the non-coding strand (to +52) than on the coding strand (to +28). In a previous study, sequences downstream of +21 were found not be important for *in vitro* transcription of the LTR (Jones *et al.*, 1986). The importance of sequences downstream from +21, however, is supported by data *in vivo* where deletions to +38 severely impair basal and induced levels of expression from the HIV LTR (Wright *et al.*, 1986). Strong interactions with only one of two DNA strands at a factor binding site have been described for other binding proteins, including TFIIIA with the 5S RNA gene of *Xenopus* (Sakonju

and Brown, 1982; Smith *et al.*, 1985) and PEB-1 with the polyoma enhancer (Piette and Yaniv, 1986). It remains to be determined whether any viral proteins such as *tat* may also bind to this region as has been speculated.

SP1 has been implicated as an important promoter element for the HIV LTR (Jones *et al.*, 1986). Mutagenesis of the third SP1 site had minimal effects upon *in vitro* transcription assays, whereas mutagenesis of either the first or second SP1 site resulted in severe reductions in *in vitro* transcription (Jones *et al.*, 1986). Site-specific mutagenesis of each site and *in vivo* transcriptional analysis are underway to determine the role of each site in transcriptional regulation and factor binding. The third SP1 binding site is likely protected by SP1 in our extracts while the two lower affinity SP1 binding sites are not protected in the presence of other HIV LTR binding proteins (Figure 3A and B). The lack of protection over these sites could result from the inhibition of SP1 binding or the transient interaction of SP1 in the presence of other HIV LTR binding proteins. It appears that the two low-affinity SP1 sites are important both *in vivo* and *in vitro* since deletion of this region results in decreased gene expression and appears to affect the binding to the TATA region (Figures 1 and 3).

Studies using insertion mutants in the SV40 promoter which alter the spacing between the TATA element and the SP1 binding sites have shown that both the distance between these elements and the position on the DNA helix were critical for *in vivo* transcription (Takahashi *et al.*, 1986). These results suggested that protein–protein interactions between factors binding to the SP1 and the TATA elements occurred. Competition studies using oligonucleotides to the high-affinity SP1 binding domain competed for binding to the third SP1 binding site (Figure 6A), but it was difficult to discern an effect on the region between −14 and −42 which contains the TATA sequence due to the position of this region near the top of the gel (Figure 6A). However, when using these same oligonucleotides in competition experiments with the LTR3 −70 fragment there was competition for binding over the region from −14 to −42 (unpublished observations). This result, combined with deletion analysis (Figure 3D), suggests a role for these SP1 binding sites in stabilizing factor binding to the region containing the TATA sequence.

The binding and regulation of cellular enhancer factors has been studied for a number of enhancer elements including both SV40 and immunoglobulin enhancers (Picard and Schaffner, 1984; Sassone-Corsi *et al.*, 1984, 1985; Scholer and Gruss, 1984, 1985; Mercola *et al.*, 1985; Davidson *et al.*, 1986; Zenke *et al.*, 1986; Wildeman *et al.*, 1984, 1986). SV40 viruses with mutated enhancer elements regain their ability to grow by *in vivo* duplication of the sequence GGGACTTTCC (Herr and Gluzmann, 1985). This sequence contains a core enhancer domain which is found in several viral enhancers (Weiher *et al.*, 1983). Deletions removing this sequence (between nucleotides 235 and 246) in the SV40 72-bp repeats result in a marked decrease in transcription in transfection experiments (Wildeman *et al.*, 1986; Zenke *et al.*, 1986). B cell extracts, as compared to HeLa cell extracts, give a larger region of DNase I protection over this sequence in the SV40 72-bp repeats (Davidson *et al.*, 1986). In the kappa enhancer, this sequence has shown binding in gel retardation assays using extracts prepared from B cells, but not other cells (Sen and Baltimore, 1986).

HIV contains two direct repeats of the sequence GGGACTTTCC (Figure 5) which are in an important transcriptional regulatory region of the LTR (Figure 1). HeLa cell extracts give complete protection over only one direct repeat in the presence of the negative regulatory element. Lymphoid extracts show complete protection over both direct repeats (F.Wu, J.Garcia, R.Mitsuyasu and R.Gaynor, submitted). The ability to detect binding to this sequence in the HIV LTR using HeLa cell extracts contrasts with previous results using the kappa enhancer (Sen and Baltimore, 1986). However, binding to this sequence in the kappa enhancer is induced in HeLa cells by treatment with phorbol esters (Sen and Baltimore, 1986). In the HIV LTR, binding to this region was shown by gel retardation assays using extracts prepared from T cells treated with phorbol esters but not with untreated T cell extracts (Nabel and Baltimore, 1987). It appears that extracts from a number of different cell lines bind to this sequence although the pattern of binding may depend on both the type and state of the cells.

The presence of negative regulatory elements has been seen in viral and cellular genes including the beta interferon gene (Goodbourn *et al.*, 1986) the c-*myc* gene, (Remmers *et al.*, 1986) and the adenovirus early region 2 promoter (Jalinot and Kedinger, 1986). The negative regulatory element in HIV may stabilize binding of the HeLa cell enhancer factor to its DNA recognition site and inhibit subsequent interaction of the enhancer with downstream binding proteins. This may prove to be one mechanism by which negative regulatory elements modulate transcription. The existence of this negative regulatory element supports earlier observations that a deletion to −167 in the HIV LTR, which falls within this binding domain, increases *tat*-induced transcription (Rosen *et al.*, 1985). However, we have not been able to demonstrate binding of factors in the LTR between −185 and −340 which was previously reported to have negative regulatory properties for heterologous promoters (Rosen *et al.*, 1985).

In addition to cellular DNA binding proteins, the viral *tat* protein is also important for transcriptional regulation of the HIV LTR. Our data and that of several recent studies (Cullen, 1986; Gendelman *et al.*, 1986; Peterlin *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.*, 1987) would suggest that one function of *tat* is to increase steady-state levels of mRNA from the HIV LTR. Whether *tat* directly or indirectly alters cellular transcription factor interactions and could be responsible for the increased *in vitro* transcription of the HIV LTR in HIV infected extracts (Okamoto and Wong-Staal, 1986) is not known.

The data presented here would support a model of interaction of cellular proteins involving both upstream and downstream regions of the viral promoter. Interactions of upstream and downstream cellular proteins have been described for the upstream factor (USF) and the II-D factor in the adenovirus major late promoter (Sawadogo and Roeder, 1985). A complete understanding of the transcriptional regulation of HIV will require the purification of each cellular binding protein, the generation of specific antibodies and the development of a reconstituted transcription system using purified components. The development of such systems should prove to be extremely important in understanding the role of cellular proteins in transcriptional regulation and provide important insights in our understanding of the biology of this deadly virus.

Materials and methods

DNA constructions

To make the five HIV LTR deletion mutants, the LTR was first cut in the upstream region with either *Pvu*II (−21), *Sfa*NI (−46), *Hae*III (−70), *Ava*I (−160) or *Tha*I (−218); the numbers in parentheses indicate the position from the cap site (Sanchez-Pescador, 1984). The fragment was then cut with *Hin*dIII (+80) and cloned into *Hinc*II/*Hin*dIII pUC19 (for DNase I footprinting). *Sma*I/*Hin*dIII fragments from these constructs were cloned into RSV CAT (Gorman *et al.*, 1983) from which the RSV sequence was removed by cutting with *Nru*I and *Hin*dIII

(for CAT assays). LTR3 −160Δ13−23 was constructed by cutting the pUC19 clone containing the −160 to +80 portion of the HIV LTR with *Bgl*II, treating with an excess of S1 nuclease, and closing with T4 DNA ligase (for DNase I footprinting). A *Sma*I/*Hin*dIII fragment from this construct was cloned into RSV CAT from which the RSV sequence was removed. All clones were sequenced.

To make the expression vector for *tat*, a *Hin*cII−*Ssp*I fragment (nucleotides 5792−6087 of the ARV-2 genome) was inserted into the *Hin*cII site of pUC19. A partial *Hin*dIII−*Bam*HI fragment from this clone was inserted into the *Hin*dIII−*Bgl*II sites of the vector RSV beta globin (Gorman *et al.*, 1982), thus replacing the globin coding sequence with the coding sequence for *tat*.

Cell lines and tissue culture conditions

HeLa cells were maintained in suspension culture in MEM with 5% newborn calf serum. HeLa cell monolayers were maintained in DMEM with 5% newborn calf serum. For transfections onto HeLa cells, the cells were split 1:2 on the day prior to the transfection and were ~70% confluent at the time of transfection.

Transfections and CAT assays

For each transfection, 5 μg of each HIV LTR3CAT construct and 5 μg of either RSV-beta globin or RSV-*tat* were used. Transfections onto 70% confluent HeLa cells were performed by the calcium phosphate method and harvested 48 h later. CAT assays were performed as described by Gorman *et al.* (1982) and each reaction was harvested at 10 min and 50 min to ensure linear conversion of the chloramphenicol with each extract. The conversion of chloramphenicol in each assay was determined by scintillation counting of both unacetylated and acetylated chloramphenicol.

S1 analysis of HIV LTR transfections

Five plates of HeLa cells were transfected with HIV LTR constructs by the calcium phosphate procedure. The plates were harvested at 48 h post-transfection, cytoplasmic RNA was prepared as described, and 50 μg was used for S1 analysis (Gaynor *et al.*, 1984). The probe was end-labeled at the *Hin*dIII site with γ-^{32}P, hybridized to 100 μg of cytoplasmic RNA at 56°C, digested with S1 nuclease, run on an 8 M urea 8% polyacryalmide gel and subjected to autoradiography.

Preparation of cellular extracts

For all extracts, a minimum of 7 ml packed cell volume was used. Nuclear extracts were prepared as described by Dignam *et al.* (1983). Extracts were dialyzed versus a buffer containing 20 mM Tris (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% glycerol. This extract was then loaded onto a heparin agarose column, washed with five column volumes of this same buffer, eluted with this buffer containing 0.5 M KCl, dialyzed and used in DNase I footprinting assays (Galas and Schmitz, 1978).

Oligonucleotide affinity column purification of the SP1

For partial purification of the SP1 protein, 10 mg of HeLa cell extract from the 0.5 M KCl eluate of a heparin agarose column was dialyzed and then applied to a 1-ml column of Sepharose CL-2B coupled with double-stranded oligonucleotides containing a high-affinity binding site of SP1 (Kadonaga and Tjian, 1986). The column was washed extensively with buffer containing 100 mM KCl and the SP1 eluted in buffer containing 1.0 M KCl. This material was dialyzed, reapplied to the column and the material from the second elution of the oligonucleotide affinity column used in DNase I footprinting of LTR3 −218 as described.

DNase I footprinting procedure

The HIV LTR fragments were also treated with T4 polymerase, cloned into the *Sma*I site of pUC19, and screened for correct orientation. Each clone was digested with *Eco*RI, treated with alkaline phosphatase, and end-labeled with γ-^{32}P to generate labeled fragments used to footprint the coding strand. To footprint the non-coding strand, the *Hin*dIII site was end-labeled. Fragments were gel-isolated (*Eco*RI−*Hae*II for the coding strand, or *Hin*dIII−*Pvu*I for the non-coding strand), electroeluted, and used for DNase I footprinting assays. One to five nanograms of end-labeled probe was added to each 50 μl reaction along with extract (0−200 μg), poly(dI)·(dC) (3 μg), and final concentrations of 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM DTT, 5% glycerol. The DNA and extract were allowed to bind for 20 min at room temperature and then the reaction volume was increased to 100 μl and final concentrations of DNase I (0.4−2.0 μg/ml), 5 mM MgCl, 2.5 mM $CaCl_2$ were added. The reaction was stopped after 30 s with phenol−chloroform, then ethanol-precipitated and loaded on a 10% polyacrylamide 8 M urea sequencing gel. G + A and C + T Maxam−Gilbert sequencing reactions were performed for each probe. All gels were then subject to autoradiography.

Oligonucleotide competition studies

Double-stranded oligonucleotides complementary to the binding domains of either the HIV LTR negative regulatory (5′-TAGCATTTCATCACA-3′), enhancer (5′-GGGACTTTCCC-3′) and untranslated (5′-CTGTACTGGGTCTCTCTGG-3′) regions were synthesized. In addition, oligonucleotides complementary to a high-affinity SP1 consensus sequence (5′-GGGGCGGGGC-3′) (Kadonaga and Tjian, 1986) or a portion of the AP1 binding site of the SV40 enhancer (5′-GGAGATGACTCTT-3′) (Lee *et al.*, 1987) were also synthesized. These oligonucleotides were annealed and ligated as described previously (Kadonaga and Tjian, 1986). Between 10 and 100 ng of ligated oligonucleotides (100- to 200-fold molar excess) were preincubated for 15 min with 100 μg of the partially purified HeLa cell extract prior to the addition of 5 ng of end-labeled LTR3 −218 fragment. After the addition of the LTR3 −218 fragment, the probe was incubated with the HeLa cell extract for 20 min prior to DNase I footprinting as described.

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