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Tax1 induction of the HTLV-I 21 bp enhancer requires cooperation between two cellular DNA-binding proteins

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Activation of the HTLV-I promoter by the viral Tax1 transactivator is mediated by a 21 bp sequence motif imperfectly repeated three times and composed of three exactly conserved domains (A, B and C from 5′ to 3′). We show here that the Tax1 response requires the integrity of the B domain and of at least one of the flanking A or C domains. We have identified three cellular proteins which bind specifically to the 21 bp motif. One of these is the already well-characterized transcription factor ATF. The other two, namely HEB1 and HEB2, are specific for the 21 bp motif. HEB1 can bind to either domain A or C, but binding of ATF and HEB2 is determined by domain B. However, neither domain B alone, nor ATF/CREB binding sites respond significantly to Tax1. We therefore propose that Tax1 induction of the 21 bp enhancer element requires interaction with the two different cellular proteins identified in this study: HEB1 and HEB2, rather than binding of the ATF factor.

Key words: ATF/DNA−protein interaction/HTLV-I/Tax1 induction

Introduction

Human T-cell leukemia virus type 1 (HTLV-I) was the first retrovirus characterized in humans (Poiesz *et al.*, 1980). It is associated with several hematological or neurological pathologies. Adult T-cell leukemia (ATL; for a review, see Wong-Staal and Gallo, 1985) and Tropical spastic paraparesis (TSP; Jacobson *et al.*, 1988) are the best characterized. Replication of the virus depends on the viral proteins Rex1 and Tax1, which regulate its genetic expression (Inoue *et al.*, 1987). These proteins are encoded by a doubly spliced 2 kb mRNA. Rex1 is a 27 kd nuclear protein which allows efficient translation of the unspliced or partially spliced intermediate viral mRNAs (Hidaka *et al.*, 1988). Tax1 is able to strongly induce the activity of the viral promoter (Felber *et al.*, 1985; Fujisawa *et al.*, 1985; Sodroski *et al.*, 1985). Tax1 can also increase the expression of different cellular genes including the interleukin 2 gene (IL2), the gene coding for the IL2 α chain receptor (Inoue *et al.*, 1986; Wano *et al.*, 1988) and the c-*fos* proto-oncogene (Fujii *et al.*, 1988; Nagata *et al.*, 1989). Activation of the two former genes could lead to an autocrine stimulation of the infected T-cells, this being the first step in the development of an ATL (Maruyama *et al.*, 1987; Yoshida and Seiki, 1987). However, it has been established in transgenic mice that the Tax1 protein exhibits oncogenic properties by itself (Nerenberg *et al.*, 1987). Tax1-activation of various cellular genes takes place at the transcriptional level and is achieved through different sequence elements (Fujii *et al.*, 1988; Ruben *et al.*, 1988). This viral protein therefore offers an interesting model for studying the coordinate regulation of specific genes. In order to understand how Tax1 exerts its activity on transcription we focused our attention on the induction of the HTLV-I viral promoter itself.

It has been shown by several groups that Tax1 activates this promoter mainly by inducing the enhancer activity of a 21 bp motif imperfectly repeated three times at positions −251, −203 and −103 with respect to the transcription start site (Fujisawa *et al.*, 1986; Shimotohno *et al.*, 1986; Brady *et al.*, 1987; Rosen *et al.*, 1987). However, the molecular mechanisms by which the Tax1 protein induces the activity of this motif remain poorly understood. No differences in DNA−proteins interactions on the viral promoter have so far been observed when comparing extracts from cells which produce the Tax1 protein with those from cells which do not (Altman *et al.*, 1988; Nyborg *et al.*, 1988). Moreover, it has been reported that the Tax1 protein was unable to bind specifically to DNA (Jeang *et al.*, 1988; Beimling and Moelling, 1989). This therefore suggests that Tax1 induction is mediated by pre-existing cellular factor(s) interacting with the 21 bp motif.

In order to identify these factors we undertook a detailed mutational analysis of the 21 bp motif. The effect of the mutations has been measured by the induced-enhancer effect both on an heterologous reporter gene and on the homologous promoter activity. We also looked for cellular factors which are able to interact with this 21 bp motif. Comparison of the Tax1-induced activity of the different mutants with their ability to bind proteins identified in this study has led to the identification of the cellular factors which are likely to mediate Tax1 induction.

Results

Sequence requirements for Tax1-induced enhancer activity

As already reported (Park *et al.*, 1988; Fujisawa *et al.*, 1989) sequence comparison of the three 21 bp repeats (I, II and III) showed that three domains are precisely conserved (Figure 1A). They will be referred to as A, B and C, respectively (Figure 1A; Fujisawa *et al.*, 1989). In order to evaluate the contribution of each of these three domains to the Tax1-induced enhancer activity of the 21 bp motif, we cloned double-stranded oligonucleotides corresponding to the most proximal (III), either wild-type or mutated,

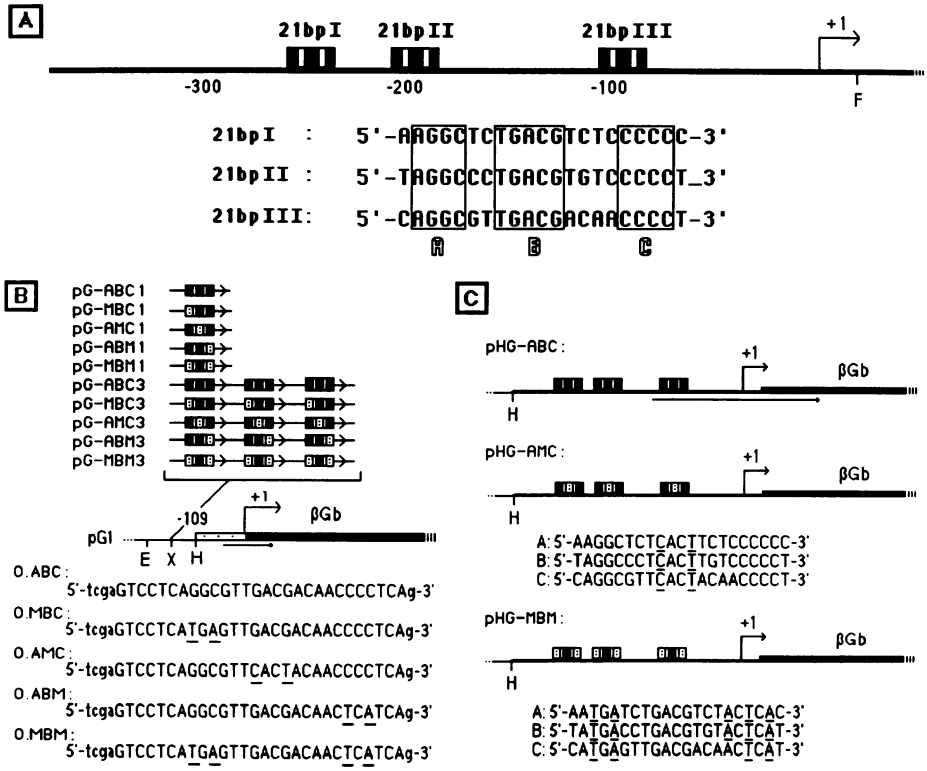

Fig. 1. (A) Schematic representation of the HTLV-I promoter. The position of the three 21 bp motifs (I, III, III) is indicated by the black and white boxes. The arrow marked +1 corresponds to the viral transcription start site. The position of the cut site for the *Fnu*dII (F) restriction enzyme is indicated. The sequence of each 21 bp motif is displayed below. The three perfectly conserved domains A, B and C are boxed. (B) Structure of the pG-ABC1 and pG-ABC3 plasmids. In this nomenclature ABC corresponds to wild-type sequence and M stands for a mutated domain. The numbers 1 or 3 indicate the number of cloned 21 bp copies. These constructs were generated as follows: each double-stranded synthetic oligonucleotide (the exact sequence is given by the top strand), bearing the protruding end of a *Xho*I site at its 5′ end and that of a *Sal*I site at its 3′ end, was ligated on itself, recut by *Xho*I and *Sal*I to give orientated multimers and cloned in the *Xho*I (X) site of plasmid pG1 (Wasylyk *et al.*, 1987). The pG1 plasmid contains the *Eco*RI(E)−*Hin*dIII(H) polylinker of M13mp12 and the rabbit β-globin gene sequence from positions −109 to +1664. Clones bearing insertion of monomers and trimers, orientated in the same way as in the viral promoter, were selected. Each clone was verified by sequencing. The different domains of the 21 bp motif are indicated by a black box (wild-type) or by a stippled box (mutated). The probe used for the S1 nuclease mapping assay is indicated by a line under the representation of the plasmids. This probe was an oligonucleotide corresponding to the β-globin sequence (non-coding strand) from position −20 to +40. (C) Structure of the pHG-ABC plasmids. These constructs were generated by cloning the *Hin*dIII−*Fnu*DII fragment of the pLTR-CAT plasmid (Fujisawa *et al.*, 1985) between the *Hin*dIII−*Pvu*III (position −9 with respect to globin +1) restriction sites of pG1. Site-directed mutagenesis of each 21 bp motif was performed as described (Kunkel, 1985) on a M13mp18 derivative bearing the entire HTLV-I promoter region, each mutant being reintroduced afterwards into the pHG constructs. The exact sequence of the different mutated 21 bp motifs is given on the viral + strand. The point mutations introduced in each 21 bp motif were identical to that used for analysing the enhancer activity on a heterologous gene except that the cytosine residue which flanks the 5′ side of the C domain in the I and II 21 bp motif was changed for an adenine to ensure a complete disruption of the stretch of cytosine which is characteristic of domain C. The different domains of the 21 bp motifs are indicated as described above.

upstream of the rabbit β-globin gene in plasmid pG1 (Figure 1B). To disrupt the natural sequence, a double point mutation was introduced in each of the three domains. The wild-type sequence was called ABC and when a domain was mutated the corresponding letter was replaced with M. It had been previously reported that the 21 bp motif was significantly active only when introduced in multiple copies (Shimotohno *et al.*, 1986; Brady *et al.*, 1987). We therefore cloned one (pG-ABC1 series) and three (pG-ABC3 series) copies of the different oligonucleotides, in the same orientation as in the viral promoter (Figure 1B). These constructs were cotransfected in HeLa cells with the plasmid pBS, which expresses the Tax1 and p21x proteins, or with the control plasmid pBx expressing only p21x. The activity of the β-globin promoter was determined by quantitative S1 nuclease mapping. Under these experimental conditions one copy of the 21 bp motif exhibited a strong Tax1-induced enhancer activity (Figure 2B, lane 2). Introduction of two additional copies of the 21 bp motif moderately increased the enhancer effect (Figure 2B, compare lanes 2 and 7). This enhancer activity was completely dependent on the presence of the Tax1 protein since plasmids pG-ABC1 and pG-ABC3 cotransfected with pBX exhibited an activity comparable to that of pG1 (Figure 2A, compare lanes 2 and 7 with lane 1).

Mutation of domain B in plasmids pG-AMC1 and pG-AMC3 completely abolished the Tax1-induced enhancer activity (Figure 2B, compare lanes 4 and 9 with lane 1). Mutation of domain A or C reduced the Tax1 activation by a factor of 10 on the monomer-containing constructs (pG-MBC1 and pG-ABM 1; Figure 2B, lanes 3 and 5) and three on the trimer-containing constructs (pG-MBC3 and pG-ABM 3; Figure 2B, lanes 8 and 10). However, when domains A and C were both mutated together (pG-MBM1 and pG-MBM3) this also led to a complete loss of the Tax1-induced enhancer activity (Figure 2B, compare lanes 6 and 11 with lane 1). These data indicate that all three domains of the 21 bp motif contribute to its induction by Tax1. Domain B is absolutely required. Mutation of domain

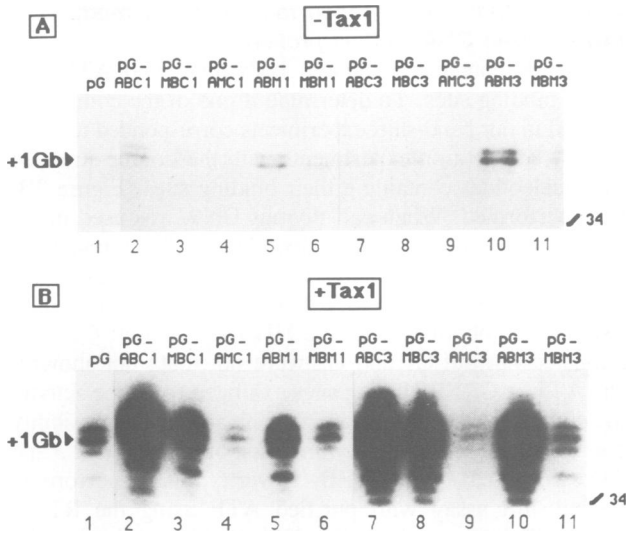

Fig. 2. Determination of the Tax1-induced enhancer activity of the mutated forms of the 21 bp motif. The different pG-ABC1 and pG-ABC3 plasmids were transfected in HeLa cells together with the pBX control plasmid (**A**) or the Tax1-expressing pBS plasmid (**B**). Plasmids pBS (Lilienbaum *et al.*, 1989) and pBX were generated by deleting *Bam*HI (position 5095)−*Sph*I (position 5126) and *Bam*HI (position 5095)−*Xho*I (position 6497) restriction fragments in the pMTPX expression vector (Inoue *et al.*, 1986). As a control plasmid pG1 was transfected in the same conditions (pG, A and B, **lanes 1**). The amount of specifically initiated β-globin RNA was determined by S1 nuclease mapping. The probe used for these experiments is described in the legend to Figure 1. +1 Gb indicates the position of the signal corresponding to RNA initiated at the β-globin gene transcription start site. The position of a 34 bp band of a pBR322−*Msp*I marker run in parallel is indicated (34).

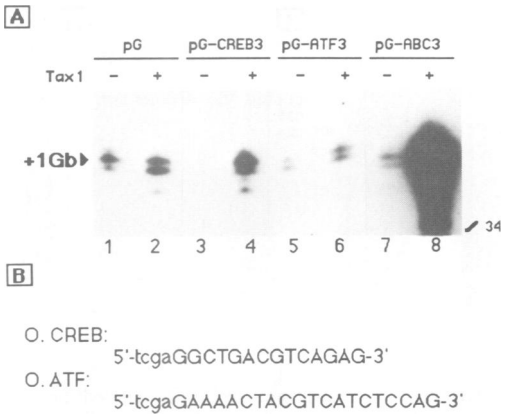

Fig. 3. Tax1 inducibility of CREB and ATF binding sites. (**A**) The basal and Tax1-induced activity of plasmids pG1 (**lanes 1** and **2**), pG-CREB3 (**lanes 3** and **4**), pG-ATF3 (**lanes 5** and **6**), pG-ABC3 (**lanes 7** and **8**) was determined by transfection in HeLa cells as described in the legend to Figure 2. Plasmids pG-CREB3 and pG-ATF3 contain orientated trimers of the rat somatostatin gene CREB binding site (Yamamoto *et al.*, 1988) and of the adenovirus EIIaE promoter ATF binding site (Jalinot *et al.*, 1988), respectively. Plasmids pG-CREB3 and pG-ATF3 were constructed by using the same protocol as for pG-ABC3 (see legend to Figure 1). (**B**) The exact sequence of the double-stranded oligonucleotides used for generating these constructs is given by the top strand.

A or C has only a partial effect. However, the presence of at least one of these two domains is also required.

We then asked whether the 21 bp motif sequences required for its enhancer activity on the β-globin gene were also required for Tax1 induction of the HTLV-I promoter. To answer this question the HTLV-I promoter sequence from position −350 to +20 was linked to the β-globin coding sequence (pHG-ABC construct; Figure 1C) and either the B domains (pHG-AMC) or the A and C domains together (pHG-MBM) were mutated. These two sets of point mutations both completely abolished the Tax1 inducibility of the HTLV-I promoter (data not shown). This clearly indicated that the effects observed with the heterologous constructs also took place in the promoter context and were not specific for a particular 21 bp motif.

Tax1 inducibility of binding sites for the ATF and CREB factors

Domain B (5′-TGACG-3′) is similar to the binding sites for two well-characterized cellular transcription factors: ATF and CREB (Jeang *et al.*, 1988; Tan *et al.*, 1989). ATF is a strong enhancer factor interacting with all the early adenovirus promoters, except E1b (SivaRaman *et al.*, 1986; Lin and Green, 1988). CREB has been identified in the rat somatostatin gene promoter (Montminy and Bilezikjian, 1987) and mediates the transcriptional response to an increase in intracellular cAMP. Since domain B contains the ATF or CREB binding site consensus sequence we asked whether genuine binding sites for these factors, present in well-studied promoters, were able to exhibit an increased enhancer activity in response to the Tax1 protein. For this purpose we placed 5′ to the β-globin promoter oligonucleotides corresponding to the ATF binding site in the adenovirus EIIaE promoter (pG-ATF3) and to the CREB binding site in the rat somatostatin promoter (pG-CREB3). These oligonucleotides were cloned as trimers in tandem repeat (Figure 3). In this construct the ATF binding site did not respond to Tax1 (Figure 3A, compare lanes 2 and 6). The CREB binding site was inactive alone (Figure 3A, compare lanes 1 and 3) but was weakly induced by Tax1 (Figure 3A, compare lanes 1 and 4). However, the induction ratio was much weaker than that observed with the 21 bp motif (Figure 3A, compare lanes 4 and 8). In conclusion, despite the sequence similarity between domain B and the ATF/CREB binding sites, the ability of these cellular transcription factors to mediate a Tax1-inducible enhancer activity on their own is either weak (CREB) or negative (ATF).

Cellular factors binding to the 21 bp motif

To understand how the different functional domains of the 21 bp motif characterized above were able to mediate a strong Tax1-induced enhancer activity, the cellular factors interacting with these domains were characterized. Lymphoid T-cells are the natural host of HTLV-I. These factors were therefore sought both in the epithelial HeLa and T-lymphoid Jurkat cells, in order to elicit if cell-specific factors could be involved in Tax1 induction. The nuclear extracts prepared with these cells were loaded onto a heparin−agarose column (Moncollin *et al.*, 1986). The fraction obtained by eluting the proteins from the column with 0.6 M KCl contained the majority of the specific DNA-binding activity (data not shown). These fractions were called JH 0.6 (Jurkat cells) and HH 0.6 (HeLa cells). In order to detect the cellular factors binding specifically to the 21 bp motif we used the electrophoretic band-shift assay. The DNA probe used in these experiments corresponded to the *Eco*RI−*Hin*dIII

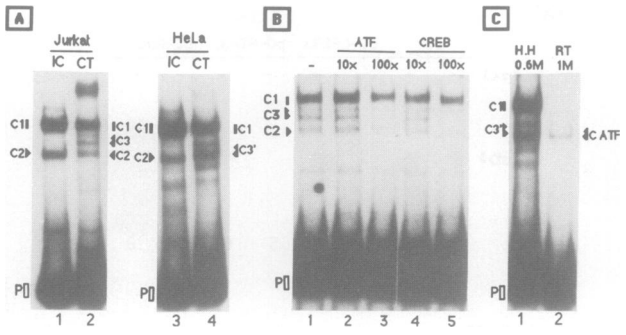

Fig. 4. Detection of the cellular proteins interacting with the 21 bp motif. (**A**) An end-labeled probe (*Eco*RI−*Hin*dIII restriction fragment of plasmid pG-ABC1, see Figure 1) was incubated either with the Jurkat cell JH 0.6 (**lanes 1** and **2**) or the HeLa cell HH 0.6 (**lanes 3** and **4**) heparin−agarose fractions. Preparation of these fractions is described in Materials and methods. Either poly(dI-dC) (**lanes 1** and **3**) or calf-thymus DNA (**lanes 2** and **4**) was used as non-specific competitor. The positions of the different specific complexes detected with the band-shift assay are indicated (C1: ■; C2: ▶; C3 and C3′: ▶). P stands for the position of the free migrating probe.
(**B**) Competition by ATF or CREB binding sites. The binding assay was performed with the JH 0.6 fraction and calf-thymus DNA. Double-stranded synthetic oligonucleotides containing the binding sites for the ATF factor in the EIIaE adenovirus promoter or for the CREB factor in the rat somatostatin promoter (see Figure 3 for the exact sequences) were used as specific competitors. These oligonucleotides were introduced in the binding reaction at a 10- (**lanes 2** and **4**) or 100-fold molar excess (**lanes 3** and **5**). The positions of complexes C1, C2 and C3 are indicated as described above. (**C**) Comigration of complex C3′ with that formed by the binding of purified ATF factor. The binding reaction was performed with the HH 0.6 (**lane 1**) or RT1M fractions (**lane 2**). Preparation of the RT1M fraction has been previously described (Jalinot *et al.*, 1988). Calf-thymus DNA was used as non-specific competitor with both fractions but only at an amount of 1 ng for the RT1M fraction. The responses of complexes C1 and C3′ are indicated as described above. The position of the ATF complex is also indicated ( ).

fragment of plasmid pG-ABC1 and contained one copy of the most proximal 21 bp motif. It is known from previous studies that the nature of the non-specific DNA competitor used in such experiments can lead to the detection of different complexes (SivaRaman and Thimmappaya, 1987). To circumvent this problem we have used both poly(dI-dC) and calf-thymus DNA as non-specific competitor. With JH 0.6 or HH 0.6 fractions and poly(dI-dC), two major complexes were detected: C1 for the upper one and C2 for the lower one (Figure 4A, lanes 1 and 3). With the JH 0.6 fraction and calf-thymus DNA C1 and C2 were still present but a weak double band, called C3, also appeared between these complexes (Figure 4A, lane 2). The smeary band which also appeared at the top of the gel was non-specific since it was not competed out by the double-stranded ABC oligonucleotide (data not shown). With the HH 0.6 fraction and calf-thymus DNA C1 was still detectable but complex C2 was replaced by a double band: C3′ (Figure 4A, lane 4). In order to rule out the possibility that one of these complexes was specific for the most proximal 21 bp motif, competition experiments with DNA fragments containing the distal 21 bp motifs (I and II; Figure 1A) were performed. Whatever the type of fraction and non-specific competitor used, this fragment efficiently competed for each of the specific complexes (data not shown). We could therefore conclude that the different complexes detected were able to form on each 21 bp motif.

Correspondence of identified factors with already characterized DNA-binding proteins

Domain B contains sequences homologous to ATF and CREB binding sites. To determine if one of the complexes detected in our band-shift experiments corresponded to these factors, competition experiments with the double-stranded oligonucleotides containing their binding sites (Figure 3B) were performed. When calf-thymus DNA was used in the binding reaction these oligonucleotides efficiently competed for the formation of complex C3 (Figure 4B, lanes 1−5). These results were obtained with the JH 0.6 fraction. Similar results were obtained with the HH 0.6 fraction: C3′ was competed out but C1 and C2 were not (data not shown). The ATF or CREB binding sites exhibited the same activity and competed at similar amounts. This raised the possibility that the factor leading to the formation of complexes C3 and C3′ was indeed ATF−CREB. To verify this we performed a band-shift assay with purified ATF using the RT1M fraction (Jalinot *et al.*, 1988; NB: in this publication the ATF factor was called EIIaE-B). The complex obtained with this fraction exactly comigrated with complex C3′ (Figure 4C). From these data we conclude that the C3 complexes correspond to the binding of ATF−CREB to the 21 bp motif. We also tried to compete out the formation of complexes C1 and C2 using sequences containing the sites for well-characterized cellular transcription factors (SV40 enhancer, immunoglobulin heavy chain gene enhancer). The results obtained have been negative and thus have not allowed the proteins leading to formation of complexes C1 and C2 to be related to any other presently known cellular transcription factor. The protein present in complexes C1 and C2 were therefore called HEB1 and HEB2, respectively.

Binding sites for HEB1 and HEB2

In order to precisely locate the binding sites for HEB1 and HEB2 DMS methylation interference studies were performed. In these experiments poly(dI-dC) was used as non-specific competitor in order to obtain only complexes C1 and C2. The results obtained with JH 0.6 and HH 0.6 were similar and showed that the binding site for HEB1 included domains B and C, and that for HEB2 mainly domain B and the 5′ border of domain C (Figure 5).

Binding of the different factors identified in this study to probes bearing the different mutations in domains A, B and C was also examined. These probes corresponded to *Eco*RI−*Hin*dIII fragments of plasmids pG-MBC1, pG-AMC1, pG-ABM1 and pG-MBM1 (Figure 1). The results presented in Figure 6 were obtained with HH 0.6 and either poly(dI-dC) or calf-thymus DNA as non-specific competitor. Similar results were obtained with JH 0.6. Mutation in domain A had no obvious effect on formation of complexes C1, C2 and C3′ (Figure 6A and B, lanes 2). Mutation of domain B slightly reduced complex C1 and led to the disappearance of complexes C2 and C3′ (Figure 6A and B, lanes 3). Mutation of domain C did not affect complexes C2 and 3′, but reduced complex C1 (Figure 6A and B, lanes 4). However, complex C1 completely disappeared when domains A and C were both mutated (Figure 6A and B, lanes 5). This observation raised the possibility that HEB1 was also able to bind to domain A. This was clearly confirmed by the results of a DMS methylation interference study on complex C1 using a probe containing a mutated domain C. The use of this probe

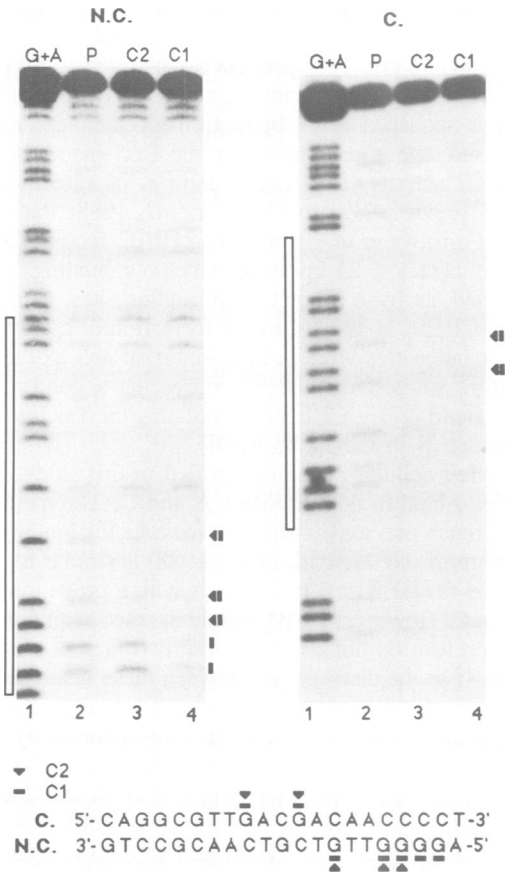

Fig. 5. Localization of the binding sites for complexes C1 and C2 by DMS methylation interference experiments. The binding reaction was performed with the JH 0.6 fraction and poly(dI-dC) as non-specific competitor. After separation on a nucleo-protein gel, DNA present in the retarded C1 and C2 complexes and in the free-migrating probe was electroeluted and treated with piperidine. After normalization of the radioactivity amounts, the different samples were loaded onto a 8% polyacrylamide sequencing gel. The pattern obtained for the free migrating probe (P, **lane 2**), the complex C1 (C1, **lane 4**) and the complex C2 (C2, **lane 3**), are given on the non-coding (N.C.) and coding (C.) strands, together with a G+A Maxam and Gilbert sequence ladder (G+A, **lane 1**). The interfering guanine residues for the C1 (■) and C2 (▼) complexes are indicated on the sequence ladders and below on the sequence of the most proximal 21 bp motif. The open box next to the G+A sequence ladder delineates the sequence given at the bottom of the gel.

showed that the detected binding site for HEB1 was then located mainly on domain A (Figure 7). These experiments established that the sequences determining the binding of ATF or HEB2 corresponded to domain B and that binding of HEB1 was allowed by either domain C or A.

Discussion

The Tax1 protein strongly activates the HTLV-I promoter by inducing the enhancer activity of its repeated 21 bp motif. This is apparent from previous studies (Fujisawa *et al.*, 1986; Shimotohno *et al.*, 1986; Brady *et al.*, 1987; Rosen *et al.*, 1987) and strengthened by our data which show that point mutations introduced in each 21 bp motif in the genuine viral promoter abolishes Tax1 induction. This Tax1 activation did not seem to be cell-specific and exhibited similar magnitudes in epithelial HeLa and T-lymphoid Jurkat cell lines (our unpublished results; Rosen *et al.*, 1985). With our sensitive S1 nuclease assay just one copy of the 21 bp motif was

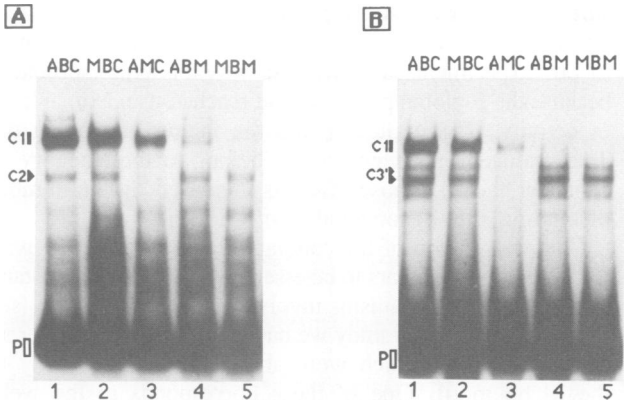

Fig. 6. Formation of the different complexes on the mutated 21 bp motifs. (**A**) The binding assay was performed with the HH 0.6 fraction and poly(dI-dC). The different probes used were *Eco*RI−*Hin*dIII fragments of plasmids pG-ABC1 (ABC, **lane 1**), pG-MBC1 (MBC, **lane 2**), pG-AMC1 (AMC, **lane 3**), pG-ABM1 (ABM, **lane 4**) and pG-MBM1 (MBM, **lane 5**). The structure of these plasmids is described in Figure 1. The positions of the C1 and C2 complexes are indicated as previously (see legend to Figure 4). (**B**) The same experiment was done with calf-thymus DNA as non-specific competitor. The positions of complexes C1 and C3′ are indicated as previously (see legend to Figure 4).

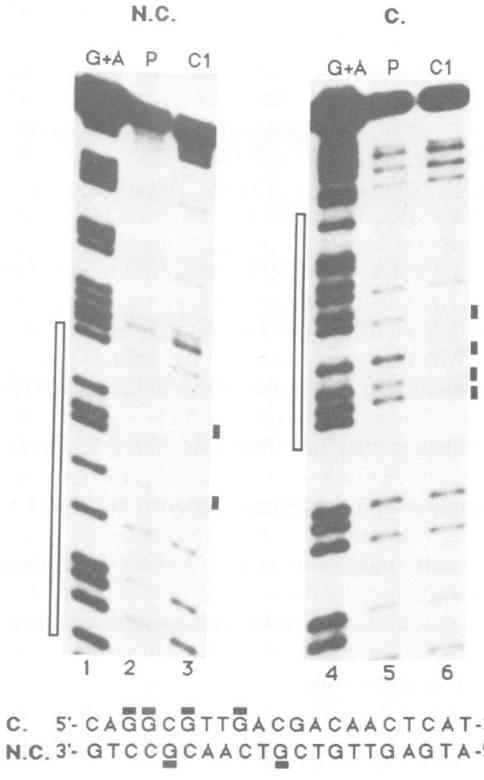

Fig. 7. Localization of the HEB1 binding site on the 21 bp motif mutated in domain C. The methylation interference experiment was performed as described in the legend to Figure 5. The binding reaction was done with the JH 0.6 fraction and poly(dI-dC) as non-specific competitor. The probe used was the *Eco*RI−*Hin*dIII restriction fragment of the pG-ABM1 plasmid (see Figure 1). The patterns obtained with the free migrating probe (P, **lanes 2** and **5**) and complex C1 (C1, **lanes 3** and **6**) are given both on the non-coding (N.C., **lanes 1−3**) and coding (C., **lanes 4−6**) strands, together with a G+A sequence ladder (G+A, **lanes 1** and **4**). The guanine residues revealed by this experiment (■) are indicated on the sequence ladder and below on the sequence of the mutated 21 bp motif. The open box next to the G+A sequence ladder delineates the sequence depicted at the bottom of the gel.

sufficient to mediate a strong response to Tax1. The effect observed was higher with an increased number of copies. In our experiments this increase was moderate, probably because the β-globin promoter had reached its maximal level of activity. This indicates that the factors mediating the Tax1-induced enhancer activity belong to the category of enhancer factors whose effect is increased by the number of binding sites (Fromental *et al.*, 1988).

Characterization of the cellular proteins interacting with the 21 bp motif appears to be essential in order to understand the molecular mechanisms involved in Tax1 induction (see Introduction). In this study we have identified three different cellular proteins which were able to bind specifically to the 21 bp motif. One of these corresponds to the well-characterized transcription factor ATF. We have shown in this paper that ATF purified following its ability to bind to the EIIaE adenovirus promoter was indeed able to interact with the 21 bp motif. The binding of this factor was also detectable in a crude fraction of a HeLa cell extract. With Jurkat cells a very similar activity was detected but the complex observed was much weaker and migrated slightly slower. This could reflect some differences in the structure of this factor in lymphoid cells. ATF is closely related to the CREB transcription factor. Both have been purified to homogeneity (Hurst and Jones, 1987; Montminy and Bilezikjian, 1987; Jalinot *et al.*, 1988; Hai *et al.*, 1988a) and from their similar binding sites and mol. wts it has been suggested that ATF and CREB are indeed the same protein (Hurst and Jones, 1987). This still remains to be established and these two factors could be members of a complex group of cellular DNA-binding proteins exhibiting the same sequence-specificity (Hai *et al.*, 1988b; Hoeffler *et al.*, 1988; Maekawa *et al.*, 1989). In our experiments the ATF and CREB binding sites both competed equally efficiently for the same complex. The CREB transcription factor mediates the transcriptional response to cAMP in a number of cellular genes (Fink *et al.*, 1988 and references therein). It has been shown that the activity of the HTLV-I promoter is induced by an increase in the intracellular cAMP (Poteat *et al.*, 1989). The *in vitro* binding of ATF−CREB to the 21 bp motif indicates that this factor is likely to mediate this response of the HTLV-I promoter to an increase in cAMP. Interestingly it has been recently reported that the 21 bp motif was poorly responsive to cAMP in Jurkat cells as compared to other cell lines (Tan *et al.*, 1989). This observation could be explained by the low intensity of the ATF−CREB complex detected with Jurkat cell extracts which indicates that this factor has either a low abundance or a weak affinity for DNA in these cells.

Although ATF−CREB is clearly able to bind to the 21 bp motif, its role in Tax1 induction seems questionable. Two recent reports have established that the exact sequence requirement of the 21 bp motif for cAMP activation and Tax1 induction are different (Fujisawa *et al.*, 1989; Nakamura *et al.*, 1989). We show here that genuine CREB or ATF binding sites respond weakly or not at all to Tax1 in comparison to the 21 bp motif. This difference of reactivity can be attributed to the sequences which flank the consensus TGACG motif. Comparison of sequences surrounding ATF or CREB binding sites indeed reveals that the A and C domains are specific for HTLV-I (Tan *et al.*, 1989). They are also conserved in HTLV-II (Shimotohno *et al.*, 1984). We have identified a cellular protein, HEB2, which interacts with domain B and part of domain C. However, similarly to ATF−CREB, binding of HEB2 to DNA appears greatly dependent on the integrity of domain B. This domain is essential for Tax1 induction since its mutation abolished the 21 bp motif induced enhancer activity on a heterologous gene, as well as the Tax1-induced HTLV-I promoter activity. This observation is in agreement with several previous reports (Jeang *et al.*, 1988; Park *et al.*, 1988; Fujisawa *et al.*, 1989). The HEB2 factor is specific for the HTLV-I 21 bp motif since its binding was not competed out by ATF or CREB binding sites. Contrary to ATF−CREB, HEB2 led to the formation of complexes of similar intensities with HeLa and Jurkat cell extracts. We therefore suggest that HEB2 represents a good alternate candidate to ATF−CREB for mediating the Tax1 activation of domain B in the 21 bp motif.

Another cellular protein identified in this study, HEB1, is able to bind to both domains A and C. The methylation interference previously reported for a factor binding to the 21 bp motif (NF21; Park *et al.*, 1988) is similar to that we detected for HEB1 over domain A with a probe mutated in domain C. However, HEB1 mainly interacts with domain C. We therefore do not know if these two factors are similar or not. If so the discrepancy between these results could be related to the different probes used in these experiments. In particular some of the experiments reported by Park *et al.* (1988) were made with a probe bearing a tandem repeat of the 21 bp motif. Mutation of one of the A and C domains reduced the Tax1 effect but mutation of both abolished it. This could indicate that HEB1 has to bind to at least one site to be active and could explain why the importance of domain A and C has been underestimated in previous studies (Jeang *et al.*, 1988). It could also explain why the mutation of just one of these domains had a reduced effect when the 21 bp motif was multimerized in tandem repeat. In this latter case there were always sites for HEB1 on both sides of domain B. Our results show that Tax1 induction requires domain B and at least one of the flanking A or C domains. We therefore propose that HEB2 acts in synergy with HEB1 to mediate the Tax1 activation of the 21 bp motif.

It has been shown that the SV40 enhancer is constituted of fundamental elements, called enhansons (Ondek *et al.*, 1988). Based on their different properties, a classification of these enhansons has been proposed. In particular some of them do not exhibit enhancer properties on their own but only when associated with others (Fromental *et al.*, 1988). Such cooperative enhanson elements have been characterized in different systems (Comb *et al.*, 1988; Mermod *et al.*, 1988). The HTLV-I 21 bp motif offers another example of an enhancer activity which results in coordinate interaction of fundamental sequence elements with specific proteins. In this case the enhancer activity is also dependent on the Tax1 protein which does not seem able to bind specifically to DNA (Beimling and Moelling, 1988). How the activity of HEB1 and HEB2 is induced by Tax1 will require further investigations. During the completion of this work it has been published that Tax1 physically interacts with a protein which binds to the HTLV-I promoter between the two proximal 21 bp repeats (Marriot *et al.*, 1989) and that protein kinase inhibitors were able to block the response of the 21 bp motif to Tax1 (Tan *et al.*, 1989). In the light of these results it will be particularly interesting to look for specific protein−protein contacts between HEB1, HEB2 and Tax1 or for

modifications of these two cellular factors in cells expressing the Tax1 protein.

In conclusion, our data show that the HTLV-I 21 bp enhancer element is able to bind several cellular proteins and represents a complex structure which must have been selected for by the virus to respond to viral as well as to cellular signals. Binding of the ATF−CREB factor could lead to the activation of the viral promoter in response to cellular stimuli such as an increase in cAMP. Two other cellular factors, HEB1 and HEB2, are likely to cooperatively mediate the response to the strong viral transactivator Tax1. Involvement of several proteins and high inducibility from a completely inactive state make the HTLV-I 21 bp enhancer element an interesting model of transcriptional control element.

Materials and methods

Transfection

HeLa cells, grown in monolayers to 40% confluence, were transfected by the calcium phosphate coprecipitation method with 2 μg of the different test plasmids, 2 μg of either the Tax1-expressing plasmid pBS or the control plasmid pBX, and 11 μg of pUC18 DNA. Preparation of total RNA by hot phenol extraction and S1 nuclease mapping were carried out as previously described (Wasylyk and Wasylyk, 1986). The S1 nuclease DNA probe was a single-stranded synthetic oligonucleotide.

Nuclear extracts and heparin−agarose fractions

Nuclear extracts of HeLa and Jurkat cells were prepared as described (Dignam *et al.*, 1983; Wildeman *et al.*, 1984) with the following modifications: the 0.42 M NaCl buffer used for extracting the proteins from the nuclei was supplemented with 10 μM $ZnCl_2$ and 0.5 mM PMSF; after centrifugation of the nuclei the supernatant was not precipitated with ammonium sulphate but directly dialysed against 20 mM HEPES, pH 7.9, 20% glycerol, 20 mM KCl, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM PMSF. For Jurkat cells special care was taken in the centrifugation steps in order to avoid lysis of the cells or nuclei. After the final dialysis the nuclear extracts were directly loaded onto a heparin−agarose column (IBF Biotechnics). Proteins were eluted in steps by washing the column with buffers containing 20 mM Tris, pH 7.9, 20% glycerol, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM PMSF and either 0.24, 0.6 or 1 M KCl. The fractions eluted by the 0.6 M KCl buffer (JH 0.6 for Jurkat cells and HH 0.6 M for HeLa cells) were dialysed against the following buffer: 20 mM Tris, pH 7.9, 10% glycerol, 50 mM KCl, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM PMSF.

Band-shift assay and methylation interference experiments

The electrophoretic band-shift assay and the methylation interference experiments were conducted as previously described (Jalinot *et al.*, 1987) except that the binding reaction and the nucleo-protein gel were carried out at 4°C. The electrophoresis buffer used for the latter was 0.2 × TBE and was not recirculated. The binding reaction was carried out by mixing 500 mM KCl (1 μl), 5 mg/ml BSA (1 μl), poly(dI-dC) or calf-thymus DNA (1 μl), water or specific competitor (1 μl), JH 0.6 or HH 0.6 fractions (4 μl) and labeled probe (10 000 c.p.m.) in 50% glycerol (2 μl). Typically the amounts of protein and non-specific competitor used were 1 μg for JH 0.6 and HH 0.6 fractions, 800 ng for poly(dI-dC) and 300 ng for calf-thymus DNA.

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