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Binding of the HTLV-I Tax1 transactivator to the inducible 21 bp enhancer is mediated by the cellular factor HEB1

Christophe Béraud, Gaël Lombard-Platet, Yvonne Michal and Pierre Jalinot

UMR 49 CNRS-ENS, Laboratoire de Biologie Moléculaire et Cellulaire, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France

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Transcription driven by the HTLV-I promoter is strongly activated by the viral transactivator protein Tax1. This effect is mediated via a 21 bp sequence which is imperfectly repeated three times in the viral promoter. We showed previously that a single 21 bp copy exhibits a strong Tax1-inducible enhancer activity and is able to bind different cellular proteins, namely ATF, HEB1 and HEB2. We have further investigated the molecular mechanism involved in the Tax1 induction of the 21 bp motif's enhancer activity by analysing Tax1 interaction with this DNA sequence. For this purpose a HeLa cell line constitutively expressing a functional Tax1 protein was established and nuclear extracts of these cells were used to perform a DNA affinity precipitation assay. This experimental approach allowed us to show that Tax1 specifically binds to the 21 bp motif. The same sequence elements of the 21 bp motif are required both for Tax1 binding and for Tax1-induced enhancer activity. Chromatographic fractionation of the HeLa tax nuclear extract showed that the binding is indirect and is mediated by the cellular factor HEB 1.

Key words: DNA affinity precipitation assay/HTLV-I/Tax1 transactivator/transcription

Introduction

Infection by the human T cell leukemia virus (HTLV-I) can lead to the development of several pathologies, in particular adult T cell leukemia (ATL) (for a review see Wong-Staal and Gallo, 1985). The viral protein Tax1 is thought to play an important role in the establishment of the HTLV-I-associated proliferative pathologies. It is indeed now established that this protein has oncogenic properties (Nerenberg *et al.*, 1987; Grassmann *et al.*, 1989; Tanaka *et al.*, 1990). The function of Tax1 in the viral cycle is to induce transcription of the proviral genome (Felber *et al.*, 1985; Fujisawa *et al.*, 1985; Sodroski *et al.*, 1985). Tax1 can also activate the expression of several cellular genes at the level of transcriptional initiation. The most significant are the interleukin 2 gene (IL2) (Wano *et al.*, 1988), the gene coding for the IL2 α chain receptor (IL2-Rα) (Inoue *et al.*, 1986) and the c-*fos* proto-oncogene (Fuji *et al.*, 1988; Nagata *et al.*, 1989). It has also been reported that the expression of the gene coding for the human β-polymerase (a DNA polymerizing enzyme involved in the repair of DNA) was repressed by Tax1 (Jeang *et al.*, 1990). Disruption of the normal regulation of the genetic expression of these important genes by Tax1 is likely to explain the oncogenic properties of this viral transactivator.

The ability of Tax1 to modify expression of a given set of cellular genes makes it an interesting model of a transcriptional regulator to study. From various studies it appears that Tax1 acts by inducing the enhancer activity of specific DNA motifs. So far the best characterized Tax1 target sequences are the binding site for the enhancer factor NF-$\varkappa$B and the 21 bp sequence present as three imperfectly repeated copies in the HTLV-I promoter (Figure 1). The NF-$\varkappa$B binding site mediates Tax1 activation of the IL2 and IL2-Rα genes. It has been shown that Tax1 causes NF-$\varkappa$B to be translocated to the nucleus in T lymphoid cells (Ballard *et al.*, 1988). This factor is normally sequestered in the cytoplasm as a complex with I-$\varkappa$B (Baeuerle and Baltimore, 1989). In the case of the 21 bp motif the mechanism involved in Tax1 activation remains unclear. The DNA–protein interactions detected on the 21 bp motif, either by DNase footprinting or electrophoretic mobility shift assay (EMSA), are similar in the presence or the absence of the Tax1 protein (Altman *et al.*, 1988; Nyborg *et al.*, 1988; Beimling and Moelling, 1990) and purified Tax1 protein does not bind specifically to DNA (Beimling and Moelling, 1989). Alignment of the three 21 bp motifs of the HTLV-I promoter shows that this sequence can be divided into three perfectly conserved domains which have been named, from 5′ to 3′, A, B and C (Fujisawa *et al.*, 1989; Montagne *et al.*, 1990). Tax1 inducibility of the 21 bp motif is lost either when the central domain B is mutated or when the flanking domains A and C are both mutated (Montagne *et al.*, 1990). From different studies it appears that several cellular proteins can interact with the 21 bp motif (Tan *et al.*, 1989a; Beimling and Moelling, 1990; Montagne *et al.*, 1990). The central domain, B, (5′-TGACG-3′) corresponds exactly to the consensus sequence for the ATF and CREB cellular factors. This sequence motif is present in a number of cellular and viral promoters. For example, in the somatostatin gene this sequence is palindromic (5′-TGACGTCA-3′) and mediates the induction of the promoter's activity in response to cAMP by association with the cellular factor CREB (Montminy *et al.*, 1986; Yamamoto *et al.*, 1988). The cDNA coding for this factor has been isolated and shows that CREB contains a basic domain leucine zipper motif (Gonzales *et al.*, 1989). In adenovirus early gene promoters the TGACG consensus motif has been shown to be involved in the EIa induction process and binds the cellular factor ATF (Lin and Green, 1988). Screening of cDNA libraries with a probe containing a trimerized ATF binding site has allowed identification of multiple leucine zipper proteins (Hai *et al.*, 1989). The same experimental approach made using the 21 bp motif revealed three proteins containing leucine zipper motifs, TREB 5, TREB 7 and TREB 36 (Yoshimura *et al.*, 1990). These two latter correspond to ATF-2 and ATF-1 respectively. In addition to ATF, we have previously

identified two cellular factors which specifically recognize the 21 bp motif: HEB1 and HEB2 (Montagne *et al.*, 1990). Binding of HEB2 is lost when domain B is mutated, whereas complete loss of HEB1 binding is achieved only when both domains A and C are mutated together. Mariott *et al.* (1989) showed that Tax1 can indirectly bind to a sequence element located between the two proximal 21 bp motifs (Marriott *et al.*, 1989). The binding is mediated by a cellular factor called TIF1 (Marriott *et al.*, 1990). However, unlike the 21 bp motif, the binding site of TIF1 does not exhibit Tax1-induced enhancer activity by itself. It has also been reported that protein kinase inhibitors are able to block the response of the 21 bp motif to Tax1 (Tan *et al.*, 1989a).

From these data two models are plausible for explaining the Tax1 induction of the 21 bp motif enhancer activity. Tax1 could induce some activating modification of one or several transcription factors interacting with the 21 bp motif. Alternatively, Tax1 could directly bind to one or several of these proteins, the combination giving rise to an active structure. By using a DNA affinity precipitation assay we tested this latter hypothesis. The results presented here show that Tax1 can indeed interact with the 21 bp motif. The sequence requirement for establishment of the complex was the same as that necessary for Tax1-induced enhancer activity of the 21 bp motif as determined by transient expression assays. Fractionation experiments allowed us to show that this interaction of Tax1 with the 21 bp motif is mediated by the cellular factor HEB1.

Results

Detection of a complex which includes Tax1 and the 21 bp motif

In order to study protein−protein interactions taking place on the 21 bp motif in the presence of the Tax1 protein, we first established a HeLa cell line expressing this viral transactivator. The Tax1 coding sequence was cloned in the vector p36/7 polyA between the human metallothionein IIA promoter and the thymidine kinase polyadenylation signal (plasmid p36/7 poly tax, Figure 2A). The plasmid p36/7 polyA contains a neomycin resistance gene under the control of the SV40 early promoter. HeLa cells were transfected with either p36/7 polyA tax or p36/7 polyA. Stable transformants were selected by treatment with G418 and cloned. The different clones were screened by using a transient expression assay. The plasmid pG-ABC3 (Montagne *et al.*, 1990), which contains three copies of the more proximal 21 bp motif (21 bp III) cloned upstream of the rabbit β-globin gene, was modified by introduction of a deletion of 15 bp from positions +1 to +16 with respect to the β-globin transcription start site (plasmid pΔG-ABC3, Figure 2A). This plasmid, pΔG-ABC3, was cotransfected with an equal amount of another plasmid bearing the rabbit β-globin sequence only (pG1, Figure 2A). The specific RNAs were analysed by quantitative S1 nuclease mapping. As expected, the RNA originating from the pΔG-ABC3 plasmid is 15 bp shorter than that corresponding to pG1. When transfection was made using clones resulting from transformation with the plasmid p36/7 polyA, both signals were of equal intensity (Figure 2B, lane 1). As previously reported, this indicated that the 21 bp motif does not exhibit any enhancer activity by itself. Among the clones resulting from transformation by p36/7 polyA tax, we selected one in which the pΔG-ABC3 signal was clearly higher than that given by PG1 (Figure 2B, lane 3). This difference was further increased by treatment of cells with Zn^{2+} cations (Figure 2B, lane 4), indicating that induction of the human metallothionein IIa promoter increased the amount of Tax1 protein expressed. This clone was designated as HeLa tax and that resulting from transformation by p36/7 polyA as HeLa neo. The presence of Tax1 protein in the HeLa tax cells was further confirmed by Western blotting experiments (Figure 2C).

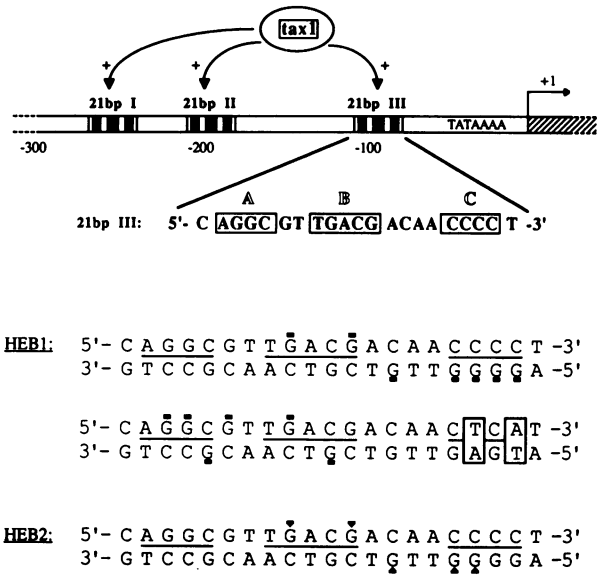

Fig. 1. Schematic representation of the HTLV-I promoter. The HTLV-I promoter contains a 21 bp motif which is repeated three times at positions −251 (21bp I), −203 (21bp II) and −103 (21bp III) with respect to the transcription start site which is indicated by the arrow marked +1. The Tax1 protein encoded by the virus strongly activates the enhancer activity of these three sequence elements. Under the representation of the promoter, the exact sequence of the more proximal 21 bp motif (21bp III) is given. Alignment of the three 21 bp motifs showed that three domains are perfectly conserved (Fujisawa *et al.*, 1989). These three domains A, B and C are boxed. Two cellular factors, HEB1 and HEB2, binding specifically to the 21 bp motif were previously identified. In EMSA tests these two factors exhibited a similar activity in nuclear extracts of both HeLa and Jurkat cells (Montagne *et al.*, 1990). The DMS methylation interference profiles of these two factors is represented (Montagne *et al.*, 1990). The interfering guanine residues are indicated by arrows for HEB2 and by rectangles for HEB1. For this latter factor another binding site was identified when the C domain was mutated. The DMS methylation interference profile obtained with the mutated probe is also indicated. The mutated nucleotides are boxed. The A, B and C domains are underlined.

The DNA−protein interactions on the 21 bp motif were first analysed by electrophoretic mobility shift assay (EMSA). Nuclear extracts prepared from either HeLa tax or HeLa neo cells gave identical patterns. Two major complexes were detected: C1 and C2. The migration and intensity of these two complexes were unchanged by nuclear extracts containing Tax1 protein (Figure 3A, lanes 1 and 2). We then performed a DNA affinity precipitation assay (DAPA). Biotinylated probes were generated using the polymerase chain reaction. The amplification of the DNA fragment was done using two primers, biotinylated at their 5′ end, and plasmids bearing three copies of the 21 bp motif, either wild type (pG-ABC3), mutated in domain B (pG-AMC3) or mutated in both domains A and C (pG-MBM3) (Montagne *et al.*, 1990). The resulting probes were named

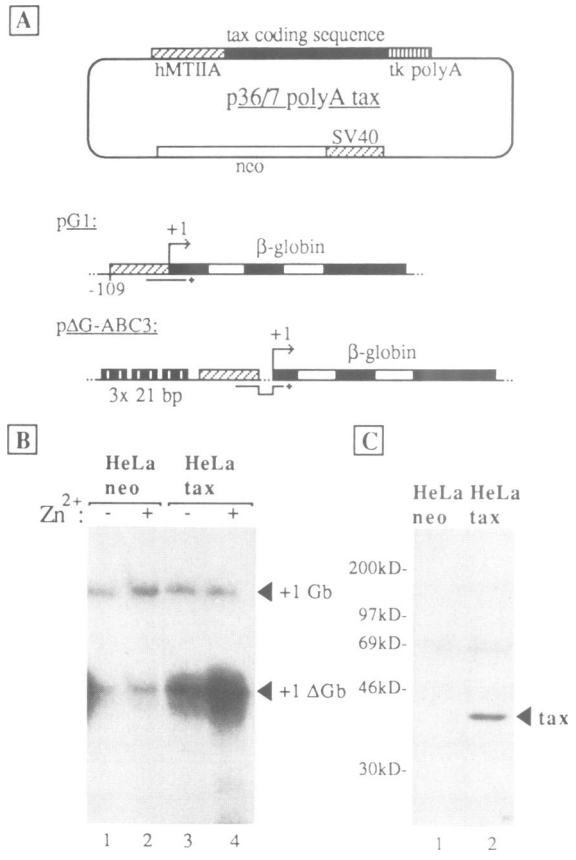

Fig. 2. Establishment of HeLa tax and HeLa neo cell lines. (**A**) Schematic representation of the plasmids. The Tax1 coding sequence deleted of the second intron sequence was generated as follows: a double-stranded synthetic oligonucleotide including the Tax1 coding sequence, from five nucleotides upstream of the initiation codon up to the *Acc*I restriction site (position 6994), and a *Aat*II restriction site was cloned in M13mp18 (plasmid pSG). The *Acc*I (position 6994)−*Aat*II (position 8038) restriction fragment from plasmid pBS (Lilienbaum *et al.*, 1990) was cloned between the *Acc*I and *Aat*II restriction sites of plasmid pSG, giving plasmid pSGT. The *Hin*dIII−*Bam*HI restriction fragment of plasmid pSGT was filled in and cloned into the vector p36/7 polyA digested by *Bam*HI and filled in (Gruffat *et al.*, 1991). The resulting plasmid, p36/7 polyA tax, contains the complete Tax1 coding sequence between the human metallothionein IIa promoter and the thymidine kinase polyadenylation signal. This plasmid also includes the neomycin resistance gene under the dependence of the SV40 early promoter. The pG1 plasmid contains the rabbit β-globin sequence from positions −109 to +1664. The plasmid pΔG-ABC3 was obtained from plasmid pG-ABC3 (Montagne *et al.*, 1990) as follows: a *Bam*HI restriction fragment containing the promoter and the beginning of the coding sequence of the β-globin gene was subcloned into the *Bam*HI restriction site of M13mp18. A deletion of the first 15 nucleotides following the β-globin transcription initiation site was introduced by *in vitro* mutagenesis (Kunkel, 1985). The mutated fragment was excised with *Bam*HI and cloned back into the vector pG-ABC3 cut with *Bam*HI. The probe used for S1 nuclease mapping is indicated by a line under the representation of the plasmids. This probe was a synthetic oligonucleotide corresponding to the β-globin sequence (non-coding strand) from position −20 to +40. (**B**) Functional test for the presence of Tax1. Isolated clones resulting from transfection of HeLa cells by p36/7 polyA or p36/7 polyA tax were cotransfected with equal amounts of the pG1 and pΔG-ABC3 plasmids. Specific RNAs produced by both constructs were analysed by S1 nuclease mapping. The results obtained with the selected clones resulting from transfection by p36/7 poly A (HeLa neo, lanes 1 and 2) and by p36/7 poly A tax (HeLa tax, lanes 3 and 4) are shown. The cells were either treated with 150 μM $ZnCl_2$ 12 h before harvesting (lanes 2 and 4) or mock-treated (lanes 1 and 3). The positions of signals corresponding to transcripts originated from plasmids pG1 (+1Gb) and pΔG-ABC3 (+1 ΔGb) are indicated. (**C**) Detection of the Tax1 protein by Western blotting. Equal amounts (30 μg) of nuclear extracts prepared from HeLa neo (lane 1) or HeLa tax (lane 2) cells were loaded on a 10% SDS−protein gel. The presence of the Tax1 protein was probed by Western blotting using a rabbit polyclonal antibody directed against Tax1 (Hanly *et al.*, 1989) and radiolabelled protein A. Positions of the different bands of a molecular weights marker run in parallel are indicated on the left side of the autoradiogram. Position of the signal corresponding to the 40 kDa Tax1 protein is marked (tax).

abc, amc and mbm respectively (Figure 3C). After incubation with the HeLa tax nuclear extract, streptavidin−agarose beads were added to precipitate the DNA−protein complexes by centrifugation. The presence of the Tax1 protein in these complexes was analysed by Western blotting. When this experiment was done with the probe abc, the presence of Tax1 in the precipitated complexes was clearly detected (Figure 3, lane 2). When the same experiment was done with either probes amc or mbm, no precipitated Tax1 was detectable (Figure 3, lanes 3 and 4). We have previously reported that these two mutations lead to a complete loss of Tax1 inducibility of the 21 bp motif, when placed either in front of a reporter gene, or in the proviral promoter context (Montagne *et al.*, 1990). We concluded from these results that Tax1 can form a specific complex with the 21 bp motif.

Tax1 binding to the 21 bp motif is indirect

To investigate further how Tax1 binds to the 21 bp motif, the HeLa tax nuclear extract was fractionated on a heparin−agarose column. Proteins were step-eluted with an increasing KCl concentration: 0.3 M (HT 0.3 fraction), 0.6 M (HT 0.6 fraction) and 1 M. DNA binding activity, tested by EMSA, was recovered in the HT 0.3 and HT 0.6 fractions. With the former, a unique band having the same mobility as the complex C1 was detected (Figure 4A, lane 3). Similarly to the original nuclear extract, HT 0.6 M led to the formation of two complexes (Figure 2A, lane 5). This indicated either that the protein leading to complex C1 was present in both HT 0.3 and HT 0.6 fractions or that two different proteins gave two complexes of the same mobility. Other fractionation experiments made with Jurkat cell extracts showed that C1 was indeed composed of two different comigrating complexes, one of which corresponds to the binding of the cellular transcription factor Sp1 (C.Béraud and G.Lombard-Platet, unpublished results). In agreement with this, when a monoclonal antibody directed against Sp1 (Courey and Tjian, 1988) was added after incubation of the DNA probe with the HeLa tax nuclear extract, a novel complex, C1′, appeared (Figure 4A, lane 2). However, complex C1, although much weaker, was still detectable. When the same experiment was done with the HT 0.3 fraction, C1 disappeared and only C1′ was observed (Figure 4A, lane 4). With the HT 0.6 fraction, addition of anti-Sp1 monoclonal antibody had no effect (Figure 4A, lane 6). From these results we concluded that Sp1 was eluted in the HT 0.3 fraction and that a different protein giving a complex of identical mobility was eluted in the HT 0.6 fraction. This latter protein was named HEB1. It coeluted in the HT 0.6 fraction with HEB2 which gave rise to the complex C2. The presence of Tax1 in the heparin−agarose fractions was probed by Western blotting and found to be in the HT 0.3 fraction (Figure 4B, lanes 1−3).

The HT 0.3 and 0.6 fractions were tested by DAPA. When

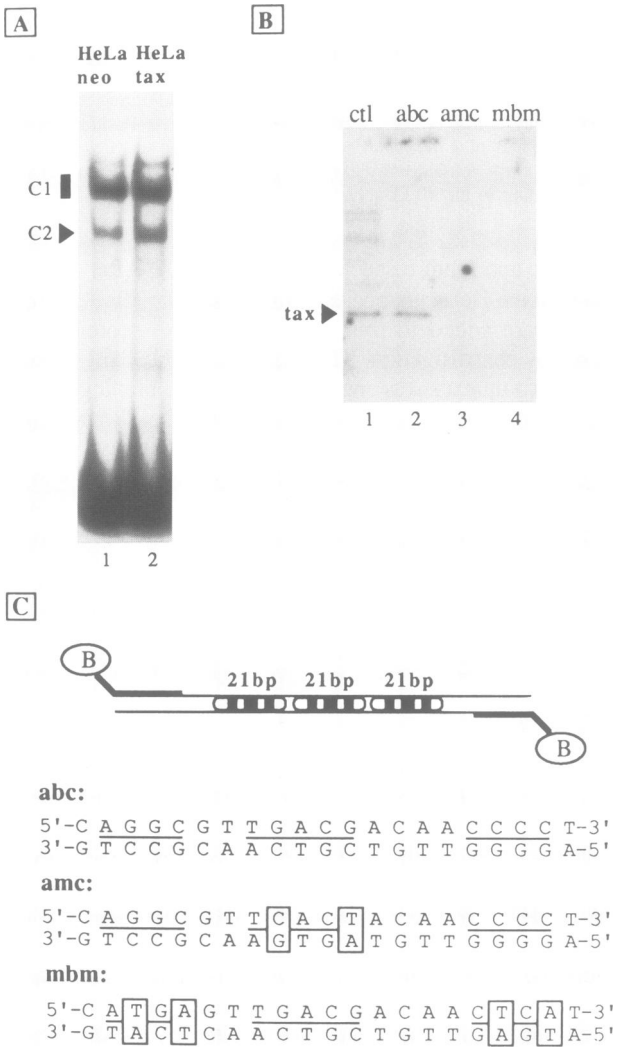

Fig. 3. Analysis of the protein–DNA complexes forming on the 21 bp motif. (**A**)Electrophoretic mobility shift assay (EMSA). Equal amounts (5 μg) of HeLa neo (lane 1) and HeLa tax (lane 2) nuclear extracts were incubated with a radiolabelled DNA probe containing a single copy of the more proximal 21 bp motif in the presence of 4 μg of poly(dI-dC) non-specific competitor. The positions of the two major complexes detected by this assay, C1 (▮) and C2 (►), are indicated. Complexes C1 and C2 resulted from the binding of the cellular factors previously named HEB1 and HEB2, respectively (Montagne *et al.*, 1990). (**B**) DNA affinity precipitation assay (DAPA). The biotinylated DNA probes abc (lane 2), amc (lane 3) and mbm (lane 4) were incubated with HeLa tax nuclear extract (175 μg) and poly(dI-dC) (7.5 μg). The DNA–protein complexes were precipitated using streptavidin–agarose beads. The DNA-associated proteins were uncoupled using SDS–PAGE loading buffer and run on a 10% SDS–protein gel. The presence of the Tax1 protein was analysed by Western blotting. The position of the signal corresponding to the Tax1 protein is indicated (tax). As a control HeLa tax nuclear extract (3.2 μg) was run in parallel on the same protein gel (lane 1). (**C**) Schematic representation of the biotinylated DNA probes. The biotinylated DNA probes were obtained from plasmids pG-ABC3, pG-AMC3 and pG-MBM3 (Montagne *et al.*, 1990) by polymerase chain reaction using two 5′-end biotinylated primers. The exact sequence of the 21 bp motif either wild type (abc), mutated in domain B (amc) or mutated in both domains A and C (mbm) is given. The mutated nucleotides are boxed.

the experiment was done with both fractions taken individually, no precipitation of Tax 1 was observed (Figure 4C, lanes 1 and 2). However, mixing of both fractions

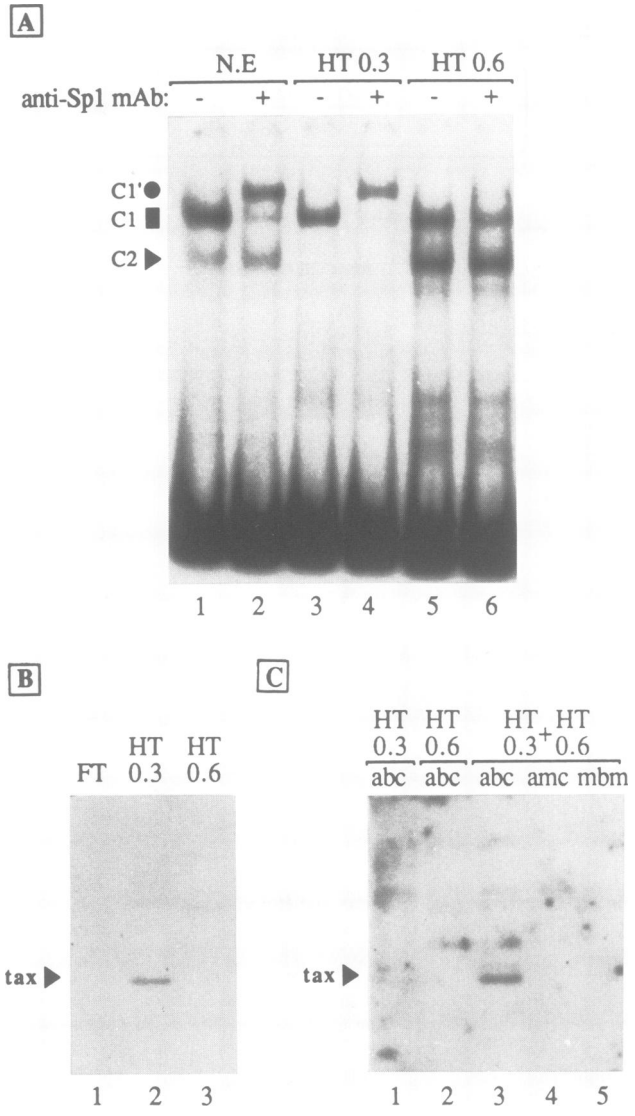

Fig. 4. (**A**) EMSA of the heparin–agarose fractions of the HeLa tax nuclear extract. HeLa tax extract was loaded on a heparin–agarose column and protein step-eluted with an increasing KCl concentration (0.3 M, 0.6 M and 1 M). The fractions eluted at 0.3 M KCl (HT 0.3 lanes 3 and 4) and 0.6 M KCl (HT 0.6, lanes 5 and 6), together with the original nuclear extract (lanes 1 and 2) were tested by EMSA. The amount of protein was 5 μg for the nuclear extract and 0.5 μg for HT 0.3 and HT 0.6. The amount of poly(dI-dC) was 2 μg for the nuclear extract and 0.3 μg for HT 0.3 and HT 0.6. The binding reactions were either directly loaded on the gel (lanes 1, 3 and 5) or preincubated before loading with a monoclonal antibody directed against Sp1 (Courey and Tjian, 1988) (lanes 2, 4 and 6). This antibody was diluted 100-fold in the binding reaction. The C1 (▮) and C2 (►) complexes are indicated together with the complex C1′ (●) resulting from addition of the anti-Sp1 antibody. (**B**) Presence of the Tax1 protein in the different fractions of the heparin–agarose column. The flow-through fraction (lane 1) of the heparin–agarose column was tested for the presence of Tax1 by Western blotting together with the HT 0.3 (lane 2) and HT 0.6 (lane 3) fractions. The position of the signal corresponding to the Tax1 protein is indicated (tax). (**C**) DAPA analysis of the heparin–agarose fractions. The HT 0.3 and HT 0.6 fractions were tested by DAPA. Each fraction was tested individually with the probe abc and the combination of both with probes abc, amc and mbm. The amount of protein was 80 μg for HT 0.3 and 30 μg for HT 0.6, 7.5 μg of poly(dI-dC) was added to each reaction. The position of the signal corresponding to the Tax1 protein is indicated (tax).

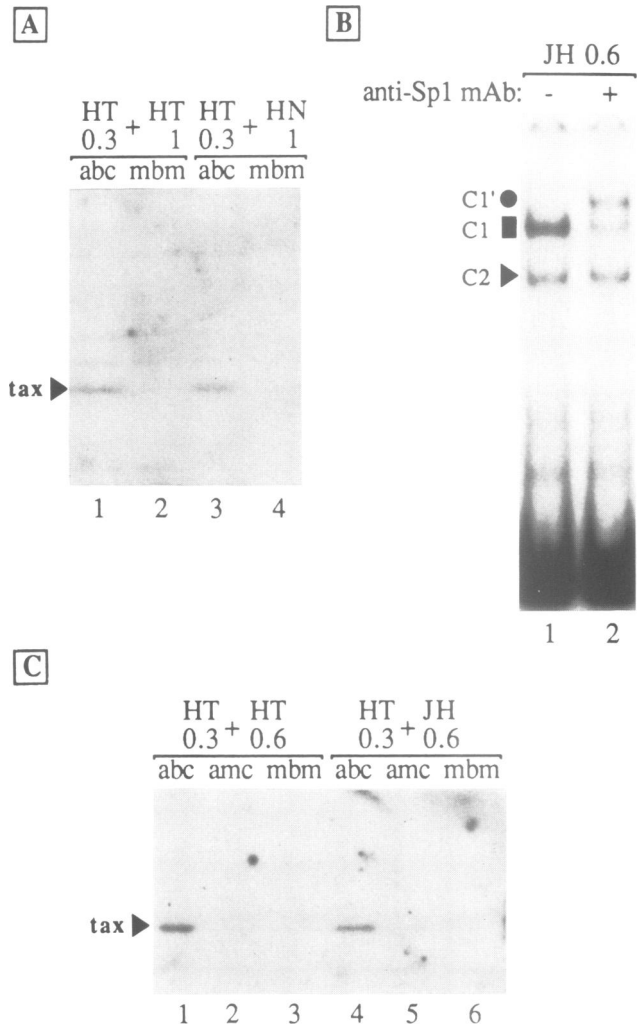

Fig. 5. Reconstitution of Tax1 binding to the 21 bp motif using fractions obtained from cells which do not express Tax1. (**A**) Nuclear extracts prepared from HeLa tax and HeLa neo cells were loaded on a heparin–agarose column. Proteins were step-eluted with an increasing KCl concentration (0.3 M and 1 M). The 0.3 M fraction from the HeLa tax nuclear extract (HT 0.3) in combination with the 1 M fraction of either the HeLa tax (HT 1, lanes 1 and 2) or HeLa neo (HN 1, lanes 3 and 4) cells was tested by DAPA. The amount of protein was 20 μg for HT 0.3 and 50 μg for HT 1 and HN 1. Five μg of poly(dI-dC) was added to each reaction. The test was made with probes abc (lanes 1 and 3) and mbm (lanes 2 and 4). The position of the signal corresponding to the Tax1 protein is indicated (tax). (**B**) A nuclear extract from Jurkat cells was fractionated on a heparin–agarose column and proteins were step-eluted with an increasing KCl concentration (0.24 M, 0.6 M and 1 M). The 0.6 M fraction (JH 0.6) was tested by EMSA either minus (lane 1) or plus (lane 2) anti-Sp1 monoclonal antibody in the binding reaction. Positions of the C1′ (●), Cl (■) and C2 (▶) complexes are indicated. (**C**) The HT 0.3 fraction in combination with either HT 0.6 (lanes 1, 2 and 3) or JH 0.6 (lanes 4, 5, and 6) was tested by DAPA. The test was performed with probes abc (lane 1 and 4), amc (lanes 2 and 5) and mbm (lanes 3 and 6). The amount of protein was 64 μg for HT 0.3 and 25 μg for HT 0.6 and JH 0.6. 10.5 μg poly(dI-dC) was added to each reaction. The position of the signal corresponding to the Tax1 protein is indicated (tax).

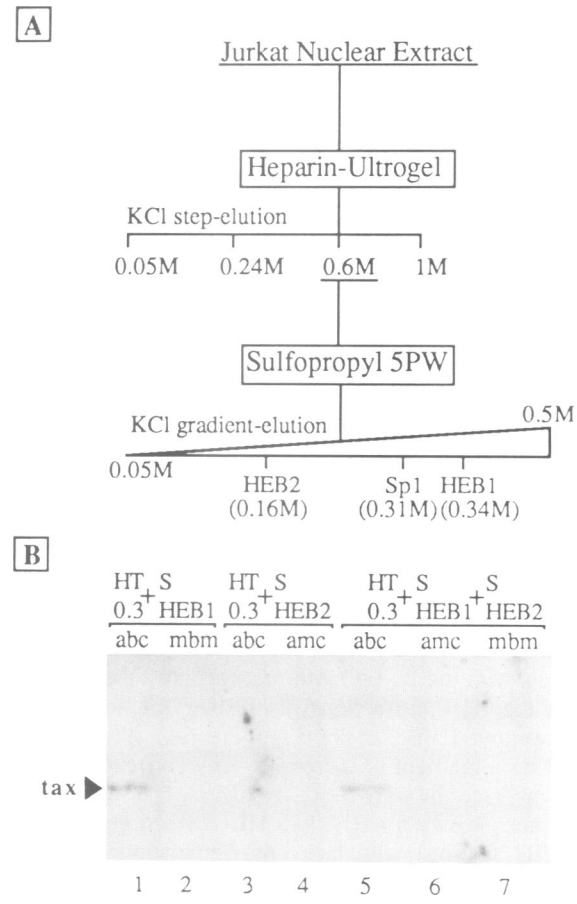

Fig. 6. Reconstitution of Tax1 binding to the 21 bp motif using separate chromatography fractions containing HEB1 and HEB2. (**A**) Purification scheme of the Jurkat nuclear extract. Nuclear extract was first fractionated on a heparin–agarose column (see legend to Figure 5). The JH 0.6 fraction was loaded onto a sulphopropyl column. The proteins were eluted by a gradient from 0.05 M to 0.5 M KCl. Each fraction was tested by EMSA. The fractions containing maximum activity for HEB1 and HEB2 factors were further analysed by DAPA. (**B**) The HT 0.3 fraction was tested in combination with the sulphopropyl fractions containing either HEB1 (S HEB1, lanes 1 and 2) or HEB2 (S HEB2, lanes 3 and 4). The mixture of HT 0.3 and S HEB1 was tested with probes abc and mbm (lanes 1 and 2), that of HT 0.3 and S HEB2 with probes abc and amc (lanes 3 and 4). The test was also performed with a mixture of the HT 0.3, S HEB1 and S HEB2 fractions with probes abc (lane 5), amc (lane 6) and mbm (lane 7). HT 0.3 (32 μg) was mixed with 100 μl of S HEB1 and/or S HEB2 and 7 μg of poly(dI-dC). The position of the signal corresponding to the Tax1 protein is indicated (tax).

reconstituted an efficient precipitation of Tax1 which was clearly specific for the probe abc as in the nuclear extract (Figure 4C, lanes 3–5). This indicated that Tax1 was unable to interact with the 21 bp motif by itself but binds to this sequence through one or several proteins contained in the HT 0.6 fraction. These proteins are presumably factors which specifically bind to the 21 bp motif. These results also indicated that Tax1 binding is not allowed by the cellular transcription factor Sp1 since it is present in the HT 0.3 fraction together with Tax1. The activity allowing Tax1 binding could have been specifically induced in the HeLa tax cells. This could have resulted from the appearance of a novel protein or the modification of a preexisting factor. To test this hypothesis we performed a DAPA by mixing the HT 0.3 fraction with fractions obtained from HeLa neo or Jurkat cells. Nuclear extracts from HeLa neo cells were fractionated on a heparin–agarose column as described above except that after the 0.3 M KCl step, a single 1 M

step was done (fraction HN 1). This was done in parallel with a HeLa tax nuclear extract (fraction HT 1). Mixing of both HT 0.3 and HT 1 fractions resulted in the specific precipitation of Tax1 (Figure 5A, lanes 1 and 2). When the HT 0.3 fraction was mixed with HN 1 a similar amount of Tax1 was specifically precipitated (Figure 5B, lanes 3 and 4). This experiment was also done with a heparin–agarose fraction of a Jurkat cells nuclear extract (fraction JH 0.6). In this case the first elution step was done at 0.24 M KCl. In contrast to HT 0.3, JH 0.6 contained both Sp1 and HEB1. Indeed, addition of the anti-Sp1 monoclonal antibody shifted part of the C1 complex (Figure 5B, lanes 1 and 2). Tested in combination with HT 0.3 by DAPA, the JH 0.6 and HT 0.6 fractions also exhibited the same activity (Figure 5C, lanes 1 and 4). These results indicate that the activity allowing Tax1 binding to the 21 bp motif which is present in the 0.6 M heparin–agarose fractions preexisted in the cell and that the constitutive presence of Tax1 does not increase this activity.

Indirect Tax1 binding to the 21 bp motif is mediated by HEB1

In order to determine the cellular protein which specifically interacts with the 21 bp motif that mediates the binding of Tax1, the JH 0.6 fraction was further fractionated on a sulphopropyl HPLC column (Figure 6A). Proteins were eluted by a gradient of increasing KCl concentration. The different fractions were tested by EMSA. Those containing maximum activity for HEB1 (S HEB1 fraction) and HEB2 (S HEB2 fraction), were tested in combination with the HT 0.3 fraction by DAPA. Mixing of S HEB1 and HT 0.3 reconstituted an efficient and specific precipitation of Tax1 (Figure 6B, lanes 1 and 2), whereas the S HEB2 fraction was found to be inactive (Figure 6B, lane 3). Addition of both S HEB1 and S HEB2 did not significantly increase the Tax1 precipitation observed with S HEB1 alone (Figure 6B, compare lanes 1 and 5). From the three proteins identified as interacting with the 21 bp motif in the JH 0.6 fraction: HEB2, Sp1 and HEB1, only the latter allowed binding of Tax1.

Since the previous characterization of HEB1 that we carried out was in fact that of both Sp1 and HEB1 together, some characteristics of the latter cellular factor were re-evaluated using the S HEB1 fraction. Addition of anti-Sp1 monoclonal antibody did not lead to the appearance of a slower migrating band (Figure 7A, compare lanes 1 and 2). This indicated that S HEB1 is free of Sp1. In agreement with what was previously found, mutation of both domains A and C led to a complete loss of HEB1 binding to the 21 bp motif (Figure 7A, lane 4). Mutation of domain B significantly decreased the intensity of this binding which, however, remained detectable (Figure 7A, lane 3). In order to locate the binding site of HEB1 precisely, a DMS methylation interference study was done using the S HEB1 fraction. The G residues identified in this study were exactly the same as those that we have previously identified and which are depicted in Figure 1 (C.Béraud, unpublished results). When the S HEB1 fraction was tested by DAPA, mutation of the central domain B led to a complete loss of Tax1 precipitation as was the case for the mutation of both domains A and C (Figure 7B, lanes 1–3). The effect of a chelating agent, 1,10-phenanthroline, was also tested by EMSA. When added in the binding reaction, this chemical agent prevented binding

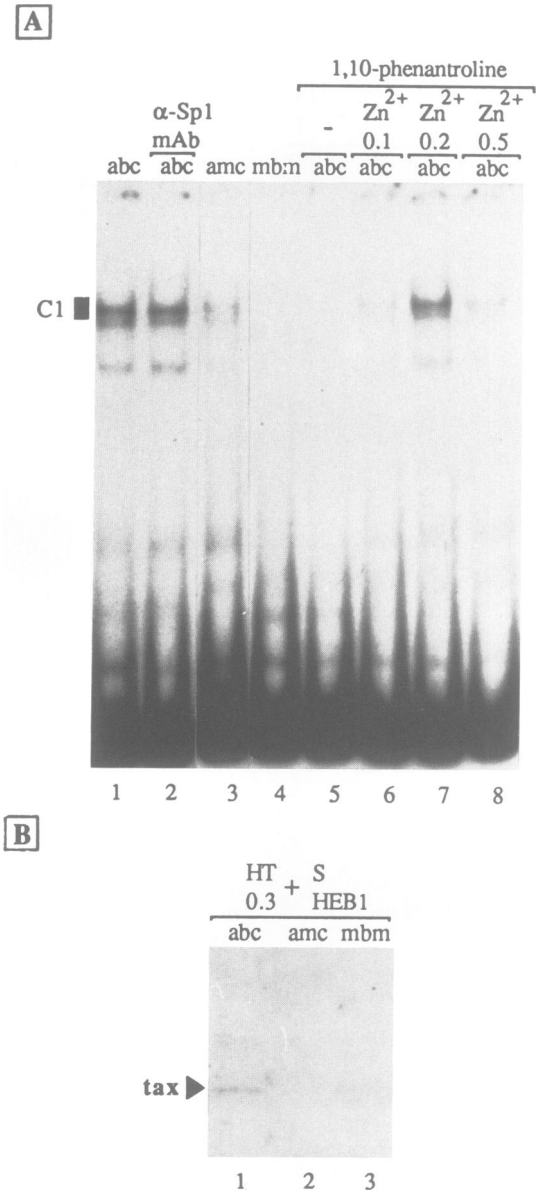

Fig. 7. (**A**) EMSA of the S HEB1 fraction. The S HEB1 fraction was tested by EMSA using probes containing one copy of the 21 bp motif which was either wild type (abc, lanes 1, 2, 5, 6, 7 and 8), mutated in domain B (amc, lane 3) or mutated in domains A and C (mbm, lane 4). These probes were *Eco*RI–*Hin*dIII fragments of plasmids pG-ABC1, pG-AMC1 and pG-MBM1 (Montagne *et al.*, 1990). The amounts of protein and poly(dI-dC) were 250 ng and 150 ng respectively. The effect of addition of anti-Sp1 monoclonal antibody to the binding reaction was analysed (lane 2). The binding reactions were also done in the presence of 1 mM 1, 10-phenanthroline either alone (lane 5) or with 0.1 mM (lane 6), 0.2 mM (lane 7) and 0.5 mM (lane 8) $ZnCl_2$. The position of complex C1 (■) is indicated. (**B**) The S HEB1 fraction mixed with the HT 0.3 fraction was tested by DAPA using the probes abc (lane 1), amc (lane 2) and mbm (lane 3). 32 μg of HT 0.3 was mixed with 100 μl of S HEB1 and 10.5 μg of poly(dI-dC). The position of the signal corresponding to the Tax1 protein is indicated (tax).

of HEB1 to the 21 bp motif. This binding was restored when Zn^{2+} cations were added together with 1,10-phenathroline. The concentration of Zn^{2+} cations required was very precise. If the concentration was too high this also led to a loss of binding (Figure 7A, lanes 6–8). This biochemical requirement for Zn^{2+} cations allowed us to distinguish

HEB1 from ATF/CREB factors as binding of these proteins or of HEB2 to the 21 bp motif was insensitive to 1,10-phenanthroline (C.Béraud, unpublished results). We conclude from these data that binding of Tax1 to the 21 bp motif is mediated by the cellular protein HEB1 which is not likely to be a member of the ATF/CREB family.

Discussion

The main feature of the 21 bp motif is the presence of the pentamer TGACG which corresponds to the consensus sequence of the binding sites for ATF/CREB factors (Tan *et al.*, 1989b). We have previously shown that ATF protein, which had been purified by testing its ability to interact with the adenovirus EIIaE promoter, interacted with the 21 bp motif (Montagne *et al.*, 1990). Tan *et al.* (1989) showed that purified proteins interacting with the 21 bp motif were able to bind various ATF or CREB binding sites (Tan *et al.*, 1989b). The HTLV-I promoter has also been shown to be inducible by cAMP through the 21 bp motif (Poteat *et al.*, 1989). Mutation of the TGACG pentamer completely abolishes the Tax1-responsiveness of the 21 bp motif, either in front of a reporter gene, or in the context of the viral promoter (Jeang *et al.*, 1988; Fujisawa *et al.*, 1989; Montagne *et al.*, 1990). All these observations have led different authors to propose that induction of the 21 bp motif enhancer activity was mediated by a ATF/CREB protein, probably by some Tax1-induced modification of the transcription factor. However, this hypothesis has not as yet received direct experimental confirmation. Furthermore, it has been shown that the sequence requirements for cAMP and Tax1 induction of the 21 bp motif are different (Nakamura *et al.*, 1989).

Other cellular proteins besides ATF/CREB factors interact with the 21 bp motif. We have previously identified a protein, called HEB2, which binds to domain B and part of domain C. Binding of this protein was abolished when the TGACG pentamer was mutated (Montagne *et al.*, 1990). Although not yet demonstrated, it is possible that HEB2 belongs to the ATF/CREB family. From the experimental data presented in this paper, it is clear that the ubiquitous cellular transcription factor Sp1 also binds to the 21 bp motif. This is rather surprising since the 21 bp motif, although including G-C rich stretches, is quite distinct from the Sp1 consensus: 5′-G/T-G-G-G-C-G-G-G/A-G/A-C/T-3′ (Kadonaga *et al.*, 1986). It is possible that the 21 bp motif contains one or several weak affinity binding sites for Sp1 and that a high abundance of this protein in the extract compensates for this low affinity. In agreement with this and in contrast to that found for HEB1 and HEB2, the binding of Sp1 to the 21 bp motif was not detected by EMSA when herring sperm DNA was used as a non-specific competitor in place of poly(dI−dC) (C.Béraud, unpublished results). Finally, another cellular protein, that we call HEB1, specifically binds to the 21 bp motif. This protein can interact with either domains B and C, or domains A and B (Montagne *et al.*, 1990), and requires Zn^{2+} cations to bind DNA. This biochemical criterion indicates that this cellular protein is not likely to be a member of the ATF/CREB family.

The experimental results presented in this paper show that Tax1 binds specifically to the 21 bp motif. This binding is indirect and its establishment requires the cellular factor HEB1. Mariott *et al.* have previously shown that a 36 kD cellular protein called TIF1 can allow the indirect binding of Tax1 to DNA (Marriott *et al.*, 1990). The TIF1 binding site is located between the two more proximal 21 bp motifs. Binding of TIF1 to its site is not competed out by an oligonucleotide containing a 21 bp motif (Marriott *et al.*, 1989). This indicates that TIF1 is different from HEB1. The TIF1 binding site does not exhibit a Tax1-induced enhancer activity by itself but increases the responsiveness of a nearby 21 bp motif (Marriott *et al.*, 1989). The presence of the TIF1 binding site near the two more proximal 21 bp motifs is likely to facilitate the functional interaction of Tax1 with these sequences. We have previously shown that mutation of either the central pentamer TGACG or of both the flanking domains A and C together, abolished the Tax1-induced enhancer activity of the 21 bp motif (Montagne *et al.*, 1990). Mutation of domains A and C prevents binding of HEB1, while mutation of the central pentamer reduces it notably. Since this latter mutation has a dramatic effect on the inducibility of the 21 bp motif, we previously proposed that the Tax1 activation involved both HEB1 and another protein interacting with the TGACG pentamer, probably HEB2. We show in this report that mutation of domain B leads to a complete loss of Tax1 binding and that this is true even when the DNA affinity precipitation assay is performed with a fraction containing only HEB1. Therefore, HEB1-mediated binding of Tax1 requires the integrity not only of the flanking domains A and C, which are required for binding HEB1, but also of the central TGACG pentamer. Therefore the sequence elements necessary for HEB1-mediated Tax1 binding are identical to those necessary for the Tax1-induced enhancer activity of the 21 bp motif. From these data two models are possible. The fully active DNA−protein complex could correspond to the 21 bp motif and the HEB1 factor associated with Tax1. The requirement for domain B can be explained in different ways. The reduced binding of HEB1 could be sufficient to prevent Tax1 association. HEB1 could also have to bind to domain B to be in a proper conformation for interacting with Tax1. Finally, with the help of HEB1, Tax1 could directly interact with domain B. Chemical crosslinking experiments conducted with purified proteins should allow to test this latter hypothesis. Alternatively, the transcriptionally active complex could include the 21 bp motif, an ATF/CREB factor and HEB1 associated with Tax1. In this second model it remains to be established if both HEB1 and an ATF/CREB protein can bind simultaneously to the 21 bp motif. Since DMS methylation interference profiles of HEB1 and ATF/CREB factors overlap on domain B, indicating that binding of one of these proteins could prevent the binding of the other, this second model seems unlikely. The role of the binding of various cellular factors to the 21 bp motif is probably to allow the response of the viral genetic expression to different cellular or viral signals rather than to create a complex structure consisting of several proteins to mediate the Tax1 effect.

Association of a viral transcriptional activator with a cellular protein binding to a DNA sequence element has been reported for adenovirus and herpes virus. By using chimeric proteins containing the GAL4 DNA binding domain linked to the coding sequence of different members of the ATF family, Liu *et al.* have shown that EIa interacts functionally with ATF2 (Liu and Green, 1990). The herpes simplex VP16 transactivator increases the enhancer activity of the TAATGARAT (R = purine) motif through interaction with

the octamer binding protein oct-1. In this latter case formation of the ternary complex consisting of the DNA motif, oct-1 and VP16 was clearly detectable by EMSA (Preston *et al.*, 1988; Stern *et al.*, 1989). This is not the case for Tax1 interaction with the 21 bp motif. This could be explained by a rapid dissociation rate of this latter complex. The DNA affinity precipitation assay has the advantage to detect complexes established at equilibrium.

Our data clearly establish that Tax1 forms a complex with a specific cellular protein binding to the target sequence of this viral transactivator. A complete understanding of the molecular mechanisms allowing the strong activation of the enhancer activity of the 21 bp motif by the Tax1 protein will now require study of the direct or indirect relationships of Tax1 with the transcriptional machinery. In this regard it would be interesting to analyse the interaction of Tax1 with a general transcription factor. An obvious candidate is the TFIID protein, but a recent *in vitro* study by Lin and Green has shown that the rate limiting step in the establishment of the transcriptional initiation complex was the association of the general transcription factor TFIIB, and that the acidic activator VP16 interacts more strongly with this factor than with TFIID (Lin and Green, 1991). The recent molecular cloning of cDNAs encoding TFIID as well as other general transcription factors (Peterson *et al.*, 1990; Kao *et al.*, 1990; Zheng *et al.*, 1990) will allow direct biochemical studies to determine how Tax1 acts on the transcriptional initiation complex.

Materials and methods

Transfection and selection of stable transformants

HeLa cells, grown in monolayers to 40% confluence, were transfected by the calcium phosphate coprecipitation method with either the Tax1-expressing plasmid p36/7 polyA tax (2 μg) or the control plasmid p36/7 polyA (2 μg) and pUC18 DNA (13 μg). 20 h after transfection cells were washed and culture medium containing 1.2 mg/ml G418 sulphate (GIBCO) was added. This medium was changed every three days and cells were grown under selection until resistant clones were visible. Clones were isolated and tested by cotransfection of the test plasmids pG1, pΔG-ABC3 (2 μg each) and pUC18 DNA (11 μg). Total RNA was prepared by the hot phenol procedure and analysed by S1 nuclease mapping as previously reported (Montagne *et al.*, 1990). One positive clone resulting from transfection by p36/7 polyA tax was selected as well as a control clone obtained by transfection of p36/7 polyA. These clones were named HeLa tax and HeLa neo respectively.

Nuclear extracts, chromatography fractions and EMSA

Preparation of nuclear extracts from HeLa tax, HeLa neo or Jurkat cells, grown in suspension, as well as their fractionation on a heparin–agarose column was carried out as previously described (Montagne *et al.*, 1990) with the following modification: the buffers used for final dialysis of the extracts and for elutions from the heparin–agarose column contained 10% glycerol in place of 20%.

After dialysis against buffer D (20 mM Tris, pH 7.9, 10% glycerol, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 10 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM PMSF), 10 mg of the JH 0.6 fraction was loaded on a sulphopropyl 5PW column. The proteins were eluted by a KCl gradient (0.05–0.5 M in buffer D) in 45 min at a flow rate of 0.7 ml/min.

EMSA was conducted as previously described (Montagne *et al.*, 1990). The end-labelled probe was the *Eco*RI–*Hin*dIII restriction fragment of the plasmid pG-ABC1, pG-AMC1 or pG-MBM1 (Montagne *et al.*, 1990). It contained one copy of the more proximal 21 bp motif. The exact amounts of extracts or fractions and of non-specific competitor [poly(dI-dC)] used in the different binding reactions are indicated in the legends to the figures.

DNA affinity precipitation assay

Two 20mer oligonucleotides flanking the *Xho*I restriction site on each strand in the plasmid pG1 were synthesized with an amine function at their 5′ end (Aminolink II, A.B.I). Coupling to biotin was done by incubating the oligonucleotide (50 μg) with a 500× molar excess of sulpho-NHS biotin (Pierce) for 3 h at 56°C in 50 mM bicarbonate buffer pH 8.5 (reaction volume: 100 μl). The biotinylated form was HPLC-purified according to standard oligonucleotide purification procedure. The DNA probes were obtained by polymerase chain reaction. The reaction was performed in a final volume of 100 μl with 1 U of *Taq* polymerase (Promega), the two biotinylated primers (0.3 μg), plasmid DNA (10 ng) and dNTPs (0.2 mM). One reaction (35 cycles) usually gave 2 μg of DNA fragment. The DNA affinity precipitation assay was performed in a total volume of 500 μl by mixing poly(dI-dC) as non-specific competitor, nuclear extracts or chromatography fractions and 2 μg of biotinylated DNA probe. The exact amounts of extracts or fractions and of non-specific competitor used in the different binding reactions are indicated in the legends to the figures. The final composition of the binding reaction was the same as that of buffer D except that the KCl concentration was 80 mM and that 0.25% Triton X-100 was included. The mix was incubated for 30 min on ice and 50 μl of streptavidin–agarose beads equilibrated in buffer D plus 0.25% Triton X-100 were added. After a 30 min incubation with agitation, the agarose beads were collected by brief centrifugation and washed twice in buffer D plus 0.25% Triton X-100. Proteins were uncoupled from the DNA probe by addition of 2× SDS–PAGE loading buffer and heating at 90°C for 5 min. After centrifugation of the agarose beads the supernatant was loaded on a 10% SDS–protein gel. Detection of the Tax1 protein was achieved by Western blotting using a rabbit polyclonal antibody directed against Tax1 (Hanly *et al.*, 1989) and radiolabelled protein A (Amersham).

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