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Characterization of a novel T lymphocyte protein which binds to a site related to steroid/thyroid hormone receptor response elements in the negative regulatory sequence of the human immunodeficiency virus long terminal repeat

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ABSTRACT

We have previously identified a T lymphocyte protein which binds to a site within the LTR of the human immunodeficiency virus type 1 (HIV-1) and exerts an inhibitory effect on virus gene expression. The palindromic site (site B) recognized by this protein is related to the palindromic binding sites of members of the steroid/thyroid hormone receptor family. Here we characterize the T cell protein binding to this site as a 100 kD protein which is most abundant in T cells and which binds to site B as a 200 kD complex. This protein is distinct from other members of the steroid/thyroid hormone receptor family including the COUP protein which has a closely related DNA binding specificity.

INTRODUCTION

The expression of the human immunodeficiency virus type 1 (HIV-1) genome is regulated by the binding of a wide variety of cellular transcription factors to specific binding sites within the long terminal repeat (LTR) region of the virus (for review see 1). Thus for example the enhancer region of the LTR (−105 to −80) binds the cellular transcription factor NF kappa B and renders the LTR responsive to stimulation by T cell activation or macrophage differentiation (2–4). Conversely the region of the LTR upstream of the enhancer has been identified as a negative regulatory element (NRE) whose removal results in an increase in the level of HIV-1 gene expression and viral replication in both Jurkat T cells and macrophages (5–7). Moreover, this region can also inhibit gene expression when linked to a heterologous promoter (8).

In an earlier study of the transcription factor binding sites within the NRE we identified a specific site (bases −328 to −347, referred to as site B) whose mutation within the LTR resulted in an increase in LTR-driven gene expression in Jurkat T cells (9). Two subsequent studies have confirmed the stimulatory effect of mutations which disrupt site B on LTR-driven gene expression both in Jurkat (10) and H9 T cells (11). Hence the binding of T cell transcription factor(s) to site B exerts an inhibitory effect on viral gene expression which may account, at least in part for the negative effect of the NRE region which was observed in earlier studies (5–7).

The major T cell protein which binds to site B recognizes a sequence in which a five base pair inverted palindrome is separated by a nine base pair spacer (9). This palindromic sequence shows strong homology to the palindromic binding sites of a number of members of the steroid/thyroid hormone receptor family of transcription factors, although these sites have a different spacing between the two halves of the palindrome (12). Such homology suggested that the major T cell site B-binding protein might be a member of the steroid/thyroid hormone receptor family. In agreement with this idea we showed that this protein could bind to the binding sites for the thyroid hormone receptor or the oestrogen receptor although less strongly than to site B itself (9). Moreover Cooney et al., (13) showed that the COUP factor, a member of the steroid/thyroid hormone receptor family could also bind to site B. Here we report the further characterization of the major T cell protein binding to site B and show that it has a molecular weight of 100kD and is distinct from the COUP protein.

MATERIALS AND METHODS

Cell culture and nuclear extract preparation

Jurkat cells were cultured in RPMI 1640 medium supplemented with penicillin, streptomycin (100 U of each) plus 10% foetal calf serum. Cells were harvested from culture by centrifugation, washed once in phosphate buffered saline before preparation of a nuclear extract. Normal peripheral blood T-lymphocytes were activated with PHA and PMA and the numbers expanded in culture with IL-2 for 5 days before harvesting. HeLa cells were cultured in DMEM with antibiotics and 10% FCS until sub-confluent, then washed twice with cold PBS and harvested with a cell-scraper into cold PBS.

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Preparation of nuclear extracts was performed as described previously (9). Nuclear extracts were finally diluted in 20mM Hepes pH 7.8, 2mM $MgCl_2$, 0.5mM DTT, 320mM NaCL. Protease inhibitors were present in all buffers.

DNA mobility shift assays

Binding reactions were performed on ice for 20 minutes with 1 μl (6−8 μg protein) of nuclear extract, 5 fmol of radiolabelled oligonucleotide probe in 10mM Tri-HCl pH 7.8, 50mM KCl, 2mM $MgCl_2$, 0.5mM DTT, 4% Ficol and 5 μg poly (dI:dC):; poly (dI:dC) in a final volume of 20 μl. After incubation the binding reaction was resolved on a 4.5% polyacryamide gel at 4°C. Following electrophoresis the gel was dried and autoradiographed. The oligonucleotides used were a site B probe, a 30 base pair sequence derived from the LTR of HIV-1 strain HXB2:- 5'TCGACAGGGGTCAGATATCCACTGACCTTC and a coup-tf probe, a 30 base pair sequence derived from the chicken ovalbumin upstream promoter (14) 5'TCGAATGGTGT-CAAGGTCAAACTTCT. Oligonucleotides were synthesized with a XhoI/SalI compatible 5' overhang. Radiolabelling was performed with α^{32}P-dCTP by filling in the overhang using AMV reverse transcriptase.

UV-crosslinking

Site B and Coup-tf oligonucleotides were synthesized with specific thymidine bases substituted by 5-bromodeoxyuridine (5-BrdU). Double stranded oligonucleotides were labelled as described and a scaled-up version of the binding reaction performed using 50fmol of labelled oligonucleotide, 5 μl nuclear extract in a final volume of 50 μl on ice for 20 minutes. The reaction mixture was then exposed to ultra-violet irradiation (310 nm) from an inverted transilluminator box at a distance of 2 cm for 30 minutes keeping the reaction cooled on ice. Following irradiation the reaction was loaded and resolved on a 4.5% polyacryamide gel. After electrophoresis, the wet gel was exposed to X-ray film to localize specific retarded bands, these were excised from the gel, heated to 100°C in ×2 SDS gel loading buffer (100 mM Tris-HCl pH 6.9, 100 mM DTT, 4% SDS, 20% glycerol, 0.1% bromophenol blue) for 10 minutes then carefully positioned in the slot of a SDS 12% polyacrylamide gel. The gel was run with ^{14}C labelled protein molecular weight markers.

Gel renaturation analysis

After SDS-polyacrylamide gel electrophoresis, gel slices from different molecular weight regions were ground and proteins eluted overnight at 4°C into 0.5 ml of 50 mM Tris/HCl (pH 7.9), 0.1% SDS, 0.1 mg/ml BSA, 1mM DTT, 0.2 mM EDTA, 0.1 mM PMSF and 2.5% glycerol. Following centrifugation the supernatant was precipitated with four volumes of acetone at −20°C for 2 hours, washed with methanol and dried. The dried pellet was dissolved in 2.5 μl of a saturated urea solution and diluted with 125 μl of 20 mM Tris/HCl (pH 7.6), 10 mM KCl, 2 mM DTT and 10 μM PMSF. Renaturation was allowed to proceed for 18 hours at 4°C (15).

Ferguson plot

The principle of the Ferguson plot has been described in detail before (16). The method was performed using the following protein standards. α-lactalbumin (14.2kd), carbonic anhydrase (29kd), chicken egg albumin (45kd), bovine serum albumin (66kd as monomer), urease (272kd as trimer). Polyacrylamide gels of 4.0, 4.5, 5.0, 5.5 and 6.0% were run using the same buffer conditions as for the gel retardation assay. On the same gels, binding reactions using site B as probe and Jurkat or HeLa nuclear extracts were run. Following electrophoresis gels were fixed, stained with Coomassie blue and destained under standard conditions. The gels were then dried under vacuum and autoradiographed. The relative mobility (Rf) of each protein standard was determined from the position of the Coomassie-stained band while the Rfs for DNA-protein complexes was determined from the autoradiograph.

RNA isolation and Northern blotting

Total RNA was prepared using the acid-guanidinium method as described by Chomczynski and Sacchi (17) 15 μg of RNA was loaded for each sample and resolved on a 1.2% agarose gel. RNA was blotted onto a nylon membrane (Genescreen Plus, DuPont) and probed with a 340 base pair fragment containing the DNA binding domain of ear-3 (18) which is identical to Coup-tf (19). The fragment was generated by PCR from an ear-3 clone and labelled by random hexanucleotide priming (20). Hybridization was carried out in 5×SSC (1.5 MNaCl, 150 mM sodium citrate) and 50% formanide at 42°C. Filters were washed in two changes of 2×SSC, 0.1% SDS for two thirty minute periods at 50°C.

RESULTS

In our earlier study (9) we established that the major T cell protein which binds to site B could also bind with lower affinity to the binding sites for the oestrogen receptor and the thyroid hormone receptor but not to that of the glucocorticoid receptor. Similarly by using purified recombinant proteins we were able to demonstrate that several previously characterized members of the steroid/thyroid hormone receptor family including the thyroid hormone receptor, the oestrogen receptor and the α, β and γ forms of the retinoic acid receptor can bind to site B although they are distinct from the major T cell protein which binds to this site (data not shown).

In view of the observation of Cooney et al., (13) that the COUP-TF member of the steroid/thyroid hormone family could bind to site B, we also compared the binding of Jurkat T cell and HeLa cell extracts to site B and a COUP binding site taken from the ovalbumin promoter. In these experiments (Figure 1a) indistinguishable binding patterns were observed using both probes. The specific complexes indicated as A, B and C were readily removed by low levels of unlabelled probe (Figure 1a) whereas the non-specific complexes were not removed by up to five hundred fold excess of unlabelled probe (data not shown).

These data indicate that very similar or identical T cell and HeLa cell proteins bind to both site B and a COUP binding site. Indeed the two major high mobility shifts observed when HeLa extracts are incubated with either of these probes (indicated as B and C in Figure 1a) are likely to be due to the COUP protein itself which has been extensively characterized in HeLa cells (21−23). Thus this protein has been shown to consist of a series of polypeptides from 43−53 kD in size, and therefore to produce two closely spaced complexes in DNA mobility shift assays (23−25). However, a complex of the same size as the major HeLa cell complex was present at a much lower level when either COUP or site B probes were incubated with the Jurkat T cell extract, the predominant complex formed with this extract being of much lower mobility (indicated as A in Figure 1a). Thus the

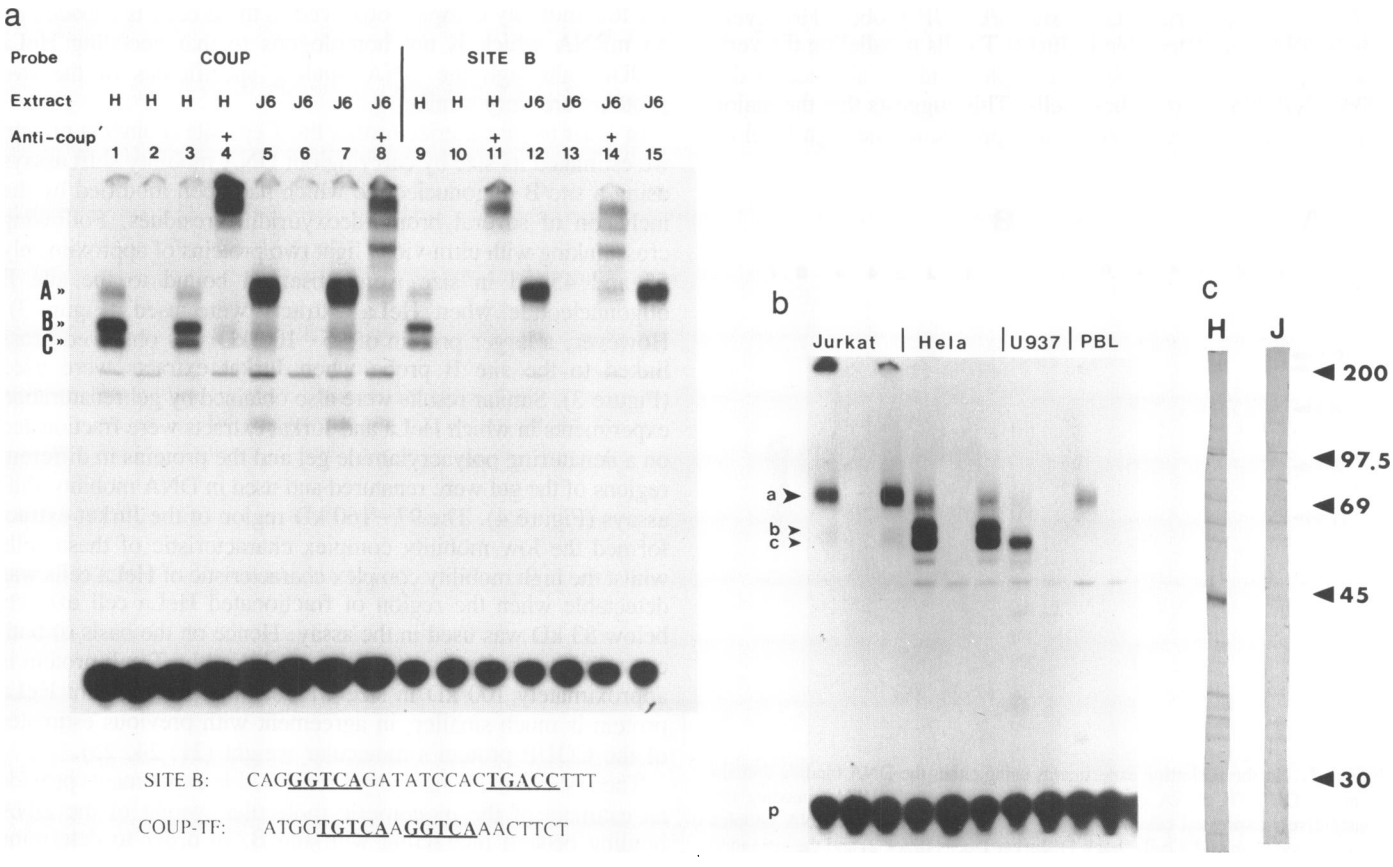

Figure 1. Panel a:- DNA mobility shift assay using the indicated COUP-TF and site B oligonucleotides and extracts from HeLa (H) or Jurkat T cells (J6). Assays were carried out in the presence of the labelled oligonucleotide and extract alone (tracks 1, 5, 9 and 12), in the presence of fifty fold excess of unlabelled probe oligonucleotide (tracks 2, 6, 10 and 13) or a similar excess of an unrelated oligonucleotide (tracks 3, 7 and 15) or with the addition of polyclonal 'anti-COUP' antibody directed against proteins which bind to the COUP-TF binding site (tracks 4, 8, 11 and 14). The arrows labelled a, b and c indicate the specific complexes forming on both probes. Panel b:- DNA mobility shift assay with the indicated extracts and a site B probe. In each case the first track shows the result with labelled probe and extract alone and the second track that in the presence of a fifty fold excess unlabelled specific competitor. In the case of the HeLa and Jurkat extracts, the results of competition with a fifty fold excess of an unlabelled unrelated oligonucleotide is shown in the third track. The arrows indicate the specific complexes forming on the probes and are labelled as in panel a. Panel c:- Western blot of HeLa (H) and Jurkat (J) proteins with a polyclonal antibody raised against all the proteins capable of binding to a COUP-TF binding site. The arrows show the positions of molecular weight makers of the indicated sizes (in kilo-daltons).

predominant T cell protein binding to site B appears to be distinct from the major COUP protein present in HeLa cells, although both proteins can bind both to site B and a COUP binding site with high affinity.

In similar experiments (Figure 1b) extracts from the U937 monocyte cell line gave a pattern on site B and COUP-TF probes similar to that of HeLa cells with a predominant high mobility complex (labelled c). In contrast activated peripheral blood lymphocytes showed a single low mobility complex (labelled a) co-migrating with that in Jurkat T cells confirming the relevance of this line in studying the T cell protein binding to Site B.

As expected, in the DNA mobility shift experiments all the proteins binding to site B or COUP-TF probes reacted with a polyclonal antiserum anti-COUP:- Figure 1a). In contrast no change in mobility was observed when pre-immune serum was added to the band shift assays (data not shown). The anti-COUP antibody was raised against the proteins retained on a column containing a COUP-TF DNA binding site (19) and will hence react with all proteins capable of binding to this site regardless of whether they are related at the protein sequence level. In Western blotting experiments (Figure 1c) this antibody detected a major 45kD protein in HeLa cells as well as a series of minor bands of up to 103kD in size. The 45kd protein was undetectable in Jurkat T cells however, providing further evidence that the HeLa COUP protein and the major T cell protein are distinct.

To investigate further the relationship of COUP and the T cell protein we probed RNA prepared from HeLa and Jurkat T cells with a cDNA clone derived from the *ear*-3 mRNA (18) which is identical to that encoding HeLa cell COUP (19). In these experiments (Figure 2a) strong hybridization was detected to two HeLa cell mRNAs of 4.5 kb and 2.5 kb in size which were also detectable in U937 cells. The size of the 4.5 kb transcript is in agreement with that previously determined for the COUP/*ear*-3 mRNA by Miyajima et al., (18) whilst the 2.5 kb mRNA may be due to cross-hybridization with the 2.5 kb transcript of the closely related *ear*-2 gene (18). No hybridization was detected with the COUP probe in mRNA prepared from Jurkat T cells or from the monocyte lines THP-1 and HL60 although these RNAs contained similar levels of the constitutively expressed mRNA encoding the calcium binding protein p68 (26: Figure 2b). Hence the COUP mRNA is detectable only in HeLa and U937 cells which form the major high mobility complex in DNA

mobility shift experiments on site B/COUP probes. However, this mRNA is undetectable in Jurkat T cells paralleling the very low level of the high mobility complex and the absence of the 45kD COUP protein in these cells. This suggests that the major T cell protein binding to site B and producing the high level of the low mobility complex observed in these cells is encoded by an mRNA which is not homologous to that encoding HeLa COUP, although the DNA binding specificities of the two proteins are very similar.

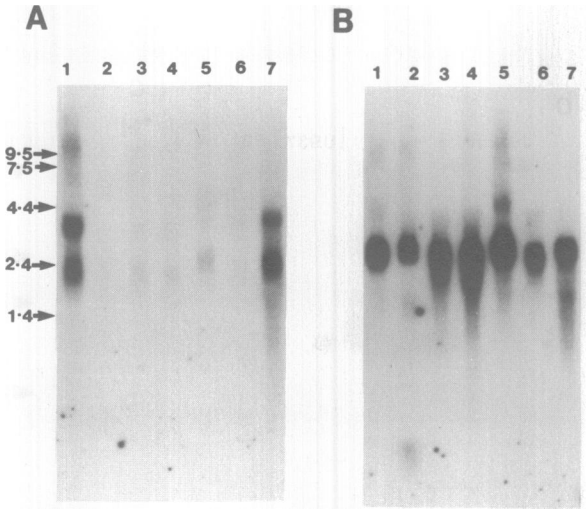

Figure 2. Northern blotting experiments using either the DNA binding domain from a COUP-TF cDNA clone (panel a) or a control cDNA encoding the constitutively expressed calcium binding protein p68 (panel b). RNA samples were derived from HeLa cells (track 1). Jurkat T cells (track 2) and the monocyte cell lines HL60 (tracks 3 and 4), THP1 (tracks 5 and 6) and U937 (track 7). For HL60 and THP1, RNA samples were prepared both prior to (tracks 3 and 5) or after (tracks 4 and 6) differentiation to a macrophage phenotype by treatment with retinoic acid. The arrows show the positions of RNA size markers of the sizes indicated (in Kilobases).

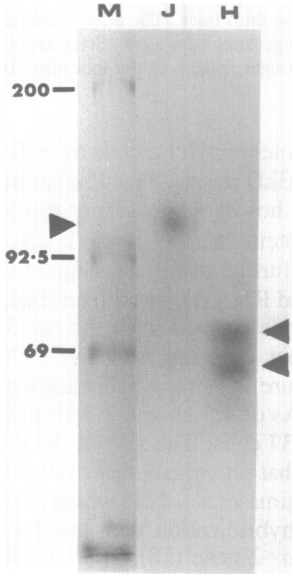

Figure 3. Ultra-violet cross linking experiment using Jurkat (J) and HeLa (H) cell extracts and a site B probe. The sizes of the two HeLa cell proteins binding to the probe (arrowed) were estimated as 45kD and 55kD and that of the Jurkat protein (arrowed) as 95–100kD by comparison to the sizes of co-electrophoresed protein molecular weight markers (track M) and subtraction of the molecular weight of the cross-linked oligonucleotide.

In order to characterize further the T cell site B binding protein we estimated its size by carrying out DNA mobility shift assays using a site B oligonucleotide which had been modified by the inclusion of several bromo-deoxyuridine residues. Following cross linking with ultra-violet light two proteins of approximately 55 and 45 kd in size were observed bound to the site B oligonucleotide when HeLa extracts were used (Figure 3). However, a larger protein of 95–100 kD was observed cross linked to the site B probe when Jurkat extracts were used (Figure 3). Similar results were also obtained by gel renaturation experiments in which HeLa and Jurkat extracts were fractionated on a denaturing polyacrylamide gel and the proteins in different regions of the gel were renatured and used in DNA mobility shift assays (Figure 4). The 97–160 kD region of the Jurkat extract formed the low mobility complex characteristic of these cells whilst the high mobility complex characteristic of HeLa cells was detectable when the region of fractionated HeLa cell extracts below 83 kD was used in the assay. Hence on the basis of both cross-linking and gel renaturation analysis, the T cell protein is approximately 100 kD in size whereas the predominant HeLa protein is much smaller, in agreement with previous estimates of the COUP protein's molecular weight (21, 24, 25).

The UV cross linking and gel renaturation experiments provide an estimate of the monomeric molecular weight of the DNA binding protein interacting with site B. In order to determine whether this protein binds as a monomer or as part of larger complex, we compared the mobility of the complex binding to site B with that of a number of proteins of known molecular weight on a series of non-denaturing gels of different acrylamide concentrations. A Ferguson plot of the resulting data (Figure 5) indicated a molecular weight of 218 kD for the major Jurkat complex and 108 kD for the HeLa cell site B DNA binding complex. A similar size of 90kD was also observed using either a gel filtration assay or glycerol gradients to determine the native

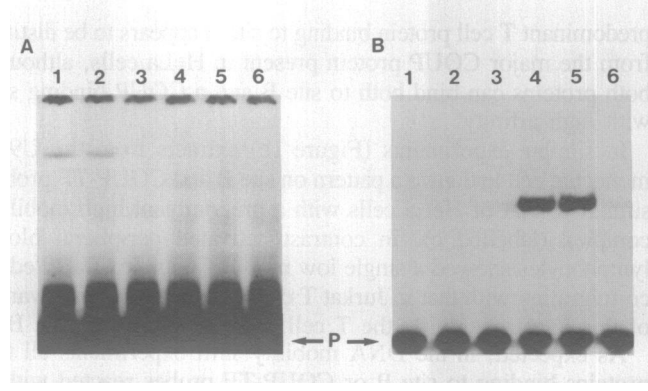

Figure 4. DNA mobility shift assay with renatured protein fractions previously separated on denaturing polyacrylamide gels. Panel A shows the result with the fractionated Jurkat extract and labelled site B probe, panel B that with fractionated HeLa extract and labelled COUP-TF probe. In each case tracks 1–3 show the result using the fraction containing proteins of 97–160kD in size, tracks 4–6 that with the fraction containing proteins below 83kD. Tracks 1 and 4 show the result with no unlabelled competitor oligonucleotide, tracks 2 and 5 that with a fifty fold excess of a non-specific competitor oligonucleotide (site A in the HIV-1 LTR) and tracks 3 and 6 that with a similar excess of the homologous competitor oligonucleotide. P indicates the position of free probe.

molecular weight of HeLa cell COUP in the absence of DNA (23, 25) suggesting that this COUP DNA binding complex also exists in solution. Therefore the T cell protein binds to site B as a larger complex which probably consists of a dimer of two approximately 100 kD proteins whilst the HeLa protein probably binds to site B as a dimer of two approximately 50 kD proteins.

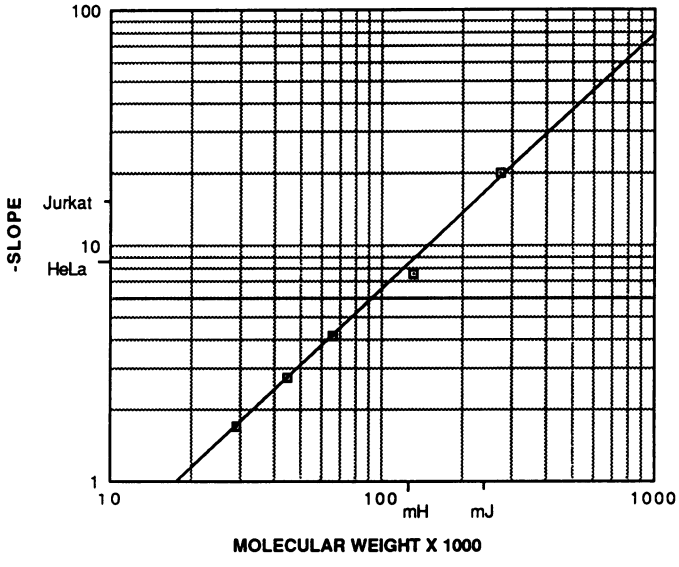

Figure 5. Ferguson plot for HeLa and Jurkat site B-binding proteins and standards of known molecular weight. The slope of the graph obtained by plotting the mobility of each protein standard in non-denaturing gels of varying polyacrylamide concentrations is subsequently plotted against its molecular weight. The molecular weight of the HeLa (mH) and Jurkat (mJ) proteins can then be calculated from the slope obtained when these proteins are similarly treated. The values obtained in this way are 240 for the Jurkat protein and 130 for the HeLa protein. Following subtraction of 22 to correct for the molecular weight of the 32 mer oligonucleotide probe this gives a value of 218 kD for the Jurkat protein complex and 108 kD for the HeLa complex.

Having established that the major T cell protein binds to the palindromic site B as a larger, possibly dimeric, complex we investigated the effect on such binding of altering the natural nine base pair spacing between the two halves of the palindrome. In these experiments (Figure 6) the degree of binding was greatly reduced by decreasing the spacing to only four bases but was effectively restored by reducing the spacing to zero or one base pair. Hence binding of the site B protein complex is dependent on the precise configuration of the two halves of the palindrome. Interestingly however, such constraints appear to be relaxed when the two halves of the palindrome are further apart since high level binding is retained when the spacing is increased to either fourteen or nineteen bases (Figure 6).

DISCUSSION

We have previously identified a site (site B) in the HIV-LTR which has a negative effect on the level of gene expression driven by the LTR in Jurkat T cells (9). This site is related to the binding sites of members of the steroid/thyroid hormone receptor family (12) and in agreement with this idea site B binds several members of this family. Similarly Cooney et al., (13) showed that the COUP factor which is a member of this family could also bind to site B in HeLa cell extracts. In agreement with this finding we show here that the major HeLa cell protein binding to site B is identical to COUP in DNA binding specificity and has a similar monomeric (45kD) and native (100kD) molecular weight to those previously reported for the HeLa COUP protein (21, 23, 25).

This COUP protein is present only at very low levels however, in Jurkat T cells where the predominant DNA binding complex is of lower mobility and is produced by the binding of a larger 100 kD protein. Cooney et al., (13) suggested that this T cell protein was a higher molecular weight form of the predominant HeLa cell COUP protein on the basis of its reactivity with a polyclonal anti-COUP antibody. However, this antibody was raised against proteins which bind to a COUP binding site (19)

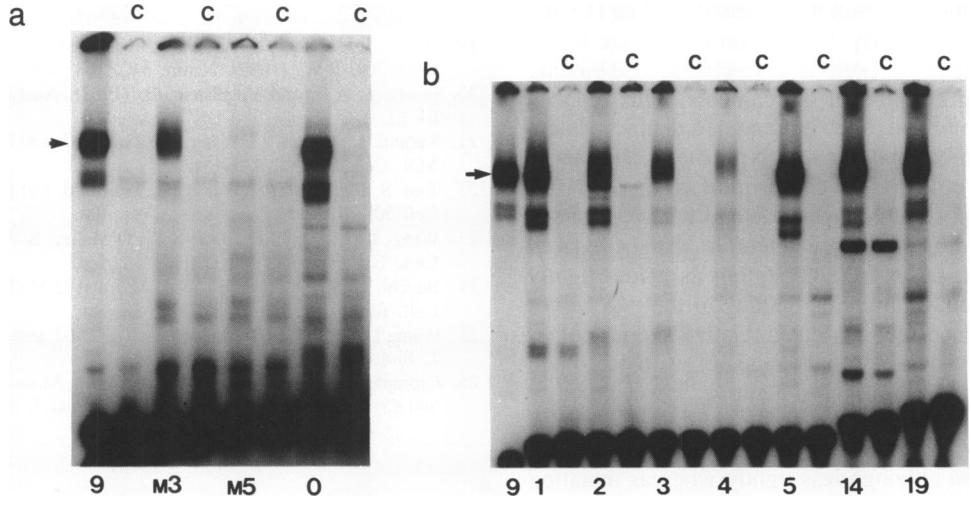

Figure 6. a and **b.** DNA mobility shift assay with site B probes containing different numbers of spacer nucleotides between the two halves of the palindromic recognition sequence and Jurkat T cell extract. Each pair of tracks shows the result with a probe containing the indicated spacing between the two halves of the palindrome either in the absence or presence (c) of a fifty fold excess of homologous competitor. Tracks labelled 9 indicate the result with a wild type site B containing a nine nucleotide spacing those labelled M3 or M5 the results with sites containing mutations in either the 3′ or 5′ half of the site B palindrome (9). The arrow indicates the position of the typical shift produced by binding of a T cell protein to wild type site B.

and would therefore detect proteins of similar binding specificity to COUP regardless of their relationship to COUP itself. We suggest that the T cell site B binding protein is unlikely to be related to HeLa cell COUP at the DNA sequence level since COUP-homologous mRNA is undetectable in Jurkat T cells which form high levels of the low mobility complex and only a low level of a complex with similar mobility to COUP. In contrast the COUP mRNA is, as expected, readily detectable in HeLa cells which express the 45 kD COUP protein at high levels. Moreover, although a low mobility complex similar to that formed with T cell extract is also formed by HeLa cells, screening of a HeLa cell cDNA library with a COUP cDNA probe did not result in the isolation of homologous cDNAs encoding the larger protein (23).

The T cell protein has a DNA binding specificity which is indistinguisable from that of the HeLa COUP protein as well as being related to that of other members of the steroid/thyroid hormone family (9). No member of this family with a size of 100 kD and the DNA binding specificity of COUP has previously been reported and this protein may therefore represent a novel member of the steroid/thyroid hormone family expressed at high levels in T cells.

The T cell protein binds to DNA as part of a larger complex of approximately 200 kD which can be detected in a Ferguson plot. Although we cannot rule out the possibility that this complex contains an additional protein, we favour the idea that it consists of a homodimer since only a single DNA binding protein is observed in UV cross linking experiments (Figure 3). The T cell homodimer is likely to form in solution since we have detected a 200 kD complex binding to site B in FPLC fractionation of T cell extracts (data not shown). These findings parallel those on the COUP protein where a 90–100kD DNA binding complex was detectable by gel filtration or glycerol gradient fractionation of HeLa extracts (23, 25), although only the monomeric 45 kD protein was detectable by UV cross-linking to DNA (21).

The binding of the T cell complex to site B appears to be dependent both on the precise sequence of the site B palindrome and the spacing between its two halves. Thus variation in sequence between different HIV isolates which affect the palindromic nature of the site result in decreased binding (13 and our unpublished data). Similarly a reduction in the size of the palindrome to four base pairs results in severely reduced binding as well as a decreased mobility of the complex. This reduced mobility complex may be due to the binding of a distinct protein different from that which binds to wild type site B. However, we observed a similar efficiency of competition for this complex with both the homologous oligonucleotide and wild type site B itself (data not shown). It is more likely therefore, that the reduced mobility is due to the same protein binding as a less tightly compact complex when the proteins binding to the two halves of the palindrome are incorrectly oriented relative to one another and hence bind both less strongly and in a more open configuration. We have also observed a similar lower mobility complex of reduced intensity when the 3′ half of the site B palindrome is mutated suggesting that this mutation may still allow the dimer to bind although less tightly whereas mutation of the 5′ half of the palindrome abolishes binding.

In summary we have shown that the major T cell protein which binds to site B is a 100kD protein which is distinct from previously characterized members of the steroid/thyroid hormone receptor family including the HeLa COUP protein which has a similar DNA binding specificity. The further characterization of the T cell protein and its potential relationship to members of the steroid/thyroid hormone receptor family will require the isolation of cDNA clones encoding this protein.

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