Interaction of DNA binding domain of HNF-3α with its transferrin enhancer DNA specific target site

Hernán Terenzi[a], Isabelle Petropoulos[a], Christine Ellouze[b], Masayuki Takahashi[b], Mario M. Zakin[a,*]

[a]*Unité d'Expression des Gènes Eucaryotes, Institut Pasteur, 28 rue du Dr. Roux, F-75724 Paris Cedex 15, France*
[b]*Groupe d'Etude Mutagénèse et Cancérogénèse, Institut Curie and URA 1342 CNRS, Bat. 110, Centre Université Paris Sud, F-91405 Orsay Cedex, France*

Received 7 June 1995

Abstract Transferrin hepato-specific gene enhancer, associated with the liver-enriched HNF-3α transcriptional factor and ubiquitous proteins, is a complex molecular edifice maintained through DNA–protein and protein–protein interactions. As a first step to understand the mechanisms responsible for its organization and activity, we have analyzed the interaction of the DNA binding domain of HNF-3α (HDBD) with a specific DNA segment present in the transferrin enhancer by different biophysical techniques. The kinetic constants of this interaction were measured using surface plasmon resonance. The HDBD–DNA interaction was also characterized by circular dichroism and fluorescence spectroscopy. HDBD binds to its specific DNA site with high affinity ($K_d \cong 10^{-8}$ M). The affinity is reduced after sequence modification of the target DNA. Size exclusion chromatography and binding stoichiometry determined by fluorescence measurements indicate that the protein is present in a monomeric form before and after interaction with the DNA. The secondary structure of the protein was not significantly altered upon binding to specific DNA. By contrast, a structural change of DNA by interaction with HDBD seems to occur.

Key words: Transferrin enhancer; DNA–protein interaction; Transcription factor; HNF-3/*fork head* DNA binding domain

1. Introduction

Hepatocyte specific gene expression is controlled in part by nuclear proteins whose concentrations are particularly enriched in liver. Among these, we found the hepatocyte nuclear factor 3 group of proteins, composed of HNF-3α, HNF-3β and HNF-3γ [1–3]. These proteins belong to a rapidly growing family, the HNF-3/*fork head* (fkh) transcription factors, that share a 110 amino acids region responsible for their interaction with DNA.

The structure of the binary complex between the HNF-3γ DNA binding domain and its DNA specific target site present in the promoter region of the transthyretin gene [3] has been solved by X-ray crystallography at 0.25 nm resolution [4]. The *fork head* domain was recognized as a variant of the helix-turn-helix (HTH) motif, containing two loops on the C-terminal side of the HTH. Direct base contacts are made by an α-helix intruding into the major groove of DNA and additional backbone contacts are provided by the loops. This variant of the HTH, with loops or wings that project over DNA, has been called 'winged helix' domain [5]. In addition, after analysis of the binding properties of a series of chimaeric fkh DNA-binding domain fusion proteins, other authors have proposed that a 20-amino acid sequence adjacent to the HNF-3 recognition α-helix influences its DNA-binding properties [6].

Recent studies have demonstrated that different fkh proteins bind to distinct DNA sites and that the specificity of protein recognition is dependent on subtle nucleotide alterations in the site [6]. It was also determined that the binding sites for four of the seven detected human fkh proteins (FREAC) share a core sequence, but differ in the positions flanking the core [7]. These flanking sequences appear to be more important in giving each protein its specificity. In this context, it is important to underline that the oligonucleotide used for the cocrystallisation with HNF-3γ did not extend beyond the 3′ border of the HNF-3γ binding core [3,4]. In addition, the two last base pairs of the oligonucleotide, which correspond to the two last positions of the HNF-3γ DNA recognition core, are different from those present in the binding site in the transthyretin promoter [3,4]. All these considerations clearly indicate that more work with different members of the protein family is required to understand the exact nature of the fkh protein–DNA complexes and how these proteins can specifically regulate their target genes.

By studying transferrin gene expression as a model for liver-specific gene expression [8], we have previously demonstrated the existence of a transferrin gene enhancer, located between nucleotides −3600 to −3300 [9]. This enhancer is composed of two functional domains (A and B), containing five enhansons named Ia, Ib, II, III and IV [10]. Characterization of the A domain, composed of enhanson Ia, and purification of liver proteins that interact with it, have shown that the decamer 5′-TGTTTGCTTT-3′ is essential for the enhancer activity and that HNF-3α specifically interacts with this DNA sequence [11]. Our results indicate that the Tf enhancer, associated with liver-enriched and ubiquitous proteins, constitutes a particular molecular edifice maintained through DNA–protein and protein–protein interactions [10]. To further understand the role of HNF-3α in the activity of this enhancer, we decided to study, as a first step, the interaction between the DNA binding domain of HNF-3α (HDBD) and its Ia specific target site. This interaction has been characterized by using surface plasmon resonance (BIAcore system, Pharmacia Biosensor), circular dichroism (CD) and fluorescence spectroscopies.

*Corresponding author. Fax: (33) (1) 40613110.

Abbreviations: HNF, hepatocyte nuclear factor; HDBD, HNF-3α DNA binding domain; Tf, transferrin; fkh, HNF-3/*fork head*; HTH, helix-turn-helix; SPR, surface plasmon resonance; CD, circular dichroism; EMSA, electrophoretic mobility shift assays.

SSDI 0014-5793(95)00767-9

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade.

Buffer employed was: 10 mM Tris-Cl pH 8.05, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 M NaCl, 0.05% Tween 20 (TEMNT).

Nucleotide sequences of oligonucleotides used in this work are as follows:

Ia, 5′ GCTCTTTGTTTGCTTTGCTTCTGTGTCAAC 3′ [11];
m2, 5′ GCTCTTatTTTGCTTTGCTTCTGTGTCAAC 3′ [11];
CR, 5′ CTGTGCTGGACTCCTTCCACTCGCGGGTCGTC 3′ [12].

2.2. Purification of the recombinant HNF-3α DNA binding domain (HDBD)

The pGEX-2T expression vector (Pharmacia) was modified to contain a *Xba*I site instead of the *Sma*I site in the cloning region, and used to express an HDBD N-terminal fusion protein with glutathione S-transferase (GST). The coding sequence of HDBD was PCR amplified from a cDNA coding for the entire sequence of HNF-3α using oligonucleotides containing *Xba*I sites in the 5′ side of the sequence to promote insertion in the *Xba*I site of the modified vector. The construction was verified by sequencing and used to transform DH5α bacteria. The cells were grown in LB medium to an absorbance of 0.5 at 600 nm, and were then induced with 0.3 mM isopropyl thio-β-D-galactoside for 4 h at 37°C under good aeration in the presence of 100 μg/ml ampicillin. Cells were harvested by centrifugation at 6000 × *g*. The pellet from 1000 ml of culture was resuspended in 20 ml of phosphate buffered saline (PBS) with 5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin and 40 μg/ml bestatin. The cells were disrupted by gentle sonication (4 cycles/20 s) on ice, triton X-100 was added to a final concentration of 10%, and the suspension centrifuged for 20 min at 15000 × *g*. The supernatant was incubated overnight at 4°C with 2 ml of glutathione-Sepharose (Pharmacia). The resin was washed with 15 volumes of PBS and finally with thrombin cleavage buffer (50 mM Tris-Cl pH 8, 150 mM NaCl and 2.5 mM $CaCl_2$). Thrombin was added (approximately 0.05% w/v) and incubation performed for 2 h at room temperature. The cleaved protein was washed out by addition of 50 mM Tris-Cl pH 8, 0.4 M NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol and applied to a Heparin Sepharose column connected to a FPLC system (HiTrap 5 ml, Pharmacia) after dilution to 50 mM NaCl in the same buffer without NaCl. The column was submitted to a linear elution gradient from 50 mM to 1 M of NaCl, in 5 column volumes; the protein was eluted at 630 mM NaCl and purified fractions were concentrated by ultrafiltration with Centricon 3 (Amicon) membranes. The concentration of protein was obtained by direct calculation of the absorbance at 280 nm, using the calculated molar extinction coefficient of 27760 $cm^{-1}M^{-1}$.

2.3. Electrophoretic mobility shift assays (EMSA)

The standard assay as well as the competition experiments were performed as previously described [11], except that the reaction temperature was 25°C instead of 4°C. The non-biotinylated Ia, m2 and CR oligonucleotides were used at a concentration of 1 nM. The complementary strands were annealed at equimolar concentration in TE by slowly cooling the hybrid to 25°C after heating at 90°C during 5 min. The CR oligonucleotide is a consensus binding site of NF1 in the human transferrin promoter [12], used as a non-specific DNA binding site for HDBD. GST-HDBD protein was used at a concentration of 20 nM.

2.4. BIAcore based kinetic analysis

BIAcore instrument and sensorchips SA5 were manufactured by Pharmacia Biosensor AB (Uppsala, Sweden).

The Tf sequences (see above) were annealed on the surface of the sensorchip, by initially passing the upper biotinylated strand, followed by the lower non-biotinylated strand. Each strand was diluted in flow buffer and passed over the surface at a rate of 5 μl/min during 1–2 min at a concentration of 10 μg/ml (upper biotinylated strand) or 100 μg/ml (lower non biotinylated strand). The hybridization level was calculated to be almost 100%.

The protein was diluted in the BIAcore flow buffer (TEMNT). Different concentrations of HDBD (40 μl) were injected at a flow rate of 5 ml/min, followed by at least 5 min wash out to measure dissociation rates. To regenerate the surface after each sample, 2 μl of 2 M NaCl were injected and proved sufficient to dissociate the DNA–protein complex.

The reaction between immobilized DNA and HDBD can be assumed to follow pseudo-first-order kinetics [13] described by the rate equation:

$$dR/dt = k_{ass}\ C\ (R_{max} - R) - k_{diss}R,$$

where dR/dt is the rate of DNA–protein complex formation in the surface, k_{ass} is the association rate constant, k_{diss} is the dissociation rate constant, R is the amount of bound HDBD, R_{max} is the maximum represor binding capacity of the surface, and C is the concenration of HDBD in solution. For each concentration of protein used the k_s (slope of the dR/dt vs R plot) was obtained and then presented as a function of the protein concentration. The slope of this curve is the k_{ass}:

$$-k_s = k_{ass}\ C + k_{diss}$$

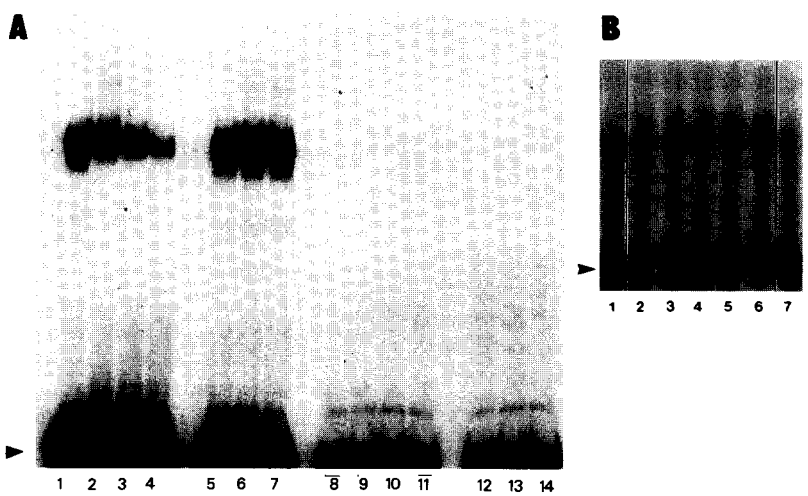

Fig. 1. EMSA assays using purified GST-HDBD and HDBD proteins. (A) The fusion protein GST-HDBD was used in EMSA assays as described in section 2. In lanes 1 to 7 the labelled probe is Ia; in lanes 8 to 14 the labelled probe is CR. Competitions were performed with 5, 50 and 250 molar excess unlabelled Ia probe (lanes 2 to 4, respectively and 9 to 11, respectively) or unlabelled CR probe (lanes 5 to 7, respectively and 12 to 14, respectively). (B) Purified HDBD at 0, 10, 50, 100, 200 and 500 nM (lanes 1 to 6, respectively) was incubated with Ia labelled probe. Lane 7 is a control with m2 labelled probe and 500 nM HDBD. The free probe is indicated by an arrow.

The k_{ass} and k_{diss} were also calculated by non-linear regression analysis (see below, section 3.3). The software BIAevaluation 2.0 (Pharmacia Biosensor) was used to perform these calculations. The K_d (equilibrium dissociation constant) was obtained as the ratio k_{diss}/k_{ass}.

2.5. Size exclusion chromatography

To calculate the relative molecular weight of native HDBD in solution a Superdex-75 column (Pharmacia) was used following manufacturers recommendations. A 25 μl sample of a 1 mM solution of HDBD in buffer TEMNT was applied to a Superdex-75 column connected to a FPLC system (Pharmacia), at 1 ml/min. The elution of HDBD was detected at 280 nm and also by SDS-PAGE analysis of the fractions. A mixture of gel filtration molecular weight markers (BSA, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa and RNAse A, 13.7 kDa) was used to obtain a calibration curve under the same elution conditions of HDBD.

2.6. Spectroscopic measurements

Circular dichroism (CD) was measured in a J-710 spectropolarimeter (Jasco, Japan) at room temperature (22 ± 2°C). The band width was 2 nm and spectra were usually averaged over 4 scans in order to improve the signal to noise ratio. The samples were measured in a 0.1 cm cell and in a low salt buffer (10 mM sodium phosphate pH 6.7 with 1 mM $MgCl_2$) to avoid excessive absorbance. (Note that Cl^- ion absorbs light below 200 nm.) In order to facilitate the comparison of spectra, the spectrum of free DNA was subtracted from the spectrum of the protein–DNA complex.

Fluorescence was measured in a FP-777 spectrofluorometer (Jasco, Japan). The measurements were performed at 20°C. The excitation wavelength was set at 295 nm with a bandwidth of 3 nm for selective excitation of tryptophan residues. The emission wavelength was usually set at 340 nm with bandwidth of 5 nm. Inner filter effect due to the additional absorption from DNA was minimized by the use of a 0.2 cm × 1 cm cell. The pathlength of incident light was thus less than 0.2 cm. The signal intensity was, however, corrected for inner filter effect and dilution of sample upon successive addition of DNA solution. The correction was made by using the change in intensity of free tryptophan fluorescence upon the addition of equivalent amount of DNA solution as a standard. The cuvette was treated by silicon (Serva) to avoid the adsorption of protein to the wall.

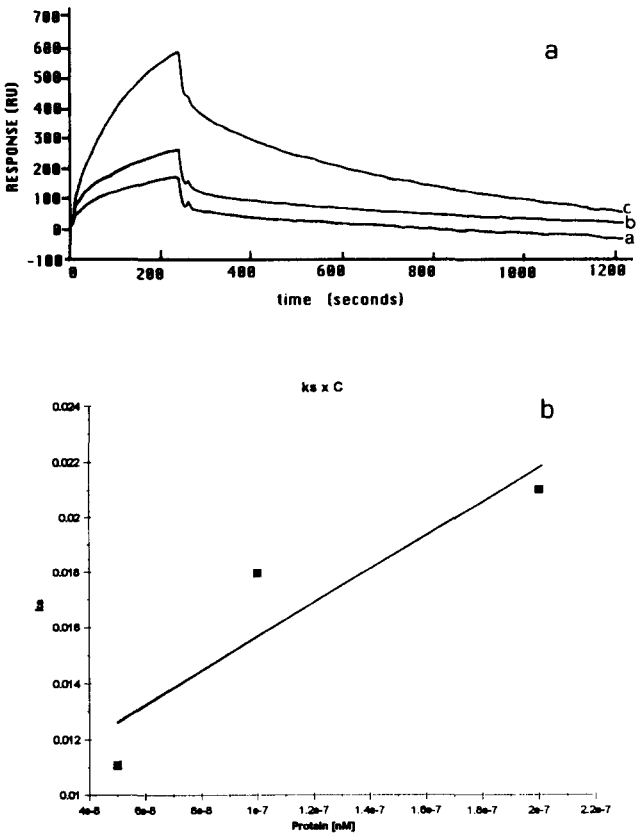

Fig. 2. Binding response of HDBD to Ia wild type DNA. (a) The curves used to calculate the values shown in Table 1 and 2 are presented. HDBD concentration used was 50, 100 and 200 nM (curves a, b and c, respectively), the buffer employed was TEMNT. (b) Plot of k_s vs concentration of HDBD. This plot represents a rate equation linearization method to determine k_{ass} as the slope of the fitted line (see section 2).

3. Results

3.1. Obtention of the recombinant DNA binding domain of HNF-3α

The amino acid sequence of the recombinant HDBD obtained in bacteria from a pGEX-2T expression vector corresponds to amino acid residues 164 to 291 of the entire HNF-3α protein [1]. The purified polypeptide is homogeneous in SDS-PAGE as a unique band at approximately 16 kDa (data not shown), matching well with the deduced molecular weight of 15284 Da, estimated from the cDNA sequence. This was confirmed by size exclusion chromatography (see below, section 3.4).

3.2. Analysis of the DNA binding properties of HDBD

To assess the functional activity of the recombinant HDBD obtained, we performed electrophoretic mobility shift assays (EMSA) with the GST-HDBD fusion protein, using the Ia DNA wild type site of the Tf gene enhancer or an unrelated DNA sequence, CR (see section 2). As showed in Fig. 1a, the formation of a specific DNA–protein complex was observed between the fusion protein and the Ia oligonucleotide, but not with the CR sequence (Fig. 1a, lanes 1–7 and 8–14, respectively). Competition experiments showed that the unlabelled Ia sequence, but not the unlabelled CR sequence, is able to compete for binding with the Ia probe inhibiting the formation of the complex. These results showed that, like the whole native protein, the GST-HDBD recombinant protein specifically interacts with the Tf Ia DNA region. This is also the case for the purified recombinant HDBD polypeptide. Indeed, HDBD interacts with the Ia probe in a protein concentration dependent manner (Fig. 1b, lanes 1–6), but not with the mutated m2 Tf enhancer sequence (Fig. 1b, lane 7).

3.3. BIAcore based kinetic analysis

The kinetic constants of DNA–protein interactions were

Table 1
Kinetic parameters of the interaction between HDBD and Ia DNA sequence

DNA	Protein conc.	k_{ass} ($M^{-1} \cdot s^{-1}$)	k_{diss} (s^{-1})	K_d (M)
Ia	50 nM	$2.34 \times 10^5 \pm 9.13 \times 10^3$	$2.13 \times 10^{-3} \pm 5.95 \times 10^{-6}$	$0.91 \times 10^{-8} \pm 0.65 \times 10^{-9}$
Ia	100 nM	$0.82 \times 10^5 \pm 5.30 \times 10^3$	$1.71 \times 10^{-3} \pm 6.42 \times 10^{-6}$	$2.09 \times 10^{-8} \pm 1.22 \times 10^{-9}$
Ia	200 nM	$0.37 \times 10^5 \pm 1.55 \times 10^3$	$1.98 \times 10^{-3} \pm 4.62 \times 10^{-6}$	$5.38 \times 10^{-8} \pm 2.98 \times 10^{-9}$

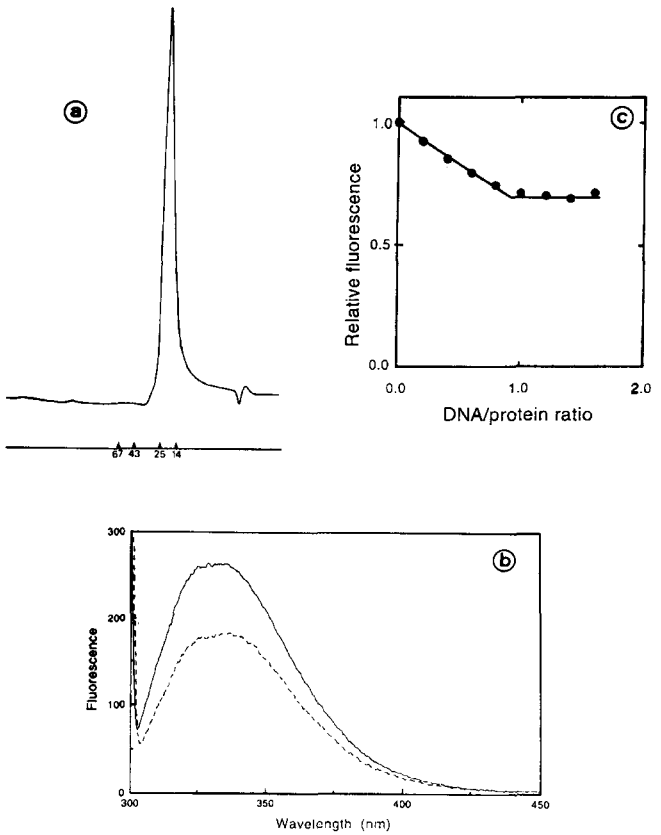

Fig. 3. One to one binding stoichiometry of HDBD to DNA. (a) Analysis of HDBD by size exclusion chromatography. Elution profile of HDBD in a Superdex-75 column running in TEMNT buffer. The elution position of molecular weight markers are shown in the bottom of the figure. (b) and (c) Fluorescence change of HDBD upon DNA binding. (b) HDBD (2 μM) emission spectra upon excitation at 295 nm, before (−) and after (− −) addition of equimolar DNA fragment spanning the Ia Tf enhancer specific site. (c) An oligonucleotide containing 30 base pairs spanning the Ia type Tf binding site for HDBD was successively added to the protein solution (2 μM) and the fluorescence measured. The variation of fluorescence signal is presented as a function of DNA fragment over protein ratio.

measured using real time changes in surface plasmon resonance with the BIAcore system. The description of the kinetic analysis has already been reported [13] (see also, section 2). The curves used to generate the rate constants of the HNF-3α interaction with the Ia Tf enhancer sequence are shown in Fig. 2a.

The curves shown in Fig. 2a were analyzed with the BIAevaluation software with a non-linear regression analysis to obtain the k_{ass} and k_{diss} for each HDBD concentration, the values are presented in Table 1. The k_{diss} was calculated from the part of the curve between 400 and 1200 s, where the data follow a 1:1 DNA–protein interaction model. The k_{ass} seems to be somewhat dependent on protein concentration, and this phenomena reflects on the calculated K_d. This could be due to a secondary binding event with similar k_{ass} to the specific interaction with the Ia site, but probably with different k_{diss} constant. To test this hypothesis the dissociation part of the curve was treated as resulting from two different binding events. The response between 320 and 1200 s was evaluated, to eliminate the interfering injection pulse. These data were treated with the BIAevaluation software using non-linear regression analysis and the two different k_{diss} obtained (k_{d1} and k_{d2}, Table 2) could represent the first rapid dissociation of the non-specific complex and the second dissociation event, one order of magnitude lower, that from the specific complex. The k_{diss} of the specific interaction calculated using this model (k_{d2}) is very similar, as expected, to the k_{diss} calculated with the single site model applied to the region between 400 and 1200 s (Table 1).

To futher verify the data the k_{ass} was calculated using a linear regression analysis of a derivative of the binding curve (see section 2). From the slope of the fitted line in the plot of k_s vs concentration of HDBD, an estimated k_{ass} value of 6.1×10^4 was obtained (Fig. 2b). This is in good agreement with the k_{ass} obtained from non-linear regression (Table 1).

3.4. HDBD is in a monomeric form before and after interaction with DNA

HDBD was analyzed by size exclusion chromatography in a Superdex-75 column. In comparison to standard globular protein markers we deduced a Kav corresponding to the calculated molecular mass of the protein, that is 15 kDa (Fig. 3a). This experiment shows that HDBD is present as a monomer in solution, as found for a *Drosophila fork head* domain by equilibrium sedimentation experiments [14].

Fig. 3b shows fluorescence emission spectra of HDBD before and after addition of the Ia Tf enhancer DNA fragment. The spectra were corrected for inner filter effect (1.2%) due to increase of absorption upon addition of DNA. The fluorescence intensity decreases about 30% upon addition of DNA. The titration of the protein by DNA shows that the fluorescence decreases linearly with increasing DNA concentration, as expected from the very high binding affinity between HDBD and Ia DNA determined above (Table 1). The fluorescence reaches a plateau at about one DNA fragment per protein molecule (Fig. 3c). This binding stoichiometry indicates that the protein, demonstrated to be present as a monomer in solution (see above), also binds to DNA in a monomeric form. In addition, these results clearly show that the protein fluorescence is quenched upon binding to DNA. Since this fluorescence change was observed upon selective excitation of tryptophan residues, we can conclude that the environment of some or all of these residues is altered by DNA binding.

3.5. Secondary structure of HDBD free or bound to DNA

The CD spectrum of the recombinant HDBD protein (Fig. 4a) shows distinct negative peaks at 225 and 210 nm and a positive peak at 190 nm, indicating the existence of a significant content in α-helical structure (15). This CD spectrum is similar to that obtained with the DNA binding domain of a *Drosophila fork head* protein [14]. Analysis of the secondary structure from the obtained HDBD spectrum shows that the content of α-helix is 30% and that of β-sheet 25%. This estimation was made by

Table 2
HDBD-Ia DNA sequence interaction

DNA	Protein concentration	k_{d1} (s^{-1})	k_{d2} (s^{-1})
Ia	50 nM	$1.35 \times 10^{-2} \pm 6.0 \times 10^{-4}$	$0.87 \times 10^{-3} \pm 3.4 \times 10^{-6}$
Ia	100 nM	$0.50 \times 10^{-2} \pm 1.3 \times 10^{-4}$	$0.53 \times 10^{-3} \pm 6.9 \times 10^{-6}$
Ia	200 nM	$0.67 \times 10^{-2} \pm 0.8 \times 10^{-4}$	$0.92 \times 10^{-3} \pm 3.4 \times 10^{-6}$

Calculation of k_{diss} using a two independent sites model.

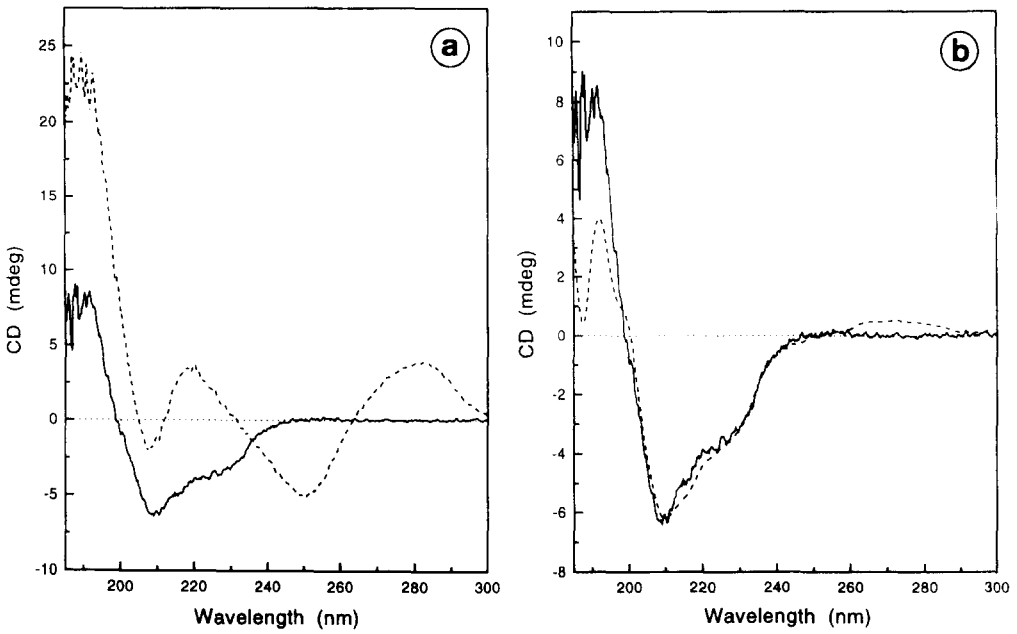

Fig. 4. CD spectra of HDBD and Ia DNA before and after interaction. The concentration of protein or DNA in each case was 10 μM. (a) CD spectra of a Ia oligonucleotide (dashed line) is shown along with that of free HDBD (solid line). (b) CD spectra of HDBD with (dashed line) and without Ia oligonucleotide (solid line) are compared.

a program provided by Jasco (Japan) using Yang's data as reference [16].

Fig. 4b shows the CD signal of an equimolar mixture of HDBD protein with Ia DNA site. The signal of free HDBD and that of the Ia-HDBD complex is similar, especially in the region between 205 nm and 250 nm (Fig. 4b), where the DNA signal is less intense than the protein signal. Thus, the spectrum of protein seems not to be drastically modified upon DNA binding. This is consistent with the fact that the calculated content of α-helix and β-sheet of HDBD alone (see above), is rather comparable to the structure of the DNA binding domain of HNF-3γ when complexed to DNA [4].

By contrast, significant signal change upon the complex formation can be observed around 280 nm and below 200 nm (Fig. 4b). A positive lobe of DNA CD at 280 nm becomes larger, and that at 190 nm becomes smaller (compare Fig. 4a and 4b). Since CD signal of DNA is larger than that of protein in this region, the signal change is probably due to a conformational change of DNA upon interaction with the protein.

4. Discussion

The transferrin enhancer presents a modular organization maintained through DNA–protein and protein–protein interactions. This enhancer interacts with a liver-enriched transcription factor HNF-3α and ubiquitous proteins [10]. As a first step to understand this complex structure, it is important to study the interaction between each of the transcriptional factors and their corresponding specific DNA binding sites. Therefore, the purpose of this work was to study the interaction between HDBD, a fragment of 128 amino acid residues containing the DNA binding domain of HNF-3α protein, and its Ia target site in the Tf enhancer.

Previous work concerning the behaviour of different HNF-3/fkh proteins in the presence of their specific DNA binding sites have shown that subtle nucleotide alterations within and in the flanking regions of the DNA target core, generate diversity in the specificity of the protein recognition [6,7]. In addition, indirect manifestations of these nucleotide alterations as DNA helicity and topology also seem to be important for the observed diversity [7]. In contrast, the amino acids involved in the DNA interaction, mainly those that make direct base contacts, are highly conserved in the fkh family. The question that may be addressed concerns the mechanisms by which these highly homologous proteins, binding to similar but different DNA sites, are able to specifically regulate their target genes.

An interesting method to study protein–DNA interactions is the recently developed BIAcore instrument that monitors surface plasmon resonance [17], permitting the real time observation of binding phenomena. The binding kinetics of two different transcription factors were recently analyzed by this method [13,18], and the results proved to be consistent with other techniques such as EMSA and filter binding assays [19].

The results presented here have shown that HDBD interacts with a strong affinity (K_d of about 10^{-8} M) with a DNA fragment spanning the Ia specific binding site (Table 1 and 2). The affinity is reduced upon the modification of the sequence of the target DNA, as expected from our previous experiments (refs. [10,11], Fig. 1 and data not shown). As shown in Table 1, the association rate constant (k_{ass}) reveals a rapid binding phenomenon. It is interesting to note that, in contrast to the k_{diss} constant, the k_{ass} is somehow dependent on protein concentration. The comparison of the two methods employed to the k_{ass} determination, namely linearized rate equations and non-linear analysis (see section 2) suggest a dependence between protein concentration and binding rate. Our suggestion to explain this phenomenon relies on the existence of a secondary non-specific binding event that was indeed observed when the dissociation

part of the curves were further analyzed (Table 2). This hypothesis was recently analyzed in experiments performed with the ETS-1 and the *lac* repressor proteins [13,18].

The HDBD-DNA interaction has been further characterized with the help of CD and fluorescence spectroscopies. Our results indicate that the protein fluorescence is quenched upon binding of HDBD to DNA (Fig. 3b). This suggest that one or more of the three tryptophan residues present in HDBD are affected by DNA binding. The titration of the protein by DNA shows that the fluorescence decreases linearly with increasing the DNA concentration, reaching a plateau at about one DNA fragment per protein molecule (Fig. 3c). This binding stoichiometry indicates that the protein binds to DNA in a monomeric form. This was also observed with the DNA binding domain of HNF-3γ. In addition, size exclusion chromatography of the protein showed that HDBD, in the absence of DNA, is also in a monomeric form in solution (Fig. 3a).

The spectroscopic analyses of the CD spectrum of HDBD (Fig. 4a) showed that the protein alone has a stable secondary structure in solution. An important observation in our work is that HDBD secondary structure seems not to be significantly modified upon DNA binding (Fig. 4b). There appears to be no induction or destabilization of an α-helix. This contrasts with the case of bZIP proteins: an induction of an α-helix upon DNA binding was observed when bZIP proteins like Fos, Jun and GCN4 were analyzed [20,21].

Interestingly, CD measurements indicate a significant change in the structure of DNA (Fig. 4a and b). Since the CD signal depends upon different structural parameters of DNA [22], physical interpretation of the CD change observed here is difficult. Nevertheless, the results suggest that the binding of HDBD alters DNA structure, and this can be one of the ways through which HNF-3α influences transcription mechanisms. Indeed, as observed by X-ray crystallography, the interaction of HNF-3γ DNA binding domain with its transthyretin promoter binding site induces a 13° bend, narrowing the major groove in contact with the protein [4]. Interestingly, using a circular permutation assay, it was recently demonstrated that binding to their target sites of the FREAC proteins, induces a bending of the DNA at an angle of 80–90° [7]. The differences observed when using the HNF-3γ or the FREAC DNA binding domains may reflect a difference in binding characteristics of the proteins or the result of the different experimental methods used. Nevertheless, our results and those of others strongly suggest that the generation of modifications of the DNA spatial organization upon binding with fkh proteins is a general property of these proteins, and may reflect a characteristic of their function as gene regulators.

To analyze some of the data presented here, we took advantage of the fact that the crystal structure of HNF-3γ DNA binding domain, although complexed to an imperfect HNF-3 DNA site, was already available [4]. Indeed, the sequence identity between the DNA binding domains of HNF-3α and HNF-3γ is 95%. On the contrary, the DNA target sites are different. It is interesting to point out that DNA sequences highly homologous or identical to the Ia Tf site were presented as low-affinity binding sites for the HNF-3γ DNA-binding domain [14]. The kinetic parameters presented in this paper indicate, on the contrary, that the HNF-3α binding domain interacts with the Ia sequence with a high affinity (Table 1). It is clear that a more thorough analysis of the structure of fkh proteins with distinct DNA recognition specificity is necessary to understand how these similar proteins regulate the expression of different target genes. However, it is also evident that, when technical limitations were overcome, these studies must be enlarged by using whole proteins instead of their isolated DNA binding domains.

Acknowledgements: We are grateful to M. Buckle for helpful discussions, and to G.N. Cohen and O. Barzu for critical reading of the manuscript. We thank S. Cot for her help in the kinetic analysis of the BIAcore experiments and E. Croullebois for typing the manuscript. This work was supported by the Centre National de la Recherche Scientifique (U.R.A. 1129 and U.R.A. 1342) and by the Commission of the European Communities (CEC Contract No. BIO2-CT93-0319). H.T. is supported by a postdoctoral fellowship from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brasil).

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