

Transcriptional regulation of the MHC class I HLA-A11 promoter by the zinc finger protein ZFX

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ABSTRACT

Regulation of the human MHC class I HLA-A11 promoter is governed by a complex array of regulatory elements. One of these elements, shown here to be critical for the transcriptional activity of the promoter, was used to screen a λ gt11 library and allowed the identification of a cDNA which coded for the zinc finger protein ZFX. ZFX was shown to bind the sequences AGGCCCCA and AGGCCCGA, located respectively at positions -271 to -263 and -242 to -234 of the HLA-A11 promoter, with similar affinities through its three C-terminal zinc fingers. ZFX⁵⁷⁵, a short isoform of ZFX, activates transcription from the HLA-A11 promoter in a Leydig cell line.

INTRODUCTION

Major histocompatibility complex (MHC) class I genes encode highly polymorphic molecules present on the surface of most nucleated vertebrate cells with the exception of some specialised cell types, such as neurones, corneal cells, pancreatic acinar cells and mature sperm cells (1).

In many instances, the presence of functional products of the human HLA-A and HLA-B loci is critical when a cell must be recognised as abnormal (either malignant or virally infected) in order to be eliminated by the immune system. Intracellular association of endogenous peptides which originate from proteolytic degradation with the polymorphic heavy chain of MHC class I molecules and the monomorphic light chain (β 2-microglobulin) is accomplished during routing to the cell membrane, where the complex can eventually be recognised by cytotoxic T lymphocytes via their T cell receptor (2).

Some viruses have evolved specific mechanisms leading to down-regulation of MHC class I in infected cells (3) and this has been shown to be critical for the elimination or survival of virally infected cells (4). Occasional evidence for the expression of MHC class I favouring the expansion of tumour cells by promoting escape from NK cell lysis has been reported (5). However, the frequent loss

of MHC class I expression in many tumours (6) and the selective loss of expression of specific alleles in some of them (7,8) is consistent with the escape of class I-negative tumour cells from an immune process operating against transformed cells which express HLA class I genes. Indeed, therapies aimed at indirectly stimulating or at re-expressing MHC class I molecules on the surface of defective cancerous cells have proved successful (9). The expression level of MHC class I genes varies among different tissues and can be regulated by various effectors, including retinoic acid (10), TNF (11), IFN (12) and hormones (13).

In many instances these effectors have been shown to act ultimately at the transcriptional level. Some proteins, such as members of the rel and STAT families (14–16), have been repeatedly implicated in the transcriptional control of MHC class I genes. They act through DNA elements which are fairly well conserved both intra- and inter-specifically. However, the rapid evolution and extensive polymorphism of MHC class I genes is not restricted to the coding portion of the genes and is also observed in their control regions (17). Although at present the implications of this fact cannot be fully appreciated, it is already known that it can account for locus- or allele-specific regulation by effectors such as IFN (18,19).

Previous analysis of transcriptional regulation of the HLA-A11 gene indicated that in addition to a conserved region which is the target of rel, STAT and IRF family factors, five other control elements within the first 337 nucleotides (nt) of the promoter also exert significant effects on transcription (20).

We report here that the mutation of one of these elements dramatically reduces transcription from the HLA-A11 promoter. Using an oligonucleotide spanning this region, we screened a HeLa λ gt11 library and isolated a partial cDNA corresponding to the C-terminal portion of the ZFX protein. The binding mode of recombinant ZFX fusion proteins to the HLA-A11 element was also characterised. We demonstrate that a short isoform of ZFX, ZFX⁵⁷⁵ (21), can be a potent transactivator of the HLA-A11 promoter. In addition to the identification of a novel transcriptional effector of the HLA class I genes, this work constitutes the first demonstration that the ZFX gene indeed encodes a *bona fide* transcription factor, as initially proposed (21,22).

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MATERIALS AND METHODS

Cloning of ZFX and plasmid construction

A cDNA library of HeLa cells in λ gt11 (Clontech) was screened essentially as described (23), except that poly(dI-dC)·(dI-dC) was used as a non-specific competitor in 500-fold excess along with an unrelated oligonucleotide in 100-fold excess in 0.5% BSA, 10 mM HEPES, pH 7.5, 50 mM KCl, 0.5 mM MgCl₂, 10 μ M ZnCl₂, 5% glycerol and hexamerised -272/-233 double-stranded oligonucleotides, labelled with [γ -³²P]ATP and used at 5 ng/ml: -272/-233C, 5'-GATCCAGGGCCAGGGCTGGCTCAGGGTCTCAGGCC-CCGAA-3'; -272/-233NC, 5'-GATCTTCGGGGCCTGAGAC-CCTGAGAGCCAGCCTGGGGCCCTG-3'. Spots superimposed on replicates were processed through secondary and tertiary screening and their inserts identified after subcloning. The full-length ZFX⁵⁷⁵ was amplified on human ovary cDNA (Clontech) with the 5' primer oligonucleotide 1 (5'-GGGAATTCGCCGCAACCGTATGATGTTCCAGACTATGCTGACACAGAGCGG-AAATTGAT-3'), which introduces a nine amino acid HA tag at the N-terminus, and the 3' primer oligonucleotide 2 (5'-CGGAATTCG-GATCCAAGGCCAATATCTCACAAACG-3') with a mixture of 1/25 Pfu (Stratagene) and Taq (BRL) (24). Amplified fragments cloned in PSG5 (Stratagene) were sequenced and found to bear a mean of two mutations per clone. A full-length cDNA was reconstituted by conventional methods, using unique internal restriction sites for *Pml*I and *Xcm*I. During the course of this work we obtained a cDNA that was lacking nucleotide 2071 (numbered according to 21); this frameshift mutation precisely located after the end of the ninth zinc finger introduces a nonsense codon at position 2111. The resulting cDNA encodes a protein deleted of its four C-terminal zinc fingers, referred to as ZFX⁵⁷⁵ Δ C. GST fusion vectors were constructed after amplification of different fragments using 100 ng of cloned ZFX cDNA as the substrate. The inserts of the various constructs were amplified as follows: using oligonucleotides 3 and 7 for GST4ZFX, oligonucleotides 4 and 7 for GST3ZFX, oligonucleotides 5 and 7 for GST2ZFX and oligonucleotides 4 and 6 for GST3ZFX Δ C: oligonucleotide 3, 5'-GGGTGATCAGGATC-CAGATCTAGCCCGTCAGTATCGGCCGA-3'; oligonucleotide 4, 5'-GGGAATTCGGATTCGGGCACAAAGGATCTTCCATT-TAG-3'; oligonucleotide 5, 5'-GGGA-TTCGGATTCGGGCAGTG-GCAGGAAAGT-3'; oligonucleotide 6, 5'-GGGGAATTCT-TAGTCTTTCGTGTGAATGGAATAACG-TGC-3'; oligonucleotide 7, 5'-CGGAATTCGGATCCAAGGCCAATATCTCA-CAAACG-3'. The 6ZFX insert was amplified using oligonucleotides 8, 5'-GCGGGATCCACCAAGAAAGCAAAC-3'; and 9: 5'-GCTCTAGAAGCTTAGGGCAGGCAACT-TC-3', digested with *Bam*HI and *Hind*III and cloned into 24KYH digested with *Bam*HI and *Hind*III (24KYH is a high copy number prokaryotic expression vector, in which expression is driven by a T7 promoter controlled by the lac repressor; details concerning its organisation are available separately on request). The resulting vector, 24KYH6ZFX, enables production of a protein beginning with MKYHHHHHHGS, followed by the amino acids translated from the sequence of the insert. An *Eco*RI fragment amplified in two steps with the oligonucleotides 10 (5'-GATGTTCCAGACTATGC-TCCCAAGAACGTCGTCCTGATGGATTCCGGCACAA-AGGATCTTCCATTTAG-3') and 7 followed by amplification with oligonucleotides 11 (5'-GGAATTCAGATCTGCCACCATGGAG-TATCCGTATGAGTTCAGACTATGCTCC-3') and 7 was cloned in the PSI vector (Promega). This produced the vector PSIN3ZF, which allowed translation of a protein beginning with

MEYYPYDVPDYAPKKRRRPDGS, followed by the three C-terminal zinc fingers. Generation of the mutated HLA-A11 promoters was achieved by two PCR steps. The first step consisted of amplifying the -337/-260 region while introducing a *Bg*III site at -266 with oligonucleotides 12 (5'-AAACTGCAGGACTCAGG-GAGACATTGAGACA-3') and 13 (5'-GAGAGCACCCGACG-AGATCTGCCCTGGGACTTCGCC-3') and separately amplifying the -270/+2 region by introducing the *Bg*III site at the same position with oligonucleotides 14 (5'-GTCCCAGGGCA-GATCTCGTGGTCTCAGGGTCTC-3') and 15 (5'-GGGGAT-CCTCGGGTCTGGGGTCTGGGG-3'). The PCR products were digested with *Bg*III and ligated. The ligation product isolated on agarose gel was digested with *Pst*I and *Bam*HI and then cloned in CAT3. The same procedure was carried out to introduce a *Xba*I site at position -234 with oligonucleotides 16 (5'-GCTCTCAGGGTC-TCTAGACCCGAAGGCGGTGT-3') and 15 and 17 (5'-ACAC-CGCCTTCGGTCTAGAGACCCTGAGAGC-3') and 12. The wild-type -337/+2, -273/+2 and -205/+2 promoters have been described previously (20); the -242/+2 promoter was constructed in the same way with oligonucleotide 18 (5'-GGGAGATCTAGGCC-CCGAAGGCGGTGTATG-3') defining the 5' boundary. All promoters were finally excised from CAT vectors by restriction enzymes *Bam*HI and *Hind*III, blunted and introduced into the *Sma*I site of the PGL3 vector (Promega).

Recombinant protein production

BL 21 DE3 bacteria bearing PGSTZFX fusions or 24KYH6ZFX were grown in L broth supplemented with 10 μ M ZnCl₂ to an OD of 0.8 at 550 nm; 0.2 mM IPTG was then added to the medium and growth continued at 37°C for 3 h. For PGSTZFX, the bacteria were centrifuged and resuspended in a 1/20 vol. of 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM MgCl₂, 10 μ M ZnCl₂, 0.1 mM PMSF, and 50 mM benzamide, sonicated on ice and centrifuged at 12 000 g for 15 min. The supernatant was adjusted to 2 mM CHAPS and applied to GSH-agarose (1 ml:10 ml supernatant); followed by extensive washing with 100 ml 20 mM Tris, pH 7.5, 200 mM NaCl, 10 μ M ZnCl₂ (buffer A), then 20 ml buffer A adjusted to 1.6 M NaCl, then again with 10 ml buffer A. The column was then resuspended in 3 vol. 50 mM glycine, pH 9.2, 1 mM CaCl₂, 10 μ M ZnCl₂ and 0.1 U/ml nuclease S7 (Boehringer). Reactions were checked by monitoring the OD variations of the supernatant (stable after 10 min) and allowed to proceed for 1 h. The column was then washed again by alternating buffer A, buffer A + 1.6 M NaCl and buffer A. The proteins were then batch eluted in 20 mM HEPES, 200 mM NaCl, 0.5 mM MgCl₂, 10 μ M ZnCl₂, 1 mM CHAPS and 5 mM GSH (1 vol.). The eluate was adjusted to 50% glycerol and stored at -20°C. In the case of 24KYH6ZFX, the cells were centrifuged, resuspended in 1/20 vol. of Tris 50 mM pH 7.5, 1 M NaCl, 5 mM benzamide and 0.1 mM PMSF and sonicated on ice. The homogenate was centrifuged at 4000 g for 5 min. The supernatant was discarded and the pellet sonicated in 50 mM Tris pH 8.9, 1% Triton X-100 and 5 mM EDTA. After centrifugation, the supernatant was discarded and the pellet re-extracted twice in the same way with 1.5 M urea, then solubilized in buffer B (6 M guanidine and 10 mM imidazole pH 7.5). The 6 M guanidine extract was loaded on NI⁺⁺-agarose equilibrated in buffer B (1/10 v/v extract) and the column was washed extensively with 10 vol. buffer B, followed by 10 vol. buffer B adjusted to 40 mM imidazole pH 7.5. Finally, the proteins were eluted with 3 vol. buffer B adjusted to 120 mM

imidazole pH 7.5, and 10 μ M ZnCl₂. The proteins adjusted to an OD of 0.4 at 280 nm were successively dialysed against 3 M guanidine, 20 mM Tris pH 7.5, 0.5 mM MgCl₂ and 10 μ M ZnCl₂ and then against the same buffer with 1.5, 0.75 and 0.4 M guanidine and finally without guanidine at 4°C. The proteins were stored at -20°C with 50% glycerol.

EMSA, DMS interference and DMS protection

EMSA and DMS interference were carried out essentially as described (20). For DMS protection, DNA binding was allowed to proceed for 15 min on ice in the presence of 1 μ g poly(dI-dC)·(dI-dC), 500 ng unlabelled heterologous oligonucleotide and 1 ng labelled fragment; 0.25 μ l DMS was then added to the 20 μ l reaction, along with a 500-fold excess of unlabelled homologous fragment. The reaction was allowed to proceed for 30 s and stopped by separation of the components by PAGE at a field strength of 10 V/cm. After 90 min migration, the free and complexed DNAs were eluted and processed through piperidine cleavage as described (25). The oligonucleotides used in these experiments were: -282/-224C, 5'-GGCGAAGTCCCAGGGCCCCAGGCGTGGC-TCTCAGGGTCTCAGGCCCCGAAGGCGGTGT-3'; -282/-224NC, 5'-TACACCGCCTTCGGGCTGAGACCCTGAGAGCC-ACGCTGGGGCCCTGGGACTTCGC-3'; -255/-224C, 5'-GCTCTAGGGTCTCAGGCCCCGAAGGCGGTGT-3'; -255/-224NC, 5'-TACACCGCCTTCGGGCTGAGACCCTAGAG-3'; -282/-250C, 5'-GGCGAAGTCCCAGGGCCCCAGGCGTGGCTCT-3'; -282/-250NC, 5'-TAGAGCCACGCTGGGGCCCTGGGACTTCGC-3'; -282/-250BIIC, 5'-GGCGAAGTCCCAGG-GCAGATCTCGTGGCTCT-3'; -282/-250BIINC, 5'-TAGAGC-CACGAGATCTGCCCTGGGACTTCGC-3'; -255/-224ATC, 5'-GCTCTAGGGTCTTCGGCCCCGAAGGCGGTGT-3'; -255/-224TANC, 5'-TACACCGCCTTCGGGGGCCAGAGA-CCCTAGAG-3'; -255/-224XbaC, oligonucleotide 16; -255/-224XbaNC, oligonucleotide 17. The results of DMS interference and protection were quantified using ImageQuant software and the Molecular Dynamics Personal Densitometer.

Cell culture and transient transfection

The JF (B-EBV) cell line was maintained and used in RPMI with 10% FCS. The TM3 cell line was obtained from ATCC and passaged in DMEM/F12 medium, 10% FCS and 5% horse serum. The cells were seeded in 10 cm² plates 24 h prior to transfection in DMEM medium without phenol red and supplemented with 2 μ M ZnCl₂ plus 10% foetal calf serum pre-treated with activated charcoal (FCSC). Transfection was performed using DOTAP (Boehringer), as recommended by the manufacturer, in a seeding medium adjusted to 2.5% FCSC. For each transfection, 2 μ g of the expression vector, 0.2 μ g of the luciferase reporter and 0.04 μ g Tk β Gal or 0.02 μ g pSV β Gal vector were used. After 6 h incubation with liposomes, the transfection medium was removed and replaced by the seeding medium for 36 h. Cells were lysed with 200 μ l of reporter lysis buffer (Promega) and luciferase activity was determined using the luciferase assay system (Promega). β -Galactosidase activity was determined according to the manufacturer, using the Galactolight kit (Tropix) downscaled 3-fold, and was used for normalisation of the luciferase activity. CAT activity was measured as previously described (20) and quantified with a β Imager 3600, using β -Vision software

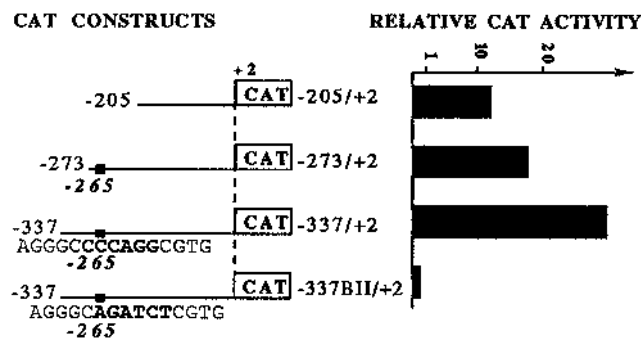


Figure 1. The -273/-205 regulatory element is essential to the activity of the HLA-A11 promoter; the numbering is according to Blanchet *et al.* (20). Activity of various reporter constructs in the JF B-EBV cell line. Ten micrograms of plasmid DNA was introduced by electroporation and CAT activity was assayed 24 h later.

(Biospace). All transactivation assays were repeated several times using at least two different plasmid preparations.

RESULTS

An element centred at position -265 is essential for high activity of the HLA-A11 promoter in various cell lines

A previous study undertaken in our laboratory has shown that the HLA-A11 promoter region -273/-205 binds effectors that activate transcription. Analysis of DNA binding activities from JF, a B-EBV cell line, and from a HeLa cell line demonstrated that several proteins come into contact with DNA in the -273/-233 region. Six bases involved in contacts with at least two different proteins in the -266 to -261 region were mutated by introduction of a *Bg*III restriction site (Fig. 1). CAT constructs driven by the mutated promoter -337/+2BII were compared with CAT constructs bearing the wild-type -337/+2 promoter.

This mutation resulted in a 20-fold reduction in promoter activity in the JF human lymphoblastoid cell line (Fig. 1). Similar results were obtained in various human cell lines originating from various tissues and in COS-7, an African green monkey kidney cell line (not shown). While previous deletion analysis undertaken in our laboratory underestimated the contribution of this region to the transcriptional regulation of the HLA-A11 promoter, this mutation clearly emphasises the key role of this element in the general activity of the promoter.

C-Terminal zinc fingers bind to the -272/-233 oligonucleotide

A λ gt11 cDNA library from HeLa cells was screened with a radiolabelled and hexamerised -272/-233 oligonucleotide encompassing the site identified by mutation. One clone was identified and found to contain an insert with 100% identity to the C-terminal region of ZFX, starting in the middle of the ninth zinc finger of ZFX (Fig. 2A). This indicated that four complete zinc fingers at the C-terminus of ZFX, which contains 13 zinc fingers, are sufficient for binding to the promoter element. Subcloning the coding portion of the λ gt11 cDNA in a PGEX vector allowed purification of the fusion protein on GSH-agarose and facilitated subsequent analysis of its DNA binding activity, whose specificity was ascertained by competition with homologous and

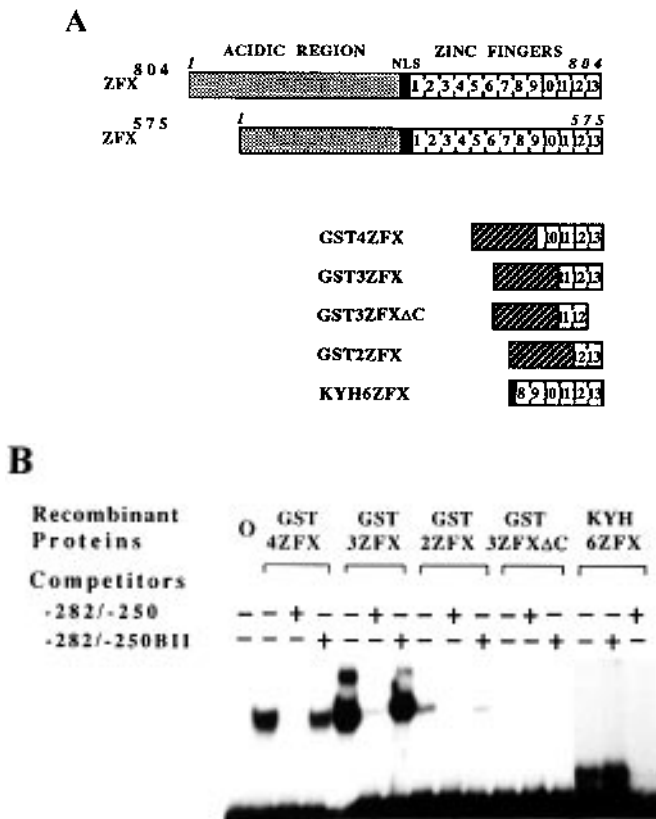


Figure 2. ZFX zinc fingers which are involved in binding to the HLA-A11 promoter DNA. (A) Organisation of the ZFX cDNA isoforms ZFX⁸⁰⁴ and ZFX⁵⁷⁵ (21) and positions of the various coding portions used to produce fusion recombinant proteins. (B) Electrophoretic mobility shift assay of the purified recombinant proteins. Ten nanograms of recombinant protein and 0.2 ng labelled oligonucleotide (–282/–224) were used in each experiment; oligonucleotides used for competition were added in 1000-fold excess.

heterologous oligonucleotides (Fig. 2B). The minimal number of zinc fingers required for DNA binding was established by constructing various fusion products. Deleting the partial ninth and the tenth zinc fingers (PGST3ZFX) significantly increased the specific activity of the DNA binding protein. Deletion of the eleventh zinc finger (PGST2ZFX) resulted in a protein of low activity, whereas retaining the eleventh and twelfth zinc fingers and deleting the thirteenth (PGST3ZFXΔC) abolished DNA binding (Fig. 2B).

The terminal three ZFX zinc fingers bind two sites centred at positions –265 and –238 of the HLA-A11 promoter

The interactions of ZFX with the HLA-A11 promoter were more precisely determined by probing the interactions of GST3ZFX with DNA by means of methylation interference. The DNA molecules eluted from the two retarded complexes were compared with the free DNA. Analysis of the upper strand from the faster migrating complex indicated a weak interference of two groups of bases separated by 25 nt, namely AGGG, from –271 to –268, and AGG, from –242 to –240 (Fig. 3A, lanes 5, 7 and 8).

The lower strand of the same complex showed contacts at GG (–267, –266) and 26 nt away at GGG (–239 to –237), (Fig. 3A, lanes 1, 3 and 4). Considering what is already known about the organisation of zinc fingers and their binding to DNA, we hypothesised that two sites are present in this region, one at –265 and the other at –238. Quantification of several interference results indicated that binding at the two sites in the faster complexes resulted in a near 50% interference at each of the two sites. This is consistent with a 1 protein/1 DNA complex in which the two DNA sites are bound with equivalent affinity. Quantification of the interference pattern of the slower complexes compared with the free DNA, which was in large excess, is fully consistent with a 2 protein/1 DNA complex, since the interference values for bases of the two sites exceeded 80% (Fig. 3A, lanes 1, 2 and 4 and 5, 6 and 8). We further investigated the relative affinities of the two sites by means of the DMS protection assay, which was carried out on the 1 protein/1 DNA complex under conditions which disfavour the 2/1 complex according to the EMSA analysis. The DNA located at the positions of the free probe (which was in large excess) and of the 1/1 complex were eluted, cleaved with piperidine and analysed on a denaturing gel (as expected, the 2/1 complex was not observed in this preparative gel; not shown). Analysis of protection data (Fig. 3B) indicated that the protected bases (–268, –241 and –240 on the upper strand and –267, –266, –239 and –238 on the lower strand) overlapped with the bases identified by interference. More surprisingly, quantification of the protection indicated that the 50% maximal protection at each site for a 1/1 complex with two equivalent binding sites was exceeded. The reactivity of the two most protected bases, –239 and –266 of the lower strand, were reduced 3.6-fold and 4.1-fold, respectively. This indicated that the 2/1 complex was most probably formed in solution at the time of contact with DMS, but that it was too unstable to be effectively caged by the gel and was dissociated into the 1/1 complex when loaded on the gel. Two bases of the non-coding strand were hyper-reactive: –236 (2.9-fold) and –264 (1.7-fold). The similar reactivity of the N7 of one guanine 5' of the two sites could have been due to a similar mobility or deformation introduced in the DNA backbone by the zinc fingers (26).

EMSA of the two sites separated on different oligonucleotides, –255/–224 (Fig. 4A) and –282/–250 (Fig. 4B), indicated that binding to each site occurred independently of the presence of the other site. As expected from the interference and protection studies, binding to the two sites was found to be within the same range of affinity. Extensive substitution of the bases at the interference positions by introduction of a *Bgl*III restriction site in the –282/–250 oligonucleotide and a *Xba*I restriction site in the –255/–224 oligonucleotide abolished the binding, as indicated by the absence of competition by these oligonucleotides (Fig. 4A and B, lanes 8–10 and 11–13, respectively). The mutation of the most 5' base identified by interference in the –255/–224 oligonucleotide at position –241, which was changed from A to T, markedly diminished the binding (Fig. 4A and B, lanes 14–16). From crystallographic and NMR studies of other zinc finger proteins complexed with their DNA targets (27–29), it was expected that this position would be in contact with the thirteenth zinc finger. The effect of this point mutation was consistent with the effect of removing the thirteenth zinc finger of ZFX from PGST3ZFX (Fig. 2B).

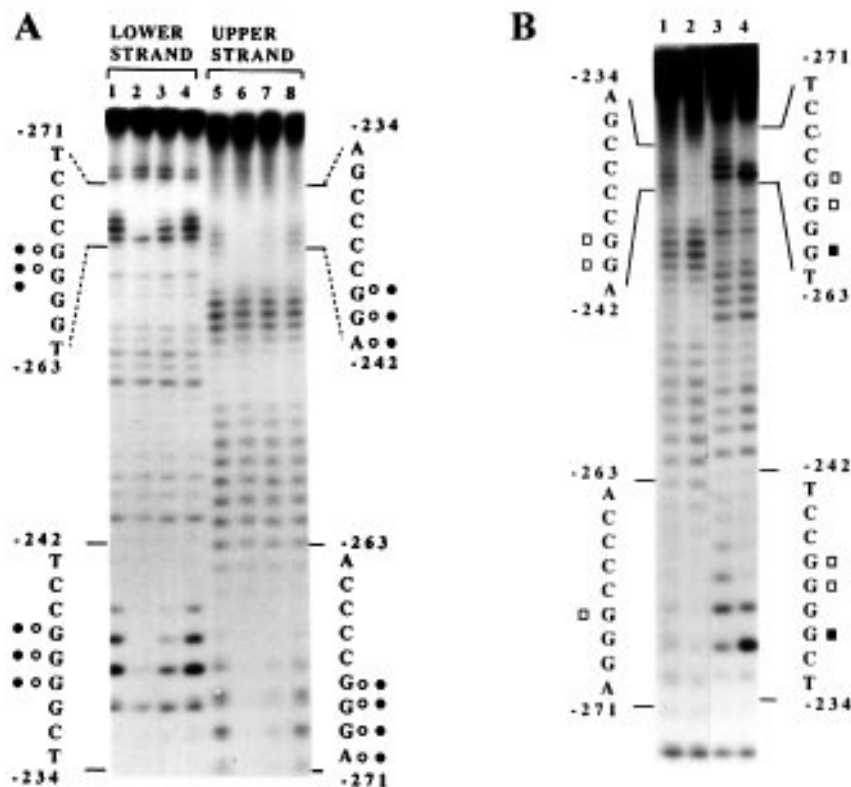


Figure 3. Identification of two binding sites within the $-282/-224$ region of the HLA-A11 promoter by DMS interference, DMS protection and EMSA. **(A)** DMS interference. The two strands were separately labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and each was annealed to the unlabelled complementary strand. DMS modification was completed prior to binding according to Materials and Methods. After a 5-fold scale-up of the EMSA reaction, the free DNA, in around 10-fold excess (lanes 1, 4, 5 and 8) and the DNA complexed in both the slower (lanes 2 and 6) and faster retarded bands (lanes 3 and 7) were eluted, cleaved and compared in a denaturing gel. Interference bases of the faster complex (○) and the slower complex (●) are indicated. **(B)** DMS protection of the same DNA probes by GST3ZFX was carried out as described in Materials and Methods. Addition of a large excess (500-fold) of unlabelled $-282/-224$ oligonucleotide along with DMS ensured that protection data are not superimposed with DMS interference occurring during the time course of the reaction. The DNA of the fast complex (lanes 2 and 4) was compared with the free DNA (lanes 1 and 3). Protected bases (□) and hyper-reactive bases (■) are indicated.

The ZFX gene encodes a transcriptional regulator which activates the HLA-A11 promoter

A cDNA which codes for ZFX⁵⁷⁵, a short isoform of ZFX, was amplified by PCR of human ovary cDNA. A full-length cDNA was cloned in the eukaryotic expression vector PSG5. The effects of the PSGZFX⁵⁷⁵ and PSG5 vectors on transcription were compared by co-transfection assay with HLA-A11 promoter-driven reporters in various cell lines (JF, Cos-7, Cos-6, HeLa, 293, T47D, TM3 and TM4; data not shown). Only the murine Leydig cell line TM3 allowed reproducible analysis of the transcriptional effects of ZFX. In this cell line, PSGZFX⁵⁷⁵ was found to stimulate transcription of the $-337/+2$, $-273/+2$ and $-242/+2$ promoter constructs by a mean value of ~ 20 -fold (Fig. 5). PSGZFX⁵⁷⁵ had a moderate effect on the $-205/+2$ promoter construct, increasing its activity 2-fold. Introduction of the mutation, which was shown to abolish binding of ZFX to the $-282/-250$ region (Fig. 4A, lanes 8–10) in the context of the $-337/+2$ promoter, reduced its basal activity by 8-fold in TM3 cells and precluded transactivation by PSGZFX⁵⁷⁵ (Fig. 5). On the other hand, modification of the downstream site in the context of the $-337/+2$ promoter by a mutation shown to abolish binding of ZFX to the $-255/-224$ region significantly affected neither the basal activity nor the stimulated activity of the promoter (Fig. 5,

$-337/+2\text{Xba}$). As expected, the stimulatory activity of ZFX⁵⁷⁵ was lost when the four C-terminal zinc fingers were deleted. Normalised promoter activities were similar with the empty PSG5 vector and with the PSGZFX⁵⁷⁵ ΔC expression vector bearing the deleted cDNA. However, a 2- to 3-fold co-stimulation of β -galactosidase and luciferase activities were reproducibly noted with this construct (not shown) and may reflect increased cell survival or increased expression of the co-introduced genes.

A minigene construct, PSIN3ZF, consisting of an initiation codon followed by a HA tag, a nuclear localisation signal and the terminal three zinc fingers, repressed the basal activity of the $-337/+2$, $-337/+2\text{Xba}$, $-273/+2$ and $-242/+2$ constructs by ~ 1.6 -fold. This suggested that the transcriptional activation seen in TM3 was not merely due to the displacement of repressor factors and that the full-length ZFX⁵⁷⁵ isoform actively contributed to the transcriptional competence of the activated promoters.

DISCUSSION

We show here that mutation of an element centred at -265 of the HLA-A11 promoter has a profound effect on its transcriptional activity. Oligonucleotides encompassing this region were used to screen a $\lambda\text{gt}11$ library; a partial cDNA was isolated and found to correspond to the C-terminal portion of ZFX. Several years ago,

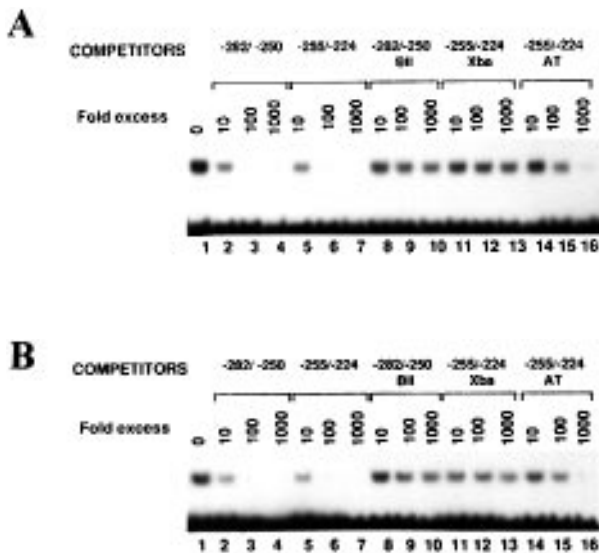


Figure 4. EMSA of GST3ZFX with labeled probes $-282/-250$ (A) and $-255/-224$ (B). Competitors used and their molar excesses are indicated. In the $-282/-250$ BII oligonucleotide bases -263 to -266 , CCCA, of the binding site were changed to AGAT. In the $-255/-224$ Xba oligonucleotide, bases -239 to -242 , AGGC, were changed to TAGA, and in $-255/-224$ AT a T replaces the A identified in DMS interference at position -242 .

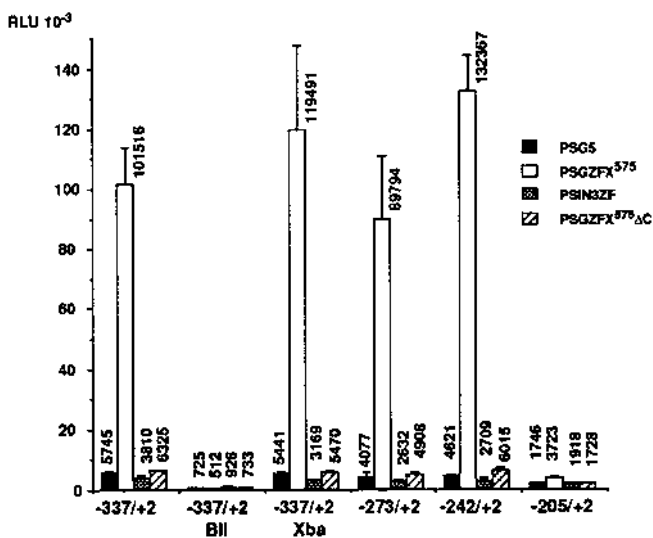


Figure 5. Regulation of different HLA-A11 promoter constructs by ZFX⁵⁷⁵, ZFX⁵⁷⁵ΔC (deleted of the DNA binding site) and N3ZF (the nuclear targeted DNA binding site protein) in TM3, a murine Leydig cell line. The $-337/+2$ BII promoter is mutated within the -265 site, as indicated in Figure 4 (oligonucleotide $-282/-250$ BII). The $-337/+2$ Xba promoter construct is mutated within the -238 site as indicated in Figure 4 (oligonucleotide $-255/-224$ Xba). Expression vectors, luciferase reporters and β -Gal normalization plasmids were introduced into cells, as described in Materials and Methods. The enzyme activities were determined 36 h later, when the cells were confluent.

ZFX was identified as an X chromosome gene that was highly homologous to ZFY, formerly thought to be a strong candidate for testis-determining factor (TDF) (30). The role of ZFY as TDF was definitively ruled out when genetic analysis and transgenic

techniques demonstrated that in fact SRY was TDF (31). The functions of both ZFY and ZFX remained elusive, although a role for them as transcription factors has been proposed on the basis of their gene sequences (21,22), and later on the basis of the transcriptional activity of constructs which fused the N-termini of ZFX and ZFY to the GAL4 DNA binding site (32). Mammalian ZFX transcripts are ubiquitous (22,33) and derive from at least four untranslated 5' exons and seven coding exons. The acidic N-terminal domain and the nuclear localisation signal are encoded by exons 5–10, while all 13 zinc fingers are encoded by exon 11. Transcripts containing all exons from 5 to 11 encode the largest ZFX protein, ZFX⁸⁰⁴, while alternative transcripts missing exon 5 encode ZFX⁵⁷⁵, a protein initiated in exon 7 (21). Other alternative transcripts have been observed in the mouse, in which exons 7 or 10 are spliced out (34). We have focused on the functional study of ZFX⁵⁷⁵ and our study conveys two messages, that, as previously hypothesised, ZFX indeed regulates transcription and that ZFX is capable of regulating HLA-A11 class I gene transcription.

ZFX⁵⁷⁵ is a transcription factor

The identification of a DNA binding site specifically recognised by the C-terminal zinc fingers of ZFX was the first step in obtaining experimental evidence of the involvement of ZFX in transcription. The terminal three zinc fingers of ZFX were shown to be sufficient for binding two sites on the HLA-A11 promoter, 7 and 8 bases of which respectively were shown to be affected in their reactivity toward DMS. This is consistent with the view that a single C₂H₂ zinc finger frequently recognises three contiguous bases (28,29). Efforts to understand the relationship between the amino acid composition of the conserved helix of C₂H₂ zinc fingers and the nature of the bases recognised have been made in several laboratories (35–40). A perfectable code is now emerging which, when applied in the three C-terminal zinc fingers of ZFX (and of ZFY, whose DNA binding properties were found to be indistinguishable from those of ZFX; not shown), predicts the A₁C₂G₃G₄X₅C₆X₇C₈A₉ recognition site in preference to T₁C₂G₃G₄X₅C₆X₇C₈T₉ (Table 1). The site determined at -265 , AGGCCCCCA, departs from this prediction at position 2, while the site at -238 , AGGCCCCGA, departs at positions 2, 4 and 8, but was experimentally found to be of similar affinity when compared with the -265 site. Interestingly, the nonamer AGGGTCTCA, found between the two sites, better matches the prediction, but no evidence of binding to this sequence was observed. Using EMSA, efficient specific DNA binding was observed with purified fusion proteins containing either six (not shown) or fewer C-terminal zinc fingers. However, we have been unable to detect the DNA binding activity of ZFX⁵⁷⁵ by EMSA of crude nuclear and cytoplasmic extracts of transfected TM3 cells or other cells (COS-7 and 293). The same holds true for the product of the PSIN3ZF construct. We suspect that an abundant inhibitor masks the DNA binding site of ZFX when the cells are disrupted. Similarly, *in vitro* transcription/translation did not allow detection of ZFX⁵⁷⁵ DNA binding activity, while the DNA binding activity of the three C-terminal zinc finger proteins was detected only very transiently soon after translation. RNase treatment of cellular extracts as well as of *in vitro* translation products were ineffective in rescuing the DNA binding activity (not shown). Nevertheless, the demonstration of transcriptional activation, which necessitates both the DNA binding site

Table 1. Prediction of the DNA binding site for the last three zinc fingers of ZFX

ZINC FINGER NUMBER	α -HELIX N-TERMINUS OF ZINC FINGERS						PREDICTED SEQUENCE 3'→5'	
	-1	1	2	3	4	5		6
11	Q	Q	S	E	L	K	K	A/C N C N G G C A/T
12	D	A	S	G	F	K	R	
13	R	P	S	E	K	N	Q	

The N-terminal sequences of each zinc finger α -helix are displayed and the three primary recognition positions of the helix are highlighted. The rules used in the present case derived mainly from Desjarlis and Berg and Choo and Klug (35,37–39), where they are extensively discussed. Numbering of α -helix positions is according to Choo and Klug (39); N indicates a position where the prediction is uncertain.

characterised in the HLA-A11 promoter and overproduction of ZFX⁵⁷⁵ in TM3 (Fig. 5), implies that, in undisrupted cells, at least a portion of the ZFX⁵⁷⁵ molecules are capable of recognising their DNA targets. In the context of the HLA-A11 promoter, the PSIN3ZF construct, which has no transactivation domain, was shown to repress transcription. However we have also identified two other promoters (not shown) that are regulated by ZFX, one of which is stimulated by PSIN3ZF and PSGZFX⁵⁷⁵, the other repressed by PSGZFX⁵⁷⁵. Thus, the ZFX gene might be able to influence transcription by a variety of mechanisms, depending on the promoter and the context provided by the cell line or tissue. In this respect, it is noteworthy that the magnitude of transactivation was much higher in TM3 than in any other cell line. The exacerbated activity of a gene borne by a sex chromosome in specialised cells of a sexual tissue suggests its involvement in sex-specific regulation. However the physiological significance of this observation remains to be clarified, and it is possible that the ubiquitous distribution of ZFX transcripts reflects a general function in cell maintenance.

Whether ZFX⁵⁷⁵ can interact in TM3 cells with an activating partner that is specifically abundant in such cells or whether a widespread inhibitor of ZFX is lacking there is at present unknown, but this issue is amenable to investigation. Our results suggest that the DNA binding activities of ZFX proteins are undetectable in EMSA of cellular extracts under the usual conditions. However, we believe that the transactivation assay conditions described here can provide a basis for comparing the transcriptional activities of the various ZFX and ZFY gene family members and their isoforms.

HLA class I regulation by ZFX

The -273/-250 region of the HLA-A11 promoter has been previously described as a binding site for AP2 and at least one other protein (20). We show here that mutating this DNA binding site results in a profound weakening of the promoter's activity in all the cell lines tested. Some of the interactions which take place at this site are therefore important for controlling transcription of the HLA-A11 promoter. We show that ZFX is able to contact some of the bases identified in our previous studies, but produces an interference pattern clearly different from any protein so far identified. ZFX also binds a second site centred at position -238. The two binding sites identified in the -273/-205 region of the HLA-A11 gene do not contribute to ZFX transcriptional activation of the various promoter

constructs in the same way. In the -337/+2 promoter context, mutation of the -265 site strongly reduced transcriptional activity and no longer allowed transactivation by ZFX. Therefore, in this case, the remaining intact site at -238 appeared to be silent. Conversely, comparison of the transactivation magnitude of the -273/+2 construct containing the two sites and the -242/+2 construct containing only the downstream site led to the conclusion that ZFX can transactivate the promoter through interactions with the -238 site and that further adjunction of the -265 site within the -273/+2 construct results in no additional increase in the transactivation magnitude. Thus, in this case, it is the -265 site which seems to be silent, although this is only the simplest interpretation of the data. These observations challenge the simple conceptions of transcriptional control regions as modular additive elements and emphasise the importance of stereospecific interactions in the assembly of transcription complexes. This matter was recently reviewed and investigated and led to the proposal of the enhanceosome as a cooperatively built up and highly ordered transcription complex (41). It is possible that sequences between -337 and -273 bind effectors which contribute to shaping the transcription complex in a different way when compared with the transcription complex assembled on the -273/+2 or -242/+2 promoters, hence the different contributions of the two binding sites in these different contexts, despite remarkably similar magnitudes of transactivation by ZFX. On the other hand, it is also possible that the Bg/III mutation at -265 has pleiotropic effects, not only precluding ZFX binding at this site but also other key effectors, or perturbing a critical local bending of DNA.

The interference pattern of ZFX obtained here at the -265 site and the AP2 interference pattern previously described at the same site (20) strongly suggest that their binding at this site is mutually exclusive. It will be of interest to study the functional consequences of AP2 and ZFX interactions on the HLA-A11 promoter by means of co-transfection studies.

The AGGGCCCCA sequence centred at -265 is well conserved among HLA-A alleles and is also found in HLA-G and HLA-E genes. However, it is less significantly conserved among HLA-B and HLA-C alleles, in which this region is quite polymorphic. This sequence is not known in the homologous position of the murine MHC class I genes. However, the whole spectrum of DNA binding recognition by ZFX is not known at present, thus it cannot be decided whether ZFX exerts an allele- or locus-specific regulation or whether it acts more generally on the various promoters of HLA Class I genes.

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