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An N- and C-terminal Truncated Isoform of Zinc Finger X-linked Duplicated C Protein Represses MHC Class II Transcription

Anastasiia Aleksandrova, **Oleksandr Galkin**, **Rupa Koneni**, and **Joseph D. Fontes***
Department of Biochemistry and Molecular Biology, 3901 Rainbow Blvd., MS3030, University of Kansas Medical Center, Kansas City, KS 66160

Abstract

The zinc finger X-linked duplicated A (ZXDA) and ZXDC proteins are both required for robust transcription of major histocompatibility complex class II (MHC II) genes. Aside from the full length ZXDC mRNA transcript, at least one additional mRNA is produced by the ZXDC gene, in which transcription initiates within the first exon and terminates within the seventh intron. The protein product produced from this transcript, which we have named ZXDC2, is truncated on both the N- and C-terminus. We demonstrate here that ZXDC2 functions to repress MHC II transcription induced in HeLa cells treated with IFN-γ. We further demonstrate that ZXDC2 interacts with both ZXDA and ZXDC, suggesting a mechanism by which ZXDC2 may inhibit MHC II transcription. These studies not only provide additional support for the role of ZXD proteins in regulating MHC II transcription, but demonstrate a unique mechanism for the synthesis of a mRNA isoform.

Keywords

Transcription factor; zinc finger proteins; MHC class II; mRNA isoforms; dominant negative; gene regulation

Introduction

The expression of major histocompatibility complex class II (MHC II) genes is regulated primarily at the level of transcription. MHC II gene promoters share a similar architecture, with four conserved cis-acting elements or “boxes” (the W, X1, X2 and Y boxes) necessary for constitutive and inducible transcription (1, 2). The X1 box is occupied by the heterotrimeric regulatory factor X (RFX) complex (3–6), the X2 box binds the cAMP response element binding protein (CREB) (7, 8) and the Y box is bound by the nuclear factor Y (NFY) heterotrimeric complex (9, 10). However, for transcription to occur, a co-activator protein known as the class II trans-activator (CIITA) must also be present (11). CIITA is the master regulator of MHC II transcription, as the DNA binding proteins appear to be constitutively present in most if not all cell types, and it is the presence of CIITA that determines whether MHC II genes are transcribed. CIITA interacts with a multitude of general transcription factors and chromatin modifying and remodeling complexes to activate transcription (12). Employing yeast two-hybrid, we recently identified a novel CIITA binding protein known as zinc finger X-linked duplicated C (ZXDC) (13). ZXDC contains ten zinc fingers and a strong transcriptional activation domain. ZXDC is the founding member of the ZXD family. The other two members, ZXDA and ZXDB, are retrogenes derived from ZXDC, and are located on the short arm of the X chromosome (14). In

*Corresponding author. jfontes@kumc.edu, Phone: +1 913 588 9848;, Fax: +1 913 588 9896.

subsequent work, we found that it is a complex of ZXDA and ZXDC that interacts with CIITA, and both ZXDA and ZXDC are necessary for robust MHC II gene transcription (13). The ZXDC protein is present at MHC II promoters, independent of the transcriptional state of these genes (13).

We describe here the characterization of a protein isoform of ZXDC we named ZXDC2. ZXDC2 arises from a unique mechanism in that transcription of the ZXDC2 mRNA initiates within the first exon of ZXDC and terminates within the seventh intron. The protein encoded by this mRNA lacks the first three zinc fingers and the entire C-terminal region required for interacting with CIITA. We show that ZXDC2 functions in a dominant negative manner, with respect to MHC II transcription, and is capable of binding to ZXDC.

Materials and Methods

Plasmids

The plasmids pCMV-FL-ZXDC and pG5-luc were previously described (15). The ZXDC2 cDNA in plasmid LafmidBA (IMAGE clone ID 39655) was obtained from a commercial distributor (InVitrogen). Portions of this cDNA were previously sequence and deposited in GenBank (accessions R51627 and R51714). We sequenced the entire cDNA and deposited the sequence in GenBank (accession FJ980275). pCMV-FL-ZXDC2 was constructed by PCR amplification of the protein coding sequences of the ZXDC2 cDNA and cloning it into plasmid pFLAG-CMV-5a. The plasmid pGAL4-ZXDC2 was constructed using the same strategy, except that the amplified ZXDC2 coding region was cloned into the plasmid pCGal4 (13).

Cell culture and plasmid transfection

The human embryonic kidney cell line 293 (HEK293; ATCC: CRL-1573) and the cervical adenocarcinoma cell line HeLa (ATCC: CRL-2) were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 500 μg/ml streptomycin. The human Burkitt's lymphoma cell line Raji (ATCC CCL-86) and its CIITA-negative derivative RJ2.2.5 (16) were maintained in RPMI-1640 media supplemented as above. Cells were transfected with plasmids as previously described (15). For the generation of the HeLa cell line stably expressing ZXDC, 1 μg of pCMV-FL-ZXDC (15) was transfected into HeLa cells and selection with 0.8 mg/ml G418 (InVitrogen) initiated 48 hours later. Clones expressing FLAG-ZXDC were identified by Western blot. In experiments with interferon gamma (IFN-γ) treatment, cells were treated 24 hours after plasmid transfection with 10 units of IFN-γ (Roche Applied Sciences, Inc., Indianapolis, IN) per ml of culture media.

Primer extension to map transcriptional start site of ZXDC2

Total RNA was isolated from HeLa, Raji, 293T, and RJ2.2.5 cells using Trizol (InVitrogen). Reverse transcription was performed using the Thermoscript RT Ssystem (Invitrogen) according to the manufacturer's instructions. The primer for reverse transcription was located within the sixth intron of ZXDC (3'UTR of ZXDC2; sequence: 5' GAATATCCTCAAGACCGAGTATGAGA). Primer extension and sequencing reactions were performed using the Thermo Sequenase Cycle Sequencing Kit (USB, Inc., Cleveland, OH) according to the manufacturer's instructions. The primer for the extension and sequencing reactions was labeled with TAMRA for fluorescent detection (5'ACCGTACTGAACTTCTTGCC). The reaction products were resolved on a 5% UREA-polyacrylamide gel. Fluorescently labeled DNA was visualized with a Typhoon 9410 imaging system (G.E. Healthcare, Inc., Piscataway, NJ).

Reverse Transcriptase PCR (RT-PCR)

Reverse transcription, PCR, detection of fluorescently labeled PCR products, and primers for ZXDC, HLA-DRA, and CIITA were as previously described (15). ZXDC2 PCR primer sequences: 5’ GTGAATGGGTCCCAGGTATG and 5’ ACTTGCCTGAGGGAGTCAGA.

Western and Northern blots and Luciferase Assays

Western blot, Northern blot and luciferase assay were performed as previously described (17). The DNA probe for ZXDC2 was derived from nucleotide position 1400–1736 of the cDNA (GenBank accession FJ980275) which is a 336 bp sequence immediately downstream of the position of the stop codon, within intron 6. This sequence shares no significant homology in the human genome outside the ZXDC gene, as determined by BLAT search of the human genome release hg19 (18). Anti-FLAG M2 antibody was purchased from Sigma Chemical (St. Louis, MO).

siRNA Transfections

All transfections were carried out in six-well dishes. HeLa cells stably expressing Flag-ZXDC, were transfected with 200 pmol of ZXDC2 siRNA (sequence 5’-CTATGCAGACGTTTCTGGTTTACAA) or negative universal control with low GC content siRNA (InVitrogen) with RNAi Max reagent (InVitrogen) according to the manufacturer’s instructions. Two serial transfections were performed at a 24 hour interval. Six hours after the second siRNA transfection, cells were either treated with 10 units of IFN-γ (Roche Applied Sciences) per ml of culture media or left untreated. RNA was harvested 24 hours after the second siRNA transfection.

Results

The ZXDC gene expresses at least two mRNA isoforms

The ZXDC gene expresses a full length mRNA spliced from a precursor RNA containing ten exons. The protein encoded for by this mRNA has 858 amino acids. Inspection of the cDNAs present in the expressed sequence tagged (est) database revealed that the ZXDC gene expresses at least one isoform that is shorter than the primary mRNA. This isoform, which we named ZXDC2 (Fig. 1A) is generated by initiation of transcription within the first exon of ZXDC and termination of transcription within the seventh intron. The protein predicted from the ZXDC2 transcript begins at a methionine that is at position 259 of the ZXDC protein. The ZXDC2 protein lacks the first three of the ten zinc fingers as well as 150 amino acids in the C-terminus of ZXDC (Fig. 1B). The C-terminal portion absent from ZXDC2 coincides, almost exactly, with the region of ZXDC we found necessary for the interaction between ZXDC and CIITA (13). There are two amino acids on the C-terminus of ZXDC2, not present in ZXDC.

We performed Northern blot with a probe generated from the 3’-UTR of ZXDC2, with a human tissue mRNA blot. The signal for ZXDC2 was relatively weak, though an mRNA was detected in several tissues, most strongly in brain, heart and testis (Fig. 1C). In heart, a second, slightly larger band was also detected. The size of the primary transcript was estimated to be approximately 4.5 kbp. As previously reported (13) a probe derived from exons 8–10 of ZXDC hybridized to one major and one minor mRNA is most tissues. One of these mRNAs was shorter (4 kbp) and one mRNA was longer (5 kbp) than the ZXDC2 mRNA detected with ZXDC2 3’UTR probe (13). The 4 kbp band appears to be a 3’ truncated message that terminates in the fourth exon (data not presented); the 5 kbp is the “full length” ZXDC mRNA containing exon 1 through exon 10 (13). In addition, the tissue specific expression patterns of all three transcripts are dissimilar.

The promoter and transcriptional start site for ZXDC2 is within the first exon of ZXDC

To confirm the transcriptional start site of ZXDC2, we performed primer extension on mRNA purified from several cell lines. mRNA was reverse transcribed with a primer located within the 3' untranslated region (UTR) of the putative ZXDC2 transcript (arrow in Fig. 1A). This position is within the sixth intron of the ZXDC gene and therefore is not present in the ZXDC mRNA. Primer extension identified a cluster of transcriptional start sites located at nucleotide positions 601 to 645 of ZXDC (GenBank accession AY936556; Fig. 2A). Genomic analyses of transcriptional start sites of human and mouse genes have shown that typically there are a cluster of transcriptional start sites spread over a 50–100 bp region (19). This region is just slightly upstream of the majority of the ZXDC2 est cDNAs in GenBank and is consistent with the ZXDC2 transcriptional start sites predicted in the DBTSS database (20). These data demonstrate that transcription of ZXDC2 initiates within the first exon of ZXDC.

To determine if the first exon of ZXDC could function as a promoter, we cloned the sequences from −642 to +39 relative to the most 3' ZXDC2 transcriptional start site (+1/+681 of ZXDC) into the plasmid pGL3 basic, which contains the firefly luciferase gene but no promoter. Transfection of this reporter plasmid into HeLa cells resulted in expression more than six fold over that of pGL3basic alone (Fig. 2B). Serial deletions from the 5' end of the ZXDC2 promoter resulted in a gradual decline in promoter activity. A reporter plasmid containing a fragment of ZXDC2 downstream of the transcriptional start site was essentially inactive (Fig. 2B). From these data we conclude that the first exon of ZXDC, upstream of the ZXDC2 transcriptional start site, can function as a transcriptional promoter,

ZXDC2 suppresses MHC II gene transcription

Since the ZXDC2 protein isoform lacks the C-terminal region necessary for binding CIITA, we reasoned that ZXDC2 may function in a dominant negative manner, suppressing the transcription of MHC II genes. To test this, we transfected HeLa cells with increasing amounts of plasmid pCMV-ZXDC2-FL. This plasmid expresses the ZXDC2 protein with a C-terminal FLAG epitope tag. Western blot with anti-FLAG on lysates from HeLa cells transfected with pCMV-ZXDC2-FL showed that a single band with approximate molecular mass of approximately 52 kDa, similar to that predicted from the amino acid sequence of ZXDC2 (49 kDa), was expressed (Fig. 3A). Interestingly, no sumoylated version of ZXDC2 was detected, though it does contain the sumoylation site (lysine at position 660 of ZXDC) we identified in ZXDC (21). Following treatment of the cells with 100 u/ml interferon gamma (IFN-γ) for 18 hours, RNA was prepared and reverse transcriptase PCR (RT-PCR) performed to detect MHC II HLA-DRA transcription. Cells transfected with empty vector had robust levels of HLA-DRA transcripts (Fig. 3B). However, HLA-DRA transcription decreased with increasing ZXDC2 expression (Fig. 3B).

siRNAs targeted to the 3'-UTR of ZXDC2 knocked down ZXDC expression as efficiently as they did ZXDC2, apparently targeting the intronic sequences of ZXDC in the unprocessed mRNA (data not presented). Therefore, to isolate the effect of ZXDC2 on MHC II transcription, we created a stable HeLa cell line expressing ZXDC from a cDNA which lacks the sequences shared with the ZXDC2 3'-UTR. Expression of ZXDC from the transgene was not affected by the ZXDC2 siRNA (data not presented). However, expression of ZXDC2 was significantly inhibited by the ZXDC-targeted siRNA but not a control siRNA (Fig. 3C). In cells where ZXDC2 was knocked down, induction of HLA-DRA transcription by IFN-γ was dramatically higher than in cells receiving the control siRNA or no siRNA at all (Fig. 3C). The levels of CIITA were unchanged regardless of the levels of ZXDC2 (Fig. 3C). We conclude from these data that ZXDC2 represses MHC II transcription.

ZXDC2 interacts with ZXDA and ZXDC

ZXDA and ZXDC form a heterodimer that is necessary for interaction with CIITA and regulation of MHC II gene transcription (15). One potential mechanism by which ZXDC2 represses MHC II transcription is by binding to ZXDA and/or ZXDC and disrupting the ZXDA-ZXDC-CIITA complex that is required for robust MHC II transcription. To determine if this mechanism may be operating, we wanted to demonstrate ZXDC2 binding to ZXDA and ZXDC. We first performed mammalian two hybrid assay with ZXDC and Gal4-ZXDC2. Gal4-ZXDC2, by itself, did not activate transcription from a reporter plasmid containing five UASg elements linked to the firefly luciferase gene (pG5-luc; Fig. 4A). This result was surprising, given that ZXDC2 protein contains the transcriptional activation domain that we mapped in ZXDC (13). Since we found that sumoylation of ZXDC is necessary for the full activity of its activation domain (21), the fact ZXDC2 is not sumoylated may partially explain this observation. The addition of increasing amounts of the plasmid pCMV-FL-ZXDC, which expresses a FLAG-tagged ZXDC protein (15), to the transfection resulted in increasing luciferase expression (Fig. 4A). This result indicated that ZXDC interacted with Gal4-ZXDC2 bound to the reporter plasmid; the activation domain of ZXDC was responsible for the observed transcription. pCMV-FL-ZXDC by itself did not activate the pG5-luc reporter plasmid (Fig. 4A).

To confirm the interaction between ZXDC and ZXDC2, we performed co-immunoprecipitation experiments. HA-tagged ZXDC was co-expressed with FLAG-tagged ZXDC2 in HEK293 cells and immunoprecipitation with anti-FLAG was performed. Western analysis of the immunoprecipitates clearly demonstrated that ZXDC was brought down by the ZXDC2 protein, confirming that the two proteins interact (Fig. 4B). We next tested whether ZXDA, which along with ZXDC forms a complex with CIITA, could also bind ZXDC2. The results of co-immunoprecipitation experiment clearly show that ZXDA also can interact with ZXDC2 (Fig. 4C).

Discussion

Our previous work demonstrated the importance of ZXDA and ZXDC, two members of the ZXD family, in the transcriptional regulation of MHC II genes (13, 15). ZXDA and ZXDC form a complex that interacts with the master regulatory protein of MHC II transcription, CIITA (15). Although the mechanism is not fully clear, it is likely that ZXDA and ZXDC are part of the MHC II enhanceosome, and their presence helps in recruiting and/stabilizing the binding of CIITA to MHC II promoters. In the present study, we have extended our findings to include the role of an isoform of ZXDC we named ZXDC2, that represses MHC II transcription. Our findings that both ZXDA and ZXDC can interact with ZXDC2 suggest that ZXDC2 may disrupt the ZXDA-ZXDC complex necessary for recruitment of CIITA to MHC II promoters. Additional studies will be necessary to confirm this model and flesh out the details of the mechanism.

ZXDC2 is produced by a unique transcriptional strategy: transcription of the mRNA initiates within the first exon of ZXDC and terminates within the seventh intron. The first exon of ZXDC can function as a promoter in reporter gene assays, and combined with our mapping of the transcriptional start site of ZXDC2, makes a strong case that the first exon of ZXDC is a *bone fide* promoter. Analyses of genome-wide promoter architecture have shown that transcriptional initiation does occur within exons, and that the usage of these initiation sites is generally tissue specific (22).

The existence of the ZXDC2 mRNA poses the question as to how an RNA polymerase II, that initiates at the ZXDC2 promoter (that is, in exon 1 of ZXDC), consistently terminates within the seventh intron instead of continuing on to the termination site after the tenth exon

of ZXDC. In recent years the close relationship between transcriptional initiation, processing and termination has been established (23–25). These links typically involve interactions between the C-terminal domain (CTD) of RNA polymerase II and various factors involved in RNA splicing, capping and polyadenylation (26). Perhaps the polymerase initiating at the ZXDC2 promoter associates with cleavage and polyadenylation factors earlier than the polymerase initiating at the ZXDC promoter.

We found that ZXDC2 dramatically represses MHC II transcription, and propose a mechanism by which ZXDC2 interacts with ZXDA and ZXDC, preventing them from participating in a transcriptionally active complex with CIITA. The implication of this model is that the regulation of expression of ZXDC2 is a critical determinant of the levels of MHC II gene transcription. Future studies will focus on regulatory pathways that control ZXDC2 transcription, and the mechanism by which this interesting mRNA isoform is synthesized.

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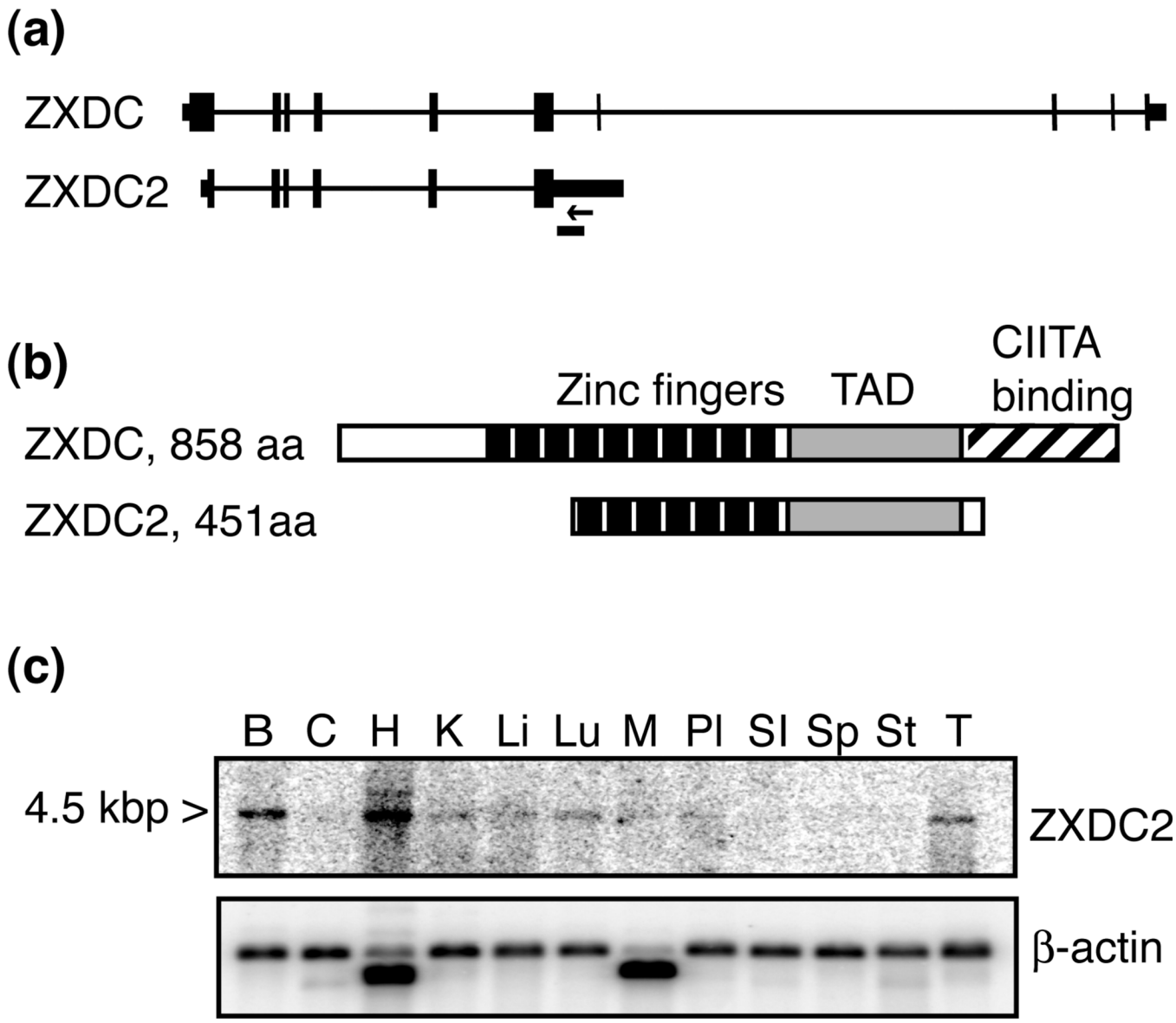

Fig. 1.

Diagram and expression of the ZXDC2 mRNA. (A) Schematic of the ZXDC gene and the portions of the gene transcribed for the ZXDC2 isoform. Boxes represent exons and thin lines represent introns. The smaller boxes are untranslated portions of exons. The arrow represents the position of the primer for reverse transcription prior to primer extension to map the transcriptional start site (see Fig. 2); the small bar below the arrow indicates the position of the probe used in the Northern blot in panel (C). (B) Schematic of ZXDC and ZXDC2 protein isoforms. Black boxes represent zinc fingers; the gray shaded area is the transcriptional activation domain (TAD) mapped in ZXDC (13); the cross-hatched area is the region necessary for ZXDC-CIITA interaction (13). (C) Northern blot of multiple human tissues. The probe was derived from the 3'-UTR of ZXDC2 cDNA (top) or the β-actin gene (bottom). B, brain; C, colon; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; Pl, placenta; SI, small intestine; Sp, spleen; St, stomach; T, testis.

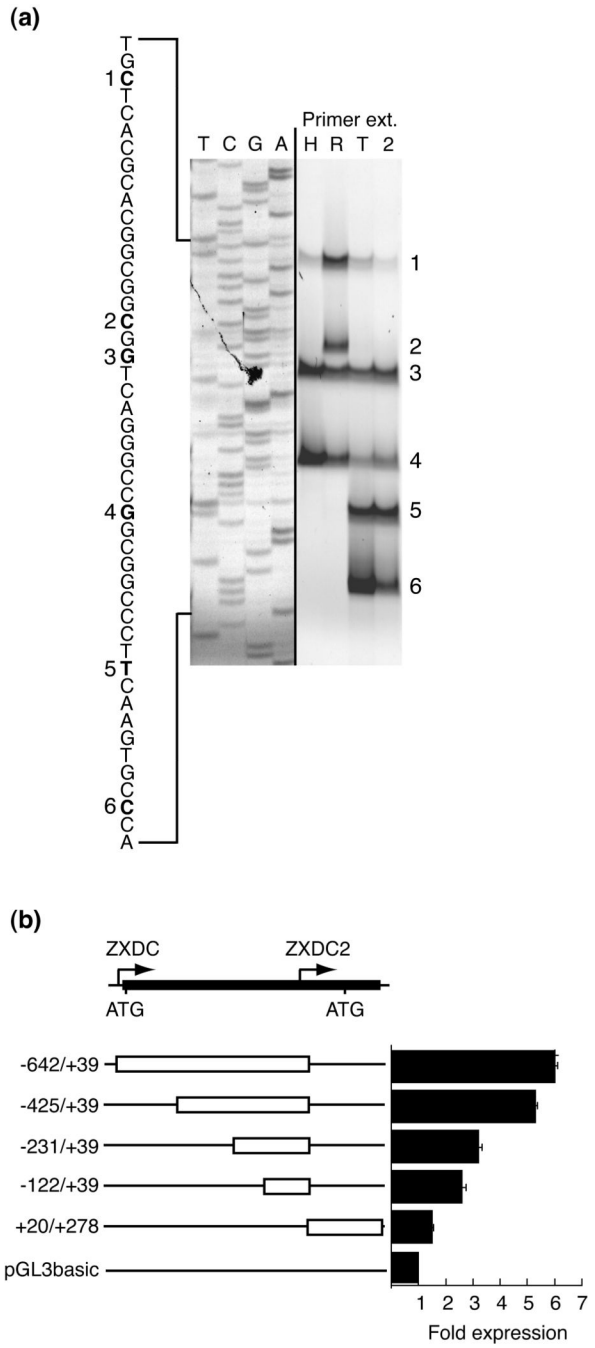

Fig. 2.

Transcription of ZXDC2 initiates within the first exon of ZXDC. (A) Primer extension analysis of ZXDC2 mRNA to identify the transcriptional start site. mRNA purified from various cell lines (H, HeLa; R, Raji; T, THP-1; 2, HEK293) was subject to reverse transcription with a gene-specific primer within the 3'-UTR of ZXDC2 (see Fig. 1 for location). A second, fluorescently labeled primer was employed for primer extension as well as a dideoxy sequencing reaction with the ZXDC cDNA. The six transcriptional start sites are numbered. (B) The first exon of ZXDC can function as a promoter. The sequence from −642 to +39 relative to the most 3' ZXDC2 transcriptional start site (+1/+681 of ZXDC) as well as serial deletions of the promoter as indicated on the figure, were amplified and cloned

into pGL3basic. HeLa cells plated in six-well dishes were transfected with either the empty vector (1 μg) or the promoter constructs (1 μg), along with 10 ng pRL-tk (expressing the *Renilla* luciferase from the HSV thymidine kinase promoter). Forty-eight hours later, firefly and *Renilla* luciferase assay was performed. Firefly luciferase was normalized to *Renilla* luciferase. Error bars are standard errors of the means for triplicate experiments. The schematic at the top depicts the relative position of the transcriptional start sites (arrows) and translational initiation codons for ZXDC and ZXDC2, within the first exon of ZXDC.

Fig. 3.

ZXDC2 represses HLA-DRA gene transcription. (A) HeLa cells plated in six-well dishes were transfected with 1 μg of empty vector or pCMV-ZXDC2-FL and Western blot performed with anti-FLAG (top panel) or anti-beta actin (bottom panel). (B) HeLa cells plated in six-well dishes were transfected with empty vector (1 μg) or increasing amounts (200, 400 or 800 ng) of pCMV-ZXDC2-FL. The amount of plasmid in each transfection was kept constant at 1 μg by inclusion of empty vector. RNA and protein were isolated and used to perform RT-PCR to detect HLA-DRA and beta-actin (top and middle panel) and Western blot with anti-FLAG (bottom panel), respectively. The RT-PCR bands were quantitated with ImageQuantTL software, and the level of HLA-DRA was normalized to the beta-actin signal

and is shown beneath the lanes (norm. expression) (C) HeLa cells stably expressing ZXDC were transfected with 200 ng siRNA directed against the 3'-UTR of ZXDC2. Transfections were performed twice, 24 hours apart. RNA was isolated and used to perform RT-PCR against HLA-DRA, ZXDC2, CIITA, and beta-actin. All primers were fluorescently labeled and PCR products detected with a Typhoon scanner (GE Healthcare). The RT-PCR bands were quantitated with ImageQuantTL software, and the levels of HLA-DRA, ZXDC2 and CIITA were normalized to the beta-actin signal and are shown beneath the lanes.

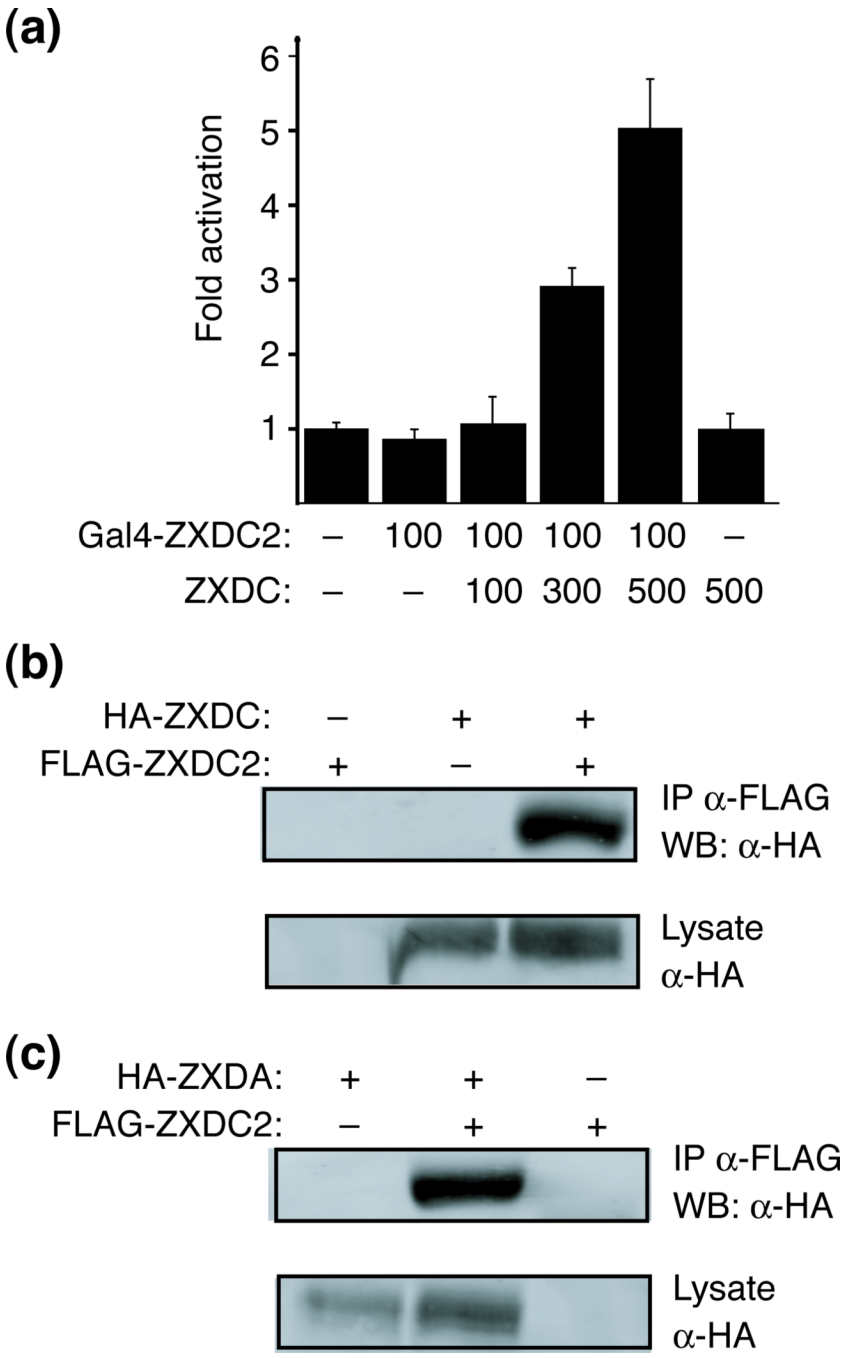

Figure 4.

ZXDC2 interacts with ZXDA and ZXDC. (A) Mammalian two-hybrid with Gal4-ZXDC2 and ZXDC. 100 ng of an expression plasmid for ZXDC2 fused to the Gal4 DNA binding domain was transfected into HeLa cells, along with the reporter plasmid pG5-luc (100 ng) pRL-tk (10 ng) and increasing amounts of ZXDC. The numerals indicate nanograms of plasmid in each transfection. The total amount of plasmid was kept constant for each transfection. Forty-eight hours later, luciferase assay was performed as in Fig. 3. Co-immunoprecipitation of ZXDC2 with (B) ZXDC and (C) ZXDA. HeLa cells plated in six-well plates were transfected with 500 ng pCMV-ZXDC2-FL and/or 500 ng pCMV-ZXDC-HA or 500 ng pCMV-ZXDA-HA. The total amount of plasmid was kept constant for each

transfection by inclusion of empty vector. Forty-eight hours later, lysates were prepared and subject to immunoprecipitation with anti-FLAG (ZXDC2). Lysates and immunoprecipitations were assayed by Western blot with the indicated antibodies.