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# The Role of Homeobox Protein Distal-Less 3 and Its Interaction with ETS2 in Regulating Bovine Interferon-Tau Gene Expression-Synergistic Transcriptional Activation with ETS2[1]

**Toshihiko Ezashi,[2,3] Padmalaya Das,[3] Rangan Gupta,[4] Angela Walker,[4] and R. Michael Roberts[3]**

*Departments of Animal Sciences[3] and Veterinary Pathobiology,[4] University of Missouri, Columbia, Missouri 65211*

## ABSTRACT

**Distal-less 3 (DLX3), a homeodomain transcription factor required for placental development in the mouse, modestly transactivates hCG-alpha subunit gene (*hCGA*) expression in human choriocarcinoma cells. Because hCG and interferon-tau (IFNT) are expressed in trophectoderm of primates and ruminants, respectively, we have tested the hypothesis that DLX3 regulates the genes for IFNT (*IFNT*). A bovine *IFNT1* promoter (−457 to +66), linked to a luciferase (*luc*) reporter, was transactivated approximately 20-fold by overexpressing DLX3 in human JAr cells. Elimination of a potential DLX3-binding site (−54 GATAATGAG −46) by either truncation or mutagenesis abolished this effect. A sequence (−59 to −44) encompassing this site bound DLX3 specifically. Coexpression of DLX3 and ETS2, which is known to be a key regulator of *IFNT* expression, increased reporter activity by more than 250-fold, whereas deletion of the established ETS2 site (−79 to −70) eliminated the ability of DLX3 to transactivate the gene. Conversely, mutation of the DLX3 site significantly reduced the transactivational effects of ETS2. Both DLX3 and ETS2 are coexpressed in JAr cells and in an IFNT-producing, bovine trophoblast cell line, CT-1. The two can be immunoprecipitated together as a complex from CT-1 cells, and RNAi-mediated, partial knockdown of DLX3 expression reduced the production of IFNT by approximately 50%. Together, these results suggest that DLX3 has a central role in controlling *IFNT* gene expression by associating with ETS2 on the *IFNT* promoter.**

*embryo, gene regulation, interferon-τ, transcription factor, trophoblast*

## INTRODUCTION

Interferon-τ (IFNT), which is produced by the early trophoblast of ruminant species such as cattle and sheep before placentation is initiated, acts in a paracrine manner on the maternal endometrium to create an environment favorable for establishment of pregnancy [1–5]. Its best-established function is to intervene in the normal cyclic regression of the maternal corpus luteum, an event that would usually occur at the end of the estrous cycle if the animal were not pregnant. Accordingly, IFNT acts in a manner analogous to that of hCG in the human, but rather than being targeted directly to the corpus luteum and having a luteotropic effect, it acts locally on the uterine endometrium and prevents the pulsatile release of the luteolytic hormone, prostaglandin $F_{2\alpha}$. As with hCG, the rapid up-regulation of IFNT expression at a time when the corpus luteum is wavering on the point of regression is essential if the pregnancy is to be maintained, whereas a less-than-robust production may contribute to embryonic loss [6]. Hence, an understanding of the transcriptional control mechanisms operating on these genes is likely to provide an insight regarding why some pregnancies are successful and why others fail.

The genes for IFNT (*IFNT*) have been cloned from several species within the suborder Ruminantia [7–10], in which they are exclusively expressed in trophoblast. Unlike the genes encoding the related IFNA (interferon-alpha, *IFNA*) and IFNB (interferon beta, *IFNB*), the *IFNT* genes are not responsive to virus, although the 5′ sequences upstream of the transcriptional start sites, which contain the main regulatory regions, are highly conserved across species through at least 400 bp [9]. A yeast single-hybrid screen, used to pinpoint proteins that bind within the proximal promoter region of *IFNT*, identified the transcription factor ETS2 as a likely regulator of *IFNT* expression in Day 13 ovine conceptuses [11]. ETS2 is the member of a large family of transcription factors that are characterized by their conserved ETS DNA-binding domain, which binds to a specific DNA motif, characterized by a core GGAA sequence. Overexpression of ETS2, but not of related transcription factors of the ETS family, transactivated a luciferase (*luc*) reporter under the control of the *IFNT* upstream regulatory region containing an ETS2-binding site (−79 to −70) in the human choriocarcinoma cell, JAr. Mutation of this DNA motif within the core GGAA sequence markedly reduced both basal and ETS2-induced activity [12]. Importantly, CSF1, a growth factor present in uterine secretions, was able to activate ETS2 via the Ras/MAP kinase signal transduction pathway and to enhance the up-regulation of *IFNT* promoters by ETS2 [13]. In addition, protein kinase A-mediated transactivation of the IFNT-regulatory region is strictly dependent on the ETS2-binding site [14]. Finally, several ovine *IFNT* "pseudogenes" that are poorly expressed lack a consensus ETS2-binding sequence in the proximal promoter element [11]. Replacement of the "mutated" and, presumably, defective ETS2-binding site in the pseudogene with 22 bp of active ETS2-binding site restores full promoter activity [15]. For these reasons, ETS2 is now generally regarded as being the pivotal transcription factor responsible for control of *IFNT* expression in trophectoderm.

Following the initial discovery that ETS2 regulates the *IFNT*, ETS2 has been shown to play a role in the regulation of several other genes that are characteristically expressed in trophectoderm, including the hCGα and hCGβ subunit genes (*hCGA* and *hCGB*) [16, 17]. The fact that deletion of the murine *Ets2* gene leads to placental failure and embryonic death as the result of defects in the development of the

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[2]Correspondence: Toshihiko Ezashi, 240h, Christopher S. Bond Life Sciences Center, Columbia, MO 65211-7310. FAX: 573 884 9394; e-mail: ezashit@missouri.edu

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ectoplacental cone and reduced expression of matrix metalloproteinases [18] suggests that ETS2 is a key factor in specifying many aspects of trophoblast function [19].

Rarely, if ever, does a single transcription factor operate alone. Most act in a combinatorial manner to provide a response that is tissue specific and regulated over time. Because ETS2 is expressed widely in adult bovine tissues (see GenBank Accession numbers BE758238.1, AW668843.1, and BE479476.1), and because IFNT expression is restricted to a single epithelial cell layer for just a few days in early pregnancy, other transcription factors must be involved in fine-tuning IFNT production. Here, we have studied a second transcription factor, the homeobox protein distal-less 3 (DLX3), a member of the distal-less family of non-Antennapedia homeobox genes, which is an established contributor to trophoblast function in mammals [20–24]. DLX3 is expressed in human and mouse trophoblast cells [25], and *Dlx3*$^{-/-}$ mice die between Embryonic Days 9.5 and 10 because of placental defects and abnormal placental vasculogenesis [22]. The protein itself consists of 287 amino acids and contains a centrally placed, DNA-binding homeodomain (Fig. 1B). It recognizes a specific sequence with a central TAAT core motif [26]. Two "signature" genes of trophoblast have been found to be regulated by DLX3—namely *hCGA* [24] and the gene for murine 3β-hydroxysteroid dehydrogenase VI (*Hsd3b6*) [23]—although others have noted that *Hsd3b6* expression is not correlated with that of DLX3 in the rodent placenta, particularly in giant cells, where most progesterone synthesis occurs [20]. These observations led us to examine the potential role of DLX3 in controlling expression of the *IFNT* and to determine whether it acts in a combinatorial manner with ETS2.

## MATERIALS AND METHODS

### Reporter Gene Constructs and Expression Plasmids

Bovine *IFNT1* (*boIFNT1*)-reporter constructs, −49*luc,* −126*luc,* and −457*luc* (containing the gene control region −49 to +50, −126 to +50, and −457 to +66, respectively), have been described previously [11, 12]. The −67*luc* construct was generated from mutated −126*luc* (−126μAP1) by *Xba*I digestion and self-ligation [13]. Site-directed mutagenesis of the homeobox (DLX3)-binding site at −53 to −50 on the −126*luc* reporters was achieved with the primers 5′-aag GCC Ttg agt acc gtc ttc (uppercase letter*s* show the mutation sites) and 5′-aag GCC Tga aaa ttt ctc tct ca and standard PCR procedures [13]. Mutagenesis of the ETS2-binding site at −79 (−126μETS2) has been described previously [12]. The mutated sequences are shown in Figures 2 and 3, respectively. The double mutation of the ETS2- and DLX3-binding sites on −126*luc* (−126μETS2/μDLX3) (Fig. 4) was generated by site-directed mutagenesis on the −126μETS2 reporter plasmid by using the primers 5′-cta gtc gtg aga gag aaa ttt tcg gGC CTt gag tac cgt ctt ccc and 5′-ggg aag acg gta ctc aAG GCc cga aaa ttt ctc tct cac gac tag and Quickchange Site-Directed Mutagenesis kit (Stratagene). Fidelity of all constructs was verified by DNA sequencing.

The DLX3 expression plasmid, *Dlx3*/pCI-neo, was a gift from Dr. Maria Morasso (Developmental Skin Biology Unit, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD) [24]. The ETS2 expression plasmids (pCGNEts2) have been described previously [11].

### Cell Cultures and Transfections

JAr cells (HTB-144; American Type Culture Collection) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 μg/ml). CT-1 cells [27] obtained from Dr. Alan Ealy (University of Florida, Gainesville, FL) were cultured in STO feeder cells conditioned medium (90% Dulbecco modified Eagle medium/F-12, 10% FBS, 1% antibiotic-antimycotic, and 10 ng/ml of fibroblast growth factor-2) on a Matrigel (Becton Dickinson)-coated surface [28]. Both DLX3 and ETS2 expression plasmids (2.6 and 0.3 μg, respectively) and 0.5 μg of the *boIFNT1* promoter-*luc* reporter constructs were transfected into JAr cells either in 60-mm dishes ($2 \times 10^5$ cells/dish) or in six-well plates ($1 \times 10^5$ cells/well with half amount of plasmid DNA described above) by the calcium phosphate method [11]. The plasmid phRG-TK (10 ng; Promega) or 50 ng of pRSVLTR-βgal were included for normalization of transfection efficiency. The expression plasmid for the constitutively active catalytic subunit (RSV-PKA) has been described previously [14]. Both PKA and ETS2 expression plasmids (1.3 and 0.2 μg, respectively) and 0.5 μg of the *IFNT-luc* reporter were transfected in six-well plates to study PKA effects. Total amounts of transfected DNA were kept constant by adding corresponding empty vectors. *Luc* reporter assays were conducted 36 h after transfection. Enzyme assays for analyses of transfection experiments were carried out as described previously [12, 13]. The activities of both firefly and *Renilla* luciferases were measured with a dual-luciferase reporter assay system (Promega). Firefly *luc* activity was normalized to either *Renilla* luciferase or β-galactosidase activity, depending on which control was used.

### Electrophoretic Mobility Shift Assay

Recombinant DLX3 protein was prepared by means of the DLX3 expression plasmid, *Dlx3*/pCI-neo, which carries the T7 promoter at the transcription start site [24]. The expression plasmid was linearized by *Not*I and was used as DNA template for in vitro transcription and translation (Promega L5040 TNT-coupled wheat germ extract system). Nuclear extracts were prepared from transfected and nontransfected JAr cells using the Nuclear Extract Kit (Active Motif). The JAr cells had been grown on 10-cm culture dishes. Cells in three dishes had been transfected with 10 μg/dish of *Dlx3*/pCI-neo plasmid DNA by means of Lipofectamine/Plus reagent (Invitrogen). Cells from three other dishes had not been transfected. A double-stranded 16-mer oligonucleotide, corresponding to nucleotide sequence −59 to −44 of the *boIFNT1* promoter, was end-labeled with [γ-$^{32}$P]ATP. The DNA probe (20 fmol, 13 879 cpm) was mixed with 2.5 μl of the in vitro-translated DLX3 and nuclear extracts from the JAr cells (7.4 μg from transfected cells and 13.4 μg from nontransfected cells). For competition binding assays, double-stranded competitor DNA (200-fold molar excess, 4 pmol) was added before incubation with the labeled probe. The DNA-binding conditions have been described elsewhere [12]. To determine that the DNA-protein complexes contained DLX3, approximately 0.1 μg of rabbit anti-DLX3 antibody [24, 29] or the same amount of an unrelated immunoglobulin G (IgG; anti-biotin antibody from Cell Signaling Technology) were added to the reaction mixture before the addition of labeled probe.

### Western Blot Analyses

Cells were extracted in Passive Lysis Buffer (Promega) or RIPA Buffer (10 mM Tris-HCl [pH 7.2], 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and 100 mM NaCl). After centrifugation to remove particulates in the extracts, soluble protein (40–50 μg/lane) was analyzed by 10–12.5% SDS-PAGE. Protein in the gels was transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). DLX3 was identified by using anti-DLX3 antibody (diluted 1:2000) from Dr. Morasso [24, 29]. The ETS2 proteins were identified on blots by use of a rabbit anti-ETS2 antiserum [17] raised against the amino-terminal fragment (amino acids between 1 and 326) of human ETS2 fused to glutathione S-transferase. The second antibody was alkaline phosphatase-conjugated anti-rabbit IgG used as described previously [12] or horseradish peroxidase-conjugated anti-rabbit or anti-sheep IgG and detected with the Phototope-HRP Western Blot Detection System (Cell Signaling Technology). Sheep anti-DLX3 polyclonal antiserum was obtained from ewes that were immunized with a purified glutathione S-transferase fusion protein containing amino acids 197–287 of the murine DLX3 protein. The IgGs of anti-ETS2 and anti-DLX3 antibodies were affinity purified using the Aminolink Plus Immobilization Kit (Thermo Scientific).

### Immunofluorescence Microscopy

The JAr and CT-1 cells were grown on coverslips and Matrigel-coated coverslips, respectively, placed in six-well tissue culture plates. After fixation (4% paraformaldehyde/PBS, 15 min) and permeabilization (1% Triton X-100/PBS, 30 min), cells on coverslips were incubated for 1 h with either 5% goat or donkey serum (depending on the secondary antibody used), followed by overnight incubation with primary antibodies (rabbit anti-ETS2 at 1:200, sheep anti-DLX3 at 1:40, mouse anti-IFNT monoclonal antibody [mAb] at 1:5) at 4°C. Secondary antibody staining was performed with fluorescein (Alexa Fluor [AF] 568, 488, and 647)-labeled goat anti-rabbit (AF 568), goat anti-mouse (AF 488), and/or donkey anti-sheep (AF 488 or AF 647) antibodies (Molecular Probes) at a 1:500 dilution. Nuclei were labeled with 4′-6-diamidino-2-phenylindole (Molecular Probes) or TO-PRO-3 iodide (Molecular Probes). Images were captured with a Bio-Rad Radiance 2000 coupled to an Olympus

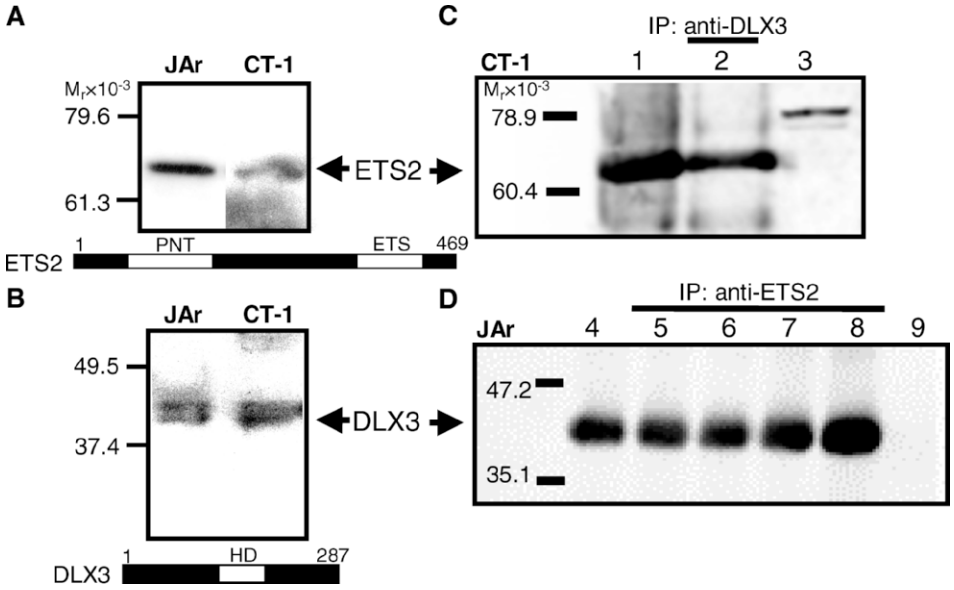


FIG. 1. Both ETS2 and DLX3 are expressed in JAr and CT-1 cell lines (**A** and **B**) and physical association exists between these transcription factors in both cell lines (**C** and **D**). Western blot analysis was conducted on JAr and CT-1 whole-cell extracts (50 μg) to determine the expression of ETS2 (**A**) and DLX3 (**B**). Molecular weight markers ($M_r \times 10^{-3}$) are shown on the left. Schematic structures of ETS2 and DLX3 proteins are presented under the blots. ETS2 is a member of ETS-domain (ETS) transcription factor family and contains a "pointed" domain (PNT) in the N-terminal region. DLX3 contains a homeodomain (HD) in its central region. The numbers represent polypeptide lengths in amino acid residues. In **C**, ETS2 is present in CT-1 cell lysates (34 μg of protein; lane 1) and can be coimmunoprecipitated with anti-DLX3 (lane 2). In lane 2, 1 mg of CT-1 cell lysate was incubated with anti-DLX3 antibody (immunoprecipitation [IP]: anti-DLX3), and the presence of ETS2 in the immune complex detected by Western blot analysis with anti-ETS2 antibody. In **D**, endogenous DLX3 in JAr cell extracts (25 μg of protein; lane 4) and analysis of proteins immunoprecipitated from 1 mg of JAr cell lysate with anti-ETS2 antibody (IP: anti-ETS2; lane 5) by immunoblot analysis with anti-DLX3 antibody are shown. Also in **D**, coimmunoprecipitation experiments also were performed with cells transfected with vectors for ETS2 (lane 6) and DLX3 (lane 7) and for both ETS2 and DLX3 (lane 8). Lanes 3 (**C**) and 9 (**D**) represent controls in which a nonspecific IgG replaced the specific antibodies used in the immunoprecipitation of the analysis.

IX70 inverted microscope or with a Zeiss LSM 510 two-photon confocal system. A hybridoma line producing an mAb against bovine IFNT was produced by the University of Missouri-Columbia Cell and Immunology Core (http://www.biotech.missouri.edu/cic/) by using recombinant bovine IFNT1a. The mAb was purified from the hybridoma culture supernatant on a protein A-agarose column (Bio-Rad) with a BioLogic Fast Protein Liquid Chromatography (Bio-Rad). The protein A column was washed with 3.2 M NaCl and 1.6 M glycine (pH 9.0), and IgG mAb was eluted with 100 mM sodium citrate (pH 3.0). The eluted fractions were neutralized with 100 mM Tris-HCl (pH 9.0) and then dialyzed in 1× PBS, 0.02% sodium azide, 1 mM PMSF, and 1 mM EDTA.

### *Tissue Immunohistochemistry*

Ovine Day 15 conceptuses were fixed in 4% paraformaldehyde for 4 h. Tissues were paraffin-embedded, and sections (thickness, 5 μm) were transferred to charged glass slides. After deparaffinization and hydration, antigen retrieval was conducted by exposing the sections to boiling "antigen unmasking solution" (citrate acid-based buffer; Vector Laboratories) for 5 min. Slides were allowed to cool for approximately 20 min at room temperature and then treated with a solution of 1.5% hydrogen peroxide in absolute methanol for 10 min at room temperature to quench endogenous peroxidases. The slides

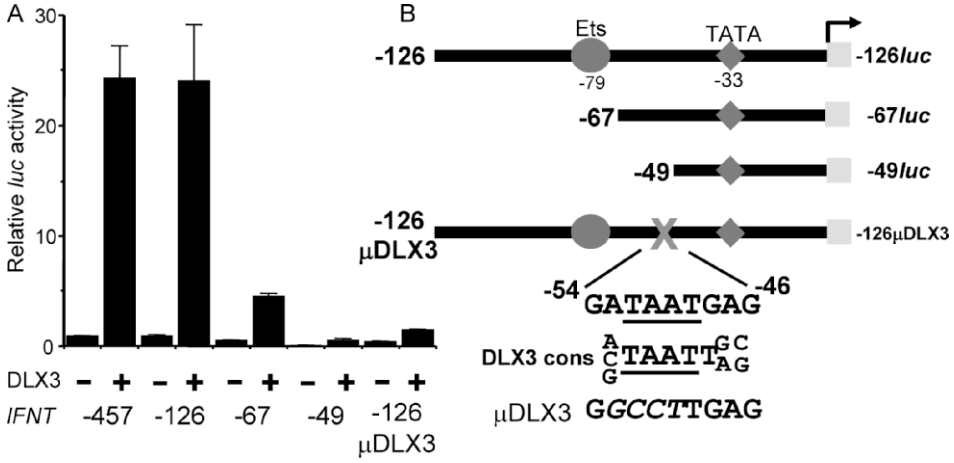


FIG. 2. A homeobox-binding site at −54/−46 serves as DLX3 responsive site in the *IFNT* regulatory region. **A**) The *IFNT* 5′-flanking region deletions (from −457 to −49 bp) and homeobox-binding site mutant (−126μDLX3) linked to *luc* reporters (0.5 μg) were transfected into human JAr choriocarcinoma cells in either the absence (−) or the presence (+) of the DLX3 expression plasmid (2.6 μg). The *IFNT* reporter activities were normalized to the activity of cotransfected reference plasmid phRG-TK (10 ng). Results are presented as the mean ± SEM, with the basal activity of −457*luc* set as 1. The representative data shown are for one of three experiments conducted in triplicate at different times. **B**) Schematic presentations of structures of the *IFNT* deletions (from −126 to −49) and the mutant reporter. The locations of the Ets binding site, TATA box, and mutation site are shown. The nucleotide sequence at −54/−46 is compared with DLX3 consensus binding sequence and the sequence of the mutated reporter.

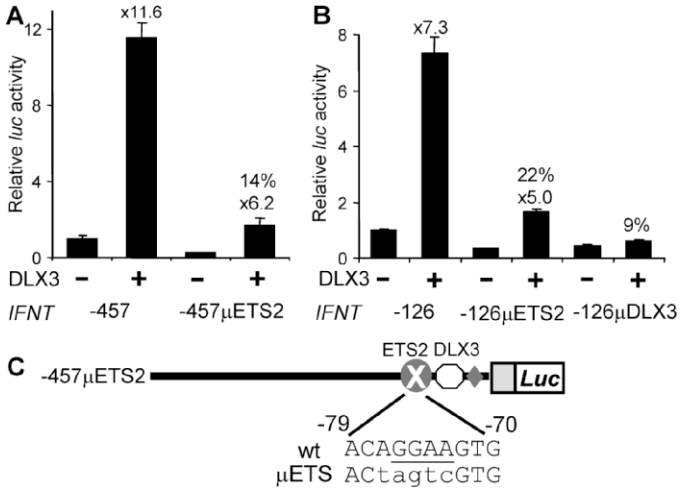


FIG. 3. Efficient DLX3 transactivation is dependent on the ETS2-binding site located at −79/−70 in the *IFNT* regulatory region. **A**) The −457*luc* and its ETS2-binding site mutant (−457μETS2) reporters (0.5 μg) were transfected into human JAr cells in either the absence (–) or the presence (+) of the DLX3 expression plasmid (2.6 μg). The *luc* activities were normalized to the activity of cotransfected reference plasmid phRG-TK (10 ng). Results are presented as the mean ± SEM, with the basal activity of −457*luc* set as 1. The fold-changes to the respective basal activity and percentages of the activities of mutant reporter to those of wild type are listed above the bar. The representative data shown are one of two experiments conducted in triplicate at different times. **B**) The −126*luc*, its ETS2-binding site mutant (−126μETS2) and its DLX3-binding site mutant (−126μDLX3) reporters (0.5 μg) were transfected into JAr cells in either the absence (–) or the presence (+) of the DLX3 expression plasmid (2.6 μg). The *luc* activities were normalized to the activity of cotransfected reference plasmid phRG-TK (10 ng). Results are presented as the mean ± SEM, with the basal activity of −126*luc* set as 1. The fold-changes to the respective basal activity and percentages of the activities of mutant reporter to those of wild type are listed above the bar. The representative data shown are one of two experiments conducted in triplicate at different times. **C**) Schematic presentations of structures of the −457μETS2 reporter. The locations of the ETS2, DLX3-binding sites, and TATA box are shown. The ETS2-binding site sequence at −79/−70 (wild type [wt]) is compared with the site-directed mutation sequence (μETS2) introduced into the mutant reporter. The same sequence mutation has been introduced into the −126μETS2 reporter.

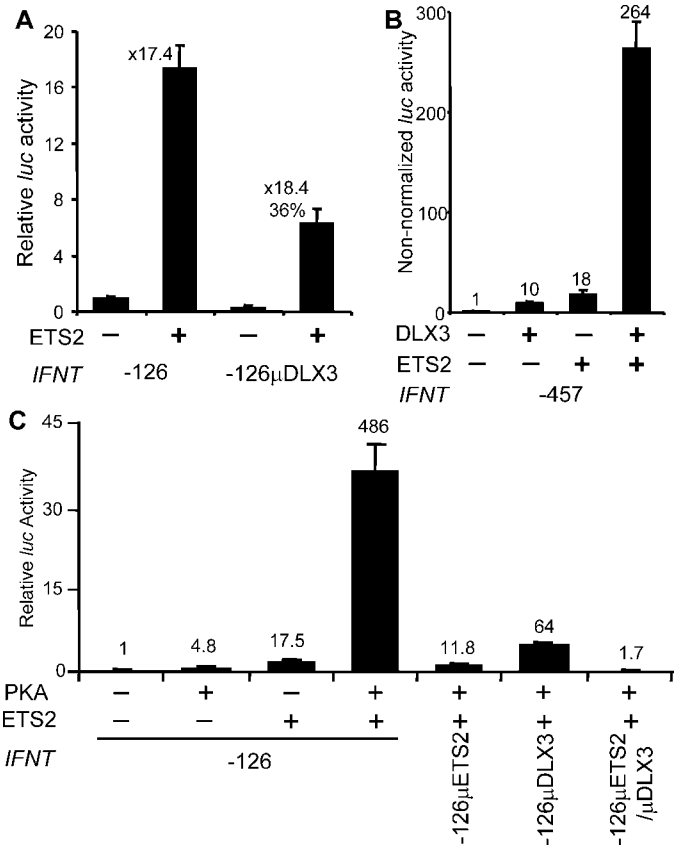


FIG. 4. Mutual cooperation of ETS2 and DLX3 in the full transactivation of *IFNT*. **A**) ETS2 is dependent on DLX3 for its full transactivation potential. The −126*luc* and its DLX3-binding site mutant (−126μDLX3) reporters (3.2 μg) were transfected into JAr cells in either the absence (–) or the presence (+) of the ETS2 expression plasmid (0.4 μg). The *luc* activities were normalized to the activity of cotransfected reference plasmid pRSVLTR-βgal (50 ng). Results are presented as the mean ± SEM, with the basal activity of −126*luc* set as 1. The fold-changes to the respective basal activity and percentages of the activities of mutant reporter to those of wild type are listed above the bar. The representative data shown are for one of three experiments conducted in triplicate at different times. **B**) DLX3 and ETS2 cooperate to up-regulate the *IFNT* reporter synergistically. The −457*luc* construct (0.5 μg) was transfected into JAr cells in either the absence (–) or presence (+) of the DLX3 (2.6 μg) or ETS2 (0.3 μg) or in the presence of the DLX3 (2.6 μg) plus ETS2 (0.3 μg) expression plasmids. The total amount of the transfected DNA was kept equal by addition of parental vectors. The results are not normalized by reference reporter activities because of a lack of suitable reference plasmid that could be used with DLX3 and ETS2 together. The *luc* activity from phRG-TK was not changed by DLX3 overexpression but was up-regulated by ETS2 overexpression. On the other hand, the β-galactosidase activity from pRSVLTR-βgal was not changed by ETS2 overexpression but was suppressed by DLX3 overexpression. Results are presented as the mean ± SEM of *luc* activities, with the basal activity of −457*luc* set as 1. The representative data shown are for one of two experiments conducted in triplicate at different times. **C**) Effects of mutating the ETS2- and DLX3-binding sites on *IFNT* reporter activity after cotransfection of the cells with ETS2 and activated PKA constructs. The −126*luc* reporter construct (−126) and its three mutated forms (−126μETS2, −126μDLX3, and −126μETS2/μDLX3) were cotransfected with expression plasmids for ETS2 and activated PKA either alone or in combination. Results are presented as the mean ± SEM of *luc* activities, with the basal activity of −126*luc* set as 1. The fold-activation values are shown above each bar.

## *Coimmunoprecipitation Analysis*

The CT-1 cells, grown as monolayers in T75 flasks, were removed by scraping and were collected by centrifugation. The cell pellet was washed with ice-cold 1× PBS, and cells were lysed in buffer containing detergents and protease inhibitors [15]. Particulate material was removed by centrifugation at 16 060 × *g*. Freshly isolated protein (1 mg) was incubated overnight with either 5 μg of affinity-purified anti-DLX3 antibody or purified control sheep IgG (Santa Cruz Biotechnology). After centrifugation, supernatants were mixed with 50 μl of protein G-agarose beads (Santa Cruz Biotechnology) for 6 h and analyzed by Western blotting with affinity-purified rabbit ETS2 (1:1000) antibody (sc-351; Santa Cruz Biotechnology) as the detection reagent.

The JAr cells (in 60-mm dishes) were transfected with 3 μg of pCGNEts2 and 3 μg of *Dlx3*/pCI-neo plasmid DNAs by using Lipofectamine/Plus reagents. For each set of reactions, the extracts were prepared from six dishes in a lysis buffer containing detergents and protease inhibitors [16]. Cell lysates were cleared by centrifugation at 16 060 × *g*, and 1 mg of freshly isolated protein was incubated overnight with either 5 μg of affinity-purified anti-ETS2 antibody (sc-351) or purified nonspecific IgG (Santa Cruz Biotechnology). After centrifugation, supernatant solutions were mixed with 50 μl of swollen, prewashed, protein G-agarose beads for 6 h. After washing, bound immune complexes were eluted in nonreducing sample buffer [17] at 80°C for 15 min. Samples were analyzed in 12% SDS gel. The immune complexes formed in the presence of ETS2 antibody were detected on Western blots with affinity-

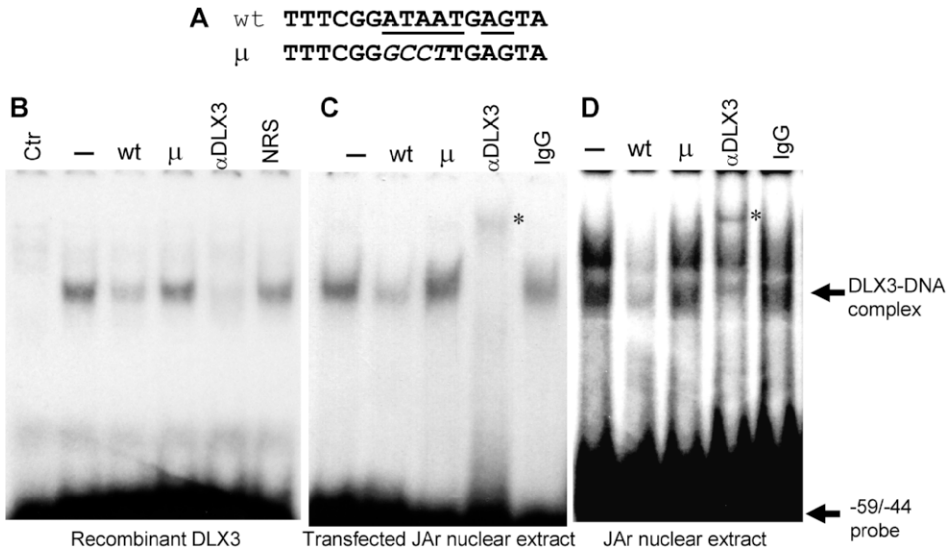


FIG. 5. DLX3 specifically binds to the homeobox-binding site at −54/−46 in the *IFNT* regulatory region. **A**) Comparison of the DNA probe sequences from −59 to −44 of the wild-type (wt) and mutant (μ) sequence. Conserved sequences to the DLX3 consensus are underlined. **B**) Electrophoretic mobility shift assay (EMSA) with in vitro-translated DLX3 protein or control protein (Ctr). The binding reactions contained radiolabeled −59/−44 probe and recombinant DLX3 protein in the absence (–) or presence of a 200-fold molar excess of either wild-type −59/−44 (wt) or the mutant (μ) duplexed oligonucleotides. Reactions represented in the two lanes on the right contained anti-DLX3 antibody (αDLX3; 0.25 μl) and normal rabbit serum (NRS; 3 μg IgG/0.25 μl), respectively. **C** and **D**) EMSA with nuclear extracts from JAr cells in which DLX3 had been overexpressed (**C**) and EMSA with nuclear extract from nontransfected cells (**D**). The binding reactions contained radiolabeled −59/−44 probe and recombinant DLX3 protein in the absence (–) or presence of a 250-fold molar excess of either wild-type −59/−44 (wt) or the mutant (μ) duplex oligonucleotides. Reactions represented in the two lanes on the right contained either rabbit anti-DLX3 antibody (αDLX3; 0.5 μl) or same amount of an unrelated rabbit IgG, respectively. The upper arrow indicates the DLX3-DNA complex; the lower arrow indicates free −59/−44 probe. The asterisk indicates supershifted DLX3-DNA complex.

purified sheep DLX3 antibody. Control immunoprecipitations were carried out with preimmune sheep IgG and analyzed in parallel. These controls also indicated the absence of IgG contamination in samples eluted from protein G-agarose beads. A 25-μg sample of protein extracted directly from the cells was analyzed in parallel to determine the relative amounts of ETS2 and DLX3 present. The DLX3 antibody was used at 1:1000 in 5% (wt/vol) nonfat dried milk dissolved in Tris-buffered saline added with 0.1% (vol/vol) Tween 20. The DLX3 bands were visualized by chemiluminescence with reagents purchased from either Cell Signaling Technology or Thermo Scientific.

## Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) analysis on chromatin purified from CT-1 cells was conducted as described previously [17]. The chromatin, prepared from approximately $10^7$ cells, was sheared by sonication and treated with a protein G-agarose bead slurry (Santa Cruz Biotechnology) to remove components that bound nonspecifically. One fifth of the precleared chromatin was saved as "total input" control. The remaining chromatin solution was divided into five portions: untreated ("no antibody" control), treated with 2 μg of sheep affinity-purified DLX3 antibody, treated with 2 μg of rabbit anti-ETS2 antibody [17], treated with 2 μg of purified nonspecific IgG, and treated with 2 μg of RNA polymerase II antibody (Active Motif). The chromatin complexes were collected on protein G-agarose, processed as described elsewhere [17], and were used as template for each PCR reaction. The primers used in the ChIP assay were 5′-tga caa acc caa att tta ttg gga aa (forward) and 5′-tct gat gat gat cgt tct aag caa gg (reverse) and were designed to amplify a region of the *IFNT* proximal promoter (−188 to +3) containing the ETS2/DLX3 enhancer region. The PCR conditions were as follows: 95°C for 3 min for one cycle, and then 32 cycles of 94°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec. The PCR products were visualized by ethidium bromide staining following electrophoresis in 2% agarose.

## RNA Interference

We first identified a *Bos taurus* cDNA (GenBank accession number NM_001081622, encoding 287 amino acids) with a sequence closely similar (90% identity in nucleotides) to that of a human DLX3 transcript (NM_005220, also 287 amino acids). Coidentity was confirmed by comparing a 158-kb length of genomic DNA from a *B. taurus* genomic clone (clone name, RP42-221D7; GenBank accession number, AC136966) with the human *DLX3* gene in terms of conserved sequence and intron/exon boundaries. Three small interfering RNA (siRNA) duplexes against the bovine sequence (NM_001081622) were designed by using the siRNA selection program at Whitehead Institute for Biomedical Research (http://jura.wi.mit.edu/bioc/siRNAext/home.php). Only one of these duplexes, r(UGG CUC AUC ACC UUC CAG A) dTdT and r(UCU GGA AGG UGA UGA GCC A) dAdA, was effective at knocking down DLX3 expression. The CT-1 cells at 30–40% confluency in six-well plates were transfected by using "lipitoid" reagent, a cationic oligopeptoid-phospholipid conjugate [30] with 50 nM siRNA duplexes specific for bovine DLX3 (Ambion) or a nonspecific control (Silencer Negative Control #1 siRNA; Ambion). The transfection method was essentially the same as described elsewhere [30], but with the following minor modifications. After the culture medium was removed, the cells were washed twice with 1 ml of OptiMEM (Invitrogen) before addition of the transfection cocktail (0.1 ml/well; lipitoid:siRNA charge ratio, 3:1) for 2 h. The transfection regent was removed, and growth medium was refreshed 24 h later. After three further days of culture, the cells were lysed and soluble proteins analyzed by Western blot analysis, with rabbit anti-DLX3 (1:2000) as the detecting antibody. The blots were then stripped (IgG Elution Buffer; Thermo Scientific) and reprobed sequentially with anti-IFNT antiserum (1:400) and anti-β-actin antibody (1:2500; Cell Signaling Technology), respectively. The anti-IFNT antiserum used was generated by immunizing rabbits with recombinant bovine IFNT through standard procedures [31]. Bound IgG was detected by chemiluminescence as described above.

Antiviral activity present in the culture media from cells transfected with siRNAs was measured in a cytopathic reduction assay as described previously [32]. Serial 3-fold dilutions of CT-1 medium samples, normalized by their cellular protein concentration, were incubated on Mardin-Darby bovine kidney cells challenged with vesicular stomatitis virus. Commercially available bovine IFNA2 (PBL InterferonSource) of known antiviral activity ($1 \times 10^6$ IU) was used as the standard. The IFNT concentration was determined by 50% protection from virus-induced cell death.

## Data Analysis

Data are presented as the mean ± SEM. Data were analyzed by one-way ANOVA followed by a Tukey multiple-comparison test to compare selected pairs of experimental groups with the PRISM statistical analysis software (version 4; GraphPad Software, Inc.). Differences of $P < 0.05$ were considered to be significant.

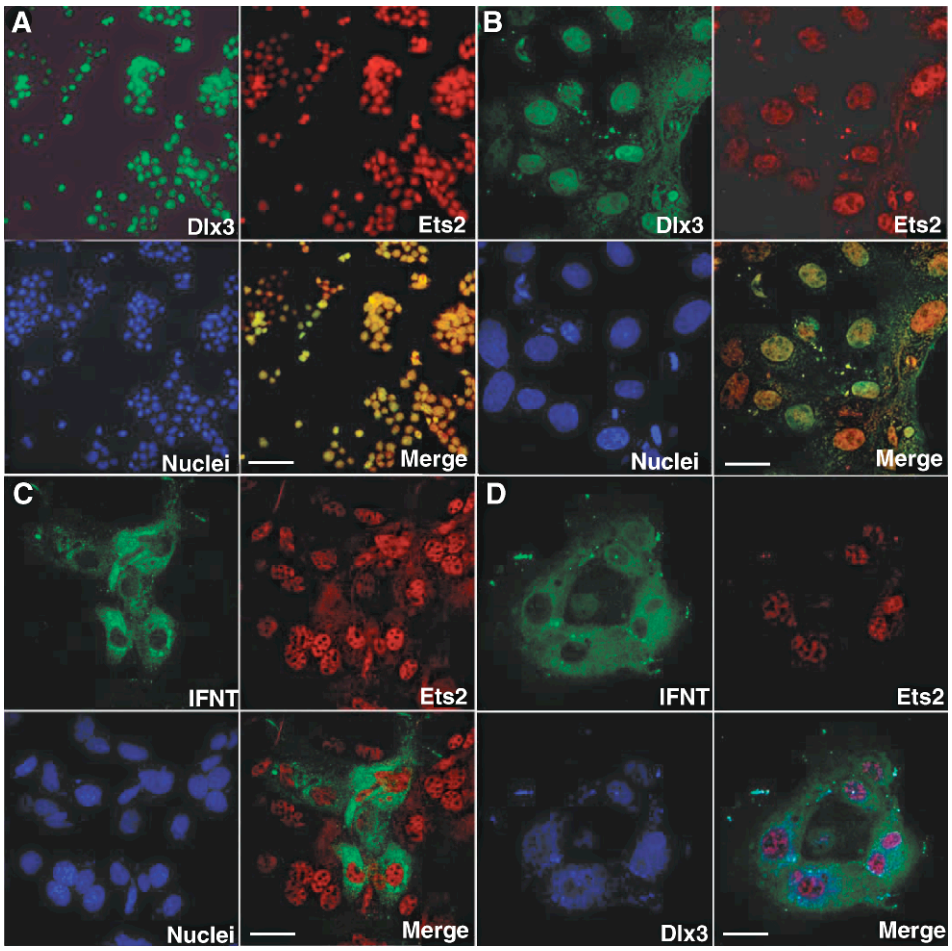


FIG. 6. Colocalization of transcription factors DLX3 and ETS2 in JAr cells (**A**) and DLX3, ETS2, and IFNT in CT-1 cells (**B–D**). **A**) DLX3 (green) and ETS2 (red) are present in the nuclei of JAr cells and colocalize as evident in the merged picture (Merge). Secondary labeling was performed with donkey anti-sheep IgG conjugated with AF 488 for anti-DLX3 and goat anti-rabbit IgG with AF 568 for anti-ETS2. Nuclei were stained with 4′-6-diamidino-2-phenylindole (blue). **B**) DLX3 (green) and ETS2 (red) colocalize in the nuclei of CT-1 cells. Procedures were identical to those in **A**. Nuclei were stained with TO-PRO-3 iodide (blue). **C**) IFNT (green) is localized to the cytoplasm of a subset of CT-1 cells, whereas ETS2 (red) is present in the nucleus. Secondary labeling was performed with goat anti-mouse IgG conjugated with AF 488 for anti-IFNT mAb and goat anti-rabbit with AF 568 for anti-ETS2 antibody. Nuclei were stained with TO-PRO-3 iodide (blue). **D**) IFNT (green), ETS2 (red), and DLX3 (blue) localization in CT-1 cells. DLX3 and ETS2 colocalize in the nuclei of cells producing IFNT (Merge). Secondary labeling for IFNT and ETS2 was as in **C**. DLX3 localization was visualized with AF 647-conjugated donkey anti-sheep IgG (blue). The controls for JAr and CT-1 cell immunocytochemistry are shown in Supplemental Figure 1. Bar = 100 μm (**A**) and 20 μm (**B–D**).

## RESULTS

### *Transactivation of* IFNT *Promoters by the Homebox Protein DLX3*

The DLX3 binds to an AT-rich region within the proximal control region of the hCGα subunit gene and, when ectopically expressed in choriocarcinoma cells, has a modest ability (~2-fold) to transactivate promoter constructs containing this sequence [24]. Because *IFNT* genes also contain a possible DLX3-binding site (Fig. 2B), with seven out of eight bases conserved relative to the consensus sequence [26], we examined whether the *IFNT* promoters can be up-regulated in response to overexpression of this homeobox transcription factor. We cotransfected the DLX3 expression plasmid and a −457 *IFNT* promoter-reporter (−457*luc*) into JAr cells. *Luc* activity increased approximately 25-fold (Fig. 2A) relative to a control without DLX3 overexpression, although considerable variation was found between experiments. DLX3 had an almost identical effect on a truncated reporter construct (−126*luc*), which contains the ETS2-binding site (−79 to −70) known to be essential for full *IFNT* expression [11–13]. Deletion of this site by further truncation to −67, however, reduced the ability of DLX3 overexpression to transactivate the *luc* reporter by almost 80% (~5-fold vs. 24-fold). Further truncation designed to remove the DLX3-binding site itself (−49*luc*) reduced the effects of ectopically expressed DLX3 by approximately 98% and virtually ablated basal activity. Mutation of the core TAAT sequence of the putative DLX3-binding site (−53 to −50) in the −126*luc* reporter construct (−126μDLX3) provided reduced basal activity (55%) compared to that observed with the wild-type −126*luc* reporter and led to complete unresponsiveness to DLX3 overexpression (Fig. 2A). Together, these data show that DLX3 can transactivate the *IFNT* promoter, that the critical sequence for DLX3 responsiveness lies between −54 and −46, and that DLX3 effects might be at least partially dependent on the presence of the established ETS2-binding site placed approximately 20 bp distal to the putative DLX3-binding site.

### *Binding of DLX3 to the* IFNT *Gene Regulatory Region*

We next determined whether DLX3 physically interacts with *IFNT* regulatory region by employing in vitro-translated DLX3 and a labeled duplex DNA probe representing the 16 bp of the *IFNT* regulatory region lying between base pairs −59 to −44 (Fig. 5A). Although a control protein (in vitro-translated luciferase) failed to bind the probe (Fig. 5B, lane 1), the recombinant DLX3 protein formed a complex (lane 2). A 200-fold molar excess of unlabeled probe competed efficiently with labeled probe in the binding reaction (lane 3), whereas an equivalent molar excess amount of a control oligonucleotide with its DLX3 binding core motif mutated (Fig. 5A, μ) was ineffective as a competitor (lane 4). Addition of anti-DLX3 antibody prevented formation of a specific complex between the wild-type probe and the protein (lane 5), whereas a control rabbit IgG had no effect (lane 6). The experiments were repeated with nuclear extracts from JAr cells in which DLX3 had been overexpressed (Fig. 5C) and from nontransfected cells (Fig. 5D). The results shown in Figure 5C essentially match those shown in Figure 5B. A specific complex with the same mobility as that formed with recombinant protein was

visible on the gel, and its mobility was disrupted ("supershifted" in the case of Fig. 5C) by the addition of anti-DLX3. A longer (17×) exposure was needed to detect the complexes formed from nuclear extracts of nontransfected cells (Fig. 5D), presumably because the DLX3 concentration was much lower in these cells. Although additional bands were revealed by this extended exposure, a complex whose formation could be inhibited by the presence of excess unlabeled probe or supershifted by anti-DLX3 was still detectable (Fig. 5D, arrow). It is unclear why anti-DLX3 prevented complex formation with recombinant protein (Fig. 5B) but caused a supershift with endogenously expressed DLX3 (Fig. 5, C and D). Nevertheless, these results indicate that both constitutively expressed and ectopically expressed DLX3 bind specifically to the −54 to −46 of the *IFNT* regulatory region.

### *Dependency of Efficient DLX3 Transactivation on the ETS2-Binding Site at −79/−70 in the* IFNT *Regulatory Region*

Although the −67*luc* promoter construct contains the intact −54/−46 DLX3-binding site, DLX3 transactivated this promoter much less efficiently ($P < 0.001$) than the −126*luc* and −457*luc* constructs (Fig. 2A). We therefore examined the effect of mutating the ETS2-binding site (−457μETS2) (Fig. 3C) on the ability of DLX3 to transactivate the −457 *IFNT* reporter. In this series of experiments, we found that DLX3 overexpression up-regulated wild-type −457*luc* by 11.6-fold relative to the basal activity (Fig. 3A). Mutation of the ETS2 site reduced basal activity by 73% and the effects of DLX3 overexpression by almost 90%. Similar cotransfection experiments with the −126*luc* and −126μETS2 constructs (Fig. 3B) provided further evidence that the DLX3 effects on the *IFNT* promoter requires the presence of the ETS2-binding site approximately two helical turns (~20 bp) upstream of the DLX3 site.

We next examined whether the reverse was true—that is, whether ETS2 transactivation was dependent on an intact DLX3 site. In this series of experiments, ETS2 overexpression was conducted according to the protocol described by Ezashi et al. [11, 12] and led to an approximately 17-fold increase in *luc* expression from the wild-type −126*luc* construct (Fig. 4A). Mutation of the DLX3-binding site reduced ($P < 0.001$) both basal and ETS2-induced *luc* activity by approximately 65%. Although overall activity was reduced for the mutant construct, the fold-change between basal and ETS2-induced activity of the −126μDLX3 and wild-type −126*luc* constructs remained approximately the same (17.4-fold vs. 18.4-fold, respectively). When we cotransfected the ETS2 and DLX3 expression constructs with the −457*luc* reporter, *luc* activity in cell extracts increased more than 250-fold, compared with the 10-fold effect of DLX3 and the 18-fold effect of ETS2 observed when the two transcription factors were examined on their own (Fig. 4B). We noted similar DLX3-ETS2 synergistic activity with the −126*luc,* −721*luc,* and −1675*luc* reporters (data not shown). These results provide evidence that the relatively weak transactivational activity exhibited by both these transcription factors can be markedly enhanced when the two are expressed together.

We recently reported the role of the PKA signal transduction pathway in regulating *IFNT* expression through the activation of ETS2 [14]. Although overexpression of the catalytic subunit of PKA alone had a modest ability to up-regulate −126*luc* (~5-fold) (Fig. 4C), coexpression with ETS2 led to a large increase in *luc* expression (~500-fold) (Fig. 4C). When the binding sites for ETS2 were mutated in the construct (−126μETS2) (Fig. 4C), *luc* expression was markedly reduced (~97%) relative to the wild-type reporter, consistent with our previous observation [14]. We then tested whether the DLX3-binding site was important for the PKA effects on *IFNT* expression. Mutation of the DLX3-binding site reduced the PKA/ETS2 effects by approximately 87%, and activity was virtually abolished when both the ETS2- and DLX3-binding sites were mutated (−126μETS2/μDLX3) (Fig. 4C). These results suggest that both DLX3 and ETS2 play crucial cooperative roles in controlling basal and PKA-induced *IFNT* expression.

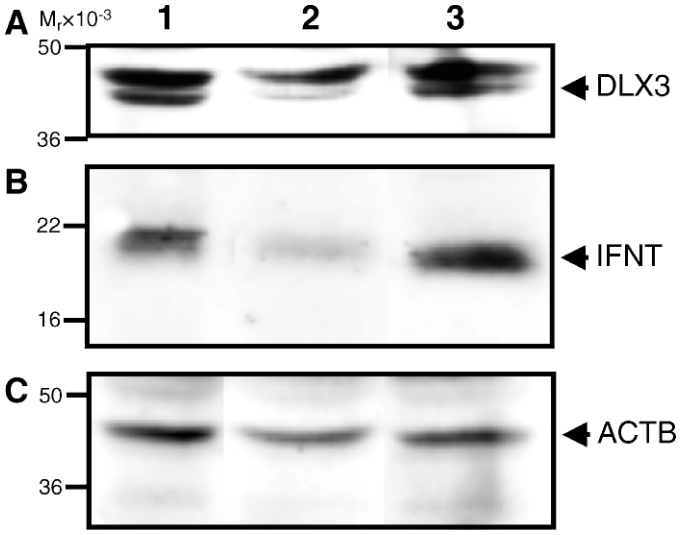


FIG. 7. Down-regulation of DLX3 expression reduces IFNT production by CT-1 cells. Cells were transfected with either a nonspecific siRNA (lane 1) or an siRNA directed against *DLX3* transcripts (lane 2) and compared with untransfected control cells (lane 3) 96 h after initial transfection. Shown are the relative amounts of DLX3, IFNT, and β-actin (ACTB) on the same Western blot (40 μg of protein) probed successively with the three detecting antibodies. The double band for DLX3 is observed consistently in CT-1 cells. It remains unclear whether this outcome is the result of posttranslational processing or of differentially spliced forms of DLX3. The broad band of IFNT with an approximate molecular weight of 21 000 Da probably results from a combination of variable glycosylation of this glycoprotein and the presence of multiple isoforms [36].

### *Expression of ETS2 and DLX3 in JAr and CT-1 Cells and in Trophectoderm Cells of Ovine Conceptuses*

Although the human genome lacks *IFNT* genes, JAr cells are permissive for expression of reporter constructs driven by transfected *IFNT* promoters, presumably because the cells express a suitable complement of required transcription factors, albeit in amounts that may not be fully optimal [33]. It is perhaps not surprising, therefore, that JAr cells express both DLX3 and ETS2, as determined by Western blot analysis of whole-cell extracts (Fig. 1, A and B) and by immunocytochemistry (Fig. 6A). Every cell observed in this experiment expressed both transcription factors within their nuclei.

We also examined DLX3 expression in the trophectoderm cell line, CT-1, which was originally derived from a bovine blastocyst outgrowth [27] and produces IFNT. Unfortunately, although CT-1 cells secrete IFNT constitutively, the cells are difficult to transfect, have demanding culture requirements, and proliferate slowly, thus making them unsuitable for the experiments described earlier with JAr cells. Both ETS2 and DLX3, however, were present in cell extracts derived from these cultures, as determined by Western blot analysis (Fig. 1, A and B), and were colocalized within the nuclei of most of the cells within the cultures (Fig. 6B). Not all CT-1 cells, however,

were producing IFNT (Fig. 6C). Rather, IFNT was confined to the cytoplasm of a minority (approximately one third) of the CT-1 cells in the culture (Fig. 6C), and all of these cells also expressed both DLX3 and ETS2 in their nuclei (Fig. 6D). These data are consistent with the notion that coexpression of ETS2 and DLX3 is necessary for the IFNT expression but is not, in itself, sufficient to ensure IFNT expression by CT-1 cells. The controls for JAr and CT-1 cell immunocytochemistry are shown in Supplemental Figure 1 (Supplemental Fig. 1 and all other supplemental data are available online at www.biolreprod.org).

Finally, DLX3 localization was examined in sections of Day 15 filamentous ovine conceptuses, which were collected when IFNT expression was high [34, 35]. The simple epithelial layer of trophectoderm was positive for DLX3 (Supplemental Fig. 2A), with relatively higher signal intensity in nuclei than in cytoplasm (Supplemental Fig. 2C), whereas sections incubated with normal rabbit serum were essentially devoid of signal (Supplemental Fig. 2, B and D).

### *Coimmunoprecipitation of DLX3 and ETS2 from JAr Cell Extracts*

The data presented so far indicate that DLX3 and ETS2 can associate on *IFNT* promoters transfected into JAr cells and, by inference, on the endogenous promoters. Here, we examined whether DLX3 and ETS2 exist together as complexes in JAr and CT-1 cells. To examine this possibility, we conducted coimmunoprecipitation experiments. Figure 1D shows that JAr cell immunocomplexes isolated by using anti-ETS2 immunoglobulin contained DLX3. Similarly CT-1 cell immunocomplexes isolated using anti-DLX3 immunoglobulin contained ETS2 (Fig. 1C). This experiment demonstrates that in these trophoblast-derived cell lines, ETS2 and DLX3 form a complex stable enough to be captured by immunoprecipitation.

We then attempted to determine whether DLX3 was associated with ETS2 on the proximal (−188 to +3) promoter region of actively transcribed *IFNT* by using ChIP assays. Sheared chromatin was prepared from bovine CT-1 cells. Sheared DNA collected in immunocomplexes after addition of affinity-purified sheep anti-DLX3, rabbit anti-ETS2 and rabbit anti-RNA polymerase II immunoglobulin, respectively, was subjected to PCR analysis with specific primers designed to amplify the proximal control region of the *IFNT* (Supplemental Fig. 3). Although the ETS2 and RNA polymerase II antibodies immunoprecipitated DNA containing the *IFNT* proximal regulatory region, the immunocomplexes brought down with DLX3 antibody failed to do so. Either DLX3 did not associate with the regulatory region of the *IFNT* promoter or the antibody was unsuitable for the ChIP analysis employed here.

### *Effect of DLX3 Silencing on IFNT Production by CT-1 Cells*

We next examined whether suppression of endogenous DLX3 expression by siRNA silencing would influence IFNT production in CT-1 cells. We employed lipitoid reagent that delivers siRNA transfection efficiently to cells that are otherwise difficult to transfect [30]. The DLX3 concentration was significantly reduced after testing one of the three *DLX3* siRNAs at 50 nM (Fig. 7A). Importantly, the intracellular concentration of IFNT was simultaneously lowered by transfection of this *DLX3* siRNA (Fig. 7B), as was the antiviral activity released into the medium (untreated control cells, $10.37 \pm 0.26 \times 10^5$ IU; cells transfected with nonspecific siRNA, $8.82 \pm 0.67 \times 10^5$ IU; cells transfected with *DLX3* siRNA, $4.07 \pm 1.41 \times 10^5$ IU), with data normalized to 90 μg of cell lysate. A second experiment provided essentially similar data, although IFNT production was considerably lower in all samples, including the untreated control.

## DISCUSSION

The results of the present study indicate that two transcription factors that have been implicated previously in the development of the mouse placenta [18, 22] act together to transactivate the proximal promoter region of the genes encoding IFNT, a major gene product of the trophectoderm of cattle for a few days during the peri-implantation phase of pregnancy. The present study also extends the concept that the signature genes of trophoblast from different species, such as *IFNT* from cattle and *hCGA* of the human, depend on a common set of transcription factors for their regulation. The presence of such transcription factors, including DLX3 and ETS2, in the trophoblast-derived cell line, JAr, probably is what makes these cells permissive for basal expression of reporters driven by *IFNT* promoters. On the other hand, overexpression of these proteins, which clearly increases the quantity of the ETS2/DLX3 complex in the cells relative to that in nontransfected controls (Fig. 1D), is necessary to achieve maximal reporter gene activity. Thus, both ETS2 and DLX3, although important for promoter transactivation, normally are present at limiting concentrations in some, if not all, cells.

The observation that the ETS2 and DLX3 effects on the *IFNT* promoter are greater than additive indicates that the two transcription factors act together synergistically. The binding sites are only two helical turns apart, and it seems probable that the proteins are partners within the same transactivational complex. The failure of the DLX3 antibody to precipitate the region of the *IFNT* promoter containing the putative DLX3-binding site, however, weakens this hypothesis. On the other hand, it is clear that DLX3 can bind to the site (Fig. 1) and can interact with ETS2 in CT-1 cells (Fig. 1C). The most likely explanation for this contradiction is that the epitope recognized by the DLX3 antibody, located between amino acids 197 and 287, is buried within the transcriptional complex or otherwise inaccessible to antibody. Another alternative is that the avidity of the antibody for its target is insufficient to retain DLX3 through the stringent washing procedures. We predict that this antibody is inappropriate for ChIP assays.

Like many other transcription factors, ETS2 is relatively promiscuous in its ability to associate with other known transcription factors, including CEBPB (C/EBPβ) [37], POU5F1 (OCT4) [12], and CREBBP/EP300 (CBP/p300) [14, 38]. It associates with CEBPB to up-regulate the gene for trophoblast Kunitz domain protein 1 (TKDP1), which has an expression pattern almost identical to that of IFNT [37]. The POU5F1 is of particular interest, because it silences both the *IFNT* [12] and *hCG* promoters [39, 40], most likely through its ability to quench ETS2 effects by coupling its POU domain with a region near the amino-terminus of ETS2 [12]. Conceivably, POU5F1 binding prevents a productive association of ETS2 with other transcriptional activators, such as DLX3. Exactly how ETS2 and DLX3 associate and whether the process is direct or involves a bridging factor, possibly a coactivator, such as CREBBP/EP300, remain to be determined. Conceivably, a less stable but still functional transactivating complex involving ETS2, DLX3, and a coactivator can form even when DLX3 is not tethered to DNA. Such a model may explain why ETS2 can up-regulate *luc* expression with the same fold-efficiency from the −126μDLX3 as the wild-type promoter (Fig. 4A). It remains unclear whether ETS2 and DLX3 need to be covalently modified to form a productive

association. The ETS2 is target for both the protein kinase A [14, 16, 17] and the Ras/MAP kinase signal transduction pathways [13, 41, 42], whereas DLX3 can be activated through protein kinase C [43]. Clearly, the proximal enhancer region of the *IFNT* can be subject to complex informational input, much of it originating from growth factors present in the maternal uterine environment in which the conceptus resides before it attaches and forms a true placenta [13, 14].

Although both ETS2 and DLX3 cause embryonic lethality when deleted from the mouse as a result of placental defects [18, 22], the factors are best known for their role in differentiation of extraplacental lineages. DLX3 is required for the proper formation of skin keratinocytes, branchial arches, teeth, and bone [44, 45]. Like DLX3 and its relatives, ETS2 and related family members appear to act as master regulatory switches during differentiation [46, 47]. In particular, ETS2 regulates the expression of some endogenous cytokeratins found in simple epithelia [48, 49] and of several genes involved in the turnover of extracellular matrix [50, 51]. Although a link between ETS2 and DLX3 in transcriptional control has not been reported previously for any known gene, our data show that the two transcription factors probably function together to regulate *IFNT* expression and might possibly partner to dictate other aspects of trophoblast development.

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