Cloning and expression of a *DAX1* homologue in the chicken embryo

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ABSTRACT

DAX1 is an unusual member of the orphan nuclear receptor family of transcription factors. Mutations in human *DAX1* cause X-linked adrenal hypoplasia congenita, while abnormal duplication of the gene is responsible for male-to-female dosage-sensitive sex reversal. Based on these and other observations, *DAX1* is thought to play a role in adrenal and gonadal development in mammals. As *DAX1* has not previously been described in any other vertebrate, a putative avian *DAX1* clone was isolated from an embryonic chicken (*Gallus domesticus*) urogenital ridge cDNA library. The expression profile of this cDNA was then examined during gonadogenesis. The clone included the conserved 3′ ligand-binding motif identified in humans and mice but the 5′ region lacked the repeat motif thought to specify a DNA-binding domain in mammals. Southern blot analysis and fluoresence *in situ* hybridisation mapping showed that the gene is autosomal, located on chromosome 1q. Sequence comparisons showed that the putative chicken DAX1 protein has 63 and 60% identity with the human and mouse proteins respectively over the region of the conserved ligand-binding domain. However, stronger identity (74%) exists with a putative alligator DAX1 sequence over the same region. Northern blotting detected a single 1·4 kb transcript in late embryonic chicken gonads, while RNase protection assays revealed expression in the embryonic gonads of both sexes during the period of sexual differentiation. Expression increased in both sexes during gonadogenesis, but was higher in females than in males. This is the first description of a *DAX1* homologue in a non-mammalian vertebrate.
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INTRODUCTION

Sex is determined genetically in higher vertebrates. In mammals, the Y-linked *SRY* gene directs the indifferent gonad to become a testis during embryogenesis (Sinclair *et al*. 1990, Koopman *et al*. 1991). The *SRY*-related gene, *SOX9*, is also necessary for testis development. *Sox9* is expressed male-specifically in mouse and chicken embryos (Kent *et al*. 1996, Morais da Silva *et al*. 1996), while mutations in the human gene can cause male-to-female sex reversal (Foster *et al*. 1994, Wagner *et al*. 1994). In addition to SRY and SOX9, the orphan nuclear receptor, SF1, participates in testicular differentiation. SF1 expression is maintained during testis development in male mouse embryos but is down-regulated in females (Ikeda *et al*. 1994), while *in vitro* studies indicate that it can regulate the gene encoding anti-Müllerian hormone (*AMH*) (Nachtigal *et al*. 1998). In contrast to our increasing understanding of testis differentiation, no ovary-determining genes have yet been identified.

The Y chromosome is clearly necessary for testis formation as it carries the *SRY* gene, but the possible role of the X chromosome in mammalian sex determination is uncertain. In 1985, Chandra suggested that mammalian sex determination may involve homologous genes carried on both the X and Y chromosomes, but where important dosage differences exist due to inactivation of one of the X chromosomes in females (Chandra 1985). More recently, the X-linked *DAX1* gene has been

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implicated in mammalian gonadal sex differentiation. Abnormal duplication of this gene (and no inactivation) blocks testis differentiation and results in male-to-female sex reversal in XY individuals (Bardoni *et al*. 1994). Meanwhile, mutations in *DAX1* are responsible for adrenal hypoplasia congenita, a condition characterised by primary adrenal insufficiency. (Hence the name, *DAX1*; dosage-sensitive sex reversal locus, adrenal hypoplasia congenita on chromosome X, number 1) (Muscatelli *et al*. 1994, Zanaria *et al*. 1994). *DAX1* is therefore implicated in the development both of the gonads and of the adrenal glands.

The murine homologue of *DAX1* (*Dax1*, also called *Ahch*) is expressed in the embryonic gonads, consistent with a role in sex determination and/or gonadal function. In the mouse, *Dax1* expression increases during ovarian differentiation but declines during testis differentiation just after peak levels of *Sry* expression (Swain *et al*. 1996). These lines of evidence have led to the suggestion that *DAX1* may operate as an ovarian-determining gene (Bardoni *et al*. 1994, Swain & Lovell-Badge 1997). While genotypically male mouse embryos carrying extra copies of *Dax1* can become sex reversed, this has only been achieved in the presence of weak alleles of *Sry* (Swain *et al*. 1998), suggesting that *DAX1* operates as an anti-testis factor under certain conditions, rather than as a natural ovarian-determining gene. In fact, it may have another role/s during gonadal differentiation. *Dax1* and *SF1* expression are co-localised in the steroidogenic lineage of developing gonads, and in the adrenals, hypothalamus and anterior pituitary (Ikeda *et al*. 1996), pointing to a pervasive endocrine function. *In vitro* studies indicate that Dax1 can repress steroidogenesis (Lalli *et al*. 1998) and antagonise SF1-mediated stimulation of the *AMH* gene (Nachtigal *et al*. 1998). Recent *Dax1* knockout studies in mice have shown that *Dax1* is not necessary for somatic differentiation of either the ovaries or testes, but spermatogenesis is impaired in the male mutants, revealing a hitherto unknown function for the gene (Yu *et al*. 1998*a*). The *in vitro* experiments and gene targeting studies suggest that, rather than having an early sex-determining role, *Dax1* may regulate hormone synthesis and gametogenesis during gonadal development.

The mammalian *DAX1* gene comprises two exons separated by an intron of approximately 3·2 kb. The gene encodes a novel orphan nuclear receptor, comprising a conserved ligand-binding domain (LBD) at the carboxy terminus but lacking the conventional zinc finger DNA-binding domain at the amino terminus. Instead, it has a 65–67 amino acid tandem repeat motif, thought to represent a novel DNA-binding domain (reviewed in Burris *et al*. 1996). Like other members of the nuclear hormone receptor superfamily, DAX1 is thought to operate as a transcription factor, although a putative ligand and target genes have not yet been identified. Given the interesting structure of *DAX1* and the question of its precise role during gonadogenesis, it is of interest to examine homologues among other vertebrates. In birds, as in mammals, sex is genetically determined, but the male is homogametic (ZZ) and the female is heterogametic (ZW). No *SRY* gene has been identified in birds, and the primary sex-determining signal is unknown. This study describes a putative avian (chicken) *DAX1* homologue and its expression pattern during gonadal sex differentiation.

MATERIALS AND METHODS

Isolation and sequencing of a chicken *DAX1* (*cDAX1*) homologue

An unamplified day 5–6 embryonic chicken urogenital system cDNA library was constructed in Lambda Zap using the UNIZAP XR vector (Stratagene, La Jolla, CA, USA). One million recombinants were screened at low stringency (2 × SSPE/0·1% SDS at 65 °C) with a 1 kb human *DAX1* cDNA probe generated by PCR. This probe included most of the coding region and was amplified using the following primers: hDAX1·1AF: 5′ CAC TGG GCA GAA CTG GGC TAC 3′, and hDAX1·1AR: 5′ CTG CAG CAT GCT GGG CTC 3′ (Zanaria *et al*. 1994). Six independent clones were autoexcised in pBluescript phagemid using helper phage. A single clone, *cDAX1/1*, containing a 1·2 kb insert was isolated and sequenced in both directions using ABI 'big dye' automated sequencing and primer walking. The other clones were smaller but identical to *chDax1/1* at the 3′ end, indicating truncated or alternative transcripts from the same locus.

Southern blot analysis

High molecular weight genomic DNA was extracted from embryonic male and female chicken livers using the lithium chloride method of Gemmell & Akiyana (1996). DNA (8 µg) was cut with HindIII or Xba1 and run on a 0·8% agarose gel overnight at 37 V. The gel was photographed and the DNA transferred to a nylon filter (Hybond N+; Amersham) under alkaline conditions. The entire 1·2 kb clone or a 250 bp RsaII subclone from the 3′ UTR were labelled with [α-^{32}P]CTP by random priming and hybridised with the filter overnight at 65 °C. The hybridisation solution was 5 × SSPE

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containing 0·5% SDS, 5 × Denhardt's solution, 100 μg/ml sheared, denatured fish sperm DNA. Following stringent washing (0·2 × SSPE/0·1% SDS at 65 °C), the filter was wrapped in plastic film, exposed to a phosphorscreen and bands quantitated on a phosphorimager (Molecular Dynamics, Kew, Victoria, Australia).

Fluoresence *in situ* hybridisation (FISH) mapping

The 1·2 kb cDNA clone was used to screen a chicken ZW genomic library constructed in λgt11. A 15 kb clone was isolated and sequenced, and found to represent the genomic copy of the putative *DAX1* cDNA (C A Smith, P S Western & A H Sinclair unpublished observations). The λ genomic clone was labelled using the Boehringer Mannheim (Sydney, NSW, Australia) biotin nick translation labelling mix (1–745–824). Metaphase chromosomes were prepared from embryonic chicken fibroblast cell lines. Cells were arrested with 100 ng/ml colcemid (Gibco BRL, Melbourne, Australia). The arrested cells were first washed in PBS and then swelled with 0·075 M KCl for 20 min at 37 °C. Cells were then pelleted at 1500 ***g*** in a bench top centrifuge and gently resuspended in fixative; methanol:acetic acid (3:1). Cells were then treated in several changes of fixative. Hybridisation and detection of the λ clone was performed as previously described (Spurdle *et al*. 1997). Hybridisation signals were visualised through a Ziess (Germany) Axioskop microscope using the appropriate UV filter and digital images were captured using a Photometrics SenSys camera. Images were processed using the V for Windows (IBM) program.

Embryo sexing, RNA isolation and Northern blot analysis

Freshly-laid chicken (*Gallus domesticus*) eggs were obtained from a commercial supplier and incubated under humid conditions at 37·8 °C. Embryos were removed from eggs, staged according to the criteria of Hamburger & Hamilton (1951) and their gonads excised. Embryos were harvested just prior to (stage 28; day 5·5), during (stages 30–38; days 6·5–9·5) and after (stage 40; day 13·5) the period of gonadal sex differentiation (reviewed in Thorne 1995). Specifically, the onset of morphological differentiation into ovaries or testes is apparent from day 6 (stage 30)

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                10          20          30              40
GGC AGA GGA CGA GCA GTG CTC GTC TCC CGG CGG GCC GGG TGG CTG AGC
 G   R   G   R   A   V   L   V   S   R   R   A   G   W   L   S

50          60            70          80          90
GGC CCC ATG GCG TGC CTG GAG CGC TGC CAC TGC TGC GCG GAC GGC CGG
 G   P   M   A   C   L   E   R   C   H   C   C   A   D   G   R

 100         110         120           130         140
CGG CAC GGC AGC ATC CTC TAC AGC ATC CTC AAG AGC CAC GAC CAG GCG
 R   H   G   S   I   L   Y   S   I   L   K   S   H   D   Q   A

    150         160         170         180           190
GCC GAG GGG CCG GGG CCG CGG CGA GGG CAG GCG GGG CGC GGC TGC TCG
 A   E   G   P   G   P   R   R   G   Q   A   G   R   G   C   S

      200         210           220         230         240
TGC GGC TCG CAG CGG CGG GTG GCC CTG AAG AGC CCG CAG GTG GTC TGC
 C   G   S   Q   R   R   V   A   L   K   S   P   Q   V   V   C

         250         260         270           280
AAA GCG GCC TCG GCC GTG CTG GTG AAG ACG CTG CGC TTC GTG CAG AAC
 K   A   A   S   A   V   L   V   K   T   L   R   F   V   Q   N

290         300           310         320         330
GTG CCC TGC TTC CAG GAG CTG CCC CTG GAC GAG CAG CTG GTG CTG GTC
 V   P   C   F   Q   E   L   P   L   D   E   Q   L   V   L   V

 340         350         360           370         380
CGC AGC TGC TGG GCG CCT CTG CTC GTG CTG GGG CTG GCG CAG GAG CGG
 R   S   C   W   A   P   L   L   V   L   G   L   A   Q   E   R

    390           400         410         420           430
GTG CAC CTA GAG ACG GTG GAG AGC GCC GAG CCC AGC ATG CTG CAG CGG
 V   H   L   E   T   V   E   S   A   E   P   S   M   L   Q   R

      440         450           460         470         480
ATC CTC ACC ACC CGG CGG CTC GGC GAG CAC GCC CCA GCT CCC GGC CGG
 I   L   T   T   R   R   L   G   E   H   A   P   A   P   G   R

         490         500         510           520
CAG CAC CCG CCC TCG GCC GGC GAG ATC CAG GCC ATC AAG GGC TTC CTG
 Q   H   P   P   S   A   G   E   I   Q   A   I   K   G   F   L

530         540           550         560         570
GCT AAG TGC TGG AGC TTG GAC ATC AGC ACC AAG GAG TAC GCC TAC CTC
 A   K   C   W   S   L   D   I   S   T   K   E   Y   A   Y   L

 580         590         600           610         620
AAG GGG ACG GTG CTC TTC AAC CCG GAT CTA CCT GGC CTG CAG TGC ACA
 K   G   T   V   L   F   N   P   D   L   P   G   L   Q   C   T

    630           640         650         660           670
CAG TAC ATT GAA GGA CTG CAG AAG GAA GCA CAG GAA GCT CTA AAT GAA
 Q   Y   I   E   G   L   Q   K   E   A   Q   E   A   L   N   E

      680         690           700         710         720
CAT GTC AGA CTC ATT CAC AGA GGT GAC CAA GCC AGA TTT GCC AAG CTG
 H   V   R   L   I   H   R   G   D   Q   A   R   F   A   K   L

         730         740         750           760
AAT GTT GTT CTA TCC TTG TTA CGA TCT ATT AAC GCT AAT GTG ATT GCT
 N   V   V   L   S   L   L   R   S   I   N   A   N   V   I   A

770         780           790         800         810
GAA CTA TTC TTC AGG CCC ATC ATT GGA TCA GTG AAC ATG GAT GAC ATG
 E   L   F   F   R   P   I   I   G   S   V   N   M   D   D   M

 820         830         840           850         860
CTT TTG GAA ATG CTT TGT GCA AAA TTA TAA agg tgt gta aag taa tga
 L   L   E   M   L   C   A   K   L   *
                                                        910
aAA TAA Atc caa tgc aaa gga act ctt aag agc aga ata gtg taa agt

                                                            960
act gta AAT AAA cta act tat tgg ttt tac ata gat agt att ttt gta

                                                  1000
ttc aat AAT AAA ata gtc ttt aag ttc tgg aat ttt tat taa aca cgg

                                                      1050
tgt ttt tgc att aag att aca gtc atc aga ata aga ttt aaa gtt gcg

                                                          1100
aag aat gtt cat atc caa agt caa ctg gct att ctt tta cca tgt cta

                                                1140
tga ata aag atc ata cat act aca aaa aaa aaa aaa aaa aa
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FIGURE 1. Nucleotide and deduced amino acid sequences of a putative chicken *DAX1* homologue. A partially conserved Kozak sequence is shown (underlined) and potential polyadenylation signals are shown in bold capitals.

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                   10        20        30        40        50
chicken    ----------------------------------------MACLER----
alligator  ------------------------------------------CVGR----
pig        MAGEDHQWQGSILYNMLMSAKQTHATREAPEARLRGSCWGCSCSSEPPVG
human      MAGENHQWQGSILYNMLMSAKQTRAAPEAPETRLVDQCWGCSCGDEPGVG
mouse      MAGEDHPWQGSILYNLLMSAKQKHASQEEREVRLGAQCWGCACGAQPVLG
           MAGE H WQGSILYN.LMSAKQ .A. E  E RL   CWGC.C  .P .G

                   60        70        80        90       100
chicken    ------C-------HCCADGR---RHGSILYSILKSHDQAAEGPGPRRGQ
alligator  ------G-------RCCAGGR---RQGSILYHILRSEEQPR-GSGP----
pig        REGQPGGPAVALLYRCCFCGEDHPRQGSILYNMLTSAKQTQETPEAPEAR
human      REGLLGGRNVALLYRCCFCGKDHPRQGSILYSMLTSAKQTYAAPKAPEAT
mouse      GERLSGGQARSLLYRCCFCGENHPRQGGILYSMLTNARQPSVATQAPRAR
            E   GG   .LLYRCCFCG. HPRQGSILYSMLTSA.Q.   .P AP A.

                  110       120       130       140       150
chicken    AG---RGCSCGSQRRVALKS------------------------------
alligator  ------GCSCGARGRGRGRSRA----------------------------
pig        LGGACWGCSCGS-EPRVGREELPGGRATVLLYRCCFCGEEHPRQGSILYS
human      LG-PCWGCSCGS-DPGVGRAGLPGGRPVALLYRCCFCGEDHPRQGSILYS
mouse      FGAPCWGCACGSAEPLVGREGLPAGQAPSLLYRCCFCGEEHPRQGSILYS
            G  CWGCSCGS .P VGR. LP G.   LLYRCCFCGE.HPRQGSILYS

                  160       170       180       190       200
chicken    --------------------------------------------------
alligator  --------------------------------------------------
pig        LLTSAKQTHVALEAPEARPGGAWWDRSYCAQRLGAREELPGGRPVTLPYR
human      LLTSSKQTHVAPAAPEARPGGAWWDRSYFAQRPGGKEALPGGRATALLYR
mouse      LLTSAQQTHVSREAPEAHRRGEWWQLSYCTQSVGGPEGLQSTQAMAFLYR
           LLTS..QTHV.  APEA.  G WW  SY .Q  G  E L    .  . YR

                  210       220       230       240       250
chicken    --------------------------------------------------
alligator  --------------------------------------------------
pig        CCFCGEDHPRQSGILCNMPMSAKQTHVAPEAQPGAPWWDPSVPAQR-VAL
human      CCFCGEDHPQQGSTLYCVPTSTNQAQAAPEERPRAPWWDTSSGALRPVAL
mouse      SYVCGEEQPQQISVASGTPVSADQTPATPQEQPRAPWWDASPGVQRLITL
           .  CGE..P.Q       P S. Q.  .P.  .P APWWD S    R ..L

                  260       270       280       290       300
chicken    --PQVVCKAASAVLVKTLRFVQNVPCFQELPLDEQLVLVRSCWAPLLVLG
alligator  -AAGGERAAAAAVLVKTLRFVKGVPCFQQLPLAEQLVLVRAAWAPLLVLG
pig        KSPQVVCEAASAGLLKTLRFVKYLPCFQVLPLDQQLVLVRSCWAPLLMLE
human      KSPQVVCEAASAGLLKTLRFVKYLPCFQVLPLDQQLVLVRNCWASLLMLE
mouse      KDPQVVCEAASAGLLKTLRFVKYLPCFQILPLDQQLVLVRSCWAPLLMLE
           K.PQVVCEAASAGLLKTLRFVKYLPCFQ.LPLDQQLVLVRSCWAPLLMLE

                  310       320       330       340       350
chicken    LAQERVHLETVESAEPSMLQRILTTRRLGEHAPAPGRQHPPS--------
alligator  LAQDGVALPAHEAAQPSMLQRILTAR------PPP---PP----------
pig        LAQDRLNFETVETLEPSLLQMILTTRRQETEGDEPPSPQPPVQPHLVLPS
human      LAQDRLQFETVEVSEPSMLQKILTTRRRETGGNEP-LPVPTLQHHLAPPA
mouse      LAQDHLHFEMMEIPETNTTQEMLTTRRQETEGPEPAEPQATEQPQMVS-A
           LAQDRL.FETVE..EPSMLQ.ILTTRR.ET GPEP  P.PP Q  ..  .

                  360       370       380       390       400
chicken    -------AGEIQAIKGFLAKCWSLDISTKEYAYLKGTVLFNPDLPGLQCT
alligator  -------AADLQALHGVLAKCWSLDIGPKEYAYLKGAVLFNPDLPGLQCT
pig        EAEHLPSVAEVQAIKGFLAKCWSLDISTKEYAYLKGTVLFNPDLPGLQCV
human      EARKVPSASQVQAIKCFLSKCWSLNISTKEYAYLKGTVLFNPDVPGLQCV
mouse      EAGHLLPAAAVQAIKSFFFKCWSLNIDTKEYAYLKGTVLFNPDLPGLQCV
           EA ..   AA.VQAIKGFLAKCWSLDISTKEYAYLKGTVLFNPDLPGLQCV

                  410       420       430       440       450
chicken    QYIEGLQKEAQEALNEHVRLIHRGDQARFAKLNVVLSLLRSINANVIAEL
alligator  QYIQGLQREAQQALNEHVRLIHRGDQARFAKLNIALSLLRSINANVIAEL
pig        KYIQGLQWGTQQILSEHIRMTHRGYQARFAELNSALFLLRFINANVLAEL
human      KYIQGLQWGTQQILSEHTRMTHQGPHDRFIELNSTLFLLRFINANVIAEL
mouse      KYIEGLQWRTQQILTEHIRMMQREYQIRSAELNSALFLLRFINSDVVTEL
           KYIQGLQW TQQIL.EH.RM.HRG QARFAELNSALFLLRFINANVIAEL

                  460       470
chicken    FFRPIIGSVNMDDMLLEMLCAKL
alligator  FFRPIIGTVNMDDMLLEMLCAKL
pig        FFRPIIGTVSMDDMMLEMLCAKL
human      FFRPIIGTVSMDDMMLEMLCTKI
mouse      FFRPIIGAVSMDDMMLEMLCAKL
           FFRPIIGTVSMDDMMLEMLCAKL
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(Van Limbough 1968, Carlon & Stahl 1985, Thorne 1995). Embryos were sexed by PCR amplification of the female-specific Xho1 repeat sequence using a small amount of genomic DNA template as described previously (Smith *et al*. 1997). RNA was extracted from pairs of gonads using the guanidinium thiocyanate method (Chomczynski & Sacchi 1987) and pooled according to sex. For Northern blotting, polyA$^+$ RNA was isolated from several tissues using oligo dT magnetic Dynabeads (Dynal, Melbourne, Australia). mRNA (2 μg) was loaded onto a 1·5% denaturing formaldehyde gel, electrophoresed overnight at 40 V, fixed by UV cross-linking and hybridised with the whole 1·2 kb *cDAX1* cDNA clone. Following high-stringency washing (0·2 × SSPE/0·2% SDS), the blot was analysed on a phosphorimager after 2 days of exposure to a phosphorscreen. The blot was then stripped in 0·5% SDS at 100 °C and re-probed with chicken glyceraldehyde-6-phosphate dehydrogenase (GAPDH) as a loading control.

RNase protection assay (RPA)

RPAs were performed on total gonadal RNA extracted from sexed embryos at stages 28, 30, 32 and 35 (days 5·5, 6·5, 7·5 and 8·5 respectively). Care was taken during dissections to avoid the neighbouring adrenal gland, which expresses *Dax1* (at least in mammals). Approximately 10 μg total RNA was used for each sex at each stage, representing six to ten pairs of gonads. A 250 bp RsaI fragment from the 3′ UTR of *cDAX1/1* in pBluescript SK$^+$ vector was used for RNase protection analysis. A 157 bp chicken *GAPDH* fragment was used as a loading control. The *GAPDH* probe was generated by PCR, using the following primers derived from the published chicken sequence: 5′ GAA GGC TGC TGC TGA TGG 3′ and 5′ TGA GCG GTG GTG AAG AGC 3′ (Panabieres *et al*. 1984). Both the putative *cDAX1* and *GAPDH* control fragments were cloned into pBluescript vector and the vectors linearised with appropriate restriction enzymes. Linearised templates were extracted twice with phenol:chloroform to remove RNases. *In vitro* transcription reactions were performed on 1 μg linear template DNA to generate cRNA antisense probes labelled with [^{32}P]UTP. Probes were labelled to a specific activity of 5×10^7–5×10^8 c.p.m./μg. RPAs were then carried out using an Ambion RPA II kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). Briefly, each sample received 1×10^5 c.p.m. cRNA probe, hybridised overnight at 42 °C and then treated with RNase A/T1. Samples were run on 5% acrylamide gels and the gels were exposed on phosphorimager screens. Relative levels of *cDAX1* expression were determined using the ImageQuant computer imaging system (Molecular Dynamics). *cDAX1* expression (normalised against *GAPDH* controls) was plotted as a percentage of maximum expression. RPAs were repeated three times on three independent series of RNA samples.

RESULTS AND DISCUSSION

Six independent clones were isolated from an embryonic chicken urogenital system cDNA library screened with a 1 kb human *DAX1* probe. The clones were purified to homogeneity through two rounds of re-screening and found to be identical at the 3′ end. Restriction mapping and sequence analysis showed that the clones were all derived from the same locus and represented truncated cDNAs (or alternative transcripts) of the same gene. The longest clone, *cDAX1/1* (1·2 kb) was selected for further analysis. This clone was sequenced in both directions by primer walking and was found to have strongest homology to mammalian *DAX1* using the BLAST search of protein and DNA databases. The cDNA and deduced amino acid sequences of *cDAX1/1*, a putative *DAX1* homologue, are shown in Fig. 1 (Gen Bank accession no. AF202991). The 1·2 kb *cDAX1/1* cDNA encodes a predicted protein of 275 amino acids. Mammalian *DAX1* cDNAs are approximately 1·8 kb in length, with an open reading frame of 1·4 kb, specifying a protein product of approximately 472 amino acids (Zanaria *et al*. 1994, Guo *et al*. 1996, Parma *et al*. 1997). The chicken sequence lacked most of the 5′ repeat motif found in mammalian *DAX1* cDNAs and thought to encode a novel DNA-binding motif. This indicates that either the chicken cDNA clone is not full length, or that the mammalian sequence is not completely conserved in the chicken. Potential polyadenylation signals and a polyA tail were found at the 3′ end of *cDAX1/1* (Fig. 1). While the chicken cDNA lacked most of the repeat motif seen in the mammals, it did have a potential transcription initiation codon and open reading frame (Fig. 1).

FIGURE 2. Alignment of chicken, alligator and mammalian DAX1 proteins. Identical amino acids are indicated by dark shading, conservative amino acid changes are indicated by light shading. The consensus sequence is shown below the aligned sequences.

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Figure 2 shows amino acid alignments for the putative *cDAX1* and those of other species. The protein shows strong homology with human, pig and mouse *DAX1* at the carboxy terminus (Zanaria *et al*. 1994, Swain *et al*. 1996, Parma *et al*. 1997). However, strongest homology is seen with a putative alligator *DAX1* sequence (P S Western, unpublished observations). This is consistent with the close phylogenetic relationship between birds and crocodilians. The region of high homology between chicken and alligator includes the conserved LBD identified in the ligand-activated nuclear receptors. Within the conserved carboxy terminus (positions 260–474), putative *cDAX1* shows 74% amino acid identity and 84% similarity with that of the alligator. Homology is somewhat lower between chicken and mammalian DAX1 proteins, the chicken showing 63 and 60% identity with the human and mouse sequences respectively. Both the chicken and alligator lack a number of residues that are present (but not well conserved) at positions 229–358 within the mammalian protein (Fig. 2).

In contrast to the highly conserved carboxy terminus, homology between the chicken and mammalian sequences is poor at the amino terminus (Fig. 2). The mammalian amino terminus includes the unusual 65–67 amino acid repeat motif thought to specify a novel DNA-binding domain. In the chicken, there is partial homology with the second of these repeat motifs (the consensus sequences RQGSILYSML at positions 75–84 and GCSCGS at positions 107–112; see Fig. 2). However, overall, homology appears low. Intriguingly, the same applies to the alligator sequence, which is strongly aligned with the mammalian sequences at the carboxy terminus but shows poor homology at the amino terminus (Fig. 2). As in the chicken, the alligator sequence is best aligned within the second repeat motif at the amino terminus (positions 70–110, Fig. 2). Northern blot analysis of late embryonic (day 15) chicken tissues revealed a single 1·4 kb transcript in ovary, testis and liver (Fig. 3). In contrast, mammalian *DAX1* transcripts are 1·7–1·9 kb in length (Zanaria *et al*. 1994, Guo *et al*. 1996, Parma *et al*. 1997). The smaller size of the chicken mRNA is consistent with the proposal that it lacks most of the unusual repeat region seen in the mammalian gene.

A schematic comparison of the mammalian and putative chicken DAX1 proteins is shown in Fig. 4. There are alternating regions of high and low homology between the chicken and human DAX1 proteins within the region of the LBD. This has also been observed in the other DAX1 sequences that have been described (Swain *et al*. 1996, Parma *et al*. 1997). Poor conservation at the amino terminus of chicken and mammalian DAX1 proteins is surprising and warrants further investigation. The presence of a potential translation start site at the 5′ end of clone *cDAX1/1*, together with a partially conserved Kozak sequence (CCCCATGG; Fig. 1) raises the possibility that the mammalian repeat motif is absent in the chicken. However, this region of the protein is thought to specify a DNA-binding domain and would therefore be critical to its function. It is possible that this function is fulfilled in chicken DAX1 by the weakly conserved single copy of the repeat motif (Fig. 2). Perhaps the non-repeat 5′ sequence in chicken and alligator is ancestral and has been duplicated during mammalian evolution. It is also possible that DAX1 does not in fact bind DNA in the chicken, although

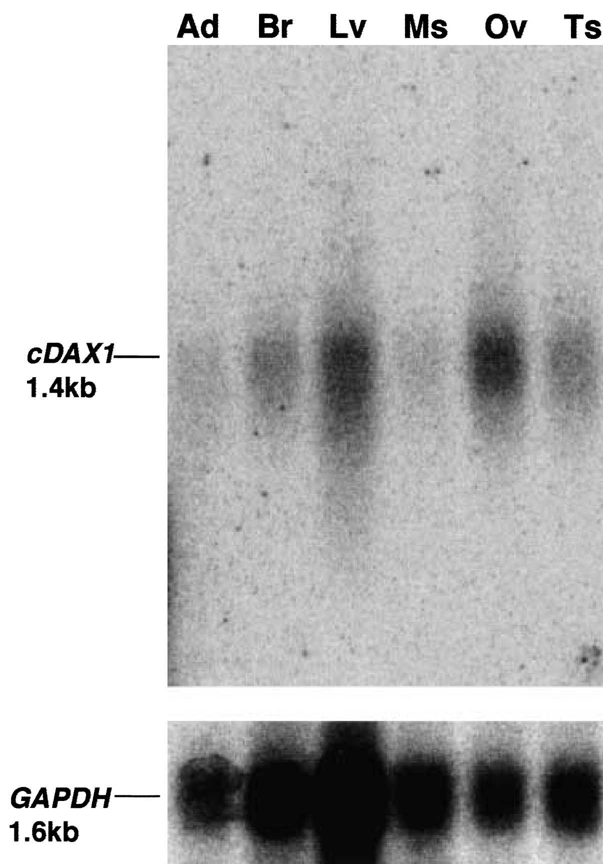

FIGURE 3. Northern blot analysis of day 15 embryonic chicken tissues hybridised with the *cDAX1* cDNA clone. After high-stringency washing, a single 1·4 kb transcript is detectable in ovary (Ov), liver (Lv), testis (Ts) and brain (Br). No clear signal is seen in the adrenal (Ad) or mesonephros (Ms). The lower panel shows *GAPDH* hybridisation as a loading control.

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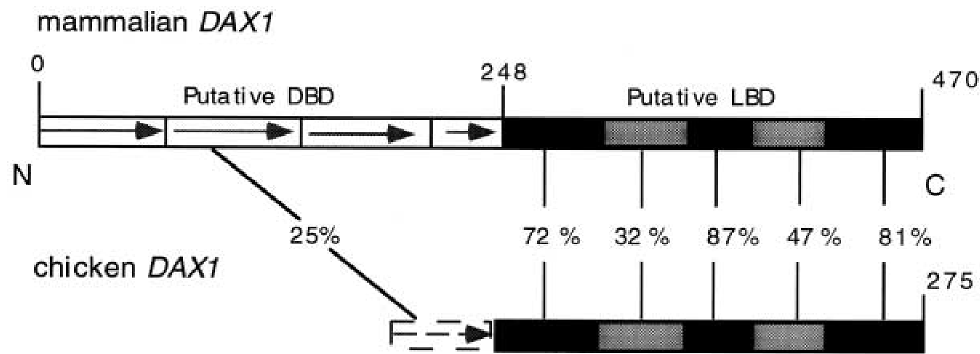

FIGURE 4. Schematic comparison of mammalian and putative chicken DAX1 protein predicted from clone *cDAX1/1*. Within the LBD, there are regions of alternating high and low homology, shown in black and grey respectively. The unusual repeat region (DBD) is absent from the chicken clone, although there is weak homology with the second mammalian repeat.

it has been shown to bind to hairpin DNA structures in mammals (Zazopoulos *et al*. 1997). The alternative possibility is that the chicken clone, *cDAX1/1*, is prematurely truncated. A *cDAX1* genomic clone is currently being examined to confirm the cDNA sequence.

Southern blots of male and female chicken DNA probed with the entire 1·2 kb cDNA clone or with a 250 bp RsaI subclone from the 3′ UTR revealed a single strong band in both sexes after high- or low-stringency washing (Fig. 5). Phosphorimaging analysis revealed equivalent band intensity between the two sexes, indicating that the gene is autosomal in chickens. FISH mapping with a 15 kb genomic clone showed that putative *cDAX1* is located on the

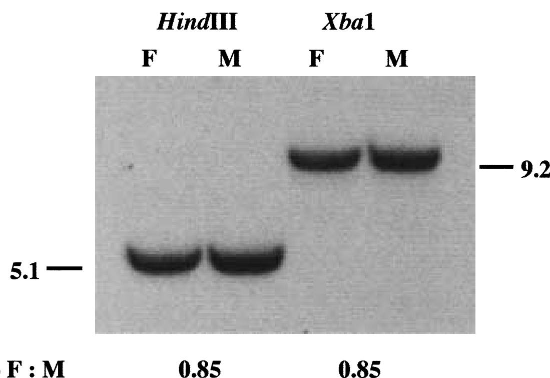

FIGURE 5. Southern blot analysis of female (F) and male (M) chicken genomic DNA digested with HindIII or Xba1 and hybridised with the 1·2 kb *cDAX1* cDNA clone. After washing at moderately high stringency (0·2 × SSPE+0·2% SDS at 65 °C), one strong band is seen, equivalent in intensity in both sexes. Molecular size of the hybridised fragments is shown (kb).

long arm of chromosome 1 (Fig. 6). Since putative *cDAX1* is not sex linked as in eutherians, it cannot participate in gonadal sex differentiation via a gene dosage mechanism as was originally postulated for mammals. In fact, *DAX1* is likely to be subject to X inactivation in humans, as XXY individuals (Klinefelter's syndrome) are male despite the extra copy of *DAX1*. Meanwhile, the marsupial *DAX1* homologue is autosomal (Pask *et al*. 1997). Under normal conditions, then, male and female mammalian embryos would be expected to have equivalent functional copies of the gene. Any sex differences in expression must therefore be attributed to differential regulation between the sexes.

RPAs showed that the putative *cDAX1* gene is expressed in both male and female chicken gonads during gonadal sex differentiation (Fig. 7). Expression was higher in females at the onset of sexual differentiation (stages 28–30; days 5·5–6·5), and it increased over development. Expression in males was somewhat lower, but it also increased over development, before declining later (stage 35; day 8·5). This pattern is broadly similar to that seen in mammals (Parma *et al*. 1997, Swain *et al*. 1996). However, *cDAX1* expression in the male does not decline at the onset of testis formation (stage 28–30), as it does in the mouse (Swain *et al*. 1996). The expression profile of this gene therefore appears to be broadly conserved in vertebrates.

Data from mammalian studies suggest that *DAX1* may play a role in the endocrine function of the developing gonads and in gametogenesis. Targeted disruption of *Dax1* in the mouse indicates that the gene is not necessary for somatic differentiation of the gonads (Yu *et al*. 1998*a*). However, in males, spermatogenic failure occurs. As *Dax1* is expressed in testicular Sertoli cells (at

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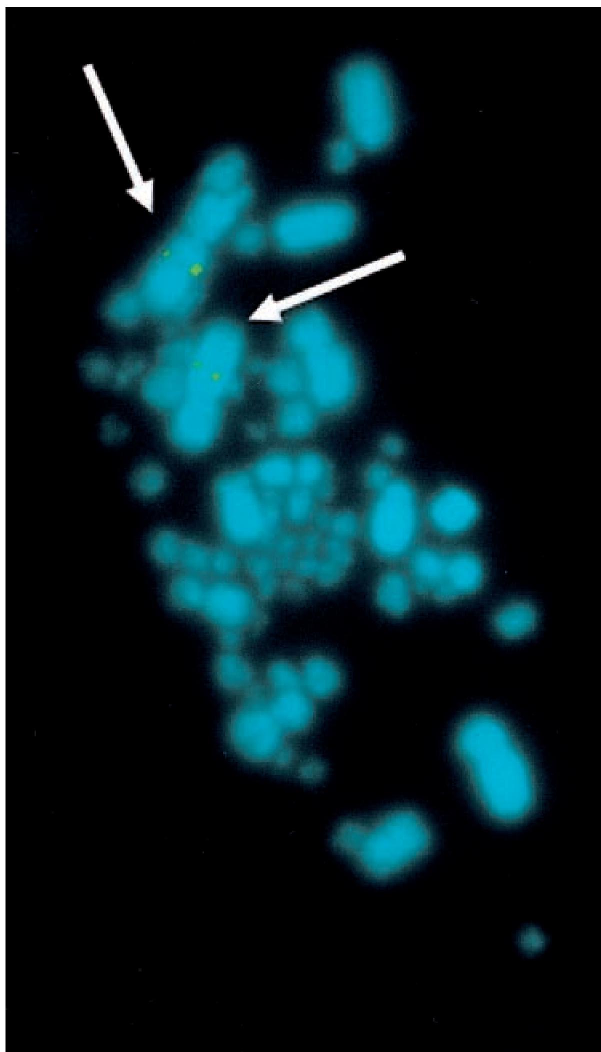
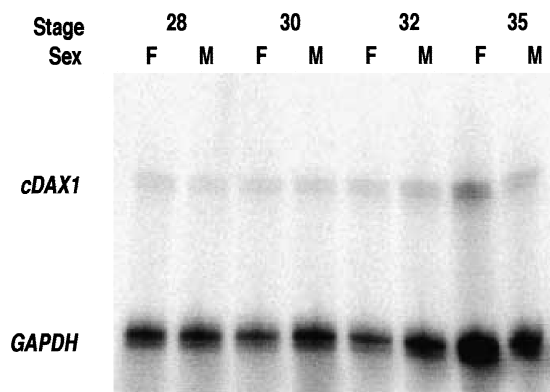


FIGURE 6. Hybridisation of 15 kb λ *cDAX1* genomic clone to chicken metaphase chromosomes. Arrows indicate localisation to chromosome 1q. Chromosomes are counterstained with DAPI (4,6-diamidino-2-phenylindole).



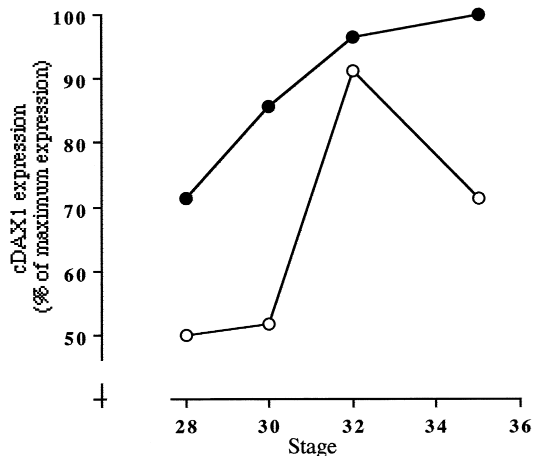


FIGURE 7. Time course of *DAX1* expression in embryonic chicken gonads, as determined by RPA. (a) Protected *cDAX1* and control *GAPDH* fragments after acrylamide gel electrophoresis for stages 28–35 in female (F) and male (M) samples. (b) Normalised *cDAX1* expression (% of maximum expression) over stages of development for female (●) and male (○) embryonic chicken gonads. The graph is based on the data shown in (a) above. Stage 28 (day 5·5) represents the onset of gonadal sex differentiation.

least in the adult rat) (Tamai *et al*. 1996), this may reflect impaired Sertoli cell–germ cell signalling. However, in embryonic rodent and human gonads, the major site of *Dax1* expression is the Leydig cell population (Ikeda *et al*. 1996, Majdic & Saunders 1996, Swain *et al*. 1996), consistent with the observation that the Leydig cells of *Dax1* knockout mice are abnormal (hyperplasic or hypertrophied) (Yu *et al*. 1998*a*). *Dax1* may therefore also regulate Leydig cell function in males. In chicken and mouse embryos, however, *Dax1* is also expressed in female embryos, and at equal or higher levels than in males (see Fig. 6; Swain *et al*. 1996). What role does it play during ovarian differentiation? Female knock-out mice appear fertile, but subtle changes in follicular structure were observed, suggesting that *Dax1* participates in folliculogenesis (Yu *et al*. 1998*a*). *Dax1* probably performs similar functions in the chicken embryo.

In mouse embryos, *Dax1* and *SF1* expression co-localise in the gonads and at other sites within

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the reproductive axis (Ikeda *et al*. 1996). This observation has led to the proposal that the two nuclear receptors interact to regulate endocrine function. Some of the functions of Dax1 appear to be antagonistic to those of SF1. In the mouse Y1 adrenocortical cell line, for example, DAX1 can block steroidogenesis by repressing several key regulators, including steroidogenic acute regultory (StAR) protein and 3β-HSD (Lalli *et al*. 1998). In contrast, *SF1* has been shown to stimulate these regulators (Parker & Schimmer 1997, Sandhoff *et al*. 1998). The exact nature of the interaction between Dax1 and SF1 is unclear; however, there is evidence that Dax1 antagonises SF1-mediated transactivation (Ito *et al*. 1997, Nachtigal *et al*. 1998). In the chicken embryo, *SF1* is expressed in both sexes during gonadogenesis and expression becoming higher in females compared with males after the onset of sexual differentiation (Smith *et al*. 1999*a*). Thus, since both *SF1* and *Dax1* expression are higher in females than in males after the onset of differentiation, the two receptors may interact to regulate the higher steroidogenic activity known to occur in the embryonic chicken ovary compared with the testis.

The factors responsible for activating *DAX1* expression in embryonic chicken gonads are unknown. A putative SF1 response element has been identified in the human *DAX1* promoter (Burris *et al*. 1995) while the mouse *Dax1* promoter is stimulated by SF1 and inhibited by COUP-TF (chicken ovalbumin upstream promoter-transcription factor) (Yu *et al*. 1998*b*). (However, *Dax1* is still expressed in the gonads of SF1 knockout mice; Ikeda *et al*. 1996.) It would be of interest to examine the expression pattern of COUP-TF during chicken gonadogenesis in parallel with *Dax1* and *SF1* expression. A recent study provides *in vitro* evidence that the Wilms' tumour suppressor gene (*WT1*) can regulate *Dax1* expression in mammals (Kim *et al*. 1999). In the chicken embryo, *WT1* is highly expressed in the gonads of both sexes from as early as day 5·5 (stage 28) (Smith *et al*. 1999*b*). *WT1* therefore fulfils the temporal and spatial requirements to be an activator of *cDAX1*, but this remains to be demonstrated.

In summary, a putative *cDAX1* cDNA clone has been isolated from an embryonic urogenital ridge cDNA library and found to have strong homology with mammalian *DAX1* at the conserved carboxy terminus. The clone lacked the unusual tandem repeat at the 5′ end. The gene appears to be a *bona fide* avian orthologue of *DAX1* and yields one strong band in both sexes by Southern blot analysis, indicating that it is not sex linked. Expression analysis during gonadogenesis reveals a similar pattern to that seen in mammals; that is, higher expression in developing female gonads compared with males. This is the first description of a *DAX1* homologue in non-mammals.

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