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Technical Article

An mRNA differential display strategy for cloning genes expressed during mouse gonad development

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ABSTRACT The mRNA differential display technique has become a popular method for isolating novel genes in a variety of biological systems including carcinogenesis, hormone regulation, plant biology and neurobiology. We have further developed the method by optimizing different steps for the use of small amounts of material, such that differential display can be used in the study of developmental biology. Our techniques include a new assay for elimination of false positive cDNA clones and a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method for the rapid analysis of differences in gene expression. This improved mRNA differential display strategy requires less than 4 μg of total RNA. We have used it for the isolation of genes which are expressed during gonad development in the mouse. One of the cDNAs found, cDNA 4.3 which corresponds to a part of the gene encoding the steroid hydroxylase 3βHSD I, was shown to be a valuable marker for adrenal development and for Leydig cell differentiation and organization during testis development.

KEY WORDS: *differential display, testis development, sex determination, Leydig cell differentiation, mouse*

Introduction

The mRNA differential display technique was developed by Liang and Pardee in 1992 and has been used to isolate novel genes and to compare gene expression in different tissues or differently treated cell lines (reviewed by Liang and Pardee, 1995; McClelland *et al.*, 1995). Several hundred articles have been published on the subject, most of them with a focus on tumorigenesis. However, only a few of them describe the application of the mRNA differential display technique in developmental biology (for example Zimmermann and Schultz, 1994; Adati *et al.*, 1995; Conway, 1995; Guimaraes *et al.*, 1995; Davis *et al.*, 1996; Mason *et al.*, 1996). This can partly be explained by the limited amount of mRNA which is available from biological material relevant for developmental biology studies and by the relative lack of technical procedures which describe the use of mRNA differential display in developmental biology (Zimmermann and Schultz, 1994; Ikonomov and Jacob, 1996). We present here an mRNA differential display method which can isolate genes expressed during gonad development in mouse. The method we develop is generally applicable for studying any developmental process.

The mRNA differential display technique permits simultaneous identification of both up- and down-regulated genes and is based on the comparative analysis of subpopulations of cDNAs which are produced by reverse transcription (RT) and polymerase chain reaction (PCR). In brief, the mRNA populations from two different tissues or cell lines are divided into four subpopulations using four different 3'-anchored oligo(dT) primers in four separate RT reactions. The cDNA fragments are then amplified using the same set of 3'-anchored primers in combination with short arbitrarily chosen 5'-primers in the presence of [α-^{33}P] dATP. Between 50 and 100 cDNA bands, in the size range 100 to 500 bp, are produced in each differential display reaction, representing the 3' end of the mRNAs. The PCR products from the two different tissues or cell lines that have been amplified with the same primer mix are then separated on denaturing polyacrylamide gels and cDNA bands with different signal intensity are candidates for cloning and further analysis.

The adult gonads, ovary and testis, emerge from precursor tissues initially common to both females and males: the indifferent gonad. This anlagen appears as a narrow band of tissue along the mesonephros, which is located on the dorsal mesentery, on either side of the dorsal aorta and neural tube in the embryo (reviewed by McElreavey *et al.*, 1995; Nordqvist, 1995; Ramkissoon and Goodfellow, 1996). The development of the indifferent gonad into either a testis or an ovary depends on the action of the sex chromosomes. In 1990, analysis of a Y-chromosome specific 35 kb DNA fragment in XX males together with mapping of the sex-

Abbreviations used in this paper: dpc, days post coitum; RT, reverse transcription; PCR, polymerase chain reaction; *Sry*, Sex-determining region Y chromosome gene; *Wt-1*, Wilms' tumor gene 1; Sf-1, Steroidogenic factor 1; *Sox, Sry*-related HMG box-containing gene; *Dax-1*, DSS-AHC critical region X, gene 1; *3βHSD I*, 3β-hydroxysteroid dehydrogenase gene 1; *CYP 17*, steroid 17α-hydroxylase gene; *HPRT*, hypoxanthine phosphoribosyltransferase gene.

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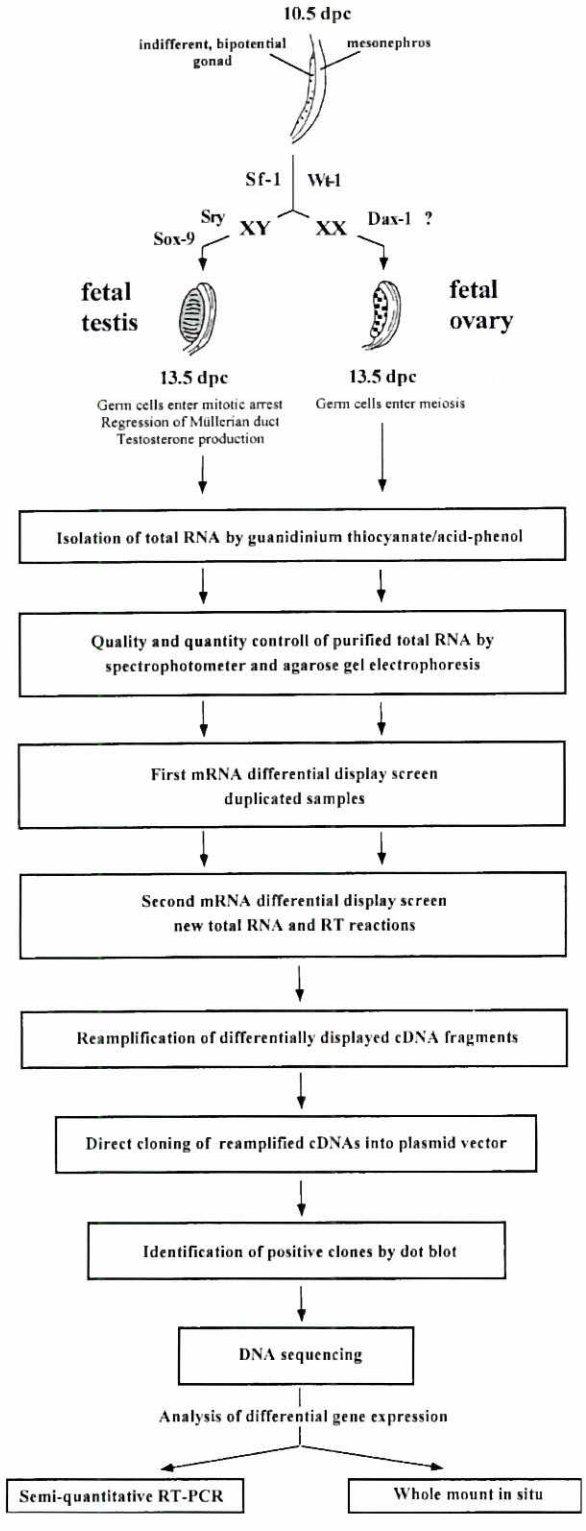

Fig. 1. Schematic flowchart of the mRNA differential display strategy. *A detailed description of each step is presented in the text (Results and Experimental Protocols). Less than 4 μg total RNA from control and test tissue is needed for an mRNA differential display screening with four different 3'-anchored oligo(dT) primers and twenty different arbitrary 5'-primers. This also includes RNA for semi-quantitative RT-PCR assaying for differences in transcription levels of genes isolated.*

determining region of the mouse chromosome led to the isolation of *SRY/Sry*, Sex-determining Region Y gene (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). This gene is conserved on the Y chromosome among almost all mammals tested. In cases of sex reversal in both humans and mice, the presence or absence of this gene correlated exactly with male or female phenotype (reviewed by Goodfellow and Lovell-Badge, 1993; Koopman, 1995). Further, XX transgenic mice carrying a 14 kb DNA fragment encoding mouse *Sry* showed normal male internal and external genitalia and normal mating behavior (Koopman *et al.*, 1991). Thus, *SRY/Sry* is the only gene on the Y chromosome which is required for testis formation in mammals.

The fruitful combination of human genetics and mouse developmental biology has led to the isolation of several other genes important for gonad development (reviewed by Schafer, 1995). Two of these other genes, Wt-*1* and *Sf-1*, are important for the development of the indifferent gonads in both females and males, as has been shown by gene targeting disruption (Kreidberg *et al.*, 1993; Luo *et al.*, 1994). The expression pattern of *Sox-9*, in combination with information from human mutations with a sex reversed phenotype, suggests that this gene is involved in Sertoli cell differentiation and probably is one member of the group of genes which orchestrates testis development (Foster *et al.*, 1994; Wagner *et al.*, 1994; Da Silva *et al.*, 1996; Kent *et al.*, 1996). *Dax-1* may be important for ovary development, since duplication of the chromosomal region containing this gene appears to override SRY and gives rise to XY females (Bardoni *et al.*, 1994; Zanaria *et al.*, 1994). In addition, mouse *Dax-1* expression coincides with a role in ovary development (Bae *et al.*, 1996; Ikeda *et al.*, 1996; Swain *et al.*, 1996).

All the genes described above encode potential transcription factors. It is therefore likely that transcription of other genes which are important for testis or ovary development will be affected, and that their differential expression will be detected by the mRNA differential display method. In order to find genes involved in gonad development and sex differentiation we have created an mRNA differential display strategy which makes it possible to isolate and clone genes from a small amount of tissue (Fig. 1). This strategy includes an optimization of the current mRNA differential display method, a novel method for avoiding false positive clones and a rapid semi-quantitative RT-PCR method to measure differences in gene expression. Using our method, less than 4 μg of total RNA is required for the screening for novel genes with 20 different arbitrary 5'-primers. We have cloned cDNA 4.3 and cDNA 80.8 from the 13.5 dpc testis mRNA pool using our method. These two cDNAs correspond to two genes which encode steroid hydroxylases, *3βHSD I* and *CYP17*. cDNA 4.3 was used as a probe in whole-mount *in situ* hybridizations and was shown to be a marker for adrenal development and for fetal Leydig cell organization during testis development.

Experimental Protocols

Tissue and RNA preparations

Gonads with attached mesonephros were dissected from embryos aged between 11.5 and 17.5 dpc, frozen directly onto dry ice and stored at -70°C. Genotypic sex was determined by staining for sex chromatin in amniotic cell nuclei as described by Palmer and Burgoyne (1991b), or by morphological examination of the gonads, looking for testis cords at 13.5 dpc and thereafter. Total RNA was

isolated from tissues by the method described by Chomczynski and Sacchi (1987) with some minor modifications. For each time point and sex, at least ten gonads with attached mesonephros were pooled and 500 µl of solution D (25 g guanidinium thiocyanate, 29.3 ml H_2O, 1.76 ml 0.75 M Na citrate pH 7.0, 2.64 ml 10% sarkosyl, 38 µl β-mercaptoethanol) was added. The tissues were homogenized by squeezing five times through a 0.8x50 sterile needle followed by ten times through a 0.6x25 sterile needle. The following solutions were added: 50 µl Na acetate (2 M, pH 4.0), 500 µl H_2O-buffered phenol, 100 µl chloroform/isoamyl alcohol (24:1). The mixture was vortexed for 1 min, chilled on ice for 15 min and centrifuged at 4°C, 12000 rpm, for 15 min. The upper phase was removed and extracted once with 500 µl chloroform/isoamyl alcohol (24:1). The RNA was precipitated by addition of 1 ml 95% ethanol, the pellet was washed with 70% ethanol, resuspended in H_2O and kept at -70°C. Since the RNA concentration and quality is critical for the following mRNA differential display, RNA samples were quantitated by absorbance at 260 nm and analyzed by 1% agarose gel electrophoresis.

DNase treatment

One µg of total RNA was added to a mixture of 5 µl DR-buffer (400 mM Tris pH 8.0, 100 mM NaCl, 60 mM $MgCl_2$, 50 mM DTT), 1 µl rRNAsin (40 U/µl, Promega, cat.# N2511), 1 µl RQ1 RNase-free DNase (1 U/µl, Promega, cat.# M6101) in a total volume of 50 µl, incubated at 37°C for 15 min, extracted once by phenol and precipitated with 10 µg glycogen (Boehringer Mannheim, cat.# 901 393). DNase treated RNA was resuspended in H_2O and stored at -70°C.

Poly(A) selection

200 µg of total RNA from female and male 13.5 dpc gonads+mesonephros were poly(A)$^+$ selected by the Poly(A) quik™ mRNA purification kit (Stratagene, cat.# 200348). Poly(A)$^+$ RNA was resuspended in 50 µl H_2O and 2 µl was used for RT, corresponding to approximately 8 µg of total RNA.

Primers

Oligonucleotide primers $T_{11}CA$, $T_{11}GC$, $T_{11}CT$, $T_{11}AG$ (denoted 3' primers) and the arbitrary decamers (denoted 5' primers), 5*2 (GCAAGTACCG), 5*3 (CCATGTCACC) and 5*20 (AAGGCCTTTA), were synthesized by the core facility at National Institute for Medical Research, London. The arbitrary sequences were selected randomly. Primers for *Sry*, *HPRT* and *3βHSD I* were synthesized by the core facility at CMB, Karolinska Institute, and are shown below in 5' to 3' direction.

5*HPRT-CCTGCTGGATTACATTAAAGCACTG,
3*HPRT-GTCAAGGGCATATCCAACAACAAAC,
5*Sry-GGTTGCAATCATAATTCTTCC,
3*Sry-CACTCCTCTGTGACACTTTAG,
5*3βHSDI-GAAGCCTTGCCAGTCACTAAC,
3*3βHSDI-GGCAAGATATGATTTAGGAC.

mRNA differential display

Reverse transcription

For each mRNA population, reverse transcription was done in four independent reactions using the four different 3' primers ($T_{11}NN$). Reactions were mixed on ice, each reaction containing 2 µg total RNA or 2 µl poly(A)$^+$ RNA and 1 µl 3'-primer (10 µM) in a total volume of 14 µl. The RNA was denatured at 70°C for 8 min and transferred back to ice. The following components were then added to each reaction: 5 µl 5xRT buffer (200 mM Tris pH 7.5, 150 mM KCl, 15 mM $MgCl_2$), 2.5 µl DTT (0.1 M), 2.5 µl dNTPs (375 µM), 0.5 µl rRNasin (40 U/µl, Promega, cat.# N2511) and 1.0 µl SuperScript II RT (200 U/µl GIBCOBRL, cat.# 18064-014). Reactions were incubated at 40°C for 1h, diluted with H_2O to 80 µl and stored at -20°C.

PCR amplification

mRNA differential display was performed essentially as described by Liang and Pardee (1992) with some modifications. The following components were mixed on ice in a PCR plate with 96 wells (Techne) or in 0.5 ml reaction tubes: 2 µl RT reaction, 2.5 µl 10xPCR buffer (500 mM KCl, 100 mM Tris pH 9.0, 1% Triton X-100), 2.5 µl $MgCl_2$ (20 mM), 0.3 µl [α-^{33}P] dATP (10 mCi/ml, DuPont NEN), 1 µl 5' primer (10 µM), 1 µl 3' primer (10 µM), 2.5 µl dNTP mix (20 µM dCTP, 20 µM dGTP, 20 µM dTTP, 14 µM dATP), 0.5 µl AmpliTaq® DNA polymerase (5 U/µl, Perkin Elmer, cat.# N801-0060). The total volume was 25 µl. Reactions were kept on ice until the PCR machine had reached 85°C and then applied onto the PCR block, denatured at 92°C for 2 min and then amplified for 22, 30 or 38 cycles at 92°C for 50 sec, 40°C for 90 sec, 72°C for 60 sec. After amplification, reactions were immediately transferred to -20°C.

Gel electrophoresis of DNA fragments

Three µl of each PCR reaction were mixed with 1.5 µl sample buffer (90% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole), denatured at 80°C for 4 min, chilled on ice and loaded onto a 5% denaturing polyacrylamide (19:1 acrylamide:bisacrylamide) gel containing 1xTBE buffer. To achieve maximal resolution of the cDNA bands, the gel was prerun for 15 min in 0.5xTBE buffer and, after loading, the samples were allowed to settle in the wells for 5 min before running the gel at a constant power of 45 W.

Detection of cDNA band patterns

Gels were transferred to filter paper, dried and exposed to X-ray film overnight at room temperature.

Recovery and reamplification of DNA bands

With help of Glogos™II Autorad Markers (Stratagene, cat.# 420202) the film was aligned with the gel and cDNA bands of interest were recovered by cutting with a razor blade. The cutting normally included some gel and filter paper, and to avoid contamination between the cDNA bands selected, a new razor blade was used for each band. The gel slice was transferred to a 1.5 ml reaction tube containing 400 µl H_2O, boiled for 15 min and centrifuged to spin down gel and filter paper. The supernatant containing the cDNA was transferred to a new tube and the DNA was precipitated with 10 µg glycogen. The pellet was dissolved in 20 µl H_2O and 10 µl was used for reamplification in a reaction volume of 50 µl. The conditions were the same as before, except that the final dNTP concentration was 40 µM instead of 2 µM and no isotope was added. One tenth of the reamplified cDNA was removed, and applied to 2% agarose gel. The remainder of the reamplified cDNA was purified using Wizard™ PCR Preps DNA Purification System (Promega, cat.# A7170).

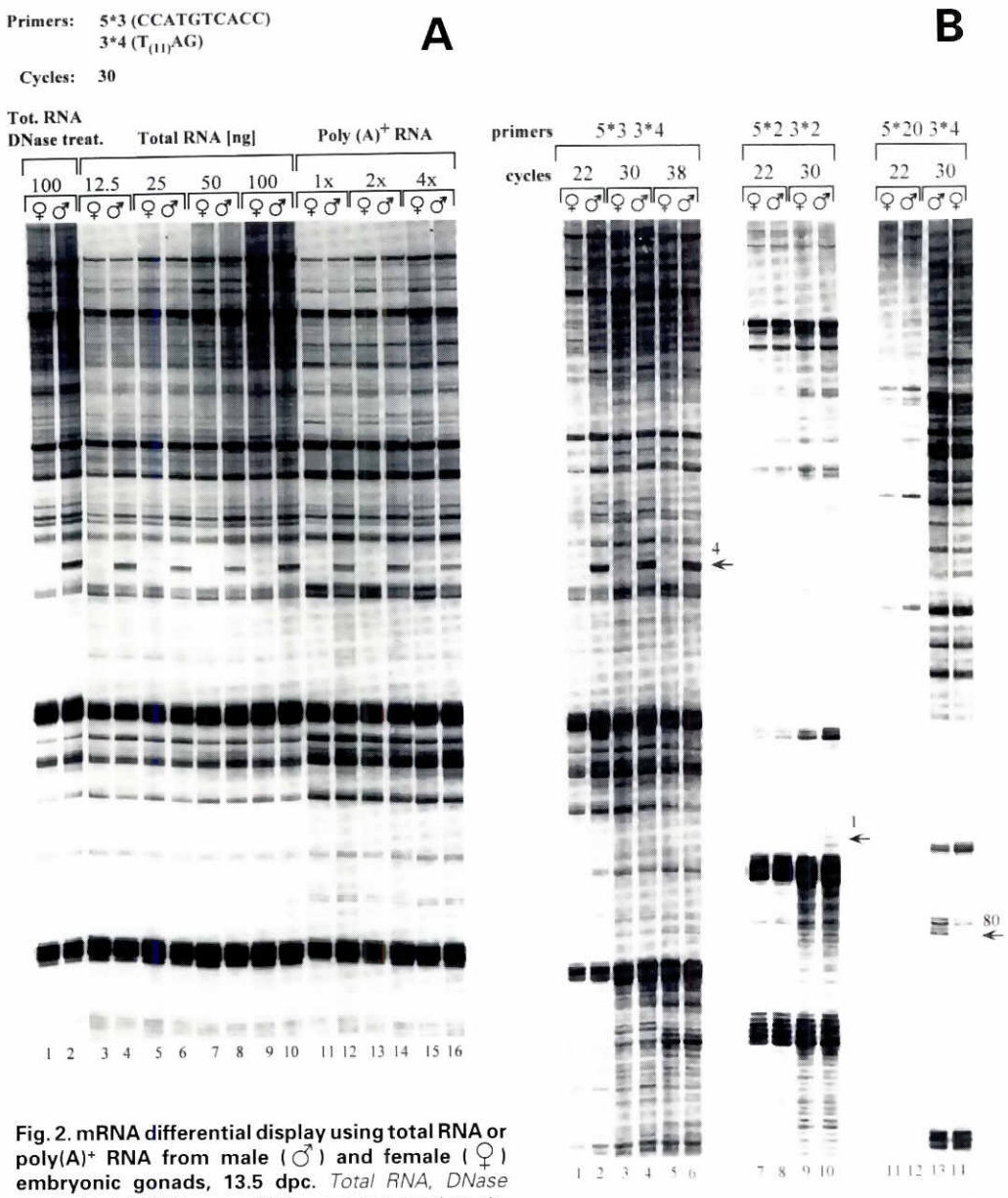

Fig. 2. mRNA differential display using total RNA or poly(A)+ RNA from male (♂) and female (♀) embryonic gonads, 13.5 dpc. *Total RNA, DNase treated total RNA and mRNA were prepared as described in Experimental Protocols.* **(A)** *The 3'-anchored oligo(dT) primer 3*4 was used for reverse transcription and in combination with the 5*3 primer, cDNAs were amplified by PCR in the presence of [α-^{33}P] dATP for 30 cycles, resolved on a denaturing polyacrylamide gel and exposed to X-ray film. Between 12.5 and 100 ng total RNA per RT-PCR reaction was used. The amount of poly(A)+ RNA was more difficult to determine. 1x corresponds to about 25 ng, 2x to 50 ng and 4x to 100 ng total RNA per RT-PCR. Some RNA seems to have been lost during the mRNA purification step.* **(B)** *mRNA differential display using different combinations of 3' and 5' primers. Reverse transcription of poly(A)+ RNA from male and female embryonic gonads, 13.5 dpc, were performed by using two different oligo(dT) primers, 3*2 and 3*4. These, in combination with 5*2, 5*3 and 5*20 were used for the subsequent PCR step, where cDNAs were amplified for 22, 30 or 38 cycles. Three differentially displayed cDNA fragments are indicated with arrows, cDNA 1, 4 and 80. Gels shown in (A and B) have been exposed overnight.*

Cloning of cDNA bands

Reamplified cDNA bands were cloned into the PCR™II vector using the TA Cloning™ Kit (Invitrogen, cat.# K2000-01). We achieved most efficient cloning using AmpliTaq DNA polymerase (Perkin Elmer) in the reamplification step. PCR products amplified with DNA Polymerase (DyNAZyme) or Taq DNA polymerase (Promega) gave lower cloning efficiency, probably due to the creation of different overhangs by these polymerases.

Dot blot

PCR of cDNA insert of single colonies

Ampicillin resistant colonies were picked and lysed in 10 µl lysis buffer (0.1 M NaOH, 0.1% SDS) for 30 min at room temperature. Cloned cDNAs were amplified from colony lysates using primers which flank the cloning site of the vector (5*TA-CCGCCAGTGTGCTGGAATTC, 3*TA-TGGATATCTGCAGAATT-CGGC). The following components were mixed on ice in a PCR plate with 96 wells (Techne): 0.5 µl colony lysate, 2.5 µl 10xPCR buffer (500 mM KCl, 100 mM Tris pH 9.0, 1% Triton X-100), 2.5 µl $MgCl_2$ (20 mM), 1 µl 5*TA primer (100 ng/µl), 1 µl 3*TA primer (100 ng/µl), 2.5 µl dNTP mix (0.5 mM), 0.5 µl AmpliTaq® DNA polymerase (5 U/µl, Perkin Elmer). The total volume was 25 µl. Reactions were kept on ice until the PCR machine had reached 85°C and then applied onto the PCR block, denatured at 92°C for 4 min and then amplified for 33 cycles at 92°C for 50 sec, 55°C for 60 sec, 72°C for 90 sec. Amplified products were analyzed by 2% agarose gel electrophoresis.

Dot blot

One µl from each colony lysate PCR was mixed with 100 µl 0.4 M NaOH, 10 mM EDTA, boiled for 10 min, transferred to ice and neutralized by adding 100 µl 2 M ammonium acetate. 90 µl of each sample was dot blotted onto duplicate nitrocellulose membranes (Hybond-C, Amersham, cat.# RPN.303E) using the Bio-Dot apparatus (BIORAD, cat.#170-6545). Membranes were then UV-cross linked and baked for 2 h in a vacuum oven at 80°C.

Probes

Probes were prepared from the original mRNA differential displays. 2 µl of the female or male mRNA differential display PCR sample was amplified in a reaction volume of 25 µl containing 2.5 µl 10xPCR buffer, 2.5 µl 20 mM $MgCl_2$, 2.5 µl 20 µM dNTPs, 3 µl

[α-^{32}P] dATP (3000 Ci/mmol, DuPont NEN), 2 µl 5' primer (10 µM), 2 µl 3' primer (10 µM), 0.5 µl AmpliTaq® DNA polymerase (5 U/µl, Perkin Elmer). Samples were denatured for 2 min at 92°C and then amplified for 5 cycles at 92°C for 50 sec, 40°C for 90 sec, 72°C for 60 sec. Probes were denatured by adding 100 µl H_2O to the PCR reactions and boiled for 10 min.

Hybridization

Filters were soaked in 2xSSC and prehybridized in hybridization mix (6xSSC, 3xDenhart's solution, 0.5% SDS) for 1 h at 60°C. Denatured probes were added and the filters incubated overnight at 60°C. Filters were washed 2x30 min in 2xSSC, 0.5% SDS at 60°C and then exposed to X-ray film.

DNA sequencing

DNA sequencing was carried out using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, cat.# 402079) and the samples were then processed on a 373 A automated DNA sequencer (Applied Biosystems).

Semi-quantitative RT-PCR

Reverse transcription

Total RNA from 11.5 dpc, 12.5 dpc, 13.5 dpc and 17.5 dpc gonads+mesonephros and adult testis (0.16-4.5 µg adult testis RNA for *HPRT* quantitative analysis and 0.5 µg male and female gonad+mesonephros RNA for *3βHSD I*, *HPRT* and *Sry* time course analysis) was mixed with 2 µl T_{11}NN primer mix (T_{11}CA, T_{11}GC, T_{11}CT, T_{11}AG, 2.5 µM of each) in a total volume of 14 µl and incubated at 70°C for 8 min. The following components were then added to each reaction: 5 µl 5xRT buffer (200 mM Tris pH 7.5, 150 mM KCl, 15 mM $MgCl_2$), 2.5 µl DTT (0.1 M), 2.5 µl dNTPs (500 µM), 0.5 µl rRNasin (40 U/µl, Promega) and 1.0 µl SuperScript II RT (200 U/µl GIBCOBRL). Reactions were incubated at 45°C for 1 h and then transferred to -20°C.

Semi-quantitative PCR

0.3 to 7.5 µl (for *HPRT* quantitative analysis) or 1.5 µl (for *Sry*, *HPRT* and *3βHSD I* analysis) of the RT reaction were mixed on ice in a PCR plate with 96 wells (Techne) together with 2.5 µl 10xPCR buffer (500 mM KCl, 100 mM Tris pH 9.0, 1% Triton X-100), 2.5 µl 20 mM $MgCl_2$, 0.3 µl [α-^{32}P] dATP (3000 Ci/mmol, DuPont NEN), 1 µl 5' primer (5*Sry or 5*3βHSDI or 5*HPRT, 100 ng/µl), 1 µl 3' primer (3*Sry or 3*3βHSDI or 3*HPRT, 100 ng/µl), 2.5 µl dNTPs (500 µM), 0.3 µl AmpliTaq® DNA polymerase (5 U/µl, Perkin Elmer). The total volume was 25 µl. Reactions were kept on ice until the PCR machine had reached 85°C and then applied onto the PCR block, denatured at 92°C for 2 min and then amplified for 24 cycles at 92°C for 60 sec, 55°C for 60 sec, 72°C for 60 sec. After the amplification, reactions were immediately transferred to -20°C.

Native polyacrylamide gel electrophoresis

Three µl of each PCR were mixed with 2 µl loading buffer (15% Ficoll, 0.2% xylene cyanole, 50 mM EDTA) and separated on a 5% native polyacrylamide (19:1 acrylamide:bisacrylamide) gel containing 1xTBE buffer. Gels were transferred to filter paper, dried and either exposed overnight to X-ray film or to PhosphorImager screens for quantitative analysis (ImageQuant software).

Whole-mount in situ hybridization

Digoxigenin-labeled antisense and sense RNA probes were prepared from linearized cDNA 4.3. *In situ* hybridizations were performed as described by Wilkinson (1992).

Results and Discussion

Here we present a strategy for the isolation of genes in developmental biology systems. This method involves four different steps; i) an mRNA differential display method which was optimized for use in developmental biology, ii) a dot blot assay for the isolation of positive cDNA clones, iii) a semi-quantitative RT-PCR assay for rapid analysis of gene expression, and iv) whole-mount *in situ* hybridization.

The procedure shown in Figure 1 needs less than 4 µg of total RNA, which can be obtained from two pairs of fetal gonads. It should therefore be possible to use this procedure for the isolation and characterization of genes in other developmental biology systems where the amount of RNA is limited.

Optimization of conditions for mRNA differential display using minute amounts of material

Total RNA was isolated from male and female gonads with attached mesonephros (gonad+mesonephros) from 13.5 dpc old mouse embryos. We chose this time point for two reasons: firstly, at 13.5 dpc the gonads have been developing for 1-2 days and genes which are involved in these processes will probably be expressed (as for *Wt-1*, *Sf-1* and *Sox-9*). Secondly, it is easy to distinguish between male and female gonads during the dissections, which means that contamination of the male RNA population by female RNAs and vice versa will be reduced to a minimum.

As a first step the amount of total RNA required from male or female 13.5 dpc gonads+mesonephros was determined. It is shown in Figure 2A that as little as 12.5 ng of total RNA gave a proper and distinct cDNA banding pattern. 12.5 ng is equivalent to the RNA from about 250 cells, based on published values of the total RNA in 8- to 16-cell and early blastocyst stages, 40 pg/cell, and in regenerating rat liver, 60 pg/cell (Piko and Clegg, 1982; Reiners and Busch, 1980). It is possible to use less RNA, even as little as 2 ng, but not all cDNA bands will be detected. This can be partially overcome by longer exposure times of the gels.

Liang *et al.* have stressed the importance of DNase treatment to avoid DNA contamination in the PCR step (Liang *et al.*, 1993). However, since the DNase treatment often results in loss of RNA we would like to omit this step. When total RNA and DNase treated total RNA were compared in the mRNA differential display, no major differences were detected in the banding pattern (Fig. 2A, lanes 1-2 and 9-10). Also, when RT was performed on total RNA without reverse transcriptase, almost no bands appeared in the mRNA differential display (data not shown). Therefore, by using a modified version of the RNA extraction method described by Chomczynski and Sacchi (1987) which reduces the amount of contaminating DNA, the DNase step could be omitted.

We also compared total RNA with poly(A)$^+$ RNA and found that the banding pattern from poly(A)$^+$ RNA was cleaner. This made it easier to detect cDNA band differences between male and female, particularly for cDNAs longer than 300bp (Fig. 2A). It has previously been recommended not to use Poly(A)$^+$ RNA since oligo(dT) contamination could give a high background smear in

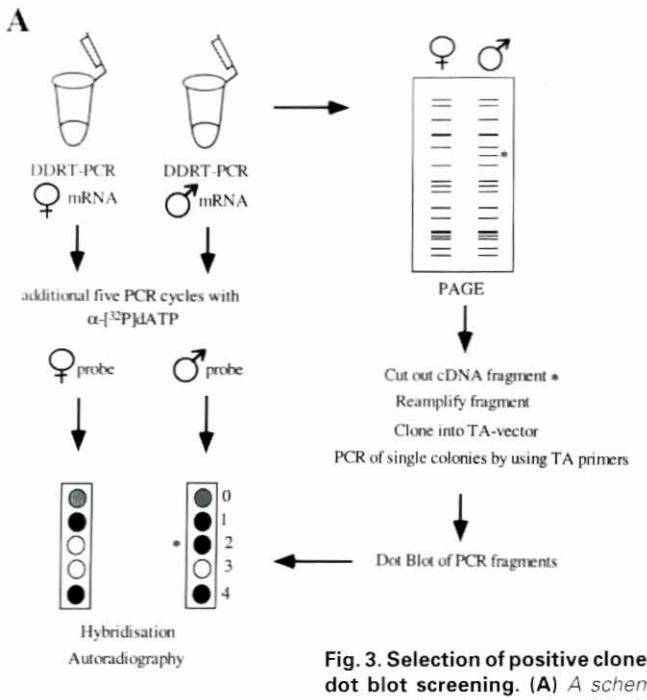

Fig. 3. Selection of positive clones by dot blot screening. (A) *A schematic presentation of the method.* **(B)** *cDNA4 (Fig. 2B) was eluted, reamplified and cloned into a TA-vector. Single colonies were picked, lysed and inserts were amplified by PCR and dot blotted onto two nitrocellulose filters. Male (♂) and female (♀) specific probes were made by extending the mRNA differential display (Fig. 2B, lanes 3 and 4) for 5 more cycles in the presence of [α-32P]dATP as described in Experimental Protocols. Two inserts, 4.0 and 4.1, contained cDNAs found in both male and female embryonic gonad. cDNA 4.3 represented an mRNA which is male specific in embryonic gonads.*

the differential display (Liang *et al.*, 1993). We have not noticed this problem in our differential display and are routinely using Poly(A)$^+$ RNA as an extra safety step to avoid picking up false positive clones containing rRNA sequences. However, if minute amounts of material are used for RNA extraction it is advisable to use total RNA since poly(A) selection often result in loss of RNA. Also, Zimmermann and Schultz were successful in using total RNA when analyzing gene expression in the preimplantation mouse embryo by mRNA differential display (Zimmermann and Schultz, 1994). In any case, one of the most important factors for a successful mRNA differential display is to have RNA of good quality and in equal amounts of the two RNA samples compared.

As suggested by Liang *et al.* (1993), the method of analyzing each set of primers in duplicate was adopted, i.e., two aliquots were removed after the RT reaction and subjected to separate PCRs. Two different numbers of rounds of PCR cycles were used, 22 cycles to detect differences in transcripts which are present in high frequency (Fig. 2B, cDNA4) and 30 cycles for detecting differences in less abundant mRNAs (Fig. 2B, cDNA1). 38 cycles was also tested and gave the same result as for 30 cycles (Fig. 2B, lanes 5 and 6). Separate experiments using the same primer pair but other mRNA batches, different *Taq* DNA polymerases and other PCR machines produced banding patterns which were nearly identical. However, separate wells in the PCR machine could amplify differently and it is important to check the uniformity of the wells to get reproducible mRNA differential displays.

To assay for differences in mRNA pattern between male and female 13.5 dpc gonads+mesonephros, a total of 20 different arbitrary 5'-primers were screened against the four different 3'-anchored oligo(dT) primers. Figure 2B displays three different primer combinations, and clearly shows that different primer pairs give different cDNA banding patterns, although the 3'-primer was the same in lanes 1-6 and 11-14. On average, there was less than one cDNA fragment which was differentially displayed per pair of primers. This seems reasonable since Liang *et al.* (1993) have reported that less than 1% of the cDNA bands appear to be expressed differentially between normal and breast cancer cells. Also, while Blanchard and Cousins (1996) found on average one differentially displayed fragment per differential display reaction when looking at intestinal mRNA regulated by dietary zinc.

Most of the time-consuming work remains after the mRNA differential display, including verification of positive clones and differences in gene expression. For this reason, a second mRNA differential display screening was performed with new RT reactions to ensure that the differences in expression patterns between the male and female RNA populations were reliable (data not shown). After this, the cDNA bands of interest, cDNA 1, 4 and 80, were cut out from the gel and reamplified.

Isolation and sequencing of three differentially displayed cDNAs

One of the major drawbacks of the mRNA differential display technique has been that the PCR products purified from gels in many cases are heterogeneous (Bauer *et al.*, 1993; Callard *et al.*, 1994; Li *et al.*, 1994; K. Nordqvist, unpublished results). Thus, one cDNA band seen on the gel can contain cDNAs of both constitutively expressed and differentially expressed genes. Several approaches have been used to circumvent this problem. These include the direct use of the reamplified cDNA fragment as a probe in Northern blot analysis, the use of Northern blot for affinity capturing of cDNAs and separation of recovered cDNA bands by a single-strand conformation polymorphism (SSCP) gel (Li *et al.*, 1994; Liang and Pardee, 1995; Mathieu-Daude *et al.*, 1996; Zhao *et al.*, 1996). However, the most commonly used procedure has been to clone reamplified cDNA into suitable plasmid vectors and to use different dot blot methods to identify the clone of interest. During the last years a flora of publications has emerged which presents technical improvements within this field (Callard *et al.*, 1994; McClelland *et al.*, 1994; Mou *et al.*, 1994; Wong and McClelland, 1994; Liu and Raghothama, 1996; Vogeli-Lange *et al.*, 1996; Zhang *et al.*, 1996). Most of these methods work excellently for finding cDNAs which represent differentially expressed genes in cell lines and larger amount of tissue, but cannot be used with a limited amount of material, as is the case in developmental biology. The advantage of the dot blot method presented here is that there is no need for extra RNA.

cDNA1, 4 and 80 were cloned into a plasmid vector (pCR™II, Invitrogen) with help of the TA-cloning procedure which takes advantage of the extra A nucleotide added by the Taq polymerase to the 3'-end of the PCR fragments. To achieve high cloning

efficiency, it was important to use AmpliTaq (Perkin Elmer) in the reamplification step. PCR products amplified with DNA Polymerase (DyNAZyme) or Taq DNA polymerase (Promega) and cloned into pCR™II gave lower cloning efficiency.

The principle of the modified dot blot assay that we developed is to make two identical nylon filters, containing PCR amplified inserts from single colonies. Male gonad specific and female gonad specific probes were made by amplifying an aliquot of the original differential display samples for five additional cycles. This was performed without reaching saturated conditions using the same set of primers as before in the presence of [α-^{32}P] dATP (Fig. 3A). The result from one dot blot experiment is shown in Figure 3B, where clone 4.3 was shown to be male specific.

Three different dot blot experiments were performed, one for each cDNA cloned, and positive clones from each dot blot were sequenced (Fig. 4). cDNA 1.2 was 153bp long and not similar to any DNA sequences in the EMBL database. cDNA 4.3 was 255bp long and identical to the non-coding 3' end of the 3β-hydroxysteroid dehydrogenase/ D^5-D^4 isomerase I (3βHSD I) gene. cDNA 80.8 was 122bp long and homologous to another steroid hydroxylase gene, *CYP17*.

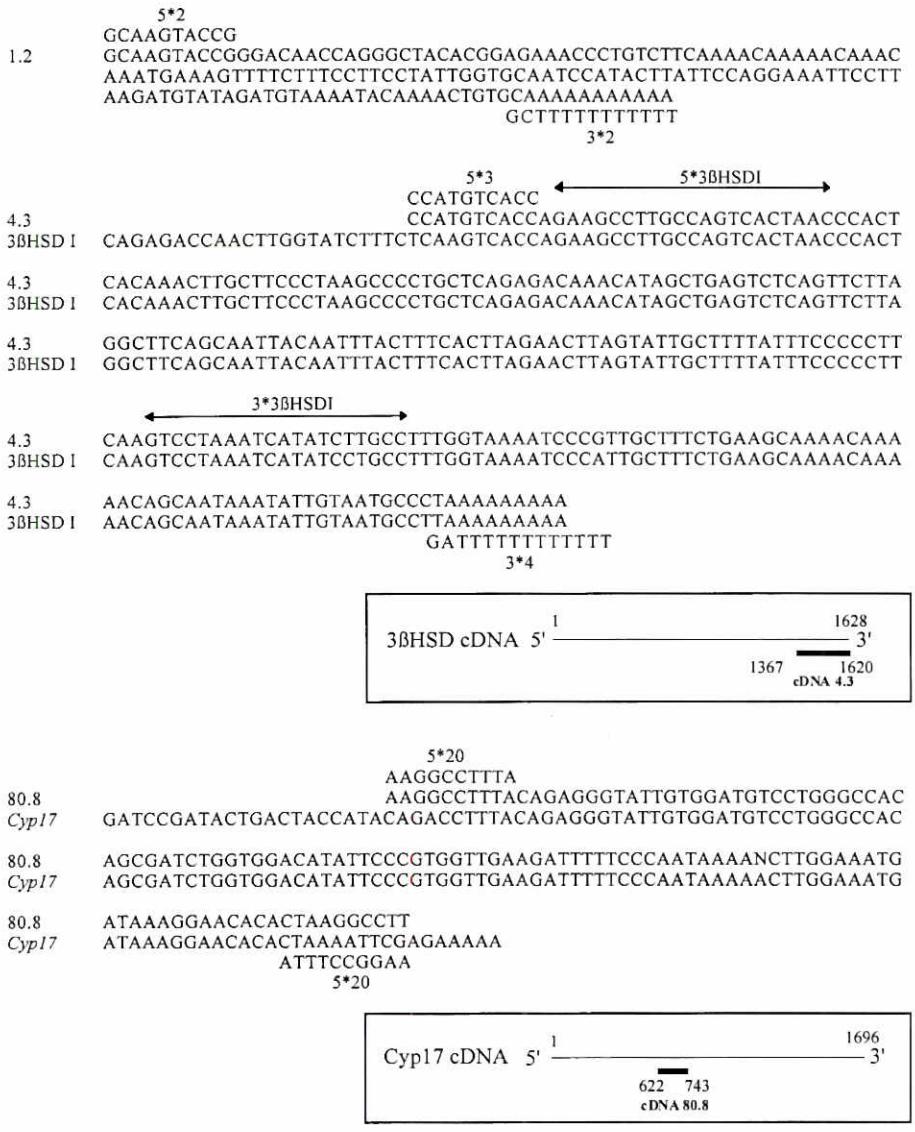

Fig. 4. Nucleotide sequence of the three differentially displayed cDNAs which are isolated. *The primers used in the mRNA differential display are depicted above and below each sequence. cDNA 1.2 shows no homology to any known gene or sequence. cDNA 4.3 and 80.8 show an almost perfect homology to two steroid hydroxylase genes, 3βHSD I and Cyp17 respectively, and the boxes show where the cDNAs are located in these genes. Primers used for the detection of 3βHSD I expression by semi-quantitative RT-PCR are indicated by arrows.*

Semi-quantitative RT-PCR assays for determining mRNA levels of genes expressed during gonad development

In previous articles describing the mRNA differential display technique, Northern blot analysis has been commonly used to confirm that a differentially displayed cDNA fragment represents a true difference in transcript levels between the samples tested (reviewed by Liang and Pardee, 1995). It is usually impossible to use Northern blot analysis when working in developmental biology, since the amount of material is so small.

To confirm the differential expression of mRNAs which correspond to cDNAs isolated by the mRNA differential display, a semi-quantitative RT-PCR assay which is simple, fast and which requires little RNA, was developed. Total RNA from female and male embryonic gonads+mesonephros were reverse transcribed with a mixture of the four different 3'-anchored oligo(dT) primers used earlier for the mRNA differential display. PCR primers were selected so that they would be located in the 5' and 3' ends of the cloned cDNA fragment. Annealing was usually carried out in the PCR at 55°C, since the reaction was successful at this temperature for most primer pairs tested. The PCR reaction was optimized to amplify only the target sequence under non-saturated conditions, and was run for 24 cycles in the presence of [α-^{32}P] dATP.

Measurements of hypoxanthine phosphoribosyltransferase *(HPRT)* gene expression showed that the RT-PCR assay gave values which were close to quantitative. Two types of experiment were carried out on HPRT. In the first experiment, different amounts of total RNA were used in the RT reactions and equal volumes from each RT reaction were then used for the PCR. Figure 5A shows that the signal obtained depended linearly on the amount used between 30 and 270 ng of total RNA per RT-PCR. In the second experiment (Fig. 5B), a fixed amount of total RNA was used in the RT reaction and different volumes of this reaction were added to the PCR. This was necessary in order to investigate whether the volume of the RT reaction was in any way limiting

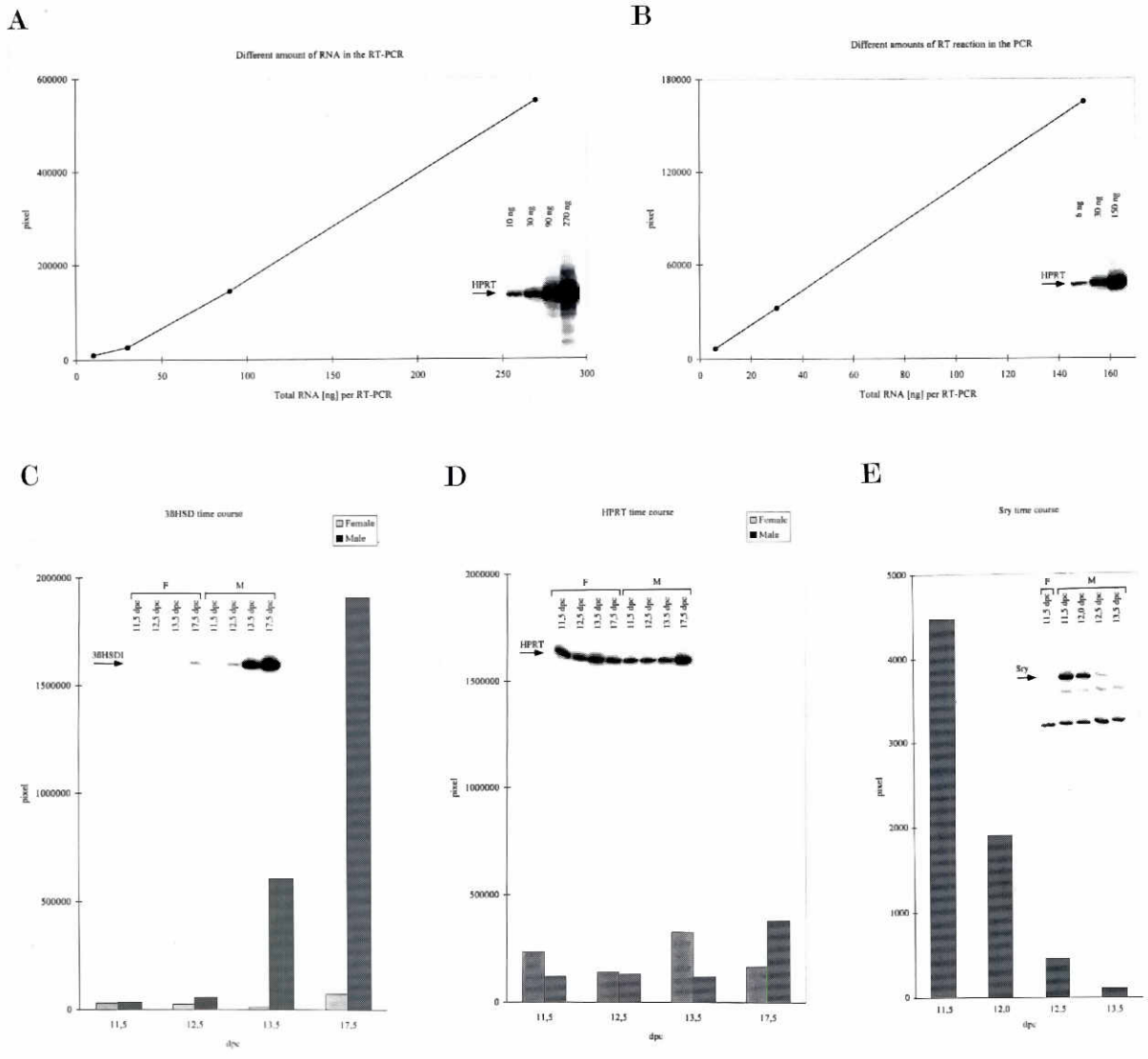

Fig. 5. Semi-quantitative RT-PCR for the analysis of *3βHSD I* and *Sry* expression. **(A)** *Control of PCR conditions. Different amounts of HPRT RNA from adult testis were reverse transcribed and introduced into PCR for 24 cycles. The PCR products were quantitated by Phosphor Imager analysis. Reactions were found to be linear between 30 and 270 ng RNA per RT-PCR.* **(B)** *0.5 μg testis RNA was reverse transcribed and different amounts of the RT mix were applied to PCR. PCR fragments representing HPRT transcripts were quantitated by Phosphor Imager analysis.* **(C)** *Time course of 3βHSD I expression. Expression was analyzed using semi-quantitative RT-PCR with the 3βHSD I specific primers depicted in Figure 4. Total RNA was prepared as described in Experimental Protocols from 11.5 to 17.5 dpc female (F) and male (M) gonads+mesonephros. 50 ng total RNA was used in each RT-PCR.* **(D)** *Time course of control, HPRT. The same RT reactions as for 3βHSD I expression were used.* **(E)** *Time course of Sry expression. The same RNA as in (C and D) was used and reverse transcribed with a Sry specific 3' primer (3*Sry). Sry expression levels were quantitated by Phosphor Imager analysis.*

the PCR. The signal depended linearly on the amount used between 10 and 150 ng of total RNA per RT-PCR. For subsequent analyses, 500 ng total RNA was used in the RT reactions, and 1/10 of the RT mix was used for each PCR, corresponding to 50 ng of total RNA per RT-PCR.

With help of the mRNA differential display method presented here, three different cDNAs were isolated from the male gonad and sequenced, cDNA 1.2, cDNA 4.3 and cDNA 80.8. cDNA 1.2 shows no homology with any known gene or DNA sequence. However, the difference in expression between male and female 13.5 dpc gonad+mesonephros was only approximately three times. cDNA 4.3 and cDNA 80.8 showed almost perfect homology with genes which encode steroid hydroxylases, *3βHSD I* and *CYP17* respectively. cDNA 4.3 was chosen for further analysis by semi-quantitative RT-PCR and whole-mount *in situ* hybridization.

Two new primers, with a GC content of about 50%, were synthesized for assaying *3βHSD I* expression during gonad development (Fig. 4, 5*3βHSDI and 3*3βHSDI). These primers were located close to the 5' and 3' ends of the cDNA4.3 sequence, excluding the AT-rich region which is at the very 3' end, and gave rise to a PCR fragment of 169bp. These primers were specific for

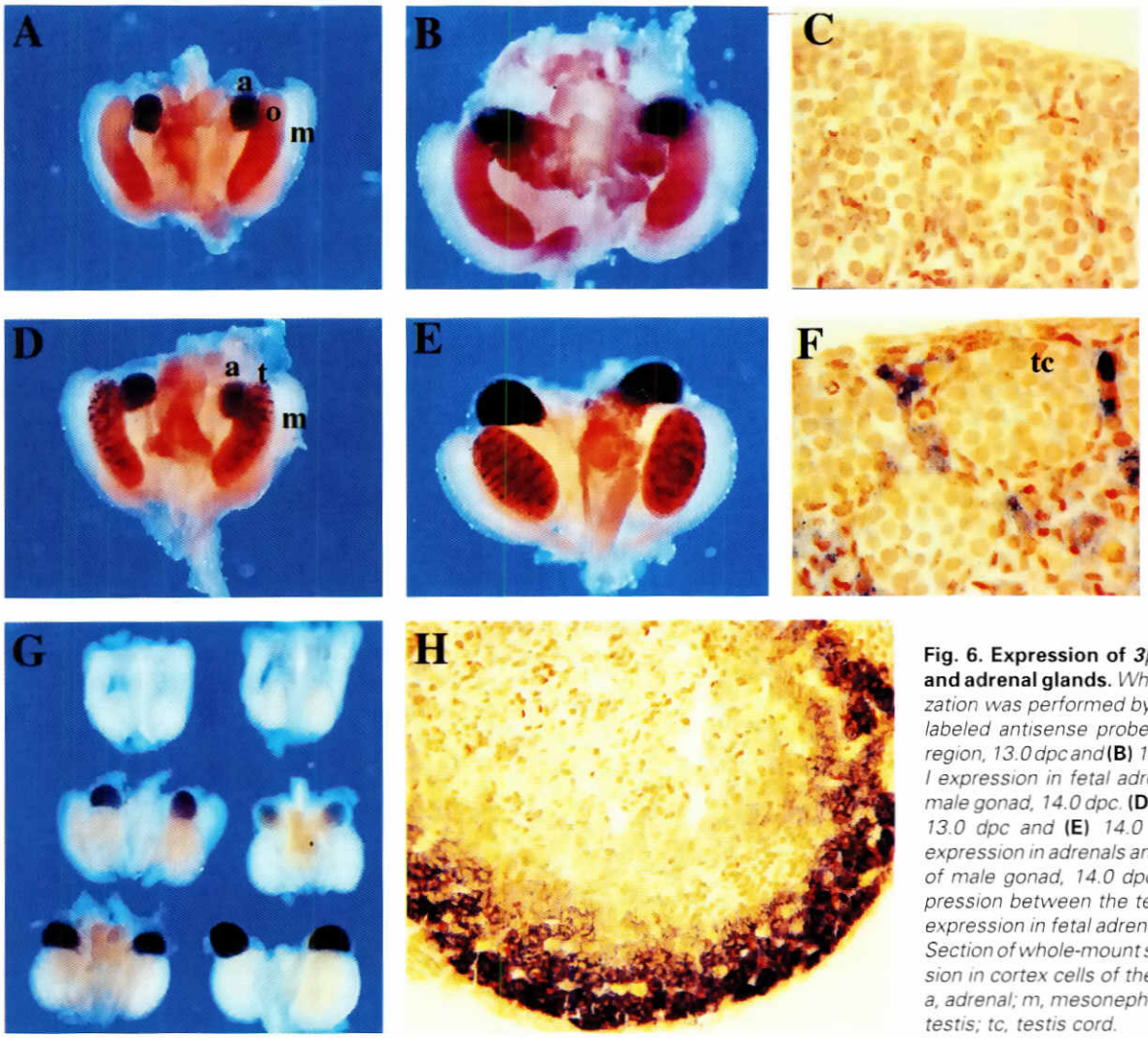

Fig. 6. Expression of *3βHSD I* in fetal gonads and adrenal glands. *Whole-mount in situ hybridization was performed by using cDNA4.3 as DIG-labeled antisense probe.* **(A)** *Female urogenital region, 13.0 dpc and* **(B)** *14.0 dpc, showing 3βHSD I expression in fetal adrenals.* **(C)** *Section of female gonad, 14.0 dpc.* **(D)** *Male urogenital region, 13.0 dpc and* **(E)** *14.0 dpc showing 3βHSD I expression in adrenals and fetal testis.* **(F)** *Section of male gonad, 14.0 dpc, showing 3βHSD I expression between the testis cords.* **(G)** *3βHSD I expression in fetal adrenals, 12.0 to 14.0 dpc.* **(H)** *Section of whole-mount showing 3βHSD I expression in cortex cells of the fetal adrenal, 14.0 dpc. a, adrenal; m, mesonephros; o, fetal ovary; t, fetal testis; tc, testis cord.*

3βHSD I and did not hybridize to transcripts from other 3βHSD genes (data not shown).

Gonads+mesonephros were removed from embryos of Parkes outbred mice (NIMR) at different time points and total RNA was extracted by the guanidinium isothiocyanate-acid phenol method. The Parkes mice were selected since detailed time courses of *Sry* and *MIS* expression from this strain have been published (Hacker *et al.*, 1995). The first time point chosen was 11.5 dpc, 14 tail somites, which is when it is first possible to remove the undifferentiated gonad+mesonephros without severe contamination of cells from the dorsal mesentery. In addition, the expression of *Sry* is a maximum at 11.5 dpc.

The semi-quantitative RT-PCR was used to compare the time courses of *3βHSD I* expression in female and male developing gonads and to compare the onset of *3βHSD I* gene expression with *Sry* expression. Figure 5C shows that very low levels of *3βHSD I* mRNA were observed at 11.5 dpc, 14 tail somites, in both male and female gonad+mesonephros. Between 12.5 and 13.5 dpc, *3βHSD I* expression increased dramatically in the male gonad+ mesonephros and at 17.5 dpc the level was about twenty times higher than at 11.5 dpc. In contrast, female gonad+-mesonephros *3βHSD I* mRNA levels stayed relatively stable throughout the time period. HPRT expression was used to check that the amount of RNA was equal at the different time points (Fig. 5D).

3βHSD is a key enzyme in the biosynthesis of steroid hormones. In mouse, five closely related genes with different expression patterns have been characterized, I to V (Bain *et al.*, 1991; Clarke *et al.*, 1993; Abbaszade *et al.*, 1995). In the adult mouse, *3βHSD I* is the only 3βHSD gene which is expressed in both gonads and adrenal glands. In fetal gonads+mesonephros we found weak *3βHSD I* expression as early as 11.5 dpc by semi-quantitative RT-PCR. The *3βHSD I* may be expressed in either the indifferent gonad or in the adrenal glands since the adrenals are not detached from the mesonephric tissue until 12.5 dpc. By separating the gonad from the mesonephros by dissection at 11.5 dpc it will be possible to investigate the source of early *3βHSD I* expression in more detail. The major increase in *3βHSD I* expression occurred between 12.5 and 13.5 dpc. Lee and Taketo (1994) found by RT-PCR that the expression of *3βHSD* starts at 13.5 dpc during their investigation of sex reversal in crosses between C57BL/6J and B6.Y^{DOM}. These

results suggest that Leydig cells begin to differentiate between 12.5 and 13.5 dpc in mouse.

The first sign of *3βHSD I* expression in female gonad occurred at 17.5 dpc, at which a slight increase in mRNA expression was detected by RT-PCR. In fetal and postnatal rat ovary, *3βHSD I* mRNA expression is first observed at day six after birth (Juneau *et al.*, 1993). By using cDNA 4.3 as a probe, it will be possible to study more accurately the time course of *3βHSD I* expression and to investigate the differentiation of steroid producing cells in developing ovary.

Sry encodes two different transcripts in mouse, a circular non-functional transcript which is expressed in the germ cells of adult testis, and a functional transcript in the somatic cells of the embryonic male gonad (Capel *et al.*, 1993; Hacker *et al.*, 1995). PCR primers for analysis of *Sry* expression by semi-quantitative RT-PCR were chosen such that they annealed only to the *Sry* transcript in the embryonic male gonad and not to the circular transcript. It was difficult to assay for *Sry* expression when using the mixture of oligo(dT) primers in the RT step (data not shown), probably due to the presence of a 3400 nt long untranslated region at the 3' end and the low abundance of the *Sry* transcript. For this reason, a *Sry* specific 3' primer was used for RT.

Sry expression was detected in male embryonic gonad at 11.5 dpc, which was the first time point examined, and decreased at 12.5 dpc, consistent with previous results from RNase protection assays (Fig. 5E; Hacker *et al.*, 1995). *Sry* is probably expressed in pre-Sertoli cells since these cells are the first male specific cells to differentiate in the fetal testis. In experiments with XX-XY chimeric mice, the Sertoli cell population is the only cell lineage which is almost exclusively composed of XY cells, suggesting a direct action of *Sry* in Sertoli cells (Palmer and Burgoyne, 1991a; Patek *et al.*, 1991). The onset of *3βHSD I* expression was later than that of *Sry*, which agrees with the hypothesis that Leydig cell differentiation is a consequence of Sertoli cell differentiation.

The semi-quantitative RT-PCR method presented here offers an easy way of looking at the expression pattern of genes which are homologous to isolated, differentially displayed cDNAs. When the amount of RNA needed has been titrated, this semi-quantitative RT-PCR assay is easier to perform than RNase protection assay which needs highly labeled RNA probes and more total RNA as starting material. With this semi-quantitative RT-PCR assay it is possible to run one RT reaction with 500 ng total RNA and then to determine the expression pattern of about ten different genes.

cDNA 4.3 is a useful marker for steroid producing cells in embryonic testis and adrenal gland

Little is known about the expression of *3βHSD I* during early testis and ovary development in the mouse. In order to investigate the localization of *3βHSD I* expression in fetal gonads and to confirm the results from the semi-quantitative RT-PCR, cDNA 4.3 was used as an antisense probe in whole-mount *in situ* hybridizations with urogenital tracts from 13.0- and 14.0-day-old embryos. No *3βHSD I* expression was detected in the gonads from female embryos or in female or male mesonephros (Fig. 6A,B,D). In contrast, the fetal adrenal glands of both sexes and the fetal testis showed strong expression. In adrenal glands, *3βHSD I* was expressed in the cortex region, which is the major site for steroid synthesis (Fig. 6G,H). In 13.0 dpc old testis, the expression is patchy and mainly concentrated in the middle and upper part of the gonad, less expression is found in the lower part (Fig. 6D). Twenty four hours later, *3βHSD I* expression is detected in the whole testis and restricted to horizontal stripes of cells (Fig. 6E). Sections of the whole-mount *in situ* 14.0 dpc testis showed that the striated appearance corresponded with expression between testis cords, probably in the Leydig cells where testosterone is produced (Fig. 6F). In summary, the whole-mount *in situ* hybridizations showed that cDNA 4.3 is a useful marker for the differentiation and organization of Leydig cells during testis development. *3βHSD I* is expressed in the male but not the female gonad at 13.5 dpc, which is consistent with results from the mRNA differential display and semi-quantitative RT-PCR. Little is known about early Leydig cell migration and differentiation and it will be interesting to analyze the three-dimensional organization of Leydig cells during testis cord formation. This could be done using confocal microscopy with cDNA 4.3 as a DIG-labeled anti-sense probe and a fluorescing antibody towards DIG.

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References

ABBASZADE, I.G., CLARKE, T.R., PARK, C.H. and PAYNE, A.H. (1995). The mouse 3 beta-hydroxysteroid dehydrogenase multigene family includes two functionally distinct groups of proteins. *Mol. Endocrinol. 9:* 1214-1222.

ADATI, N., ITO, T., KOGA, C., KITO, K., SAKAKI, Y. and SHIOKAWA, K. (1995). Differential display analysis of gene expression in developing embryos of *Xenopus laevis*. *Biochim. Biophys. Acta 1262:* 43-51.

BAE, D.S., SCHAEFER, M.L., PARTAN, B.W. and MUGLIA, L. (1996). Characterization of the mouse Dax-1 gene reveals evolutionary conservation of a unique amino-terminal motif and widespread expression in mouse tissue. *Endocrinology 137:* 3921-3927.

BAIN, P.A., YOO, M., CLARKE, T., HAMMOND, S.H. and PAYNE, A.H. (1991). Multiple forms of mouse 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase and differential expression in gonads, adrenal glands, liver, and kidneys of both sexes. *Proc. Natl. Acad. Sci. USA 88:* 8870-8874.

BARDONI, B., ZANARIA, E., GUIOLI, S., FLORIDIA, G., WORLEY, K.C., TONINI, G., FERRANTE, E., CHIUMELLO, G., McCABE, E.R.B., FRACCARO, M., ZUFFARDI, O. and CAMERINO, G. (1994). A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. *Nature Genet. 7:* 497-501.

BAUER, D., MÜLLER, H., REICH, J., RIEDEL, H., AHRENKIEL, V., WARTHOE, P. and STRAUSS, M. (1993). Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Res. 21:* 4272-4280.

BLANCHARD, R.K. and COUSINS, R.J. (1996). Differential display of intestinal mRNAs regulated by dietary zinc. *Proc. Natl. Acad. Sci. USA 93:* 6863-6868.

CALLARD, D., LESCURE, B. and MAZZOLINI, L. (1994). A method for the elimination of false positives generated by the mRNA differential display technique. *Biotechniques 16:* 1096-1097.

CAPEL, B., SWAIN, A., NICOLIS, S., HACKER, A., WALTER, M., KOOPMAN, P., GOODFELLOW, P. and LOVELL-BADGE, R. (1993). Circular transcripts of the testis-determining gene *Sry* in adult mouse testis. *Cell 73:* 1019-1030.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation

by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162:* 156-159.

CLARKE, T.R., BAIN, P.A., GRECO, T.L. and PAYNE, A.H. (1993). A novel mouse kidney 3β-hydroxysteroid dehydrogenase complementary DNA encodes a 3-ketosteroid reductase instead of 3β-hydroxysteroid dehydrogenase/delta5-delta4-isomerase. *Mol. Endocrinol. 7:* 1569-1578.

CONWAY, G. (1995). A novel gene expressed during zebrafish gastrulation identified by differential RNA display. *Mech. Dev. 52:* 383-391.

DASILVA, S.M., HACKER, A., HARLEY, V., GOODFELLOW, P., SWAIN, A. and LOVELL-BADGE, R. (1996). Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nature Genet. 14:* 62-68.

DAVIS Jr, W., De SOUSA, P.A. and SCHULTZ, R.M. (1996). Transient expression of translation initiation factor eIF-4C during the 2-cell stage of the preimplantation mouse embryo: identification by mRNA differential display and the role of DNA replication in zygotic gene activation. *Dev. Biol. 174:* 190-201.

FOSTER, J.W., DOMINGUEZ-STEGLICH, M.A., GUIOLI, S., KWOK, G., WELLER, P.A., STEVANOVIC, M., WEISSENBACH, J., MANSOUR, S., YOUNG, I.D., GOODFELLOW, P.N., BROOK, J.D. and SCHAFER, A.J. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature 372:* 525-530.

GOODFELLOW, P.N. and LOVELL-BADGE, R. (1993). SRY and sex determination in mammals. *Annu. Rev. Genet. 27:* 71-92.

GUBBAY, J., COLLIGNON, J., KOOPMAN, P., CAPEL, B., ECONOMOU, A., MÜNSTERBERG, A., VIVIAN, N., GOODFELLOW, P. and LOVELL-BADGE, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature 346:* 245-250.

GUIMARAES, M.J., BAZAN, J.F., ZLOTNIK, A., WILES, M.V., GRIMALDI, J.C., LEE, F. and McCLANAHAN, T. (1995). A new approach to the study of haematopoietic development in the yolk sac and embryoid bodies. *Development 121:* 3335-3346.

HACKER, A., CAPEL, B., GOODFELLOW, P. and LOVELL-BADGE, R. (1995). Expression of *Sry*, the mouse sex determining gene. *Development 121:* 1603-1614.

IKEDA, Y., SWAIN, A., WEBER, T., HENTGES, K., ZANARIA, E., LALLI, E., TAMAI, K., SASSONE-CORSI, P., LOVELL-BADGE, R., CAMERINO, G. and PARKER, K. (1996). Steroidogenic Factor 1 and Dax-1 colocalize in multiple cell lineages: Potential links in endocrine development. *Mol. Endocrinol. 10:* 1261-1272.

IKONOMOV, O.C. and JACOB, M.H. (1996). Differential display protocol with selected primers that preferentially isolates mRNA of moderate- to low-abundance in a microscopic system. *Biotechniques 20:* 1030-1042.

JUNEAU, C., DUPONT, E., LUU-THE, V., LABRIE, F. and PELLETIER, G. (1993). Ontogenesis of 3 beta-hydroxysteroid dehydrogenase delta 5-delta 4 isomerase in the rat ovary as studied by immunocytochemistry and *in situ* hybridization. *Biol. Reprod. 48:* 226-34.

KENT, J., WHEATLEY, S.C. ANDREWS, J.E., SINCLAIR, A.H. and KOOPMAN, P. (1996). A male-specific role for *Sox9* in vertebrate sex determination. *Development 122:* 2813-2822.

KOOPMAN, P. (1995). The molecular biology of SRY and its role in sex determination in mammals. *Reprod. Fertil. Dev. 7:* 713-722.

KOOPMAN, P., GUBBAY, J., VIVIAN, N., GOODFELLOW, P. and LOVELL-BADGE, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature 351:* 117-121.

KREIDBERG, J.A., SARIOLA, H., LORING, J.M., MAEDA, M., PELLETIER, J., HOUSMAN, D. and JAENISCH, R. (1993). WT-1 is required for early kidney development. *Cell 74:* 679-691.

LEE, C.H. and TAKETO, T. (1994). Normal onset, but prolonged expression, of *Sry* gene in the B6.Y^{DOM} sex-reversed mouse gonad. *Dev. Biol. 165:* 442-452.

LI, F., BARNATHAN, E.S. and KARIKO, K. (1994). Rapid method for screening and cloning cDNAs generated in differential mRNA display: application of northern blot for affinity capturing of cDNAs. *Nucleic Acids Res. 22:* 1764-1765.

LIANG, P. and PARDEE, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science 257:* 967-971.

LIANG, P. and PARDEE, A.B. (1995). Recent advances in differential display. *Curr. Opin. Immunol. 7:* 274-280.

LIANG, P., AVERBOUKH, L. and PARDEE, A.B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Res. 21:* 3269-3275.

LIU, C.M. and RAGHOTHAMA, K.G. (1996). Practical method for cloning cDNAs generated in an mRNA differential display. *Biotechniques 20:* 576-579.

LUO, X.R., IKEDA, Y.Y. and PARKER, K.L. (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell 77:* 481-490.

MASON, C., LAKE, M., NEBREDA, A. and OLD, R. (1996). A novel map kinase phosphatase is localised in the branchial arch region and tail tip of *Xenopus* embryos and is inducible by retinoic acid. *Mech. Dev. 55:* 133-144.

MATHIEU-DAUDE, F., CHENG, R., WELSH, J. and McCLELLAND, M. (1996). Screening of differentially amplified cDNA products from RNA arbitrarily primed PCR fingerprints using single strand conformation polymorphism (SSCP) gels. *Nucleic Acids Res. 24:* 1504-1507.

McCLELLAND, M., MATHIEU-DAUDE, F. and WELSH, J. (1995). RNA fingerprinting and differential display using arbitrarily primed PCR. *Trends Genet. 11:* 242-246.

McCLELLAND, M., RALPH, D., CHENG, R. and WELSH, J. (1994). Interactions among regulators of RNA abundance characterized using RNA fingerprinting by arbitrarily primed PCR. *Nucleic Acids Res. 22:* 4419-4431.

McELREAVEY, K., BARBAUX, S., ION, A. and FELLOUS, M. (1995). The genetic basis of murine and human sex determination: a review. *Heredity 75:* 599-611.

MOU, L., MILLER, H., LI, J., WANG, E. and CHALIFOUR, L. (1994). Improvements to the differential display method for gene analysis. *Biochem. Biophys. Res. Commun. 199:* 564-569.

NORDQVIST, K. (1995). Sex differentiation – gonadogenesis and novel genes. *Int. J. Dev. Biol. 39:* 727-736.

PALMER, S.J. and BURGOYNE, P.S. (1991a). *In situ* analysis of fetal, prepuberal and adult XX-XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. *Development 112:* 265-268.

PALMER, S.J. and BURGOYNE, P.S. (1991b). The Mus musculus domesticus Tdy allele acts later than the Mus musculus musculus Tdy allele: a basis for XY sex-reversal in C57BL/6-Y^{POS} mice. *Development 113:* 709-714.

PATEK, C.E., KERR, J.B., GOSDEN, R.G., JONES, K.W., HARDY, K., MUGGLETON-HARRIS, A.L., HANDYSIDE, A.H., WHITTINGHAM, D.G. and HOOPER, M.L. (1991). Sex, chimeras, fertility and sex determination in the mouse. *Development 113:* 311-325.

PIKO, L. and CLEGG, K.B. (1982). Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. *Dev. Biol. 89:* 362-378.

RAMKISSOON, Y. and GOODFELLOW, P. (1996). Early steps in mammalian sex determination. *Curr. Opin. Genet. Dev. 6:* 316-321.

REINERS Jr, J.J. and BUSCH, H. (1980). Transcriptional and post-transcriptional modulation of cytoplasmic ribonucleic acids in regenerating liver and Novikoff hepatoma. *Biochemistry 19:* 833-841.

SCHAFER, A.J. (1995). Sex determination and its pathology in man. *Adv. Genet. 33:* 275-329.

SINCLAIR, A.H., BERTA, P., PALMER, M.S., HAWKINS, J.R., GRIFFITHS, B.L., SMITH, M.J., FOSTER, J.W., FRISCHAUF, A.M., LOVELL-BADGE, R. and GOODFELLOW, P.N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature 346:* 240-244.

SWAIN, A., ZANARIA, E., HACKER, A., LOVELL-BADGE, R. and CAMERINO, G. (1996). Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nature Genet. 12:* 404-409.

VOGELI-LANGE, R., BÜRCKERT, N., BOLLER, T. and WIEMKEN, A. (1996). Rapid selection and classification of positive clones generated by mRNA differential display. *Nucleic Acids Res. 24:* 1385-1386.

WAGNER, T., WIRTH, J., MEYER, J., ZABEL, B., HELD, M., ZIMMER, J., PASANTES, J., BRICARELLI, F.D., KEUTEL, J., HUSTERT, E., *et al.* (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9*. *Cell 79:* 1111-1120.

WILKINSON, D.G. (Ed.) (1992). Whole mount *in situ* hybridisation of vertebrate embryos. In *In situ Hybridisation: A Practical Approach*. IRL Press, Oxford, pp. 75-83.

WONG, K.K. and McCLELLAND, M. (1994). Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA. *Proc. Natl. Acad. Sci. USA 91:* 639-643.

ZANARIA, E., MUSCATELLI, F., BARDONI, B., STROM, T.M., GUIOLI, S., GUO, W., LALLI, E., MOSER, C., WALKER, A.P., McCABE, E.R., *et al.* (1994). An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature 372:* 635-641.

ZHANG, H., ZHANG, R. and LIANG, P. (1996). Differential screening of gene expression difference enriched by differential display. *Nucleic Acids Res. 24:* 2454-2455.

ZHAO, S., OOI, S.L., YANG, F.C. and PARDEE, A.B. (1996). Three methods for identification of true positive cloned cDNA fragment in differential display. *Biotechniques 20:* 400-404.

ZIMMERMANN, J.W. and SCHULTZ, R.M. (1994). Analysis of gene expression in the preimplantation mouse embryo: use of mRNA differential display. *Proc. Natl. Acad. Sci. USA 91:* 5456-5460.

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