ANALYSIS OF GENE EXPRESSION IN THE DEVELOPING OVARY AND TESTIS OF THE CHICK EMBRYO.

Lynn HAINES, Derek McBRIDE, Michael CLINTON
Division of Development and Reproduction, Roslin Institute, Roslin (Edinburgh), Midlothian EH25 9PS. Scotland.

In mammals, sex depends upon the inheritance of a heteromorphic pair of sex chromosomes, X and Y, with the dominant Y chromosome in males leading to the formation of testes. However, this mechanism of sex determination can not operate in all vertebrates, for example, in most avian species the female is the heterogametic sex (ZW) and the male the homogametic sex (ZZ). At present, it is unclear whether ovary development is the result of a dominant W chromosome or that the gonads develop under a Z chromosome dosage mechanism (1). The gonadal primordia, the genital ridges, appear in the chick embryo at day 4 of development and superficial morphological differences are apparent between males and females by day 8. Sex determination is thought to occur around day 6 of development (fig 1) (2).

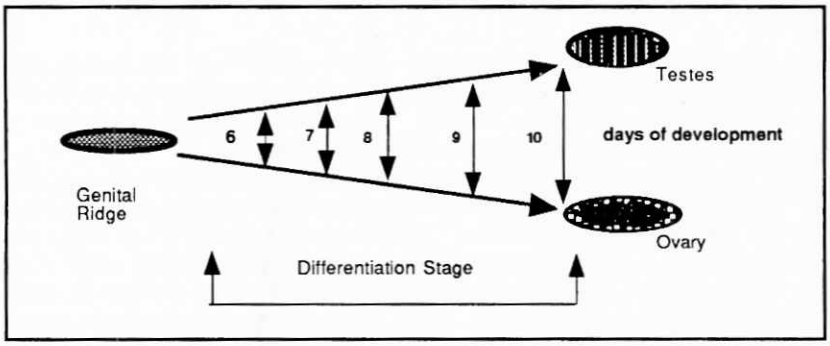

Fig 1. Diagram representing the initial developmental period in the chick gonad.

Gene expression was studied from day 4 to day 10 of incubation, to isolate genes involved in ovarian and testicular differentiation, in addition to genes involved in the sex determination process. The technique utilised to study gene expression in the developing chick gonad is differential display (fig 2). Basically, RNA is extracted from the tissue of interest. Reverse transcription is carried out, using modified oligo dT primers targeted against the poly A tail. This cDNA is amplified by a low stringency PCR reaction, that uses the original oligo dT primer in combination with a primer of arbitrary sequence. The PCR products are visualised as bands on an acrylamide gel (3).

Methods.

Genital ridges were dissected from day 4 to day 10 chick embryos. W-PCR was carried out to sex the embryos, by amplifying a repeat on the W-chromosome (female specific) (4). RNA was extracted from male genital ridges and female genital ridges at different stages of development. 5µg of RNA, from different developmental stages, was used to make cDNA by reverse transcription using a modified oligo dT primer which targeted the poly A tail (Pharmacia Biotech First Strand cDNA Synthesis kit). Differential display PCR was performed on a fraction of the resulting cDNA, using the same oligo dT primer in combination with nine primers of arbitrary sequence, and in the presence of ^{35}S-dATP (fig. 3). PCR products were separated on a native acrylamide gel and visualised by autoradiography. Bands were excised from the gel, re-amplified using the same primer pairs as in the original amplification. The DNA from each candidate band was cloned and sequenced (Sequenase Version 2.0, Amersham International). These sequences were compared to nucleotide and protein databases (BLAST analysis package) (5).

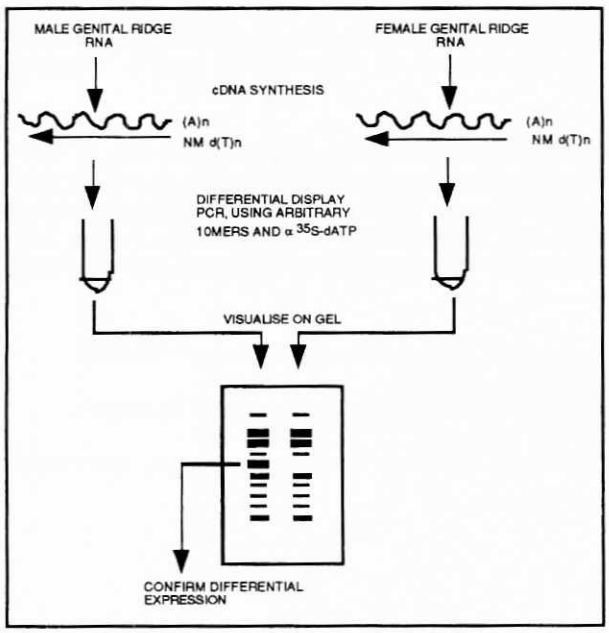

Fig. 2 Diagram of Differential Display.

Northern analysis was carried out by electrophoresing 10µg genital ridge RNA from males and females for each developmental day being studied, and RNA was transferred by capillary action onto a nylon membrane (Hybond-N, Amersham International). The RNA was fixed by baking the membrane at 80°C for two hours. The blot was probed with radiolabelled DNA insert (^{32}P- dCTP) obtained from the display gel. Hybridisation was carried out at 60°C overnight. RNA dot blots were performed by dotting 4µg genital ridge RNA, from males and females from each developmental day being studied, onto a nylon membrane. Fixation of RNA and hybridisation was carried out as for Northern analysis.

Results and Discussion.

Ten candidate clones have been excised from the display gels. They have been re-amplified, sub-cloned and sequenced. Five of these clones gave significant homology to genes in the databases. These were: clone 1 - RhoA, a GTP-binding protein involved in regulation of the actin cytoskeleton and changes in cell morphology and growth (6 and 7); clone 3 - rho-globin, an embryonic form of B-globin; clone 6 - *mef-2*, a tissue specific transcription factor so far

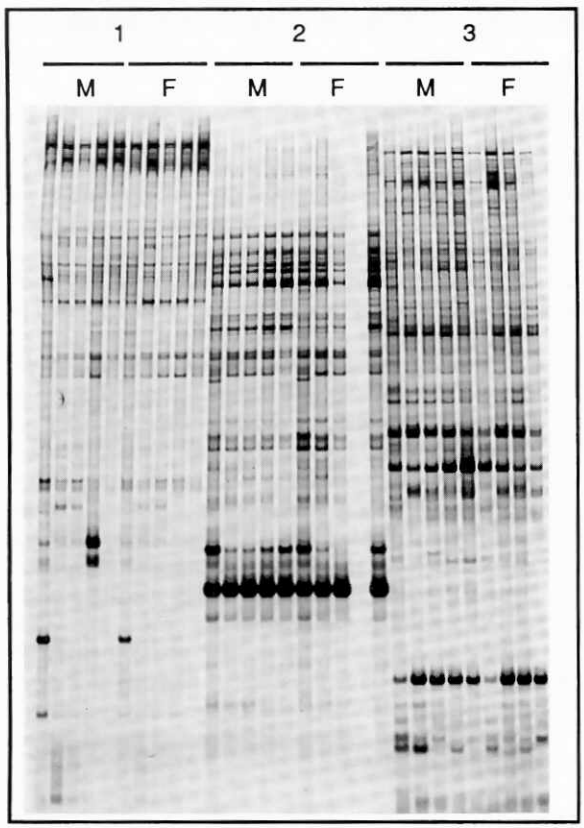

Fig 3. Example of a display gel. M = male samples from day4, 5, 6, 8 and 9 of incubation. F = female samples from day 4, 5, 6, 8 and 9 of incubation. 1, 2 and 3 represent the three different primer combinations.

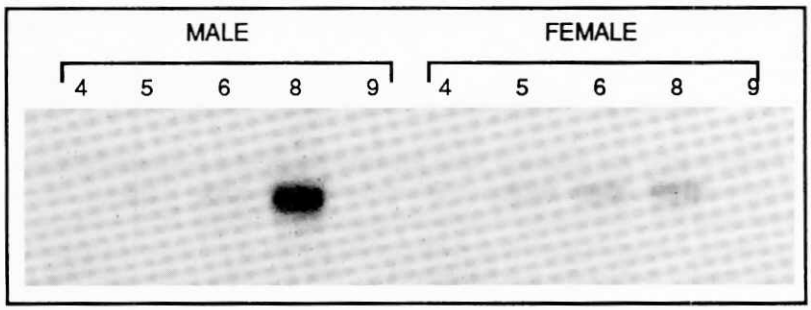

Fig 4. Northern analysis of a transcript isolated from a differential display gel. 4, 5, 6, 8 and 9 indicate days of incubation from either male of female genital ridge RNA.

only identified in muscle; clone 8 - TBP-1, an ATPase that forms part of the 26S proteasome complex; clone 9 - chaperonin t-complex protein-1, which assists in protein folding. The remaining five clones gave no significant homology to sequences in the databases.

We have begun to perform differential expression confirmation studies. To date we have confirmed differential expression for three of the clones by Northern analysis. Clone 1 (91% homology to RhoA at amino acid level) gave a stage specific signal by Northern analysis that matched the display pattern, with higher levels present in day 4 male and female genital different gonad development. Clone 2 shows a sexually dimorphic pattern of expression, replicating the pattern on the display gel, with higher levels of expression in day 8 male genital ridge. Clone 3 has higher levels of expression in day 5 males and females, matching the display expression pattern. This expression data corresponds with the well documented data that rho-globin is expressed in primitive erythrocytes, by day 5 of development primitive begin to be replaced with definitive erythroctytes that do not express rho-globin (8).

Transcripts # 6 and # 7 were also analysed by Northern hybridisation, however no signals were obtained. A number of the transcripts we excise from the gel will be of low abundance and not detectable by Northern analysis. Alternative methods such as ribonuclease protection assays will have to be performed to confirm differential expression.

Conclusions.

We have shown that differential display is an effective method to isolate differentially expressed developmental genes. This is borne out by clone 3 (100% sequence homology to rho-globin). The banding pattern on the display gel matches that by Northern analysis, which concurs with well documented expression data of this embryonic gene. Of the remaining two clones that have been confirmed, one (clone 2) has a sexually dimorphic pattern of expression suggesting a possible function in the testis differentiation pathway. The other clone (clone 1) is a GTP-binding protein that may have a function at the indifferent gonad stage of development.

References.

1. Halverson, J. and Dvorak, J. (1993). Genetic control of sex determination in birds and the potential for its manipulation. Poultry Science. 72:890-896.
2. Venzke, D. (1954). The morphogenesis of the indifferent gonad of chicken embryos. Am. J. Vet. Res. 15:300-307.
3. Clinton, M. (1994). A rapid protcol for sexing chick embryos (*Gallus g. domesticus*). Animal Genetics. 25:361-362.
4. Liang, P. and Pardee, A. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science. 257:967-971.
5. Altschul, S., Gish, W., Miller, W., Meyers, E. and Lipman, D. (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-410.
6. Vojtek, A. and Cooper, J. (1995). Rho family members: activators of MAP kinase cascades. Cell. 82:527-529.
7. Drgonova, J., Drgon, T., Tanaka, K., Koller,R., Chen, G-C., Ford, R., Chan, C., Takai, Y. and Cabib, E. (1996). Rho 1P, a yeast protein at the interface between cell polarization and morphogenesis. Science. 272:277-279.
8. Bruns, G. and Ingram, V. (1973). Erythropoesis in the chick embryo. Phil. Trans. R. Soc. Lon. B. 266:225-305.