Sexually dimorphic expression of protease nexin-1 and vanin-1 in the developing mouse gonad prior to overt differentiation suggests a role in mammalian sexual development

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The mammalian sex-determining pathway is controlled by the presence or absence of SRY expression in the embryonic gonad. Expression of SRY in males is believed to initiate a pathway of gene expression resulting in testis development. In the absence of SRY, ovary development ensues. Several genes have now been placed in this pathway but our understanding of it is far from complete and several functional classes of protein appear to be absent. Sex-determining genes frequently exhibit sexually dimorphic patterns of expression in the developing gonad both before and after overt differentiation of the testis or ovary. In order to identify additional sex-determining or gonadal differentiation genes we have examined gene expression in the developing gonads of the mouse using cDNA microarrays constructed from a normalized urogenital ridge library. We screened for genes exhibiting sexually dimorphic patterns of expression in the gonad at 12.5 and 13.5 days post-coitum, after overt gonad differentiation, by comparing complex cDNA probes derived from male and female gonadal tissue at these stages on microarrays. Using in situ hybridization analysis we show here that two genes identified by this screen, protease nexin-1 (Pn-1) and vanin-1 (Vnn1), exhibit male-specific expression prior to overt gonadal differentiation and are detected in the somatic portion of the developing gonad, suggesting a possible direct link to the testis-determining pathway for both genes.

INTRODUCTION

The mammalian gonads, the testis and ovary, are derived from the sexually dimorphic development of an initially indifferent (or bipotential) gonadal primordium, the genital ridge. Genetic and developmental studies have revealed that the fate of the developing genital ridge is under the control of the Y-linked sex-determining gene SRY (1,2). In the mouse, Sry transcription in the male (XY) genital ridge begins at 10.5 days post-coitum (d.p.c.), reaching a peak at 11.5 d.p.c., prior to overt differentiation, before becoming silenced by 13.0 d.p.c. (3,4). It is likely that expression of SRY in the supporting cell lineage of the bipotential gonad is responsible for the commitment of those cells to the Sertoli cell (i.e. testicular) fate. In the absence of SRY expression, the indifferent gonad becomes committed to the ovarian fate. Although much evidence exists that the DNA-binding protein SRY acts as an architectural transcription factor (5), the nature of the cascade of putative transcriptional events initiated by it remains undetermined.

Since the identification of SRY as the testis-determining gene, several additional genes have been isolated with a role in sex determination, i.e. loci whose mutation or inappropriate expression is associated with some degree of sex reversal in humans and/or mice. These include SOX9 (6–8), WNT4 (9), DAX1 (10,11), WT1 (12) and SF1 (13,14). Several other genes expressed by testicular Sertoli cells have been implicated in fetal gonad differentiation and function, including the anti-Müllerian hormone (AMH) (15), the hedgehog family member DHH (16), GATA-4 (17) and DMRT1 (18). Analyses of the expression of all these genes have demonstrated that they are transcribed in a sexually dimorphic fashion in the somatic lineages of the developing gonad. In the case of sex-determining genes such as SRY and SOX9 this sexual dimorphism in expression precedes overt gonad differentiation. However, sex-determining genes often exhibit a sexually dimorphic pattern of expression well after overt testis and ovary differentiation, as in the case of SOX9, DAX1 and WNT4. Of the genes discussed above only WNT4, DHH and AMH are not transcription factors. Thus, it is likely that the known sex-determining genes under-represent the different functional
classes of proteins required for the development of the mammalian gonads. Attempts have been made to identify additional sex-determining genes based on expression assays. Differential display reverse transcriptase–polymerase chain reaction (RT–PCR) on male and female fetal gonadal RNA identified the male-specific (Y-linked) gene *Uty* (19) and the male-specific gene *testatin* (20). However, it is clear that gaps still exist in our understanding of the sex-determining pathway and this situation can only be improved by the identification of all the genes that play a role in this developmental process.

Here we describe an attempt to identify additional sex-determining and/or gonad differentiation genes in the mouse utilizing cDNA microarrays. Our strategy was to identify genes expressed in a sexually dimorphic fashion in the developing mouse gonad at 12.5 and 13.5 d.p.c. At these developmental stages it was possible to collect routinely enough gonadal tissue to perform repeat microarray experiments. Based on the expression profiles of known sex-determining genes discussed above, we reasoned that molecules identified at 12.5 and 13.5 d.p.c. on the basis of sexually dimorphic expression in the gonads would sometimes exhibit the same sexual dimorphism at earlier gonadal stages, pre-dating overt gonad differentiation. This class of gene provides a potential link to the genetic pathway of sex determination. Thus, our aim was to analyze in more detail the expression of any molecules identified in our microarray screen exhibiting a sexually dimorphic expression pattern at 12.5 and 13.5 d.p.c. Recent advances in cDNA microarray technology have provided the means to perform rapid and sensitive studies of gene expression on large numbers of genes printed robotically onto glass slides (21–23). Whilst cDNA microarrays have been utilized primarily for expression profiling in lower eukaryotes (23), we describe here their application to a mammalian developmental model.

RESULTS

Generation and sample sequencing of a normalized mouse urogenital ridge (NMUR) cDNA library

As a screen for sex-determining/gonad differentiation genes we aimed to identify transcripts exhibiting sexually dimorphic patterns of expression in the developing gonads of female and male mice utilizing cDNA microarrays. In order to generate a set of cDNA elements for microarraying that would be the most relevant to our biological system, we prepared a normalized cDNA library from 11.5 and 12.5 d.p.c. NMUR (see Materials and Methods). Equal amounts of male and female RNA were used in the generation of this library to ensure good representation of genes expressed from both developing gonads. The library was subjected to two rounds of normalization (24) in order to minimize redundancy of clones selected as elements on our microarrays. Sample sequencing of the 5′ end of 2086 NMUR cDNA clones by the IMAGE Consortium/WashU EST sequencing program showed the sample to contain 1024 UniGenes and 907 novel (unclassified) clones. Thus, NMUR represents a low redundancy clone-set of transcripts found in the developing mouse gonad and mesonephros.

Figure 1. Examination of transcription in the developing mouse UGR using cDNA microarrays. (a) Close-up of a region of an array showing results for several control genes after hybridization with fluorescently labelled female (Cy5/green) and male (Cy3/red) target cDNA from 12.5 d.p.c. UGRs. The control genes are: X1, *Sry*; X2, *Oct4*; X3, 17α-hydroxylase; Y1, *Sox9*; Y2, *Wnt4*; Y3, β-HSD; Z1, *Dax1*; Z2, Dhh; and Z3, *Gata4*. (b) A scatter plot (logarithmic scale) depicting the relative intensities of the Cy5 (y-axis) and Cy3 (x-axis) signals for each cDNA on a 1152 element microarray hybridized with the targets described in (a). Those genes which showed a consistent sexual dimorphism in expression in this series of experiments are numbered. The gene *Pn-1* is expressed in a male-enhanced fashion, similar to the control genes described in (a). For the purpose of clarity, pre-labelled positive control spots (‘landing lights’) have been removed from the scatterplots shown here. (c) Scatter plot showing intensities of Cy5 (y-axis) Cy3 (x-axis) signals for a microarray with 2352 elements hybridized with female and male 13.5 d.p.c. gonadal target RNA. Both *Pn-1* and *Vmn1* exhibit male-enhanced expression.
Two series of microarray experiments were performed. The first series used 1152 NMUR cDNA clones. PCR products from these clones were printed onto glass slides (see Materials and Methods) and hybridized with fluorescently labelled total cDNA derived from male and female gonad–mesonephros pairs dissected from 12.5 d.p.c. embryos. All elements were printed onto each slide in duplicate and all hybridizations were performed in duplicate. It was important to analyse the behaviour of control genes on these arrays, particularly those known to be expressed in a sexually dimorphic fashion at 12.5 d.p.c., since we were searching for genes showing a similarly clear male–female dimorphism. Figure 1 shows a close-up of a section of the microarray including the control genes Sox9, Wnt4, 3β-HSD, 17α-OH, Dhh and Oct4. A variety of assays has shown that Sox9, 3β-HSD, 17α-OH and Dhh are expressed in a male-specific fashion in the gonad at 12.5 d.p.c. (7–9,16). The microarray data consistently support these observations. Wnt4 is expressed prominently in the developing ovary at 12.5 d.p.c. but not in the developing testis (9). These observations are also supported by the microarray data. As expected, the primordial germ cell marker, Oct4, reliably shows no significant expression differences between males and females. Figure 1b represents the microarray data as a scatter plot, showing male versus female hybridization intensities (Cy5/Cy3 ratios) for each element. In our screen, we sought molecules that exhibited a similar profile of expression to the control genes, which consistently showed at least a 3-fold greater expression in either males or females. Only one additional gene, the serine protease inhibitor protease-nexin 1 (Pn-1) (also known as glia-derived nexin 1) (Fig. 1b) fulfilled these criteria in this first series of experiments.

The second series of experiments was performed on microarrays printed with an expanded set (2352) of clones. These were hybridized with fluorescently labelled total cDNA derived from sub-dissected male and female gonads (minus the mesonephros) extracted from 13.5 d.p.c. embryos. Female- and male-enhanced control genes included on these microarrays gave the expected signals, as did Pn-1 (Fig. 1c), indicating that the sexual dimorphism observed at 12.5 d.p.c. was gonadal in origin, not mesonephric. Two additional genes also showed a consistent male bias in the 13.5 d.p.c. gonad, vanin-1 (Vnn1) and Gli2 (Fig. 1c). The Gli2 gene was present on the smaller microarrays employed in the first series of experiments but no significant sexual dimorphism was observed so this gene was not studied further. Vnn1 was not present on the smaller microarrays so it was examined further by wholemount in situ hybridization.

Microarray analysis

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Analysis of Pn-1 and Vnn1 gonadal NMUR expression by in situ hybridization

Figure 2 shows a developmental series of male and female gonads ranging from 11.5 to 13.5 d.p.c., revealing the spatio-temporal profile of Pn-1 expression. Pn-1 transcription is restricted to the male gonad at all stages and precedes overt differentiation of the testis. At 13.5 and 12.5 d.p.c. expression is prominent within the testis cords (Fig. 2a and b). At 11.5 d.p.c. expression is restricted to the male gonad (Fig. 2c and d). Expression is first observed at the 15 tail-somite stage, equivalent to ~11–11.25 d.p.c. (Fig. 2e). If Pn-1 is linked directly to the testis determination pathway one would predict expression in the Sertoli cell lineage of the developing testis. Sectioning of the 13.5 d.p.c. testis revealed expression throughout the testis cord, with highest levels at the periphery where Sertoli cells abut the basement membrane (Fig. 2f). In contrast, the Sertoli cell marker Sox9 is expressed almost exclusively at the periphery of the testis cord (Fig. 2g). The expression of Pn-1 in the somatic component of the testis cord was confirmed by examining its transcription in developing testes dissected from embryos homozygous for the extreme allele of dominant spotting (W/We) at 12.5 d.p.c. Homozygous W/We gonads lack germ cells (25), as shown by the absence of the germ cell-specific marker Oct4. However, Pn-1 transcripts are detected in W/We gonads demonstrating that its expression is not germ cell dependent.

The expression of Vnn1 is shown in Figure 3. As expected from the microarray data, expression is prominent in the testis at 13.5 d.p.c., with no expression in the ovary at the same stage (Fig. 3a). This sexual dimorphism is also observed at 12.5 d.p.c. (Fig. 3b). At both stages expression is localized to the testis cords. The earliest point at which expression is observed in the developing male gonad is at the 26 tail-somite stage, immediately prior to overt testis differentiation (Fig. 3c). This corresponds to ~12.0–12.25 d.p.c. Sectioning of the testis at 13.5 d.p.c. reveals a concentration of signal in the region of the Sertoli cells at the periphery of the cord (Fig. 3d). Little or no expression is visible in primordial germ cells. Vnn1 expression is also observed in developing testes from 12.5 d.p.c. W/We
embryos, confirming its expression in the somatic cell lineage of the testis cords (Fig. 4).

**DISCUSSION**

The use of cDNA- or oligonucleotide-based microarrays to examine transcription in mammals has, in the main, been restricted to RNA extracted from specific cell types or cell lines (26–29). Here we have shown that this technology is applicable to whole developing organs. In the context of using complex tissues for expression profiling, it is interesting to note that the expression of both genes detected by this screen is cell-type restricted. Thus, the contribution of particular cell types to a microarray expression profile can be readily discerned and concerns about background noise from other cell types obscuring such signals seem to be unfounded, consistent with other published observations (30). The methodologies employed here are applicable to other developmental systems where tissue sources are comparably restricted. Whilst suitable normalized cDNA libraries may not be available for all such systems, this problem will soon be circumvented by the provision of large minimal (non-redundant) sets of genes. The advantages of a microarray approach over other differential screening procedures include the possibility of analysing multiple developmental time-points on a number of genetic backgrounds.

The screen described here has allowed the identification of two potentially important gonadogenesis genes from a limited number of clones using moderate amounts of starting total RNA. Both of the genes identified, Vnn1 and Pn-1, are expressed in a sexually dimorphic fashion both after and before overt gonad differentiation and each is detected in the Sertoli (supporting) cell lineage of the testis. Each one encodes a protein that will contribute to our understanding of the early molecular events in gonadogenesis. Neither are transcription factors, unlike most known sex-determining genes, therefore these new candidate genes will facilitate the investigation of other cellular processes in testis development. The chromosomal locations of PN-1 (2q33–35 and MMU 1) and VNN1 (6q23–24 and MMU 10) are not associated with reports of abnormalities in sexual development in humans or mice.

Vnn1 (vascular non-inflammatory molecule-1) is a glycosylphosphatidylinositol (GPI)-anchored cell surface molecule involved in the homing of bone marrow cells to the thymus (31). Antibody against vanin-1 blocks thymus colonization by haematopoietic progenitor cells and interferes with lympho-stromal cell adhesion. It is believed that vanin-1, which is expressed in thymic epithelial cells, regulates late adhesion steps of thymus homing under physiological, non-inflammatory conditions. A closely related molecule found in humans is also known to regulate β1 integrin-mediated cell adhesion, in vitro transendothelial migration and motility of neutrophils (32).

We describe here the expression of Vnn1 in an epithelial cell type of the developing testis, the Sertoli cell. A role in the regulation of cell migration might exist for vanin-1 in this developmental context. It is known that several somatic gonadal cell types have their origin in either the mesonephros or coelomic epithelium (33,34). In the case of three cell types (peritubular myoid cells, vascular endothelial cells and myoepithelial cells) this migration from the mesonephros occurs before 11.5 d.p.c. in a male-specific fashion as an indirect consequence of Sry expression (33). It has been suggested that migrating peritubular myoid cell precursors might drive testis cord formation via interaction with cells within the gonad (35). The close physical association between peritubular myoid cells and Sertoli cells suggests that it is likely that some interaction between these two cell types is required for normal cord formation. Given the expression of Vnn1 in the Sertoli cell lineage just prior to cord formation and its role in regulating cell homing in other contexts, it is possible that vanin-1 regulates Sertoli cell association with (and adhesion to) migrating peritubular myoid cells. However, other Sertoli cell functions at this stage cannot be excluded, including those related to germ cell development. Further functional studies will be required to address the role of vanin-1 in gonadogenesis, including the possibility that its transcription is regulated by SRY, SOX9 or one of the other testis-determining transcription factors.

Protease nixin-1 (PN-1) is a serine protease inhibitor that can modulate the proteolytic activity of thrombin, tPA, uPA and trypsin. It is widely expressed in the embryo, most prominently in the developing nervous system (36). Here we report the male-specific expression of Pn-1 in the developing mouse gonad from as early as 11.25 d.p.c., over 24 h before overt testis differentiation. Whilst it is not possible to determine the cell-type specificity of Pn-1 expression at this early stage, it is expressed in the somatic component of the testis cord (the Sertoli cells) at both 12.5 and 13.5 d.p.c. The promoter region of the rat PN-1 gene contains a binding site for the protein encoded by the Wilms tumour-associated gene (WT1) (37). The WT1 gene is expressed in Sertoli cells and has been implicated in male genital abnormalities in Denys–Drash syndrome patients (12). It is not known if this WT1 binding site is conserved in the mouse Pn-1 promoter or whether it is required for gonad-specific expression of Pn-1. However, the location (Sertoli cells) and timing of onset (11.25 d.p.c.) of Pn-1 expression in the developing male gonad make it a candidate for regulation by any of the known male-determining transcription factors.

The onset of expression of Pn-1 in the developing male gonad at least 24 h prior to overt testis differentiation suggests an important role for PN-1 in the early events of testis determination and/or differentiation. It has been postulated that during the development of the mesencephalon and myelencephalon, regulation of proteolysis by PN-1 may serve to preserve the location of cells before migration or differentiation (36). The structural basis of testis cord formation is the deposition of a basal lamina surrounding the Sertoli cells of the cord. In vitro experiments have shown that peritubular myoid cells and Sertoli cells cooperate to produce this basal lamina (38). A protease inhibitor such as PN-1, secreted by pre-Sertoli/Sertoli cells, could act to maintain the integrity of the basal lamina during the earliest stages of cord formation and through subsequent testis development.

It should now be possible to use RNA amplification techniques (39) to profile gonadal transcription on larger microarrays with increased sensitivity at earlier time-points, including 10.5 and 11.5 d.p.c. In addition, using NMUR it will be possible to examine expression in the developing mesonephros, another organ with a sexually dimorphic fate in the developing embryo. These experiments are currently ongoing.
**MATERIALS AND METHODS**

**NMUR cDNA library**

A normalized cDNA library was prepared from urogenital ridges (UGRs) dissected from 11.5 and 12.5 d.p.c. embryos. Each UGR consists of a gonad and attached mesonephros, and each embryo contains a pair of UGRs. Approximately 150 pairs of UGRs were dissected from 11.5 d.p.c. embryos. At this stage embryonic sex was determined by staining the amnion for the presence of Barr bodies (40).Poly(A)^+ RNA was extracted from the sexed pools of tissue (Micro mRNA Purification Kit, Amersham Pharmacia, Little Chalfont, UK) and equal proportions of male and female RNA were combined. At 12.5 d.p.c. sexing of embryos was possible by the presence or absence of testis cords. RNA from 11.5 d.p.c. UGR material was then combined with that from 12.5 d.p.c. dissections in a 3:1 ratio. A second round of poly(A)^+ purification was performed prior to library synthesis. The cDNA library was constructed and normalized according to previously published procedures (24).

Samples of the library (NMUR) were supplied to the IMAGE/WashU sequencing programme for sample sequencing. Following transformation, 3072 NMUR colonies were picked and arrayed into 384-well plates. At the time of publication, 2086 of these clones have been partially sequenced (see mouse UniGene entry Library 144 at http://www.ncbi.nlm.nih.gov/UniGene/browse.cgi?ORG=Mm ). A copy of these primary plates was obtained and used to generate cDNA products (elements) for the microarray experiments. Additional NMUR clones were prepared in-house by transformation and colony picking. A panel of 12 control genes (Sry, Sox9, Wnt4, Dhh, Oct4, Gata4, Pax2, Pax8, Dax1, Amh, 17α-hydroxylase, 3β-HSD) was also collected for inclusion on the microarrays. These control genes are known to be expressed during urogenital development, some in a sexually dimorphic fashion.

**Production of cDNA microarrays**

Plasmid DNA was prepared for all NMUR and control clones. Approximately 10 ng of plasmid DNA was used as a template in a 100 µl PCR using 5 U Tag polymerase (AB Technologies, Epsom, UK) and 30 pmol of universal vector primers (M13F: 5′-GTGTTTCCAGTCCGAC-3′, M13R 5′-ACAGGAAC-AGCTATAGC-3′). Cycling conditions were: 1× 94°C for 3 min and 35 cycles of 94°C for 45 s, 48°C for 20 s and 72°C for 3 min. Products were analysed by gel electrophoresis to determine the success and quality of the PCR. Each product was then precipitated with iso-propanol and resuspended in 20 µl of 4× SSC, 0.2% sarkosyl. DNA samples were robotically arrayed in duplicate onto poly-l-lysine-coated slides using a Genetic MicroSystems 417 microarrayer (GRI, Braintree, UK). Poly-l-lysine slides were prepared and submitted to post-array fixation using published methods (22).

**RNA target preparation and hybridization**

Total RNA was purified using the Qiagen RNeasy RNA mini preparation kit (Crawley, UK). RNA was labelled by the incorporation of Cy3- or Cy5-labelled nucleotides into first-strand cDNA using reverse transcriptase. Total RNA (50 µg) was mixed with 4 µg of oligo(dT)~15 in a volume of 25 µl and heated to 70°C for 10 min. The mixture was then cooled to room temperature and the following reagents added: 10 µl of 5X first strand synthesis buffer, 5 µl 0.1 M DTT, 1 µl low-C dNTP mix (25 mM dATP, dGTP, dTTP), 2 µl of 2.5 mM dCTP, 4 µl 1 mM Cy3- or Cy5-dCTP (Amersham Pharmacia), 1.5 µl (3 U) RNAguard (Pharmacia) and 2 µl (2 U) of reverse transcriptase (SuperScript II, Life Technologies, Paisley, UK). In all experiments, male-derived RNA was labelled with Cy3-dCTP and female-derived RNA was labelled with Cy5-dCTP.

After cDNA synthesis, the RNA was removed by alkaline hydrolysis. The reaction mix was then neutralized and purified by ethanol precipitation. The labelled cDNA was resuspended in 50 µl of hybridization solution [6x SSC, 0.5% SDS, 50% formamide, 0.25 µg/µl Cot1 DNA, 0.4 µg/µl poly(dA)] and was denatured by heating to 80°C for 10 min. It was then cooled to 42°C for 1 h. The hybridization solution was then applied to the microarray, covered with a coverslip and placed into a hybridization chamber (Telechem, Sunnyvale, CA) overnight at 42°C. After hybridization, the arrays were washed in 0.5 1 of 0.2× SSC/0.5% SDS for 2 min followed by 0.5 1 of 0.2× SSC for a further 2 min.

Cy3 and Cy5 hybridization signals were collected using a GMS 418 dual colour laser array scanner and data interpreted using the Imagine 2.0 software (GRI). The signals were normalized by comparing the expression of the entire population of cDNA elements such that the median ratio of Cy3 to Cy5 signal was 1.0. Experiments were performed in duplicate using independently prepared RNA preparations. Each array element was spotted in duplicate so that four data points were obtained for each gene.

**Embryo collection and wholemount in situ hybridization**

Timed matings of female (C3H) and male (101) breeding pairs were used to generate staged mouse embryos. Pairs were set up at ~3 p.m. and vaginal plugs were checked the following morning. Noon on the day of the plug was counted as 0.5 d.p.c. Accurate staging was performed by counting tail somites posterior to the hindlimb bud. For wholemount in situ analysis embryonic urogenital organs were dissected into sterile 1× phosphate buffered saline (PBS), 0.2% bovine serum albumin and fixed in 4% paraformaldehyde overnight. NMUR cDNA clones (Wnt1, accession no. AI115468, and Pn-1, accession no. AI115537) were linearized with EcoRI and an anti-sense digoxigenin-labelled probe generated using T7 RNA polymerase. Wholomount in situ hybridization was performed according to published methods (7). Embryonic material was photographed first on agarose in PBS and then in 100% glycerol under coverslips using a photomicroscope (Nikon, Kingston, UK) with Fuji 64T film. Embryonic material was frozen in OCT compound and sectioned at 14-µm thickness on a cryostat (Leica, Milton Keynes, UK). Sections were then mounted under coverslips in Aquamount (BDH, Lutterworth, UK). Brightfield photographs were taken using an Axioshot (Zeiss, Herts, UK) with a digital camera.

**RT–PCR**

RT–PCR was performed using the Superscript 1-Step RT–PCR kit (Life Technologies). Pn-1 primers were, 5′-TGAAGATGGGAAC-CAAGCTT-3′ and 5′-GAAATCTTCTTACAGATCG-3′;
Oct4 primers were 5′-CTCAGCCTTAAAGACATGTT-3′ and 5′-TTCCTCTGTGACCTCCCTT-3′; vanin-1 primers were 5′-TGGGGATTGGTCTGCTTCA-3′ and 5′-GTAATCT- TATCCTAAGTTG-3′. W4 heterozygous females and males were intercrossed to generate W/W embryos. Embryos were collected at 12.5 d.p.c. and male gonads were dissected away from the attached mesonephros for RNA extraction. The rest of the embryo was used for Southern analysis in order to identify W/W homozygotes. DNA was restricted with BglII and Southern blots were probed with a radiolabelled c-kit cDNA clone (1).

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REFERENCES


