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A Genomic Clone of *Zfy-1* from a Y^{DOM} Mouse Strain Detects Post-Meiotic Gene Expression of *Zfy* in Testes

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In order to obtain a genomic clone of *Zfy-1* from a Y chromosome of Mus musculus domesticus (Y^{DOM}) origin, we cloned size-fractionated SJL/J DNA in EMBL-4 and selected colonies which hybridized to pDP1007, a human zinc finger Y clone. The specificity of the clone in hybridizations to mouse and human DNA and partial sequencing confirmed that the clone (subcloned as pGZfy1D) was of *Zfy-1* origin. Studies on the expression during testicular development of mRNAs hybridizing to the clone suggested that the gene is expressed post-meiotically.

Recently, David Page and his colleagues have isolated a 1.3 kb piece of genomic DNA (cloned as pDP1007) from the short arm of the human Y chromosome which is highly conserved in all male placental mammals but absent in females of the same species (1). The sequence is located within interval 1A2 (about 140 kb long or 0.2% of the Y chromosome) and is present in most XX males and missing in some XY females. The 1.3 kb fragment appears to be an open reading frame for a gene encoding 13 Cys-Cys/His-His zinc fingers. A similar structure was first described in frog transcription factor IIIA of *Xenopus* (2). This human zinc finger protein may prove to be the putative testis determining factor (TDF) which, by binding in a sequence specific manner to either DNA or RNA, would regulate transcription and so induce the bipotential fetal gonads in a male embryo to develop as testes.

In the majority of placental mammals tested there is only one copy of TDF found on the Y chromosome (called *ZFY* for zinc finger on the Y chromosome) and only one copy on the X chromosome (*ZFX*) (1). However, in mice, there are two male specific or Y linked copies (3,4). Both copies are found within the sex determining region on the short arm of the murine Y chromosome. The two copies are distinguished as *Zfy-1* and *Zfy-2*; *Zfy-2* also encodes a zinc finger

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protein (5). Transcripts hybridizing to *Zfy-1* and *Zfy-2* were detected in adult mouse testis RNA, suggesting that both sequences are transcribed in this tissue (5). In sex reversed mice (*Sxr*), sterile XX, *Sxr* "males" have both *Zfy-1* and *Zfy-2* sequences detectable by Southern analysis (3,4). XX, *Sxr'* male mice, however, have only *Zfy-1*. In mice, therefore, it seems that only *Zfy-1* is a good candidate for the sex determining gene.

In the two subspecies of mice, *Mus musculus domesticus* and *Mus musculus musculus*, the Y chromosomes are genetically distinct and are distinguished as Y^{DOM} and Y^{MUS} respectively (6). There are differences between the two Y chromosomes: the mating of an F1 male Y^{DOM} mouse (from a male Y^{DOM} X female inbred C57BL/6J cross) with a female C57BL/6J (this inbred stain has Y^{MUS}) mouse results in sex reversed XY offspring that develop either as females with two ovaries or as hermaphrodites. This sex reversal must be Y linked because only Y^{DOM} males pass it on to their offspring. It is postulated that a difference between *Tdy* (the murine homologue of TDF) on Y^{DOM} and *Tdy* on Y^{MUS} does not permit Y^{DOM} to interact properly with certain homozygous autosomal loci in the inbred C57BL/6 strain, thus causing sex reversed offspring.

The fact that *Zfy-1* may be sufficient to induce maleness in mice led us to attempt to clone and characterize this sequence. We chose to do this from a strain with Y^{DOM} in order to later be able to compare it to *Zfy-1* from Y^{MUS} to ascertain if it is possible that the Y^{DOM} versus Y^{MUS} effect detected in the C57BL/6J strain is inherent in *Zfy-1*. Northern analyses suggest that the sequence is expressed late during spermatogenesis.

MATERIALS AND METHODS

Molecular cloning of *Zfy-1*: A mouse genomic library was constructed from *Eco*RI arms of the EMBL-4 (7) derivative of lambda phage (Stratagene), using *Eco*RI digested 10-12 kb size-selected SJL/J male mouse DNA extracted from liver. Size-selected DNA was isolated from low melt agarose (SeaPlaque GTG) after 3 days of electrophoresis at 50V (0.6% 250mL gel). The library was screened using the human genomic insert of plasmid pDP1007 labelled using the radiolabelling technique of Feinberg and Vogelstein (8) with ^{32}P dCTP (Amersham). Filters were washed at room temperature for 20 min in 2XSSC, 1%SDS and then at 65C for 15 min intervals to a final concentration of 0.5XSSC, 1%SDS.

Isolation of *Zfy-1* insert and molecular subcloning as pGZfy1D: Positive plaques detected by hybridization with pDP1007 were picked and suspended in 1 mL SM buffer and 75uL chloroform. Phage DNA was isolated by a modified plate lysate method (9). The final DNA isolation was from 100 ul 1% low melt agarose blocks (10). The *Zfy-1* insert was recovered from pure phage DNA by *Eco*RI digestion to give an 11.2 kb fragment. The 2 kb subfragment from a *Hind*III digestion of the 11.2 kb *Zfy-1* insert was isolated from low melt agarose after gel electrophoresis. The vector, a pGEM4 derivative with a mouse metallothionine promoter inserted, was linearized with *Hind*III, dephosphorylated, and ligated with T4 ligase at 4C for 2 days. JM109 cells were made competent by the $CaCl_2$ method. Transformed cells were plated on LB agar with 75 ug/mL of ampicillin.

Southern analyses: AKR/J genomic DNA was digested with EcoRI, electrophoresed and transferred to nylon by the standard procedure. Probes pDP1007 and the 2 kb *Hind*III subfragment of pGZfy1D were labeled by oligopriming (8) and hybridized in 0.5 M Tris HCl, pH7.5; 25 mM EDTA; 2.5 ug/ml heparin; 0.5% sodium pyrophosphate; and 2.5% sarcosyl. Posthybridization washes were performed in 0.04 M Na_2HPO_4, pH7.2; 0.5% SDS, 1 mM EDTA for 20 min. at R.T. and then @ 65° C for 15 min. intervals to a final concentration of 0.25 X posthybridization wash solution.

Genomic PCR sequences analysis of *Zfy-1*: The 1 kb fragment flanked by primers derived from the human TDF region sequence (sense: TAT CCT TGC ATG ATT TGT GG, positions 111-130 of pDP1007; antisense: GGC ATC TGT AGT GCT ATA CT, positions 1096-1115 of pDP1007) was enzymatically amplified using the 2 kb *Hind*III subfragment of the 11.2 kb *Zfy-1 Eco*RI fragment as a template. Standard conditions of PCR were used: 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min in a reaction buffer containing 50 mM KCl, 10mM Tris HCl pH8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 units of Taq polymerase (Perkin Elmer Cetus), each dNTP at 200 uM and each primer at 1 uM per 100 uL reaction mixture. When the reaction was completed, the whole reaction mixture was run on a 1% low melting point agarose gel (SeaPlaque GTG) in TBE buffer in the presence of 0.0002% ethidium bromide. The band corresponding to the specific product was excised from the gel. DNA was isolated from the agarose, precipitated and used for reamplification of the 1 kb fragment. The final pure PCR product was dissolved in a small volume of water. The purified DNA was then sequenced directly using the dideoxytermination method and Sequenase (U.S. Biochem. Corp.) and reagents as provided in the Sequenase T7 DNA Polymerase kit. The U.S. Biochem. Corp. procedure for sequencing with PCR was used. The samples were electrophoresed in a 6.0% polyacrylamide, 7M urea sequencing gel, fixed, dried and exposed to X-ray film (Kodak XAR-5) for 2-7 days.

Northern analysis: Total RNA was prepared from decapsulated testes of random bred, CD1 mice of defined age by the guanidinium-isothiocyanate procedure (11). Preparations of about 90% pure spermatocytes and similarly pure spermatids were prepared from adult testes as previously described (12). Poly A^+ RNA was prepared from total RNA using one pass over oligo(dT) cellulose (13). RNA was denatured and resolved on 1% agarose-formaldehyde gels (13) or denatured with glyoxal and dimethylsulfoxide and resolved on 1% agarose gels. After transfer to nylon supports hybridization with oligo-primed ^{32}P-labelled probes was as described for Southern analysis.

Hybridization of Northerns with oligonucleotide probes: Antisense 17-mer oligodeoxynucleotides were made specific for *Zfy-1* and *Zfy-2* from the published sequences of *Zfy-1* and reported base changes in *Zfy-2* (5). The sequence TAG GAG TGG CGA CAC TG provided the antisense to positions 2255-2271 of *Zfy-2* and TAG GAG TGT CGA CAC TG to *Zfy-1* (since *Zfy-2* has an 18 bp deletion compared to *Zfy-1* the numbering is not the same, ref. 14). These probes were end-labeled with gamma-^{32}P-ATP and T4 polynucleotide kinase (13). Hybridization was in 5X SSPE, 0.1%SDS at 47C. The filters were washed extensively in 6X SSC at 0C and for 1 min at 47C.

RESULTS AND DISCUSSION

The mouse *Zfy-1* sequence was cloned from a Y^{DOM} size-selected male mouse genomic library and retrieved with the human Y chromosomal probe pDP1007. The sequence obtained was shown to correspond to *Zfy* by Southern analysis. Under identical stringency washes, hybridization with pDP1007 and pGZfy1D to an *Eco*RI digested mouse filter give variant band patterns (Fig. 1). The human Y

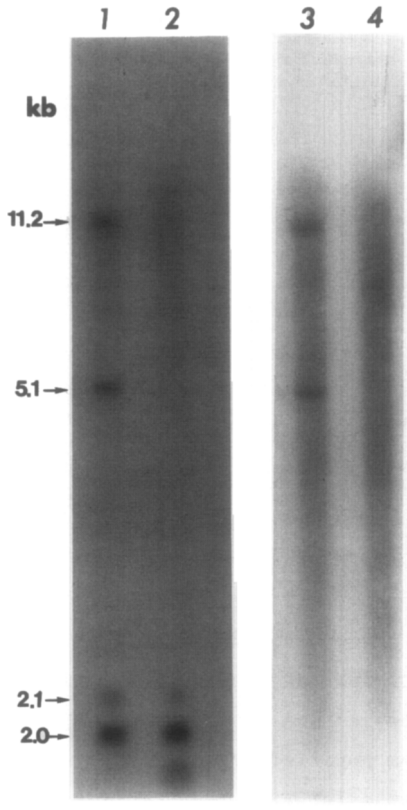

Figure 1. Hybridization of probes pDP1007 and pGZfy1D to EcoRI digested AKR/J mouse DNA. Lane 1: male, pDP1007; lane 2: female, pDP1007, lane 3: male, pGZfy1D; lane 4: female, pGZfy1D.

chromosomal sequence pDP1007 shows homology to four mouse sequences: the male specific 11.2 kb *Zfy-1* and 5.5 kb *Zfy-2*, and the male/female shared 2.3 kb *Zfx* and 2.0 kb *Zfa* (zinc finger protein on autosome 10). Mouse *Zfy-1* subclone pGZfy1D, however, hybridizes only to the male specific sequences of *Zfy-1* and *Zfy-2*.

To further identify pGZfy1D as *Zfy-1*, partial polymerase chain reaction (PCR) sequencing was performed. The first 228 base pairs sequenced from the 5' primer were identical to positions 1410-1637 of a published *Zfy-1* cDNA (15, subspecies of mice not indicated).

In order to determine when during testicular development *Zfy* related sequences are expressed, we first studied a development curve to detect the time of appearance of sequences in male mice of various postnatal ages. This approach allows one to determine at which stage of germ cell development transcripts first appear since only very early spermatocytes are present one week postnatally while mature spermatocytes are present at two weeks and spermatids first make their appearance at three weeks of age. With 20 ug of total RNA from mice in these stages, only very faint hybridization to a 3.3 kb transcript was seen in adult testis, the stage when all of the above mentioned

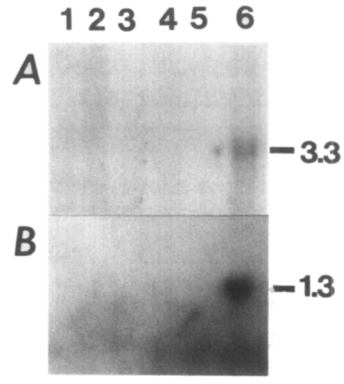

Figure 2. Northern analysis of testicular RNAs with pGZfy1D (A) and pPM459 (LDH-X, B). Lane 1: 7 days testes, total RNA; lane 2: 14 day testes total RNA; lane 3: 21 day testes, total RNA; lane 4: adult testes, total RNA, lane 5: purified spermatocytes, poly A^+ RNA; lane 6: purified spermatids, poly A^+ RNA. Sizes indicated in kb.

spermatogenic cells are present abundantly. Thus, we separated cells from mature testis in order to further elucidate the time of appearance of this transcript. Purified poly A^+ RNA from purified spermatocytes does not give a signal while poly A^+ from purified spermatids gives a clear hybridization signal at the 3.3 kb transcript (Fig. 2). This pattern is identical to the pattern found with the probe for LDH-X, a mRNA known to be synthesized commencing at pachytene and to have continued synthesis during the spermatid stage (16). We used synthetic oligonucleotides to try and determine if the transcript selected was due to *Zfy-1* or to *Zfy-2*. We find a very faint hybridization signal at the 3.3 kb transcript size with the *Zfy-1* oligonucleotide but not with the *Zfy-2* oligodeoxynucleotide (data not shown). The results, therefore, suggest that the postmeiotic, 3.3 kb transcript is from *Zfy-1*.

Our results do not elucidate a possible role of *Zfy-1* in sex determination. It has been controversial whether or not *Zfy* is expressed in gonadal ridges although expression of a ZFY cDNA in human fetal testes has been observed (17). *Zfy-2* expression in adult mouse testes has been reported (5) but an analysis of the time of expression has not been performed. We are intrigued to find that *Zfy* joins a large number of putative DNA-binding proteins that are expressed post-meiotically. For years it was a genetic maxim that genes were not expressed after meiosis (reviewed in 18) but this maxim was disproved when new messages were found to appear after meiosis (19). In fact, an analysis of spermatogenesis revealed that, of messages detected by 2D gel analysis of in vitro translation mixtures, there were nearly twice as many that first appeared after meiosis as were specific to spermatocytes (20). It has now become clear that a number of DNA-binding and other proteins are

first expressed in testes at or near the time of meiosis. The DNA binding proteins include ones containing homeoboxes (21), nuclear located c-oncogenes such as cfos (22), and, of course, protamines (23). It is interesting that another zinc finger protein (of unidentified function) is also expressed at this time (24). The roles of these many meiotically and post-meiotically expressed proteins in spermatogenesis are yet to be determined.

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