POLYMERASE CHAIN REACTION (PCR)-DIFFERENTIAL DISPLAY IDENTIFIES A SUBSET OF STAGE-DEPENDENT AND CADMIUM-REGULATED GENES DURING SPERMATOGENESIS IN *SQUALUS ACANTHIAS*

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The increasing incidence of male reproductive disorders in humans and wildlife has been ascribed to accumulation of hormonally active agents (HAAs) in the environment, but clear cause-and-effect relationships remain to be established. A program of research in this laboratory has identified the spiny dogfish shark (*Squalus acanthias*) as an advantageous model for determining which stages of development, physiological processes, and genes are targeted by known or suspected spermatotoxicants (Callard, G.V., *et al.*, In: Boekelheide, K., *et al.*, eds., Comprehensive Toxicology, Vol. 10, Section 1, Elsevier, NY. pp. 235-247, 1997). In previous studies using this model, we reported that cadmium (Cd), an established mammalian spermatotoxicant, is taken up and retained preferentially in stem cell and spermatogonial stages *in vivo*, where it increases the percentage of germinal clones undergoing apoptosis (Betka, M., *et al.*, *Biol. Reprod.* 60:147-157, 1999). In these early stages, as compared to later in development, a higher percentage of the retained Cd is associated with cell nuclear subfractions, indicative of nuclear-located binding sites. One mechanism by which Cd can disrupt cellular processes is by altering gene transcription via activation of metal receptor binding to specific DNA motifs termed metal response elements in the regulatory regions of target genes (Carvan M.J., *et al.*, *Arch. Biochem. Biophys.* 376:320-327, 2000). A second possibility is that Cd mimics or blocks normal estrogen regulated gene transcription by activating nuclear estrogen receptors that, in turn, interact with estrogen response elements (Stoica A., *et al.*, *Mol. Endocrinol.* 14:545-543, 2000). Interestingly, the majority of estrogen receptor binding activity (Callard, G.V., *et al.*, *Endocrinology* 117:1328-1335, 1985) and the highest amount of estrogen receptor mRNA (Sikora R. and Callard, G., unpublished data) are found in premeiotic regions of shark testis.

To identify Cd-sensitive genes, the polymerase chain reaction-differential display (PCR-DD) method of mRNA fingerprinting was applied (GenHunter, Nashville TN). An important advantage of PCR-DD is that it allows simultaneous recognition of up- and down-regulated mRNAs as a function of treatment and/or developmental stage. Poly (A+) RNA was prepared from dissected testicular tissues in defined stages: germinal zone (GZ, stem cells - early spermatogonia), premeiotic (PrM, mid- to late-spermatogonia); meiotic (M, spermatocytes – early spermatids); and postmeiotic (PoM, elongating – mature spermatids) and reverse transcribed by standard methods. Five primer sets were used to obtain a total of 49 stage-dependent and 39 Cd-responsive bands. The intensity of one band (termed G1-3) varied by stage (PrM >> GZ; undetectable, M and PoM) and was approximately 5- to 10-fold greater in GZ and PrM (but not other) stages of animals that received a single injection of $CdCl_2$ (5 mg/kg body weight) 3 d earlier as compared to matched controls (not shown). Cloning and sequence analysis identified the excised G1-3 band as a ~400 kb fragment of the control region of the mitochondrial (mt) genome previously reported in GenBank (accession #18134; Rasmussen, A.S., and Arnason, U., *J. Mol. Evol.* 48:118-123, 1999; Fig. 1). Northern analysis using the isolated DNA as a probe revealed 6 hybridizing bands, of which an abundant 1.5 kb mRNA and

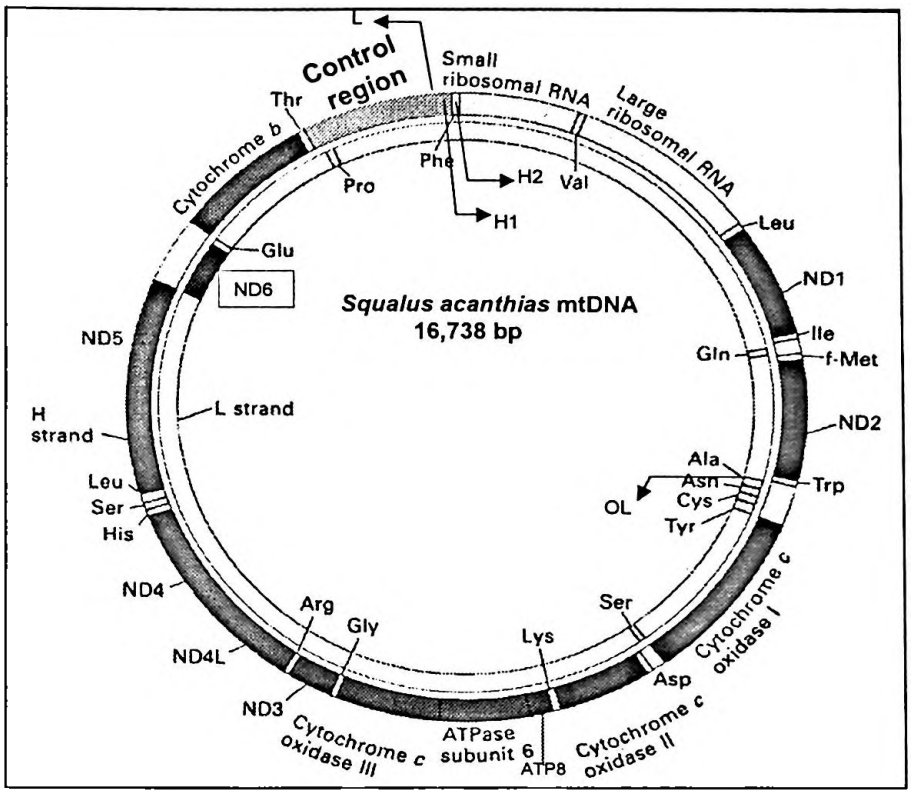

Figure 1. The organization of mtDNA in the shark *Squalus acanthias*. The isolated DNA sequence (G1-3) was located within the control region on the H-strand (medium grey). Also shown are protein-encoding sequences on each of the two strands (black), including cytochrome oxidase subunits I, II, and III, sequences encoding ribosomal RNAs (light grey) and transfer RNAs (clear), and transcription start sites off the H-strand (arrows H1, H2 = clockwise). The heavy strand encodes a polycistronic primary RNA (II) that includes sequences of 11 species of mRNA with the G1-3 containing control region at the 3'-terminus. It is processed by endonucleases to yield mRNAs that are subsequently polyadenylated. Regulation is known to be exerted at the level of transcription initiation, transcript II processing, polyadenylation, and mRNA stability. Adapted from Darnell *et al.* (Molecular Cell Biology, W.H. Freeman, NY. pp. 928-930, 1986) with shark-specific information from Rasmussen & Arnason (*op cit.*).

a lower abundance 2.5 kb mRNA showed the same stage- and Cd-dependence as PCR-DD (not shown). The remaining bands were stage-dependent but unaffected by Cd. 5'-RACE using a primer specific for the isolated G1-3 DNA yielded a 1.0 kb sequence with Thr-transfer RNA immediately upstream of the control region. We infer from these results that the multiple bands seen on Northern blots are different sized 3'-end fragments of the partially processed transcript II (refer to legend, Fig. 1). RT-PCR analysis of GZ, PrM, M and PoM stages of control and Cd-treated animals using gene-specific primer sets for cytochrome oxidase subunits (Cox I, II and III) showed all three mRNAs were greater in GZ of Cd-treated than untreated controls but the

fold-difference and stage-related differences in band intensity were not as predicted by PCR-DD and Northern analysis (not shown). We interpret this to mean that PCR-DD and Northern analysis of primary and partially processed transcripts containing the control region (G1-3) provide an estimate of ongoing transcriptional activity on the H strand of mtDNA, whereas RT-PCR estimates steady state levels of specific mRNAs which are dependent not only on ongoing rates of transcription but also on multiple posttranscriptional regulatory processes (refer to legend, Fig. 1), and on the amount of Cox-specific mRNAs remaining from synthesis in all preceding developmental stages.

The selectivity of Cd's actions on mtDNA expression in GZ and PrM stages implies a functional relationship to the Cd action pathway rather than a general toxicant-induced increase in energy demand on the mitochondrial genes. Of the twelve proteins encoded by mtDNA, the cytochrome oxidase subunits are especially interesting due to their involvement in caspase activation leading to apoptosis (Kluck, R.M., *et al.*, *J. Cell Biol.* 147:809-822, 1999), and their reported stage-dependence and androgen responsiveness in rodent testis (Ku C.Y., *et al.*, *Mol Endocrinol* 5:1669-1676, 1991; Saunders, P.T., *et al.*, *Biol. Reprod.* 48:57-67, 1993). Although further analysis of mRNAs found by PCR-DD to differ in Cd-treated and untreated animals is in progress, initial results demonstrate the feasibility of PCR-DD for identification of developmentally programmed and toxicant sensitive genes during spermatogenesis in the shark testis model. A goal of continuing research is to identify molecular markers of spermatotoxicant effect with utility for identifying chemical exposures *in vitro* and *in vivo*, for extrapolation to other species including man, and as an entry point to elucidate downstream intratesticular action pathways. Supported by NIEHS P42 ES07381 and EPA R825434.