Discovery of a spermatogenesis stage-specific ornithine decarboxylase antizyme: Antizyme 3

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Communicated by Raymond L. White, University of Utah, Salt Lake City, UT, February 8, 2000 (received for review November 10, 1999)

Previous studies with mice overproducing ornithine decarboxylase have demonstrated the importance of polyamine homeostasis for normal mammalian spermatogenesis. The present study introduces a likely key player in the maintenance of proper polyamine homeostasis during spermatogenesis. Antizyme 3 is a paralog of mammalian ornithine decarboxylase antizymes. Like its previously described counterparts, antizymes 1 and 2, it inhibits ornithine decarboxylase, which catalyzes the synthesis of putrescine. Earlier work has shown that the coding sequences for antizymes 1 and 2 are in two different, partially overlapping reading frames. Ribosomes translate the first reading frame, and just before the stop codon for that frame, they shift to the second reading frame to synthesize a trans-frame product. The efficiency of this frameshifting depends on polyamine concentration, creating an autoregulatory circuit. Antizyme 3 cDNA has the same arrangement of reading frames and a potential shift site with definite, although limited, homology to its evolutionarily distant antizyme 1 and 2 counterparts. In contrast to antizymes 1 and 2, which are widely expressed throughout the body, antizyme 3 transcription is restricted to testis germ cells. Expression starts early in spermiogenesis and finishes in the late spermatid phase. The potential significance of antizyme 3 expression during spermatogenesis is discussed in this paper.

From its initial isolation as a mysterious substance in 1678, it gradually became widely believed that spermine was uniquely present in semen, although as early as 1878 it was found in many mammalian tissues (1). Spermine is synthesized from spermidine, for which the precursor is putrescine, which in turn comes from decarboxylation of ornithine (2). In mammals, ornithine decarboxylase (ODC; EC 4.1.1.17) (see reviews in refs. 3 and 4) is in very low abundance (0.0003 to 0.01% of cellular protein; ref. 5) and, with a half-life of 10–20 min, is one of the most short-lived mammalian enzymes (6). The originally characterized antizyme (later termed antizyme 1) binds to ODC, inactivates it (see reviews in refs. 7 and 8), and, in a catalytic manner, targets it for degradation by the 26S proteosome without ubiquitination (9, 10). The half-life of ODC complexed with antizyme 1 is <5 min (11). Antizyme 1 also inhibits the transporter required for the uptake of extracellular polyamines (12, 13). The progression from the sequencing of a partial cDNA for antizyme 1 (14) to a complete cDNA sequence (15), together with earlier studies (16), led to the interesting finding by Matsufuji and colleagues (see review in ref. 17) that the coding sequence is in two different reading frames with ribosomes required to shift reading frames to synthesize functional antizyme 1 (15, 18). The efficiency of ribosomal frameshifting is responsive to polyamine levels, and frameshifting is the sensor for an autoregulatory circuit. The structure (19) and mapping (20) of the antizyme 1 gene have been described (21), and studies on its transcriptional control are in progress. A protein that binds to antizyme 1 with a greater affinity than ODC and that also inhibits antizyme 1 may function to stabilize ODC by trapping antizyme 1 (22, 23), but further work is required to ascertain the protein's biological role.

Recently, a second mammalian antizyme, antizyme 2, has been identified (24), and the structure of its encoding gene (25) and properties (26) have been studied. Like antizyme 1, its expression requires ribosomal frameshifting at the last codon of its first ORF (24). Antizyme 2 mRNA is 16-fold less abundant than that of antizyme 1 (24). Both antizymes 1 and 2 mRNAs are present in the numerous mammalian tissues tested (24). In this paper, we show that there is a third mammalian antizyme, but that its mRNA is highly restricted to testicular germ cells.

In transgenic mice expressing human ODC, Halmekytö and colleagues (27–29) found that ODC activity was grossly elevated in nearly all tissues examined, but only in testis and brain was the level of putrescine higher than in nontransgenic littermates. The concentration of putrescine in testes of transgenic males was 5 to 20 times higher, and in brain, 3 times higher than that found in controls, with just a 2-fold increase in the half-life of transgene-derived ODC in testes as compared with that of the corresponding mouse enzyme (30). We considered that the elevated levels of putrescine in testes, and to a lesser extent in brain, might be caused by a less permeable blood/tissue barrier than that found in other tissues. In addition, transgenic males had reduced fertility or were infertile (27). They showed varying levels of resemblance to one of the two forms of the "Sertoli cell only" syndrome of infertile men (27). In this form of human infertility, nursing Sertoli cells are intact, but germinal epithelium and maturing sperm cells are greatly reduced (31, 32). Apart from the testis and just one of the male accessory sexual organs, the preputial glands, all other tissues examined appeared histologically normal.

A specific dependence of type B spermatogonia on putrescine (33) and the deleterious effects of high levels of testicular putrescine may make the control of testicular ODC a fertile ground for investigation of spermatogenesis.

Materials and Methods

Northern Blots and *in Situ* Hybridization. Northern blots containing poly(A)$^+$ RNA from 16 adult human tissues were purchased from CLONTECH. A human antizyme 3 fragment including the entire coding region was PCR amplified. A radiolabeled probe was made from this fragment by incorporating [^{32}P]dCTP with random-primed labeling kit (Ambion, Austin, TX). Both Northern blots were probed simultaneously according to the manufacturer's specifications.

For mouse antizyme 1 and 3 *in situ* hybridization, fragments including the entire coding region were PCR amplified (with a T7 promoter as part of either the sense or the antisense primer). Digoxigenin-labeled sense and antisense probes were generated

Abbreviations: ODC, ornithine decarboxylase; GST, glutathione *S*-transferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF175297 (mouse) and AF175296 (human)].

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.070055897. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.070055897

separately by using T7 RNA polymerase and the digoxigenin RNA labeling kit (Roche Diagnostics).

Paraffin-embedded sections from adult mouse testes sections were deparaffinized by heating and by two consecutive incubations in xylene. After dehydration in a graded series of ethanol concentrations, the sections were fixed in 4% paraformaldehyde for 2 h. The sections were proteinase K-digested (6 μg/ml for 20 min at room temperature), refixed in 4% paraformaldehyde, and dehydrated. Control sections were RNase-treated (40 μg/ml at 37°C for 30 min), washed, and dehydrated. Subsequent hybridization and washing were performed by following standard protocols (34). Digoxigenin was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Dako) visualized with the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate. Sections were counterstained with Meyer's hematoxylin.

In situ probing for antizyme 1 was done in parallel with probing for antizyme 3, so that all steps in the hybridization procedure would be identical and the results could be directly comparable.

Staging (from stages I to XII) was performed according to the criteria described by Leblond and Clermont (35). Staging originally was described for guinea pig and was later confirmed for mouse spermatogenesis and spermiogenesis (35).

DNA Manipulation and Sequencing. The plasmids for glutathione *S*-transferase (GST)-antizymes 1 and 3 expression were made by two-step PCR, amplifying mouse antizymes 1 and 3 (all but the first codon of ORF1 through the downstream ORF2) in such a way as to delete the U of the UGA stop codon of ORF1. The PCR products were cloned into the *Eco*RI and *Xho*I restriction sites of the pGEX-5X-3 plasmid. All DNA clones were sequenced with automated sequencing machines (ABI 100; Applied Biosystems).

ODC Antizyme Assays. Five plates of 90% confluent MCT cells (36) were induced with fresh medium for 6 h. The plates were frozen and thawed twice with the subsequent addition of 0.5 ml of homogenizing buffer (25 mM Tris·HCl, pH 7.4/0.25 M sucrose/1 mM DTT/20 μM pyridoxal phosphate/2 mM EDTA). The cells were collected and sonicated. The sonicated lysate was centrifuged (13,000 rpm for 15 min; Sorvall SS34 rotor), and the supernatant was collected. Extracts were dialyzed overnight in dialysis buffer (25 mM Tris·HCl, pH 7.4/1 mM DTT/20 μM pyridoxal phosphate/0.1 mM EDTA). Extract (25 μl) was used for each ODC assay. ODC activity was assayed by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine (Amersham Pharmacia) as described (37). Each reaction was for 1 h. Preincubation of the mouse extract with 0.1 mM difluoromethylornithine for 15 min led to >99% inhibition of $^{14}CO_2$ release.

Results

Identification of a Third Mammalian Paralog of Antizyme. A BLAST search in the expressed sequence tag database identified several mouse and human sequences that were distinct yet similar to the previously described mouse and human antizyme gene sequences. Two cDNA clones [one from mice (GenBank accession no. AA183473) and one from humans (GenBank accession no. AA448519)] containing the most complete 5′ sequences were obtained from the IMAGE Consortium and sequenced in their entirety. The completely sequenced cDNAs were submitted to GenBank and were given accession numbers AF175297 (mouse) and AF175296 (human). As with all known eukaryotic antizymes, this latest antizyme (antizyme 3) gene lacks an appropriate translation initiation codon in the longest ORF. Instead, just as with the other antizymes, the initiation codon apparently is present in an upstream ORF (ORF1), partially overlapping the downstream ORF (ORF2) in such a way that +1 ribosomal frameshifting in the overlap would result in the synthesis of full-length protein. Analyses of the cDNA sequences of antizymes from various organisms have shown that the most highly conserved region of antizyme at the nucleotide level immediately surrounds the end of ORF1, just where +1 ribosomal frameshifting occurs for mammalian antizyme 1 (24, 39). Frameshifting occurs at the UCC codon immediately preceding the UGA stop codon of ORF1 (15). With antizyme 1, sequences in close proximity to the UCCUGA site are essential for efficient frameshifting (15). Conservation of this region in other antizymes leads to the inference that this is the site of +1 frameshifting in the other antizymes. Analysis of the equivalent region in antizyme 3 shows that its ORF1 also ends with UCCUGA and that, overall, 16 nucleotides in the vicinity are identical to the consensus (Fig. 1*A*). For these reasons, our working model is that +1 frameshifting is required for expression of antizyme 3 and that this frameshifting occurs at a position analogous to that found in mammalian antizymes 1 and 2. With this model, the predicted lengths of mouse and human antizyme 3 proteins are 195 and 187 aa, respectively. The human protein is 86% identical and 90% similar to mouse antizyme 3. The mouse antizyme 3 protein has a small triplication (insertion) of 5 aa near the end of ORF1, which is not present in the human protein. Human antizyme 3 protein is 47% similar and 31% identical to human antizyme 1, and 44% similar and 29% identical to human antizyme 2. Essentially all similarity of antizyme 3 to antizymes 1 and 2 resides in ORF2 (Fig. 1*B*).

Phylogenetic analysis of the known eukaryotic antizyme proteins indicates that mammalian antizyme 3 diverged from the other two mammalian antizymes early in vertebrate evolution (Fig. 1*C*).

Biochemical Assay on Mouse Antizyme 3. The mouse antizyme 3 protein was tested for antizyme (ODC-inhibitory) activity by using a gene fusion with GST. In this construct, ORF1 and ORF2 of the antizyme are fused in-frame by deleting the T nucleotide that encodes the U of the stop codon of ORF1. This GST-antizyme fusion protein was expressed in *Escherichia coli* and purified by affinity chromatography. ODC-inhibitory activity was assayed by incubating the recombinant antizyme protein with mouse tissue culture crude extract. In parallel, the same assay was used with recombinant GST-mouse antizyme 1 protein. The results (Fig. 2) show that both recombinant antizyme 1 and 3 proteins can inhibit mouse ODC. The inhibition by antizyme 3 (inhibition constant K_I = 27.7 nM) shows higher apparent K_I than that shown by antizyme 1 (K_I = 4.5 nM). This 6.2-fold difference in apparent K_I may reflect real biochemical differences between the two proteins or it may be because of the slightly different properties of the GST fusion proteins (for example, reflecting differences in the size of ORF1 and associated spacer effect).

Expression of Antizyme 3 mRNA. All antizymes for which the transcriptional pattern of expression is known (this includes mammalian antizymes 1 and 2) are widely expressed throughout the organism in question. However, initial analysis suggested that antizyme 3 might be an exception. After the complete cDNA sequence was obtained for mouse and human antizyme 3, computer-generated analysis showed that all expressed sequence tag entries (>30; both human and mouse) corresponding to this gene come either from testis-specific libraries or from pooled libraries in which at least one component is testis-derived. To investigate the possibility that antizyme 3 is expressed only in testis, Northern analysis was performed on mRNA from 16 human tissues. The results (Fig. 3) reveal an mRNA with a size

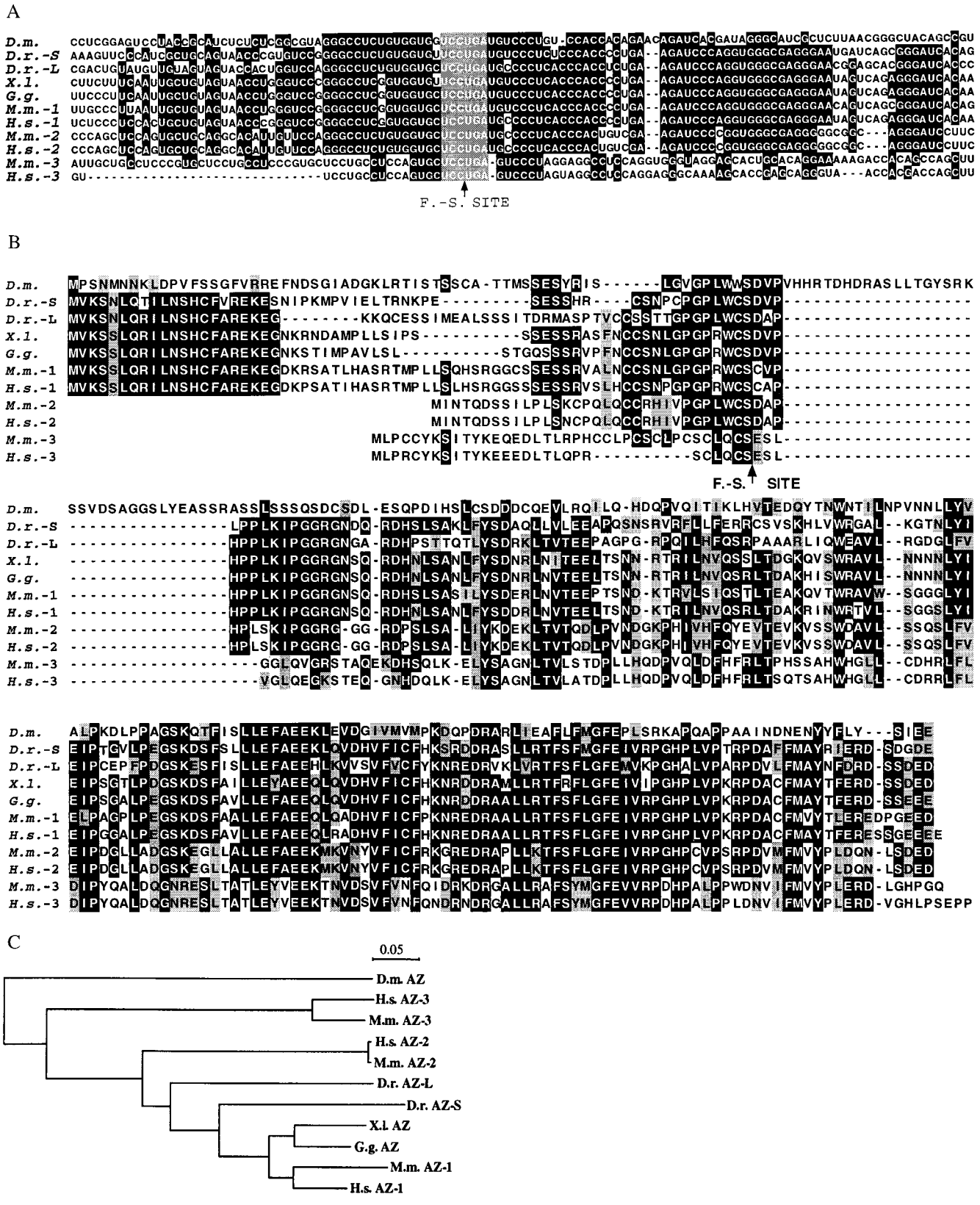

Fig. 1. Comparison between the mouse and human genes for antizyme 3 and the other known antizymes. *D.m.*, *Drosophila melanogaster*; *D.r.*, *Danio rerio* (*S*, short; *L*, long); *X.l.*, *Xenopus laevis*; *G.g.*, *Gallus gallus*; *M.m.*, *Mus musculus*; *H.s.*, *Homo sapiens*. (*A*) Comparison of the nucleotide sequences of the frameshift sites of different antizyme genes. Black background indicates nucleotide identity among at least seven antizyme genes. The frameshift (F.-S.) site is indicated with an arrow, and the UCCUGA sequence is shown with a gray background. (*B*) Comparison of the protein sequences of antizymes from different organisms. A black background indicates amino acid identity among at least five proteins. Gray background indicates amino acid similarity among at least six proteins. The arrow indicates the position of the frameshift site. (*C*) Unrooted phylogenetic tree of the antizyme proteins drawn with the CLUSTALX program.

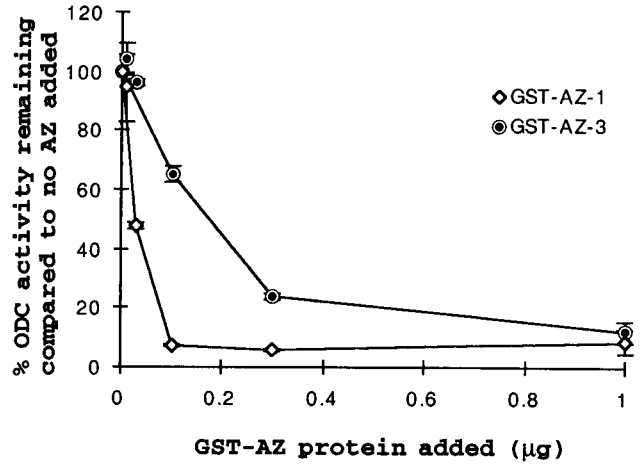

Fig. 2. Inhibition of mouse ODC by mouse antizymes 1 and 3. Various amounts of affinity-purified GST-antizymes (GST-AZs) 1 and 3 were mixed with mouse ODC-active extract and assayed for ODC activity. ODC activity of 100% is equal to 29.7 nmol of product per mg of total protein × h.

of 1.1 kb that is detectable only in testis. (Overexposure of the same gels hints of antizyme 3 expression in the prostate. If this signal is genuine, it is more than 200 times weaker than in testis.)

In situ hybridization was performed on mouse testis to determine the specific pattern of expression of antizyme 3. The results are shown in Fig. 4. Antizyme 3 transcripts were not observed in cells that are in early stages of spermatogenesis (spermatogonial and spermatocytic phases). Specific expression was found in cells during spermatogenesis as soon as the proacrosomal granules appeared in spermatids (Golgi phase). Expression is prominent in the head cap. The level of expression in the seminiferous tubes was highest during stages VIII to XII (Fig. 4*I*), disappeared during the late spermatid phase, and was absent in the spermatozoa. Residual signal was present in the cytoplasm of the "residual bodies" left behind by the mature sperm. No staining was detected in Leydig cells, Sertoli cells, or vasculature. To evaluate the potential redundancy of antizyme 3 in testis, an *in situ* probing for antizyme 1 expression was done under identical conditions as for antizyme 3. The results (Fig. 4 *F* and *G*) indicate that antizyme 1 expression levels in testes are near or below the detection limits of the technique and, thus, several orders of magnitude lower than those for antizyme 3.

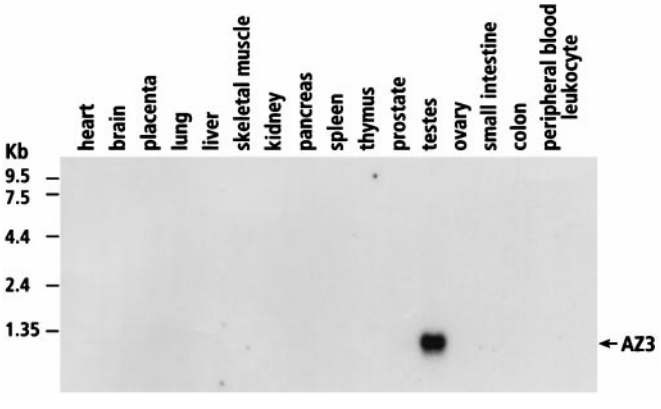

Fig. 3. Northern blot analysis of poly(A)$^+$ mRNA from 16 human tissues probed with random-labeled human antizyme 3 probe. The arrow indicates the position of the antizyme 3 (AZ3) signal.

Discussion

The discovery of a different antizyme has implications for the mechanism and evolution of programmed frameshifting, the control of ODC activity, and the regulation of polyamine levels, as well as for spermatogenesis and fertility. Antizyme 3 gene organization is highly suggestive of the requirement of programmed frameshifting for its expression. The fact that antizyme 3 has an ORF1 of approximately the same length as in antizymes 1 and 2, coupled with an ORF2 in the +1 frame but lacking an obvious start codon, is consistent with this hypothesis. Strikingly, the core sequence for antizyme frameshifting in antizyme 3 mRNA is conserved from *Schizosaccharomyces pombe* to *Caenorhabditis elegans* (38) to *Drosophila melanogaster* (39) to *Xenopus laevis* (40) to mammalian antizymes 1 and 2. Notably, however, a 3′ pseudoknot of the type shown to be important for antizyme 1 frameshifting has not been found in antizyme 3. This feature is also absent in lower eukaryotic antizymes, although at least in *S. pombe*, a different 3′ sequence has evolved to stimulate the programmed frameshifting (I.P.I., unpublished data).

Antizyme 3 protein is similar to vertebrate antizymes 1 and 2 only in its ORF2-encoded segment. Early studies with a partial cDNA of antizyme 1 showed that the ORF2-encoded segment is active on its own for the interaction with ODC (16). The domains of mammalian antizyme 1 interacting with ODC have been defined (8, 41). One region (amino acids 121–227) is necessary, and sufficient, for binding to and inhibition of ODC. This region is the most highly conserved between antizymes 1 and 3 (39% identity and 58% similarity between the two human paralogs), and *in vitro* assays demonstrate that antizyme 3 inhibits ODC (Fig. 2). A second region of antizyme 1 (amino acids 69–112) has been implicated in being essential for destabilization of ODC protein. This region of the protein is less conserved between antizymes 1 and 3 (17% identity and 27% similarity between the two human paralogs), raising the possibility that antizyme 3 may not be able to accelerate the degradation of ODC.

If the only function of ORF1 is to set up a frameshifting sensor of cytosolic polyamine levels for the regulatory purpose of ensuring that the amount of antizyme synthesized is appropriate for the cellular polyamine levels, then one might expect ORF1 to be short, just as it is in the parallel case of the frameshifting required for *E. coli* release factor 2 expression (42, 43). There are two forms of mammalian antizyme 1. Synthesis of one form initiates at the first AUG of ORF1, and it has a mitochondrial localization signal in its ORF1-encoded segment, resulting in at least some of the protein being imported into mitochondria (44). Synthesis of the other form of antizyme 1 initiates at a second AUG within ORF1. It lacks the mitochondrial localization signal and differs from the first form both in its response to physiological changes and in its half-life (44). Antizymes 2 and 3 do not have the localization signal, and the reason why ORF1 is longer than necessary for just frameshifting regulatory purposes raises the possibility that its product has an additional function. The N-terminal 20 amino acids of all vertebrate orthologs of antizyme 1 are very highly conserved (Fig. 1*B*). This sequence is not at all conserved in antizymes 2 and 3, again implying that this region of the protein might have evolved different functions in the different antizyme paralogs.

ODC mRNA and protein are present at some level in all testicular cells (Leydig, Sertoli, spermatogenic cells, etc.), but the levels of expression vary greatly. Several studies have shown that ODC expression during sperm development increases sharply (from earlier background levels) and peaks in late pachytene spermatocytes and early round spermatids (45, 46). In later stages of spermatid development and in spermatozoa, ODC

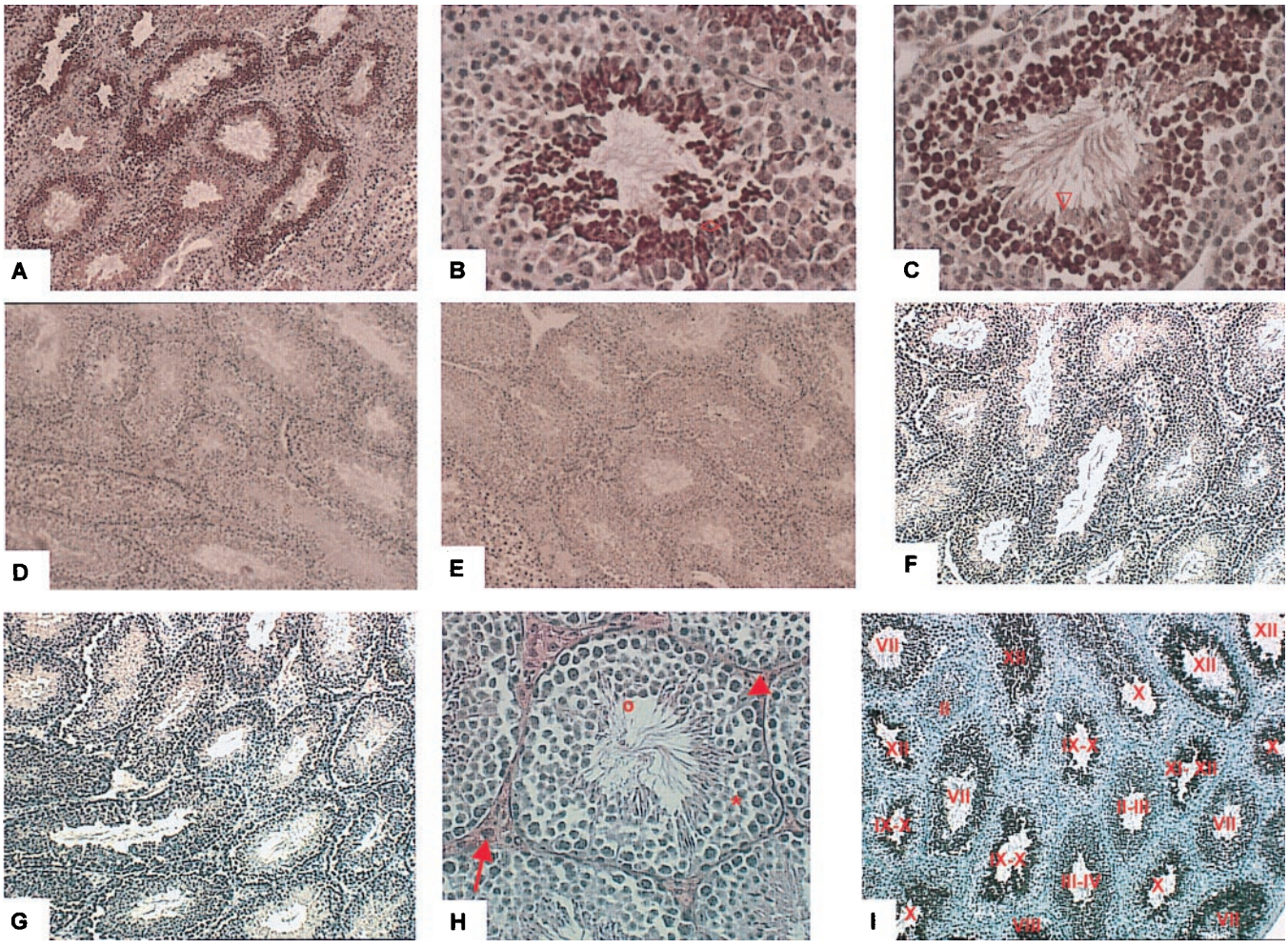

Fig. 4. *In situ* hybridization of antizyme 3. (*A*) Antizyme 3 expression is restricted to the luminal regions of seminiferous tubules. No staining is observed in Leydig cells, Sertoli cells, myoid cells, or the vasculature. (*B* and *C*) High magnification (×400) shows antizyme 3 expression during spermiogenesis as early as the proacrosomal granules appear in spermatids during the Golgi phase. Staining disappears during the late spermatid phase and is absent in spermatozoa. No staining is observed in the RNase-treated tissue section (*E*) or when the sense probe is used (*D*). (*F* and *G*) Hybridization with antizyme 1-specific antisense (*F*) and sense (*G*) probes. (*H*) Hematoxylin/eosin staining of seminiferous tubules showing spermatocytes (star), spermatozoa (circle), Leydig cells (arrow), and Sertoli cells (arrowhead), indicating the specificity of the hybridization. (*I*) Antizyme 3 expression is highest during stages VIII to XII (indicated by Roman numerals).

expression falls back to background levels. By contrast, no antizyme 3 mRNA expression is detected before early spermatid stages of spermatogenesis or in any of the nonspermatogenic cells of testes. Antizyme 3 mRNA expression is maximal during the middle and late stages (stages VIII through XII) of spermatid development, and then it disappears again in the spermatozoa. It appears that the antizyme 3 wave of expression follows the wave of high ODC expression during spermatogenesis. This pattern of expression of the two genes implies that the physiological role of antizyme 3 is to quickly "extinguish" (prevent overaccumulation of) ODC activity after the stage at which ODC plays its role in spermatogenesis, most likely late spermatocytic/early spermatidal phase. By the same type of experiments (*in situ* hybridization), antizyme 1 expression is essentially nondetectable in testicular tissues (specifically during spermatogenesis). This finding is consistent with our previous observations demonstrating that of 50 human tissues examined, antizyme 1 mRNA is least abundant in testis. Because antizyme 2 mRNA was found to be even less abundant in testis by the same experiment (dot-blot analysis), its expression there would also be expected to be below the sensitivity threshold of *in situ* hybridization. From the fact that antizyme 3 is expressed only in testis and then only in the spermatid phase, whereas antizymes 1 and 2 are only negligibly (if at all) expressed during spermatogenesis, we conclude that antizyme 3 has evolved specifically to provide spatial and temporal regulation of ODC during spermatogenesis.

The role of ODC and, by extension, polyamines in spermatogenesis is not clear. Several functions have been proposed. These functions include possible roles in DNA synthesis and packaging during meiosis or regulation of transcription in haploid spermatogenic cells. Whatever their roles in spermatogenesis, there is a good indication of what might be the physiological consequences of extending high levels of ODC expression past its normal stage in sperm development. As mentioned above, the main phenotype of transgenic mice overproducing ODC is male infertility and an associated alteration in seminiferous epithelial morphology. In such animals there is a significant reduction of mature spermatozoa. In fact, this deleterious effect is observed in all postmeiotic cells. Perhaps, in these cells the normally occurring antizyme 3 is "swamped out" by the extra ODC, which leads to excessive

polyamine (putrescine) accumulation and premature cell death. If this hypothesis is correct, mammals (mice in particular) lacking this gene should be male infertile with a specific morphology of the seminiferous epithelium (i.e., normal Sertoli cells, spermatogonia, and spermatocytes, but reduced or absent spermatids and spermatozoa). If this phenotype is confirmed, the antizyme 3 gene will become a candidate for heritable forms of human male infertility with similar testicular morphology (certain types of Sertoli cell only syndrome).

Our findings are consistent with the previous conclusion that polyamines play an important and special role during late meiosis and early spermiogenesis.

This paper is dedicated to Drs. Shin-Ichi Hayashi, Senya Matsufuji, and Philip Coffino for their roles in establishing the reality of antizyme despite skepticism. We thank Dr. Senya Matsufuji for stimulating our interest in antizyme and Jasmine Parvaz for technical help. This work was supported by a grant from the Department of Energy to R.F.G. (DEFG03-99ER62732) and a National Institutes of Health grant to J.F.A. (RO1-GM48152). A.R. is a recipient of a Boehringer Ingelheim fellowship, and D.A.T. is a U.S. Department of Veterans Affairs Investigator.

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