Rossel M, Schuffenecker I, Schlumberger M, Bonnardel C, Modigliani E, Gardet P, Navarro J, et al (1995) Detection of a germline mutation at codon 918 of the *RET* proto-oncogene in French MEN 2B families. Hum Genet 95:403–406

Sapienza C (1994) Parental origin effects, genome imprinting, and sex-ratio distortion: double or nothing? Am J Hum Genet 55:1073–1075

Sapienza C, Hall G (1994) Genome imprinting in human disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 7th ed. McGraw-Hill, Baltimore, pp

Schuffenecker I, Billaud M, Calender A, Chambe B, Ginet N, Calmettes C, Modigliani E, et al (1994) *RET* proto-oncogene mutations in French MEN 2A and FMTC families. Hum Mol Genet 3:1939–1943

Trupp M, Arenas E, Fainzilber M, Nilsson A-S, Sieber B-A, Grigoriou M, Kilkenny C, et al (1996) Functional receptor for glial cell-line derived neurotrophic factor (GDNF) encoded by the c-*ret* proto-oncogene. Nature 381:785–789

Wohllk N, Cote GJ, Bugalho MMJ, Ordonez N, Evans D, Goepfert H, Khorana S, et al (1996) Relevance of *RET* proto-oncogene analysis in sporadic medullary thyroid carcinoma. J Clin Endocrinol Metab 81:3740–3745

Address for correspondence and reprints: Dr. Isabelle Schuffenecker or Dr. Gilbert M. Lenoir, Laboratoire de Génétique, Hôpital Edouard Herriot, 1 Place d'Arsonval, 69437 Lyon cedex 03, France.
© 1997 by The American Society of Human Genetics. All rights reserved.
0002-9297/97/6001-0032$02.00

Am. J. Hum. Genet. 60:237–241, 1997

Expression of *DAZ*, an *Azoospermia Factor* Candidate, in Human Spermatogonia

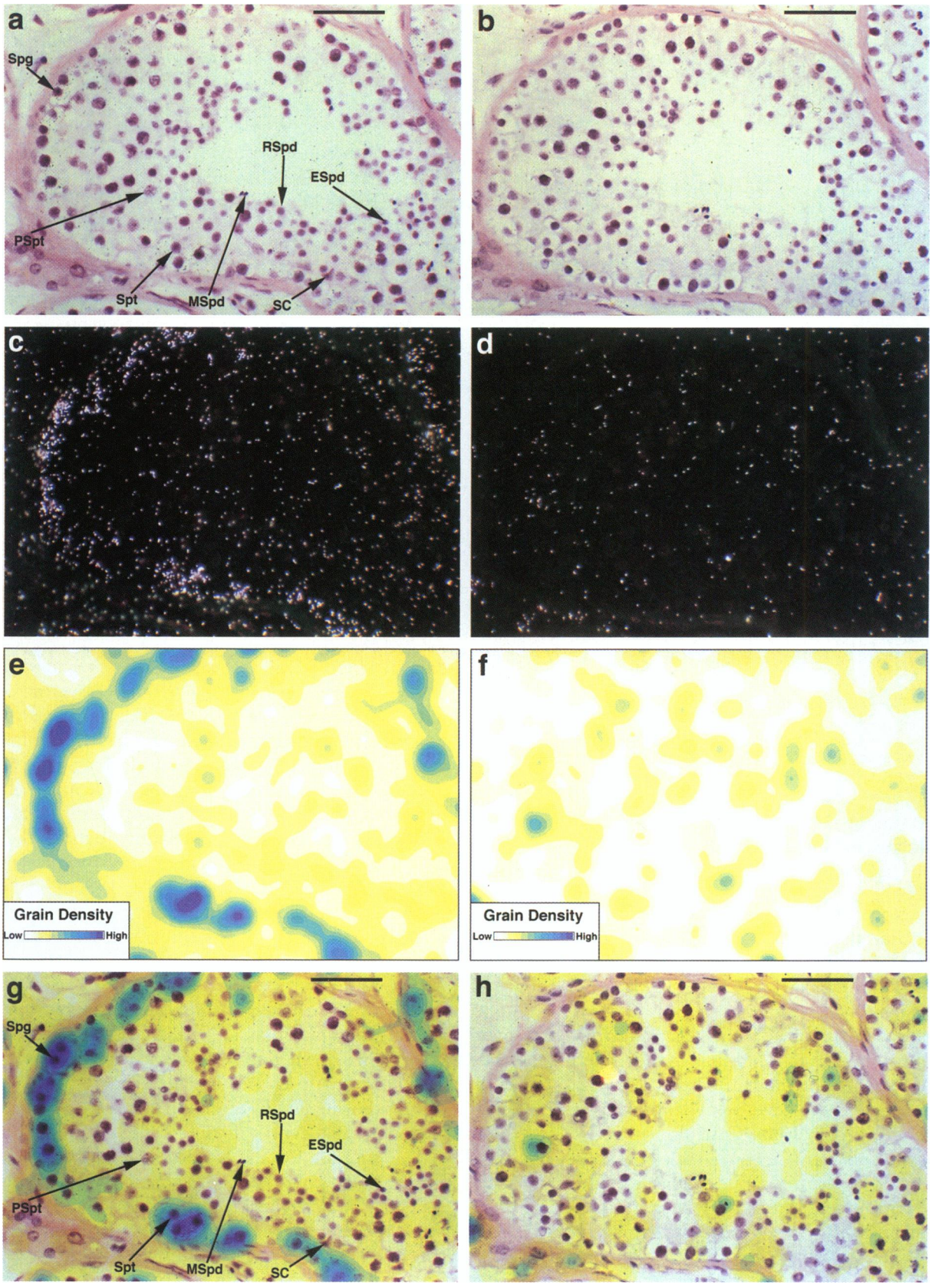
To the Editor:

Three percent to 4% of men have severe defects in sperm production that result in infertility (van Zyl et al. 1975; Hull et al. 1985). Most of these men are otherwise healthy, and the cause of their spermatogenic failure is rarely identified with certainty. Little is known about possible contributions from genetic factors, but some cases may be due to mutations that disrupt male germ-cell development without affecting the soma. Such "pure male sterile" genes have been identified in invertebrates (Ellis and Kimble 1994; Castrillan et al. 1993). In humans, no pure sterile factor—male or female—has been unequivocally defined at the biochemical level, although some critical genetic loci have been mapped.

The *azoospermia factor* (*AZF*), found on the long arm of the Y chromosome, may be the most thoroughly studied pure male sterile locus in humans. In 1976, Tiepolo and Zuffardi reported microscopic deletions of Yq in six men with azoospermia (no sperm in semen). On the basis of these findings, they proposed that Yq carries an *AZF* gene or gene complex required for spermatogenesis (Tiepolo and Zuffardi 1976). Recently, Tiepolo and Zuffardi's hypothesis has received strong experimental support (Ma et al. 1992; Kobayashi et al. 1994; Najmabadi et al. 1996; Vogt et al. 1996). In particular, 13% of men with nonobstructive azoospermia were found to have deletions of a consistent, specific portion of Yq—an "*AZF* region" (Reijo et al. 1995). The deletions were not present in the affected individuals' fathers but had arisen de novo, establishing that the deletions were the cause of azoospermia rather than an incidental finding.

De novo deletions of this *AZF* region have been found to result in a wide range of spermatogenic defects. In some *AZF*-deleted individuals, testis biopsies revealed the complete absence of germ cells ("Sertoli cell–only syndrome"). In other individuals, early spermatogenic cells were observed ("testicular maturation arrest"). In two of the latter individuals, spermatogenesis had sometimes progressed through meiosis to the stage of condensed spermatids (Reijo et al. 1995). Indeed, we have shown recently that deletion of *AZF* is compatible with completion of spermatogenesis, albeit at greatly reduced output: two men with severe oligozoospermia (markedly reduced but nonzero semen sperm counts) were found to have de novo, *AZF*-region deletions similar or identical to those found in unrelated azoospermic men (Reijo et al. 1996*a*). Thus, spermatogenesis can, in some instances, proceed without *AZF*. However, spermatogenic output is severely diminished in the absence of *AZF*, and in some cases germ cells (even spermatogonial stem cells) are found to be completely lacking. As we and our colleagues have suggested, this wide array of *AZF* phenotypes might be explained most simply by a defect (with variable expressivity) in spermatogonia, the stem cells that are the source of the spermatogenic lineages in the adult testis (Reijo et al. 1995, 1996*a*).

The molecular identity of *AZF* is not yet known with certainty, but much experimental evidence has accumulated in favor of *DAZ* (*deleted in azoospermia*), a multicopy gene cluster located in this *AZF* region (Reijo et al. 1995; Saxena et al. 1996). As revealed by northern blotting of human tissue RNAs, *DAZ* is transcribed specifically in testis. On the basis of sequence comparisons, *DAZ* appears to encode an RNA-binding protein. Although no human *DAZ* point mutants have been reported, recent genetic findings in *Drosophila* provide strong support for the hypothesis that *DAZ* is *AZF*. Eberhart and colleagues have characterized a *Drosophila* gene, *boule,* whose product shows striking amino acid similarity to the human DAZ protein, especially in the RNA-binding domain. Just as with human *AZF*, loss-of-function mutations in *Drosophila boule* disrupt spermatogenesis and result in azoospermia but spare the soma (Eberhart et al. 1996). Thus, the *Drosophila* ho-


a
Spg
RSpd
ESpd
PSpt
Spt
MSpd
SC
b
c
d
e
Grain Density
Low
High
f
Grain Density
Low
High
g
Spg
RSpd
ESpd
PSpt
Spt
MSpd
SC
h

mologue of *DAZ* is a pure male sterile gene with striking parallels to *AZF*.

Like *Drosophila boule*, human *DAZ* is expressed specifically in the testis (Reijo et al. 1995; Eberhart et al. 1996). Given the prospect that *DAZ* might be *AZF*, we set out to study its expression in the human testis in detail. We were interested in determining (i) whether *DAZ* is expressed in the somatic cells of the testis, in germ cells, or both and (ii) whether the pattern of *DAZ* expression would be consistent with the array of spermatogenic defects observed in men with deletions of *AZF*.

To localize *DAZ* transcripts, radio-labeled oligonucleotide probes were hybridized to sections of normal human adult testes, essentially as described by Mutter and colleagues (1993). Testes were obtained from the Department of Pathology, Brigham and Women's Hospital, Boston, following institutional review board approval. The tissues originated from three men with prostate cancer who had undergone elective orchiectomies. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. All testes appeared histologically normal, with active spermatogenesis. Five-micron sections were cut from paraffin-embedded testes, dewaxed in xylene, and rehydrated in graded ethanols to water. Two synthetic antisense probes, each 50 nt in length, were selected from the published *DAZ* cDNA sequence (Reijo et al. 1995). Probe A (GGCAACTGATATCCAGTGGTGACCTGAAATGGTGAACTTGGATAAGCAGG) was chosen from within a 72-bp segment that is tandemly repeated seven times in the *DAZ* coding sequence. Probe B (CTTCGGTCCACAGATTTCTCCTTTGCTCCCCAACAGGGCACTGCGGTGGC) was also chosen from coding sequence, but outside the tandem repeats. Oligonucleotides complementary to probes A and B were used as negative controls. All four probes were purified on acrylamide gels and then end-labeled with ^{35}S-α-dATP by use of terminal deoxynucleotidyl transferase. Hybridizations were carried out overnight in a humidified chamber at 42°C using a solution of 4 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate [pH 7.4]), 50% formamide, 1 × Denhardt's (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% BSA) 1% sarcosyl, 0.2 M sodium phosphate (pH 7.0), 10% dextran sulfate, 250 μg/ml yeast tRNA, 500 μg/ml salmon sperm DNA, 200 mM DTT, and 10^6 cpm/100 μl of ^{35}S-α-dATP end-labeled oligonucleotide. Sections were then washed four times for 15 min each in 1 × SSC at 55°C. After drying, slides were dipped in 50% NTB-2 emulsion (Kodak diluted with water), stored at 4°C for 10–176 d, and developed. Images of autoradiographic grain density were electronically processed with commercial software (Adobe Photoshop). In brief, conventional dark-field images were inverted and brightness levels adjusted so that autoradiographic silver grains appeared black on a white background. This inverted dark-field image was then subjected to "Gaussian-blur" filtering, by which signal intensities in individual pixels are locally averaged so as to produce continuous intensity contours.

Lengthy exposures were required for optimal signal detection: 87 d for probe A and 176 d for probe B. The signal-to-noise ratio obtained with probe A, derived from a seven-fold tandem repeat within the *DAZ* transcript, was somewhat better than that obtained when probe B was used, derived from a nonrepeated portion of the transcript. However, both probes revealed a similar distribution of *DAZ* transcripts, and multiple exposures on testis sections from three men yielded consistent results. As shown in figure 1, using probe A, transcripts were detected just inside the perimeter of seminiferous tubules—not around the entire circumference of the tubule, but in discrete patches (similar results obtained with probe B; not shown). Image processing (fig. 1*e, f*) facilitated identification and classification of individual cells underlying areas of intense signal. Spermatogonia were clearly the source of most of the intense signals. Occasional early (leptotene/zygotene) primary spermatocytes had similarly high levels of transcripts, but transcripts were not detected in later (pachytene) primary spermatocytes with either probe. Neither probe revealed the presence of *DAZ* transcripts in other spermatogenic cells or in the somatic cells of the testis (e.g., Sertoli, Leydig, peritubular cells).

Our findings with human Y-chromosomal *DAZ*—that it appears to be expressed exclusively in germ cells and most abundantly in spermatogonia (fig. 1)—may extend to autosomal homologues found in humans and mice. In both humans and mice, there exists an autosomal *DAZ* homologue (*DAZH* in humans; *Dazh*/*Dazla* in mice) that is expressed in testes and, at a much lower level, in ovaries (Cooke et al. 1996; Reijo et al. 1996*b*; Saxena et al., in press). The absence of *Dazh* transcripts

Figure 1 Hybridization of *DAZ* probe to human adult testis. In the left column (panels *a, c, e,* and *g*) are images of a seminiferous tubule probed with antisense probe A. In the *right* column (panels *b, d, f,* and *h*) are images of an adjacent tissue section probed with the complementary sense probe. *a* and *b,* Bright-field photographs, sections stained with hematoxylin and eosin; autoradiographic grains dimly visible in background. *c* and *d,* Dark-field photographs of the same field, highlighting autoradiographic silver grains. *e* and *f,* Autoradiographic grain density contours produced by subjecting dark-field images (panels *c* and *d*) to Gaussian blur filtering (see text). *g* and *h,* Grain-density contours (panels *e* and *f*) overlaid onto bright-field histology (panels *a* and *b*). Cell types: Spg = spermatogonium; Spt = leptotene/zygotine primary spermatocyte; PSpt = pachytene primary spermatocyte; RSpd = round spermatid; ESpd = elongating spermatid; MSpd = mature spermatid; and SC = Sertoli cell. All images at 400× magnification; bar = 50 μm.

in germ-cell–deficient mice suggests that expression of this autosomal gene is restricted to germ cells in both testes and ovaries (Reijo et al. 1996*b*; J. Seligman, R. Reijo, D. C. Page, unpublished data). Specifically, autosomal mouse *Dazh* appears to be transcribed in spermatogonia, as indicated by developmental northern-blotting studies (Reijo et al. 1996*b*)—like human Y-chromosomal *DAZ*.

A portion of the testis in situ signal we observed with *DAZ* probes may have originated from *DAZH*. If so, then the specificity of the observed hybridization pattern (fig. 1) suggests that it may describe the expression of both Y-chromosomal *DAZ* and its autosomal homologue.

Although many mammalian genes are expressed in the testis, *DAZ*'s restricted pattern of expression is unusual. Numerous genes are expressed exclusively in male germ cells (e.g., protamines and testis-specific isoforms of tubulins, histones, and lactate dehydrogenase), mostly in meiotic or postmeiotic cells, but not in the earliest stages of the spermatogenic pathway (Distel et al. 1984; Drabent et al. 1991; Salehi-Ashtiani and Goldberg 1993; Wykes et al. 1995). Other genes are expressed in premeiotic germ cells (e.g., c-myc, c-kit, fos, jun, MAGE -1 and -4, hsp60, and FSH β), but also in one or more somatic tissues (Koji et al. 1988; Manova et al. 1990; Kurata et al. 1993; Markkula et al. 1995; Meinhardt et al. 1995; Takahashi et al. 1995). The specificity of *DAZ* expression in spermatogonia and their immediate derivatives, early primary spermatocytes, suggests that human *DAZ* functions in the first phases of spermatogenic differentiation—or earlier, in the maintenance of the spermatogonial stem-cell populations.

As we have pointed out elsewhere (Reijo et al. 1995), the phenotypes associated with deletions of *AZF* suggest that it may function in spermatogonia—where we have now shown *DAZ* to be expressed. Additional evidence that *DAZ* may be *AZF* is found in *Drosophila boule*, a homologue of *DAZ* and, like *AZF*, a pure male sterile locus. The products of the human *DAZ* and *Drosophila boule* genes exhibit remarkable amino acid sequence similarity (Reijo et al. 1995; Eberhart et al. 1996). We have now established that the human and *Drosophila* genes also have similar expression patterns: both are transcribed exclusively in male germ cells.

In addition to providing evidence for the molecular identity of *AZF*, our findings suggest possible avenues for future studies of spermatogonia. Spermatogonia play a central role in human heredity, genetic disease, and evolution. They are the source of all male gametes and, as some have suggested, may be the cells in which most germ-line mutations arise (Haldane 1947; Ketterling et al. 1993; Shimmin et al. 1993). Given these observations, it is remarkable how little is known about the molecular biology of this stem-cell population (Hecht 1995). The availability of molecular markers like *DAZ* may help remedy this situation.

DOUGLAS B. MENKE,[1] GEORGE L. MUTTER,[2] AND DAVID C. PAGE[1]

[1]*Howard Hughes Medical Institute, Whitehead Institute, and Department of Biology, Massachusetts Institute of Technology, and* [2]*Department of Pathology, Brigham and Women's Hospital, Boston*

Acknowledgments

We thank Kevin Boynton for instruction on tissue sectioning, Charles Tilford for advice on image processing, and Paul Bain, Karin Jegalian, Bruce Lahn, Renee Reijo, Chao Sun, and Charles Tilford for comments on the manuscript. This study was supported by the National Institutes of Health.

References

Castrillon DH, Gonczy P, Alexander S, Rawson R, Eberhart CG, Viswanathan S, Di Nardo S, et al (1993) Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster:* characterization of male-sterile mutants generated by single P element mutagenesis. Genetics 135:489–505

Cooke HJ, Lee M, Kerr S, Ruggiu M (1996) A murine homologue of the human *DAZ* gene is autosomal and expressed only in male and female gonads. Hum Mol Genet 5:513–516

Distel RJ, Kleene KC, Hecht NB (1984) Haploid expression of a mouse testis alpha-tubulin gene. Science 224:68–70

Drabent B, Kardalinou E, Doenecke D (1991) Structure and expression of the human gene encoding testicular H1 histone (H1t). Gene 103:263–268

Eberhart CG, Maines JZ, Wasserman SA (1996) Meiotic cell cycle requirement for a fly homologue of human *deleted in azoospermia.* Nature 381:783–785

Ellis RE, Kimble J (1994) Control of germ cell differentiation in *Caenorhabditis elegans.* Ciba Found Symp 182:179–188

Haldane JBS (1947) The mutation rate for haemophilia and its segregation ratios in males and females. Ann Eugen 13: 262–271

Hecht NB (1995) The making of a spermatozoon: a molecular perspective. Dev Genet 16:95–103

Hull MGR, Glazener CMA, Kelly NJ, Conway DI, Foster PA, Hinton RA, Coulson C, et al (1985) Population study of causes, treatment, and outcome of infertility. Br Med J 291: 1693–1697

Ketterling RP, Vielhaber E, Bottema CDK, Schaid DJ, Cohen MP, Sexauer CL, Sommer SS (1993) Germ-line origins of mutation in families with hemophilia B: the sex ratio varies with the type of mutation. Am J Hum Genet 52:152–166

Kobayashi K, Mizuno K, Hida A, Komaki R, Tomita K, Matsushita I, Namiki M, et al (1994) PCR analysis of the Y chromosome long arm in azoospermic patients: evidence for a second locus required for spermatogenesis. Hum Mol Genet 3:1965–1967

Koji T, Izumi S, Tanno M, Moriuchi T, Nakane PK (1988)

Localization *in situ* of c-myc mRNA and c-myc protein in adult mouse testis. Histochem J 20:551–557
Kurata S, Matsumoto M, Hoshi M, Nakajima H (1993) Transcriptional control of the heme oxygenase gene during mouse spermatogenesis. Eur J Biochem 217:633–638
Ma K, Sharkey A, Kirsch S, Vogt P, Keil R, Hargreave TB, McBeath S, et al (1992) Towards the molecular localisation of the AZF locus: mapping of microdeletions in azoospermic men within 14 subintervals of interval 6 of the human Y chromosome. Hum Mol Genet 1:29–33
Manova K, Nocka K, Besmer P, Bachvarova RF (1990) Gonadal expression of *c-kit* encoded at the *W* locus of the mouse. Development 110:1057–1069
Markkula M, Hamalainen T, Loune E, Huhtaniemi I (1995) The follicle-stimulating hormone (FSH) beta- and common alpha-subunits are expressed in mouse testis, as determined in wild-type mice and those transgenic for the FSH beta-subunit/herpes simplex virus thymidine kinase fusion gene. Endocrinology 136:4769–4775
Meinhardt A, Parvinen M, Bacher M, Aumuller G, Hakovirta H, Yagi A, Seitz J (1995) Expression of mitochondrial heat shock protein 60 in distinct cell types and defined stages of rat seminiferous epithelium. Biol Reprod 52:798–807
Mutter GL, Stewart CL, Chaponot ML, Pomponio RJ (1993) Oppositely imprinted genes H19 and insulin-like growth factor 2 are coexpressed in human androgenetic trophoblast. Am J Hum Genet 53:1096–1102
Najmabadi H, Huang V, Yen P, Subbarao MN, Bhasin D, Banaag L, Naseeruddin S, et al (1996) Substantial prevalence of Microdeletions of the Y-chromosome in infertile men with idiopathic azoospermia and oligozoospermia detected using a sequence-tagged site-based mapping strategy. J Clin Endocrinol Metab 84:1347–1352
Reijo R, Alagappan RK, Patrizio P, Page DC (1996*a*) Severe oligozoospermia resulting from deletions of the *Azoospermia Factor* gene on the Y chromosome. Lancet 347: 1290–1293
Reijo R, Lee T-Y, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, et al (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 10:383–393
Reijo R, Seligman J, Dinulos MB, Jaffe T, Brown LG, Disteche CM, Page DC (1996*b*). Mouse autosomal homolog of *DAZ*, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. Genomics 35: 346–352
Salehi-Ashtiani K, Golberg E (1993) Differences in regulation of testis specific lactate dehydrogenase in rat and mouse occur at multiple levels. Mol Reprod Dev 35:1–7
Saxena R, Brown LG, Hawkins T, Alagappan RK, Skaletsky H, Reeve MP, Reijo R, et al (1996) Origins of the *DAZ* gene cluster on the human Y chromosome: an autosomal gene was transposed, then repeatedly amplified and pruned. Nat Genet 14:292–299
Shimmin LC, Chang BHJ, Li WH (1993) Male-driven evolution of DNA sequences. Nature 362:745–747
Takahashi K, Shichijo S, Noguchi M, Hirohata M, Itoh K (1995) Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res 55:3478–3482
Tiepolo L, Zuffardi O (1976) Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Genet 34:119–124
van Zyl JA, Menkveld R, van Kotze TJ, Retief AE, van Niekerk WA (1975) Oligozoospermia: a seven-year survey of the incidence, chromosomal aberrations, treatment and pregnancy rate. Int J Fertil 20:129–132
Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Kohn FM, et al (1996) Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. Hum Mol Genet 5:933–943
Wykes SM, Nelson JE, Visscher DW, Djakiew D, Krawetz SA (1995) Coordinate expression of the PRM1, PRM2, and TNP2 multigene locus in human testis. DNA Cell Biol 14: 155–161

Address for correspondence and reprints: Dr. David C. Page, Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142.
© 1997 by The American Society of Human Genetics. All rights reserved.
0002-9297/97/6001-0033$02.00

Am. J. Hum. Genet. 60:241–244, 1997

mtDNA and Y Chromosome–Specific Polymorphisms in Modern Ojibwa: Implications about the Origin of Their Gene Pool

To the Editor:

The Ojibwa, the second-largest Native American group in Canada, are an Algonkian-speaking people, who have the greatest concentration in the province of Ontario (Szathmary et al. 1974). Their geographic distribution has been established in the last two centuries. During this period, the population has been divided into two main branches that have accumulated some cultural (Dunning 1959) and genetic differentiation (Szathmary et al. 1974). The northern Ojibwa appear to have been least influenced by Europeans, whereas the southeastern Ojibwa of the Lake Huron region appear to have been exposed to a great European influence. On the basis of genetic analysis of some serum and red-cell protein polymorphisms, Szathmary and Reed (1972) and Szathmary et al. (1974) were able to reveal the presence of "Caucasian" alleles in the southeastern Ojibwa and to give an estimate of Caucasian admixture of ~30%; however, more recent data on other autosomal locus polymorphisms indicate that the genetic admixture may be as great as 50% (D. E. C. Cole and L. A. Rubin, unpublished data).

Previous studies of mtDNA variation have shown that the Ojibwa are the Native American group with the highest proportion (~25%) of mtDNAs not belonging to haplogroups A, B, C, and D (Torroni et al. 1993). These additional mtDNAs, which are all defined by the