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Regulation of 'haploid expressed genes' in male germ cells

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Introduction

Spermatogenesis offers an experimental system whereby the gene expression of eukaryotic cells with tetraploid, diploid, and haploid chromosome complements can be compared. Starting from a population of stem cells, the diploid spermatogonia follow one of two lineages. One subpopulation of cells initiates a differentiation process ultimately leading to the spermatozoon while a second, presumably distinct, subpopulation of spermatogonia enters a pathway that maintains and repopulates the stem cells of the testis. The cells destined to become spermatozoa undergo several spermatogonial divisions. The last complete replication of DNA during spermatogenesis, in the preleptotene primary spermatocyte, heralds the start of meiosis. During the lengthy interval of meiotic prophase, homologous chromosomes synapse and genetic recombination occurs, producing the genetic diversity required for survival of a species. Following meiotic recombination, the 4N spermatocytes divide twice without any DNA synthesis, producing first the diploid secondary spermatocytes and then the haploid spermatids. During spermiogenesis, a period lasting about 14 days in the mouse, the round spermatid is transformed into the spermatozoon. Transcription terminates during mid-spermiogenesis as the nucleus exchanges its somatic-like nucleosome structure for the nucleoprotamine structure of the mammalian spermatozoon. Concomitant with the unique chromatin alterations of the spermatid, an acrosome develops in the nucleus and an axoneme and tail are assembled. The resulting sperm shapes are species-specific.

Many of the differentiative events of the later stages of spermatogenesis are dependent upon gene products solely expressed from the haploid genome. A sizable number of testis-specific or testis-enriched marker proteins have been shown to be primarily or solely transcribed and translated in post-meiotic male germ cells (Goldberg, 1977; Hecht, 1986). We have been especially interested in the regulation of genes that encode structural proteins of the developing male gamete. Included in this group are several DNA-binding proteins such as the transition proteins and the protamines, as well as cytoplasmic structural proteins such as isotypes of actin and tubulin (reviewed by Hecht, 1986). Proto-oncogenes such as int-1, pim-1 and c-mos are also initially expressed during spermiogenesis (reviewed by Propst *et al.*, 1988). Using transition protein 1 (TP1), protamine 1 and protamine 2 as model 'haploid' genes, most of the discussion in this article focusses on mechanisms that regulate the temporal expression of genes in the mammalian testis.

Identifying haploid expressed genes

Several different approaches have been used to identify and isolate post-meiotically expressed genes. In my laboratory DNA probes for transition protein 1 and the two protamines have been identified by differentially screening a mouse testicular cDNA library prepared from sexually mature animals (Kleene *et al.*, 1983). Differential screening of this library with radiolabelled cDNAs prepared against poly(A)$^+$ RNA from meiotic pachytene spermatocytes or from post-meiotic round spermatids identified a group of cDNAs that hybridized solely or preferentially with

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round spermatid cDNAs. Additional analyses revealed that the RNA transcripts coded for by the cDNAs were not expressed in liver, brain, cultured Sertoli cells or in the prepubertal testes of 17-day-old mice (an age at which spermatogenesis has proceeded to meiosis). The putative post-meiotic cDNAs were subsequently shown by DNA sequence analysis to code for transition protein 1 (Kleene *et al.*, 1988) and the two protamines (Kleene *et al.*, 1985; Yelick *et al.*, 1987). The post-meiotic temporal expression of these 3 genes was established by Northern blot analysis of RNA prepared from isolated populations of testicular cell types (Kleene *et al.*, 1984; Yelick *et al.*, 1989) and from testicular RNA prepared from prepubertal mice of several different ages as they advance from birth to sexual maturity (Hecht *et al.*, 1986). Recently, in-situ hybridization of testicular sections has confirmed that, in the mouse and rat, the transition protein 1 and protamine 1 and 2 genes are solely expressed from the haploid genome (Hecht & Penschow, 1987; Mali *et al.*, 1988). Using a similar approach, studies from other laboratories have identified a large number of additional genes (Dudley *et al.*, 1984), including transition protein 2 (Kleene & Flynn, 1987) and t complex polypeptide 1 (TCP-1) (Willison *et al.*, 1986, 1989) that are temporally expressed in post-meiotic male germ cells.

A second approach that has proved successful in identifying post-meiotically expressed genes of the testis has been to utilize cDNA or genomic DNA clones for genes whose expression has been examined in other tissues as probes for screening testis cDNA libraries. Proto-oncogenes such as c-mos (Goldman *et al.*, 1987; Mutter & Wolgemuth, 1987; Propst, 1987), int-1 (Shackleford & Varmus, 1987) and pim-1 (Sorrentino *et al.*, 1988) have demonstrated male germ cell expression that appears haploid-specific in mammals such as the mouse. Other genes such as the proto-oncogene c-abl have been shown to express a transcript that is of novel size in haploid male germ cells (Ponzetto & Wolgemuth, 1985). A member of the heat shock 70 gene family also appears to produce a novel transcript during spermiogenesis (Zakeri *et al.*, 1988).

Screening testicular cDNA libraries with coding region probes for widely expressed genes has also proved successful in isolating cDNAs for novel or unique testicular isotypes of many proteins. This approach has led to the identification of genes and probes for testicular histone variants (Cole *et al.*, 1986; Kim *et al.*, 1987; Moss *et al.*, 1989), α-tubulin (Distel *et al.*, 1984; Hecht *et al.*, 1988), a smooth muscle γ-actin (Waters *et al.*, 1985; Kim *et al.*, 1989), and the testicular isozymes for cytochrome c (Goldberg *et al.*, 1977; Virbasius & Scarpulla, 1988) and phosphoglycerate kinase 2 (Erickson *et al.*, 1980; Gold *et al.*, 1983; McCarrey & Thomas, 1987).

The rapidly increasing number of post-meiotic genes that are being identified establishes that the haploid genome of the spermatid does not simply continue transcribing genes activated during meiosis but initiates the transcription of genes programmed for specific activation during spermiogenesis (Table 1).

Table 1. Post-meiotic genes of the testis

Protein	Reference
Transition protein 1	Heidaran & Kistler (1987a, b); Kleene *et al.* (1988)
Transition protein 2	Kleene & Flynn (1987)
Protamine 1	Kleene *et al.* (1985)
Protamine 2	Yelick *et al.* (1987)
Int-1	Shackleford & Varmus (1987)
t complex polypeptide 1 (TCP1)	Silver (1985); Willison *et al.* (1986, 1989)
c-mos	Goldman *et al.* (1987); Propst *et al.* (1988)
pim 1	Sorrentino *et al.* (1988)
Regulatory subunit type II protein kinase	Oyen *et al.* (1988)
c-abl (mRNA size variant only)	Ponzetto & Wolgemuth (1985)
Smooth muscle γ-actin	Kim *et al.* (1989)

The above list indicates some of the testicular gene products believed to be exclusively transcribed from the post-meiotic genome.

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Chromatin transitions during spermatogenesis: histones → transition proteins → protamines

Marked changes in the shape and composition of the gamete nucleus occur as spermatogenesis proceeds in mammals (reviewed by Meistrich, 1989). The structural organization of the chromatin of spermatogonia and early spermatocytes is composed primarily of the histones present in most somatic cells. During meiotic prophase, many new histone variants have been detected in the chromatin. Recently, a post meiotic H2B histone variant has also been identified (Challoner *et al.*, 1989; Moss *et al.*, 1989). Some of these histone variants are unique to the testis whereas others are also found in other tissues. As the differentiation of the male germ cell advances into spermiogenesis, several transition proteins replace the histones in the chromatin and are in turn replaced by the protamines, producing the nucleoprotamine of the spermatozoon. These chromatin changes which convert a transcriptionally active nucleus to the quiescent nucleus of the spermatozoon, require the temporally regulated expression of a group of DNA-binding proteins.

In the mouse, two distinct transition proteins, TP1 and TP2, and two distinct protamines (P1 and P2) have so far been identified. The transition proteins 1 and 2 are small highly basic proteins that are unique to the testis and are believed to contribute to, but perhaps not initiate, the termination of RNA synthesis during spermiogenesis (Kistler *et al.*, 1975, 1976). DNA sequence analyses of transition protein cDNA and genomic clones from the mouse (Kleene & Flynn, 1987; Kleene *et al.*, 1988) and rat (Cole & Kistler, 1987; Heidaran & Kistler, 1987a; Heidaran *et al.*, 1988) have established that TP1 and TP2 are distinct. The existence of additional transition proteins currently identified only as protein bands in polyacrylamide gels remains to be established. We cannot exclude the possibility that post-translational modifications of TP1, TP2 or other proteins have created the additional electrophoretic bands (Meistrich *et al.*, 1981). Towards the end of spermiogenesis (steps 12 to 15 in the mouse), the transition proteins are displaced by the two mouse protamines, the predominant proteins of the mammalian sperm nucleus.

The spermatozoa of most mammals contain either the P1 protamine or a mixture of the P1 and P2 protamines (reviewed by Balhorn, 1989; Hecht, 1989a, b). Both protamines are found in the

```
                            10                    20                   30                    40                 50
Mouse     A R Y R C C R S K S R S R C R R R R R   R C R R R R R R   C C R R R R R R     C C R R R R S   Y T   I R C K K Y
Rat       - - - - - - - - - - - - - - - - - - -   - - - - - - - -   - - - - - - - -     - - - - - - -   - -   F - - - R -
Boar      - - - - - - - - H - - - - - - P - - -   - - - - - - - -   - - P - - - -(-)A V - - - -         - - V - - - R R C
Human     - - - - - - - - Q - - - - Y Y - Q - Q   - S - - - - - -   S - Q T - - - A M R - - - P -       - R   P - - R R H
Bull      - - - - - - L T H - G - - - - - - - -   - R C - - - - -   R F G - - - - - R V - - - -         - - V - - - T R Q
Ram       - - - - - - L T H - - - - - - - - - -   - R C - - - - -   R F G - - - - - R V - - - -         - - V V - - T R Q
Goat      - - - - - - L T H - - - - - - - - - -   - R C - - - - -   R F G - - - - - R V - - - -         - - V V - - T R Q
Stallion  - - - - - - - - Q - Q - - - - - - - -   - R C - - - - -   R S V - Q - -     V - - - -         - - V L - - R R R R
Rabbit    V - - - - - - - Q - - - - - - - - - -   - - - - - - - -   - - Q - - - V -   K - - - -     T   - -   L - - R R -

Trout                       M P - - - - S***P V - - - - - * R V S - - - - - -     G G - - - -
```

Fig. 1. Comparison of the mammalian P1 protamine and trout protamine sequences. Spaces have been introduced to best align the protamines; – indicates identical amino acid; * indicates regions of nucleotide heterogeneity in six trout protamine genes. A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I =Ile; K = Lys; L = Leu: M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; Y = Tyr. Sources of sequences are: mouse (Kleene *et al.*, 1985; Bellve *et al.*, 1988); rat (Ammer & Henschen, 1988); boar (Tobita *et al.*, 1983; Maier *et al.*, 1988); human (McKay *et al.*, 1985; Ammer *et al.*, 1986; Lee *et al.*, 1987b); bull (Mazrimas *et al.*, 1986); ram (Ammer & Henschen, 1988); goat (Ammer & Henschen, 1988); stallion (Belaiche *et al.*, 1987; Ammer & Henschen, 1987); rabbit (Ammer & Henschen, 1987); and trout (Dixon *et al.*, 1985).

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(a) Mouse TP1

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                                  1                                  10
                        Met Ser Thr Ser Arg Lys Leu Lys Thr His Gly Met Arg Arg Gly Lys Asn
            5'    GAAAGTACC ATG TCG ACC AGC CGC AAG CTA AAG ACT CAT GGC ATG AGG AGA GGC AAG AAC
                 -9         1                                  30

                20                                  30
    Arg Ala Pro His Lys Gly Val Lys Arg Gly Gly Ser Lys Arg Lys Tyr Arg Lys Ser Val Leu Lys Ser
    CGA GCT CCT CAC AAG GGC GTC AAG AGA GGT GGA AGC AAG AGA AAA TAC CGG AAG AGC GTC CTG AAA AGT
              60                                  90                                  120
    40                                  50
    Arg Lys Arg Gly Asp Asp Ala Ser Arg Asn Tyr Arg Ser His Leu ***
    AGG AAA CGG GGC GAT GAT GCA AGT CGC AAT TAC CGA TCC CAC TTG TGA TGCGGCAATG AGCTCTGCCC
                                       150                                 180


    TGGTGGTCTT CAAACAACAC GGGGCAGGAG CATGAGGACA TCAGAGGGGG ACTGCCAAAG AGATCTGAAG TTAGACCAAA
                          210                                 240

    AGCCAAAGAT CCTATCAGAG TGGGTAAATG CCAGTCGTGA CGAAATTCGG AATGTATATG TTGGCTGTTT CTCCCCAACA
  270                                 300                                 330

    TCTCAATAAC ATTTTGAAAA CAAATAAAAT TGTGAAAAAC AAAAAAAA       3'
              360                 390
```

(b) Mouse P1

```
                                                                                    1
                                                                                  Met Ala Arg
5'   GACCCCTGCTCACAGGTTGGCTGGCTCGACCCAGGTGGTGTCCCCTGCTCTGAGCCAGCTCCCGGCCAAGCCAGCACC ATG GCC AGA
    -78                -60                -30                                      1
                                    10                                  20
    Tyr Arg Cys Cys Arg Ser Lys Ser Arg Ser Arg Cys Arg Arg Arg Arg Arg Arg Cys Arg Arg Arg Arg
    TAC CGA TGC TGC CGC AGC AAA AGC AGG AGC AGA TGC CGC CGT CGC AGA CGA AGA TGT CGC AGA CGG AGG
                              30                                  60
                    30                                  40
    Arg Arg Cys Cys Arg Arg Arg Arg Arg Arg Cys Cys Arg Arg Arg Arg Ser Tyr Thr Ile Arg Cys Lys
    AGG CGA TGC TGC CGG CGG AGG AGG CGA AGA TGC TGC CGT CGC CGC CGC TCA TAC ACC ATA AGG TGT AAA
              90                                  120
      50
    Lys Tyr ***
    AAA TAC TAG ATGCACAGAATAGCAAGTCCATCAAAACTCCTGCGTGAGAATTTTACCAGACTTCAAGAGCATCTCGCCACATCTTGAA
    150                                 180                                 210

    AAATGCCACCGCCGATGAAAAACAGGAGCCTGCTAAGGAACAATGCCACCTGCCAATAAATGTTGAAAACTCAAAAAAAAAAAAAAAAAAA  3'
      240                               270                                 300                       326
```

(c) Mouse P2

```
          Met Val Arg Tyr Arg Met Arg Ser Pro Ser Glu Gly Pro His Gln Gly Pro Gly Gln Asp
    5'  CC ATG GTT CGC TAC CGA ATG AGG AGC CCC AGT GAG GGT CCG CAC CAG GGG CCT GGA CAA GAC
          1                                   30                                  60

    20                                  30                                  40
    His Glu Arg Glu Glu Gln Gly Gln Gly Gln Gly Leu Ser Pro Glu Arg Val Glu Asp Tyr Gly Arg
    CAT GAA CGC GAG GAG CAG GGG CAG GGG CAA GGG CTG AGC CCA GAG CGC GTA GAG GAC TAT GGG AGG
                                        90                                  120

                                  50                                  60
    Thr His Arg Gly His His His His Arg His Arg Arg Cys Ser Arg Lys Arg Leu His Arg Ile His
    ACA CAC AGG GGC CAC CAC CAC CAC AGA CAC AGG CGC TGC TCT CGT AAG AGG CTA CAT AGG ATC CAC
                                150                                 180

                          70                                  80
    Lys Arg Arg Arg Ser Cys Arg Arg Arg Arg Arg His Ser Cys Arg His Arg Arg Arg His Arg Arg
    AAG AGG CGT CGG TCA TGC AGA AGG CGG AGG AGA CAC TCC TGC CGC CAC AGG AGG CGG CAT CGC AGA
                        210                                 240

                  90                                  100
    Gly Cys Arg Arg Ser Arg Arg Arg Arg Arg Cys Arg Cys Arg Lys Cys Arg Arg His His His ***
    GGC TGC AGA AGA TCC CGA AGG AGG AGG AGA TGC AGG TGC AGG AAA TGT AGG AGG CAC CAT CAC TAA
                270                                 300

    GCCTCCCCAGGCCTGTCCATTCTGCCTGGAGCCAAGGAAGTCACTTGCCCAAGGAATAGTCACCTGCCCAAGCAACATCATGTGAGGC
        330                               360                                 390

    CACACCACCATTCCATGTCGATGTCTGAGCCCTGAGCTGCCAAGGAGCCACGAGATCTGAGTACTGAGCAAAGCCACCTGCCAAATAAA
                420                 450                                 480

    GCTTGACACGAGAAAAAAAAAAAAAAAAA  3'
            510
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sperm nuclei of the mouse, human, stallion and certain hamsters and primates, whereas only the P1 protamine is detectable in mature spermatozoa of species such as the bull or rat (Calvin, 1976). Considering the essential role of protamine in compacting the nucleus to produce a functional spermatozoon, this variability is somewhat surprising. Moreover, the absence of P2 in the spermatozoa of many species is of considerable interest since sequences homologous to the mouse protamine 2 gene have been detected in the genomic DNAs of mammals whose spermatozoa contain no detectable P2 (Johnson *et al.*, 1988b). The recently characterized rat protamine 2 gene shows specific nucleotide differences from the mouse protamine 2 gene that may reflect regulatory differences leading to the differential expression of P2 in mouse and rat (Tanhauser & Hecht, 1989; Bunick *et al.*, 1990a).

In the mouse, both protamines are single copy genes that are closely linked genetically on the proximal end of mouse chromosome 16 (Hecht *et al.*, 1986; Reeves *et al.*, 1989). In the human, protamine 1 is also located on chromosome 16 (Reeves *et al.*, 1989). In view of the tight linkage of protamines 1 and 2 in mouse and hamster, it will be interesting to see whether the human protamine 2 gene(s) are also closely linked to the protamine 1 gene.

The P1 protamine is a 50- or 49-amino acid molecule with a central highly basic core of polyarginines, a highly conserved N-terminus, and a highly variable C-terminus (Fig. 1). Although the N termini of P1 are virtually invariant among mammals, substantial differences are seen among species for the remainder of the protein (Figs 1, 2). Compared to histones, the mammalian protamines appear to be evolving rapidly.

Although the P2 protamines also contain over 50% arginine, they differ considerably from P1 protamines in size, composition and sequence. The P2 protamine isolated from mouse spermatozoa is 63 amino acids long and has been shown to be synthesized as a precursor of 106 amino acids (Yelick *et al.*, 1987) (Fig. 2). The mouse P2 precursor is sequentially processed in the nucleus of the elongating spermatid (Balhorn *et al.*, 1984). In human spermatozoa, two P2 protamine variants exist (Ammer *et al.*, 1986; McKay *et al.*, 1986) (Fig. 3). Although clearly showing similarity to the mouse P2, they differ in sequence and size, being proteins of 57 and 54 amino acids, with the smaller human P2 protamine lacking a tripeptide at the N terminus. Whether both human protamine 2 sequences are transcribed from one gene is not known. The protamine 2 gene from the rat codes for a putative precursor of protamine 2 (Bower *et al.*, 1987) and shows high homology to the mouse protamine 2 gene (Bunick *et al.*, 1990a) (Fig. 4). Why the mouse protamine 2 and presumably other protamine 2 proteins are translated as precursor molecules requiring post-translational processing while protamine 1 requires none is not apparent. Reasons as to why no mature rat protamine 2 has been found in rat spermatozoa will be considered later.

Both mammalian P1 and P2 protamines are rich in the amino acids arginine and cysteine. The P2 protamine of the mouse also contains about 20% histidine, an amino acid absent in the mouse P1 protamine. While the histidines and cysteines in protamine 2 do not form zinc fingers as has been found for many other DNA-binding proteins, the combination of these amino acids in protamine 2 suggests that metal chelation could occur in the nucleus of the mammalian spermatozoon (Kvist & Eliasson, 1978).

Fig. 2. Sequences of cDNAs and their predicted amino acids for mouse transition protein 1 and mouse protamines 1 and 2. The predicted amino acid sequences are presented above the nucleotides. Amino acids are numbered beginning with serine for TP1, alanine for P1, and valine for P2 because the initiating methionine is removed from the termini of these proteins. The vertical line after amino acid 43 of the P2 sequence indicates the site of the final cleavage producing the mature form of mP2 found in spermatozoa. The termination codons, TGA for TP1, TAG for P1, and TAA for P2, are denoted by asterisks. The canonical hexanucleotide AATAAA is underlined in each cDNA sequence. The common protamine 3′ untranslated repeat sequence is denoted by a line over it.

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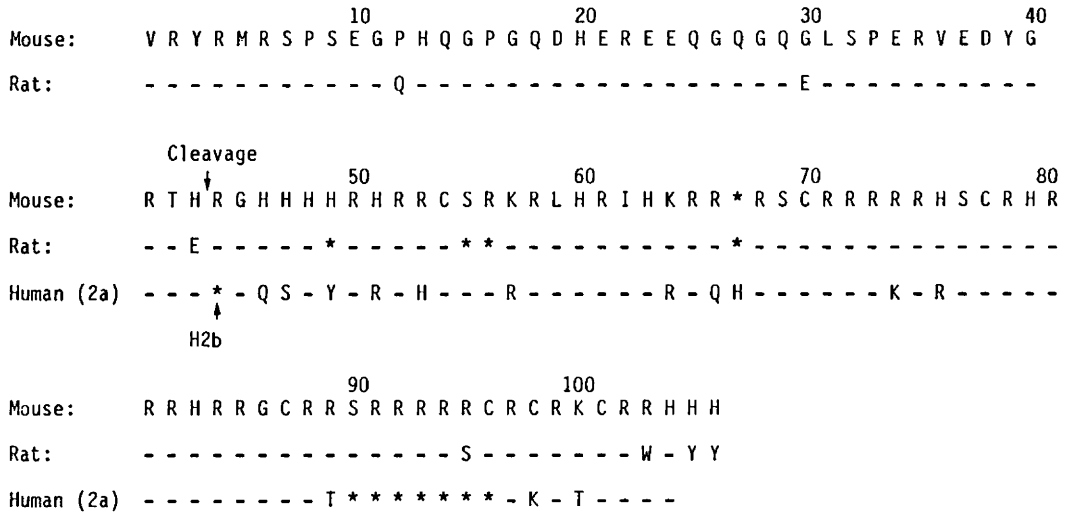

Fig. 3. Protamine 2 sequences for mouse, rat and man. – indicates identical amino acid; * indicates deleted amino acid; A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; Y = Tyr. Sources of sequences are: mouse (Yelick *et al.*, 1987); rat (Tanhauser & Hecht, 1989); human (McKay *et al.*, 1986; Ammer *et al.*, 1986; Domenjoud *et al.*, 1988).

Post-meiotic expression of transition protein 1 and the protamines

It has been demonstrated in several laboratories that the transition proteins and protamines are synthesized towards the end of spermiogenesis (Bellve *et al.*, 1975; Geremia *et al.*, 1976; Mayer & Zirkin, 1979; Mayer *et al.*, 1981; Meistrich *et al.*, 1981; Bhatnagar *et al.*, 1983). To define when the genes for these proteins are transcribed in the mouse, we have utilized cDNAs for the mouse TP1 and P1 and P2 as probes for hybridization studies. Northern blot analyses of RNA extracts of the testes of prepubertal mice indicate that the TP1 and protamine mRNAs are detectable around Day 22, a time when 8–10% of the testicular germ cells have differentiated to round spermatids (Hecht *et al.*, 1986). Additional studies with RNA samples from isolated populations of pachytene spermatocytes, round spermatids, elongating spermatids and residual bodies have confirmed that no TP1 or protamine mRNAs are detectable in primary spermatocytes although abundant amounts of TP1 and P1 and P2 mRNAs are found in all the post-meiotic RNA samples (Kleene *et al.*, 1984; Yelick *et al.*, 1989).

Although we are able to distinguish between events in meiotic and post-meiotic cell types, Northern blot hybridization analyses of RNAs prepared from populations of isolated spermatogenic cells are limited by the cell types obtained and the purity of the preparations. To define better the precise times of spermiogenesis when transition protein 1 and protamine 1 and 2 mRNAs are present, we have used in-situ hybridization techniques (Hecht & Penschow, 1987; Mali *et al.*, 1988). In the rat, the P1 and P2 mRNAs are first detectable in the middle of step 7 of spermiogenesis during substage VIIc. The mRNA levels are high in step 7–14 spermatids, decrease in step 15–16 spermatids and are virtually undetectable in step 17–19 spermatids. In the rat, TP1 mRNAs can be detected during step 6 of spermiogenesis and levels of TP1 mRNA decrease during spermiogenesis before the protamine mRNAs decline. Considering that protamines replace transition proteins during spermiogenesis, the earlier appearance and disappearance of TP1 mRNAs detected by in-situ hybridization are consistent with the pattern of protein synthesis for these DNA-binding

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proteins. The presence of TP1 and P1 and P2 mRNAs long before their respective proteins are synthesized argues for translational regulatory controls, a topic that will be discussed below.

Having established the post-meiotic expression of the mouse transition protein 1 and the two protamine genes, we have attempted to use these representative haploid genes to investigate the control mechanisms for temporal gene regulation in the mammalian testis. Our efforts have focussed on DNA structural changes produced by methylation and demethylation of cytosines in the DNA and on the role(s) *cis*-acting DNA elements and *trans*-acting factors play in regulating transcription.

Regulation at the DNA level

The regulatory roles of DNA methylation in eukaryotic gene expression are a topic of controversy (reviewed by Cedar, 1988). Although an extensive literature correlating reduced levels of methylation and increased transcriptional activities of genes exists, there are exceptions (Razin & Szyf, 1984). Adding to this confusion, many methylation studies attempt to relate transcriptional events occurring in one individual cell type to methylation changes found in DNA that was prepared from the mixed population of cell types found in most tissues.

The testis provides an exellent system to relate methylation changes and gene expression. In addition to the availability of cDNA and genomic DNA probes for numerous temporally well defined genes, it is possible to dissect the changing methylation states of testicular DNAs by analysis of DNA prepared from highly purified populations of cell types ranging from type A spermatogonia to spermatozoa. We have compared the methylation states of several meiotically and post-meiotically expressed testicular genes to genes constitutively expressed in the testis by using the methylation-sensitive and methylation-insensitive restriction enzymes, Hpa II, Hha I, and Msp I (unpublished). Using Southern blots for analysis, the mouse TP1, P1 and P2 genes show different methylation patterns in testicular and somatic tissues. The sites monitored by the restriction enzymes in the coding and 5′-flanking regions of the TP1 gene were predominantly methylated in spleen DNA, whereas in testis DNA the sites became progressively less methylated between Days 6 and 22 after birth and were mostly unmethylated in testis DNA of adult mice. In contrast, the sites detected by the same enzymes in the coding and flanking regions of the mouse P1 and P2 genes were predominantly methylated in the testis of adult mice. Analysis of DNAs from isolated testicular cell types revealed that the methylation changes in the TP1, P1 and P2 genes occurred in germ cells between the type B spermatogonial stage and pachytene spermatocytes. Control genes showed no methylation changes in DNA from prepubertal and adult testes. These results demonstrate that a pre-meiotic switching of methylation patterns for temporally expressed testicular genes occurs early during germ cell development in the testis. The fact that the demethylation of the mouse TP1 gene and the increased methylation states of the P1 and P2 genes occur before their expression suggests that complex chromatin regulatory changes underlie methylation regulation in the mammalian testis.

Transcriptional regulation

The interaction between *cis*-acting DNA regulatory elements of a gene and *trans*-acting factors plays a prominent role in the regulation of transcription in eukaryotic cells. To define the *cis*-acting DNA elements of the protamine genes, genomic clones for mouse protamines 1 and 2 have been isolated and sequenced (Johnson *et al.*, 1988a). Although the coding regions of the protamine 1 and 2 genes share little sequence homology, there are several similar or identical sequences in the 5′-flanking regions and one strongly conserved sequence in the 3′ untranslated region of the protamine genes. Directly upstream and adjacent to the AATAAA polyadenylation signal is a nucleotide sequence which is highly conserved between P1 and P2 (Fig. 2). Of the nucleotides in the sequence, $GA^{A}_{G}CAA^{T}_{A}GCCACCTGCC$, 15 are identical in both protamine genes. This sequence, which

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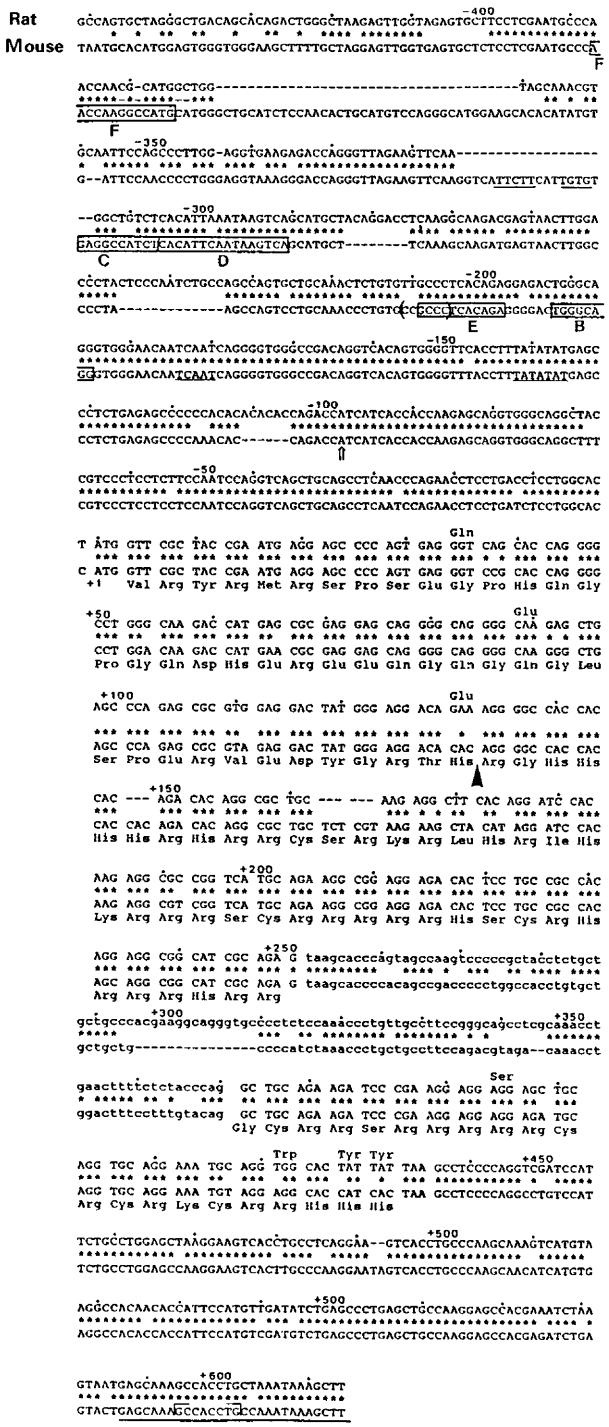

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contains an octamer from an immunoglobulin enhancer, has been maintained in the protamine 1 gene of mouse (Kleene *et al.*, 1985), human (Lee *et al.*, 1987b), boar (Maier *et al.*, 1988) and bull (Lee *et al.*, 1987a; Krawetz *et al.*, 1987, 1988), and in the protamine 2 genes of mouse (Yelick *et al.*, 1987; Johnson *et al.*, 1988a), rat (Tanhauser & Hecht, 1989) and human (Domenjoud *et al.*, 1988).

To provide a functional assay for putative control elements in the conserved 5′-flanking sequences, fusion genes containing potential mouse P1 or P2 control sequences linked to reporter genes were introduced into mice by microinjection into fertilized eggs. The transgenic mice that were produced were analysed to assess whether the constructs contained specific regulatory elements which could direct both tissue-specific and temporal transcription in the testis. Constructs containing a 880-nucleotide 5′-flanking sequence in a 2·4 kb DNA fragment of mouse protamine 1 (Peschon *et al.*, 1987) or an 859-nucleotide 5′-flanking sequence for protamine 2 linked to the proto-oncogene myc or the SV40 T antigen (Stewart *et al.*, 1988) were tested by injection into fertilized mouse eggs. The transgenes exhibited regulated transcription in round spermatids, indicating that the 5′-flanking regions of the mouse P1 or P2 protamine genes contain the *cis*-acting elements essential for post-meiotic transcription.

To help characterize testicular *trans*-acting factors that presumably recognize the *cis*-acting elements of the protamine genes, we are following two complementary approaches. Using DNA gel retardation and DNA footprinting techniques, we have sought to define DNA–protein interactions. To date we have found by gel retardation studies that several DNA fragments subcloned from the 859-nucleotide 5′-flanking region of the protamine 2 gene bind protein and are retarded during migration in DNA gel shift assays (P. A. Johnson & N. B. Hecht, unpublished observation). Footprinting analysis will help to define the specific DNA sequences recognized by the putative *trans*-acting factors.

A second approach, in-vitro transcription of testicular and somatic gene constructs in testicular extracts, provides a more functional assay for critical testicular DNA–protein interactions. Using transcriptionally active extracts prepared from whole mouse or rat testes or isolated seminiferous tubules, sequences essential for protamine gene transcription *in vitro* have been determined for the mouse protamine 2 gene (Bunick *et al.*, 1990b). Sequential deletion of regions of the 5′-flanking region of the protamine 2 gene reveals that sequences required for positive promotion of the gene lie within the −170 to −82 nucleotides upstream from the start of transcription (Dignam *et al.*, 1983). The testis-derived transcription system appears unique among mammalian in-vitro systems in its temperature optimum. Adult testis extracts give maximal RNA synthesis at 20°C whereas a prepubertal testis extract favours 30°C. The availability of an in-vitro transcription system that mimics in-vivo gene expression in the testis, the testis offers a powerful means to define testicular transcriptional regulatory factors.

Translational regulation

The required synthesis of many sperm proteins towards the end of spermatogenesis long after transcription has terminated necessitates the storage and translational regulation of mRNA. It is likely that the mechanisms regulating expression of the transition proteins and protamines will be representative of the many testicular proteins synthesized in the later stages of spermiogenesis. Analysis of testicular extracts reveals that the vast majority of the mouse TP1, P1 and P2 mRNAs are present in the ribonucleoprotein (non-polysomal) fractions of a testicular extract (Kleene *et al.*,

Fig. 4. Sequences of the protamine 2 genes of rat (R) and mouse (M). The nucleotide numbers are for the rat. Potential upstream promoter sequences are boxed and indicated by a letter. +1 indicates the start of transcription. The triangle indicates the processing site for the mature form of the mouse protamine 2. Where the protein sequences between the rat and mouse genes differ, the rat amino acid is written above its sequence. Note: In contrast to Fig. 3, in this figure asterisks (*) indicate identity and dashes (–) indicate deletions. (Modified from Bunick *et al.*, 1990a).

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1984; Yelick *et al.*, 1989). Moreover, analysis of the cellular distribution of mRNAs from isolated populations of cell types demonstrates that mouse TP1, P1 and P2 mRNAs are first detected in round spermatids with mRNA sizes of 600, 580 and 830 nucleotides, respectively. In elongating spermatids, the cell type in which the DNA binding proteins are synthesized, the mRNAs decrease in length. The mouse TP1 mRNAs are heterogeneous in size, varying from 480 to 600 nucleotides while mouse P1 and P2 mRNAs decrease to about 450 and 700 nucleotides, respectively. The shortened transcripts for all 3 genes are found on polysomes whereas the longer mRNAs are present in the ribonucleoprotein post-monosomal fraction of polysome gradients. We conclude that the mouse TP1, P1 and P2 mRNAs are initially transcribed during mid-spermiogenesis and stored as ribonucleoproteins in the cytoplasm of round spermatids until translation 3–7 days later. The translated forms of TP1 and the protamine mRNAs are shortened as a result of partial deadenylation of the mRNAs. The changes in mRNA length between stored and translated mRNAs are not unique to the mouse or its DNA-binding proteins. Similar reductions of protamine mRNA length during spermiogenesis have been seen in trout (Dixon *et al.*, 1985), rat (Bower *et al.*, 1987) and hamster (Bower *et al.*, 1987), and for other testicular genes in rodents (N. B. Hecht, unpublished observations).

Although the relationship between translational activation and partial deadenylation is not understood, the 3′ untranslated region of the protamine mRNA seems to be essential for temporal expression during spermiogenesis. This has been elegantly established from transgenic mouse studies with a protamine 1 promoter and a human growth hormone reporter gene (Braun *et al.*, 1989b). The protamine 1 promoter directs transciption of this construct to the round spermatid. Depending upon the 3′ untranslated sequence of the construct, the mRNA is either translated immediately or stored. For example, when the 3′ untranslated region from growth hormone is substituted for the 3′ untranslated region of protamine, the mRNAs are translated immediately in round spermatids. Replacement of the 3′ untranslated region with the P1 3′ untranslated region delays the translation of the growth hormone mRNA until later in spermiogenesis, to the same time that protamine proteins are normally synthesized. These studies suggest that information for temporal expression is present in the 3′ untranslated regions of protamine mRNAs.

Sequence analysis of several protamine 1 and protamine 2 cDNAs or genes has revealed a conserved sequence straddling the AATAAA polyadenylation signal in the 3′ untranslated region of the protamine molecules. Included in this sequence of about 24 nucleotides is the octamer E motif sequence of GCCACCTG (Staudt *et al.*, 1988). Although the precise function of this sequence has not been established, the strong conservation of this nucleotide sequence in a gene whose coding region is highly variable among mammals suggests that it is important.

Why is protamine 2 absent in rat spermatozoa?

Although the rat and mouse are closely related in terms of evolutionary time, the composition of their sperm nuclei differs substantially. Mouse spermatozoa contain both protamine 1 and 2 (in a ratio of 1:2) whereas only protamine 1 has been detected in rat spermatozoa (Calvin, 1976; Balhorn *et al.*, 1984). This difference is not based upon genomic differences between mouse and rat because the rat genome contains a gene that codes for a putative protamine 2 precursor (Tanhauser & Hecht, 1989). In fact, sequence comparison of the mouse and rat protamine 2 precursor genes indicates that, at both the nucleotide and amino acid sequence levels, there is greater than 90% similarity between these genes (Fig. 4) (Bunick *et al.*, 1990a). Why then do we not find protamine 2 in rat spermatozoa? We believe that the absence of rat protamine 2 protein in spermatozoa is due to a reduced level of protamine 2 precursor mRNA and perhaps also to an inability of the rat testis to process completely the rat protamine 2 precursor (see below).

To determine whether the rat protamine 2 precursor gene is transcribed, RNA was isolated from rat testes and hybridized to the mouse protamine cDNAs. Comparison of protamine 1 and 2

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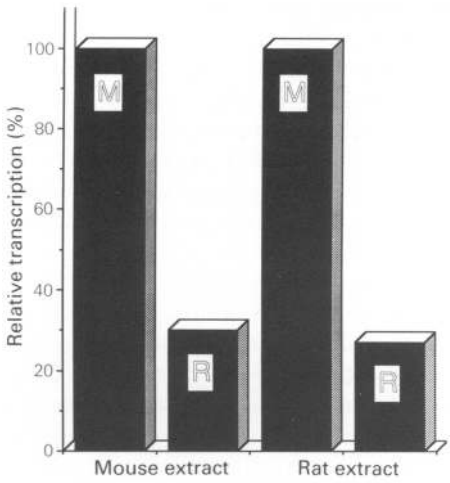

Fig. 5. Comparison of template abilities of rat and mouse protamine genes *in vitro*. M represents a mouse construct, R represents a rat construct. Equimolar amounts of rat and mouse protamine 2 template are added to mouse or rat testicular extracts prepared from sexually mature animals. Transcription conditions are as described by Bunick *et al.* (1990a). Variation between 3 independent assays was less than 10%.

mRNA levels in testicular extracts prepared from sexually mature mice and rats reveals that approximately equal amounts of protamine 1 mRNA are present in mouse and rat, while rat protamine 2 precursor mRNA is present at a level greatly reduced compared to that of mouse protamine 2 precursor (Bower *et al.*, 1987). This reduction in the steady state level of rat protamine 2 precursor mRNA suggests that transcription is reduced and/or that the rat protamine 2 precursor mRNA is less stable.

To evaluate possible transcriptional differences between the mouse and rat protamine 2 gene promoters, in-vitro transcription in rat and mouse testicular extracts was compared. When equivalent constructs of the rat or mouse protamine 2 genes are used as templates for in-vitro transcription, mouse and rat testicular extracts both support transcription. However, when equimolar amounts of rat and mouse protamine 2 templates are jointly transcribed in the same reaction mixture, the mouse template is transcribed 3–4-fold better than the equivalent rat template (Bunick *et al.*, 1990a) (Fig. 5). The in-vitro level of transcription of the rat protamine 2 gene ($\sim$30% of the amount of the mouse protamine 2 gene) is substantially higher than the approximately 3–5% in-vivo steady state levels of rat protamine 2 precursor determined by Northern blot analysis (Bower *et al.*, 1987). This disparity could reflect a real difference between transcription *in vitro* and *in vivo* or may be due to a higher turnover rate for the rat protamine 2 percursor mRNA *in vivo*.

The presence of rat protamine 2 precursor mRNA on polysomes and the detection of a rat protamine 2 precursor protein in epididymal spermatozoa suggests that protamine 2 precursor is synthesized in the rat (Bunick *et al.*, 1990a). Comparison of the predicted amino acid sequence of mouse and rat protamine 2 reveals differences in three amino acids in the putative precursor part of the protamine 2 molecule. Compared to the mouse, the proline, glycine and histidine at amino acid positions 12, 30 and 43 are replaced by glutamine, glutamate, and glutamate in the rat. Amino acids 12 and 43 serve as known processing sites for the mouse protamine 2 precursor (R. Balhorn, personal communication). The fact that the rat and mouse protamine 1 differ by two amino acids out of 50 suggests that the protamine 2s are also likely to be very similar. Assuming that the rat protamine 2 precursor requires processing at the same sequence sites used by the mouse, the amino acid substitutions at processing sites could explain the absence of any detectable mature protamine 2 in rat spermatozoa.

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Significance of haploid gene expression

The segregation of chromosomes during spermatogenesis produces post-meiotic haploid cells that are genetically distinct. Transcription from the genome of the post-meiotic spermatid would lead to differential expression and presumably transmission of specific alleles to each gamete. This differential haploid gene expression has been used to explain the transmission ratio distortion in t-haplotypes, a phenomenon in which male mice heterozygous for a complete t-haplotype transmit the t-haplotype chromosome with frequencies far above the distribution predicted by Mendelian inheritance (Silver, 1985).

Although genetically distinct, the maturing haploid male germ cells develop in a syncytium. Intracellular bridges, about 1 μm in diameter, connect the cells thereby ensuring synchronous development of clones of cells (Dym & Fawcett, 1971). Until recently it was not known whether macromolecules could pass between the cytoplasm of connected cells. Using a transgenic mouse with a construct containing the protamine 1 promoter and a human growth hormone reporter gene, Braun *et al.* (1989a) have demonstrated in hemizygous transgenic mice that the human growth hormone reporter sequence of the transgene is shared among genetically distinct spermatids. Moreover in-situ hybridization analyses suggest that human growth hormone mRNAs move through the intercellular syncytial bridges. If human growth hormone mRNAs are representative of all testicular transcripts, products of post-meiotic genes should be equally distributed among spermatids. Whether this means that all post-meiotic gene products will be shared by developing gametes remains to be established.

Conclusions

The development and differentiation of eukaryotic cells is dependent upon the ordered temporal expression of groups of gene products. In the testis, a continuum of cells differentiate predictably in a well ordered sequence of events. Diverse biochemical studies have identified and defined the time of expression of numerous testicular marker proteins. The three DNA-binding proteins, transition protein 1 and protamine 1 and 2, represent three abundant nuclear structural proteins that are expressed post-meiotically. All three are initially transcribed from the haploid genome and are translationally regulated during spermiogenesis. Comparative regulatory studies between these three post-meiotic genes and some of the many additional sperm proteins involved in the massive structural changes during spermiogenesis will help to define gene-specific, stage-specific, cell type-specific and general regulatory mechanisms for male germ cells. Such analyses will provide an important understanding of the control mechanisms for developmental gene expression in the mammalian testis.

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