Characterization of the testes-specific *pim-1* transcript in rat

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ABSTRACT

The *pim-1* proto-oncogene encodes a serine/threonine protein kinase and is expressed in cells of hemato-lymphoid origin and in the germ cell lineages. In somatic cells, the *pim-1* gene is expressed as a 2.8 kb transcript while a shorter sized transcript (2.3 kb) is expressed in rat testes. We have determined that the shorter testes-specific *pim-1* transcript arises through the use of an alternate polyadenylation signal present in the 3' untranslated region of the gene. This alternate polyadenylation event results in the removal of an A/U-rich regulatory element located in the 3' untranslated region of the *pim-1* gene. This A/U-rich motif has been shown by a number of laboratories to destabilize the transcripts of genes that contain this sequence. Consistent with these findings, we have demonstrated that the shortened testes-specific *pim-1* transcript is more stable than the longer A/U-rich containing somatic transcript. We suggest that the functional significance of different sized *pim-1* transcripts may be directly related to their different stabilities and that the greater stability of the testes-specific transcript may be essential for the translational delay observed in post-meiotic male germ cells.

INTRODUCTION

Many proto-oncogenes have been recognized as regulatory elements in signal transduction pathways. Although the function of proto-oncogenes is generally assumed to involve the mediation of proliferative signals, some proto-oncogenes have also been shown to be expressed and regulated during cellular differentiation. Recent evidence suggests that the *pim-1* proto-oncogene, which encodes a serine/threonine kinase (1—3), may be involved in the signal transduction of both mitogenic (4—7) and differentiation (7,8) pathways. The *pim-1* proto-oncogene was first identified by its frequent activation by proviral insertion in murine leukemia virus-induced T cell lymphomas (9,10). In a majority of the tumors (>70%), provirus was found to preferentially integrate in the 3' untranslated region of the *pim-1* gene (11). This preferential integration may play a crucial role in cellular transformation as proviral integration in the 3'

untranslated region results in the removal of A/U-rich sequences implicated in the mRNA destabilization of other A/U-rich containing genes (12—14). Elimination of this destabilizing motif in the *pim-1* gene may directly contribute to the higher steady state levels of *pim-1* mRNA observed in MULV-induced lymphomas by increasing the stability of truncated *pim-1* transcripts. The over-expression of *pim-1* mRNA has also been observed in many myeloid and lymphoid leukemias (15—17) and may be intimately linked to oncogenic cell transformation. In addition to the potential destabilizing role in MuLV-induced T-cell lymphomas, the A/U-rich motif has also recently been implicated in the modulation of *pim-1* mRNA stability in mitogen stimulated lymphocytes (7).

Expression of the *pim-1* gene is restricted to germ cells (8,18) and to the hemato-lymphoid cell lineages (10,15,16). The *pim-1* gene is expressed as a 2.3—2.4 kb transcript in the rat and mouse testes and as a 2.8 kb transcript in somatic tissues. The testes-specific *pim-1* transcript has been shown to be selectively expressed in haploid post-meiotic early spermatids (8). This developmentally regulated stage-specific expression of the *pim-1* gene suggests an involvement of the Pim-1 kinase in the signal transduction events associated with normal germ cell maturation.

In addition to the *pim-1* gene, a large number of other genes including abl (18), t-fer (19), calmodulin (20), and the cAMP-dependent protein kinase (PKA) regulatory and catalytic subunits (21,22) have also been shown to express testes-specific transcripts that are shorter than the somatic transcripts. This pattern suggests that mechanisms specific to germ cells may alter pre-mRNA processing or the specificity of transcription. Here we report the cloning and characterization of the testes-specific 2.3 kb rat *pim-1* transcript and demonstrate that the shorter size of the testes-specific transcript is due to the use of an alternate polyadenylation site in the gene. The use of an alternate polyadenylation site in the testes-specific *pim-1* gene effectively removes the destabilizing A/U-rich sequences normally present in the longer 2.8 kb somatic *pim-1* transcript. In addition, in actinomycin D experiments, we observed that the shorter testes-specific *pim-1* transcript is substantially more stable than the longer somatic A/U-rich containing rat *pim-1* transcript. We suggest that the higher stability of *pim-1* transcripts expressed in germ cells is related to the deletion of the destabilizing A/U-rich sequence motif.

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Furthermore, we postulate that the increased stability of testes-specific *pim-1* transcript may be essential for the characteristic translational delay observed in post-meiotic germ cells (23–25).

MATERIALS AND METHODS

Library screening

A lambda gt11 adult rat testes cDNA library (a generous gift from Dr. Michael Griswold, Washington State University) and a lambda gt10 adult rat testes cDNA library (obtained through Clonetech, Palo Alto, CA) were screened for *pim-1* cDNA clones using a human *pim-1* cDNA hybridization probe (26). Approximately 7.5×10^5 phage plaques from the lambda gt11 library and 1.6×10^6 plaques from the Clonetech library were transferred to nitrocellulose filters, denatured, neutralized, baked, and prehybridized for at least 4 h in 30% formamide, 5×SSPE, 1×Denhardts, 0.1% SDS, and 20 µg/ml denatured salmon sperm DNA at 42°C. Filters were subsequently hybridized overnight with a radiolabeled 2669 bp EcoR1 fragment of the human *pim-1* cDNA (26). Filters were washed in 2×SSC, 0.5% sarcosyl, and 0.5% sodium pyrophosphate at 50°C and autoradiographed. Approximately forty-two positive clones were identified and the cloned inserts were removed from lambda vector DNA using the polymerase chain reaction as previously described (27). Briefly, PCR DNA amplification was performed using primers constructed to vector regions flanking the EcoR1 cloning site and template DNA was prepared by lysing a single phage plaque in 200 µl of water. A typical reaction consisted of a 50 µl aliquot of lysed phage template, 50 pmol of each primer, 0.2mM each dNTP, 10 µl of 10×Promega (Madison, WI) reaction buffer, and 2.5 units of Promega *Taq* DNA polymerase. The reaction mixture was heated to 95°C for 2.5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 5 min. PCR products were purified on a 0.7% low melting agarose gel and blunt-end ligated into the EcoRV site of Bluescript plasmid (Stratagene, La Jolla, CA).

PCR and 3′RACE

3′RACE (rapid amplification of cDNA ends) as previously described (28), was performed on rat germ cell RNA. An adapter-dT primer (5′ GACTCGAGTCGACATCGA-TTTTTTTTTT-TTTTTTT 3′) was used to synthesize cDNA. The reaction contained 2 µg of total rat germ cell RNA, 10 pmol of primer, 1 U of AMV (Promega) reverse transcriptase, 0.2 mM dNTP's, 10 µl of 10 reaction buffer, and 1 U RNasin (Promega) and was carried out at 42°C for 45 min. PCR was subsequently performed on 10 µl of the cDNA reaction in the presence of 0.2 mM dNTP's, 10 µl of 10×buffer, 2.5 U Promega *Taq* DNA polymerase, and 30 pmol of each primer. PCR primers consisted of an antisense adapter primer (5′ GACTCGAGTCGACATCG 3′) identical to the 5′ end of the adapter-dT primer and a sense rat *pim-1* primer (5′ GCGACATCAAGGACGAG 3′) corresponding to nucleotides #500–517 (see Fig. 2). The reaction was first heated to 94°C for 2.5 min, and then 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 3 min were performed on a Perkin Elmer Cetus DNA Thermal Cycler. The specific PCR product was identified by Southern blot analysis (29) and subsequently purified on low melting agarose and blunt-end cloned into the EcoRV site of the Bluescript plasmid (Stratagene). PCR amplification of RNA was similarly used to generate a *pim-1* clone spanning the coding region (clone e in

Fig. 1) as described above but with the following modifications. cDNA was synthesized using 0.2 µg of an oligo-dT primer and the PCR reaction was performed using an anti-sense primer specific to the rat *pim-1* gene corresponding to nucleotides #1198–1215 (5′ TTCCATGACAGGGCTCT 3′). The sense primer was chosen from conserved mouse and human *pim-1* sequences that span the ATG initiation codon (5′ GGG<u>ATG</u>CT-CTTGTCCAA 3′).

DNA sequencing

The dideoxy chain termination DNA sequencing method was used employing a commercial kit (Sequenase Version 2.0, United States Biochemical Corporation, Cleveland, Ohio).

Computer analysis

Nucleotide sequence data was analyzed on the program package from the University of Wisconsin Genetics Computer Group (30) using the Washington State VADAMS computer Facility.

Germ cell isolation

Germ cells were isolated from decapsulated testes of adult Sprague-Dawley rats using a collagenase and trypsin treatment as described (31). Germ cells were cultured in 75 ml flasks at a density of 3×10^6 cells/ml in Ham's F12 medium (Flow Laboratories, Inc., McLean, VA) supplemented with 6 mM pyruvate and 3 mM lactose. Cells were maintained at 33°C in 5% CO_2 and used immediately in actinomycin D chase experiments. Transcriptional inhibition by actinomycin D was verified by measuring [^{3}H] uridine incorporation. 1×10^6 isolated germ cells incorporated an average of 139 cpm in 2 h following actinomycin D treatment while control cells incorporated an average of 7649 cpm. The purity of germ cell preparations (>95%) was determined by scoring cells under a light microscope following staining with hematoxylin and eosin.

Sertoli cell isolation

Sertoli cells were isolated from adult Sprague-Dawley rats as described previously (43). Briefly, testes were decapsulated and incubated in a trypsin/DNAse solution. The tubule pellet was subsequently treated with collagenase and filtered through a 100 µm nylon mesh. The filtrate was centrifuged and the Sertoli cells cultured in Ham's F12 medium at 34°C in 5% CO_2 for 5 days.

Lymphocyte isolation and culture

Lymphocytes were isolated from the spleens of Sprague-Dawley rats as previously described (32). Briefly, cell suspensions were obtained by tissue mincing and passage through steel mesh screens. Cells were cultured at 4×10^6 cells/ml in RPMI (Flow Laboratories, Inc., McLean, VA) supplemented with 5% fetal calf serum (Tissue Culture Biologicals, Tulare, CA) and stimulated with 5 µg/ml Con A (Sigma Chemical Co., St. Louis, MO), a T cell mitogen, for 48 h.

Transcriptional inhibition with Actinomycin D

The half-life of *pim-1* transcripts were determined, as previously described (7), by measuring the levels of message remaining in cells after treatment with 10 µg/ml of actinomycin D (Sigma, St. Louis, MO) for varying lengths of time. Actinomycin D treatment was found to inhibit *de novo* transcription (>92%) without adversely affecting short term cell viability.
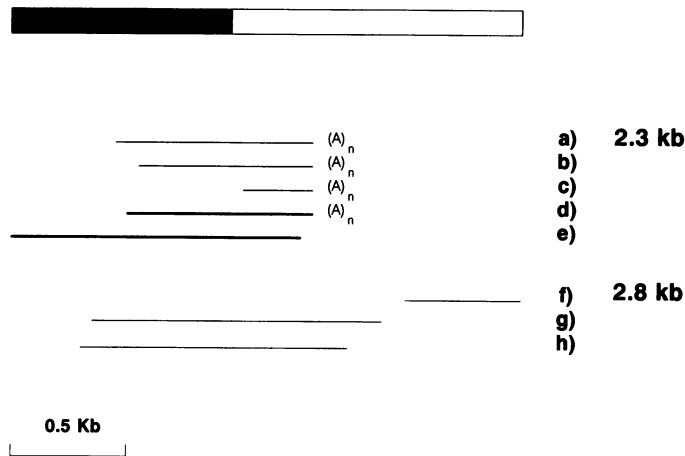
Fig. 1. Structure of the rat *pim-1* cDNA clones. Schematic representations of both the 2.3 and 2.8 kb *pim-1* clones are presented. At the top of the figure, the protein coding region of the rat *pim-1* transcript is indicated by the filled box and the 3' untranslated region as an open box. Clones a−e represent the 2.3 kb *pim-1* transcript. Clones a−c were obtained from rat testes cDNA libraries and are polyadenylated at nucleotide # 1302. Clone d was generated by a 3'RA-CE PCR procedure and is polyadenylated at nucleotide # 1302. Clone f was PCR-generated and contains the entire coding region. Clones f−h contain sequences unique to the 2.8 kb *pim-1* transcript and were obtained from rat testes cDNA libraries.

Northern blot analysis

Total RNA was isolated by the guanidinium method described elsewhere (33). Briefly, cells were solubilized in a 4M guanidinium isothiocyanate solution and then gently layered over a 5.7M cesium chloride cushion. Samples were ultracentrifuged at 150,000×g for 18 h in a SW 41 Beckman rotor and the RNA pellet resuspended and quantified. RNA (20 μg/lane) was electrophoresed in a 1.25% agarose/formaldehyde gel, transferred to a nylon membrane (Nytran) and immobilized by UV cross-linking. Blots were prehybridized in 50% formamide, 5×SSC, 50 mM KPO_4 (pH 8.0), 5×Denhardt's, 0.1% SDS, and 100 μg/ml salmon sperm DNA for a minimum of 4 h at 42°C and hybridized in 50% formamide, 5×SSC, 20 mM KPO_4 (pH 6.5), 1×Denhardt's, 0.1% SDS, and 100 μg/ml salmon sperm DNA using [^{32}P] dATP labeled probes. Following overnight hybridization at 42°C, filters were washed up to 59°C in 0.1% SDS and 0.1×SSC and autoradiographed at −80°C on pre-flashed film. The following probes were used; the pSVc-*myc*-1 plasmid containing the second and third exons of the cellular mouse *myc* gene (34), the 1250 bp *Pst*1 fragment from the chicken β-tubulin cDNA (35), the entire human actin cDNA (36), the 2900 bp *Dra*1/*Eco*R1 fragment of the human *pim-1* cDNA (26), and the entire mouse regulatory subunit (R1α) of the cAMP-dependent protein kinase cDNA (37).

RESULTS

Cloning of the rat testes-specific *pim-1* cDNA

Northern blot analysis has revealed the existence of two forms of *pim-1* mRNA transcripts in both mice and rats (7,8). In somatic cells, the *pim-1* gene encodes a 2.8 kb transcript while a 2.3 kb mRNA is expressed in rat testes. The existence of two *pim-1* transcripts of differing sizes could be the result of cross-hybridization of two distinct but closely related genes, or the

alternative splicing of a single transcript, or the use of an alternate promoter, or possibly the use of an alternate polyadenylation signal. To characterize the testes-specific *pim-1* transcript, *pim-1* cDNA molecules were cloned from two independent adult rat testes cDNA libraries using the human *pim-1* cDNA as a probe (26). More than 40 positive clones were identified and, of these, the longest clones were sequenced and are shown in Fig. 1. Based on sequence analysis, two distinct classes of *pim-1* clones were identified. The first class of clones (Fig. 1; a, b, and c) were found to be highly homologous to the human *pim-1* cDNA, ~90% in the coding region and ~61% in the 3' untranslated region. These clones are all polyadenylated at nucleotide position # 1302 and contain a potential ATTAAA poly-(A) addition signal 12 bp upstream of the site of polyadenylation. This site, however, occurs hundreds of nucleotides upstream of the position predicted by sequence comparison of the human 2.8 kb *pim-1* cDNA. The second class of rat *pim-1* clones identified from the rat testes cDNA libraries (see Fig. 1; f, g, and h) are identical and colinear in sequence to the first class of clones up to the poly-A site at nucleotide # 1302. However, this second class of rat *pim-1* clones do not terminate in a poly-A tail at nucleotide position # 1302, but rather, contain several hundred additional base pairs of 3' untranslated sequence that is highly homologous to the 3' untranslated region of the human 2.8 kb *pim-1* cDNA. In addition, clone (f) contains several tandem copies of the conserved transcript destabilizing A/U-rich motif present in the 3' untranslated regions of the mouse and human *pim-1* genes and ends at the same relative nucleotide position as identified for human *pim-1* cDNA clones (11,16).

The sequence analysis of clones a−h and the colinearity of the two classes of rat *pim-1* clones up to the upstream polyadenylation site strongly suggest that the short 2.3 kb testes-specific *pim-1* transcript arises from alternate polyadenylation of a precursor mRNA common to both the 2.8 kb and 2.3 kb transcripts. To further demonstrate the use of an alternate testes-specific polyadenylation signal in the shorter 2.3 kb *pim-1* transcript, the 3'RACE (rapid amplification of 3' ends) technique was used to extend rat *pim-1* cDNA in the 3' direction (through the poly-(A) tail). cDNA was generated from total rat germ cell RNA using an oligo-dT-adapter primer (see materials and methods) and PCR amplification was performed on germ cell cDNA using a 3' adapter primer and a homologous 5' rat *pim-1* primer derived from sequence information from clone (a) (Fig. 1). An 807 bp fragment was generated, subcloned into Bluescript and sequenced. As shown in Fig. 1d, the polyadenylated, PCR-generated 3'RACE product also terminates exactly at nucleotide position # 1302. In addition, this 3'RACE product is identical in sequence to the overlapping regions of clones a−c. This independent method supports the original hypothesis that the 2.3 kb testes-specific *pim-1* transcript utilizes an alternate upstream polyadenylation signal.

To further verify that the protein coding region of the rat 2.3 kb *pim-1* transcript is intact relative to the human and mouse *pim-1* transcripts, we used PCR to generate a cDNA clone spanning the entire protein coding region (Fig. 1e). The 5' primer was chosen from conserved mouse and human sequences spanning the initiation codon and the 3' primer was an antisense sequence representing nucleotides # 1198−1215. PCR was performed on oligo-dT primed rat germ cell RNA and a 1218 bp product was generated. Three independent clones were sequenced and the overlapping regions were found to be identical to clones a−d.

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M  L  L  S  K  I  N  S  L  A  H  L  R  A  A  P  C  N  D
gggatgctcttgtccaagatcaactccctggcccacctgcgcgcagcccttgcaacgac      60

L  H  A  N  K  L  A  P  G  K  E  K  E  P  L  E  S  Q  Y  Q
ctgcacgccaacaagctggcgccgggcaaagagaaggagccctggagtcgcagtaccag    120

V  G  P  L  L  G  S  G  G  F  G  S  V  Y  S  G  I  R  V  A
gtgggcccgctgttgggcagcggtggcttcggctcggtctactcgggcatccgcgtcgcc   180

D  N  L  P  V  A  I  K  H  V  E  K  D  R  I  S  D  W  G  E
gacaacttgccggtggccatcaagcacgtggagaaggaccggatttccgactgggggaa    240

L  P  N  G  T  R  V  P  M  E  V  V  L  L  K  K  V  S  S  G
ctgcccaacggcacccgagtgcccatggaagtggtcctgctgaagaaggtgagctcgggc   300

F  S  G  V  I  R  L  L  D  W  F  E  R  P  D  S  F  V  L  I
ttctcgggcgtcattagacttctggactggttcgagaggcccgatagtttcgtgctgatc   360

L  E  R  P  E  P  V  Q  D  L  F  D  F  I  T  E  R  G  A  L
ctggagaggcccgaacccgtgcaagacctcttcgacttcatcaccgagcgaggagccctc   420

Q  E  E  L  A  R  S  F  F  W  Q  V  L  E  A  V  R  H  C  H
caggaggagctggcccggagcttcttctggcaggtgctggaggccgtgcggcattgccac   480

N  C  G  V  L  H  R  D  I  K  D  E  N  I  L  I  D  L  N  R
aactgcgggggttctccaccgcgacatcaaggacgagaacatcttaatcgacctgaaccgc   540

G  E  L  K  L  I  D  F  G  S  G  A  L  L  K  D  T  V  Y  T
ggcgaactcaaactcatcgacttcgggtcggggcgctgctcaaggacacagtctacacg     600

D  F  D  G  T  R  V  Y  S  P  P  E  W  I  R  Y  H  R  Y  H
gactttgacggaacccgagtgtacagtcctccagagtggattcgctaccatcgctaccac   660

G  R  S  A  A  V  W  S  L  G  I  L  L  Y  D  M  V  C  G  D
ggcaggtcggctgctgtttggtccctggggatcctgctctatgacatggtctgcggagat   720

I  P  F  E  H  D  E  E  I  V  K  G  Q  V  Y  F  R  Q  R  V
attccatttgagcacgacgaagagatcgtcaagggccaagtgtactttaggcaaagggtc   780

S  S  E  C  Q  H  L  I  R  W  C  L  S  L  R  P  S  D  R  P
tcttcagaatgtcaacatcttattagatggtgcctgtccctgagaccatcggaccggccc   840

S  F  E  E  I  Q  N  H  P  W  M  Q  D  V  L  L  P  Q  A  T
tcctttgaagaaatccagaaccatccgtggatgcaggatgttctcctgccccaggccacc   900

A  E  I  H  L  H  S  L  S  P  S  P  S  K  *
gccgagattcatctgcacagcctgtcaccatcacccagcaaatagcagccattctgtcag   960

accctccaggggaagagagagcttgtctgctggcctccaacaggaccctgctctacgatgc  1020

agggacagaaatgacaactcattccaggctccggggtccctggagcaacctccctcaagg   1080

agaagagactagttcactcgtcctggaccccgctttgcccctcacagactcagtggcgtc   1140

cagtgtggctggcgtccgcagagtcccgggtgttggggggggaggtgggagtgggtcaga   1200

gccctgtcatggaactttagtcaccatggagactgtgggtcaccaagatgggccagggta   1260

ggggaaaaacatttggggggtgggattaaaaactagcaccat  1302
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Fig. 2. Nucleotide and amino acid sequence of the rat 2.3 kb testes specific *pim-1* cDNA. The nucleotide numbers are given at the right with numbering beginning 3 nucleotides 5' of the initiation codon. The putative alternate polyadenylation site is underlined and nucleotide 1302 (underlined) represents the last nucleotide present in both the shorter and longer *pim-1* transcripts.

```
                                            ****  *  *
rat     MLLSKINSLAHLRAAPCNDLHANKLAPGKEKEPLESQYQVGPLLGSGGFG    50
mouse                R        T
human                         T

        *           ** *
rat     SVYSGIRVADNLPVAIKHVEKDRISDWGELPNGTRVPMEVVLLKKVSSGF   100
mouse                                                     D
human        S

        *
rat     SGVIRLLDWFERPDSFVLILERPEPVQDLFDFITERGALQEELARSFFWQ   150
mouse                                              D  G
human

        ***     *         *  ***
rat     VLEAVRHCHNCGVLHRDIKDENILIDLNRGELKLIDFGSGALLKDTVYTD   200
mouse                          S    I
human

        *       **         *       *  *
rat     FDGTRVYSPPEWIRYHRYHGRSAAVWSLGILLYDMVCGDIPFEHDEEIVK   250
mouse                                                    I
human                                                    IR

              *         ** *
rat     GQVYFRQRVSSECQHLIRWCLSLRPSDRPSFEEIQNHPWMQDVLLPQATA   300
mouse       F  T         K              R        GD    AS
human       F                  A        T                 E

rat     EIHLHSLSPSPSK*    314
mouse           GS
human           G
```

Fig. 3. Comparison of the derived amino acid sequences of the rat, mouse, and human Pim-1 proteins. Amino acids are numbered starting with 1 at the initiation codon. Positions where the mouse and human amino acids differ from the rat sequence are displayed. Asterisks indicate areas of Pim-1 homology with other protein kinases (38,39).
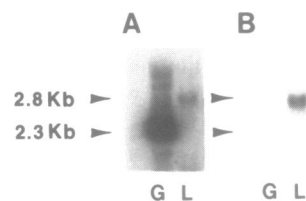
Fig. 4. Northern blot analysis of 2.3 and 2.8 kb *pim-1* transcripts. 20 μg of total RNA was isolated from adult rat germ cells (G) and rat spleen lymphocytes (L) and analyzed for hybridization to *pim-1* probes by Northern blot analysis. *A*. The 2.6 kb human *pim-1* cDNA clone (26) containing both coding and 3' untranslated sequences was radiolabeled and used as a probe. *B*. The Northern blot in *A* was stripped and rehybridized with the 2.8 kb rat *pim-1* cDNA clone (clone f; fig. 1) spanning the 3' untranslated region coding for the destabilizing A/U-rich motif.

The sequence of the 2.3 kb rat testes-specific *pim-1* transcript is presented in Fig. 2 and was obtained by overlapping clones a−e. Any sequence discrepancies between the PCR-generated clones were eliminated by sequencing three independent colonies from each cloned PCR fragment. As shown in Fig. 2, the 2.3 kb rat *pim-1* cDNA contains a large open reading frame of 945 nucleotides and a 3' untranslated region of 357 nucleotides. Homology between the rat and the human and mouse coding regions, which contain exactly the same length of nucleotides is 90% and 94% respectively at the nucleotide level.

The predicted rat Pim-1 protein, as shown in Fig. 3, shares a high degree of homology to the mouse (94%) and human (96%) proteins. Human (2,3) and mouse (1) Pim-1 proteins have been demonstrated to be protein serine/threonine kinases. Regions that are highly conserved between various kinases (38,39) and human (12,16,26) and mouse (11) Pim-1 kinases are also conserved in the rat homolog (Fig. 3). Specifically, the LGXGXXG amino acid motif at positions 44 to 67 thought to be involved in ATP binding is conserved between the rat, mouse, and human species. As also shown in Fig. 3, other conserved protein kinase regions in the C-terminal portion of the human and mouse Pim-1 protein are likewise conserved in the rat protein. Based on these

homologies, we predict that the rat Pim-1 protein is also likely to be a protein serine/threonine kinase.

Cell specific differences in *pim-1* transcript sizes

Sequence analysis of *pim-1* cDNA clones indicate that the use of alternative polyadenylation sites gives rise to the smaller sized *pim-1* transcripts expressed in rat germ cells and that the second class of cDNA clones (f−h) represent sequences unique to the longer somatic 2.8 kb *pim-1* transcripts. Evidence that further substantiates these observations was obtained from Northern blot analysis of rat germ cell and lymphocyte RNA. As shown in Fig. 4a and consistent with our previous findings, both the testes-specific 2.3 kb *pim-1* transcript and the somatic 2.8 kb *pim-1* transcript expressed in lymphocytes hybridize to a full length *pim-1* cDNA probe containing both protein coding and 3' untranslated sequences. This blot was stripped and re-probed with the 560 bp rat *pim-1* cDNA identified as clone (f) in Fig. 1. This clone contains only the 3' untranslated sequences unique to the
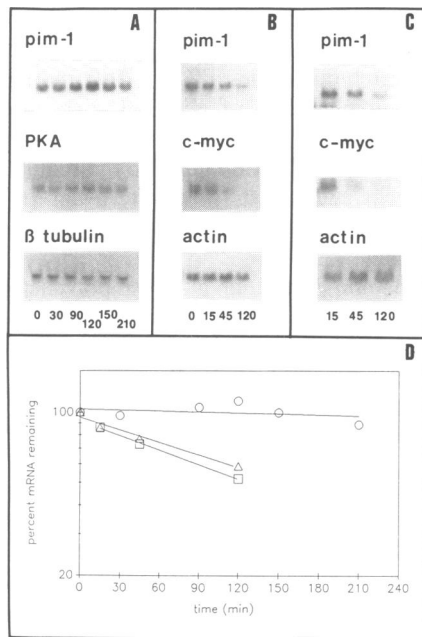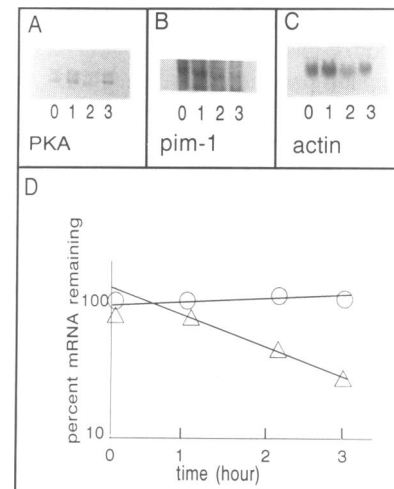
Fig. 5. Half-life comparisons of the rat 2.3 and 2.8 kb *pim-1* transcripts. Germ cells were isolated from adult rat testes and lymphocytes were isolated from rat spleen. Cells were treated with actinomycin D for various times and the mRNA levels remaining at each time point assessed by Northern blot analysis. 20 μg of total RNA were loaded per lane and blots were hybridized with various probes as indicated. *A*. Northern blot analysis of *pim-1* mRNA stability in rat testes germ cells. *B & C*. Northern blot analysis of *pim-1* mRNA stability in lymphocytes isolated from rat spleen. *D*. Graphical representation of *pim-1* mRNA stability was obtained by densitometric scanning. The amount of mRNA observed at the first time point was normalized to 100% and the percentage of remaining signal plotted vs. time (min) of actinomycin D treatment. A linear film response was verified by scanning at least two different exposures. Key: O – – O, rat testes germ cells; △ – –△, rat spleen lymphocytes; □ – –□, rat spleen lymphocytes.

Fig. 6. Half-life comparisons of somatic *pim-1* and PKA-R1α transcripts in Sertoli cells. Sertoli cells were treated with actinomycin D and the mRNA remaining at each time point analyzed by Northern blot analysis. 20 μg of total RNA were loaded per lane and blots were hybridized with various probes as indicated. A. Northern blot analysis of *pim-1* mRNA stability. B. mRNA stability of PKA-R1α transcripts (3.2kb and 2.9 kb). C. Northern blot analysis of actin mRNA stability. D. Graphical representation of mRNA stability was determined by densitometric scanning as described for Fig. 5. The amount of RNA remaining at each time point was normalized to actin. Key: △ – –△, *pim-1* mRNA; O – – O, PKA-R1α mRNA (3.2 kb transcript).

2.8 kb *pim-1* transcript including the A/U-rich motif implicated in mRNA destabilization. Fig. 4b shows that only the longer somatic 2.8 kb *pim-1* transcript hybridizes to this probe. These results clearly demonstrate that the 2.3 kb testes-specific *pim-1* transcript contains a deletion in the 3' untranslated region and is missing the destabilizing A/U-rich motif.

pim-1 mRNA stability

To investigate the contribution of the A/U-rich sequence motif on *pim-1* mRNA stability, we compared the stability of the rat 2.3 kb testes-specific *pim-1* transcript to the 2.8 kb *pim-1* transcript expressed in rat lymphocytes. As demonstrated by Northern blot analysis in Fig. 5, we found that the 2.3 kb *pim-1* transcript was very stable with no appreciable decrease in RNA levels apparent up to 210 min after the addition of actinomycin D. Transcriptional inhibition following actinomycin D treatment was verified by measuring [³H] uridine incorporation into RNA. In contrast, the A/U-rich sequence containing 2.8 kb *pim-1* transcript expressed in mitogen stimulated rat splenic lymphocytes consistently showed a much shorter half-life ($T_{1/2}$ ~135 min; n=3) while actin levels appeared almost constant. In these cells, transcriptional inhibition was verified by confirming the reported half-life of c-*myc* as ~30 min.

In Fig. 5, we also examined the half-life of the type 1α regulatory subunit (R1α) of cyclic AMP-dependent protein kinase (PKA). Similar to the *pim-1* gene, the PKA-R1α gene has

recently been shown to express a truncated testes-specific transcript that arises through an alternate polyadenylation event (21). In addition, potential transcript destabilizing A/U-rich sequences are also removed from the shorter PKA-R1α transcript by an alternate polyadenylation process. As shown in Fig. 5, the half-life of the 1.7 kb PKA-R1α transcript expressed in germ cells is nearly identical to the stable *pim-1* transcripts observed in these cells. To determine if the longer PKA-R1α transcripts (3.2kb and 2.9kb) expressed in somatic cells have a shorter half-life, we analyzed mRNA stability in cultured rat Sertoli cells (Fig. 6). In contrast to the *pim-1* gene, the somatic PKA-R1α transcripts in Sertoli cells are very stable with essentially no decrease in message levels up to 3 h after the addition of actinomycin D. In these same cells, however, the stability of the *pim-1* transcript was substantially shorter and similar to that observed in rat spleen cells ($T_{1/2}$ ~120 min). These results indicate that although the PKA-R1α gene contains numerous A/U-rich regions scattered throughout the 3' untranslated region (21), that these sequences are distinct from the A/U-rich motif present in the *pim-1* gene and are not sufficient to mediate mRNA instability.

DISCUSSION

We have provided strong evidence that the rat 2.3 kb and 2.8 kb *pim-1* transcripts differ in their length of 3' untranslated sequences and that the smaller testes-specific transcript arises from the use of an alternate upstream polyadenylation site. In this report we have described the isolation of two classes of rat *pim-1* cDNA clones that are identical in their overlapping sequences but differ in their length of 3' untranslated sequence. The two classes of clones are colinear up to an upstream polyadenylation site located at nucleotide position #1302. The

shorter testes-specific class of clones are polyadenylated at nucleotide position #1302 while the longer class of cDNA clones expressed in somatic tissue extend through this upstream poly-A site and contain several hundred bases of additional 3' untranslated sequence. Specifically, the longer 2.8 kb transcript contains the A/U-rich motif implicated in mRNA destabilization while the shorter 2.3 kb message does not.

The most reasonable and likely interpretation of our results is that the shorter testes-specific transcript arises through an alternate polyadenylation event rather than alternate splicing. However, direct proof of alternate polyadenylation must await sequence comparison to a rat genomic *pim-1* clone. Nevertheless, this conclusion is consistent with the results from three different experimental approaches. Taken together, the sequence analysis and colinearity of clones obtained from testes cDNA libraries and from the PCR generated 3' extension of *pim-1* cDNA (3' RACE) (Fig. 1) as well as data obtained from Northern blot analysis (Fig. 4) all strongly support an alternate polyadenylation of the shorter testes-specific *pim-1* transcript.

The site of poly-(A) addition in the 2.3 kb *pim-1* transcript is somewhat unusual in that it is not preceded by the highly conserved hexanucleotide AAUAAA. As shown in Fig. 2, however, the sequence AUUAAA is present 12 nucleotides upstream of the poly-(A) addition site. This sequence has been previously recognized as an active variant of the conserved hexanucleotide (40).

The use of atypical polyadenylation sites has recently been reported for a number of other genes that express shorter testes-specific transcripts. Similar to the 2.3 kb *pim-1* transcript, the alternate polyadenylation of the rat testes-specific 1.6 kb PKA-R11β transcript (cAMP-dependent protein kinase regulatory subunit) also uses the AUUAAA variant (22). However, the smaller germ-cell specific transcripts of the human PKA-R11β (22), mouse c-abl (18) and rat calmodulin genes (20) use different variants of the polyadenylation signal, UAUAA, UACAAA, and GAUAAA respectively. An emerging pattern of expression of smaller sized transcripts in male germ cells generated through atypical upstream polyadenylation sites suggests the use of alternate mechanisms of polyadenylation specific to germ cells. The exact nature of these mechanisms is unknown but may involve the use of germ cell specific snRNPs (small nuclear ribonucleoprotein) that recognize variants of the polyadenylation signal located upstream of the normal somatic polyadenylation site. The use of snRNPs in the cleavage and subsequent poly-(A) addition of RNA has been previously suggested (40,41) and may occur through the base pairing of the RNA portion of the snRNP and sequences surrounding the polyadenylation signal.

The alternate polyadenylation of the testes-specific *pim-1* (see Fig. 2), PKA-R1α (21), c-abl (18), PKA-R11α and -R11β (22), and calmodulin (20) genes, does not affect the protein coding regions but does result in the deletion of 3' untranslated sequences. The functional significance of the removal of 3' untranslated sequences by the testes-specific mechanisms of alternate polyadenylation may be in the removal of sequences involved in message destabilization. We have shown that the alternate polyadenylation of the shorter testes-specific *pim-1* transcript results in the removal of A/U-rich sequences located in the 3' untranslated region of the somatic 2.8 kb *pim-1* transcript (see Fig. 1 and Fig. 4a & b). These sequences have been previously shown to be involved in the destabilization of several other transiently expressed genes (13,14,42). Our results with actinomycin D chase experiments indicate that the shorter *pim-1*

transcript expressed in germ cells was substantially more stable ($T_{1/2}$ > >3.5 h) than the longer A/U-rich containing transcript expressed in rat lymphocytes ($T_{1/2}$ ~140 min) (Fig. 5) and implicate the involvement of the A/U-rich motif in these differential stabilities. Conclusive evidence demonstrating the postulated role of the A/U-rich motif in the regulation of RNA stability in germ cells must, however, await further studies involving the transfection of the short 2.3 kb *pim-1* transcript into somatic cells.

Similar to the *pim-1* gene, an alternate polyadenylation event results in a smaller sized PKA-R1α testes-specific transcript. In addition, potential A/U-rich sequences have been identified in the 3' untranslated region of the PKA-R1α gene. The longer transcript contains numerous A/U-rich sequence monomers scattered throughout the 3' untranslated region while the shorter transcript contains only two A/U-rich monomers. Based on this consideration, it has been suggested that the shorter testes-specific PKA-R1α transcript may be more stable than the longer message (21). We have addressed this question by comparing mRNA stability of the shorter *pim-1* and the PKA-R1α transcripts expressed in germ cells to the longer messages expressed in Sertoli cells. We observed that the both the smaller *pim-1* and PKA-R1α transcripts expressed in germ cells were extremely stable ($T_{1/2}$ > > 3.5 h)(Fig. 5). Somewhat unexpectedly, the longer PKA-R1α transcripts expressed in Sertoli cells were also very stable ($T1/2$ > > 3h) while the half-life of the 2.8 kb *pim-1* transcript was markedly shorter ($T_{1/2}$ ~120 min) (Fig. 6). These results suggest possible functional differences between the A/U-rich sequences in the *pim-1* gene and the PKA-R1α gene. An obvious structural difference between the A/U-rich sequences of these genes is that the *pim-1* gene contains four tandemly linked repeats of the AUUUA motif while the PKA-R1α gene contains only monomers of the AUUUA motif (or variations thereof). Recent evidence suggests that certain protein factors which bind A/U sequences require several tandemly linked repeats of the AUUUA motif to effectively bind (44,45). Therefore, the putative protein factors in Sertoli cells which mediate mRNA destabilization/stabilization in the *pim-1* transcript may simply be unable to bind to the A/U-rich sequences in the PKA-R1α transcripts.

Similar studies involving the analysis of c-abl mRNA half-life demonstrate that the shorter c-abl transcript expressed in mouse spermatids is substantially more stable than the somatic transcript (18). Although the A/U-rich motif is not present in the 3' untranslated region of the somatic c-abl, PKA-R11α, PKA-R11β, and calmodulin transcripts, this does not preclude the presence of other, as of yet unrecognized, sequences that may be similarly involved in message destabilization. We suggest that shorter sized-mRNAs which lack destabilizing sequences may be preferentially expressed in germ cells because of their higher stability. Evidence suggests that mouse protamine transcripts are stored for days after the cessation of transcription in the late stages of spermatid differentiation and are later translated during the final stages of spermiogenesis (23). The characteristic cessation of transcription in spermatids beyond the stage of nuclear condensation (step 12 of spermiogenesis)(25) suggests that the stabilization of transcripts by the removal of destabilizing sequences would ensure adequate transcripts for translation in the subsequent stages of spermatogenic differentiation.

In conclusion, we have demonstrated that the rat 2.3 kb *pim-1* transcript arises from an alternate polyadenylation event which results in the removal of 3' untranslated sequences including an

A/U-rich motif implicated in mRNA destabilization. We have observed that the shorter 2.3 kb testes-specific transcript is more stable than the longer A/U-rich containing 2.8 kb somatic transcript and suggest that the functional significance of different sizes of *pim-1* transcripts is related, in part, to their different stabilities. We further postulate that the increased stability of the testes-specific *pim-1* transcript may be essential for the characteristic translational delay observed in post-meiotic germ cells.

REFERENCES

1. Saris,C.J.M., Domen,J. and Berns,A. (1991) *EMBO J.*, **10**, 655–664.
2. Padma,R. and Nagarajan,L. (1991) *Cancer Res.*, **51**, 2486–2489.
3. Hoover,D., Friedmann,M., Reeves,R. and Magnuson,N.S. (1991) *J. Biol. Chem.*, **266**, 14018–14023.
4. Dautry,F., Weil,D., Yu,J. and Dautry-Varsat,A. (1988) *J. Biol. Chem.*, **263**, 17615–17620.
5. Cooper,G.M. (1990) *Oncogenes*. Jones and Bartlett Publishers, Boston, MA. pp. 213–223.
6. Hunter,T. (1991) *Cell*, **64**, 249–270.
7. Wingett,D., Reeves,R. and Magnuson,N.S. (1991) *J. Immun.*, **147**, 3653–3659.
8. Sorrentino,V., McKinney,M.D.,Giorgi,M., Geremia,R. and Fleissner,E. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2191–2195.
9. Cuypers,H.T., Selten,G., Quint,W., Zijistra,M. Maandag,E.R., Boelens,W., van Wezenbeek,P., Melief,C. and Berns,A. (1981) *Cell*, **37**, 141–150.
10. Selten,G., Cuypers,H.T. and Berns,A. (1985) *EMBO J.*, **4**, 1798–1798.
11. Selten,G., Cuypers,H.T., Boelens,W., Maandag,E.R., Verbeek,J., Domen,J., van Beveren,C. and Berns,A. (1986) *Cell*, **46**, 603–611.
12. Domen,J., Von Lindern,M., Hermans,A., Breuer,M., Grosveld,G. and Berns,A. (1987) *Oncogene Res.*, **1**, 103–112.
13. Shaw,G.and Kamen,R.A. (1986) *Cell*, **46**, 659–667.
14. Reeves,R., Elton,T., Nissen,M. Lehn,D. and Johnson,K. (1986) *Proc. Natl. Acad. Sci.*USA, **84**, 6531–6535.
15. Nagarajan,L., Louie,E., Tsujimoto,Y., Ar-Rushdi,A., Huebner,K. and Croce,C.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2556–2560.
16. Meeker,T.C., Nagarajan,L., Ar-Rushdi,A., Rovera,G., Heubner,K. and Croce,C.M. (1987) *Oncogene Res.*, **1**, 87–101.
17. Amson,R., Sigaux,F., Przedborski,S., Flandrin,G., Givol,D. and Telerman,A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8857–8861.
18. Meijer,D., Hermans,A., von Lindern,M. van Agthoven,T., de Klein,A., Mackenbach,P., Grootegoed,A., Talarico,D., Valle,G.D. and Grosveld,G. (1987) *EMBO J.*, **6**, 4041–4048.
19. Fischman,K., Edman,J.C., Shackleford,G.M., Turner,J.A., Rutter,W.J. and Nir,U. (1990) *Mol. Cell. Biol.*, **10**. 146–153.
20. Slaughter,G.R. and Means,A.R. (1989) *Mol. Endocrinol.*, **3**, 1569–1578.
21. Sandberg,M., Skalhegg,B. and Jahnsen,T. (1990) *Biochem. Biophy. Res. Comm.*, **167**,323–330.
22. Oyen,O., Myklebust,F., Scott,J.D., Cadd,G.G., McKnight,G.S., Hansson,V. and Jahnsen,T. (1990) *Biol. Reprod.*, **43**, 46–54.
23. Hecht,N.B. (1986) In Stefanini,M. (ed.), *Molecular and Cellular Endocrinology of the Testis*. Elsevier Science Publishers B.V., Amsterdam, pp. 199–213.
24. Heidaran,M.A. and Kistler,W.S. (1987). *J. Biol. Chem.*, **262**, 13309–13315.
25. Geremia,R., Boitani,C., Conti,M. and Monesi,V. (1977) *Cell Differ.*, **5**, 343–355.
26. Reeves,R., Spies,G.A., Kiefer,M., Barr,P.J. and Power,M. (1990) *Gene*, **90**, 303–307.
27. Dorfman,D.M., Zon,L.I. and Orkin,S.H. (1989) *BioTechniques*, **7**, 569–570.
28. Frohman,M.A. (1990) In Innins,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (ed.), *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, CA. pp. 28–38.
29. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) In: *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor University Press, Cold Spring Harbor.
30. Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
31. Bellve,A.R., Cavicchia,J.C., Millette,C.F., O'Brien,C.A., Bhatnagar,Y.M. and Dym,M. (1977) *J. Cell. Biol.*, **74**, 68–85.
32. Mishell,B.B. and Shiigi,S.M. (1980) *Selected Methods in Cellular Immunology*. W. H. Freeman and Co., San Francisco, CA. pp. 13–18.
33. Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1989) *Current Protocols in Molecular Biology*, Greene Publishing Associated and Wiley-Interscience, New York, pp. 4.2.1.
34. Land,H., Pwada,L.F. and Weinberg,R.A. (1983) *Nature*, **304**, 596–602.
35. Cleveland,S.W., Lopata,M.A., McDonald,R.J., Cowan,N.J., Rutter,W.J. and Kirschner,M.W. (1980) *Cell*, **20**, 95–105.
36. Hanukoglu,I., Tanese,N. and Fuchs,E. (1983) *J. Mol. Biol.*, **163**, 673–678.
37. Uhler,M.D., Carmichael,D.F., Lee,D.C., Chrivia,J.C., Krebs,E.G. and McKnight,G.S. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1300–1304.
38. Van Breveren,C. and Verma,I.M. (1985) *Curr. Topics Mocrobiol. and Immunol.*, **123**, 73–98.
39. Hunter,T. and Cooper,J.A. (1985) *Ann. Rev. Biochem,*, **54**, 897–930.
40. Wickens,M. (1990) *Trends Biochem. Sci.*, **15**, 277–281.
41. Berget,S.M. (1984) *Nature*, **309**, 179–181.
42. Reeves,R. and Magnuson,N.S. (1990) *Prog. Nucleic Acid Res. Mol. Biol.*, **38**, 241–282.
43. Dorrington,J.H., Roller,N.F. and Fritz,I.B. (1975) *Mol. Cell Endocr.*, **3**, 57–70.
44. Malter,J. (1989) *Science*, **240**, 664–665.
45. Bohjanen,P.R., Petryniak,B., June,C.H., Thompson,C.B. (1992) *J. Biol. Chem.*, **267**, 6302–6309.