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Expression specificity of the mouse exonuclease 1 (*mExo1*) gene

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ABSTRACT

Genetic recombination involves either the homologous exchange of nearly identical chromosome regions or the direct alignment, annealing and ligation of processed DNA ends. These mechanisms are involved in repairing potentially lethal or mutagenic DNA damage and generating genetic diversity within the meiotic cell population and antibody repertoire. We report here the identification of a mouse gene, termed *mExo1* for mouse exonuclease 1, which encodes a ~92 kDa protein that shares homology to proteins of the *RAD2* nuclease family, most notably human 5′ to 3′ exonuclease Hex1/hExo1, yeast exonuclease 1 (Exo1) proteins and *Drosophila melanogaster* Tosca. The *mExo1* gene maps to distal chromosome 1, consistent with the recent mapping of the orthologous *HEX1*/*hEXO1* gene to chromosome 1q42–q43. *mExo1* is expressed prominently in testis, an area of active homologous recombination, and spleen, a prominent lymphoid tissue. An increased level of *mExo1* mRNA was observed during a stage of testis development where cells that are actively involved in meiotic recombination arise first and represent a significant proportion of the germ cell population. Comparative evaluation of the expression patterns of the human and mouse genes, combined with previous biochemical and yeast genetic studies, indicate that the Exo1-like proteins are important contributors to chromosome processing during mammalian DNA repair and recombination.

INTRODUCTION

Nucleases are required for the successful execution of most DNA repair and recombination events (1). Furthermore, nuclease activities are involved in cellular processes such as DNA replication and apoptosis. While the major nucleases that operate to remove target damage during base excision and nucleotide excision repair have been identified, the primary nucleases functioning during mammalian recombination and mismatch repair are less well defined.

In recombination, nucleolytic processing is essential for generating single-stranded DNA regions required for annealing during DNA end-joining (non-homologous recombination) and for strand invasion during the exchange of sequences between homologous chromosomes (2). Recent studies have found that three human proteins, Mre11, Hex1/hExo1 and hRad1, exhibit potentially relevant nuclease activities (3–6). Moreover, mutations in the corresponding genes of yeast *Saccharomyces cerevisiae* (*MRE11*, *EXO1* and *RAD17*, respectively) render these cells defective in certain aspects of recombination and DNA repair (7–9). The combination of these results suggest that hMre11, Hex1/hExo1 and hRad1 (named after the *Schizosaccharomyces pombe RAD1* gene product, the protein equivalent to *S.cerevisiae* Rad17) are likely crucial factors in the chromosomal processing of mammalian recombination.

Analysis thus far has found that most DNA recombination intermediates exist as single-stranded 3′-overhangs, suggesting that a majority of the nucleolytic processing takes place in a 5′ to 3′ direction (10). The hRad1 protein (6) and a homologous protein Rec1 from *Ustilago maydis* (11) have been shown to function as 3′ to 5′ exonucleases, a polarity opposite of what would presumably be needed for double-strand break processing [it is worth noting that there is some discrepancy in the literature as to whether hRad1 possesses a nuclease activity (12), and further investigation to explain this disparity is needed]. Moreover, despite the original belief that the Mre11 protein complex (comprised of Mre11, Rad50 and Xrs2; 13) functions as a 5′ to 3′ exonuclease (based on yeast genetic studies and chromosome content analysis), recent studies have shown that purified human Mre11 protein, by itself or in its complex, exhibits a 3′ to 5′ exonuclease function, as well as single-stranded endonuclease and hairpin cutting activities (3,4). To explain the yeast genetic studies (10), it has been proposed that an associated helicase acts to unwind the target DNA end, permitting the endonuclease activity of the Mre11/Rad50 complex to clip the 5′ single-stranded region (13) and generate a 5′ to 3′ recessed end. More recent analysis has shown that the *NBS1* gene product promotes strand separation and facilitates the single-stranded endonuclease activity of Mre11 (14). However, this nuclease incision activity, in cooperation with Rad50 and Nbs1, appears to cut in a 3′ to 5′

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direction and generates only a short single-stranded DNA segment, inconsistent with the extensive (~600 nt) 5′ to 3′ resection known to occur *in vivo* (10,15). In total, these data indicate a need for an additional nuclease(s) to create the more extensive 5′ to 3′ resection required for recombination.

A recently identified human protein similar to Exo1 of *S.cerevisiae* and *S.pombe*, termed Hex1/hExo1, has been shown to exhibit a 5′ to 3′ exonuclease activity (5) and to interact with Msh2 (16), a factor that functions in both mismatch repair and homologous recombination (17). In addition, mutations in *Exo1* of *S.cerevisiae* or *S.pombe* lead to defects in mismatch repair (18), meiotic and mitotic recombination (19) and UV-damage repair (20). The combination of these biochemical and genetic studies suggest that the Exo1-like proteins act as important nucleases during processes of repair and recombination. Further, expression analysis in *Drosophila melanogaster* has revealed that the Exo1 homolog, *TOS*, is expressed highest in the developing oocyte, a region of active meiotic recombination (21). As a step towards defining the biological contribution(s) of the mammalian Exo1-like proteins, which share notable similarity to the proteins of the *RAD2* nuclease family (22), we have isolated a mouse cDNA encoding the Exo1 equivalent (termed *mExo1*) and have examined the expression specificity of the corresponding gene. Our findings are discussed in relationship to the human gene expression pattern, and suggest a role for the mammalian Exo1 proteins in homologous recombination and events that occur during haematopoiesis.

MATERIALS AND METHODS

Isolation of the *mExo1* cDNA

Four independent *mExo1* cDNA clones were isolated from a pachytene spermatocyte library (23) constructed in the Lambda-ZAP vector (Stratagene, La Jolla, CA) using a radio-labeled PCR fragment of the *HEX1* cDNA (5) as a probe and standard hybridization techniques. Following successive rounds of hybridization of nylon filter plaque lifts in 1× buffer (24) containing 45% formamide, plaque purified positive cDNA isolates were converted to phagemids according to the manufacturer's *in vivo* excision protocol (Stratagene). Phagemid DNA (pBS*mExo1*) was purified using the Qiagen Midi Prep system. The entire nucleotide sequence of the mouse cDNA clone was determined using the Primer Island™ *in vitro* transposition system (Perkin Elmer, Foster City, CA) as described elsewhere (5).

Interspecific backcross mapping of the *mExo1* gene

cDNA probes were mapped by following the segregation of restriction fragment length variants (RFLVs) in progeny of a *Mus musculus* × *Mus spretus* interspecific backcross (IB) ([C3Hf/Rl-*Mgf*^{Sl2ENURg}/+ × *M.spretus*) × C3Hf/Rl]; 25,26) using Southern blot hybridization methods (24). A 7 kb *Hin*cII restriction fragment was detected with the *mExo1* probe in *M.spretus* DNA; the same probe detected a 2.1 kb *Hin*cII fragment in DNA of C3Hf mice (which represents the *M.musculus* parent used to create this cross). The segregation pattern of *mExo1 M.spretus* and *M.musculus* RFLVs was traced in 120 IB progeny and compared to segregation patterns produced by other gene markers typed in the same IB panel. The position of the nearby *Spna1* gene was determined using a human α-spectrin cDNA probe (ATCC no. 61900), which detected a 4.6 kb *Taq*I RFLV in *M.spretus* DNA samples and a 6.0 kb fragment in *Taq*I-digested C3Hf DNA. *Taq*I fragments, of 2.8 and 6.0 kb, were detected in *M.spretus* and C3Hf DNA samples, respectively, with an EST probe (IMAGE no. 423176) of the *Hlx* gene; a 9 kb *Taq*I RFLV detected with a *Cr2* cDNA probe (mCr21-1, ATCC no. 63066) was used to trace the inheritance of *M.spretus* alleles (against a background of 3.5 kb C3Hf fragments). Segregation pattern data were analyzed and stored using Map Manager data analysis software (27).

Northern blot analysis

Hybridizations with Clontech Northern blots were performed for 2 h at 55°C in ExpressHyb Solution (Clontech, Palo Alto, CA) using either 3′- or 5′-end fragments from *HEX1* or *mExo1* labeled with the Megaprime DNA Labeling System (Amersham, Piscataway, NJ) and [α-^{32}P]dCTP (Amersham). Blots were washed once at room temperature for 20 min and twice at 50°C for 20 min with 50 mM $NaPO_4$ (pH 7.4), 0.5% SDS, 1 mM EDTA. Images were obtained using a Molecular Dynamics phosphorimager Storm 860 and autoradiography. Blots were normalized with β-actin (Clontech).

For the mouse testis developmental blot, RNA was isolated from postnatal testis at 8, 14, 17, 20, 28, 35 and 42 days of age using an poly(A)+ RNA isolation kit (5 prime-3 prime, Inc., Boulder, CO). Similar amounts of RNA (2 μg) from each developmental stage were separated on denaturing gels and blotted using standard methods (28). Northern blots of poly(A)+ RNA were hybridized to randomly primed radio-labeled *mExo1* probes (29) for 20 h at 42°C in 1× hybridization buffer (24) containing 50% formamide. Blots were washed once in 2× SSC, 1% SDS for 30 min at room temperature, and once in 0.2× SSC, 0.1% SDS for 20 min at 68°C.

RT–PCR analysis

Total RNAs were isolated from mouse spleen, thymus, lymph node, brain and bone marrow using the RNA isolation kit and used for cDNA synthesis with the Clontech Advantage™ RT-for PCR Kit. Following cDNA synthesis, PCR reactions with primers specific to a 3′-portion of *mExo1* cDNA (5′-GGCC-TAAACACGTCGAG-3′; 5′-CTCACTGATCTTCGTCTG-3′) were carried out for 30 cycles, and the DNA products were analyzed on a 2% agarose TBE gel (28).

RESULTS

Features of the mExo1 protein

A mouse pachytene spermatocyte cDNA library was screened (23) using a fragment of the human *HEX1* cDNA (5) as a probe to isolate a mouse cDNA equivalent termed *mExo1* (accession no. AJ238213). The encoded protein has a theoretical pI of 8.9, a predicted molecular weight of 92 032 Da and a consensus nuclear localization signal (KPRP) at position 416–419. Alignment of the mExo1 amino acid sequence with the two human Exo1-like proteins (which are products of alternatively spliced mRNAs; 16,30) found that the mouse protein maintains the full C-terminal extension and shares ~72% identity to the longer human protein (Fig. 1A). Most of the sequence divergence between mExo1 and human Exo1-like proteins, independent of

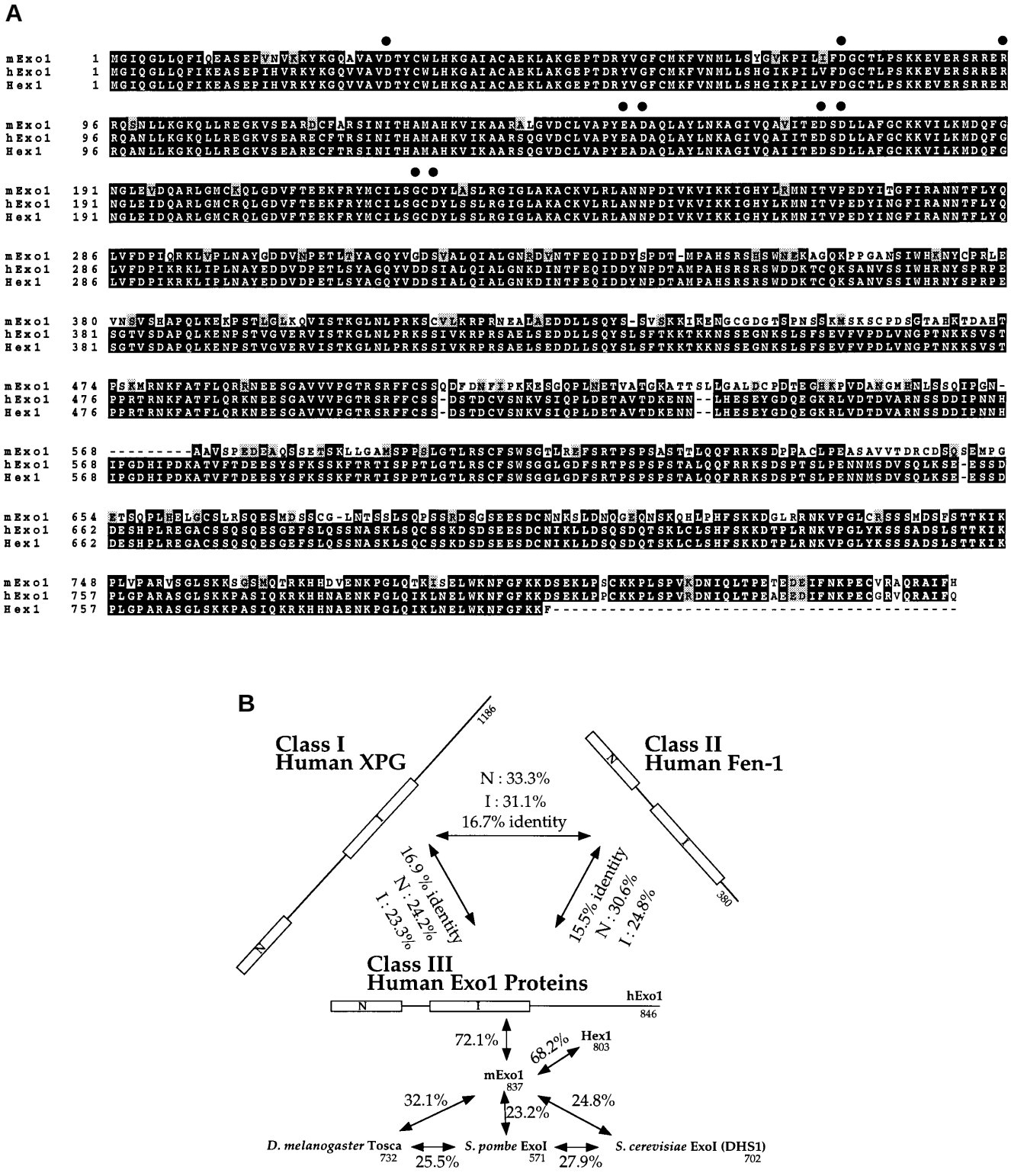

Figure 1. Alignment of the mammalian exonuclease 1 proteins and sequence comparison of the RAD2 nuclease family. (**A**) Amino acid sequence alignment of mExo1, hExo1 (16,30) and Hex1 (5). The solid circles represent amino acid residues that are identical to active site residues of T4 RNase H (46). Black indicates regions of identity, gray regions of similarity, and white regions of non-conservation. Dashes represent gaps. Note: hExo1 is equivalent to hExo1b and Hex1 is equivalent to hExo1a (30). (**B**) Schematic representation of the three human *RAD2* classes. The relative positions of the N and I nuclease domains are boxed and indicated in bold. Percent identity (as determined by Network Protein Sequence Analysis) across the entire length of the protein, or across the N and I nuclease domains, is indicated. Arrows note the proteins being compared. Length in amino acids for each protein is noted below each respective protein.

the C-terminal differences, occurs in the central region of these proteins. A cDNA splice variant encoding a shortened form of the mouse protein was not found in EST database searches.

The mammalian Exo1-like proteins show similarity to members of the *RAD2* nuclease family (Fig. 1B), which includes the structure-specific nucleases, XPG (Class I) and Fen1 (Class II), the bacteriophage exonucleases T4 RNase H and D15, and the small fragments of *E.coli*, *Streptococcus pneumoniae* and *Thermus aquaticus* DNA polymerases (22). This conservation is largely restricted to the core nuclease elements, the N (N-terminal) and I (Internal) domains. The highest similarity for mExo1 was observed with the *RAD2* Class III group that includes *D.melanogaster* Tosca and the yeast Exo1 proteins (Fig. 1B).

Chromosomal mapping of *mExo1*

To map the *mExo1* gene, we studied the segregation of *Mus spretus and Mus musculus* alleles in an IB panel (25). By comparing the segregation pattern of *mExo1* alleles with those of more than 400 other genes that had been typed in the same IB system, we were able to locate the *mExo1* gene to the distal portion of mouse chromosome 1 (Mmu1). The *mExo1* gene is located between the gene encoding alpha spectrin 1 (*Spna1*) and H2.0-like homeobox gene *Hlx*, mapping ~1.7 and 5.8 cM away from those two genes, respectively (Fig. 2, top).

Although distal mouse chromosome 1 is related along its length to human chromosome 1q (H1q), significant differences in gene order have been observed when comparing positions of related mouse and human loci within these syntenically homologous regions (31,32). For example, *SPTA1* (the human ortholog of *Spna1*), *HLX* and *CR2* are located in H1q21, H1q42–q41 and H1q32, respectively, indicating that the chromosomal segment containing *Hlx* and *Cr2* is inverted in order relative to *Spta1* and other Mmu1 loci when compared to orthologous genes in the human genome (Fig. 2, bottom). Since *mExo1* is located close to *Spna1* in mouse, but *HEX1/hExo1* maps to human chromosome 1q42–q43 (5,16,30), these results suggest that *mExo1* is contained within and located near the end of the evolutionarily inverted segment.

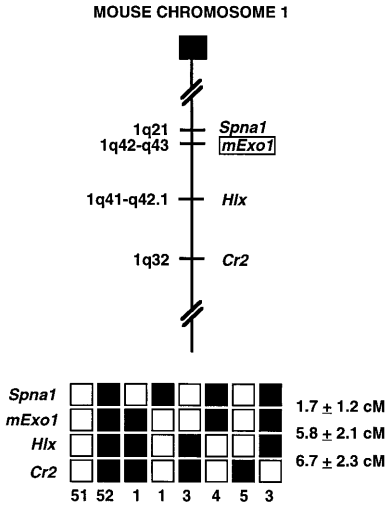

Figure 2. The *mExo1* gene is located in distal mouse chromosome 1. (**Top**) Partial map of distal mouse chromosome 1. A partial map of Mmu1 is shown, with position of the centromere indicated by a black box. Positions of *mExo1* and other Mmu1 genes are indicated by tick marks, with gene symbols shown on the right. Numbers at left report the published chromosomal positions of human orthologs of each of the four mouse genes (47). (**Bottom**) Segregation pattern of *mExo1* compared to that of other Mmu1 gene markers. The figure summarizes the segregation of *M.spretus* (S) and *M.musculus* (M) alleles of the *mExo1* gene in a 120-member IB panel. Each column represents one of the eight specific types of chromosomes detected in the 120 progeny; white and black boxes represent M or S alleles detected for gene markers and are indicated to the left of each row, respectively. Numbers at the bottom of each column report the number of animals found to carry each of the eight chromosome types. Numbers at the right between rows indicate the calculated distance between pairs of markers, in centimorgans (cM), and are reported with associated standard errors.

Expression specificity of the *mExo1* transcript

Using 5′ and 3′ fragment probes of the *mExo1* cDNA, we examined the tissue-specific expression of the mouse gene. Northern blot analysis revealed that the *mExo1* transcript is expressed highest in testis and spleen with lower expression observed in lung (Fig. 3A). To expand on these northern blot studies and evaluate potential qualitative expression similarities with the human gene, *mExo1* expression was monitored by RT–PCR in several relevant tissues. These studies found that *mExo1* mRNA is present at significant levels in thymus, lymph node and spleen (Fig. 3B), but at a lesser extent in brain, embryo body (separated from heads) and bone marrow, a pattern not fully consistent with that observed in human northern blot studies (5,30). Bone marrow expression was observed in mouse upon a second PCR reaction (data not shown). An estimate of the relative quantity of *mExo1* and *hEXO1/HEX1* mRNA in various tissues as determined by northern blot studies is shown in Figure 3C.

To investigate a possible role in spermatogenesis, the pattern of *mExo1* gene expression was examined in testis around the time of puberty. Since the first round of spermatogenesis occurs synchronously during testis maturation, it is possible to correlate changes in gene expression during this period with changes in the relative proportions of specific germ cell types in the testis (33). Mitotically dividing spermatogonia are the only germ cell type that is present in 8-day-old testis, with the bulk of cellular proliferation occurring between days 0 and 8 (all other germ cell types are non-proliferating). Meiotic spermatocytes are present in the testis at day 10, but only represent a small fraction of the spermatogenic cells at this stage of development. However, by day 17, spermatocytes constitute 33% of the seminiferous epithelium. The first cohort of pachytene cells, in which recombination occurs, appears between days 14 and 16 of postnatal development. After day 20 of age, the fraction of meiotic cells in the testis decreases as the number of post-meiotic germ cells steadily increases. Northern blot analysis and subsequent densometric analysis indicated that *mExo1* is expressed in testis throughout postnatal development, but that steady-state transcript levels are elevated at days 14–20, with a gradual decrease during this 6-day period and thereafter (Fig. 4).

DISCUSSION

The N-terminal portion of the mExo1 protein displays significant similarity to the *RAD2* nuclease domain found in several

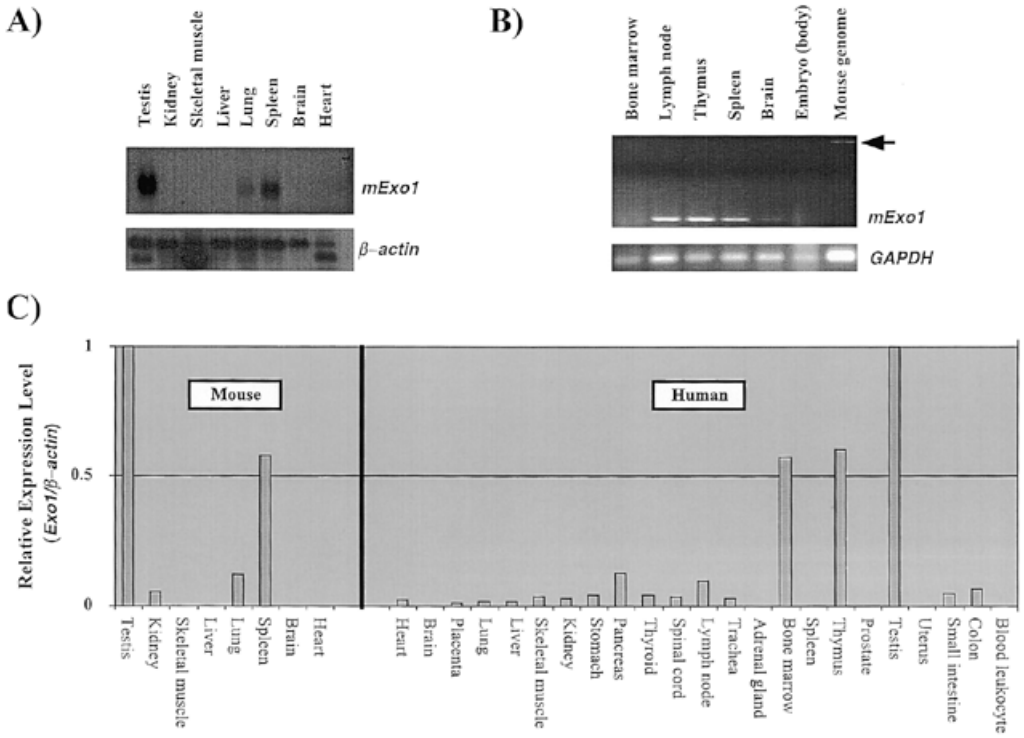

Figure 3. Expression specificity of *mExo1* and *HEX1*/*hEXO1* mRNA. (**A**) Northern blot analysis of *mExo1* mRNA expression in various tissues. Images were obtained by autoradiography. The β-*actin* control probe of the same blot is shown below. (**B**) RT–PCR analysis of *mExo1* expression. PCR products were analyzed as described in Materials and Methods, and images of ethidium bromide stained gels were obtained using Alphaimager™2000. The positive control for cDNA synthesis was GAPDH. As a control for DNA contamination, mouse chromosomal DNA (mouse genome) was used as the template. Genomic DNA PCR product is indicated by an arrow. Position of cDNA products is indicated by *mExo1*. (**C**) Quantitative comparison of the mouse and human gene expression patterns. Values were obtained by phosphorimager analysis and were normalized to values obtained for the β-*actin* control probe. Where two β-*actin* signals (alternatively splice β-*actin* transcripts) are observed, normalization was performed relative to the quantitative value obtained for both transcripts. Source and tissues from which RNA was isolated are indicated.

phage, prokaryotic and eukaryotic nucleases, with highest similarity to the Exo1-like, Class III proteins. mExo1 also maintains the core nuclease elements (the N and I domains) and many of the critical enzymatic residues (22) associated with the nuclease activities of the other *RAD2* family members. We have found that Hex1/hExo1 and its *RAD2* domain exhibit 5′ to 3′ exonuclease and 5′-flap endonuclease activities (5, B.-I.Lee and D.M.Wilson III, manuscript submitted). These nuclease functions likely operate during repair and recombination to excise mismatched nucleotides, resolve specific DNA intermediates or generate recombination-friendly DNA ends. Such activities are consistent with the DNA metabolic defects observed with the yeast *exo1* mutants, which have been found to display a mutator phenotype consistent with a mismatch repair defect as well as defects in mitotic and meiotic recombination (8,34). It has been recently reported that Hex1/hExo1 also maintains an RNAse H activity that may contribute to DNA replication (35).

The C-terminal region of the *RAD2* class III proteins is highly divergent, and it is this domain that likely dictates with which factors the various Exo1-like proteins interact, and thus to which cellular processes they contribute. Recent studies have reported an association of hMsh2, a factor that recognizes both mispaired nucleotides and Holliday junctions (17), with Hex1/hExo1 (16), and others have shown that this interaction occurs between the C-terminal domains of these proteins (L.Rasmussen, M.Rasmussen, B.-I.Lee, D.M.Wilson III and H.C.Bisgaard, unpublished result). This observation is consistent with the finding that the yeast Msh2 and Exo1 proteins associate (34), and suggests a cooperative role for these proteins in mismatch repair and homologous recombination.

The high expression level seen in testis for both the mouse and human genes suggests that the mammalian Exo1-like proteins likely contribute to homologous recombination events. A more detailed look at *mExo1* mRNA levels during testis development revealed a slightly higher level of expression during a period marked by a peak in the proportion of meiotic germ cells within the seminiferous tubules. Furthermore, the *mExo1* expression pattern parallels the appearance of pachytene cells, which are actively involved in recombination. Combined with the previous yeast genetic studies, these studies provide further evidence that the Exo1-like proteins operate during recombination processes. Worth noting is the observation that the human *MSH5* gene, a mutS homolog that is required for meiotic crossing over in *S.cerevisiae*, exhibits a nearly identical tissue-specific expression pattern to that

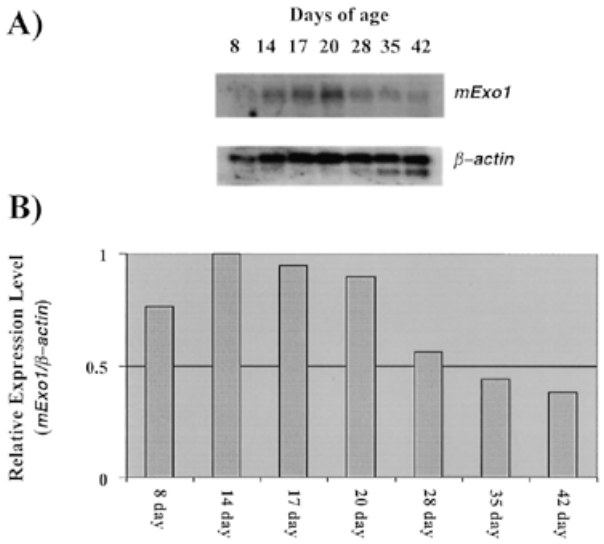

Figure 4. Expression of *mExo1* in testis during postnatal development. (**A**) Northern blot analysis of *mExo1* mRNA expression during testis development. Images were obtained by autoradiography. The β-*actin* control probe is shown below. (**B**) Quantitative analysis of the changes in steady-state levels of *mExo1* mRNA during testes development. Hybridization signals were measured by densitometric methods and were normalized to signals obtained with the β-*actin* control probe as described in Figure 3. In (A), the smaller transcript is observed only in post-meiotic germ cells (round and elongating spermatids) but not in earlier cell type. The larger transcript is the predominant form and is present in both somatic and germ cells. The age of the animal from which the testis was isolated is indicated in days.

observed for *HEX1/hEXO1* (36,37). It will now be interesting to determine whether the mammalian Exo1-like proteins interact with double-strand break processing factors to facilitate 5′ to 3′ resection and efficient recombination.

The mouse and human genes were found to be expressed highly in lymphoid tissues (38), although slight differences in the relative levels of mRNA exist between the two species, particularly between spleen and bone marrow. Given this subtle expression variation, it seems possible that the biological functions of the mammalian proteins have slightly diverged. Nonetheless, due to the high transcript levels detected in several lymphoid tissues, the mammalian Exo1-like proteins appear to function in yet unidentified processes related to haematopoiesis.

Interspecific backcross mapping results place *mExo1* in the distal portion of mouse chromosome 1, ~1.8 cM distal to the alpha spectrin gene. This position is consistent with the location of the human gene counterpart, which was mapped to the syntenically homologous region of human chromosome 1q42–q43 (5). Such a location suggests a potential link between the *mExo1* gene and the classic mouse mutation, *icthyosis (ic)*, which has been mapped to the same genetic interval (39). *Icthyosis* mutant mice are sterile and short lived, have rough, scaly skin and short, very thin or absent hair; in these respects they resemble human patients afflicted with trichothiodystrophy (TTD), a disease known to be caused by mutations in certain DNA repair genes [*ERCC2* (40); *ERCC3* (41)]. In addition, defects in nuclear morphology and abnormal DNA content have been noted in brain, leukocytes and epidermal cells of *ic* mutant mice (42–45). Further investigation into the possible link of *mExo1* mutations and the icthyosis phenotype are underway.

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