Novel Repeat Elements Direct Rat Proenkephalin Transcription during Spermatogenesis*

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Feng Liu, John Tokeson‡, Stephan P. Persengiev, Karl Ebert§, and Daniel L. Kilpatrick¶

From the Neurobiology Group, Worcester Foundation for Biomedical Research, Shrewsbury, Massachusetts 01545 and the §Department of Anatomy and Cell Biology, Tufts University School of Veterinary Medicine, North Grafton, Massachusetts 01536

Spermatogenesis consists of a well defined developmental program involving a series of proliferative and differentiative stages. These events can be divided into three phases: 1) mitosis involving spermatogonial cell types, 2) meiotic division and differentiation of spermatocytes into haploid spermatids, and 3) spermiogenesis in which spermatids morphologically differentiate into spermatozoa. Successful integration of these phases requires the sequential expression of different subsets of genes and involves, in part, stage-dependent transcription from spermatogenic cell-specific promoters. Developmental regulation of distinct and sometimes cell-specific transcription factors appears to be a critical mechanism controlling this spermatogenic program. For example, a number of proliferation-associated transcriptional regulators, including e-fos, c-jun, and c-myc, are expressed exclusively in spermatogonial cell types (1, 2). Similarly, germ cell-specific forms of CREB and CREM, as well as novel mRNAs for Sp1, are expressed during meiotic and/or postmeiotic stages (3–5).

Recent studies have begun to shed light on the role of transcriptional regulators during specific phases of sperm development. CREM, a spermatogenic cell-specific CREM isoform, functions as a transcriptional activator of promoters containing cAMP response elements (CREs) (6). Expression of CREM protein is reportedly restricted to postmeiotic (spermatid) stages, suggesting a specific role during spermiogenesis (4). Consistent with this, it has been shown to activate transcription from a number of haploid-specific promoters that contain CREs, including those for RT7, protamine-1, and transition protein-1 (6–8). In addition, CREM-deficient mice are infertile due to the failure to complete spermiogenesis beyond the round spermatid stage, and they lack testicular expression of several haploid-specific, CRE-dependent genes (9, 10). Clearly, while CREM has a critical role in regulating spermiogenesis via CRE-containing promoters, additional transcription pathways are required for appropriate sperm maturation. For example, several germ cell promoters are active during meiosis alone or in both late meiotic and early postmeiotic germ cells, including those for histones H1 and H2B, lactate dehydrogenase c, Pdha-2, proacrosin, and pgk-2 (11–16). Further, formation of pachytene spermatocytes and early (round) spermatids is not interrupted in CREM-deficient mice, and proacrosin expression persists in these mutants (9, 10). Thus, CREM-independent transcriptional mechanisms clearly are important for meiosis and haploid cell formation.

The proenkephalin gene, which codes for enkephalin-containing opioid peptides, is expressed in a stage-dependent manner during rat and mouse spermatogenesis (17, 18). Opioid peptides have been implicated in paracrine interactions between germ cells and Sertoli cells that may be important for maintenance of spermatogenesis (19, 20). Proenkephalin expression occurs initially at low levels in early meiotic stages and then increases markedly in late pachytene spermatocytes and early (round) spermatids and declines thereafter. These cells use an independent, alternate promoter within the single copy proenkephalin gene to synthesize a novel 1700-nt germ cell-specific mRNA (21). Previous studies have demonstrated that the rat proenkephalin germ line promoter is contained within a 500-bp TATA-less sequence located within the first somatic
5'-Flanking Elements Mediate Proenkephalin Germ Line Promoter

introns (22). While the upstream somatic promoter contains functional CRE elements (23), the germ line proenkephalin promoter lacks such consensus sequences. Since this promoter is highly active in both pachytene spermatocytes and round spermatids, its characterization should provide valuable insight into CRE-independent transcriptional mechanisms governing formation of late meiotic and early haploid cells. Here we report the identification of novel cis-acting repeat elements required for expression of the rat germ line proenkephalin promoter using a transgenic mouse model. The existence of germ cell-specific nuclear factors interacting with these elements is also demonstrated.

MATERIALS AND METHODS

DNA Constructs—Previous transgenic studies (22) used a rat proenkephalin chloramphenicol acetyltransferase (CAT) fusion construct encompassing the germ cell proenkephalin start site region as well as adjacent 3'- and 5'-sequences (RPKCAT0.5). Derivatives of this gene were generated as follows (see Fig. 1). RPKCAT0.5_del is RPKCAT0.5.3BSM were prepared by isolation of HindIII-MspI and HindIII-BsmI fragments, respectively, from RPKCAT0.5 and subcloning into the promoterless RPKCAT vector using HindIII and Smal sites. This was made by deletion of the first 100 bp from RPKCAT0.5 using T7f and Xhol and re-ligation of blunt ends. Constructs RPKCAT0.5-3_Del, RPKCAT0.4-3_Del, RPKCAT5_Del, RPKCAT0.45, and RPKCAT0.45-3_Del were all generated using the polymerase chain reaction (PCR). Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA) was used in 20 cycle reactions. The DNA sequences of 5'-primers were as follows: 0.5'-3_Del, GATCTACCATG- ATGATCGGCATGCCTCAACCTTCT; 0.4-3_Del, CAGTACCGTGCA- GAATCTGCTCAACGCAAGCG; and 0.45-3_Del, ACTACCA- TCAGATGCCGCTCTCGCCGCGGGCG; and 0.5'-5_Del, GATCTACCATCAT- GATCGAACAGGAGGATCGCCCA. The 3'-primer used for each construct was T3f and Del construct was TCCTGTCCTTCTCGAGGGCGGCG, while that used for constructs 0.5'-5_Del and 0.45_Del was GCTTGCTCCAGATG- CTGAGGCT. Restriction sites within the RPKCAT vector used for subcloning of PCR products were as follows: 0.5-3_Del, 0.5-5_Del, 0.45-3_Del for ClaI_SmaI, and 0.4-3_Del for XhoI_SmaI.

The transgene GCP1mut was prepared by annealing sense and antisense oligonucleotides containing GCP1 sequences that extended to the third repeat element at its 3'-end where a T7f site is located and that contained mutations in the second and third repeats (shown in lower case): GCCTTGGCGCTTCTCCCCAGGGCAGAGATTGTCCCTGCTGGTCCTGCTCCAGAATCTTGCCCT; and GCP1mut23, GCGCCTGCTCCCGAGCCGCGAACTCATTGTTGCTCTCCAGAATCTTGCCCT; and GCP1mut123, GCGCCTGCTCCCGAGCCGCGAACTCATTGTTGCTCTCCAGAATCTTGCCCT. GC- P1mut1, GCP1mut2, GCP1mut3, and GCP1mut23 oligonucleotides contained the same mutated bases of the indicated repeat elements as GCP1mut123. TATA-mut sequences were used as nonspecific competitor DNA: GGCGGGGGAGAAAAGGGGT. Additional competitor DNAs used were: CRE, AGAGATTGCGCTGAGTTGAGACGCG; CREmut, AGAGATTGCGCTGAGTTGAGACGCG; and Gli, GAAAGATTGCGCTGAGTTGAGACGCG.

Autoradiographs of competition experiments using wild-type and mutated GCP1 sequences were quantified using densitometric scanning (PDI, Huntington Station, NY).

Sequence Analysis—A search for transcription factor binding elements within rat proenkephalin promoter sequences was performed using the TSSites program (Genetics Computer Group, Madison, WI).

RESULTS

Functional Mapping of Downstream Sequences within the Testis-specific Proenkephalin Promoter—Previous work demonstrated that a fusion gene construct (RPKCAT0.5) containing 500 bp of the rat proenkephalin gene linked to the reporter gene CAT was sufficient for cell- and stage-specific expression of the germ line proenkephalin promoter (22). RPKCAT0.5 contains the transcriptional start site region for the rat promoter as well as neighboring 5'- and 3'-sequences (Fig. 1). To further map cis-elements that specify spermatogenic cell transcription, a series of proenkephalin-CAT constructs containing different portions of this 500 bp promoter sequence were generated and tested in transgenic mice (Fig. 1). summarizing the results for a series of nine different constructs in comparison with RPKCAT0.5. Constructs RPKCAT0.5_delSP (−118 to +312), RPKCAT0.5_delSP (−118 to +168), RPKCAT0.5 (which also contains the upstream somatic promoter), and RPKCAT0.5-3_Del (−118 to +62) comprise sequential deletions of the 3' promoter region. All four constructs actively

performed as described previously (25). Briefly, radiolabeled antisense RNA probes were generated from linearized plasmids using [α-32P]UTP and either T7 or T3 RNA polymerase. The RNA probes were annealed to total RNA in a final volume of 30 μl of hybridization buffer and then digested with 40 μg/ml Rnase A and 2 μg/ml Rnase T1. Samples were treated with proteinase K (20 mg/ml) and then extracted, precipitated, and analyzed on 5% polyacrylamide sequencing gels. Riboprobes vectors for CAT (pcAT) sequences and the rat proenkephalin germ line start site region (pAv) have been previously described (25). The vector p0.65-3_DelCAT was used for start site analysis of the RPKCAT-3_Del constructs. It was prepared by subcloning a Xhol/EcoRI fragment from RPKCAT0.45-3_Del into the same sites within pB8-8K (−) (Stratagene, La Jolla, CA).

Preparation of Germ Cells and Nuclear Extracts—Spermatogenic cells enriched in pachytene spermatocytes and spermatids were prepared by enzymatic digestion of adult transgenic mouse testes essentially as described by Bellve et al. (27). For nuclear extracts, enriched spermatogenic cells were isolated from adult rat testes and homogenized in the presence of a protease inhibitor mixture (5 μg/ml each aprotinin, pepstatin A, and leupeptin, 2 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride) (22). Nuclei were isolated on sucrose gradients, and nuclear proteins were extracted as described previously (22, 28) and stored at −70 °C.

Gel Mobility Shift Assays—Gel-shift assays were performed according to standard protocols modified for the 25–1 ng of 32P-labeled DNA (oligonucleotide or genomic) was incubated on ice for 20 min with 2 μg of nuclear extract in a total reaction buffer volume of 14 μl. The reaction buffer contained 10 mM HEPES (pH 7.9), 30 mM NaCl, 10% (v/v) glycerol, 0.5 mM dithiothreitol, 6 mM MgCl2, 6 mM spermidine, 0.5 μg of sonicated salmon sperm DNA, and 0.5 μg of poly(dA·dT). DNA-protein complexes were resolved on 4% non-denaturing polyacrylamide gels using low ionic strength buffer containing 12.5 mM Tris borate (pH 8.0) and 0.25 mM EDTA. Competition assays were performed by addition of excess amounts of unlabeled oligonucleotide into the reaction mixture.

Double-stranded oligonucleotides and genomic restriction fragments containing 5' overhangs were labeled with [α-32P]dCTP by fill in reactions as described by Maniyan. Oligonucleotides were prepared by restriction digestion of pRPKCAT0.5 (22). DNA sequences of oligonucleotide probes were as follows: GCP1, GCCCTTGCTGCCGCGCACGCAGACACTGATTGCTTGCTCCAGAATCTTGCCCT; and GCP1-mut123, GCCCTTGCTGCCGCGAACATAGTGCATTGCTCCAGAATCTTGCCCT. GCP1mut1, GCP1mut2, GCP1mut3, and GCP1mut23 oligonucleotides contained the same mutated bases of the indicated repeat elements as GCP1mut123. TATA-mut sequences were used as nonspecific competitor DNA: GGCGGGGGAGAAAAGGGGT. Additional competitor DNAs used were: CRE, AGAGATTGCGCTGAGTTGAGACGCG; CREmut, AGAGATTGCGCTGAGTTGAGACGCG; and Gli, GAAAGATTGCGCTGAGTTGAGACGCG.

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expressed CAT transcripts in testes of transgenic mice but not in somatic tissues (kidney, liver, heart, or cerebellum) as determined by RNase protection analysis of total RNA using an antisense riboprobe to CAT sequences. In each case, a 250-nt CAT protection fragment was selectively detected in testis RNA (Fig. 2; data not shown). To confirm that the transgenes were expressed in the male germ line, RNase protection was performed using RNA from enriched preparations of pachytene spermatocytes and round spermatids, stages that express rat proenkephalin in high amounts. CAT transcripts were abun-
dantly detected in these germ cells for all four transgen-
es (Fig. 3A and data not shown). The level of transgene expression in germ cells was equivalent to that for the original RPKCAT0.5 construct, as shown for RPKCAT0.5–3Del (Fig. 3A).

Transcriptional initiation of the 1.7-kilobase proenkephalin mRNAs in rat and mouse testis occurs at multiple sites spanning a 35-bp region within the germ line promoter. RNase protection using probes encompassing this region was performed to determine whether transcription of the four transgenes initiated from these same sites. Analysis of testicular RNA from RPKCAT0.5–3Del mice using the p0.45–3′DELCAT riboprobe generated multiple protection fragments of approximately 300, 309, 320, and 334 nt that agree well with the predicted sizes for products initiated from the appropriate start sites (plus column). The minus column shows the number of transgenic lines that did not express CAT either in testis or germ cells, but not in somatic tissues, and that were initiated from the appropriate start sites (data not shown). This result agrees well with the sizes predicted for transscripts initiated from the 3′-Del transgenes, as shown in the schematic in panel C, C, the region encompassed by the p0.45–3′DELCAT riboprobe is shown together with the four observed protection fragments generated from the initiation region (In) below it.

or 105 bp (−118 to −14) (RPKCAT0.4) of the 5′-flanking region resulted in complete loss of detectable CAT transcripts in testes of transgenic mice (Figs. 1 and 4). For all but one transgenic line, CAT expression was also absent in somatic tissues of mice harboring these transgenes. One RPKCAT0.4 line exhibited expression in multiple somatic tissues as well as testis (data not shown). This appears to reflect the fortuitous insertion of the transgene adjacent to or within a ubiquitously active tran-

Analysis of 5′-Flanking Sequences—In contrast to the above findings, deletion of either 128 (−118 to +10) (RPKCAT5′-Del)
The specific protection products are shown by the bracket. Rat testis (RT) and non-testis mouse testis (MT) served as positive and negative controls, respectively. The transgenic lines tested are given by the number above each lane. T, testis; C, kidney.

The preceding findings indicated that nuclear protein interactions with GCP1 sequences are critical for activity of the proenkephalin germ line promoter during spermatogenesis. Sequence analysis did not reveal any consensus binding sites for known transcription factors within this region. However, the GCP1 sequence does contain three direct repeats (CTCCA/CG) (Fig. 7A) that could function to regulate transcription. The possible involvement of these repeated sequences in factor binding was, therefore, examined using gel-shift analysis. GCP1 sequences form a major and additional minor complexes of higher mobility with rat testis and germ cell nuclear extracts that are distinct from those formed with liver nuclear proteins (Fig. 7B). The relative abundance of the higher mobility complexes varied in different experiments (Fig. 7, C and D), and they may reflect probe heterogeneity and/or partial proteolysis in some experiments. Competition experiments demonstrated that binding of germ cell factors to GCP1 was DNA sequence-specific (Fig. 7C). This analysis also re-

Previous experiments detected multiple sites within the 500 bp proenkephalin germ line promoter that were bound by nuclear proteins from spermatogenic cells (22). One of these sites, termed GCP1 (–54 to –4), was contained within the functional 105 bp 5′-flanking sequence, immediately upstream of the initiation region (Fig. 1). To determine whether binding within this region was functionally important, another transgene (RPKCAT0.45) was tested in which the 51-bp GCP1 sequence was selectively added back to the RPKCAT0.4 transgene. Readi-

Novel Spermatogenic Cell Nuclear Factors Specifically Interact with GCP1—The preceding findings indicated that nuclear protein interactions with GCP1 sequences are critical for activity of the proenkephalin germ line promoter during spermatogenesis. Sequence analysis did not reveal any consensus binding sites for known transcription factors within this region. However, the GCP1 sequence does contain three direct repeats (CTCCA/CG) (Fig. 7A) that could function to regulate transcription. The possible involvement of these repeated sequences in factor binding was, therefore, examined using gel-shift analysis. GCP1 sequences form a major and additional minor complexes of higher mobility with rat testis and germ cell nuclear extracts that are distinct from those formed with liver nuclear proteins (Fig. 7B). The relative abundance of the higher mobility complexes varied in different experiments (Fig. 7, C and D), and they may reflect probe heterogeneity and/or partial proteolysis in some experiments. Competition experiments demonstrated that binding of germ cell factors to GCP1 was DNA sequence-specific (Fig. 7C). This analysis also re-
revealed that GCP1 sequences were not recognized by CRE-binding proteins present in spermatogenic cell nuclear extracts (Fig. 7C). Further, mutation of the three direct repeats abolished the binding of germ cell factors (Fig. 7D), suggesting the direct involvement of these elements in GCP1/protein interactions.

Functional Analysis of GCP1—Additional studies of the repeat sequences were performed to determine their relative contributions to factor binding by GCP1. Mutation of the first repeat alone had a negligible effect on this interaction (Fig. 8). However, mutation of either the second or third repeat resulted in partial loss of binding activity (50–65% decrease) and mutation of both resulted in essentially complete disruption of the GCP1/protein complex (Fig. 8; see also Fig. 7D). Mutation of repeat 1 together with either the second or third repeat did not further reduce GCP1 binding activity, nor did deletion of the 5′-end of GCP1 including the first repeat (Fig. 8). Thus, the second and third repeats, which are identical in sequence and differ from the first repeat in the fifth position (A instead of C), are required for maximal binding of GCP1 to germ cell nuclear factors. It is possible that the first repeat is inactive because of this single base difference in the fifth position or because of its positioning within GCP1, or both.

To examine the functional significance of these in vitro protein interactions with GCP1 sequences, an additional transgene, containing mutations of the second and third repeats identical to those used in gel-shift analysis, was constructed and tested (GCP1mut; Fig. 1). Mutation of these two sites resulted in complete loss of transgene expression in mouse testis and did not produce abnormal expression in somatic tissues (Fig. 9). These findings indicate that the interactions between spermatogenic cell nuclear proteins and the second and third repeat elements within GCP1 are critical for proenkephalin germ line promoter activity.

DISCUSSION

Spermatogenic cells are characterized by the production of a large number of unique transcript forms often encoding ubiquitously expressed proteins (30). Some of these mRNAs encode germ cell-specific isoforms that may perform specialized functions during spermatogenesis (30). In other cases, novel untranslated sequences are present that may have specific regulatory roles, as in the translational control of testis-specific superoxide dismutase-1 mRNA involving its 5′-untranslated region (31). Transcription from an alternate spermatogenic cell-specific promoter is often involved in generation of these unique transcripts (32–34), as for rat and mouse proenkephalin. Alternate promoters expressed in somatic cells frequently function selectively during cell development and differentiation (35). It is therefore possible that some germ cell-specific transcripts reflect the necessity for an alternate promoter that provides appropriate cell-specific and developmentally regul-
moter and that a 500-bp region could mediate cell- and stage-specific expression (22). In this study, we have demonstrated that a 116-bp region encompassing the start site region is sufficient for appropriate transcription of the proenkephalin promoter in somatic cells and spermatocytes and that a 51-bp 5′-flanking sequence (GCP1) is absolutely required for its activity. These data are consistent with the presence of one or more cell-specific transactivators in these spermatogenic stages that interact with specific elements in GCP1. At present, there is no evidence for the involvement of transcriptional repression in somatic cells since deletion analyses did not result in ectopic transgene expression in somatic tissues. However, a role for silencer elements located elsewhere within the minimal promoter region cannot be ruled out. This is relevant since repression has been implicated in spermatogenic cell transcription of the c-mos, lactate dehydrogenase c, Tctex-1, and histone H2b promoters (12, 36–38).

Recent studies have begun to characterize transcriptional regulation during spermatogenesis using transgenic, cell transfection and in vitro transcription approaches (7, 11, 15, 22, 32, 39–44). While numerous germ cell-specific promoters have been analyzed, much remains to be learned regarding the specific transcriptional mechanisms mediating stage-dependent gene expression in the male germ line. A critical role for CREM proteins and CRE-dependent promoter regulation in mediating spermiogenesis has been established (9, 10). However, additional mechanisms appear necessary for formation of meiotic and early postmeiotic germ cells. In fact, several testis-specific DNA binding proteins apparently unrelated to CREM have been implicated in promoter regulation in these spermatogenic stages. For example, novel ets-domain-like proteins appear to regulate the pgk-2 promoter in mouse germ cells (39), and a testis-specific protein recognizes a novel palindromic regulatory element involved in testis-specific transcription of the mouse lactate dehydrogenase c gene (37). Both of these promoters function during spermatocyte and spermatid stages. In addition, testis-specific factors bind to regulatory elements (TE1 and TE2) unrelated to CREs within the spermatocyte-specific histone H1t promoter (45). However, the identities and functional roles for these and other putative germ cell-specific trans-factors, such as Tet-1 (46), are generally unknown.

The proenkephalin germ line promoter also becomes highly active in pachytene spermatocytes, and the 51-bp GCP1 regulatory region does not contain CRE-like sequences and is not responsive to CREM or CREB in transient co-transfection assays (data not shown). In addition, proenkephalin expression is not reduced in testes of CREM-deficient mice. Therefore, the proenkephalin gene is also regulated by CREM-independent mechanisms during spermatogenesis. Consistent with this, germ cell-specific nuclear proteins distinct from CREM and other CRE-binding proteins specifically bind to this region. Factor binding involves a novel sequence (CTCCAG) repeated twice within GCP1, and more importantly, mutation of these two elements abolishes proenkephalin germ line promoter activity. Interestingly, this repeat resembles sequences within the lactate dehydrogenase c palindromic element that are required for germ cell-specific transcription (CTCTCG) (37). In both promoters, these sequences are located just upstream of the start sites, and in both cases, germ cell proteins bind to DNA segments containing these elements. Similarly, the testis-specific element TE2 found within the rat histone H1t promoter also contains sequences resembling the GCP1 repeats (CCCGAG) (45). It is therefore possible that sequences related to the repeat elements within GCP1 may regulate multiple germ cell-specific promoters expressed during late meiosis. Repeated elements are often required for proper DNA binding or enhanced activation by trans-acting factors. For example, DNA binding by thyroid hormone requires appropriately spaced direct repeats (47), and Sp1 exhibits synergistic promoter activation when multiple binding sites are present (48).

Functional analysis of other sequences within the 116-bp promoter region is an important goal of future analyses. For example, initiation regions are often critical for activation of TATA-less promoters (49) although sequences resembling typical initiator (Inr) elements are not found within the start site region of the proenkephalin germ line promoter. In addition, another region is present within the defined minimal promoter (GCP2) that binds germ cell-specific nuclear factors and contains a palindromic GC-box (see Fig. 6B). Finally, characterization of the GCP1 binding proteins, their developmental expression, and potential interactions with other promoters active during late meiosis are clearly important to pursue.

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