The RHOX5 Homeodomain Protein Mediates Transcriptional Repression of the Netrin-1 Receptor Gene Unc5c

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The X-linked mouse Rhox gene cluster contains more than 30 homeobox genes that are candidates to regulate multiple steps in male and female gametogenesis. The founding member of the Rhox gene cluster, Rhox5, is an androgen-dependent gene expressed in Sertoli cells that promotes the survival and differentiation of the adjacent male germ cells. Here, we report the first identification and characterization of a Rhox5-regulated gene. This gene, Unc5c, encodes a pro-apoptotic receptor with tumor suppressor activity that we found is negatively regulated by Rhox5 in the testis in vivo. Transfection analyses in cell lines of different origin indicated that Rhox5-dependent down-regulation of Unc5c requires another Sertoli cell-specific cofactor. Examination of other mouse Rhox family members revealed that mouse Rhox2 and Rhox3 also have the ability to down-regulate Unc5c expression. The human RHOX protein PEPP2 (RHOXF2) also had this ability, indicating that Unc5c repression is a conserved RHOX-dependent response. Deletion analysis identified a Rhox5-responsive element in the Unc5c 5′-untranslated region. Although 5′-untranslated regions typically house post-transcriptional elements, several lines of evidence indicated that Rhox5 down-regulates Unc5c at the transcriptional level. The repression of Unc5c expression by Rhox5 may, in part, mediate the pro-survival function of Rhox5 in the testis, as we found that Unc5c mutant mice have decreased germ cell apoptosis in the testis. Along with our other data, these findings led us to propose a model in which Rhox5 is a negative regulator upstream of Unc5c in a Sertoli-cell pathway that promotes germ-cell survival.

Homeobox genes were originally identified as genes responsible for embryonic mutant phenotypes in Drosophila melanogaster (1). Homeobox genes have since been identified in a wide variety of eukaryotic species ranging from Saccharomyces cerevisiae to mammals (2, 3). The signature feature of all homeobox genes is a ~180-nt motif encoding a 60-amino acid helix-turn-helix DNA-binding domain called a homeodomain that enables homeobox proteins to act as transcription factors. Homeodomain-containing transcription factors regulate a large number of embryonic developmental events, including anterior-posterior axial identity and organogenesis (4).

Although the roles of homeobox transcription factors in embryonic development have been intensely studied for over two decades, their functions in controlling post-embryonic developmental events have only begun to be scrutinized. Homeobox transcription factors likely have a role in postnatal and adult developmental events because they are expressed in the cell types involved. Indeed, studies have shown that the homeobox genes HoxA9, Hoxc13, and Pdx1 are crucial for hematopoiesis, hair growth, and gut homeostasis, respectively (5–7). Another post-embryonic event that is likely regulated by homeobox genes is spermatogenesis. This idea is supported by the fact that >50 of the ~200 known mouse homeobox genes are expressed in the testis and other male reproductive organs (8–16). However, the roles of most of these genes in postnatal and adult events in the male reproductive tract are not yet clear because efforts to elucidate their roles using knockout mice have been clouded by putative functional redundancies and embryonic lethality (10). Instead, most progress has been made in demonstrating the role of homeobox genes in the formation of the male reproductive tract during embryogenesis (15–18).

To date, the only mammalian homeobox gene known to have a role in spermatogenesis is Pem (Rhox5), the founding member of the reproductive homeobox on the X chromosome (Rhox) gene cluster. In mice, this cluster contains >30 homeobox genes that are selectively expressed in reproductive tissues (8, 11–14, 19). Individual Rhox genes display unique patterns of expression during postnatal testes development and in different adult reproductive tissues, suggesting that they have unique roles in the reproductive tract (8). The human RHOX genes PEPP1/OTEX (RHOXF1) and PEPP2 (RHOXF2) are also selectively expressed in the testis, suggesting that Rhox genes have conserved roles in the reproductive tract (20, 21).

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3866 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 283 • NUMBER 7 • FEBRUARY 15, 2008

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 7, pp. 3866 –3876, February 15, 2008
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Targeted deletion of Rhox5 in mice results in male subfertility, marked by increased germ-cell apoptosis, reduced sperm number, and reduced sperm motility (8). Rhox5-null mice have not only increased numbers of apoptotic germ cells at seminiferous epithelial cycle stages that normally contain dying germ cells (stages I–IV and stage XII) but also apoptotic germ cells at stages that normally lack dying germ cells (stages V–XI). Because Rhox5 is not detectably expressed in germ cells but instead is expressed in the adjacent Sertoli nurse cells, the increased germ-cell apoptosis in Rhox5-null mice is most likely caused by loss of a Sertoli cell-expressed RHOX5-dependent survival factor (9, 22, 23).

To identify genes that convey RHOX5-mediated germ cell survival, we set out to identify genes regulated by Rhox5 in Sertoli cells. A wide range of strategies have been used to screen downstream targets of homeobox transcription factors, but remarkably few targets have been identified to date (24–26). This is probably the result of many factors, including the complexity of regulatory networks under the control of homeobox genes and the fact that the full-length cis elements bound by homeobox transcription factors have been difficult to identify; most homeobox proteins appear to acquire DNA-binding specificity because of their interactions with cofactors in vivo (27–29). To identify Rhox5-regulated genes, we chose to use microarray analysis to screen for transcripts differentially expressed in response to physiological levels of Rhox5 in the Sertoli cell line 15P-1. We report here that one RHOX5-regulated gene encodes the netrin receptor Unc5C, a type-I transmembrane protein of the immunoglobulin superfamily that has pro-apoptotic activity (30–32). The ligand for Unc5C is netrin-1, which governs axon migration in the brain by triggering chemorepulsion (33). Although Unc5C and other Unc5 family members have been well studied in the brain, their function and regulation in other organs have not been explored. Here, we report that the Unc5c gene is expressed in Sertoli cells in the testis. We demonstrate that Unc5c is negatively regulated by Rhox5 in Sertoli cells, provide evidence that this regulation occurs at the transcriptional level, and identify Unc5c cis sequences and RHOX5 domains responsible for this down-regulation. We also show that Sertoli cell-specific cofactors have a role in this down-regulation and compare the ability of RHOX5 to down-regulate Unc5c transcription with that of other RHOX family members. These results led us to posit that RHOX5’s germ cell-survival function is mediated in part by its ability to down-regulate Unc5C, a pro-apoptotic molecule (31, 34, 35). Consistent with this notion, we found that Unc5c mutant mice have decreased male germ-cell apoptosis. Taken together, our results suggest the existence of a cell-survival pathway regulated by the RHOX5 homeobox transcription factor in the testis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—The 15P-1, MSC-1, TM4, HeLa, MME, and 10T1/2 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and both penicillin and streptomycin at 50 mg/ml. The MLTC-1 Leydig cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and both penicillin and streptomycin at 50 mg/ml. The cells were grown at 37 °C in 5% CO2 atmosphere and split when ~80% confluent.

The concentration of plasmids used for transfection was determined independently using both a fluorometer and analytical gel electrophoresis. For transient transfection analysis, cells were plated in 12-well dishes the day before transfection at a density of ~5×105 cells per well in 0.5 ml of serum-free medium. On the day of transfection, 1–2 μg of plasmid DNA was diluted with 150 μl of serum-free medium and mixed with 150 μl of serum-free media containing 6 μl of Lipofectamine (Invitrogen), incubated at room temperature for 30 min, and the DNA-lipid complex was added to the wells and incubated at 37 °C in a CO2 incubator for 5 h. Cells were harvested 36 h post-transfection for luciferase assay using the Dual Luciferase Assay System (Promega, Madison, WI), following the manufacturer’s instructions. The relative luciferase activity was calculated by normalizing against the co-transfected internal control pRL-TK. The results shown are the mean ± S.E. of three independent transfection experiments.

**Plasmids**—The full-length Unc5c-P vector (G-708) was made by PCR-amplifying Unc5c sequences with the primers MDA-2231 and -2232, cloning into the pGEM-Teasy vector (Promega, Madison, WI), releasing the insert using the restriction enzymes Xhol and HindIII, and cloning into the reporter vector pGL3 (Promega Inc.). The Unc5c-m1 (G-782) plasmid was made in an analogous way using the primers MDA-2949 and -2232. The Unc5c-m1 construct was then used as template to generate the deletion constructs Unc5c-m2 (G-758), Unc5c-m3 (G-762), Unc5c-m4 (G-802), Unc5c-m5 (G-787), Unc5c-m6 (G-803), and Unc5c-m7 (G-788) by PCR-mediated deletions (36), using the antisense primer MDA-2515 in combination with the sense primers MDA-2516, -2511, -2530, -2490, -2531, and -2491, respectively. Unc5c-m8 (G-829) and Unc5c-m9 (G-830) were generated by inverse PCR using an antisense primer MDA-2703 in combination with the sense primers MDA-2531 and -2490, respectively. Unc5c-m12 (G-852) and Unc5c-m10 (G-838) were generated by inverse PCR using the antisense primers MDA-2722 and -2711 in combination with the sense primer MDA-2950. Unc5c-m13 (G-860) was amplified by inverse PCR with the primers MDA-2743 and -2711. Unc5c-m14 (G-880) and Unc5c-m15 (G-902) were generated by introducing a single copy of the 100 nt of Unc5c 5’-UTR immediately preceding the start with 100 nt of ampicillin gene sequences (starting 6 nt downstream of the start ATG). Unc5c-m14 (G-880) and Unc5c-m15 (G-902) were generated by introducing a single copy of the 100 nt Unc5c 5’-UTR into the Nhel-Xhol site of Unc5c-m10. RHOX5-ΔHD (Pem-298), which encodes the truncated RHOX5 protein lacking the homeodomain, was generated with primers MDA-159 and -160. The mammalian expression vectors encoding RHOX1 (R-113), RHOX2 (R-114), RHOX3 (R-115), RHOX4 (R-116), RHOX6 (R-118), RHOX8 (R-119), RHOX9 (R-120), RHOX10 (R-121), RHOX11 (R-122), RHOX12 (R-123), RHOX11 (R-125), and RHOF2 (R-124) were generated by amplifying the respective cDNAs by RT-PCR from testis RNA and subcloning them into pcDNA5 (Invitrogen). The sequence of the manipulated region and insert/vector junctions in all plasmids was confirmed by DNA sequencing.
RNA and Protein Analysis—Total cellular RNA was prepared from cell lines and tissues as described previously by guanidinium isothiocyanate lysis and centrifugation over a 5.7 M CsCl cushion (37). Ribonuclease protection analysis was performed as described previously (9) using a probe that contained 500 nt of the Unc5c 5′-UTR and 98 nt of Unc5c coding sequence. The β-actin probe contains 135–169 nt of human β-actin exon 3 (GenBank™ accession number X00351). A set of RNA size markers generated from the century ladder template (Ambion, San Antonio, TX) was included in all gels. Real-time reverse transcriptase (RT)-PCR analysis was performed as described previously (8) using primers MDA-1287 and -1904 to detect mature mRNA, and primers MDA-2755 and -2756 to detect precursor mRNA. Standard RT-PCR analysis was performed by first synthesizing cDNA using the same protocol as in real-time RT-PCR. An antisense oligonucleotide MDA-2480 (g) in combination with the sense oligonucleotides MDA-2706 (a), MDA-2743 (b), MDA-2491 (c), MDA-2530 (d), MDA-2451 (e), and MDA-2584 (f), were used to specifically amplify the 5′-flanking region of the Unc5c promoter-driven luciferase transgene (Table 1).

For Western blot analysis, the protein concentration in total cell lysates was determined using the Bio-Rad DC protein assay. 20 μg of total cell lysates was electrophoresed in 10% SDS-PAGE gels, transferred to Hybond ECL nitrocellulose (American Biosciences), and probed with antibody against RHOX5, FLAG (Sigma), or β-actin (Sigma). The membranes were given three 10-min washes with 0.1% Tween 20 in phosphate-buffered saline at room temperature and then incubated for 45 min at room temperature with the 2nd antibody (ECL kit anti-rabbit or anti-mouse from Amersham Biosciences) at a dilution of 1:5,000. The membrane was developed by using the ECL-Plus reagent according to manufacturer’s protocol (Amersham Biosciences). The membranes were developed by using the ECL-Plus reagent according to manufacturer’s protocol (Amersham Biosciences).

**RESULTS**

The *Rhox5* Homeobox Gene Negatively Regulates Unc5c Expression in Sertoli Cells—Using microarray analysis, we identified genes differentially expressed in response to forced expression of physiological levels of *Rhox5* in the 15P-1 Sertoli cell line. We were able to take this gain-of-function approach because 15P-1 cells lack detectable *Rhox5* expression. By stably transfecting 15P-1 cells with an expression vector harboring *Rhox5* cDNA sequences, we selected for 15P-1 cell clones that had *Rhox5* mRNA levels similar to that in Sertoli cells in vivo. Microarray analysis of two cell clones expressing *Rhox5* with 2 control cell clones lacking *Rhox5* expression revealed that ~90 transcripts were up-regulated and ~90 transcripts were down-regulated by 2-fold or more in response to *Rhox5* (p < 0.05).5 One of the genes that we identified as negatively regulated by *Rhox5* in 15P-1 cells was the netrin receptor gene *Unc5c*. This gene was of interest to us because it is known to promote apoptosis (31), an event also regulated by *Rhox5* (8). Quantitative real-time RT-PCR (QPCR) analysis demonstrated that two independent 15P-1 cell clones expressing *Rhox5* had dramatically decreased levels of *Unc5c* mRNA compared with two *Rhox5*-negative 15P-1 cell clones (Fig. 1A). The same pattern of *Unc5c* expression was seen in other 15P-1 cell clones that either express *Rhox5* or not (data not shown).

To determine whether *Unc5c* is regulated by *Rhox5* in vivo, QPCR was performed on total testes cellular RNA from *Rhox5-

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null and control littermate mice. We examined testes from postnatal day 12 (P12), which express high levels of Rhox5 mRNA and Rhox5 protein (9, 22) and have a high proportion of Sertoli cells (40, 41). This analysis showed that Rhox5-null testes had increased Unc5c mRNA levels (Fig. 1B), indicating that Unc5c expression is repressed by Rhox5 in vivo, just as it is in 15P-1 cells. Note that Rhox5-null mice expressed a low level of Rhox5 mRNA (~100-fold less than in wild type), which is consistent with the fact that the targeted deletion in these mice does not disrupt known transcriptional elements but instead the Rhox5 homeodomain (22).

Because Rhox5 is specifically expressed in Sertoli cells in the testis (9), we examined whether Unc5c is regulated by Rhox5 in these cells. To do this, we purified cell fractions from adult testes enriched for Sertoli and interstitial (i.e. Leydig) cells using a modified protocol involving gravity sedimentation, enzymatic treatments, and finally hypotonic shock to eliminate contaminating germ cells. Although it is not possible to generate entirely pure populations of these cell types, our protocol yielded fractions that were substantially enriched, based on their relative expression of the Sertoli and Leydig cell markers Gata1 and P450scc, respectively (Fig. 2A). QPCR analysis indicated that Unc5c mRNA was expressed in both the Sertoli cell and the interstitial cell fractions from adult testes (Fig. 2A). Likewise, Unc5c mRNA was expressed in both the Sertoli and interstitial cell fractions prepared from P12 testes (Fig. 2B, note that P12 testes have a higher proportion of these somatic cell types as compared with adult testes (40, 41), and thus the enrichment for markers was lower than for adult testes). In contrast, Rhox5 mRNA was preferentially expressed in the Sertoli cell fraction from both P12 and adult testes (Fig. 2, A and B). The low Rhox5 mRNA signal in the interstitial cell fraction from P12 testes is probably the result of residual Sertoli cells in this fraction, because the Sertoli cell-specific marker, Gata1 (42), had the same expression profile as Rhox5 (Fig. 2B). Further evidence that Rhox5 is not expressed in interstitial cells is immunohistochemical analysis of postnatal and adult testes demonstrating that Rhox5 protein is only detectable in Sertoli cells, not Leydig or other interstitial cells (22, 23). To determine whether Rhox5 regulates Unc5c expression only in Sertoli cells or in both Sertoli and interstitial cells, we purified these cell subsets from wild-type and Rhox5-null mice. This analysis indicated that Unc5c mRNA expression was up-regulated in both the Sertoli and interstitial cell fractions from Rhox5-null mice, as compared with littermate control mice (Fig. 2C). Collectively, the data suggest that the regulation of Unc5c by Rhox5 in the testis is complex, involving different pathways in interstitial and Sertoli cells.

Rhox5-mediated Repression of Unc5c in Sertoli Cells—To identify sequences responsible for Rhox5-mediated repression of Unc5c expression, we amplified Unc5c genomic sequences extending from 1 kb upstream of the previously reported transcription start site (TSS) (30) to 115 nt downstream of the Unc5c TSS. This genomic fragment was cloned into the pGL3-basic firefly luciferase reporter vector and transfected into 15P-1 cells together with an internal control Renilla luciferase reporter construct (pRL-TK) and a Rhox5 expression plasmid. We found that the Rhox5 expression plasmid inhibited luciferase expression from the Unc5c promoter-containing plasmid (Unc5c-P) in a dose-dependent manner (Fig. 3A). Whereas the repression was not as dramatic as observed for the endogenous Unc5c gene (Fig. 1A), it was reproducible and statistically significant (p < 0.05).

Homeobox transcription factors typically require their homeodomain region to bind DNA and thereby regulate the transcription of most of their targets (43). However, there have been instances of homeobox transcription factors acting independently of their homeodomain by "piggy-backing" on other transcriptional factors (44). To determine whether its homeodomain is required for RHOX5 to repress Unc5c expression, we constructed a mutant Rhox5 expression plasmid encoding a truncated RHOX5 protein that has the intact N-terminal part and C-terminal part of RHOX5 but lacks the entire homeodomain (HD) (Fig. 3B). Transient transfection analysis indicated that this truncated RHOX5 protein lost its ability to repress Unc5c expression (Fig. 3A). The inability of this RHOX truncation mutant to repress gene expression was not the result of its instability, because it was expressed at similar levels as the wild-type protein, as shown by Western blot analysis (Fig. 3B). We
conclude that RHOX5 requires its homeodomain region to repress Unc5c expression.

To assess whether Rhox5 is sufficient to repress Unc5c expression or whether it requires cell type-specific cofactors, we performed transient transfection analysis in cell lines of different tissue origins. We found that the Rhox5 expression vector was only able to inhibit Unc5c expression in the Sertoli cell lines (15P-1 in Fig. 3A, and MSC-1 and TM4 in Fig. 3C) but not in the four non-Sertoli cell lines tested, including those of both epithelial and mesenchymal origin (Fig. 3C). This indicated that Rhox5-mediated repression requires one or more cofactors expressed in Sertoli cells but not in any of the other cell types we tested. As expected, the construct expressing RHOX5 lacking the homeodomain (RHOX5/ΔHD) did not have a significant effect on Unc5c expression in any of the Sertoli cell lines. However, unexpectedly, it significantly increased Unc5c expression in the MLTC-1 Leydig cell line. Although we do not know the
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To identify the sequences responsible for RHOX5-mediated repression, we analyzed the same mutants for expression when cotransfected with a RHOX5 expression vector. As a control, we also cotransfected cells with an expression vector encoding a homeodomain-deleted version of RHOX5 (described in Fig. 3B). Deletions removing all sequences up to −85 (m1 to m4) had no effect on RHOX5-mediated repression, demonstrating that sequences conferring repression were downstream of −85. In contrast, the deletion leaving only 35 nt of 5'-flanking sequence (construct m5) lost RHOX5-mediated repression. Although this suggested the possibility that the region between −85 and −35 houses a RHOX5-responsive element, a more likely explanation is that the −35 construct lost the ability to be efficiently transcribed and thus indirectly lost the ability to be repressed. Consistent with this latter interpretation is that luciferase activity from the −35 construct (m5) is even less than that of the −85 construct (m4) when cotransfected with the RHOX5 expression vector. Furthermore, the −85 to −35 region cannot harbor an essential RHOX5-responsive element, because deletion of this entire region did not prevent RHOX5-mediated repression in construct m8.

Analysis of these mutants, along with even more severe truncation mutants (m6, m7, and m9), suggested the possibility that RHOX5-responsive sequences are not present in the Unc5c promoter. An alternative hypothesis was that RHOX5-responsive sequences are instead in the Unc5c 5'-UTR, as our reporter constructs harbored 115 nt of the Unc5c 5'-UTR. To test this possibility, we made deletions in the 5'-UTR region (Fig. 4B). We found that deletion of most of the 5'-UTR (between +15 and +115, construct m10), allowed luciferase expression but prevented RHOX5-mediated repression. This provided evidence...
that the Unc5c 5'-UTR does indeed harbor sequences mediating Rhox5-mediated repression. Insertion of a heterologous 100-nt sequence in place of the Unc5c 5'-UTR sequences (construct m11) did not confer Rhox5-mediated repression, indicating specificity. Deletion of either the 3' half (between +70 and +115, construct m12) or the 5' half (between +15 and +75, construct m13) did not prevent Rhox5-mediated repression, suggesting that redundant Rhox5-responsive elements exist in the 5'-UTR region. To distinguish between the +15 and +115 regions having transcriptional versus post-transcriptional effects, we moved this region upstream of the TSS. We reasoned that, if it was an mRNA element acting post-transcriptionally, it could not have an effect at this site. Alternatively, if it was a transcriptional enhancer it would be predicted to function when moved upstream of the TSS. Consistent with the latter possibility, we found that placement of the +15 to +115 region upstream of the TSS (construct m14) conferred the ability to respond to Rhox5. Interestingly, it did not confer this property when introduced in the opposite orientation (construct m15), suggesting that its function is orientation-specific.

Rhox5 Regulates Unc5c at the Transcriptional Level—Our finding that the Rhox5-responsive region is in the 5'-UTR was surprising because 5'-UTRs typically harbor sequences involved in post-transcriptional regulation, not transcriptional regulation. To determine whether the previously mapped TSS for Unc5c in brain (30, 32) was also the TSS in testis, we used ribonuclease protection analysis using a probe spanning exon 1 and the region upstream (Fig. 5, A and B). A band of ~210 nt, which matches the length expected, based on the previously mapped transcription start site (30). Note that a lower exposure is shown for the RNA century ladder. β-actin RNA levels serve as an internal control. C, top: RT-PCR analysis of total cellular RNA from 15P-1 cells transiently transfected with the Unc5c-m1 plasmid (Fig. 4A), using the forward primers shown and a reverse primer in the luciferase gene (primer g; see "Experimental Procedures"). Bottom: genomic organization of the 5'-end of the mouse Unc5c gene. The location of primer used for QPCR are indicated; the black boxes denote Unc5c exons. Bottom: QPCR analysis, performed and quantified as described in Fig. 1A, on the indicated 15P-1 cell clones. Unc5c pre-mRNA and mature mRNA levels were determined using primer pairs A/B and C/D, respectively.

FIGURE 5. Rhox5 regulates Unc5c at the transcriptional level. A, Unc5c 5'-flanking and 5'-coding regions. The location of the probe used for ribonuclease protection analysis (shown in panel B) is shaded; the location of primers used for RT-PCR analysis (shown in panel C) are underlined. The 5'-end of Unc5c mRNA, as previously determined by the 5' rapid amplification of cloned ends method (30), is indicated by an asterisk. The translated region is in bold. B, ribonuclease protection analysis of total cellular RNA (50 μg) isolated from 15P-1 cells, adult testis, and adult brain. The size of the protected Unc5c mRNA band was ~210 nt, which matches the length expected, based on the previously mapped transcription start site (30). Note that a lower exposure is shown for the RNA century ladder. β-actin RNA levels serve as an internal control. C, top: RT-PCR analysis of total cellular RNA from 15P-1 cells transiently transfected with the Unc5c-m1 plasmid (Fig. 4A), using the forward primers shown and a reverse primer in the luciferase gene (primer g; see "Experimental Procedures"). Bottom: genomic organization of the 5'-end of the mouse Unc5c gene. The location of primer used for QPCR are indicated; the black boxes denote Unc5c exons. Bottom: QPCR analysis, performed and quantified as described in Fig. 1A, on the indicated 15P-1 cell clones. Unc5c pre-mRNA and mature mRNA levels were determined using primer pairs A/B and C/D, respectively.
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The human region syntenic with the mouse Rhox cluster harbors two RHOX genes that have been well characterized: RHOXF1 and RHOXF2 (20, 21). To test the functional role of RHOXF1 and RHOXF2, we cotransfected expression vectors encoding them with the Unc5c-P reporter construct. We found that RHOXF2, but not RHOXF1, significantly repressed Unc5c-driven reporter expression (Fig. 6C). This provided evidence that Unc5c transcriptional repression is a conserved response. It also shows that, as with mouse RHOX proteins, human RHOX proteins differ in their ability to regulate Unc5c.

Unc5c Promotes Germ Cell Apoptosis—UNC5C has been reported to be pro-apoptotic in tissue-culture cells (31). We hypothesized that UNC5C also promotes the apoptosis of germ cells in vivo. This follows from our finding the Unc5c is negatively regulated by Rhox5 (Fig. 1), a germ cell apoptosis inhibitor in vivo (8). To test this hypothesis, we used the TUNEL assay to detect apoptotic cells in testes from Unc5c-mutant mice (30, 32). In adult testes from these Unc5c−/− mice, we observed a modest but statistically significant decrease in the number of apoptotic cells per seminiferous tubule cross-section as compared with control littermate (Unc5c+/+) mice (Fig. 7A). Unc5c−/− adult testes also had a decreased number of tubules containing apoptotic cells (Fig. 7A). As in wild-type testes, the apoptotic cells in Unc5c−/− testes were primarily spermatocytes, based on the presence of a meiotic spindle or decondensed chromatid and their basal or near-basal position in seminiferous tubules.

We also examined apoptotic cells in testes from P12 Unc5c−/− mice. At this postnatal age, Rhox5 mRNA and RHOX5 protein are highly expressed (8, 22) and inhibit Unc5c expression (Fig. 1B). Using TUNEL, we observed that testes from P12 Unc5c−/− mice had much fewer apoptotic germ cells than did control littermate mice (Fig. 7, B and C). The decrease in apoptotic germ cell frequency in these postnatal Unc5c−/− testes was more dramatic than in adult Unc5c−/− testes (compare Fig. 7B with Fig. 7A). As with adult testes, P12 testes had decreased number of apoptotic germ cells per tubule cross-section and the number of tubules harboring apoptotic cells (Fig. 7B). Collectively, these data indicate that UNC5C promotes germ cell apoptosis in the testis.

DISCUSSION

Herein, we report the first identification and characterization of a gene regulated by Rhox5, a homeobox gene isolated almost 20 years ago by subtraction hybridization (45, 46). This Rhox5-regulated gene, Unc5c, encodes a netrin receptor pre-

FIGURE 6. Regulation of Unc5c by other mouse Rhox family members and human RHOXF2. A, promoter activity of the Unc5c-P reporter (Fig. 2A) in 15P-1 cells cotransfected with FLAG-tagged expression constructs encoding the indicated mouse RHOX proteins, performed and quantified as in Fig. 3A, B. Western blot analysis of the RHOX expression constructs used for the analysis in panel A. Extracts were prepared from 293T cells 48 h post-transfection, and the Western blots were probed with anti-FLAG antisera (Sigma). The second lane of each blot was loaded with extract from untransfected cells, which serves as a negative control. The blot was stripped and reprobed with a β-actin antisera as an internal control. C, promoter activity of the Unc5c-P reporter, performed as in panel A with the indicated human RHOX expression plasmids. Two-tailed Student’s t test: *, p < 0.05, **, p < 0.005.
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![Graph showing quantitative analysis of TUNEL-positive tubules and cells in adult mice testes from Unc5c+/+ and Unc5c−/− mice.](image1)

![Graph showing quantitative analysis of TUNEL-positive tubules and cells in P12 mice testes from Unc5c+/+ and Unc5c−/− mice.](image2)

![Graph showing quantitative analysis of TUNEL-positive tubules and cells in UncSh3+/− and UncSh3−/− mice.](image3)

FIGURE 7. Unc5c promotes testicular germ cell apoptosis. A, quantitative analysis of TUNEL-positive tubules and cells in adult mice testes from Unc5c+/+ and Unc5c−/− mice. The data represent the mean ± S.E. from a total of six tissue sections (**, p < 0.005). B, quantitative analysis of TUNEL-positive tubules and cells from P12 mice testes. The data represent the mean ± S.E. of a total of 13 tissue sections from three Unc5c+/+, two Unc5c+/−, and two Unc5c−/− mice (*, p < 0.02; **, p < 0.005; ***, p < 0.001). C, TUNEL analysis of apoptotic germ cells in P12 testes sections. Apoptotic germ cells were assessed by immunofluorescence with the ApopTag (green). Nuclei are stained with propidium iodide (red).

RHOX5 is known to have crucial roles in neuronal development (47). We found that the expression of Unc5c is negatively regulated by Rhox5 in Sertoli cells both in vivo and in vitro (Fig. 1, A and B), and we mapped a Rhox5-responsive cis-regulatory element in the Unc5c 5′-UTR (Fig. 4, A and B). The location of this Rhox5-responsive element was surprising because 5′-UTRs typically house post-transcriptional elements controlling RNA stability or translation. Nevertheless, three lines of evidence supported that Rhox5 regulates Unc5c expression at the transcriptional level. First, the RHOX5 DNA-binding (homeodomain) region was required for RHOX5 to down-regulate Unc5c promoter-driven reporter expression (Fig. 3A). Second, the level of Unc5c precursor transcripts were strongly reduced in response to Rhox5 (Fig. 5D), a result consistent with reduced transcription, not destabilized mature mRNA. Third, insertion of the Rhox5-responsive element at a site upstream of transcriptional initiation in a reporter construct conferred the ability to respond to Rhox5 (Fig. 4B). This effect was specific, because insertion of a heterologous sequence did not confer Rhox5 responsiveness. To our knowledge, only a few transcriptional response elements have been identified in 5′-UTRs. For example, the gene encoding macrophage migration inhibitory factor is transcriptionally induced in response to hypoxia by virtue of an element in its 5′-UTR (48). Similarly, the Nex1, Pur, and CD28 genes harbor 5′-UTR elements necessary for their transcriptional control (49–51).

Although most Rhox family members did not share with Rhox5 the ability to regulate Unc5c transcription, we found that Rhox2 and Rhox3 did have this activity (Fig. 6A). This suggests the possibility that these three homeobox genes have partially redundant functions. A likely site for redundancy is the Sertoli cell, because all three are expressed in Sertoli cells (8). Furthermore, Rhox2 and Rhox3 have very similar postnatal expression patterns in the tests, and Rhox3 is coexpressed with these two at a midpoint of postnatal development (8). Complicating this picture is the recent discovery that the mouse genome harbors eight paralogs of both Rhox2 and Rhox3 that are almost identical in sequence (11–14). Thus, it is possible that 16 genes in addition to Rhox5 have the ability to regulate Unc5c transcription. Rhox2 and Rhox3 genes are also coexpressed with Rhox5 in the epididymis, ovary, and placenta, extending the possibility of redundancy to these other tissues (8, 12). This putative redundancy could explain why Rhox5-null male mice are subfertile rather than infertile (8) and why Rhox5-null female mice have no obvious defects in either ovary or placenta (22).

The homeodomain third helix harbors four key amino acids (at positions 47, 50, 51, and 54) responsible for making base-specific contacts with DNA (52). Surprisingly, none of the four amino acids at these positions in RHOX5 is the same as in RHOX2 or RHOX3 (8). This is particularly surprising given that RHOX proteins harboring the same amino acids as RHOX5 at some of these four positions (e.g. RHOX8 is identical with RHOX5 at three of four positions) lacked the ability to regulate Unc5c (Fig. 6A). One explanation for this apparent paradox is that RHOX2 and RHOX3 use different amino acids than RHOX5 to bind to the same DNA sequence. Another possibility is that RHOX2 and RHOX3 uniquely recruit cofactors that confer the ability to bind the same sequence as RHOX5 and/or efficiently recruit other coregulatory molecules necessary for transcriptional control of Unc5c. By analogy, HOX homeobox proteins have been shown to depend on cofactors to increase their binding affinity and/or define their DNA-binding specificity (53–55). Recently, several RHOX5-binding proteins have been identified, including the tumor suppressor menin, the cell-cycle regulator CDC37, the MyoD inhibitor domain-containing protein MDFIC, and the pro-survival molecule prosaposin (56–59), but their effect on RHOX5 function remains to be determined. Our study suggests that one of the cofactors...
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responsible for regulating the Unc5c gene is Sertoli cell-specific, because we found that Rhox5 was only capable of regulating Unc5c transcription in Sertoli cell lines, not other cell lines (Fig. 3C).

The regulation of Unc5c by RHOX proteins is likely to be a conserved response, because we demonstrated that the human RHOXF2 gene repressed the mouse Unc5c promoter (Fig. 6C). Although we did not examine the regulation of the human orthologue, UNC5C, we suspect that the regulation will be similar, because the human and mouse UNC5C proteins are 96.7% identical in sequence and the human and mouse UNC5C 5′-UTR regions are 75% identical in sequence (32). We found that RHOXF2 was selective in its ability to repress Unc5c transcription because the other well characterized gene in the human RHOX gene cluster, RHOXF1 (21), had little or no activity (Fig. 6C). There are also four other known RHOX-related genes in the human genome. Two are in the RHOX gene cluster; one of these (RHOXF1B) is related in sequence with RHOXF1 but encodes a shorter protein, and the other (RHOXF2B) encodes a protein identical in sequence with that encoded by RHOXF2 (21). The other two RHOX-related genes are ARX and ESXR1, which have functional roles in the brain and placenta, respectively (60–62). Both are on the X chromosome, but far distant from the RHOX gene cluster, probably as a result of one or more translocation events. Evidence that all six human RHOX genes derived from a common ancestor is that they all have a 46-nt exon encoding the middle portion of the homeodomain, a signature feature of RHOX genes (8, 14, 21). The ESXR1 and ARX genes arose before the divergence of pri-mates and rodents, whereas the genes in the RHOX cluster are likely to have had a complex evolutionary history, making it impossible at this point to assign orthologs between primates and rodents. All four of the well characterized human RHOX genes are expressed in the testes (20, 21, 60, 61). RHOXF1 is also expressed in epididymis, ESXR1 is expressed in placenta, and ARX is expressed in forebrain (21, 60, 61). It remains for future studies to determine whether RHOX genes regulate the expression of UNC5C in these other tissues.

Unc5c was first identified as the gene responsible for C. elegans mutants with abnormal cell migration (63) and was later shown to correspond to several genes in higher organisms, including three genes in mammals that all encode netrin-binding proteins by virtue of their immunoglobulin-like extracellular domains (30, 64). In mice, mutations in the Unc5c gene cause cerebellar and midbrain defects, apparently as a result of abnormal neuronal migration. Although the molecular mechanisms underlying these defects remain to be determined, it may be the result of the loss of the ability to respond to netrin, because Unc5c is a member of the netrin receptor family and all UNC5 family members have been shown to bind to Netrin-1 (30, 32, 65, 66). Although Netrin-1 can have a chemoattractant effect when bound by one of its receptors, deleted in colorectal cancer, its binding to UNC5C and other UNC5 family members triggers chemorepulsion of growing axons (67).

UNC5C belong to a unique class of ligand-dependent recep-

tors that promote the development of cells when bound by their cognate ligands, but elicit apoptosis in their absence (35, 68). Although the exact mechanism underlying the pro-apoptotic activity of these molecules is still under investigation, it is likely to occur through their C-terminal death domain. This death domain is released by caspase 3 in vitro, and mutations in the cleavage site prevent the pro-apoptic activity of these receptors in transfected cells (31). RHOX5 also has a role in apoptosis because Rhox5-null mice have an increased number of apoptotic germ cells in the testes (8). Apoptosis is crucial for spermatogenesis, because it permits the appropriate number of germ cells to survive in seminiferous tubules for efficient spermatogenesis. Two germ cell apoptotic waves occur during spermatogenesis in mice; the early wave is during the first round of spermatogenesis (first 4 weeks after birth), and the late wave occurs primarily in stages I and XII–XIV of the seminiferous epithelial cycle in the adult testis (69, 70). Inhibition of the early apoptotic wave by disrupting the pro-apoptotic gene Bax or overexpressing the anti-apoptotic Bclxl or Bcl2 genes in mice causes the accumulation of spermatagonia and infertility later in life (69, 71). Rhox5 also appears to be important for controlling this balance of germ cell survival versus death, as loss of Rhox5 causes an increased frequency of germ cell apoptosis in the adult testis (8). Germ cells that normally die (stage I–IV spermatogenesis and stage XI spermatocytes) are more numerous in Rhox5-null mice, and a subset of germ cells that do not normally die (stage V–XI spermatocytes) also undergo apoptosis in Rhox5-null mice. Because Rhox5 is first expressed early during the first round of spermatogenesis (postnatal day 8) (72), it seems likely that Rhox5 is also involved in protecting germ cells from apoptosis during the first round of spermatogenesis, although this has not been experimentally tested. The fact that Rhox5 inhibits both germ cell apoptosis and the expression of the pro-apoptotic gene Unc5c led us to hypothesize that Unc5c functions to promote germ cell apoptosis in the testis. Indeed, we found less apoptotic germ cells in both postnatal and adult Unc5c-mutant mice testes (Fig. 7). Based on these results, we propose a model in which Unc5c is downstream of Rhox5 in a cell survival pathway operating in the testis. It remains for future experiments to further test this model experimentally and to determine whether this putative regulatory pathway operates in cell types besides germ cells in vivo.

Acknowledgments—We thank Thomas Jucius (Jackson Laboratories) for collecting and fixing testes tissues from Unc5c mutant and control mice, Miriam Buttigieg for tissue collection from B6 mice, and Drs. Dineshkumar Dandekar and Wai-kin Chan for helpful advice throughout the course of this project.

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