Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 5813–5817, August 1987
Developmental Biology

Differential expression of the mouse homeobox-containing gene *Hox-1.4* during male germ cell differentiation and embryonic development

(spermatogenesis/embryogenesis)

DEBRA J. WOLGEMUTH*†‡, CAROLYN M. VIVIANO*, ELENA GIZANG-GINSBERG*, MICHAEL A. FROHMAN§, ALEXANDRA L. JOYNER§¶, AND GAIL R. MARTIN§

*Department of Genetics and Development and †The Center for Reproductive Sciences, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032; and §Department of Anatomy, University of California, San Francisco, CA 94143

Communicated by Don W. Fawcett, May 8, 1987

ABSTRACT ***Hox-1.4* is a mouse homeobox-containing gene (initially identified as *HBT-1*), whose expression appears to be testis-specific in the adult animal. Examination of *Hox-1.4* transcripts in RNA from testes of mutant mice deficient in germ cells confirms that *Hox-1.4* expression within the testis is germ cell-specific. Enriched populations of spermatogenic cells were used to localize the expression of *Hox-1.4* specifically to germ cells that have entered into and progressed beyond the meiotic prophase stage of differentiation and to demonstrate the presence of two different size *Hox-1.4* transcripts. Examination of RNA from teratocarcinoma cell cultures and mouse embryos at 10.5–16.5 days of gestation demonstrated the presence of several *Hox-1.4* transcripts, which are larger than those present in germ cells. In the midgestation fetus, *Hox-1.4* expression is most abundant in the spinal cord.**

Mammalian germ cells undergo a unique and precise series of changes during differentiation. Certain of these events are common to both the male and female germ cells, such as genetic recombination and reduction division. "Chromosomal imprinting" of these cells also occurs during passage through the germ line and appears to be integral to the ability of the male and female gametes to support embryogenesis (1–3). Unique to male germ cell differentiation is the renewal of the mitotic stem cell population in the adult and the occurrence of the major morphogenetic events after the cells are haploid (4).

One approach to understanding the different processes that are involved in gametogenesis is to identify genes that are expressed in developing germ cells and to determine their functions. Most genes identified in this manner are likely to be involved in determining the structural features of germ cells or in controlling their metabolic activity. In addition, some genes may play regulatory roles in the developmental process itself, and these are of particular interest.

The identification of the homeobox in genes that regulate *Drosophila* development (5, 6) has provided a means for isolating potential developmental regulatory genes from higher organisms. The 180-base-pair (bp) homeobox domain is highly conserved both in a number of *Drosophila* genes known to play regulatory roles during development and in the genomes of a number of higher organisms, including mammals (7). Although the functions of the homeobox-containing genes identified in mammals are still unknown, most of these genes have been shown to be expressed during various stages of embryogenesis (8–18).

Using the homeobox domain as a probe, we sought to identify possible regulatory genes involved in germ cell development. Specifically, the 180-bp homeobox domain from the Antennapedia gene of *Drosophila* was used to screen a mouse testicular cDNA library, and a homeobox-containing gene, previously termed *HBT-1*, was isolated (19). This gene, now designated *Hox-1.4*,‖ is expressed specifically and at high abundance in the testis (8, 9, 19). *Hox-1.4* is a member of a cluster of at least six homeobox-containing genes, the *Hox-1* complex, which maps to mouse chromosome 6 (9, 21).

Hox-1.4 transcripts of ≈1.4 kilobases (kb) in length were detected in adult testis but not in testes of embryos at 17–20 days of gestation (19) or in neonatal (day 7–10 postpartum) testes (8, 19). Embryonic and immature testes contain germ cells in all of the mitotic stem cell stages, as well as all the somatic cell types found in the adult testis, including Leydig cells and Sertoli cells, but they do not contain meiotic germ cells. This suggests that expression of *Hox-1.4* is limited to germ cells that have reached meiotic stages.

The meiotic stages of spermatogenesis involve many specialized substages. The goal of the present study was to confirm the germ cell specificity of expression of *Hox-1.4* and to determine at which spermatogenic stage(s) *Hox-1.4* transcripts can be detected. We further studied *Hox-1.4* by assaying for its expression in the developing mouse embryo.

MATERIALS AND METHODS

Sources of RNA. Testes were dissected from mature male mice homozygous for the atriochosis allele (*at/at*) (ATEB/Le a/a dat/deb; The Jackson Laboratory) and from their heterozygous (*at/+*) and wild-type (+/+) littermates or from wild-type Swiss Webster mice (Camm Research Animals, Wayne, NJ). Enriched populations of spermatogenic cells were prepared from testicular cell suspensions from Swiss Webster mice by sedimentation at unit gravity through gradients of 2–4% bovine serum albumin according to our previously described procedures (22). Total RNA was extracted from the testes or separated cells using the LiCl precipitation method of Cathala *et al.* (23).

Mouse embryos at various stages of gestation were obtained by mating random-bred ICR animals (Simenson Lab-

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‡To whom reprint requests should be addressed at: Department of Genetics and Development, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.
¶Present address: Division of Molecular and Developmental Biology, Mt. Sinai Hospital Research Institute, 600 University Avenue, Toronto, ON, Canada M5G 1X5.

‖The terminology used to designate various mouse homeobox-containing genes conforms with that suggested recently (20). Specifically, *Hox-1.4* is used to designate the homeobox-containing gene that was previously termed *HBT-1* (19), *MH3* (8), and *Hox-1-3* (9).

oratories, Gilroy, CA). The day on which the vaginal plug was detected was considered 0.5 day of gestation. Embryos at the designated stages were removed from the implantation sites and separated from the extraembryonic membranes. Total RNA was extracted using the guanidinium thiocyanate/$CsCl_2$ method of Chirgwin *et al.* (24), with minor modifications, from whole fetuses or from seven fetal and two extraembryonic tissue compartments that were dissected from fetuses at 12.5 days of gestation as described by Joyner and Martin (14).

RNA was similarly extracted from PSA-1 teratocarcinoma stem cells maintained in the undifferentiated state or cultured under conditions that result in embryoid bodies at stages 1, 2, and 3 of differentiation as described by Joyner *et al.* (13).

Probes for Hybridization. The following probes were used in this analysis: (*i*) pHBT-1-3′, a subclone of the original 225-bp cDNA clone pHBT-1 derived by *Mbo* II–*Eco*RI digestion of pHBT-1 (19), which contains flanking sequences 3′ to the homeobox; (*ii*) p2181 B2 4a, a genomic clone of *Hox-1.4* containing ≈800 bp of sequences immediately 3′ to the homeobox (25); and (*iii*) pA1, a chicken β-actin cDNA clone (ref. 26; the gift of D. Cleveland).

Blot-Hybridization Analysis. All experimental procedures were carried out according to procedures described by Maniatis *et al.* (27), unless otherwise specified. Poly(A)$^+$ RNA was selected according to Aviv and Leder (28). In most experiments, either total RNA, poly(A)$^+$ RNA, or poly(A)$^-$ ("flow-through") RNA was fractionated by electrophoresis on denaturing 1% agarose/2.2 M formaldehyde gels at room temperature. Higher resolution denaturing formaldehyde gels contained agarose at a concentration of 1.5%, and electrophoresis was performed at 4°C. Following transfer of the RNA samples to nitrocellulose or GeneScreen membranes, the blots were hybridized with the DNA probes labeled by nick-translation with ^{32}P-labeled dNTPs as previously described by Wolgemuth *et al.* (19) or Joyner *et al.* (13). Transcript sizes were estimated by comparison with RNA size standard markers from Bethesda Research Laboratories.

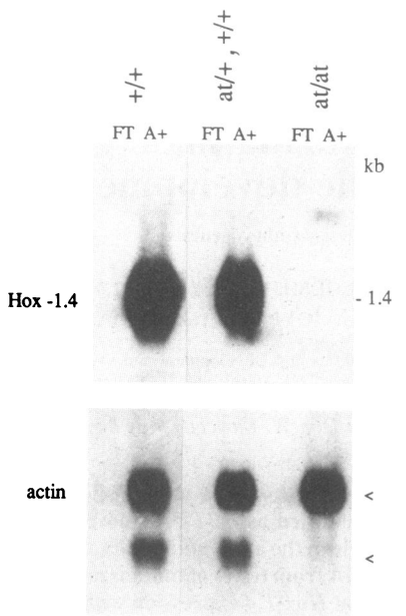

FIG. 1. *Hox-1.4* transcripts in adult testes from mouse strains defective in spermatogenesis. (*Upper*) Testicular RNA samples were fractionated on a 1% agarose/2.2 M formaldehyde gel and analyzed by blot hybridization with ^{32}P-labeled pHBT-1-3′. The lanes labeled A+ each contain 10 μg of poly(A)$^+$ RNA, and those labeled FT (flow-through) each contain 25 μg of RNA that did not bind to the oligo(dT) column. +/+, RNA samples from wild-type mice; at/at, RNA from homozygous animals; at/+ and +/+, RNA samples obtained from the littermates of the homozygous *at/at* animals, which include phenotypically indistinguishable individuals of both the *at*/+ and +/+ genotypes. The faint band detected in the at/at lane represents a residual actin transcript remaining from a previous hybridization. (*Lower*) The blot was washed and rehybridized again with the probe for β-actin as a positive control. The lines indicate the position of the ≈2.1- and ≈1.65-kb actin transcripts detected in testicular tissues.

RESULTS

***Hox-1.4* Transcripts in Testicular Cells.** Blot-hybridization analysis of RNA from the testes of mature males homozygous for the mutant atrichosis (*at*) allele was performed to confirm that expression of the *Hox-1.4* gene is limited to germ cells. The rationale for choosing this particular mutation among those known to affect germ cell development is discussed elsewhere (29). Briefly, testes from mature males homozygous for the *at* allele are devoid of germ cells (30) but contain somatic cells, including Leydig cells and Sertoli cells, which appear to be normal (31, 32). Heterozygotes (*at*/+) contain the complete germ cell lineage and are fully fertile.

RNA was isolated from testes of homozygous (*at/at*) males and from their normal heterozygous (*at*/+) and wild-type (+/+) littermates and was hybridized with a probe that contains *Hox-1.4*-specific sequences 3′ to the homeobox. No *Hox-1.4* transcripts were detected in RNA from the testes of homozygous (*at/at*) males (Fig. 1). In contrast, *Hox-1.4* transcripts of ≈1.4 kb in length were readily detected in RNA from testes of their *at*/+ and +/+ littermates. A control hybridization of the same filter with a β-actin cDNA probe showed discrete bands in all poly(A)$^+$ RNA samples (Fig. 1). Two β-actin transcripts of ≈2.1 and ≈1.65 kb in length were detected in the *at*/+ and +/+ RNA, but only the larger transcript was detected in the *at/at* sample. This is consistent with previous observations that a variant β-actin mRNA of 1.65 kb is found in the testis and is associated with the presence of germ cells (25, 33).

Having thus demonstrated that *Hox-1.4* expression is limited to germ cells, we sought to determine more precisely its stage specificity of expression during spermatogenesis. Our previous studies had shown that *Hox-1.4* transcripts are not detected in embryonic and immature testes but are present in adult testes (19), suggesting that *Hox-1.4* expression is limited to germ cells that have entered the meiotic stages of differentiation. Moreover, we noted in our blot-hybridization analysis that the testicular *Hox-1.4* transcripts were always detected as a rather broad band (see Fig. 1), suggesting that more than one size class of *Hox-1.4* transcripts might be present in testicular RNA.

To examine these possibilities, highly enriched populations of germ cells at three stages of differentiation were obtained from adult, wild-type males (see *Materials and Methods* and ref. 22). RNAs were isolated from the various cell populations and analyzed by blot hybridization after fractionation in 1.5% agarose gels. *Hox-1.4* transcripts were detected in poly(A)$^+$ RNAs from all three populations of cells: meiotic prophase spermatocytes (predominantly in the pachytene stage of meiosis), early spermatids, and a mixture of cytoplasmic fragments from elongating spermatids and of residual bodies (Fig. 2). This analysis also revealed the presence of two distinct *Hox-1.4* transcripts in these cells, one of ≈1.35 kb and a second of ≈1.45 kb in length. The 1.35-kb transcript was most abundant in RNAs isolated from the meiotic prophase spermatocyte fraction, whereas the 1.45-kb transcript was predominant in the early spermatid fraction and the fraction containing the cytoplasmic fragments and residual bodies.

Expression of *Hox-1.4* During Embryogenesis. Although postnatal *Hox-1.4* expression appears to be specific to male germ cells that have entered meiosis, it was of interest to

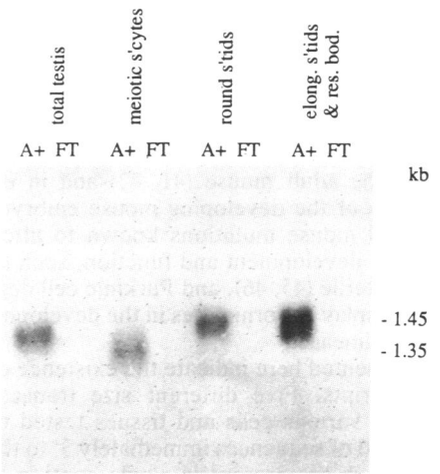

FIG. 2. *Hox-1.4* transcripts in enriched populations of spermatogenic cells. RNA was isolated from enriched populations of spermatogenic cells separated by sedimentation at unit gravity (22). Poly(A)$^+$ (A+) and flow-through (FT) RNAs were fractionated in a 1.5% agarose/2.2 M formaldehyde gel and analyzed by blot hybridization with ^{32}P-labeled p2181 B2 4a. The poly(A)$^+$ RNA samples each contain 5 μg of RNA and the FT samples contain 30 μg of RNA. The source of RNA for the samples is indicated and consists of total adult testis, enriched meiotic prophase spermatocytes (>78% pure), enriched early (round) spermatids (>80% pure), and enriched cytoplasmic fragments from elongating spermatids and residual bodies (>90% pure).

determine if this gene might also be expressed in the developing embryo. Since embryos at early stages are difficult to obtain in sufficiently large numbers, we assayed the embryonal carcinoma cell line PSA-1 for the presence of *Hox-1.4* transcripts. When maintained in the undifferentiated state *in vitro*, these cells provide a model system for the inner cell mass of the mouse blastocyst (3.5 days of gestation). PSA-1 cells at stages 1, 2, and 3 of differentiation *in vitro* provide cell cultures that have features in common with mouse embryos at stages prior to (4.5–6.5 days of gestation), during (6.5–8.5 days), and after (8.5–10.5 days) gastrulation, respectively (13, 34–36).

Blot-hybridization analysis of poly(A)$^+$ RNA from the PSA-1 cell cultures revealed that a *Hox-1.4* transcript ≈2.0 kb in length is present at low abundance in the undifferentiated cells. As the cells progress through stages 1, 2, and 3 of differentiation *in vitro*, the abundance of this transcript appears to decrease (Fig. 3). When poly(A)$^+$ RNA from whole fetuses at 10.5–16.5 days of gestation was analyzed, a *Hox-1.4* transcript of ≈1.7 kb in length was detected in all samples (Fig. 3). The RNA blots were rehybridized with a β-actin probe as a control for the quality and relative amounts of RNA in each of the samples (Fig. 3).

To examine the embryonic tissue specificity of *Hox-1.4* expression, conceptuses at day 12.5 of gestation were dissected into seven fetal and two extraembryonic compartments (14), and total RNA from each sample was analyzed (Fig. 4). The ≈1.7-kb *Hox-1.4* transcript was found to be most abundant in RNA from the spinal cord sample, which consisted of spinal cord and spinal ganglia but no spinal column. The spinal cord sample also contained a low level of a slightly larger transcript of ≈2.4 kb in length. The ≈1.7-kb transcript was also detected, at much lower abundance, in RNA from samples of "face," viscera, limbs, and carcass. No *Hox-1.4* transcripts were detected in either the anterior and posterior brain samples or in the extraembryonic tissue samples (i.e., the placenta and yolk sac). A direct comparison of the embryonic and germ cell *Hox-1.4* transcripts is shown in Fig. 4 *Upper Right*, confirming the relative size differences observed.

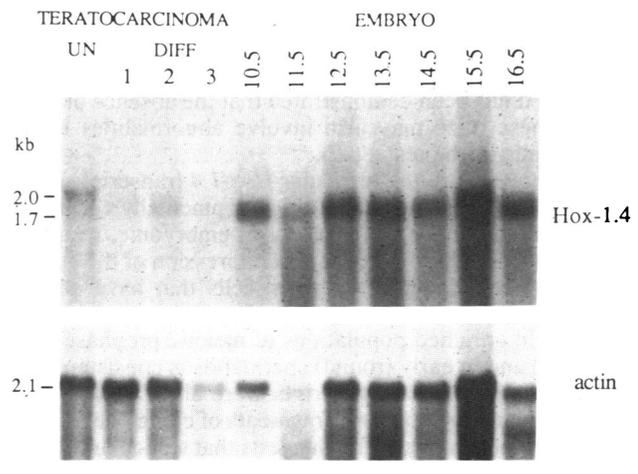

FIG. 3. *Hox-1.4* transcripts in teratocarcinoma cells and mouse embryos at various stages of gestation. (*Upper*) Poly(A)$^+$ RNA (5 μg per lane) isolated from PSA-1 teratocarcinoma stem cells maintained in the undifferentiated state (UN) and at three stages of differentiation (DIFF 1, 2, 3; see ref. 13) and from whole embryos at the days of gestation indicated was analyzed by blot hybridization under conditions of high stringency using the *Hox-1.4* probe, p2181 B2 4a. (*Lower*) The blot was subsequently washed and rehybridized with a probe for β-actin.

DISCUSSION

Among the mammalian homeobox-containing genes studied thus far, the *Hox-1.4* gene exhibits a unique tissue specificity of expression in the adult mouse. Transcripts from this gene appear to be specific to the testis and have not been detected in other adult organs examined to date (8, 9, 19). Moreover, in the testis, *Hox-1.4* transcripts are restricted to the germ cells. The strongest evidence supporting this conclusion is provided by our present studies on the *at* mutant strain. Males homozygous for the *at* mutation are devoid of germ cells and concomitantly lack *Hox-1.4* transcripts. Somatic cells in *at/at* mice have been characterized with respect to

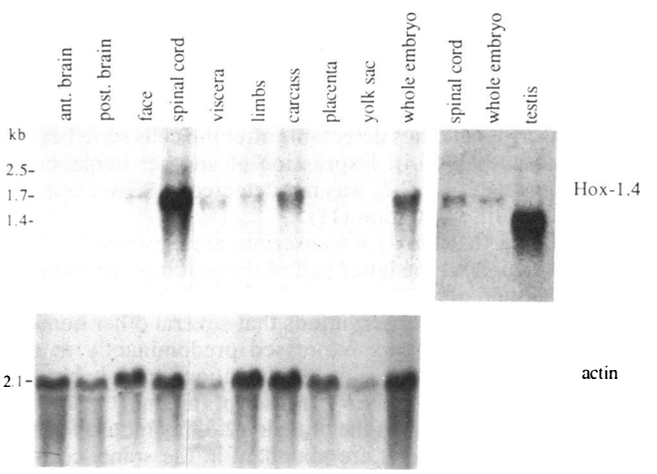

FIG. 4. Tissue specificity of *Hox-1.4* expression in midgestation embryos. (*Upper Left*) The seven fetal and two extraembryonic tissues indicated were dissected from mouse conceptuses at 12.5 days of gestation (14). Blot-hybridization analysis was performed using total RNA (15 μg per lane) for each tissue, as well as from whole embryos, with the p2181 B2 4a *Hox-1.4* probe. (*Lower*) The blot was subsequently washed and rehybridized with a probe for β-actin. (*Upper Right*) For direct comparison of the sizes of the *Hox-1.4* transcripts found in embryos and male germ cells, a sample of total RNA from embryonic spinal cord (5 μg), from whole embryos (10 μg), and poly(A)$^+$ RNA from adult testis (1 μg) was analyzed by blot hybridization with the p2181 B2 4a probe.

steroid production and appear to be relatively normal (31). *Hox-1.4* transcripts have also been reported to be absent in testes from mice carrying mutations at the steel locus (8). However, it has been demonstrated that the absence of germ cells in these mice may also involve abnormalities in the somatic cells of the testis (37).

The expression of the testicular *Hox-1.4* transcripts is not only cell type-specific but also developmentally regulated. The absence of *Hox-1.4* transcripts in embryonic, neonatal, and prepuberal testes suggests that expression of the gene in the adult testis is limited to germ cells that have entered meiosis (8, 19). Our finding that *Hox-1.4* transcripts are abundant in enriched populations of meiotic prophase spermatocytes and in early (round) spermatids is consistent with this conclusion. These transcripts were also found in fractions containing cytoplasmic fragments of elongated spermatids and residual bodies. This suggests that testicular *Hox-1.4* transcripts are quite stable, since these spermatogenic stages are transcriptionally inactive (4).

Although the highly restricted tissue specificity of *Hox-1.4* expression in the adult is unique among homeobox-containing genes studied thus far, other *Hox* loci are expressed in the adult testis. For example, *Hox-1.1* transcripts were found to be very abundant in the testis, but they were also readily detected in kidney, brain, and several other tissues, and *Hox-1.2* transcripts were detected in both testis and kidney (10). *Hox-1.3* transcripts were also detected in adult testis, among other tissues (38). It is interesting to note that these genes are found in the same cluster, *Hox-1* (20). Although the observations to date are limited, it is nonetheless intriguing to speculate that *Hox-1* genes may function in the differentiation of the male germ line.

Our observations on the pattern of *Hox-1.4* expression in PSA-1 cells and in embryos reveal interesting differences as well as similarities with what is known about the expression of other homeobox-containing genes during embryonic development. The finding that a 2.0-kb *Hox-1.4* transcript is present in RNA from undifferentiated PSA-1 cells suggests that this transcript may be expressed in the early embryo, presumably in the inner cell mass of the blastocyst. The *En-1* and *En-2* homeobox-containing genes are also expressed in undifferentiated PSA-1 cell cultures. However, *Hox-1.4* transcripts decrease in abundance as the cells differentiate, whereas one *En-1* and one *En-2* transcript are uniformly abundant in all PSA-1 cell cultures examined, and a second *En-1* transcript becomes detectable after the cells have begun to differentiate (13, 14). Expression of another homeobox-containing gene, *Hox-2.1*, was not detected in PSA-1 cells at any stage of differentiation (11).

The finding that *Hox-1.4* transcripts are expressed in the embryo throughout the latter half of the gestation period and are most abundant in the spinal cord of 12.5-day fetuses is interesting in light of observations that several other homeobox-containing genes are expressed predominantly in the central nervous system. Blot-hybridization analysis has revealed that both *En-1* and *En-2* transcripts are most abundant in the posterior portion of the brain in 12.5-day mouse fetuses (14), *Hox-2.1* transcripts are abundant in the spinal cord of mouse (12) and human (15) embryos, and the HHO.c13 human homeobox-containing gene is also expressed abundantly in the brain and spinal cord of human embryos (16). Moreover, RNA localized by *in situ* hybridization analysis has demonstrated that the *Hox-1.5* gene is expressed in the neural tube of the 9.5-day mouse embryo (17) and that the *Hox-3* gene is expressed in the anterior region of the spinal cord of neonatal mice (18).

Although it is difficult to draw conclusions about the significance of the common expression of *Hox-1.4* in the testis and central nervous system, it is interesting to note that other genes have been reported that exhibit a similar pattern of expression. For example, the neuropeptide hormone genes proopiomelanocortin and proenkephalin are expressed with unique developmental and cellular patterns of expression in male gonads (29, 39, 40). The protooncogene *int-1* exhibits the most striking parallel with *Hox-1.4* in its pattern of expression. *int-1* is expressed uniquely in postmeiotic male germ cells in the adult mouse (41, 42) and in the central nervous system of the developing mouse embryo (42, 43). Finally, several mouse mutations known to affect central nervous system development and function, such as quaking (44), pink eye sterile (45, 46), and Purkinje cell degeneration (47, 48), also display abnormalities in the development of the male germ cell lineage.

The data presented here indicate the existence of multiple *Hox-1.4* transcripts. Five different size transcripts were detected in the various cells and tissues tested by using a probe comprised of sequences immediately 3′ to the *Hox-1.4* homeobox, which are present in single copy in the mouse genome (ref. 19; D.J.W., unpublished observations). The two shortest transcripts, 1.35 and 1.45 kb in length, were detected in RNA from developing germs cells. A 1.7-kb transcript is detected in fetuses from 10.5 to 16.5 days of gestation and appears to be most abundant in the embryonic spinal cord. A 2.4-kb transcript was also detected in the embryonic spinal cord, but it was present in very low abundance. Finally, a transcript of 2.0 kb in length is detected in RNA from undifferentiated PSA-1 teratocarcinoma cells and may represent a *Hox-1.4* RNA species that is transcribed in periimplantation embryos. Data consistent with these findings have been reported by Duboule *et al.* (9), who observed a *Hox-1.4* transcript estimated to be 1.2 kb in length in RNA from adult testis and a *Hox-1.4* transcript estimated to be 1.45 kb in length in RNA from whole mouse embryos. In contrast, Rubin *et al.* (8) did not detect size differences between embryonic and testicular *Hox-1.4* transcripts.

At present, nothing is known about the structures of the different size transcripts, except that they are recognized by the same sequences 3′ to the *Hox-1.4* homeobox. Data from the study by Duboule *et al.* (9) suggest that the testicular transcripts but not the embryonic transcripts are either initiated or contain a splice site a short distance 5′ to the homeobox. Elucidation of the structural differences among the different size transcripts will require analysis of cDNA clones encoding each of them and a determination of their relationship to the genomic DNA in the region surrounding the *Hox-1.4* homeobox. Such studies, and others directed at understanding the functional differences among the *Hox-1.4* transcripts identified here, will contribute to an understanding of the role this gene may play in male germ cell and embryonic development.

We would like to thank Margaret Mostoller, George Grills, Ann Eakin, and Michael Richman for technical assistance and Dr. G. Shackleford for providing several RNA samples. We are grateful to Dr. Richard Palmiter and to our laboratory colleagues, especially Dr. Rita Duggal and Dr. George Mutter, for helpful discussions and critical readings of the manuscript. This study was supported by National Institutes of Health Grants HD18122 (D.J.W.) and HD20959 (G.R.M.). D.J.W. was the recipient of a Research Career Scientist Award from the Irma T. Hirschl Trust. A.L.J. was supported by a postdoctoral fellowship from the Medical Research Council of Canada. M.A.F. is supported by a postdoctoral fellowship from the American Cancer Society.

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