BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 104–112 (1996)
Article No. 0019

Expression of Multiple *C-kit* Receptor Messenger Ribonucleic Acid Transcripts during Postnatal Development of the Rat Testis

Sher Ali,*,[1] Neelakanta Ravindranath,* Meng-Chun Jia,* Neal Musto,* Tohru Tsujimura,† Yukihiko Kitamura,† and Martin Dym*,[2]

**Department of Cell Biology, Georgetown University Medical Center, 3900 Reservoir Road, NW, , Washington, DC 20007; and †Department of Pathology, Osaka University Medical School, Yamada-oka 2-2, Suita, Osaka, 565, Japan*

Received November 21, 1995

The *c-kit* protooncogene is a transmembrane tyrosine kinase receptor expressed during gametogenesis. Using the polymerase chain reaction (PCR), we have identified the *c-kit* receptor mRNA transcripts in the rat testis and studied their expression during postnatal development of the testis. Five different transcripts were identified using sets of primers encoding within the extracellular domain. Two transcripts were obtained from primer sets encoding regions within the cytoplasmic domain and the primer set encoding the entire length of the *c-kit* receptor. We have compared the levels of expression of these transcripts on different days during postnatal development. The level of expression of a particular transcript varied depending upon the developmental stage of the testis. In summary, our results suggest that multiple forms of mRNAs exist for the *c-kit* receptor in the rat testis, and they are regulated differentially during postnatal development.

The *c-kit* receptor is a cell surface receptor expressed in the gonads of both sexes (1–3). In the testis, *c-kit* receptor has been localized to Leydig cells (1,4) and spermatogonia (2,4,5). The intracellular kinase domain of the *c-kit* receptor is tyrosine-phosphorylated in rat type A spermatogonia (5) in response to the kit ligand (stem cell factor, steel factor) produced by testicular Sertoli cells (6). In the mouse testis, the expression of the *c-kit* receptor has been demonstrated throughout all stages of male germ cell development after birth (2,7). The c-kit receptor is a single copy gene and was first cloned from a mouse brain cDNA (8). In both mouse and human tissues, two major *c-kit* receptor isoforms have been described (9–11). In mature mouse testis, a 5.5 Kb *c-kit* receptor mRNA is expressed at high levels in spermatogonia and two mRNA transcripts of 3.2 and 2.3 Kb are expressed in spermatids (7). The full length coding sequence for the *c-kit* receptor is 3.8 Kb. Therefore, the smaller transcripts presumably code for the truncated versions of the *c-kit* receptor (12). Little is known about the mechanisms of the testis-specific and developmentally regulated expression of *c-kit* receptor mRNAs. Also it is not clear as to which truncated transcript is more abundant in the testes at a specific stage of development, nor do we know the exact number of transcripts in the testes. In addition, we do not know whether these different transcripts have any biological significance. We undertook the present study as an initial step towards better characterizing the *c-kit* receptor mRNA species and their regulation during postnatal development of the rat testis.

MATERIALS AND METHODS

RNA isolation. Testes from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 20, 30, 40 day old, and adult rats were obtained and total RNA was isolated using the Tri-X reagent RNA isolation kit (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions.

First strand cDNA synthesis. The first strand of cDNA was synthesized following the protocol of the Stratascript RT/PCR kit (Stratagene, San Diego, CA). Ten μg of total RNA were reconstituted in 38 μl of diethylpyrocarbonate-treated water (DEPC-water) and 3 μl of random hexamer oligo primers (300 ng) were added to each reaction tube. The tubes were

[1] Present Address: National Institute of Immunology, New Delhi, India.
[2] To whom correspondence should be addressed. Fax: (202) 687-1823.

0006-291X/96 $12.00

incubated at 65° C for 5 min and then cooled slowly to room temperature for about 10 min. To each tube, 5 μl of 10X first strand synthesis buffer, 1 μl of RNAse block nuclease inhibitor, 5 μl of dNTPS (10 mM each), and 1 μl of RNAse H-reverse transcriptase (50 units/μl) were added. The reactants were mixed gently and incubated at 37°C for 1 hr. The tubes were further incubated at 95°C for 5 min to inactivate the enzymes. The reaction mixtures were then placed on ice, or stored at −20°C until used.

Polymerase chain reaction (PCR). The PCR amplification was carried out in a 100 μl reaction volume containing 10 μl of reaction buffer, 5 μl of 10 mM dNTPs each, 50 pmole of each primer, 4-5 μl of template DNA, 1 μl of Taq Polymerase (Perkin Elmer Cetus), and 1 μl of Taq extender PCR additive (Stratagene, San Diego, CA). The reaction mixture was overlaid with an equal volume of mineral oil. Prior to PCR cyclic amplification, the reaction mixtures were incubated at 95°C for 4-5 min, and then cooled at room temperature for 5 min. PCR amplification of the cDNA was performed in a Thermal Cycler (McCoy) for a total of 35 cycles followed by an eventual extension at 72°C for 8 min. Depending upon the sets of primers used, their respective optimal denaturation, annealing and extension temperatures were adjusted. The samples were soaked at 4°C until used for analysis.

To ascertain the quality of the first strand of cDNA synthesized, a control PCR with a set of primers for ubiquitously expressed β-actin (Stratagene, San Diego, CA) was performed. The amplified product corresponded to the expected size (245 bp). Seven sets of oligonucleotide primers were designed based on the published rat *c-kit* receptor cDNA sequence (13), and the details of these oligonucleotide primers are shown in Fig. 1. The positions of the forward and reverse primers, and the sizes of the resultant amplified products are also included. Using these different sets of primers, testicular cDNA was amplified following the protocol described above.

Southern hybridization. The PCR products were separated on 1.5% agarose gel using 1 × TAE Buffer. The smaller size products, less than 500 base pairs, were separated on 2% agarose gel. The DNA was transferred onto Duralon membrane (Stratagene, San Diego, CA) following the capillary transfer method. The blots were then hybridized overnight with ^{32}P-labeled *c-kit* receptor cDNA lacking the primer sequences. After hybridization, blots were subjected to stringency washes and exposed for 5–15 mins or longer to obtain optimal signals.

The rat *c-kit* cDNA used for hybridization of PCR products was a full-length probe of 3816 bp extending from −44 bases upstream of the start codon to the 3′-untranslated region of the message (13). The fragments lacking primer sequences were generated using appropriate restriction enzymes.

RESULTS

RT-PCR Analysis for C-kit Receptor mRNA Expression during Postnatal Development of the Rat Testis

The results of Southern analysis of PCR products obtained using different sets of primers for the *c-kit* receptor throughout postnatal development of the rat testis are described below. To exclude the possibility that variations between different ages were due to the difference in quantity of PCR product present in all the lanes, verification was done by hybridization for β-actin. PCR product for β-actin was of the expected size (245 bp), and there were no significant differences in quantity throughout the postnatal period and in the adult. For analytical purposes, the relative intensities of the hybridization signals (intensity of each band of c-kit receptor transcript divided by the intensity of the respective β-actin signal) were calculated by assigning a relative intensity value of 1.0 arbitrary units to the values obtained for each transcript on day 1 or 2.

PCR with the Primer Sets Designed to Amplify Regions in the Extracellular Domain of the C-kit Receptor

The primer set of 1 and 3 (see Fig. 1) from nucleotide 17 to 867 gave rise to the expected 850 bp product, and additional products of 400 bp, 600 bp, and 1.5 Kb (Fig. 2, top panel). The levels of 400 bp transcript increased significantly by day 15 and declined to basal levels by day 40, and in adults. Although the 600 bp transcript followed the same course, the levels on day 40 and in the adult were much higher in comparison to the 400 bp transcript. The levels of the 850 bp product increased progressively from day 1 to day 17, and stayed at that level through day 40 and in the adult. However, the level of the 1.5 Kb transcript was very low between days 1–5, and then progressively increased from day 6 to day 17, and declined to the level seen on day 9 by day 40 and in the adult (Fig. 2, bottom panel).

Similarly, primers 2 and 3 (Fig. 1) spanning the nucleotide 73 to 867 also revealed four bands in the range of 400 bp to 1.2 Kb (Fig. 3, top panel). The expected 794 bp product (Fig. 3, bottom,

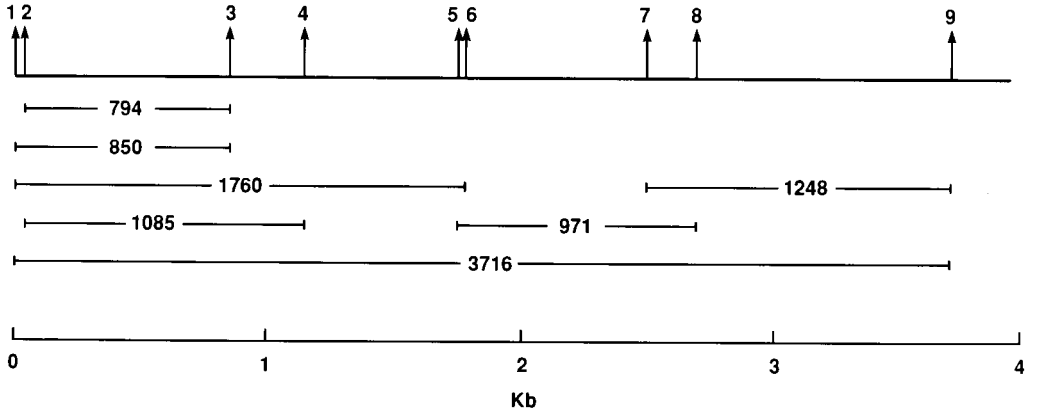

FIG. 1. Schematic representation of reverse transcriptase polymerase chain reaction (RT-PCR) with different sets of primers. The relative positions of sense and antisense primers are shown in the map. The vertical arrows from left to right (1–9) show the position of sense and antisense nucleotides used for the PCR reaction. Primer numbers **1** (GAGCTCAGAGTCTAGCGCAG) refer to nucleotide position 17, **2** (TGCTCTGCGTCCTGTTGGTC) to 73, **3** (CCTGGCGTTCGTAATTGAAGTC) to 867, **4** (TGCTTTGGTTGTCGGATTTG) to 1158, **5** (AGGAGATAAATGGGAACAATTATGT) to 1732, **6** (AGCTGCGTTGGGTCTATGTA) to 1777, **7** (TAGCCAGAGACATCAGGAATGA) to 2485, **8** (CTTCCTTGATCATCTTGTAAAACTT) to 2569 and **9** (GTATGAACGCATACAACGTA) to 3733, respectively. A set of β actin primers (GTGGGCCGCTCTAGACACCA and CGGTTGGCCTTAGGGTTCAGGGGGG) (Stratagene, San Diego, CA) was used as positive control. The horizontal lines show the orientation of the oligo primers and the numbers flanked by the lines show the product length.

panel) and the 600 bp transcript were expressed throughout the postnatal period and in the adult, however, the level of expression was high on day 3, declined significantly by day 9 below the levels observed on day 1, and then increased significantly by day 10 and maintained a steady level from day 10 to day 40 and in the adult. Interestingly, two additional transcripts of 400 bp and 1.2 Kb were strongly expressed between days 10–20 only.

PCR for *c-kit* performed with the primers 2 and 4 gave the expected product of 1085 bp, and two additional bands of 1.6 Kb and 1.8 Kb (Fig. 4, top panel). Quantitative analysis of the 1085 bp band by densitometry revealed that the levels of this mRNA transcript were low on day 2, increased 10 fold by day 15, remained steady until day 30, declined to very low levels on day 40, and was barely detectable in adult testes (Fig. 4, bottom panel). Of the two additional transcripts observed, the 1.8 Kb transcript was expressed steadily from day 2 to day 40 of age, whereas the 1.6 Kb transcript was expressed only on day 20, 30, and 40 (Fig. 4).

PCR with the Primer Set Amplifying Both Extracellular and Transmembrane Regions of C-kit Receptor

The primer set 1 and 6 (Fig. 1) spanning nucleotides 17 through 1777 revealed multiple bands in the range of 600 bp to 3.1 kb (Fig. 5, top panel). The expression of the expected 1760 bp band was fluctuating throughout the postnatal period (Fig. 5, bottom panel). However, the expression was significantly higher on days 13 and 20. Of the other four discernible bands (600 bp, 1.2 Kb, 1.5 Kb and 3.1 Kb), the levels of the 600 bp, 1.2 Kb and 1.5 Kb transcripts increased progressively from day 2 to day 30, and then declined slightly on day 40 and in the adult. Although the expression of the 3.1 Kb transcript was similar to the other 3 transcripts, its levels were significantly reduced on day 40 and in the adult.

PCR with the Primer Sets Designed to Amplify Regions within the Cytoplasmic Domain Including 3′ Untranslated Sequences of C-kit Receptor

PCR amplification with primers 5 and 8 (Fig. 1) from nucleotide 1732 to 2703 revealed two major bands. Besides the 971 bp expected product, another low molecular size band of 400 bp was

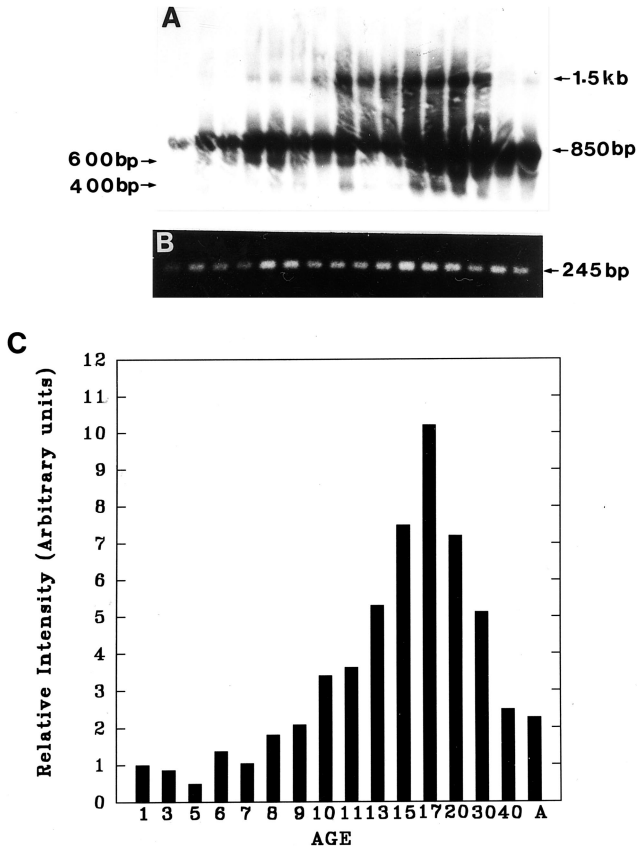

FIG. 2. RT-PCR amplification of *c-kit* receptor gene segments from rat testis during the postnatal period using sense and antisense primers (1 and 3) corresponding to the rat brain *c-kit* cDNA. Total RNA isolated from 1- to 40-day-old and adult rats were reverse transcribed and then amplified by PCR. Aliquots of PCR product were run on a 1.5% agarose gel containing ethidium bromide, transferred to nylon membranes, and hybridized with ^{32}P labeled with rat *c-kit* cDNA lacking primer sequences (A). The reverse transcribed total RNA was also amplified using specific primers for β-actin, and the products were run on a 2% agarose gel and stained with ethidium bromide (B). Intensity of the 1.5 Kb *c-kit* transcript relative to β-actin signal is shown in (C). The data were normalized by assigning a value of 1.0 arbitrary unit to the ratio obtained for each transcript on postnatal day 1. The results shown are representative of 3 different experiments.

seen in all the samples. The level of expression of both was uniform throughout the postnatal period and in the adult.

The set of oligo primers 7 and 9 (Fig. 1) spanning the cDNA from 2485 to 3733 gave the expected size band of 1248 bp and an additional band of approximately 600 bp. Both these transcripts were expressed uniformly in immature and in adult testes.

PCR with the Primer Set Amplifying Sequence Encoding for the Full-Length C-kit Receptor

PCR performed with primers extending between nucleotide 17 to 3733 (primers 1 and 9, Fig. 1), revealed the expected size product of 3716 bp and an additional band of 1.1 kb (Fig. 6, top panel). Both the transcripts were expressed throughout the postnatal period and in adult testis. However, the maximal expression was observed on day 15. The levels of both transcripts were low on day 40 (Fig. 6, bottom, panel). Adult testes showed basal levels of expression seen on day 1.

DISCUSSION

Although the *c-kit* receptor has been cloned from the rat brain (13) and its expression and phosphorylation in response to kit-ligand in rat spermatogonia has been reported (5), very little is

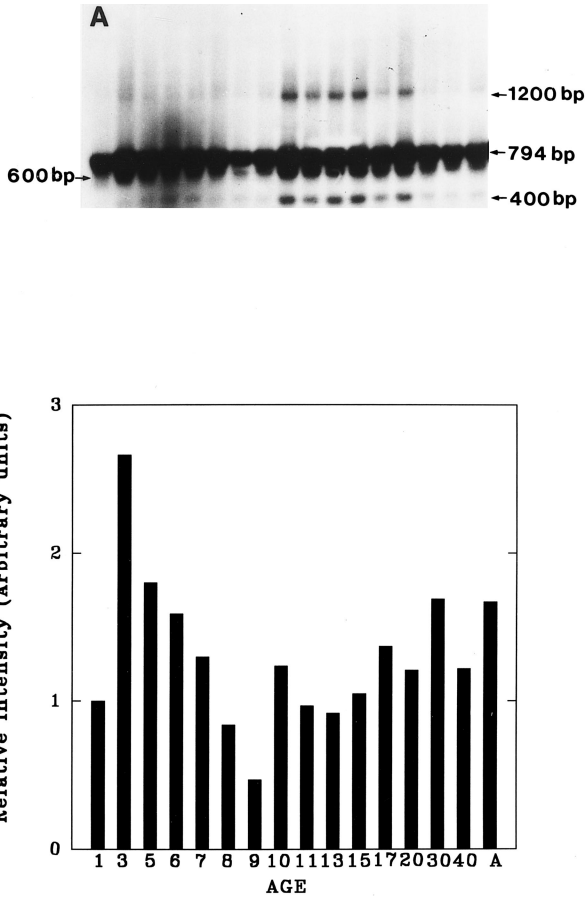

FIG. 3. RT-PCR amplification of *c-kit* receptor gene segments from rat testis during the postnatal period using sense and antisense primers (2 and 3) corresponding to the rat brain *c-kit* cDNA. Total RNA isolated from 1- to 40-day-old and adult rats were reverse transcribed and then amplified by PCR. Aliquots of PCR product were run on 1.5% agarose gel containing ethidium bromide, transferred to nylon membranes, and hybridized with ^{32}P-labeled rat *c-kit* cDNA lacking primer sequences (A). The reverse transcribed total RNA was also amplified using specific primers for β-actin, and the products were run on 1.5% agarose gel and stained with ethidium bromide. The intensity of the 794-bp *c-kit* transcript relative to β-actin signal is shown in (B). Data were normalized by assigning a value of 1.0 arbitrary unit to the ratio obtained for each transcript on postnatal day 1. The results shown are representative of 3 different experiments.

known about the messenger ribonucleic acids encoding the *c-kit* receptor and their regulation during postnatal development in the rat testis. The present study reveals the existence of multiple *c-kit* receptor messenger ribonucleic acids using reverse transcriptase-polymerase chain reaction analyses of *c-kit* receptor mRNAs isolated from postnatal and adult rat testes.

RT-PCR analysis using different primer sets specific for sequences coding the extracellular domain of the receptor revealed that at least 5 mRNA transcripts are present in testes obtained from 1–30 day old rats (Figs. 3–6). Since the testes from 1–3 day old rats consists of supporting cells (Sertoli cells and myoid cells), gonocytes, and precursor Leydig cells (14), the transcripts observed (one or two) must have originated from either gonocytes and/or precursor Leydig cells. However, Manova et al (2) failed to localize the *c-kit* receptor in gonocytes by *in situ* hybridization, and Yoshinaga et al (4) reported weak or faint expression of *c-kit* in gonocytes in testis from neonatal mouse. They concluded that in mouse, gonocytes and primitive type A spermatogonia do not express the *c-kit* receptor. Based on this conclusion, it could be presumed that these transcripts have

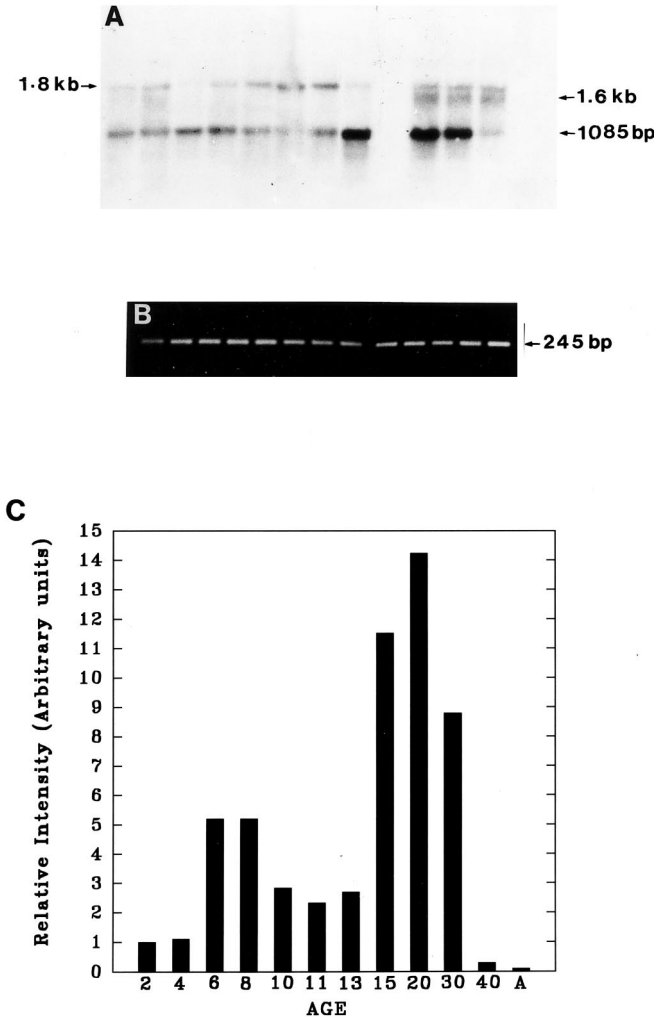

FIG. 4. RT-PCR amplification of *c-kit* receptor gene segments from rat testis during the postnatal period using sense and antisense primers (2 and 4) corresponding to the rat brain *c-kit* cDNA. Total RNA isolated from 2- to 40-day-old and adult rats were reverse transcribed and then amplified by PCR. Aliquots of PCR product were run on 1.5% agarose gel containing ethidium bromide, transferred to nylon membranes, and hybridized with ^{32}P-labeled rat *c-kit* cDNA lacking primer sequences (A). The reverse transcribed total RNA was also amplified using specific primers for < gkb>-actin, and the products were run on 1.5% agarose gel and stained with ethidium bromide (B). The intensity of the 1085-bp *c-kit* transcript relative to the β-actin signal is shown in (C). The data were normalized by assigning a value of 1.0 arbitrary unit to the ratio obtained for each transcript on postnatal day 2. The results shown are representative of 3 different experiments.

originated from precursor Leydig cells. Between days 3 and 10, the number of transcripts obtained with different sets of primers increased to either 3 or 5. During this period, primitive spermatogonia proliferate giving rise to type A spermatogonia (14). A few intermediate and type B spermatogonia are also formed. The newer transcripts observed could be attributed to the expression of *c-kit* receptor by type A spermatogonial cells. Both Manova et al (2) and Yoshinaga et al (4) observed labeling of type A spermatogonial cells and Leydig cells between days 6–12 postnatal in mouse. Further, they observed labeling of type B spermatogonia and preleptotene spermatocytes from day 13 to 19 postnatal in mouse. Analogous to their finding, the expression of different transcripts between day 10–20 postnatal in the rat testis was high. At this time period in rats, at least two

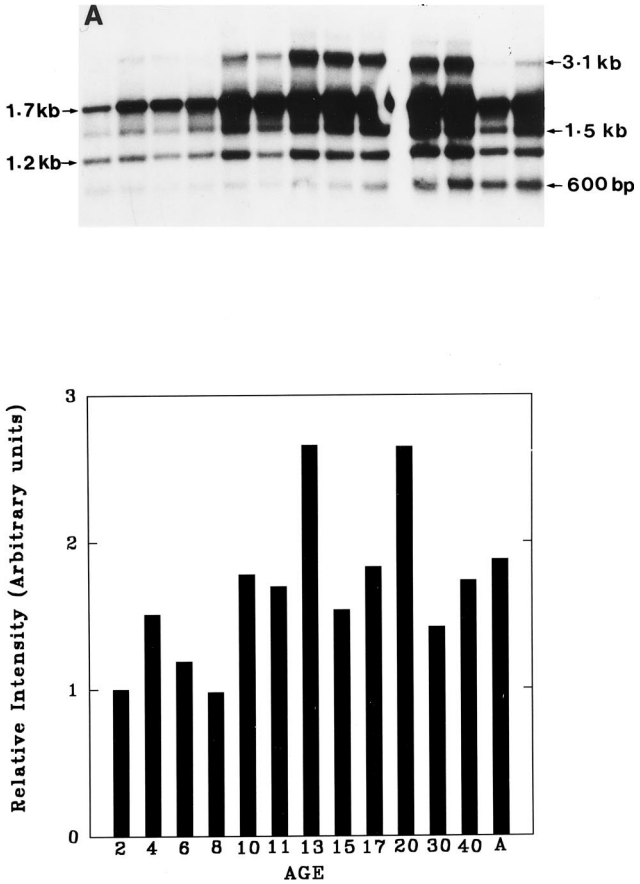

FIG. 5. RT-PCR amplification of *c-kit* receptor gene segments from rat testis during the postnatal period using sense and antisense primers (1 and 6) corresponding to the rat brain *c-kit* cDNA. Total RNA isolated from 2- to 40-day-old and adult rats were reverse transcribed and then amplified by PCR. Aliquots of PCR product were run on a 1.5% agarose gel containing ethidium bromide, transferred to nylon membranes, and hybridized with ^{32}P-labeled rat *c-kit* cDNA lacking primer sequences (A). The reverse transcribed total RNA was also amplified using specific primers for β-actin, and the products were run on a 1.5% agarose gel and stained with ethidium bromide. Intensity of the 1760-bp *c-kit* transcript relative to β-actin signal is shown in (B). The data were normalized by assigning a value of 1.0 arbitrary unit to the ratio obtained for each transcript on postnatal day 2. The results shown are representative of 3 different experiments.

generations of germ cells consisting of type A spermatogonia, intermediate type spermatogonia, type B spermatogonia, and spermatocytes (leptotene/zygotene) are present (14). Since all the three spermatogonial cell types have been shown to express the *c-kit* receptor in mouse (2,4), it is possible that different cell types may express different form of the *c-kit* receptor. The relative number of spermatocytes/spermatids to spermatogonial cells increases enormously beyond post-natal day 20. Since total RNA from the whole testis was used in the present study, only those mRNA transcripts which are in substantial quantity are amplified.

Interestingly, PCR using a set of primers (1 & 6) which amplified sequences between nucleotide 17 and 1777 encoding the entire extracellular domain and transmembrane domain, and a very small portion of the cytoplasmic domain upstream of the kinase region revealed 5 transcripts. Since all these transcripts corresponded with one or the other transcripts detected with primer sets which did not amplify the coding sequences for the transmembrane domain and a portion of the cytoplasmic domain, it is possible that all the transcripts originated from the alternate splicing of mRNA sequences within the extracellular domain. The most surprising observation was that when we used

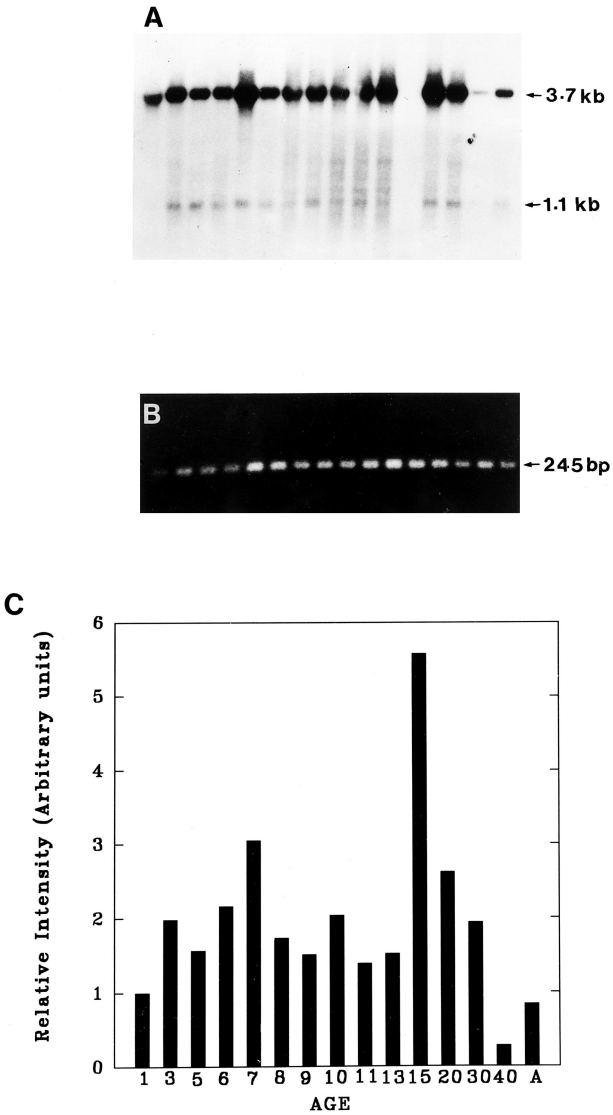

FIG. 6. RT-PCR amplification of *c-kit* receptor gene segments from rat testis during the postnatal period using sense and antisense primers (1 and 9) corresponding to the rat brain *c-kit* cDNA. Total RNA isolated from 1- to 40-day-old and adult rats were reverse transcribed and then amplified by PCR. Aliquots of PCR product were run on a 1.5% agarose gel containing ethidium bromide, transferred to nylon membranes, and hybridized with ^{32}P-labeled rat *c-kit* cDNA lacking primer sequences (A). The reverse-transcribed total RNA was also amplified using specific primers for β-actin, and the products were run on a 1.5% agarose gel and stained with ethidium bromide (B). The intensity of the 3716-bp *c-kit* transcript relative to β-actin signal is shown in (C). The data were normalized by assigning a value of 1.0 arbitrary unit to the ratio obtained for each transcript on postnatal day 1. The results shown are representative of 3 different experiments.

primer sets which amplified sequences encoding for the cytoplasmic domain with or without 3′ end untranslated sequences, only two transcripts were observed. The larger transcript was of the expected size product, and therefore contained the entire coding sequence for the cytoplasmic domain. The smaller one must apparently be lacking some of the intervening sequences. A 3.2 Kb *c-kit* mRNA transcript encoding a truncated receptor consisting of C-terminal 190 aminoacids of the *c-kit* protein has been described (12). This protein lacks the entire extracellular and transmembrane domains and the kinase insert region of the cytoplasmic domain. However, this transcript has

been cloned from post-meiotic germ cells, and its expression in mouse spermatids has been demonstrated (7). In addition a 2.3 Kb transcript has also been reported in mouse spermatids (7). Although the smaller transcript could represent one of these proteins, the expression of this transcript during early postnatal development before the appearance of post-meiotic germ cells suggests that either this transcript is different from those reported or the expression of this transcript during early stages of development is too low to be detected by Northern blot analysis. Interestingly, only two transcripts were observed when PCR was performed with primers 1 and 9 (nucleotide sequences between 17 and 3777) using Taq extender polymerase (Stratagene, San Diego, CA). These two transcripts may again represent the two alternate forms of *c-kit* described for different tissues which have in-frame addition or deletion of codons 510–513 encoding four aminoacids in the extracellular domain (9,11,15), or the two transcripts with addition or deletion of sequences in the cytoplasmic domain as described above in our study.

In conclusion, we have identified for the first time multiple *c-kit* receptor mRNA transcripts in the rat testis which originate as a result of addition or deletion of sequences in the extracellular or cytoplasmic domains, and demonstrated their differential expression during postnatal development of testis. The cloning and sequence analysis of these transcripts are underway. Further studies to determine the cell-specific expression of these transcripts within the testis and their biological significance are necessary to establish the role of the *c-kit* receptor in spermatogenesis.

ACKNOWLEDGMENTS

This work was supported by NIH Grant No. P01 HD24633 to M.D. Financial assistance to S.A. from an INDO-US Science and Technology Fellowship Program is gratefully acknowledged.

REFERENCES

1. Orr-Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y., and Lonai, P. (1990). *Development* **109,** 911–923.
2. Manova, K., Nocka, K., Besmer, P., and Bachvarova, R. F. (1990). *Development* **110,** 1057–1069.
3. Horie, K., Tatakura, K., Taii, S., Narimoto, K., Hoda, Y., Nishikawa, S., Nakayama, H., Fujita, J., and Mori, T. (1991). *Biol Reprod* **45,** 547–552.
4. Yoshinaga, K., Nishikawa, S, Ogawa, M., Hayashi, S., Kunisada, T., and Fujimoto, T. (1991). *Development* **113,** 689–699.
5. Dym, M., Jia, M. C., Dirami, G., Price, J. M., Rabin, S. J., Mocchetti, I., and Ravindranath, N. (1995). *Biol Reprod* **52,** 8–19.
6. Rossi, P., Albanesi, C., Grimaldi, P., and Geremia, R. (1991). *Biochem Biophys Res Commun* **176,** 910–914.
7. Sorrentino, V., Giorgi, M., Geremia, R., Besmer, P., and Rossi, P. (1991) *Oncogene* **6,** 149–151.
8. Qiu, F., Ray, P., Brown, K., Baker, P. E., Jhanwar, S., Ruddle, F. H., and Besmer, P. (1988). *Embo J* **7,** 1003–1011.
9. Reith, A. D., Ellis, C., Lyman, S. D., Anderson, D. M., Williams, D. E., Bernstein, A., and Pawson, T. (1991). *Embo J* **10,** 2451–2459.
10. Vandenbark, G. R., deCastro, C. M., Taylor, H., Dew-Knight, S., and Kaufman, R. E. (1992). *Oncogene* **7,** 1259–1266.
11. Giebel, L. B., Strunk, K. M., Holmes, S. A., and Spritz, R. A. (1992). *Oncogene* **7,** 2207–2217.
12. Rossi, P., Marziali, G., Albanesi, C., Charlesworth, A., Geremia, R., and Sorrentino, V. (1992). *Dev Biol* **152,** 203–207.
13. Tsujimura, T., Hirota, S., Nomura, S., Niwa, Y., Yamazaki, M., Tono, T., Morii, E., Kim, H. M., Kindo, K., Nishimune, Y., and Kitamura, Y. (1991). *Blood* **78,** 1942–1946.
14. Clermont, Y., and Perey, B. (1957). *Amer J Anat* **100,** 241–267.
15. Yarden, Y., Kuang, W.-J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T. J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987). *Embo J* **6,** 3341–3351.