

The proximal *cis*-acting elements Sp1, Sp3 and E2F regulate mouse *mer* gene transcription in Sertoli cells

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Mer belongs to the Tyro 3 family of receptor tyrosine kinases (RTKs). Together with *Axl* and *Rse*, the three RTKs are believed to play important functional roles in the male gonads because gene knockout male mice lacking all of these receptors are infertile. In the present study, postnatal expression of *Axl* and *Rse* in mouse testes decreased during maturation while expression of *Mer* increased age-dependently during testicular development. To investigate the transcriptional regulation of gene expression in the testis, a ≈ 1.5 kb fragment of the 5' flanking sequence of *Mer* was isolated. The sequence lacks a typical TATA or CAAT box. 5' RACE revealed that the putative major transcriptional start site of *Mer* is located at +102 bp upstream of the translation initiation site. Using transient transfections of luciferase reporter constructs driven by various lengths of the 5' flanking sequence, the gene segment $-321/+126$ showed the highest transcriptional activity in a mouse Sertoli cell line (TM4). DNAase I footprinting experiments revealed four

footprints within the region from -321 to -26 , including three binding sites for the transcriptional factor Specificity protein 1 (Sp1) and one for an unknown transcriptional factor. Electrophoretic mobility shift assay (EMSA), supershift assay, mutation studies and cotransfection demonstrated that those Sp1 *cis*-acting motifs interacted either with Sp1 or Sp1/Sp3, depending on location and the nearby nucleotide sequences. An E2F binding site which down-regulates *Mer* transcription, as revealed by EMSA, deletion and mutation studies, was identified downstream in the proximity of the promoter. Taking all of these data together, the study has demonstrated that Sp1, Sp3, E2F and probably another unknown transcriptional factor play a critical role in regulating the proximal promoter activities of *Mer*.

Keywords: E2F; *Mer* gene; receptor tyrosine kinases; Sertoli cells; Sp1 and Sp3.

Receptor tyrosine kinases (RTKs) are cell surface receptors that contain intrinsic protein tyrosine kinase activity in their cytoplasmic regions. They are responsible for transmitting signals from the extracellular environment into the cell cytoplasm following binding of peptide growth factors [1,2]. Interactions involving these molecules are critical in regulating cell survival, proliferation and differentiation. The RTKs *Axl*, *Rse* and *Mer* are classified into the Tyro 3 RTK subfamily. Receptors in the Tyro 3 subfamily share a distinctive extracellular region of two immunoglobulin-related domains linked to two fibronectin type III repeats. The growth-arrest specific gene 6 (*Gas6*), which is capable of protecting cells from apoptosis, has been identified as the common ligand for *Axl* [3], *Rse* [4], and *Mer* [5,6].

Axl, *Rse* and *Mer* are widely expressed in adult tissues and present in considerable amounts in neural, lymphoid, vascular and reproductive tissues [7–9], ensuring their significant biological roles in multiple tissues [5,10–16]. Previous studies in our laboratory using cell culture and RT/PCR have demonstrated that *Rse*, *Mer* and *Gas6* are expressed in the Sertoli cells and the expression of *Gas6* was

responsive to forskolin [17]. By the use of the gene knockout mice model, null mutation in all three receptors (*Mer*^{−/−} *Axl*^{−/−} *Rse*^{−/−}) severely affected male gonadal functions but imposed less significant detrimental effects to other tissues and organs. However, deletion of any single receptor or any combination of two receptors resulted no detectable defect in fertility [18]. These findings suggest that these three receptors are essential regulators of spermatogenesis and that their functions in gonadal development can be compensated for by each other. It is likely that *Gas6* may exert its biological effect through these receptors and may also be essential for the tropic maintenance of diverse cell types in the testis. Because of their importance in the testis, molecular mechanisms underlying specific transcription and expression efficacy of the Tyro 3 family genes are critical for the maintenance of normal testicular functions.

The Tyro 3 family genes are expressed also in other tissues such as lymphoid and the vascular tissues [7–9]. More recently, their presence was found to be significant in modulating the activity of antigen-presenting cells during an immune response and contributing to the normal regulatory of the immune system [15,19]. Such findings also raise questions about the nature of *cis*-regulatory elements and cognate *trans*-acting factors that confer either testicular or extratesticular expression to the Tyro 3 family genes.

In this study, Northern blot analysis showed that the developmental expression pattern of *Mer* in mouse testes was different from the other members of the Tyro 3 family. To gain insight into the molecular mechanism regulating

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Mer expression in the testis, we have cloned and characterized the mouse Mer promoter, and identified Sp1, Sp3 and E2F as important contributors to the proximal Mer promoter function in a mouse Sertoli cell line (TM4) which is known to have Mer expression [17].

MATERIALS AND METHODS

RNA extraction

Total RNA was prepared from tissues using Trizol reagent, as suggested by the manufacturer (Gibco BRL Life Technologies). The concentration of RNA was determined by spectrophotometry at 260 nm, and its integrity was assessed by agarose gel electrophoresis. Polyadenylated RNA was prepared by oligo(dT) affinity chromatography using the PolyAtract System IV (Promega).

Northern blot analysis

Northern blot analysis was performed as described previously [20]. To detect the expression of tyrosine kinase receptors *Rse*, *Axl* and *Mer* and their ligand *Gas6*, cDNA probes were produced by RT-PCR. The primers used were as follows: 5'-TGTCTGCGAATGGAAGTGGAGAAC-3' (*Rse*, sense) and 5'-CAGGCTGTTGCTACCTCCCTTACT-3' (*Rse*, antisense), which generated a 663-bp *Rse* PCR product; 5'-TGTGCAGCCCATAAGGACACACAG-3' (*Axl*, sense) and 5'-ATGGTGGCTGTGCGGAGGTGGTGA-3' (*Axl*, antisense), which generated a 595-bp *Axl* PCR product; 5'-TGTCCAAGGGTGTACATATCAACAT-3' (*Mer*, sense) and 5'-AGCCGAGGATGATGAACATAGAGT-3' (*Mer*, antisense), which generated a 700-bp *Mer* PCR product; 5'-CGGCATTCCCTTCAAGGAGAGT-3' (*Gas6*, sense) and 5'-CTCAACTGCCAGGACCACCAACT-3' (*Gas6*, antisense), which generated a 397-bp *Gas6* PCR product; and 5'-TCCGCTGCAGTCCGTTCAAGTCTT-3' [ribosomal protein S16 (S16), sense] and 5'-GCCAACTTCTTGATTGCGAGCG-3' (S16, antisense), which generated a 384-bp S16 product. Total RNA (25 µg per sample, except for *Mer* where 60 µg was used), isolated from testes of BALB/c mice using Trizol as described above, was resolved by electrophoresis on a 1% agarose/formaldehyde gel and transferred by capillary blotting to a nylon membrane (Hybond-N, Amersham Life Science). RNA was cross-linked by exposure to ultraviolet light (Ultraviolet Cross-linker, Amersham Life Science), and the blotted membranes were prehybridized for 5 h in 50% deionized formamide at 42 °C. Membranes were hybridized under the same conditions for 24 h in fresh hybridization solution containing either an α -³²P-labelled *Rse*, *Axl*, *Mer*, or *Gas6* cDNA probe. The membranes were then washed three times for 10 min each with 2×0.3 mol·L⁻¹ sodium chloride and 30 mmol·L⁻¹ sodium citrate (NaCl/Cit pH 7.0) containing 0.1% SDS at room temperature, and then washed twice for 5 min each with $1 \times$ NaCl/Cit containing 0.1% SDS at 65 °C. Finally membranes were washed with $0.1 \times$ NaCl/Cit containing 0.1% SDS at room temperature for 10 min. The same blot was washed and reprobed with a α -³²P-labelled S16 cDNA probe to confirm the integrity of the RNA samples. Radioactivity of hybridized mRNA species was measured by phosphor-imaging scanning (STORM 860,

Molecular Dynamics) and visualized by autoradiography with double intensifying screens and Kodak X-OMAT AR films at -80 °C for 4 h to 10 days. The time was dependent on the abundance of the target mRNA.

5' RACE

5' RACE was performed using the Marathon cDNA Amplification Kit (Clontech Laboratories Inc.). Polyadenylated RNA (1 µg) from testis of BALB/c mouse was reversibly transcribed into cDNA. A specially designed adapter sequence provided in the Marathon kit was ligated to the ends of the cDNA, and the adapter primer served as the forward primer. An antisense gene-specific primer (5'-GTGCCCCGAGCAATTCCTTTCCATCTTTCC-3') derived from nucleotides 426-455 in the *Mer* cDNA (GenBank accession no. MMU21301), and antisense gene-specific primer (5'-CGCAACAGGAGGTAGGAGCTTTGATGCTG-3') derived from nucleotides 288-316, served as the outer and nested primers, respectively. Major PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced.

Identification of the *Mer* 5'-flanking sequence

The 5'-flanking sequence of *Mer* was obtained using the protocol described in the Universal GenomeWalker Kit (Clontech Laboratories, Inc.). Briefly, five separate walker libraries were constructed by ligating a specially designed adapter sequence to mouse genomic DNA (Clontech Laboratories, Inc.), and each was digested by a different restriction enzyme. The antisense gene-specific primers were designed from the *Mer* exon 1 sequence. The outer primer (5'-GGAGCAGCAGCAGCCCCAGTAGCAGT-3') was complementary to nucleotides 64-90 in the *Mer* cDNA. The nested primer (5'-CCAGTAGCAGTGGGGCCAGAACA-3') was complementary to nucleotides 51-74. Major PCR products amplified with the adapter primers were cloned into pGEM-T Easy vector and sequenced. Gene-specific primers designed for subsequent walkings were as follows: 5'-AGATCTCGCCAGTCGCCGAGGGCGCGTGCGAA-3' (complementary to +12/+37 in Fig. 2A) and 5'-AGATCTGAGTGGCAGTGGCGAGTTGGGGATCGCA-3' (-53/-26). Various portions of the 5' flanking region were cloned into pGL3 Basic (Promega) for analysis of their transcriptional activities.

In vitro deoxyribonuclease I (DNase I) footprinting

Nuclear extracts were prepared from TM4 cells as described previously [21]. Regions to be footprinted were amplified by appropriate pairs of primers where the antisense primers were end-labelled with [γ -³²P]deoxy-ATP and T4 polynucleotide kinase (Gibco BRL Life Technologies). Unincorporated radio-labelled nucleotides were removed with a MicroSpin G-25 column (Amersham Pharmacia Biotech Inc.), and the radio-labelled DNA fragments were further gel purified. Approximately 30 000 c.p.m. of end-labelled DNA was digested with 0.45 U Dnase I (FPLC pure, Amersham Pharmacia Biotech, Inc.) in an *in vitro* footprinting assay [22]. The DNase I digestions were terminated, and the products were treated with proteinase K before analysis on an 8% urea/acrylamide DNA sequencing gel

along side a Maxam–Gilbert sequencing reaction of the footprinted fragment [23]. The resulting gel was examined by autoradiography.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides used in the assay were GSA1: 5'-CATTCTGCCCCGCCCTCCA-3'/3'-GTAA GACGGGGCGGGGAGGT-5'; GSA2: 5'-ATCCTCCC CTTCCCGCCCCCTCCTCCAGTTC-3'/3'-TAGGAGG GGAAGGGCGGGGAGGAGGTCAG-5'; GSA3: 5'-TCCCCCTTCCCGCCCCCTGTC-3'/3'-AGGGGAA GGGCGGGGACAG-5' and GSA4: 5'-CAGCAGGCGC CAGAGTG-3'/3'-GTCGTCCGCGGTCTCAC-5'. These oligonucleotides were end-labelled with [γ - 32 P]deoxy-ATP using T4 kinase (Gibco BRL Life Technologies). Between 3.5 μ g and 10 μ g TM4 nuclear extract was incubated in the presence or absence of an excess of unlabelled competitor oligonucleotide (50–500 fold excess) in a final volume of 15 μ L containing 10 mM Hepes pH 7.6, 50 mM KCl, 25 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol and 1 μ g poly(dI:dC) (Amersham Pharmacia Biotech, Inc.). After a 15-min incubation at room temperature, approximately 30 000 c.p.m. γ - 32 P-labelled double-stranded oligonucleotides were added and incubated on ice for 30 min and then at room temperature for a further 30 min. Reactions using antibodies (1 μ g) were performed as above, with a final addition of antibody and incubation on ice for 30 min. The reactions were separated by polyacrylamide gel electrophoresis (6%), and analysed by autoradiography.

Site-directed mutation analysis

Site-directed mutagenesis was performed according to the three-step PCR-mutagenesis method [24]. Four mutagenic oligonucleotides SM1: 5'-GGAGGGTGGAGGGGCTTG GCAGAATGGAACCTT-3' (–212/–243), SM2: 5'-ACTGG AGGAGGGGGCTTGAAGGGGAGGATCCA-3' (–166/–197), SM3: 5'-GGAGTGGACAGGGGCTTGAAGGG GGAAGGGCA-3' (–113/–144), and SM4: 5'-GCACTG CCACTCTGGATCCTGCTGCCCGGGCG-3' (–37/–6) were synthesized where each consisted of two mutated bases at the middle (in italic) and 15 complementary bases at the 5' and 3' ends. The mutation primers MP-B: 5'-GG AGTACTAACCCTGGCCTAGCAAAATAGGCTGTCC C-3' and MP-C: 5'-GGAGTACTAACCCTGGCCTTT ATGTTTTTGGCGTCTTCCA-3' were designed from the universal primer sequences of pGL3 Basic vector (RVprimer3 and GLprimer2, respectively), with the 17 bp mutation sequence (in italic) at the 5' end. Another mutation primer designed from the italicized 17 bp mutation sequence was designated MP-D. The proximal promoter region spanning from +126 to –321 of the *Mer* flanking sequence was subcloned into the pGL3 Basic vector to produce pGL3/(–321/+126) which was used as the template for the first and second PCR reactions. After the first PCR reaction, a product defined by the SM1-3 and the MP-B, or the SM4 and the MP-C was produced and purified from 1.5% agarose gel electrophoresis (SephaglasTM Bandprep Kit, Amersham Pharmacia Biotech, Inc.). In step two, a single cycle of PCR reaction was performed using the original pGL3/(–321/+126) as the template and the product from the first PCR reaction as the primer. In step

three, MP-D and either GLprimer2 (first PCR reaction using SM1-3), or RVprimer3 (first PCR reaction using SM4) were added in the final PCR reaction. The mutation primers MP-B, MP-C, MP-D and the universal primers of pGL3 Basic vector were utilized to conduct site-directed mutagenesis on small DNA fragments subcloned into the pGL3 Basic vector. The DNA sequences of the mutation clones were confirmed by base sequencing using the ABI Prism 3100 Genetic Analyser (Applied Biosystems, CA, USA).

Cell culture and transfections

The culture media and reagents used for tissue culture experiments were obtained from Gibco BRL Life Technologies. Mouse Sertoli (TM4) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose), supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U·mL^{–1} penicillin, and 100 μ g·mL^{–1} streptomycin. Reporter constructs were transfected using LipofectAMINE (Gibco BRL Life Technologies), according to protocol suggested by the manufacturer. Briefly, cells were plated in six-well plates at approximately 2×10^5 cells per well for 24 h before transfection. Before addition of DNA/liposome complexes, cells were rinsed with serum-free DMEM. For each transfection, 1 μ g reporter constructs were cotransfected with 0.5 μ g pSV- β -galactosidase control vector (Promega) in 1 mL serum-free DMEM by incubation at 37 °C for 5 h. An equal volume of DMEM containing 20% fetal bovine serum was then added, and the cells were incubated overnight at 37 °C. The culture medium with the DNA/liposome mixture was replaced by DMEM containing 10% fetal bovine serum on the following day. Forty-eight hours after the start of transfection, cells were rinsed twice with NaCl/P_i (10 mM sodium phosphate and 0.15 M NaCl pH 7.5) and harvested by Reporter Lysis Buffer (Promega). For luciferase assays, cell extracts (20 μ L) were mixed with 100 μ L luciferase assay reagent (Promega) for detection in a luminometer. For β -galactosidase assays, cell extracts (150 μ L) were mixed with an equal volume of assay 2 \times buffer (Promega) and then incubated at 37 °C until it became yellow. The reaction was stopped by the addition of 500 μ L 1 M sodium carbonate. Absorbance at 420 nm was measured and used to correct for transfection efficiency. Relative luciferase activities were calculated by dividing luciferase light units by attenuance reading from the β -galactosidase assay. Fold increases in the relative luciferase activities of various constructs were determined in relation to the background luciferase activity of the promoterless pGL3 Basic. All transfection experiments were performed in duplicate and were repeated at least three times.

RESULTS

Northern blot analysis reveals the difference in the expression pattern between *Mer* and the other Tyro 3 family members (*Axl* and *Rse*) in mouse testes during maturation

Northern blot analysis was used to characterize the expression pattern of the Tyro 3 family members and their common ligand, Gas6, in mouse testes during maturation. When total RNA from mouse testes of different ages

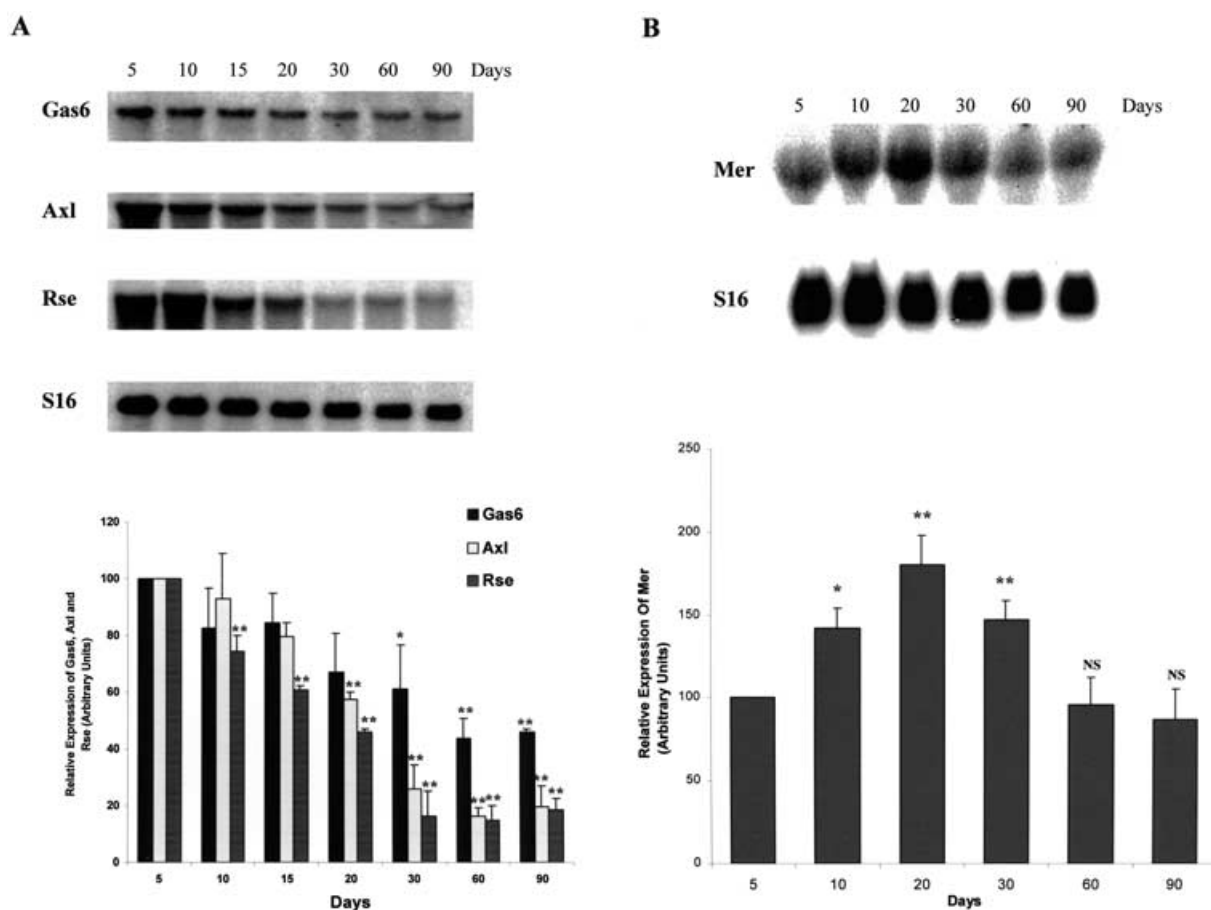


Fig. 1. Northern blot analysis of tyrosine kinase receptors *Axl*, *Mer* and *Rse*, and their common ligand *Gas6* in mouse testes during postnatal development. Total RNA was extracted from BALB/c mouse testes at the different ages indicated. (A) Twenty-five μ g RNA was resolved on a 1% agarose/formaldehyde gel, transferred onto nylon membrane in 20 \times NaCl/Cit and hybridized with the corresponding α - 32 P-labelled cDNA probe at 42 $^{\circ}$ C overnight. The 2.9-kb *Gas6* RNA, the 4.1-kb *Axl* RNA and the 3.8-kb *Rse* RNAs were detected after 1 week of autoradiographic exposure at -80° C as shown in the upper panel. Each blot was then washed and re-probed with α - 32 P-labelled *S16* cDNA and a typical *S16* expression gel is shown. The graph shows *Gas6*, *Axl* and *Rse* expression in Northern blots quantified by densitometry and normalized against *S16* expression detected in the same application. (B) Sixty μ g RNA was used for Northern blot analysis of *Mer*. A 4.4-kb *Mer* transcript was detected after 2 weeks of autoradiographic exposure at -80° C as shown in the upper panel. The same blot was washed and re-probed with α - 32 P-labelled *S16* cDNA. The graph in the lower panel shows *Mer* expression in Northern blots measured by densitometry. Expression was normalized against *S16* expression detected in the same application. Results show the mean \pm SEM of three independent experiments of different animals. The mean was significantly different from the 5-day-old mouse at * P < 0.05 and ** P < 0.01. NS, Not significantly different from the 5-day-old mice.

ranging from 5 days to 90 days was analysed by Northern blotting, a single predominant band of 4.1 kb *Axl*, 3.8 kb *Rse* or 2.9 kb *Gas6* was detected in all ages of mouse testes examined (Fig. 1A). The trends for both *Axl* and *Rse* expression during development were similar in that they remained high before puberty and significantly decreased after 20 days of age. On the other hand, the expression of *Gas6* decreased only slightly during maturation. However, *Mer* was not detectable in 25 μ g total RNA, and the reason may be due to its low mRNA level in mouse testes. As such, the amount of total RNA used for Northern blot analysis was increased to 60 μ g, and a single predominant band of 4.4 kb *Mer* mRNA was detected in all ages of mouse testes examined (Fig. 1B). Unlike the expressions of *Axl* and *Rse*, *Mer* expression increased from 5 days of age, attained the highest peak at around 20 days, and declined steadily thereafter up to 90 days of age. Because of its unique expression pattern in mouse testes, which coincides with

testicular development and onset of spermatogenesis, we next examined the transcriptional regulation of *Mer*.

Potential regulatory elements in the mouse *Mer* 5'-flanking region

To identify potential sequence elements involved in the transcriptional regulation of *Mer*, we isolated a region of 1489 bp 5' to the translation initiation codon (GenBank accession no: AF517125). A preliminary 5'-deletion analysis of luciferase expression showed that the proximal 628 bp of this region (-527 bp relative to the putative transcription start site) was able to exhibit the optimal promoter activity (data not shown). This sequence does not contain a typical TATA box, a CAAT box or an initiator sequence (Fig. 2A). 5' RACE was performed to map the transcription start sites. Nucleotide sequencing of the 5' RACE products amplified using transcripts isolated from mouse testis and *Mer*

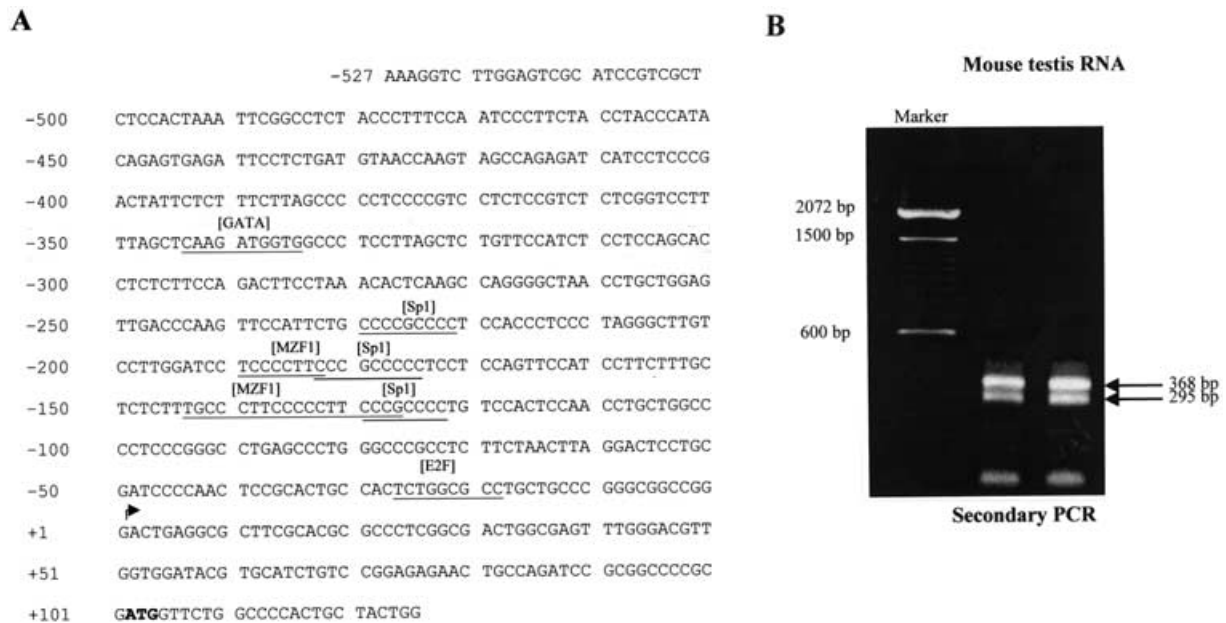


Fig. 2. Title. (A) The partial nucleotide sequence of the mouse *Mer* 5'-flanking region. An ≈ 1.5 kb sequence upstream of the translation start site was obtained by two rounds of genomic walking (GenBank accession no. AF 517125) and the proximal 628-bp region (relative to the translation initiation codon) is shown. Upstream nucleotides are marked as negative numbers. The translation initiation codon is shown in bold. Potential binding sites for transcription factors GATA, Sp1, MZF1 and E2F are underlined and indicated. (B) Identification of mouse *Mer* transcription start site by 5' RACE. Two major PCR products were generated by two rounds of 5' RACE. The fragments were cloned and sequenced. Two sites located at 29 and 102 bp upstream of the translation start site were mapped. The latter position was chosen as the putative major transcription start site and designated as +1 in (A).

cDNA-specific primers revealed that this TATA-less promoter initiated transcription from several start sites including two that were located at +29 and +102 bp upstream from the translation initiation codon (Fig. 2B). As the +102 bp position resulted a stronger band in the PCR gel and was located at a more GC-rich flanking region, it was chosen as the putative major transcription start site and designated as +1, as shown in Fig. 2A. A computer-assisted search using TFSEARCH [25] revealed several putative binding sites for transcription factors including GATA, multiple Sp1 and MZF1, E2F (Fig. 2A).

Transcriptional activity of the *Mer* 5'-flanking region

To determine which segment(s) of the proximal 5' flanking region of *Mer* is important for transcription in Sertoli cells, TM4 cells were transfected with the *Mer* promoter constructs outlined in Fig. 3A, and activity from the luciferase reporter gene was measured. The results indicate that the promoter sequence (−527/+126) exhibited a 10-fold increase in relative luciferase activity when compared with the promoterless luciferase vector pGL3 Basic (Fig. 3A). When the same fragment was inserted in a reverse direction the promoter activity dropped dramatically, which implies that some of the *cis*-elements within this region are functionally unidirectional. Of the five constructs of various lengths flanking from +126 to −527, the maximal activity (13-fold) was provided by the −321/+126 region. 5' deletion from −321 to −181 decreased this maximal activity significantly, suggesting that an approximate 140-bp segment of the 5' flanking region of *Mer* located between −321 and −181 is involved in promoting transcription in TM4

Sertoli cells. This region contains two DNA motifs known to bind the transcription factor Sp1 (CCCGCC) and one DNA motif for Myeloid Zinc Finger Gene 1 (MZF1) (TCCCCTT) (see Fig. 2A). The gene segment −181/+126 also maintains a moderate transcription activity (sixfold), and within this region it contains one DNA motif for Sp1 and one for MZF1. It is suggested that these three Sp1 motifs exert interplays with each other to give maximal transcription activity.

Apart from the 5' deletion constructs, 3' deletion constructs were made for transfection studies. The results in Fig. 3B show that transcriptional activity increased gradually when the sequence was deleted from +126 to −26 relative to the putative transcription start site. Within the region between +1 to −26, a DNA motif for E2F was found, which was known to interact with Sp1 for transcriptional regulation [26].

TM4 Sertoli cell nuclear protein interactions with the *Mer* 5'-flanking region

To analyse the −321/+126 region of the *Mer* proximal promoter, an *in vitro* DNase I footprinting assay was performed using a nuclear extract prepared from TM4 Sertoli cells. The results revealed four footprints within the region from −321 to −26, including three Sp1 and one novel *cis*-element (Fig. 4). In addition, double-stranded oligonucleotides of those footprint sequences containing Sp1 *cis*-element produced shifted bands with TM4 nuclear proteins in an EMSA, and addition of excess unlabelled oligonucleotides demonstrated the specificity of these interactions (Fig. 5).

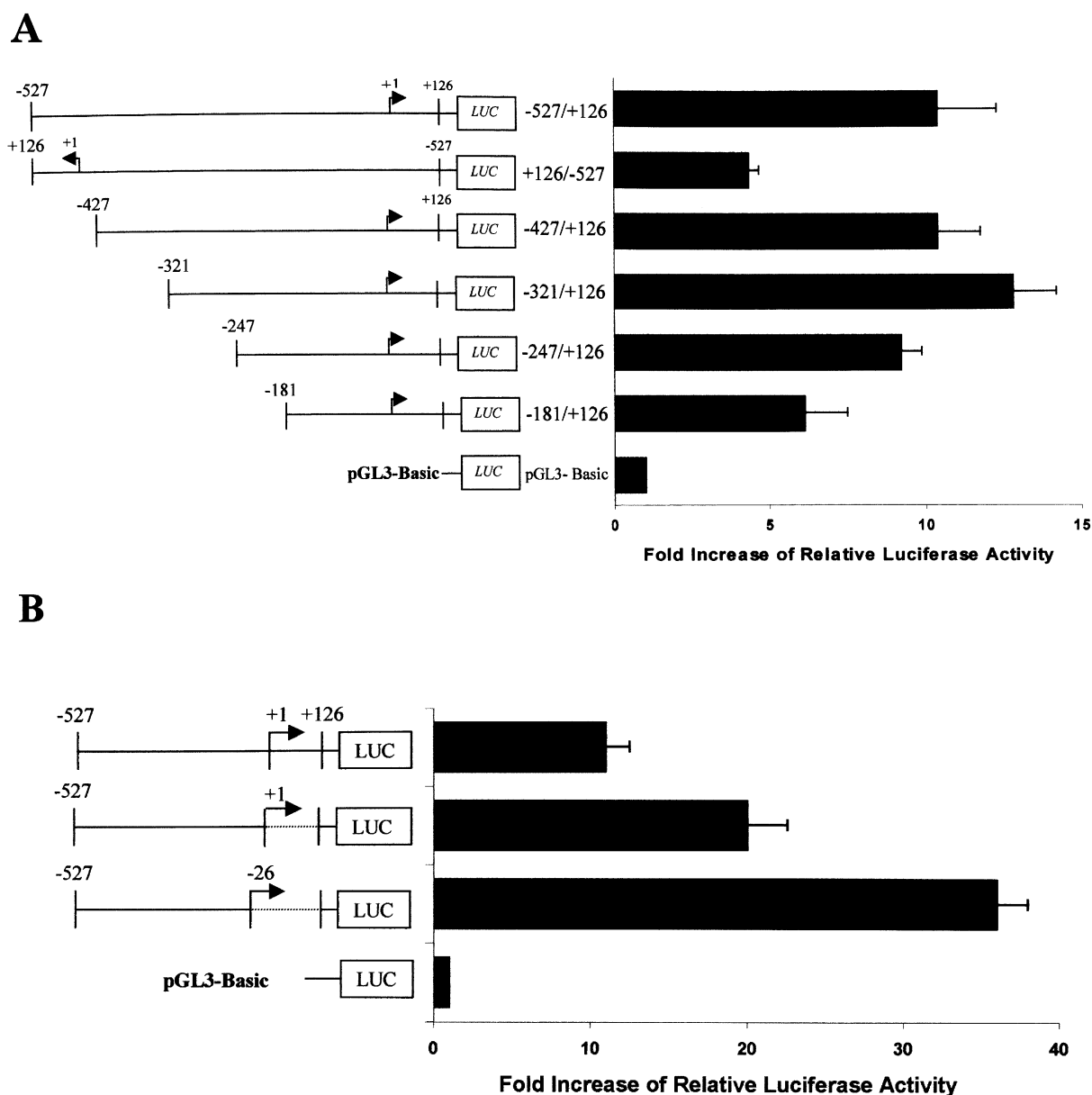
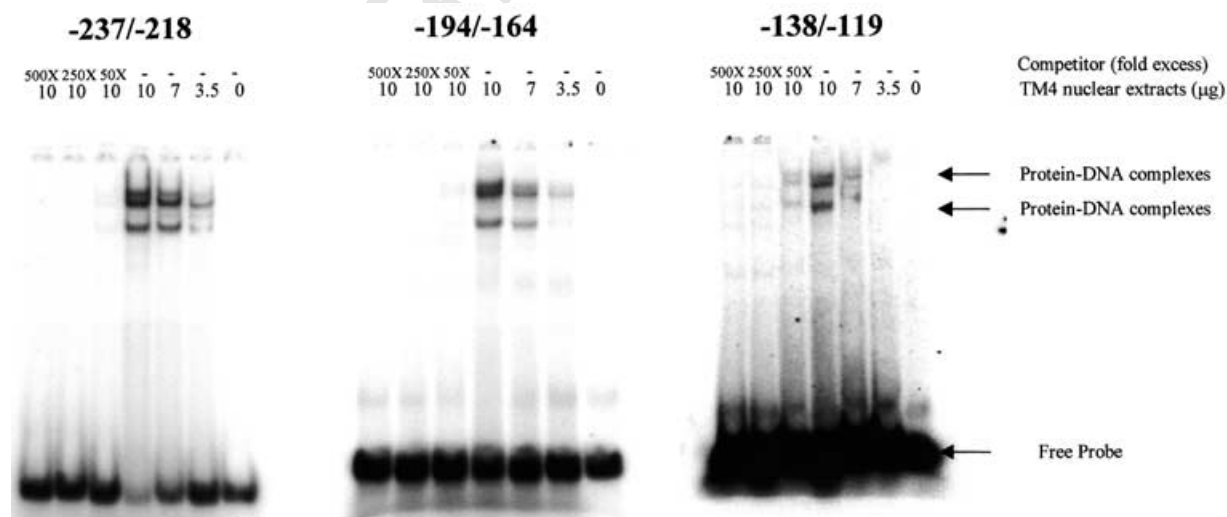
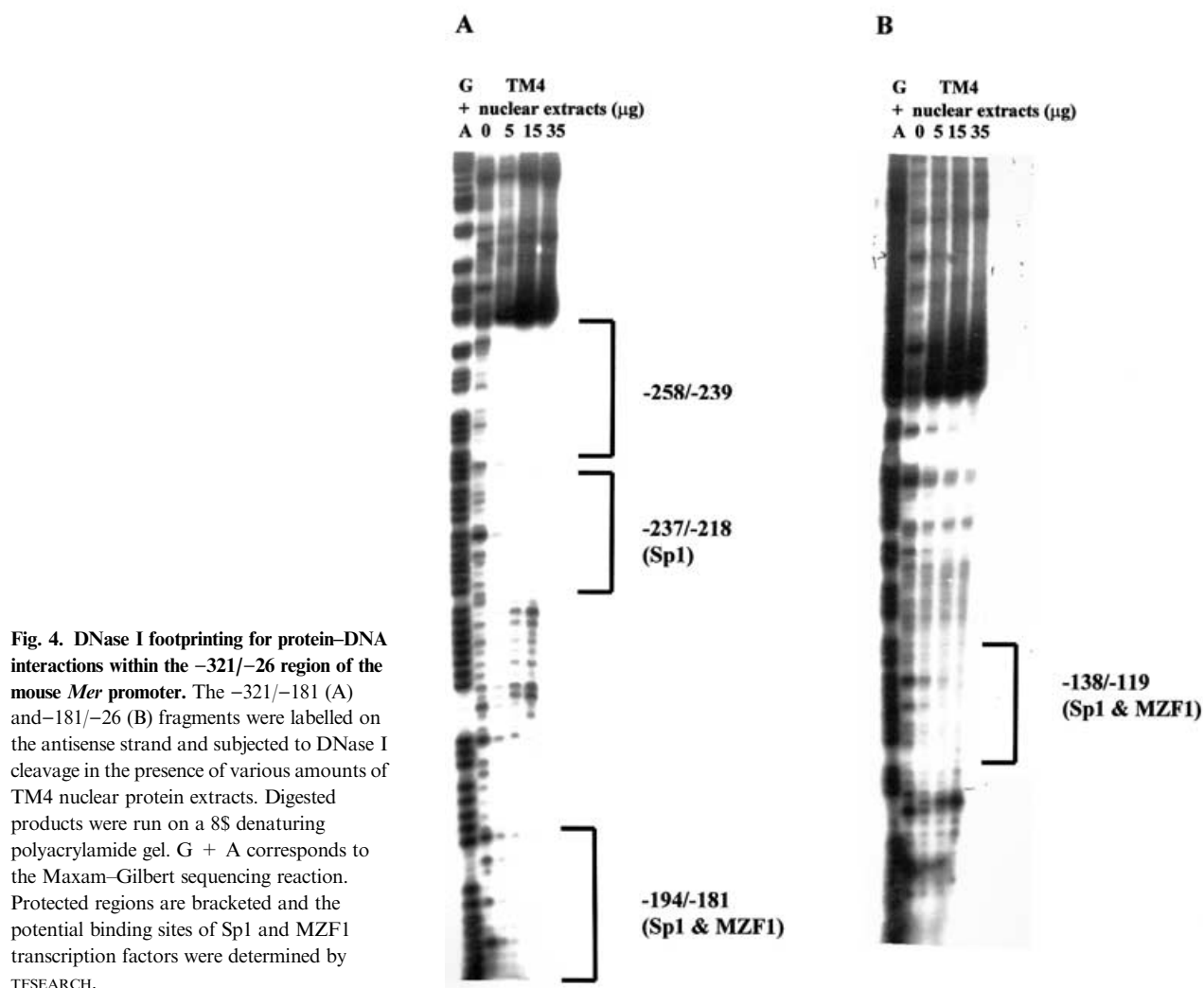


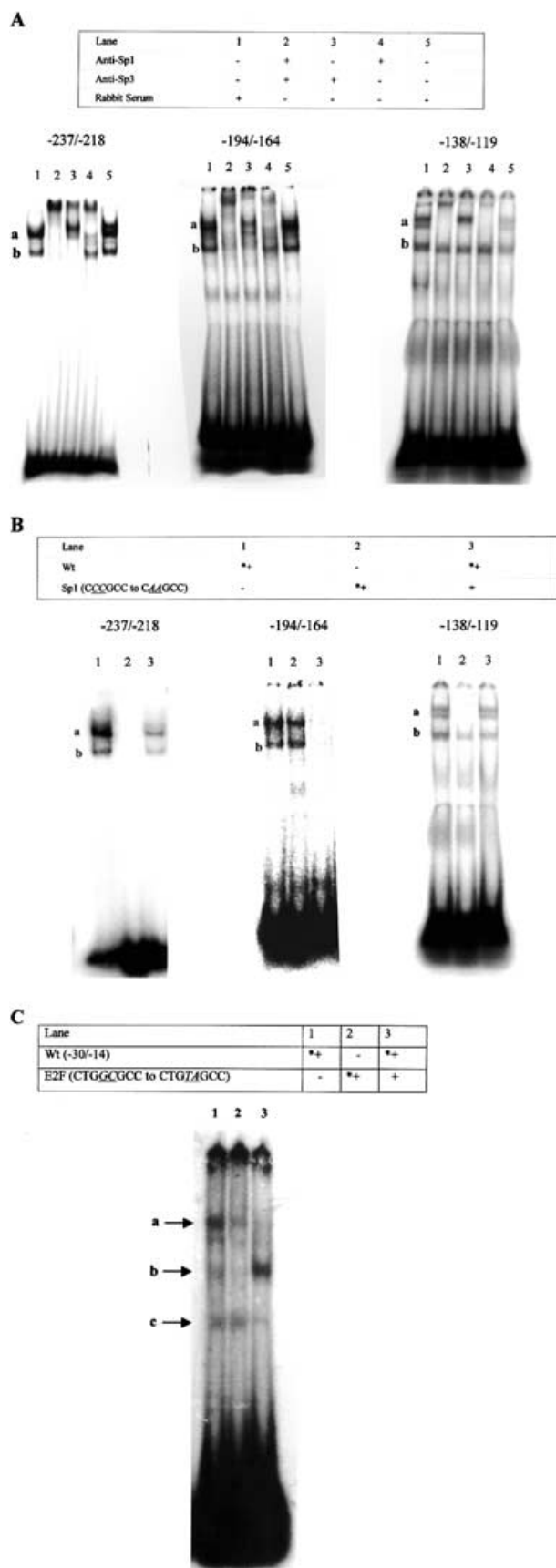
Fig. 3. Transient luciferase expression analysis of 5'- and 3'-deletions within the mouse *Mer* 5' flanking region. (A) Transcriptional activities of various 5'-deletion constructs tested in TM4 cells and (B) Effect of 3'-deletion on transcriptional activities. Luciferase expression plasmids were generated by inserting various portions of the 5'-flanking sequence of *Mer* up to and beyond the translation initiation codon at +102 (A), or various 3' deletion constructs (B) into promoterless luciferase vector pGL3 Basic as shown in the left-hand panels. Arrows represent the orientation of the promoter and the position of transcription start site at +1 is indicated. Fold increase of relative luciferase activities of the constructs were determined in relation to the background luciferase activity of the promoterless pGL3 Basic. All promoter activity of each construct was normalized against β -galactosidase activities produced by the pSV- β -galactosidase vector which serves as an internal control. Results are means \pm SD of three separate transfections performed in duplicate.

Sertoli cells nuclear proteins Sp1 and Sp3 bind Sp1 DNA binding motif of the *Mer* promoter

TM4 nuclear proteins were shown to interact with the three footprint regions which contain Sp1 DNA binding motifs. As three of the footprint regions contain not only Sp1 binding motif but also MZF1 binding motif, we wanted to prove further that the shifted bands were due to the Sp transcriptional factor family. Therefore, the protein-DNA

complexes shown in Fig. 5 were further analysed using antibodies directed to Sp1 or Sp3 nuclear proteins. The results shown in Fig. 6A confirm that for both segments -237/-218 and -194/-164, the two shifted bands, a and b, were due to the binding of Sp1 and Sp3, respectively, while for the segment -138/-119, the slower migrating protein-DNA complex a contained Sp1 but the faster migrating complex b did not contain either Sp1 or Sp3. It is suggested that it may involve other transcriptional factor bindings.





To further confirm that Sp1 and related family members constitute the major portion of nuclear protein binding to these three footprint sequences, TM4 cell nuclear extracts were incubated with these three oligonucleotides, each containing a double point mutation in the Sp1 *cis*-acting motif. Under our expectation, for the segment -237/-218, all shifted bands were eliminated when the Sp1 *cis*-acting motif was mutated (Fig. 6B), and also for the segment -138/-119 where the upper band was eliminated. The results of these two segments shown in Fig. 6B were consistent with the previous supershift assay using Sp1- and Sp3-specific antibodies (Fig. 6A). Interestingly, for the region -194/-164, the shifted bands cannot be eliminated after introducing mutation into the Sp1 motif. Together with the result of supershift assay, it may imply that in addition to Sp1 and Sp3, other unknown transcriptional factors may exist to interact directly with this region.

In addition, the deletion analysis shown in Fig. 3C suggests that the increase in the promoter activity may be due to the removal of the E2F *cis*-acting element. To prove that the transcriptional factor E2F is involved in the interaction with this motif, EMSA was performed and the results showed that double-stranded oligonucleotide of the region -30/-14 containing the E2F *cis*-element produced shifted bands (a, b and c) with TM4 nuclear proteins (lanes 1 and 3, Fig. 6C). When a double point mutation was introduced to the E2F *cis*-acting motif, the protein-DNA complex b was eliminated (lane 2, Fig. 6C), suggesting that it may be due to the binding of E2F transcription factor to this motif.

Effect of Sp1, Sp3 and E2F on *Mer* promoter activity in Sertoli cells

Double point mutations were introduced into each of the Sp1 and E2F *cis*-acting elements of the pGL3/(-321/+126) construct (upper panel, Fig. 7A). These constructs were then transfected into Sertoli TM4 cells to initiate the transcription of the luciferase reporter genes. The results showed that mutation of either one of the two distal Sp1 binding motifs resulted in a dominant reduction of

Fig. 6. Title. (A) Antibodies to Sp1 and Sp3 bind to the protein-DNA complex of the mouse *Mer* promoter. EMSA was performed using TM4 nuclear protein extracts and a labelled oligonucleotide containing bases -237/-218, -194/-164 or -138/-119 of the mouse *Mer* promoter. Antibodies to transcription factors were added to some of the binding reactions as indicated. The two un-shifted protein complexes observed to bind the wild-type oligonucleotide (lanes 1 and 5) are labelled as a and b. (B and C) Mutation of Sp1 (B) and E2F (C) binding sequences abolishes protein-DNA binding. EMSA was performed using TM4 nuclear protein extracts and mutant oligonucleotides spanning bases -237/-218, -194/-164 or -138/-119 (Sp1) and -30/-14 (E2F) of the mouse *Mer* promoter. The two protein complexes identified are labelled a and b in (B), and a, b and c in (C). Lanes 1 and 2 show the results of binding reactions using radioactively labeled (*) oligonucleotides containing the wild-type and mutated sequences, respectively. Lane 3 shows the result of the binding reactions in which nuclear extracts were preincubated with un-labelled, mutated oligonucleotides prior to the addition of labelled (*) wild-type oligonucleotide.

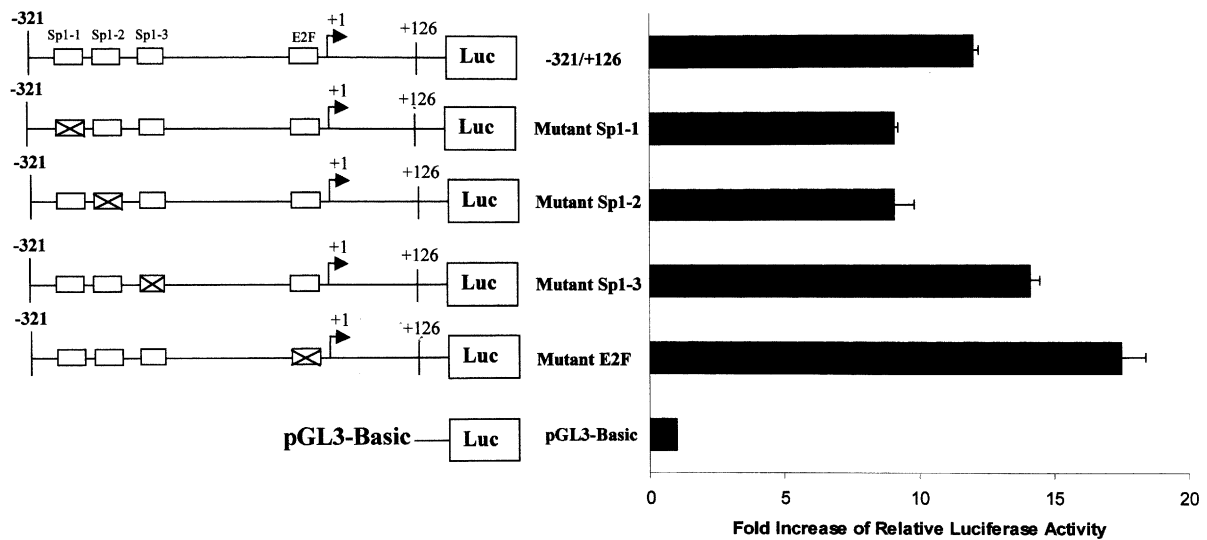
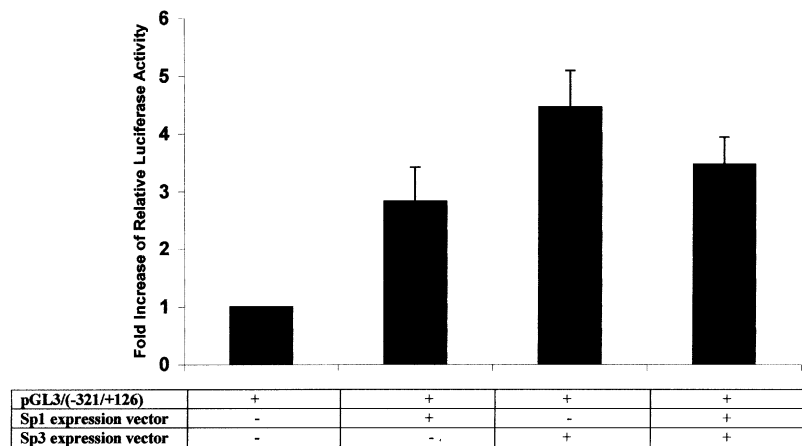
A**B**

Fig. 7. Title. (A) Effect of mutation of Sp1 and E2F *cis*-acting motifs on *Mer* promoter activity. Sertoli cells were transfected with the wild-type pGL3/(-321/+126) reporter gene constructs or the constructs containing double point mutations at the Sp1 and E2F *cis*-acting motifs as shown in the left-hand panel. (B) Over-expression of Sp1 or Sp3 in TM4 cells increases transcription from the *Mer* promoter. TM4 cells were cotransfected with the pGL3/(-321/+126) reporter gene constructs plus 1 µg of the Sp1 or the Sp3 expression vector, or both. Sp1 as well as Sp3 increase transcription of mouse *Mer*.

transcriptional activity. In contrast, mutation of the proximal Sp1 or the E2F motif caused a slight and moderate enhancing effect on the promoter activity, respectively (Fig. 7A). Thus, it is believable that these Sp1, Sp3 and E2F binding sequences on the *Mer* promoter play a significant role in regulating the transcription of *Mer*. The effects of Sp1 and Sp3 over-expression on the transcriptional activity of the *Mer* promoter in Sertoli cells were further investigated. Cells were transfected with the pGL3/(-321/+126) construct plus either the Sp1 or the Sp3 expression vector, or both. The results demonstrated that over-expression of Sp1 and Sp3 led to increased transcription from the *Mer* proximal promoter (Fig. 7B).

DISCUSSION

Mer is a member of the mammalian Tyro 3 receptor tyrosine kinase family, and it is widely expressed in tissues of epithelial and reproductive origins and cells of the immune system [27]. For the reproductive tissue, recent studies have shown that the Tyro 3 receptors are playing a significant role in spermatogenesis. In this study, it was shown that the postnatal expression of *Mer* in mouse testes is distinct from that of other members of the Tyro 3 RTK family. While both *Axl* and *Rse* show higher expression just after birth, expression of *Mer* increases constantly and attains the highest peak at around 20 days of age. As

spermatogenesis of mice begins at this age and at 30 days old, mice become sexually mature [28], the results seem to imply that the change in the patterns of *Mer* as well as *Axl/Rse* expression may be due to the increasing number of germ cells or to the onset of the influence of hormones involved in spermatogenesis. The possible reasons for modulating the expression of these receptors in the testis have yet to be defined. Unlike *Axl* and *Rse*, *Mer* mRNA was difficult to detect by Northern blotting, which might be due to its relatively low abundance in the testis [18]. This difference may suggest that these receptors are playing different roles in developing testicular functions. Because the *Mer* mRNA expression showed an age-dependent increase during testicular maturation which was different from those of the other members of Tyro 3 subfamily, we next sought to investigate the transcriptional regulation of *Mer* in Sertoli cells. In this study, the TM4 cell line was used as an *in vitro* model cell line. This cell line is derived from immature testes of normal 11- to 13-day-old BALB/c mice [29]. It is a continuous and nontransformed cell line and has been demonstrated to share morphological and functional properties with resident Sertoli cells *in situ* [30,31]. It has also been used in this and other laboratories as an *in vitro* cell model in transcriptional [32–34] and other functional studies [17,35].

To identify and characterize the 5' upstream regulatory region of *Mer*, an ≈ 1.5 kb fragment of the 5' flanking sequence of the gene was isolated and characterized. Like other RTK promoter sequences [36–38], *Mer* contains no TATA box, CAAT box, or initiator sequences. However, together with other common features, such as multiple transcription start sites and the presence of multiple GC box consensus sequence, the *Mer* 5' flanking region displays features typical of a TATA-less type promoter. Various deletion constructs driving the luciferase reporter gene in TM4 cells were used to analyse the promoter activity along the 5' flanking region of *Mer*. The result demonstrated that the gene segment $-321/+126$ contained the highest transcriptional activity in TM4 cells. Within this region, four protein-binding sites were detected by DNase I footprinting and gel shift assays. Three of them contained Sp1 *cis*-acting motifs, which are located at the $-237/-218$, $-194/-164$ and $-138/-119$ positions. Mutation studies revealed that Sp1 binding sites at the regions of $-237/-218$ and $-194/-164$ are responsible for up-regulation of promoter activity; while for the site at the latter region, Sp1 may interact with other unknown transcription factors to repress the *Mer* promoter activity. Thus, it suggests that Sp1 can play a role as either an activator or a repressor dependent on the nearby nucleotide sequence and the cellular context. The consensus sequence for Sp1 binding (CCCGCC) has been identified in the promoter regions of a variety of genes although its role in gene regulation is not fully understood [39]. The site is known to bind Sp1, an ≈ 100 kDa zinc-requiring transcription factor, and binding results in increased transcription of the associated gene [40–42]. Sp1 is essential for development in mice, as *Sp1*^{−/−} embryos have retarded growth and die early in gestation [43]. Some studies have suggested that Sp1 is involved in cell differentiation [44] as it has been found in highest levels in haematopoietic stem cells, foetal cells, and spermatids [43]. Others have speculated that it is a major transcription factor for housekeeping genes, as the Sp1 consensus sequence is commonly found in the promoter

region of these genes [45]. However, recent studies and our own data indicate that many mammalian gene types are controlled by Sp1, including genes for structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, and surface receptors [39,45]. Our report adds *Mer* to this growing list of relevant genes regulated by Sp1.

We have shown that apart from Sp1, Sp3 is also present in the protein complexes bound to the Sp1 *cis*-acting motifs in the *Mer* promoter by the Supershift assay using the Sp1 and Sp3 antibodies. Interestingly, Sp3 did not bind to all of the three Sp1 *cis*-acting motifs, but only to the two located at $-237/-218$ and $-194/-164$. Furthermore, by using sequence mutation introduced into the Sp1 *cis*-acting motifs, we have shown that the protein-binding site situated at $-194/-164$ is not merely a result of direct binding of Sp1 or Sp3, but that it may involve binding of other unknown transcriptional factors, even though it contains a Sp1 *cis*-acting element. Over-expression of Sp1 and Sp3 by cotransfection with Sp1 and Sp3 cDNAs leads to a further increase of transcription from the *Mer* promoter/enhancer region. In other studies, Sp3 has been shown to function either as an inhibitor [46] or as an activator [47] of gene transcription. Sp3 activity is therefore dependent on both the promoter characteristics and the cellular context.

As such, our findings have demonstrated that Sp1 and Sp3 seem to be necessary for promoting the transcription of *Mer* in mouse Sertoli cells. Other studies have shown that Sp1 acts co-operatively with other transcription factors such as AP-1 [48], EGR1 [49,50], NFkB [51] and STAT 1 [52]. Besides, E2F has been shown to interact physically with Sp1 to activate the human DNA polymerase α promoter and to exert a slightly greater than additive effect on the mouse thymidylate synthetase promoter; both of these promoters are TATA-less and have single binding sites for Sp1 and E2F [26]. It is noted that, like these genes, *Mer* also has an E2F binding site sequence located between +1 and −26. Our data show that after deletion of the sequence from +1 to −26 or mutation of the E2F binding site, transcriptional activity of *Mer* increased markedly (Figs 3C and 7A), suggesting that the relatively low *Mer* mRNA expression in the testis when compared with other Tyro 3 RTKs may putatively be due to interaction of E2F with Sp1 to suppress the transcription of *Mer* in Sertoli cells. Other studies have shown the involvement of TATA-less genes containing E2F and Sp1 binding sites in DNA replication and cell growth control, in which E2F has been found to act either as a transcriptional repressor or as an activator on these promoters, depending on the growth state or cell cycle stage of the cells [53–57]. However, in the case of *Mer*, the complexity of regulation by these two factors and the potential importance of the Sp1 *cis*-acting motifs in transcriptional regulation will be of future interest.

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