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Characterization of the Promoter of the Murine *mac25* Gene

Noriyuki Kanemitsu,*,† Mitsuo V. Kato,* Tsuneharu Miki,† Sei Komatsu,‡,§,¶ Yasushi Okazaki,‡,§ Yoshihide Hayashizaki,‡,§,¶ and Toshiyuki Sakai*

**Department of Preventive Medicine and †Department of Urology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan; ‡Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation (JST), Japan; §Laboratory for Genome Exploration Research Group, RIKEN Genomic Science Center (GSC) and Genome Science Laboratory, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba 305-0074, Japan; and ¶Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-0006, Japan*

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It is important to know the regulation of the expression of the *mac25* gene because of its reduced expression in several cancer cells and of its induction by some hormonal factors. We cloned the promoter region of the murine *mac25* gene and found five repeats of CCAAT sequences, four Sp1 sites, a TATA-like sequence, and an initiator (INR) sequence. Analysis using luciferase reporter plasmids indicated that CCAAT repeats have a strong enhancer activity and the second to fourth Sp1 sites are essential for basal activity of the expression of the *mac25* gene. The 1 kb region that contains the promoter and exon 1 of the *mac25* gene was in a typical CpG island. As hypermethylation and reduced expression of the *mac25* gene were reported in murine liver tumors, methylation of this CpG island may be directly associated with the expression of the *mac25* gene and tumorigenesis.

Key Words: **promoter; *mac25;* genome region; mouse; transcription factor; CpG island; luciferase.**

The human *mac25* gene was initially cloned as a gene, in which the expression is reduced in meningioma (1). The human gene was also cloned as a gene associated with cellular senescence (2). The murine homologue was also cloned as a gene, which has growth-suppressor activity on osteosarcoma cells (3). The expression of the *mac25* gene is also reduced in breast cancer (4), prostate cancer and osteosarcoma (5) and uterine leiomyomata (6). The malignant phenotype of prostate cancer cells was suppressed by the introduction of the *mac25* gene (7).

The human *mac25* gene was mapped to the chromosome 4q12 (2, 4). Human chromosome 4 induces cellular senescence of cervical carcinoma (HeLa), bladder carcinoma (J82), and glioblastoma (T98G) cell lines (8). Loss of heterozygosity (LOH) on chromosome 4q has been detected in hepatocellular carcinoma (9–11), breast cancer (12), bladder carcinoma (13), cervical carcinoma (14, 15), lung cancer (16, 17), esophageal adenocarcinoma (18), papillary thyroid cancer (19), head and neck squamous cell carcinoma (20), and childhood acute lymphoblastic leukemia (21).

The murine *mac25* gene was mapped to a region of mouse chromosome 5 (*D5Rik124*) (22, 23). The *mac25* gene is also hypermethylated, and the expression was reduced in murine liver tumors (23). Therefore, the *mac25* gene may have a tumor-suppressor activity against various cancers in human and mouse.

The N-terminal region of mac25 protein is highly homologous to insulin-like growth factor binding protein (IGFBP) (2). In fact, the gene was also cloned as a low-affinity IGFBP (24). The central region of mac25 protein is also highly homologous to follistatin, an activin-binding protein (3, 25, 26). On the other hand, the C-terminal region of mac25 protein is homologous to the fibroblast growth factor receptor (FGFR), especially in immunoglobulin-like motif (26). Therefore, the product of the *mac25* gene may regulate various signal transductions and that may result in tumor suppression, growth suppression, and cellular senescence.

The expression of the *mac25* gene is up-regulated by retinoic acid (2), cortisol (27) and the parathyroid hormone (28). Because the expression of the *mac25* gene is reduced in various cancer cells, it is very important to determine the mechanism of the expression of the *mac25* gene. In this study, we analyzed the genomic region around the exon 1 of the murine *mac25* gene and characterized the promoter region of the gene.

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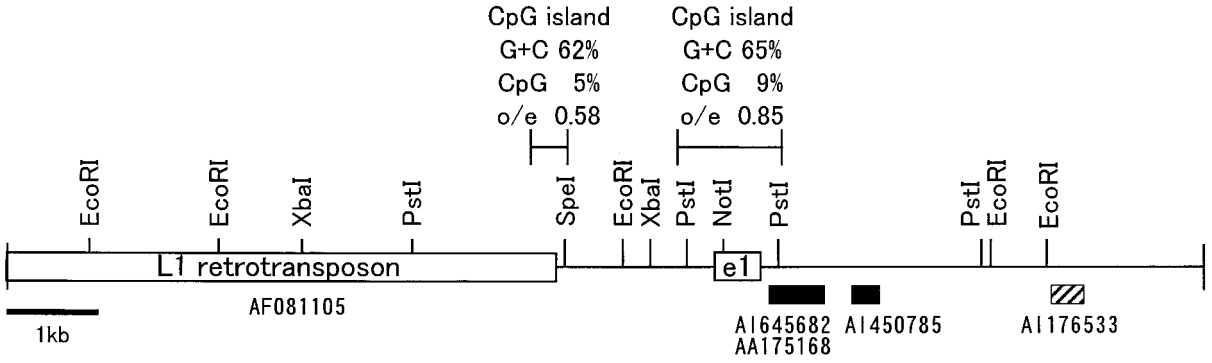

FIG. 1. Summary of the insert of phage clone M33 that contains the genomic region around the exon 1 of the murine *mac25* gene. In the approximately 13 kb insert, an L1 retrotransposon (indicated in a white long bar), which is highly homologous to Tf subfamily L1Md-Tf6 (Accession No. AF081105) was found at the upstream of the *mac25* gene. The direction of this L1 retrotransposon is opposite to that of the *mac25* gene. The exon 1 of the *mac25* gene is indicated as e1 in the box. In the intron 1, three regions homologous to mouse and rat EST sequences were found (black and hatched bars, respectively). Accession numbers of ESTs are mentioned below. Two CpG islands are observed. One is in the promoter region of L1 transposon (G + C 62%, CpG 5%, observed/expected CpG ratio 0.58) and the other is in the approximately 1 kb region that contains exon 1 of the *mac25* gene (G + C 65%, CpG 9%, observed/expected CpG ratio 0.85).

MATERIALS AND METHODS

DNA sequencing. An insert of the phage M33 (23) was subcloned into plasmid vectors and the sequences were determined by the dideoxy-chain termination method, using a BigDye Terminator Cycle Sequencing ready reaction (PE Applied Biosystems, Perkin–Elmer Japan, Chiba, Japan) and 310 genetic analyzer (PE Applied Biosystems). Sequences were assembled using the software Auto-Assembler (PE Applied Biosystems).

Reporter analysis. The promoter region of the *mac25* gene was cloned into pGVB2 (Toyo Ink Inc., Tokyo, Japan), which included firefly luciferase. Breast cancer cell line MCF7 was cultured in DMEM (GIBCO-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum. MCF7 cells were transfected with plasmid DNA by the DEAE-dextran method, as mentioned previously (29). Luciferase activity was analyzed using the PicaGene luminescence kit (Toyo Ink Inc.). Relative luciferase activity was standardized by protein quantity of each cell lysates.

Construction of the mutant of CCAAT, Sp1, TATA and INR. The reporter plasmid with a mutation in CCAAT site was constructed inserting the PCR fragment amplified using mutated primer, CCAAT/AS: AAG TTA ACC TTT CAG ATG AGA GA.

Reporter plasmids with mutations in Sp1, TATA, and INR sites were constructed by substitution with annealed oligonucleotides listed below.

Sp1-2m/S: GAT CCC GGG CGG GCG AAG CGG TTT TGG CAG CGG GCT GAC TCT CCG CCC C; Sp1-2m/AS: GGG GCG GAG AGT CAG CCC GCT GCC AAA ACC GCT TCG CCC GCC CGG; Sp1-3m/S: GAT CCC GGG CGG GCG AAG CGG GGC GGG CAG CGG GCT GAC TCT AAA ACC C; Sp1-3m/AS: GGG TTT TAG AGT CAG CCC GCT GCC CGC CCC GCT TCG CCC GCC CGG; Sp1-4m/S: GGG GAG GGC TCC CAC GGA GCA CCG GTT TTG GCA GCG GCT TTA AGG GTT GGC CTC CGG GAC CCG GAC TGC TCA CTC GCG CGT G; Sp1-4m/AS: CTA GCA CGC GCG AGT GAG CAG TCC GGG TCC CGG AGG CCA ACC CTT AAA GCC GCT GCC AAA ACC GGT GCT CCG TGG GAG CCC TCC CC; Sp1-2,3m/S: GAT CCC GGG CGG GCG AAG CGG TTT TGG CAG CGG GCT GAC TCT AAA ACC C; Sp1-2,3m/AS: GGG TTT TAG AGT CAG CCC GCT GCC AAA ACC GCT TCG CCC GCC CGG; mTATA/S:GGG GAG GGC TCC CAC GGA GCA CCG GGC GGG GCA GCG GCC GCA AGG GTT GGC CTC CGG GAC CCG GAC TGC TCA CTC GCG CGT G; mTATA/AS: CTA GCA CGC GCG AGT GAG CAG TCC GGG TCC CGG AGG CCA ACC CTT GCG GCC GCT GCC CCG CCC GGT GCT CCG TGG GAG CCC TCC CC; mINR/S: GGG GAG GGC TCC CAC GGA GCA CCG GGC GGG GCA GCG GCT TTA AGG GTT GGC CTC CGG GAC CCG GAC TGC AGG CGC GCG CGT G; mINR/AS: CTA GCA CGC GCG CGC CTG CAG TCC GGG TCC CGG AGG CCA ACC CTT AAA GCC GCT GCC CCG CCC GGT GCT CCG TGG GAG CCC TCC CC; mTATAmINR/S: GGG GAG GGC TCC CAC GGA GCA CCG GGC GGG GCA GCG GCC GCA AGG GTT GGC CTC CGG GAC CCG GAC TGC AGG CGC GCG CGT G; mTATAmINR/AS: CTA GCA CGC GCG CGC CTG CAG TCC GGG TCC CGG AGG CCA ACC CTT GCG GCC GCT GCC CCG CCC GGT GCT CCG TGG GAG CCC TCC CC.

RESULTS AND DISCUSSION

Structure of Murine Genomic Clone M33 Containing the Exon 1 Region of the mac25 Gene

Figure 1 summarizes the structure of the murine genomic region (M33) of the *mac25* gene. We sequenced a phage genomic clone (M33) containing about 13 kb insert. We found an L1 retrotransposon, which is highly homologous to Tf subfamily L1Md-Tf6 (Accession No. AF081105) at the upstream of the *mac25* gene. The direction of this L1 transposon was opposite to that of the *mac25* gene. The genomic clone contained only exon 1 of the *mac25* gene. In the intron 1, three regions homologous to mouse and rat EST sequences were found, suggesting that some other transcriptional units may exist in intron 1. Two CpG islands were observed. One was in the promoter region of L1 transposon (G + C 62%, CpG 5%, observed/expected CpG ratio 0.58) and the other was in the approximately 1 kb region that contained exon 1 of the *mac25* gene (G + C 65%, CpG 9%, observed/expected CpG ratio 0.85). While the observed/expected CpG ratio of the promoter of L1 transposon was very marginal, the CpG island may associate with inactivation of the retrotransoposon. As hypermethylation of the *mac25* gene was reported in murine liver tumors (23), methylation of this CpG island around the exon 1 may associate with expression of the *mac25* gene and tumorigenesis.

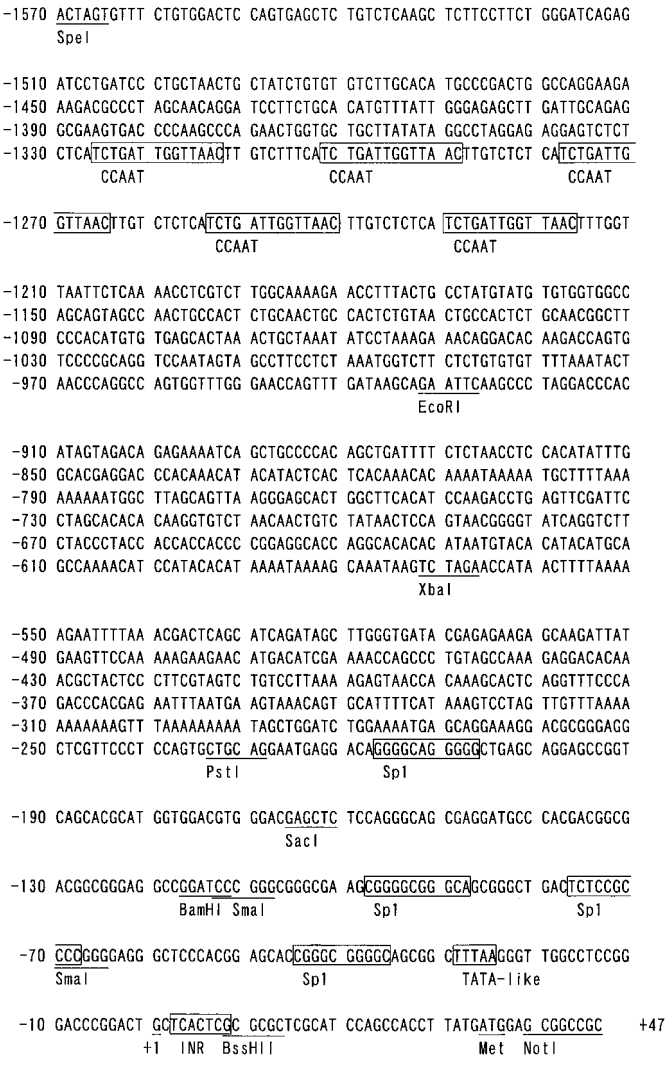

FIG. 2. Nucleotide sequence of the murine *mac25* gene. Restriction enzyme sites are underlined with the name of the enzyme. Putative transcription factor binding sequences and initiator sequence (CCAAT, Sp1, TATA-like and INR) are boxed. The transcription initiation site is indicated as +1. The translation initiation site is indicated as Met with an underline.

Characterization of the Promoter Region of the mac25 Gene

In the promoter region of *mac25* gene, we found five repeats of CCAAT (NFY, c/EBP) sequences, four Sp1 sites, a TATA-like sequence and an initiator (INR) sequence (Fig. 2). The transcriptional start site was determined by the result of the longest cDNA that was cloned by the cap site trapped method (30). The initiator (INR) consensus (PyPyAN(A/T)PyPy, Py = pyrimidine; Ref. 31) sequence with one base mismatch (TCACTCg, from +3 to +9 in Fig. 2) was also found just after the initiation site. A TATA-like sequence (TTTAA) was found at −29 to −25. TATA boxes are located at 25–30 bp upstream of its transcriptional initiation site in the majority of the eukaryotic promoters (31). The structure of the murine *mac25* gene may be very reasonable for the expression.

Although it was reported that the *mac25* gene is up-regulated by retinoic acid (2), cortisol (27) and the parathyroid hormone (28), we could not find any such hormone receptor responsive elements in the promoter region of the *mac25* gene. Regulation of the *mac25* gene by hormonal factors may be indirect or mediated by other novel mechanisms. In fact, the promoter of the gene for cyclin-dependent kinase inhibitor p27 lacks vitamin D3 responsive element (VDRE), although the *p27* gene is up-regulated by vitamin D3 (32). They also reported that NFY and Sp1 are important for induction of the *p27* gene by vitamin D3 (32). The promoter sequence of the *mac25* gene contains exactly NFY (CCAAT) and Sp1 sequences. We also observed that the *mac25* gene is up-regulated by vitamin D3 (Kanemitsu *et al.*, unpublished data). Therefore, the *mac25* gene may be up-regulated by such novel mechanism in the response to hormonal factors.

Promoter Activity of the mac25 Gene

The promoter regions of the *mac25* gene were cloned into the luciferase reporter vector (pGVB2), and luciferase activity was analyzed in several deletion mutants (Figs. 3 and 4). The longest promoter construct SpeI-Luc had about 1052-fold activity of the parental vector pGVB2 (Fig. 3A). The activity was significantly ($P < 0.05$) reduced in three deletion mutants (EcoRI-Luc, XbaI-Luc, and PstI-Luc). However, the activity of these three mutants was still significantly ($P < 0.001$) higher than the parental vector. The promoter region from SpeI to EcoRI contained five CCAAT repeats (Figs. 2 and 3). These CCAAT repeats may have a strong enhancer activity for the promoter of the *mac25* gene. To check whether the CCAAT repeats really act as the enhancer, we constructed the reporter plasmids that had reduced number of CCAAT sequence (Fig. 3B). The luciferase activity depended on the number of CCAAT sequences (Fig. 3B). The activity of the reporter that had a mutant CCAAT sequence was same as deleted mutant EcoRI-Luc. Therefore, the five CCAAT repeats have the strong enhancer activity for the promoter of the *mac25* gene.

Because the *Pst*I-Luc mutant still had higher activity than the parental vector (Fig. 3A), shorter mutants were constructed and analyzed (Fig. 4A). No reduction of the activity was observed in SacI-Luc and BamHI-Luc compared with PstI-Luc. However, two deletion mutants (SacIΔ-Luc and SmaI-Luc) showed a significantly reduced level ($P < 0.05$ and $P < 0.001$, respectively) of the activity, which was almost the same activity as that of the parental vector (Fig. 4A). SmaI-Luc contained one Sp1, one TATA-like and one INR site (Figs. 2 and 4). However, that is not enough for basal

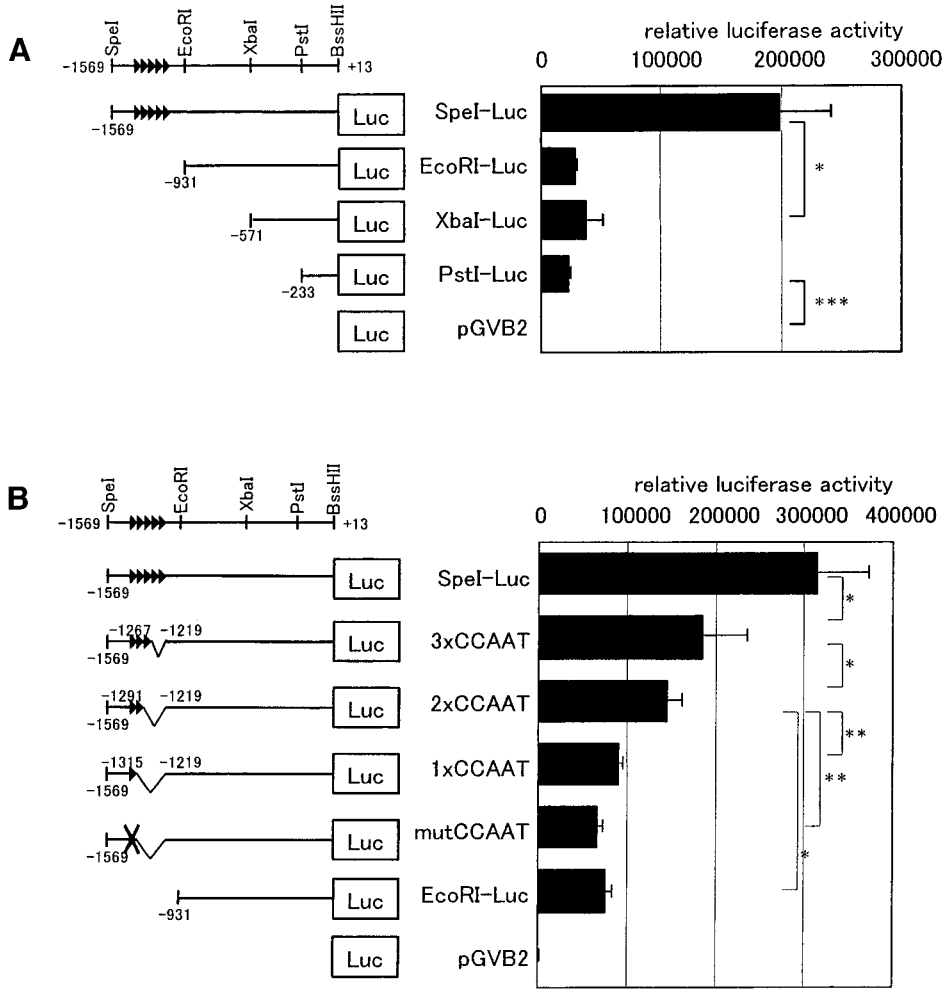

FIG. 3. (A) Analysis of the reporter activity of the deletion mutants of the murine *mac25* gene promoter. Averages (and standard deviations) of three independent samples are shown. The longest promoter construct SpeI-Luc has about 1052-fold activity of the parental vector pGVB2. Although the activity was reduced in three deletion mutants (EcoRI-Luc, XbaI-Luc, and PstI-Luc) significantly ($P < 0.05$), the activity of these three mutants was still significantly ($P < 0.001$) higher than the parental vector. The promoter region from SpeI to EcoRI, which contains five CCAAT repeats (indicated by arrowhead; see also Fig. 2), had a strong enhancer activity for the promoter of the *mac25* gene. PstI-Luc, which contains four Sp1 sites, one TATA-like site and an INR sequence (Fig. 2), still has a minimum promoter activity. (B) Analysis of the reporter activity of the CCAAT- mutants of the murine *mac25* gene promoter. 3× CCAAT, three repeats of CCAAT sequence; 2× CCAAT, two repeats; 1× CCAAT, one CCAAT sequence; mutCCAAT, one mutant CCAAT sequence. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

promoter activity of the *mac25* gene (Fig. 4A). The promoter region from *Sma*I (−110) to SmaI (−67) contained two Sp1 sites. Therefore, these Sp1 sites may be important for basal activity of the promoter of the *mac25* gene. To know which Sp1 site is important for basal activity of the promoter, the reporter plasmids that had mutations in the second, third and fourth Sp1 site were constructed (Fig. 4B). The luciferase activity decreased in the reporters that had a mutation in Sp1 sites (Fig. 4B). These Sp1 sites seem to contribute to the basal activity of the promoter of the *mac25* gene synergistically. To know the effect of the mutations of Sp1 sites on the full-length promoter, these mutations were introduced to the full-length promoter (Fig. 4C). Since the full-length reporter with three mutations in Sp1 sites (SpeI2,3,4m) showed the same activity as that of shorter plasmid with tree mutations in Sp1 sites (SacI2,3,4m), these Sp1 sites may be indispensable for the basal activity of the promoter of the *mac25* gene (Fig. 4C).

The reporters with mutations in TATA-like sequence and INR were constructed (Fig. 5). The luciferase activities of these mutants (mTATA, mINR, mTATAmINR) were significantly lower than that of the wild-type reporter (SacI-Luc). However, the activities of these mutants were not suppressed completely and still higher than Sp1 triple mutant (2,3,4m). Therefore, Sp1 sites may contribute to the basal promoter activity much

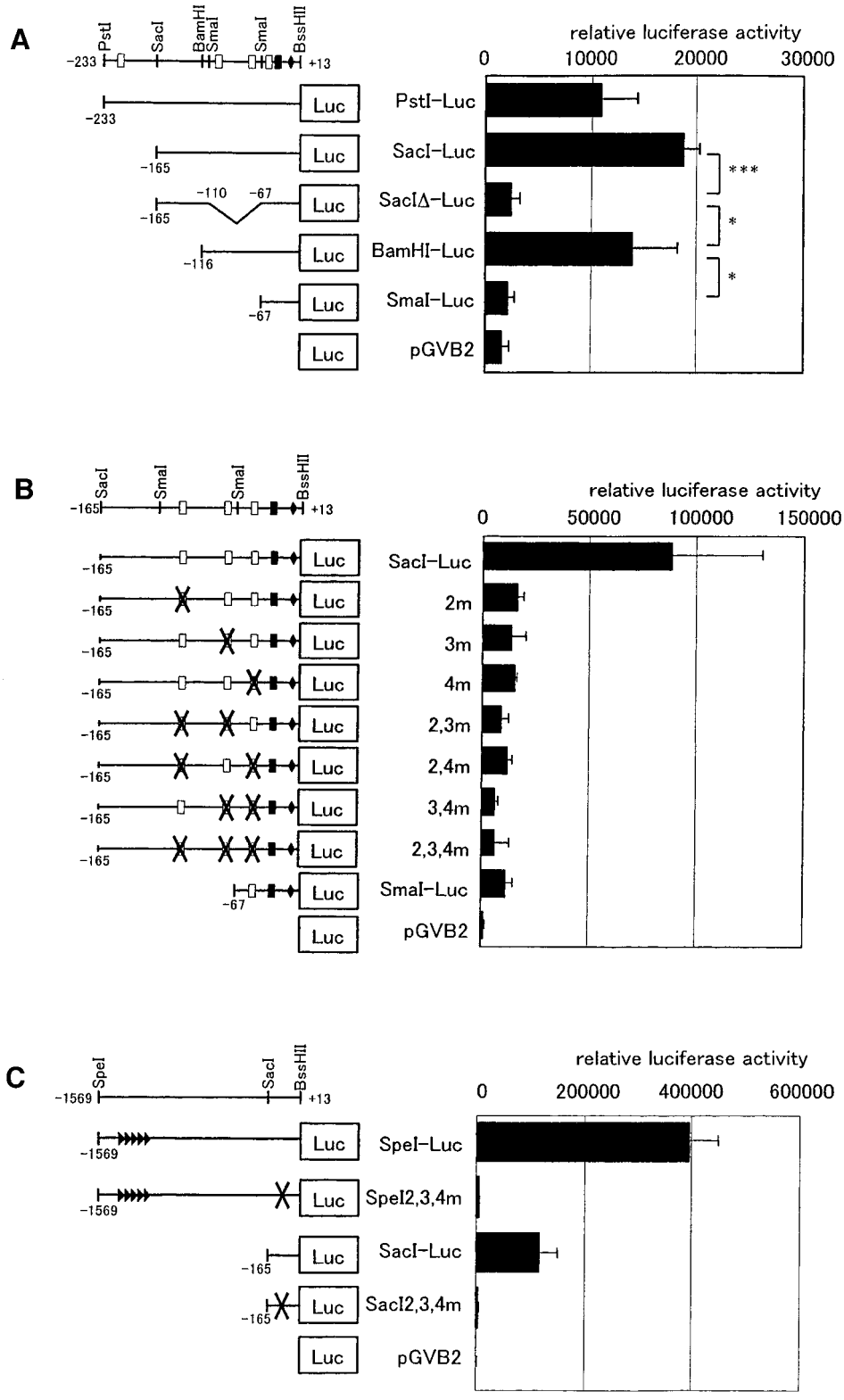

FIG. 4. (A) Detailed analysis of the minimum promoter activity of the *mac25* gene. Averages (and standard deviations) of three independent samples are shown. Although no reduction of the activity was observed in SacI-Luc and BamHI-Luc compared with PstI-Luc, two deletion mutants (SacIΔ-Luc and SmaI-Luc) showed a significantly reduced level ($P < 0.05$ and $P < 0.001$, respectively) of the activity, which was almost the same activity as that of the parental vector. The promoter region from SmaI (−110) to SmaI (−67), which contains two Sp1 sites, is important for basal activity of the promoter of the *mac25* gene. Sp1, TATA-like and INR sequences are indicated by open boxes, a closed box and a closed diamond, respectively. (B) Analysis of the reporter activity of the Sp1− mutants of the murine *mac25* gene promoter. 2m, mutation in second Sp1 site; 3m, third Sp1; 4m, fourth Sp1; 2,3m, mutations in second and third Sp1; 2,4m, second and fourth; 3,4m, third and fourth; 2,3,4m, mutations in all three Sp1 sites. (C) Analysis of the full-length reporter activity of the Sp1− mutants of the murine *mac25* gene promoter. SpeI2,3,4m is the full-length reporter plasmid that has three mutations in second to fourth Sp1 sites. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

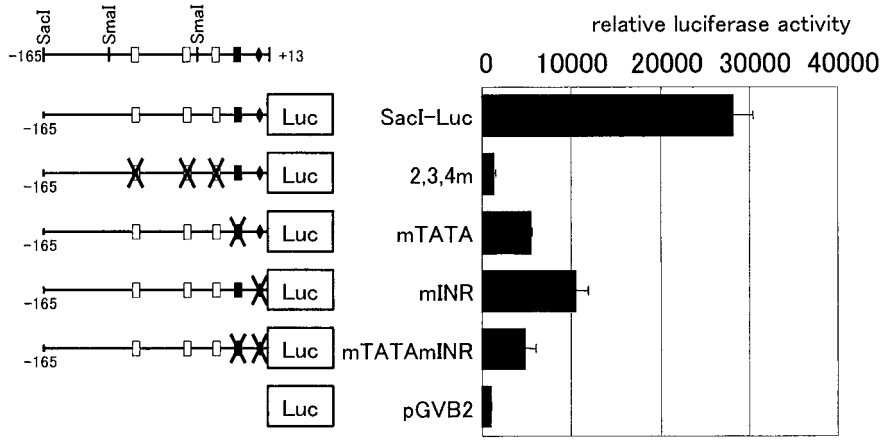

FIG. 5. Analysis of the reporter activity of the TATA-like and INR- mutants of the murine *mac25* gene promoter. 2,3,4m, mutations in three Sp1 sites; mTATA, mutation in TATA-like sequence; mINR, mutation in INR sequence; mTATAmINR, mutations both in TATA-like and INR sequences.

more than TATA-like and INR sequences although these TATA-like and INR sequences exactly contribute to the basal promoter activity.

In this report, we characterized the promoter region of the *mac25* gene. The control of the expression of the *mac25* gene is very important for cancer therapy and prevention, because of the increasing amount of reports about the reduction of the expression of the *mac25* gene in various human cancers (1, 4–6). Hypermethylation and down-regulation of the *mac25* gene in murine liver tumor were also reported (23). While some factors (retinoic acid, cortisol and parathyroid hormone) increase the expression of the *mac25* gene (2, 27, 28), the regulated elements in the promoter region of the *mac25* gene have not been determined. The identification of such regulators may be important not only to determine the mechanism of the gene expression, but also to identify a clue for cancer therapy and prevention.

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