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The DNA-Binding and Transcriptional Activities of MAZ, a Myc-Associated Zinc Finger Protein, Are Regulated by Casein Kinase II

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***M*yc-*a*ssociated *z*inc finger protein (MAZ) is a transcription factor that contains proline-rich, alanine repeats and six C_2H_2-type zinc finger motifs, as well as five putative sites of phosphorylation by *c*asein *k*inase II (CKII). Site-specific mutagenesis of MAZ revealed that the serine residue at position 480 was the major site of phosphorylation by CKII both *in vitro* and *in vivo*. Phosphorylation of MAZ by CKII at this serine residue was required for maximum binding of MAZ to the pyrimidine-rich DNA of the *n*uclease-*h*ypersensitive *e*lement (NHE) in the 5′-end promoter region of the c-*myc* gene. Mutation of serine at position 480 to alanine eliminated the DNA-binding activity of MAZ to this element. Moreover, the mutated MAZ was unable to enhance the expression of luciferase encoded by a c-*myc* promoter/luciferase reporter gene in HeLa cells in the presence of CKII. These results suggest that phosphorylation of the serine residue at position 480 of MAZ by CKII can control the function of MAZ by altering its DNA-binding activity.**

The c-*myc* gene encodes a sequence-specific DNA-binding protein that plays an important role in the regulation of progression of the cell cycle, proliferation, differentiation and apoptosis (1). Transcription of the c-*myc* gene is controled by two closely spaced promoters, designated P1 and P2. The mRNA transcribed from the P1 promoter accounts for 10–25% of all transcripts of c-*myc* mRNA and mRNA transcribed from the P2 promoter accounts for 75–90% of c-*myc* transcripts (1–3). Regulation of the expression of c-*myc* occurs at multiple levels, which include the intiation, the termination and the attenuation of transcription (1, 4). Optimal initiation of transcription from the P2 promoter requires the ME1a2, E2F and ME1a1 elements in the P2 promoter (5, 6). Several transcription factors, including Sp1 (7), the Myc-associated zinc-finger protein MAZ (= ZF87; refs. 8, 9) and its murine homolog Pur-1 [purine-binding factor (10)], and E2F (11), bind to these elements at least *in vitro*. MAZ was identified as a transcription factor that binds to the GA box (GGGAGGG) in the ME1a1 element (6, 8, 9) with higher affinity than it does to the ME1a2 element (6, 8, 10 and 12). MAZ also binds to the GA box of the attenuator region of P2 within the first exon of the c-*myc* gene (13). In addition, MAZ-binding sites are present between the closely spaced human genes for complement C2 and factor B (9); between human genes for g11 and C4 (13); within an intron of the murine gene for IgM-D (13); in the promoter regions of human or murine genes for CD4 (14), for serotonin 1a receptor (15), for serum amyloid A (16, 17) and for endothelial nitric oxide synthetase (18), as well as in the adenovirus major late promoter (19). The murine homolog of MAZ, Pur-1, binds to the GAGA boxes of rat genes for insulin I and II and of the human gene for islet amyloid polypeptide (10). An insulin-linked polymorphic region (ILPR) also contains binding sites for Pur-1 (20). The cDNAs for MAZ (= ZF87 = Pur-1), a DNA-binding protein, have been cloned (8–10, 21, 22). We previously isolated the cDNA (MAZi) for MAZ from human pancreatic islet cells (21). MAZ is widely expressed in the mammalian organs such as brain, heart, liver, lung and spleen (21, 23–26). The increased and aberrant transcription of the gene for MAZ has been demonstrated during the terminal phase of chronic myelogenous leukemia (27). We (21–23) and others (2, 3) found that MAZ bound the pyrimidine-rich DNA of the

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nuclease-hypersensitive element (NHE) in the 5′-end promoter region of c-*myc* gene as well as ME1a1 site. Both elements are reported to be critical for the increased expression of c-*myc* gene (2, 3, 21, 25). We reported that this primidine-rich NHE of 5′-end promoter region of the c-*myc* gene formed the unique structures of single, double and triple-helix conformation (GGC-typed triple helix) of the DNA *in vitro* and *t*riple-*h*elix *zi*nc *f*inger protein-1 (THZif-1), a transcription factor of MAZ family, strongly bound this unique structure; especially single stranded and GGC-typed triple stranded structure of this NHE of the c-*myc* gene. Therefore, we focused here the comparative DNA-binding function of phosphorylated MAZ variant proteins described below, to these unique structures of the primidine-rich DNA of c-*myc* NHE *in vitro*.

Analysis of the nucleotide sequence of the gene for MAZ suggested that MAZ might have multiple potential sites for phosphorylation by casein kinase II (CKII), protein kinase C (PKC) and tyrosine kinase (TK). CKII is of particular interest since it is a ubiquitous, heterotetrameric serine/threonine protein kinase that consists of two catalytic (α and α') and two regulatory (β) subunits (28). CKII phosphorylates and regulates the activities of a variety of transcription factors, which include activators, repressors and viral factors (28, 29). In the present study, we found that CKII was able to phosphorylate the serine residue only at position 480 in the carboxy-terminal region of MAZ. Such phosphorylation resulted in an increase in the DNA-binding of MAZ to the primidine-rich c-*myc* NHE and transcriptional activities of MAZ on the c-*myc* reporter gene. Our observations suggest that CKII might be one of the key factors that regulates the function of MAZ.

MATERIALS AND METHODS

Plasmids. pTH315 was constructed as described previously (21). The cDNA for MAZ was inserted into the *Bgl*II/*Bam*HI site of the histidine-tagged plasmid, pEBVHis C (Invitrogen Co., Carlsbad, CA, USA) and the *Eco*RI site of the glutathione S-transferase fusion construct pGEX-2T (Amersham Pharmacia Biotech., Uppsala, Sweden). Two MAZ mutants (MAZ-S-A and MAZ-S-D) were generated by conversion of Ser at position 480 (TCG) to Ala (GCA) and Asp (GAT), respectively, using a kit for *in vitro* mutagenesis (Boehringer-Mannheim, Mannheim, Germany) with addition of the *Kpn*I linker. Plasmids that encoded wild-type MAZ and the two mutant forms of MAZ, based on the vector pEBVHis C, were digested with *Kpn*I/*Bam*HI and *Bgl*II/*Hin*dIII and appropriate fragments were subcloned into the respective sites of the vector pEGFP-C3 (CLONTECH Inc., Palo Alto, CA, USA) to generate plasmids that encoded a green fluorescent protein-fused (GFP-fused) MAZ construct and corresponding mutated constructs, namely pEGFP-C3MAZ, pEGFP-C3MAZ-S-A and pEGFP-C3MAZ-S-D, respectively. The c-*myc* luciferase reporter plasmids pCG362-5/HP (P1 + P2 promoter) and pblueLUC (control), were prepared as described elsewhere (30).

Synthesis of oligodeoxynucleotides and preparation of single-stranded, double-stranded and triple-stranded DNA probes. Oligodeoxynucleotides were synthesized by standard phosphoroamidite chemistry and purified by reverse-phase chromatography on an Oligonucleotide Purification/Elution Cartridge (Cruachem Inc., Glasgow, Scotland). Concentrations were determined in terms of absorbance at 260 nm. The integrity of oligodeoxynucleotides was verified by 5′-end radiolabeling with [γ-^{32}P]ATP (5,000 mCi/mmol; Amersham Pharmacia Biotech.) and T4 polynucleotide kinase (Toyobo, Kyoto, Japan) with subsequent gel electrophoresis (20% polyacrylamide) under denaturing conditions and nucleotide sequencing. The sequences of the oligonucleotide probes for binding studies were as follows: 1) c-*myc* NHE-G (purine-rich strand), 5′-ggggagggtggggagggtggggaaggtggggagga-3′; and 2) c-*myc* NHE-C (pyrimidine-rich strand), 5′-tcctccccaccttccccaccctccccaccctcccc-3′. DNA probes corresponding to single-stranded (ss), double-stranded (ds) and triple-stranded (ts) c-*myc* NHE were prepared as described by Boles and Hogan (31) and Sakatsume *et al.* (22). Samples were subjected to electrophoresis in polyacrylamide gels prepared with 89 mM Tris (pH 7.4), 89 mM boric acid and 5 mM $MgCl_2$, and then the various DNA probes were extracted from the gels (21, 22).

Metabolic radiolabeling of cells and DNA-binding assay. For metabolic labeling, HeLa cells were incubated for 3 h with 500 μCi of [^{32}P]-orthophosphate (300 μCi/ml; Amersham Pharmacia Biotech.) in phosphate-free Dulbecco's modified Eagle medium (DMEM) or with 400 μCi of [^{35}S]-methionine (50 μCi/ml; Amersham Pharmacia Biotech.) in methionine-free DMEM. Cells were lysed by incubation at 4°C for 30 min in lysis buffer [50 mM Tris (pH 8.0), 250 mM NaCl, 30 mM sodium pyrophosphate, 20 mM sodium phosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF)] supplemented with 1 μg/ml leupeptin, 0.5 μg/ml pepstatin and 1 μg/ml aprotinin. Lysates were centrifuged at 30,000 × g and the supernatants were stored at −80°C. Cell lysates were treated with Ni-NTA agarose beads (Qiagen GmbH, Hilden, Germany) for isolation of proteins. The beads were then washed three times with phosphate-buffered saline (PBS). Proteins were solubilized in 2× sample buffer for SDS-PAGE (22), boiled for 5 min, fractionated by SDS-PAGE (10% polyacrylamide) and visualized by exposure to X-ray film. For DNA-binding assays, cell lysates were incubated with the appropriate biotin-labeled single-, double-, or triple-stranded (ss-, ds-, ts-) c-*myc* NHE DNA probe according to the protocol from the manufacturer of the assay system (Dynal, Oslo, Norway; ref. 22). The amount of MAZ bound to c-*myc* NHE-C that had been immobilized on beads (Dynabeads M-280 streptavidin; Dynal) was determined after fractionation by SDS-PAGE (10% polyacrylamide) and subsequent autoradiography.

Assays of luciferase activity. HeLa cells (1×10^5) that had been grown in DMEM with 10% FCS were transfected, using the TransFast system (Promega Corp., Madison, WI, USA), with 1.0 μg of DNA of each c-*myc*-luciferase construct or with pblueLUC as a control, as well as with 1.0 μg of DNA of pEBVHis C MAZ, pEBVHis C MAZ-S-A, or pEBVHis C MAZ-S-D, as indicated in Fig. 4B. After incubation for 48 h, cells were harvested, lysed in lysis buffer and assayed for luciferase activity according to the protocol from Promega. Concentrations of proteins in lysates were determined with a kit from Bio-Rad (Hercules, CA, USA) with bovine serum albumin (fraction V) as the standard. Transfection efficiencies were normalized in terms of β-galactosidase (β-gal) activity as described elsewhere (30).

Fluorescence microscopy. HeLa cells were transfected with 2.5 μg of DNA of pEGFP-C3MAZ, pEGFP-C3MAZ-S-A or pEGFP-C3MAZ-S-D, prepared as described above, at 37°C for 1.0 h using the TransFast system. After 48 h, cells were washed twice with PBS and GFP-positive cells were visualized under a fluorescence microscope (IX70; Olympus, Tokyo, Japan). Approximately 20% of cells were GFP-positive. Whole-cell extracts were subjected to SDS-PAGE (10% acrylamide) and then proteins were electrotransferred to a nitrocellulose membrane. Polyclonal antibodies against GFP from *Aequaria victoria* (CLONTECH; diluted 1:1,000) were used as primary antibodies and horseradish peroxidase-conjugated antibodies raised in goat against rabbit IgG (Amersham Pharmacia Biotech.; diluted

1:2,500) were used as second antibodies for detection of the expression of each GFP-fused construct with the ECL detection system (Amersham Pharmacia Biotech.).

Phosphorylation of MAZ by casein kinase II (CKII). Reaction mixtures (20 μl) contained binding buffer [10 mM Tris (pH 7.8), 5 mM $MgCl_2$, 1 mM spermidine, 10% sucrose, 25 μM $ZnCl_2$, 5 mM dithiothreitol and 1 μg poly(dI-dC) (Amersham Pharmacia Biotech.)] supplemented with a 100 μM solution of ATP that contained [γ-^{32}P] ATP (30-60 μCi/mmole), and 50 ng of GST-fusion protein. In some experiments, non-radioactive ATP was used to phosphorylate MAZ protein. Casein kinase II (New England Biolabs Inc., Beverly, MA, USA) was added, and after incubation at 37°C for 10 min, reactions were quenched by the addition of sample buffer for SDS-PAGE. In some experiments, active CKII enzymatic proteins were purified as described elsewhere (29). Products were fractionated by electrophoresis on an SDS-polyacrylamide (10%) gel. Gels were dried prior to exposure to X-ray film.

Purification of fusion proteins, CKII-mediated phosphorylation and electrophoretic mobility shift assay (EMSA). Derivatives of pGEX-2T-MAZ were introduced into *E. coli* AD202 and the synthesis of each fusion protein was induced by addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside to the culture medium. Proteins were purified with glutathione-agarose beads (Amersham Pharmacia Biotech.) as described elsewhere (21, 22). Phosphorylation of GST-MAZ and the mutant proteins was performed as described previously (22) for examination of the effects of phosphorylation on binding of the proteins to DNA in EMSAs (21, 22).

Western blotting analysis. Cells were lysed and fractionated by SDS-PAGE (10% polyacrylamide) and then proteins were electroblotted onto a membrane as described elsewhere (25, 26). The membrane was incubated with 1,000-fold-diluted antibodies against his-tag (QIAexpress^{TMRGS}.His; QIAGEN Gmbh). After the membrane had been washed, antibodies that had bound to the membrane were detected with horseradish peroxidase-conjugated antibodies raised in goat against mouse immunoglobulin (Zymed Laboratories, South San Francisco, CA, USA) and ECL detection reagents.

RESULTS AND DISCUSSION

The nucleotide sequence of the cDNA for MAZ suggests the presence in MAZ of putative sites of phosphorylation by several protein kinases, such as *c*asein *k*inase II (CKII), *t*yrosine *k*inase (TK) and *p*rotein *k*inase *C* (PKC) (32–34). In an attempt to identify the exact location of the sites of phosphorylation of MAZ, we performed phosphorylation assays *in vitro* and found that MAZ was efficiently phosphorylated by CKII, by TK and by PKC *in vitro* (data not shown). We also found that CKII-phosphorylated MAZ protein showed the strongest DNA-binding to the pyrimidine-rich DNA of the nuclease-hypersensitive element (NHE) in the promoter region of the c-*myc* gene as compared to those of phosphorylated MAZ proteins by TK or PKC *in vitro*. (data not shown). Thus, we focused our attention on the phosphorylation of MAZ by CKII.

Five putative sites of phosphorylation of MAZ by CKII, namely, the amino acids at position 146, *S*TVD; at position 204, *S*ALE; at position 325, *S*HSD; at position 372, *S*RPD; and at position 480, *S*GAE, were found in the deduced amino acid sequence of MAZ. We constructed GST-fusion proteins and examined whether the fusions with wild-type and mutant forms of MAZ in which each serine residues was replaced by alanine, could be phosphorylated by CKII. We found that the phosphorylation of MAZ protein by CKII was detected in the serine residue only at position 480 *in vitro* (data not shown). Thus, we studied the effects of the CKII-mediated phosphorylation of MAZ protein at position 480 on the DNA-binding activity and the transcription activity of the c-*myc* gene.

The wild-type GST-MAZ fusion protein was phosphorylated *in vitro* by CKII, but the mutated GST-MAZ-S-A and GST-MAZ-S-D fusion proteins, in which the serine residue at position 480 was replaced either by alanine or by aspartic acid, were not (Fig. 1A). Thus, the serine residue at position 480 appeared to be the major residue that was phosphorylated by CKII *in vitro* (Fig. 1A). The levels of expression of GST-MAZ and the mutant forms were similar, as estimated by SDS-PAGE (10% polyacrylamide; Fig. 1B). We examined the DNA-binding activities of CKII-phosphorylated GST-MAZ protein and the mutated forms *in vitro* by an EMSA (Fig. 1C). As expected, the intensity of the shifted band was significantly enhanced in the case of CKII-phosphorylated GST-MAZ protein. By contrast, GST-MAZ-S-A did not bind strongly to the ds c-*myc* NHE-GC DNA probe even after treatment with CKII. Moreover, the DNA-binding activity of GST-MAZ-S-D, which gave a similar intensity of the shifted band to untreated GST-MAZ, was not significantly affected by CKII-mediated phosphorylation (data not shown).

We next examined the phosphorylation of MAZ *in vivo* by radiolabeling HeLa cells that had been transfected with the expression plasmids that encoded MAZ and two mutant forms of MAZ. We constructed expression plasmids that encoded mutant MAZ proteins in which the serine residue at position 480 was replaced either by alanine (S-A) or by aspartic acid (S-D) (pEBVHis C MAZ-S-A and pEBVHis C MAZ-S-D) to compare them with wild-type (WT) MAZ (encoded by pEBVHis C MAZ). As shown in Fig. 2A, a protein of approximately 58 kDa in immunoprecipitates was preferentially radiolabeled with [^{32}P]-orthophosphate. The relative levels of expression of wild-type (pEBVHis C MAZ-WT) and mutant (pEBVHis C MAZ-S-A and pEBVHis C MAZ-S-D) MAZ were examined by Western blotting with the antibodies against the histidine-tag. Two bands, corresponding to rapidly and slowly migrating forms of MAZ, were detected on gels. The slowly migrating MAZ protein might have represented modified forms of MAZ, generated by farnesylation, palmitylation, glycosylation or other modifications (data not shown; panel b in Fig. 2A). [^{35}S]-Methionine-radiolabeled wild-type MAZ and mutated MAZ (encoded by pEBVHis C MAZ-S-D) were recovered from extracts of HeLa cells after incubation with biotin-labeled pyrimidine-rich single-stranded (ss) and double-stranded (ds) DNA probes that corresponded to the

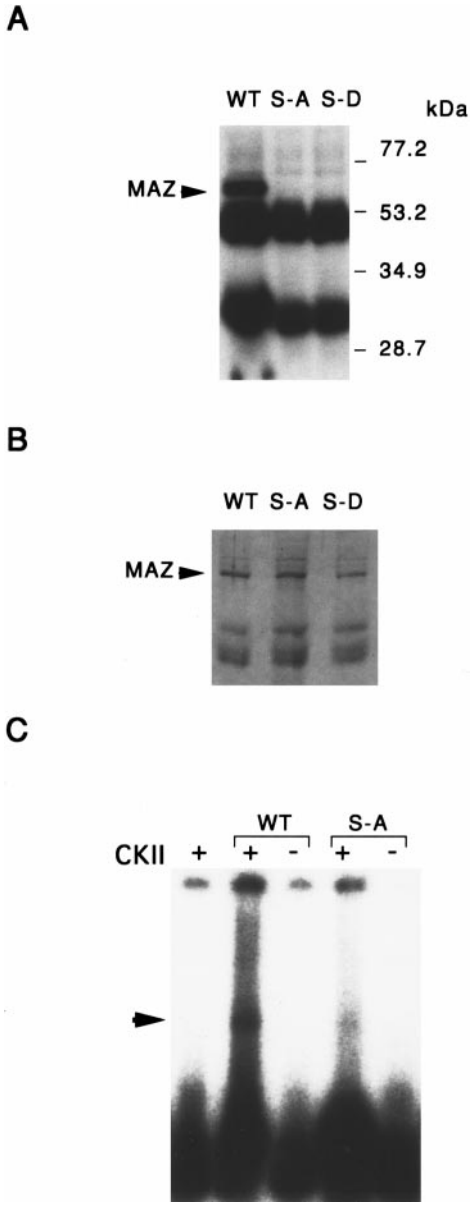

FIG. 1. Phosphorylation of MAZ by casein kinase II of the serine residue at position 480. (A) CKII-mediated phosphorylation of GST-MAZ derivatives. GST-tagged MAZ fusion proteins, namely, the wild type (GST-MAZ; WT) and the mutants GST MAZ-S-A (S-A) and GST MAZ-S-D (S-D), were used as substrates in assays *in vitro* with CKII as described in Materials and Methods. All reaction mixtures contained 100 μM ATP, which included 10 μCi [γ-^{32}P]ATP, and all were incubated at 37°C for 10 min and then subjected to SDS-PAGE. Proteins were visualized by autoradiography. The position of MAZ is indicated on the left of the photograph. (B) Comparison of the protein levels of GST-MAZ, GST-MAZ-S-A and GST-MAZ-S-D by SDS-PAGE (10% polyacrylamide gel). (C) EMSA of binding activity to double-stranded c-*myc* NHE GC DNA of GST-MAZ fusion proteins that had been phosphorylated or not phosphorylated by CKII (ref. 29). Two hundred ng of GST-MAZ protein or a mutant protein were incubated with [^{32}P]-radiolabeled ds c-*myc* NHE-GC oligodeoxynucleotide (ODN) and EMSA was performed as described in Materials and Methods. Complexes were resolved on a nondenaturing 5% gel and visualized by exposure to X-ray film for 2 h. Lane 1, 500 ng of CKII; lane 2, phosphorylated GST-MAZ protein; lane 3, non-phosphorylated GST-MAZ; lane 4, phosphorylated GST-MAZ-S-A; lane 5, non-phosphorylated GST-MAZ-S-A. CKII, casein kinase II, B, DNA-protein complex, F, free DNA probe.

nuclease-hypersensitive element (NHE) of c-*myc* (Fig. 2B, panels a and c), as well as with a biotin-labeled triple-stranded (ts) c-*myc* NHE-GGC probe (data not shown). Of the two forms of MAZ, it was the rapidly migrating form of MAZ that clearly had strong DNA-binding activity as compared with the slowly migrating form (Fig. 2A, panel b, and Fig. 2B, panel a). At present we do not know the significance of the different DNA-binding activities of these two forms of MAZ.

No binding of DNA by MAZ was detected with the purine-rich ss c-*myc* NHE-G DNA as probe (panel b in Fig. 2B). However, MAZ bound weakly to ds c-*myc* NHE-GC DNA (panel c). In contrast to the wild type MAZ, the corresponding [^{35}S]-methionine-labeled mutant MAZ was not recovered from extracts of cells that harbored pEBVHis C MAZ-S-A plasmid (panels a and c in Fig. 2B). Therefore, the serine residue at position 480 of MAZ appeared to be critical for the binding of MAZ to the NHE of c-*myc* gene *in vivo*.

We next examined the DNA-binding activities of the wild-type and mutant GST-MAZ fusion proteins that had been phosphorylated by CKII, using ss c-*myc* NHE, ds c-*myc* NHE and ts c-*myc* NHE as probes. As shown in Fig. 3, as compared to the wild type, the GST-MAZ-S-A mutant protein exhibited a three-fold reduction in DNA-binding activity with the ss c-*myc* pyrimidine-rich NHE (ss c-*myc* NHE-C) as probe. By contrast, the GST-MAZ-S-D mutant protein gave a result similar to the wild type (panel A). Addition of the corresponding unlabeled oligodeoxynucleotides to reaction mixtures revealed competition for binding by unlabeled ss c-*myc* NHE-C (panel C) but not by unlabeled ss c-*myc* NHE-G (22, data not shown). Moreover, GST-MAZ did not bind to the ss c-*myc* NHE-G probe (panel B). The DNA-binding activity of GST-MAZ-S-A was significantly reduced when ds c-*myc* NHE-GC and ts c-*myc* NHE-GGC were used as probes (panels D and F). Wild-type GST-MAZ and mutant proteins bound to neither the ss c-*myc* NHE-G DNA probe nor the ts c-*myc* NHE-CGC DNA probe (panels B and G). The results using the wild type GST-MAZ protein are mostly identical to our previous reports of the DNA-binding studies of THZif-1, a member of MAZ family (22). Thus, we conclude that MAZ protein also showed the similar DNA-binding function against the unique structures of this c-*myc* NHE to those of THZif-1 (22).

To explore the subcellular localization of the wild-type and mutant MAZ proteins, we transfected cells with plasmids that encoded green fluorescent protein (GFP) fused to wild-type or mutant MAZ. After transfection, we examined the subcellular localization of each GFP-MAZ protein by fluorescence microscopy. As

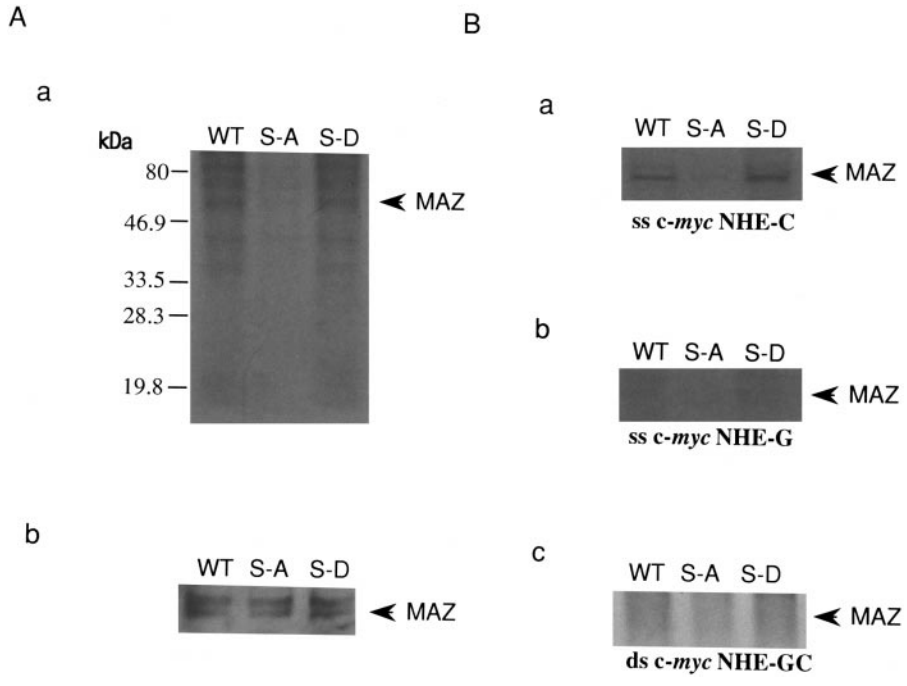

FIG. 2. Phosphorylation of MAZ in HeLa cells transfected with a histidine-tagged MAZ expression vector. (A) The expression vectors for histidine-tagged MAZ (pEBVHis C MAZ) and mutant forms of MAZ derived from it (pEBVHis C MAZ-S-A and pEBVHis C MAZ-S-D) were introduced into HeLa cells and stably transfected clones were isolated as described elsewhere (18). HeLa cells were labeled metabolically by incubation with 500 μCi of [^{32}P]-orthophosphate for 3 h. Cells were harvested and lysed as described in Materials and Methods. Cell lysates were incubated with Ni-NTA agarose beads and bound proteins were released and subjected to SDS-PAGE with subsequent autoradiography (panel a). Western blotting was performed with antibodies against the histidine-tag and revealed that equivalent concentrations of wild-type MAZ and mutant MAZ proteins had been generated in HeLa cells transfected with the respective MAZ expression vectors (panel b). (B) Lysates from [^{35}S]-methionine labeled HeLa cells were incubated for 60 min with biotin-labeled ss c-*myc* NHE-C (panel a), ss c-*myc* NHE-G (panel b) and ds c-*myc* NHE-GC (panel c) bound to Dynabeads M-280 streptavidin, as described in Materials and Methods. The amounts of MAZ or mutant MAZ bound to beads were analyzed by SDS-PAGE and autoradiography. The position of MAZ is indicated on the right of each photograph.

shown in Fig. 4A, the wild-type and mutated MAZ proteins fused to GFP remained in nuclei. Thus, the mutation at position 480 in the MAZ protein did not affect the localization of the GFP-MAZ protein within the cells. In addition, the levels of expression of wild-type and mutated GFP-MAZ were found to be very similar when evaluated by Western blotting with antibodies agaisnt GFP (data not shown). The transfection efficiency of each GFP-MAZ expression vector was similar and approximately 20 to 30%. Thus, we speculate the wild-type and mutants of MAZ protein were localized within the nuclei although we can't rule out a possibility that these observations are due to the indirect effect of GFP-fusion proteins. We observed the similar localization of *myc*-tagged wild type and mutant MAZ proteins (data not shown). The transfection efficiencies of each construct were almost the similar, too.

We next examined the effect of a mutation at position 480 of MAZ on the activation of transcription of the c-*myc* gene in the presence and in the absence of plasmids that encoded CKII (α, α' and β). We introduced reporter constructs driven by the native P1 and P2 promoters (pCG362-5/HP) of the c-*myc* gene into HeLa cells and assayed the resultant luciferase activities (Fig. 4B). The luciferase activity under the control of the P1 and P2 promoters was significant after cotransfection with a plasmid that encoded wild-type MAZ. However, the luciferase activity due to pCG362-5/HP was reduced by about 50% after cotransfection with pEBVHis C MAZ-S-A, as compared to pEBVHis C MAZ. Transfection of pEBVHis C MAZ-S-D resulted in moderate induction of luciferase activity. Cotransfection with the plasmids that encoded CKII resulted in approximately 1.6-fold enhancement of the luciferase activity of the c-*myc* reporter gene in the case of wild type MAZ construct (Fig. 4B). However, the effects of CKII were not significant in the case of both mutated MAZ constructs (Fig. 4B). Thus, the putative site of phosphorylation by CKII of MAZ at position 480 appears to play a key role in regulation of the expression of the c-*myc* gene.

Our results indicate that the phosphorylation status of a serine residue in the carboxy-terminal region of MAZ is important for regulation of the expression of the c-*myc* gene. Elimination of any possible phosphor-

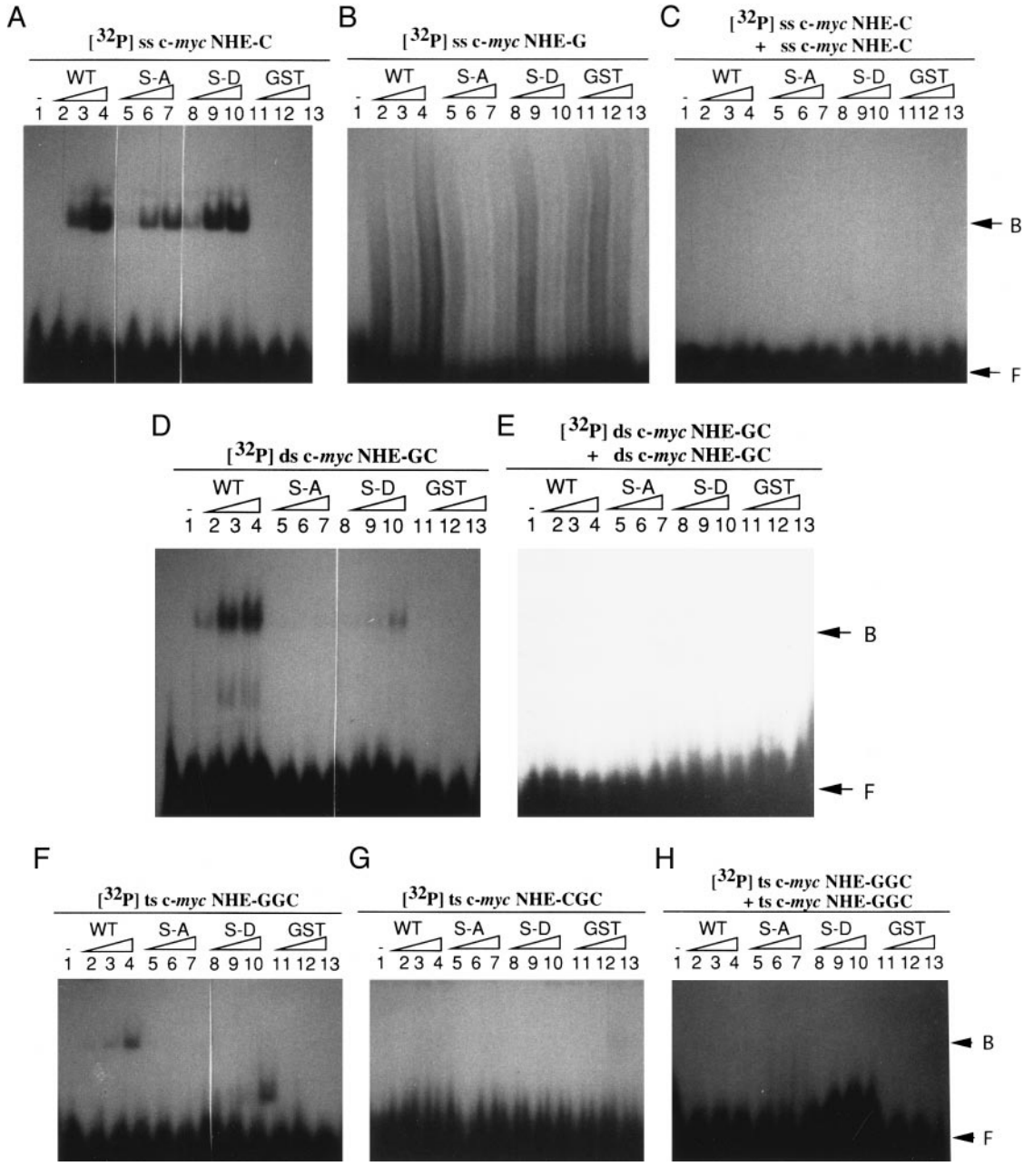

FIG. 3. Electrophoretic mobility shift assays (EMSAs) of the binding of GST-MAZ fusion proteins to single-stranded, double-stranded and triple-stranded c-*myc* NHE DNA. (A), (B) and (C): Indicated amounts of GST-MAZ (lanes 2–4), of mutant protein (lanes 5–10) and of GST control protein (lanes 11–13) were incubated with [^{32}P]-radiolabeled ss c-*myc* NHE-C oligodeoxynucleotide (ODN) in the absence of (panel A) or in the presence of (panel C) 500 ng unlabeled ss c-*myc* NHE-C ODN or with [^{32}P] ss c-*myc* NHE-G ODN (panel B) at 4°C for 60 min. Resultant complexes were resolved on nondenaturing 5% gels and visualized by exposure to X-ray film for 12 h. The concentrations of MAZ, of mutant proteins and of GST were as follows: lane 1, no added protein; lanes 2, 5, 8 and 11, 60 ng; lanes 3, 6, 9 and 12, 180 ng; lanes 4, 7, 10 and 13, 240 ng, respectively. (D) and (E): Binding of GST-MAZ (lanes 2–4), the mutant proteins (lanes 5–10) and GST (lane 11–13) to [^{32}P]-radiolabeled ds c-*myc* NHE-GC ODN in the absence (panel D) or presence (panel E) of 1 μg of unlabeled ds c-*myc* NHE-GC ODN at 4°C for 60 min. Resultant complexes were resolved in nondenaturing 5% gels, with subsequent exposure to X-ray film for 12 h. The concentrations of protein were as follows: lane 1, no added protein; lanes 2, 5, 8 and 11, 260 ng; lanes 3, 6, 9 and 12, 700 ng; lanes 4, 7, 10 and 13, 1,200 ng, respectively. (F), (G) and (H): Binding of GST-MAZ (lanes 2–4), the mutant proteins (lanes 5–10) and GST (lanes 11–13) to [^{32}P]-radiolabeled ts c-*myc* NHE-GGC ODN in the absence (panel F) or presence (panel H) of 2 μg of unlabeled ts c-*myc* NHE-GGC ODN as competitor and to [^{32}P]-radiolabeled ts c-*myc* NHE-CGC (panel G) at 4°C for 60 min. Resultant DNA-protein complexes were resolved in nondenaturing 5% gel, with subsequent exposure to X-ray film for 12 h. (F) and (H): Lane 1, no added protein; lanes 2, 5, 8 and 11, 60 ng; lanes 3, 6, 9 and 12, 180 ng; lanes 4, 7, 10 and 13, 300 ng. (G) Lane 1, no added protein; lanes 2, 5, 8 and 11, 240 ng; lanes 3, 6, 9 and 12, 700 ng; lanes 4, 7, 11, and 13, 1200 ng. B, DNA-protein complex; F, free DNA probe. GST-MAZ and the mutant proteins were phosphorylated by CKII *in vitro* (ref. 29) and used for EMSAs.

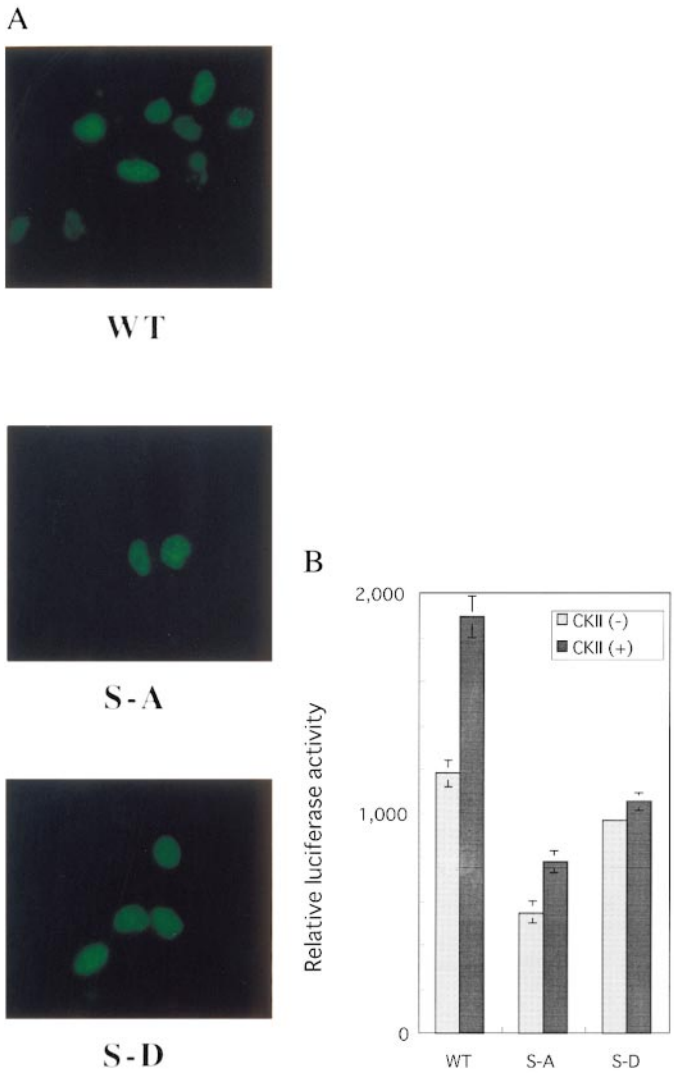

FIG. 4. Localization in cells of MAZ and the mutant MAZ proteins and transcriptional activation of the c-*myc* promoter. (A) Localization of wild-type GFP-MAZ and GFP fusion proteins with mutant forms of MAZ. HeLa cells were transfected with GFP fusion constructs as described Materials and Methods. After 48 h, cells were examined by fluorescence microscopy (×400) to localize signals due to GFP. The majority of the GFP fusion proteins were localized in nuclei. (B) Transcriptional activation of a c-*myc* promoter/luciferase reporter gene by MAZ and mutated forms of MAZ in the presence and in the absence of a cotransfected CKII expression vectors (α, α' and β). HeLa cells were transfected with 1.0 μg of pEBVHis C MAZ, pEBVHis C MAZ-S-A or pEBVHis C MAZ-S-D together with 1.0 μg of pCG362–5/HP (P1 and P2 promoter) reporter or pblueLuc in the presence and in the absence of plasmids that encoded casein kinase II (subunits α, α' and β; 1.0 μg each). Cells were lysed and luciferase activity was analyzed as described in Materials and Methods. The average of results from three independent experiments and the standard deviation for each value are indicated.

ylation at this site by mutation of the serine residue at position 480 to alanine had a significant effect on the CKII-dependent activation of transcription by MAZ. Replacement of this serine residue by aspartic acid resulted in an increase in the DNA-binding of MAZ (Figs. 1A, 2 and 3), and these activities resembled those observed when this site had been phosphorylated, an indication that phosphorylation might augment the DNA-binding activity by introducing a constitutive negative charge, as suggested previously (35). However, we did not detect the induced transcription of c-*myc* promoter-reporter gene by MAZ-S-D in the presence of CKII as compared to that of wild type MAZ (Fig. 4B). This indicates that the CKII-dependent phosphorylation of serine residue at position 480 of MAZ protein might be critical for the transcriptional activation induced by MAZ. Phosphorylation of this site enhanced the binding to the NHE of c-*myc* (Fig. 2). The results of the site-directed mutagenesis of this putative phosphorylation site strongly suggest that the conformation and/or phosphorylation status of its central core might be critical for the binding of MAZ to DNA. This site, which corresponds to a CKII recognition site, is conserved in all known members of the MAZ family, in organisms from mouse to human. This observation is consistent with the hypothesis that this site is important for the functions of MAZ.

CKII phosphorylates a variety of transcription factors, such as activators, repressors, basal transcription factors, cofactors and viral transactivators, which regulate the transcription of the target genes *in vitro* and *in vivo* (28, 29). The transcription factor Sp 1 is one of the factors that are phosphorylated by CKII (36). It has been reported that the ME1a1 element and the primidine-rich DNA of NHE in the 5′-end promoter region of c-*myc* gene are recognized by MAZ and Sp 1 (2, 22). Both factors contain similar C2H2-type zinc finger motifs as DNA-binding domains. Phosphorylation of the carboxyl terminus of Sp 1 by CKII resulted in a decrease of the DNA-binding activity of Sp 1 (36). By contrast, we demonstrated here that phosphorylation of a specific carboxy terminal residue of MAZ by CKII increased the DNA-binding activity of MAZ and selectively regulated MAZ-dependent transactivation. Thus, within the same *cis*-element of the c-*myc* promoter, both DNA-binding factors are targets of CKII-mediated phosphorylation, having with opposite effects in terms of the DNA-binding activity and the modulation of the transcription of c-*myc* by the respective factors. One possible model of the control of transcription of c-*myc* involves the recruitment of CKII to the promoter DNA and the subsequent phosphorylation of MAZ, Sp 1 and/or other factors that are bound to nearby elements only at the time when and at the site where each is operative. Further investigations of CKII-mediated interactions between transcription factors and the basal transcriptional apparatus should lead to a better understanding of the molecular mechanisms of the CKII-mediated regulation of transcription.

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