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Molecular cloning of MSSP-2, a c-*myc* gene single-strand binding protein: characterization of binding specificity and DNA replication activity

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ABSTRACT

We have previously reported the human cDNA encoding MSSP-1, a sequence-specific double- and single-stranded DNA binding protein [Negishi, Nishita, Saëgusa, Kakizaki, Galli, Kihara, Tamai, Miyajima, Iguchi-Ariga and Ariga (1994) *Oncogene*, 9, 1133–1143]. MSSP-1 binds to a DNA replication origin/transcriptional enhancer of the human c-*myc* gene and has turned out to be identical with Scr2, a human protein which complements the defect of cdc2 kinase in *S.pombe* [Kataoka and Nojima (1994) *Nucleic Acid Res.*, 22, 2687–2693]. We have cloned the cDNA for MSSP-2, another member of the MSSP family of proteins. The MSSP-2 cDNA shares highly homologous sequences with MSSP-1 cDNA, except for the insertion of 48 bp coding 16 amino acids near the C-terminus. Like MSSP-1, MSSP-2 has RNP-1 consensus sequences. The results of the experiments using bacterially expressed MSSP-2, and its deletion mutants, as histidine fusion proteins suggested that the binding specificity of MSSP-2 to double- and single-stranded DNA is the same as that of MSSP-1, and that the RNP consensus sequences are required for the DNA binding of the protein. MSSP-2 stimulated the DNA replication of an SV40-derived plasmid containing the binding sequence for MSSP-1 or -2. MSSP-2 is hence suggested to play an important role in regulation of DNA replication.

INTRODUCTION

Expression of the proto-oncogene c-*myc* is delicately regulated, and a variety of *cis* and *trans* elements are suggested to be involved in the regulation (for reviews see 1, 2). We have identified a putative DNA replication origin/transcriptional enhancer upstream from the human c-*myc* gene (3, 4). The sequence of 21 bp essential for replication and transcription therein, containing an AT-rich element TCTCTTA, stimulated SV40 DNA replication (5). Moreover, we isolated viable mutants of SV40, in which the original AT-rich sequence adjacent to the SV40 origin core was substituted by TCTCTTA or similar sequences (6). The 21 bp sequence was bound by a c-*myc* protein complex not only in the double-stranded but also in the single-stranded form (7). Several proteins were identified as directly binding to either strand of the 21 bp sequence and were named MSSP (*m*yc single-strand binding proteins). We then cloned a cDNA encoding one of the MSSP family proteins, MSSP-1, and found that MSSP-1 binds to both double- and single-stranded DNA in a sequence-specific manner (8). MSSP-1 stimulated the replication of a derivative of SV40 DNA which contained the MSSP-1 binding sequence. Another group has independently cloned human cDNAs identical or similar to that for MSSP-1. The encoded proteins, Scr2 and Scr3, complemented the defect of cdc2 kinase in *S.pombe* (9). From the results of genetic analyses, Scr2 and Scr3 are suggested to be involved downstream of cdc2 and cdc13, respectively, in the regulation of cell cycle movement, especially from G_1 to S phase, in *S.pombe* (for reviews see 10, 11). MSSP-1, Scr2, and Scr3 commonly contain a novel RNA binding motif, RNP-1, which appears to be important in the functions of other proteins containing the motif (12).

In this report, we have cloned the human cDNA encoding another MSSP family protein, MSSP-2. The sequence of the MSSP-2 cDNA was almost identical to that of MSSP-1 cDNA, except for an extra 48 nucleotides near the 3′ end. In DNA/protein binding reactions using MSSP-2 expressed in *E.coli* as a histidine fusion protein, as well as in transfection experiments in human HeLa cells, MSSP-2 is indistinguishable from MSSP-1 in terms of the DNA binding specificity and the promoting activity on DNA replication. The results using various deletion mutants of MSSP-2 suggest that the RNP motifs are required for DNA binding.

MATERIALS AND METHODS

Plasmids and antibody

The *Eco*RI fragment containing the entire cDNA for MSSP-1 or MSSP-2 was inserted in the *Eco*RI site of pMT-1, a plasmid carrying the metallothionein promoter. The plasmids constructed

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were named pMT-MSSP-1 or pMT-MSSP-2, respectively. An anti-MSSP-1 polyclonal antibody was prepared by immunizing New Zealand rabbits with the purified GST−MSSP-1 fusion protein expressed in *E.coli*. The IgG fraction was purified from the anti-MSSP-1 rabbit serum using protein A−agarose. The anti-MSSP-1 antibody recognized both MSSP-1 and MSSP-2.

cDNA cloning of MSSP-2

A human placenta cDNA library in λgt11 was purchased from Clontech Inc. The whole MSSP-1 cDNA was labeled with [^{32}P]dCTP by random primer and Klenow fragment and used as a probe for cDNA screening. Approximately 1×10^5 plaques were screened for DNA−DNA hybridization with the MSSP-1 probe under moderate stringency (in 30% formamide at 37°C), and the hybridized filters were washed twice at 37°C in a buffer containing 3× SSC and 0.1% SDS, and then twice at 37°C in a buffer of 0.1× SSC and 0.1% SDS. After the third round of the screening, two positive plaques were obtained. The cDNA from neither plaques contained the N-terminal portion of the gene. The subsequent screening was carried out by use of the N-terminal-lacking cDNA as a probe, yielding a clone possessing the intact coding sequence of a protein. The cDNA insert was amplified by polymerase chain reaction (PCR) with the forward and reverse primers for λgt11 vector. The amplified DNA was digested with *Eco*RI and recloned into the *Eco*RI site of pGEM4Z. The resultant clone was designated pGEM-MSSP-2. All the sequences were determined by the chain termination method using various deletion mutants from the N-terminus prepared by the standard method by use of exonuclease III and mung bean nuclease (13).

Construction of plasmids and expression of MSSP-2−(His)$_6$ fusion protein in *E.coli*

pET-MSSP-2, an expression plasmid of a fusion protein of MSSP-2 and 6 histidines under the control of the T7 promoter, was constructed using PCR. Sequences of the two primers used were as follows: MSSP-ATG primer, 5′-ACCCCATGGCCCC-TCCCAGT-3′; MSSP-His primer, 5′-GTAGTCCATGGCTA-GTGGTGGTGGTGGTGGTGGTTATTAGGTTGAAA-3′. The reaction in 50 μl solution contained 10 ng of pGEM-MSSP-2, 10 pmol each of MSSP-ATG primer and MSSP-His primer, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus). The reactions were carried out first for 5 min at 94°C, and then for 30 cycles of 1 min at 94°C followed by 2 min at 55°C, and finally for 3 min at 72°C. The PCR product was directly inserted in pCRII (Invitrogen), and the insert was recovered from the plasmid by digestion with *Eco*RI and partially digested with *Nco*I. pET-MSSP-2 was prepared by inserting the *Nco*I fragment containing the MSSP-2 cDNA in the *Nco*I site of pET-11d.

Expression and purification of a MSSP-2−(His)$_6$ fusion protein in *E.coli* was carried out following the protocol of QIAGEN Inc. Briefly, a single colony of *E.coli* BL21 (DE3) transformed with pET-MSSP-2 was cultured overnight in L broth containing 100 μg/ml of ampicillin, and then for 3−5 h after addition of IPTG to 1 mM. The bacteria were collected and lysed in buffer B containing 8 M urea, 0.1 M Na phosphate, and 10 mM Tris−HCl, pH 8.0. After centrifugation, the supernatant containing soluble proteins was applied to Ni−NTA resin (QIAGEN Inc.) and mixed gently for 30 min at room temperature. After centrifugation, the resin was washed three times with a buffer containing 8 M urea, 0.1 M Na phosphate, and 10 mM Tris−HCl (pH 6.3), and the proteins bound to the resin were eluted with a buffer containing 250 mM imidazole.

Construction of deletion mutants of MSSP-2

Deletion mutants of MSSP-2 (see Fig. 3) were constructed by PCR. The sequences of the oligonucleotides used for PCR were as follows: RNP-A upper, 5′-GAACAAATGCGACAGCCC-TG-3′; RNP-A lower, 5′-CAGGGCTGTCGCATTTGTTC-3′; RNP-B upper, 5′-TGGTACAAGTGAATCAACAG-3′; RNP-B lower, 5′-CTGTTGATTCACTTGTACCAC-3′; C-terminus upper, 5′-GCAGATGAGTACATACATGC-3′; C-terminus lower, 5′-GCATGTATGTACTCATCTGC-3′; pET primer (nucleotides 285−303 in pET-11d), 5′-CTCAGCTTCCTTT-CGG-3′. The conditions for PCR were the same as those for pET-MSSP-2 preparation. First PCR contained 10 ng of pET-MSSP-2, 10 pmol each of the T7 promoter and the lower primers (or the pET and upper primers), and 2.5 U of Taq DNA polymerase. One hundred nanograms each of the amplified fragments, 10 pmol each of the T7 promoter and the pET primers were used for second PCR. The amplified fragment was digested with *Xba*I and *Bam*HI and inserted between the *Xba*I and the *Bam*HI sites of pET-11d.

Band shift assay

Oligonucleotides were chemically synthesized. For single-stranded DNA probes, oligonucleotides were labelled with [γ-^{32}P]ATP and polynucleotide kinase. For double-stranded DNA probes, complementary plus and minus strand oligonucleotides were heated at 100°C for 5 min prior to annealing at room temperature for 15 min. Annealed DNAs were labelled with [α-^{32}P]dCTP and Klenow fragment. The sequences of the oligonucleotides used are shown in Fig. 2. Binding reactions were carried out by incubating 20−40 fmol of labelled probe ($3-5\times10^4$ c.p.m.) with 0.1−0.4 mg of MSSP-2−(His)$_6$ fusion protein for 15 min at room temperature in a buffer containing 1 mM EDTA, 4% Ficoll 400, 1 mM DTT, 4 mg/ml of bovine serum albumin and 50 mM KCl. The reactions were electrophoresed through a 4% polyacrylamide gel (acrylamide:bisacrylamide ratio, 29:1) in 0.25× TBE (1× TBE is 90 mM Tris, 90 mM borate, and 2.5 mM EDTA) buffer. The gel was dried and autoradiographed.

Establishment of cell lines expressing MSSP-1 and MSSP-2

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. The cells when ~50% confluent in a 10 cm dish were co-transfected with 1 μg of pMT-MSSP-1 or pMT-MSSP-2 and 50 ng of pLTR-Hgr, an expression vector of the hygromycin B resistance gene linked to the Rouse sarcoma virus long terminal repeat, by the calcium phosphate precipitation method (14), and cultured in medium containing 100 μg/ml of hygromycin B. About 2 weeks after transfection, the colonies resistant to hygromycin B were isolated using penicillin cups. The resistant cell clones were cultured in the presence of the drug until they became more than 10^4. The clones possessing the intact form of the cDNA of MSSP-1 or MSSP-2 linked to the metallothionein promoter were identified as follows: total cellular DNAs were extracted from the resistant clones (13), digested with various restriction enzymes, transferred to a nitrocellulose filter by the method of Southern (15), and hybridized with labelled MSSP-1 or MSSP-2 cDNA, or the metallothionein promoter region. To examine the expression of

MSSP-1 or -2 in the established cell lines, the cells when ~50% confluent in a 6 cm dish were cultured in the presence or absence of 100 or 200 mM $ZnSO_4$ for 24 h. Whole cell extracts were prepared by incubating the cells with 0.5% NP40 for 20 min at 0°C, separating in a 10 or 12.5% polyacrylamide gel containing SDS in a Tris−glycine buffer, and transferring to a nitrocellulose filter. The filter was reacted with the anti-MSSP polyclonal antibody and the antibody-coupled proteins were visualized with an ECL detection system (Amersham Co. Ltd). Two cell lines, 1H9 and 2H1, expressing MSSP-1 and MSSP-2, respectively, were used for DNA replication assays.

DNA replication assay

pSVmycØ, described previously (6), was used as a reporter plasmid. Ten nanograms of pSVmycØ and 200 ng of pUCcibiH prepared from *dam*⁻ *E.coli* cells (16) were transfected into 1H9 or 2H1 cells 60% confluent in a 6 cm dish together with various amounts of pEF-321T, an SV40 T antigen expression vector, by the calcium phosphate precipitation method (14). Forty eight hours after transfection, low molecular weight DNA was extracted from the cells by the Hirt method (17), digested with *Hin*dIII and *Dpn*I, electrophoresed and transferred to a nitrocellulose filter (15), and hybridized with ^{32}P-labelled pUC19 under highly stringent conditions.

RESULTS AND DISCUSSION

cDNA cloning of MSSP-2

A human cDNA library in λgt11 was screened by the labelled probe of whole MSSP-1 cDNA under moderately stringent conditions. Approximately 10^5 plaques were screened and two positive clones were obtained. Because neither clone contained a putative initiation codon, a second set of screening was carried out using labelled short cDNA probe. Finally a positive clone including an initiation codon was obtained and the entire nucleotide sequence was determined (EMBL accession no. X77494). The cDNA obtained contained 1698 nucleotides, encoding 389 amino acids. MSSP-2 shared extensive homologous sequences with MSSP-1, except for an extra 16 amino acids (numbers 241−259 in MSSP-2) and two nucleotides substitutions (Fig. 1). Since there exists a stop codon in the region upstream from the initiation ATG, the clone was considered to contain the whole coding sequence. Another group have independently cloned two human cDNAs identical or quite similar to MSSP-1. The clones, scr2 and scr3, complemented the defect of cdc2 in *S.pombe* (5). As shown by the alignment in Fig. 1B, the four clones possess two copies of the RNA binding motif, RNP-1. The amino acid sequences around the motifs are nearly identical, while the sequences in the N- and C-terminal regions are less conserved.

Figure 1. Comparison of amino acid sequences among the MSSP/Scr family. The amino acid sequences of MSSP-1, MSSP-2, Scr2 and Scr3 are represented in the standard one letter abbreviations. The identical amino acids among four proteins are indicated with dots. The positions of the RNP1 consensus motifs are marked.

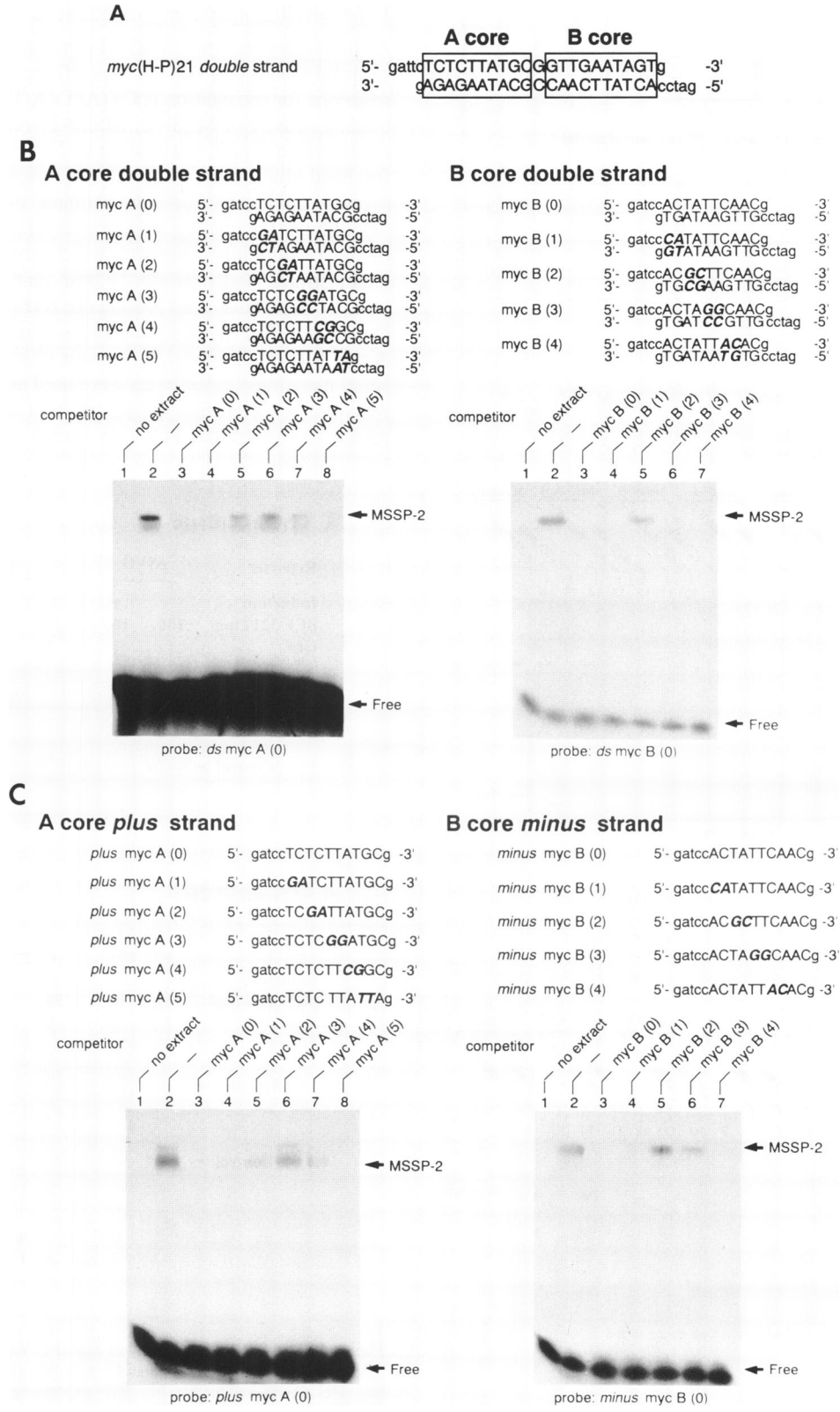

Figure 2. DNA binding specificity of MSSP-2. **(A)** The 21 base pairs, present in a putative DNA replication origin/transcriptional enhancer upstream from the human c-*myc* gene, was divided into two parts, core A and core B. **(B)** Band shift assays with double-stranded DNA probes using purified MSSP-2−$(His)_6$ fusion protein. Oligonucleotides corresponding to core A and core B in a double-stranded form were labelled with [^{32}P]dCTP and used for probes in band shift assays as described in Materials and Methods. **(C)** Band shift assays with single-stranded DNA probes using purified MSSP-2−$(His)_6$ fusion protein. Oligonucleotides corresponding to core A plus or core B minus strand were labelled with [^{32}P]ATP and used as probes in band shift assays as described in Materials and Methods. Nucleotide sequences of various oligonucleotides used as competitors are shown in the upper panels of (B) and (C). Non-labelled competitor oligonucleotides were added to reaction mixtures at a 100 molar excess of probe. Arrows 'MSSP-2' and 'Free' indicate the positions of the MSSP-2−DNA complex and free probe, respectively.

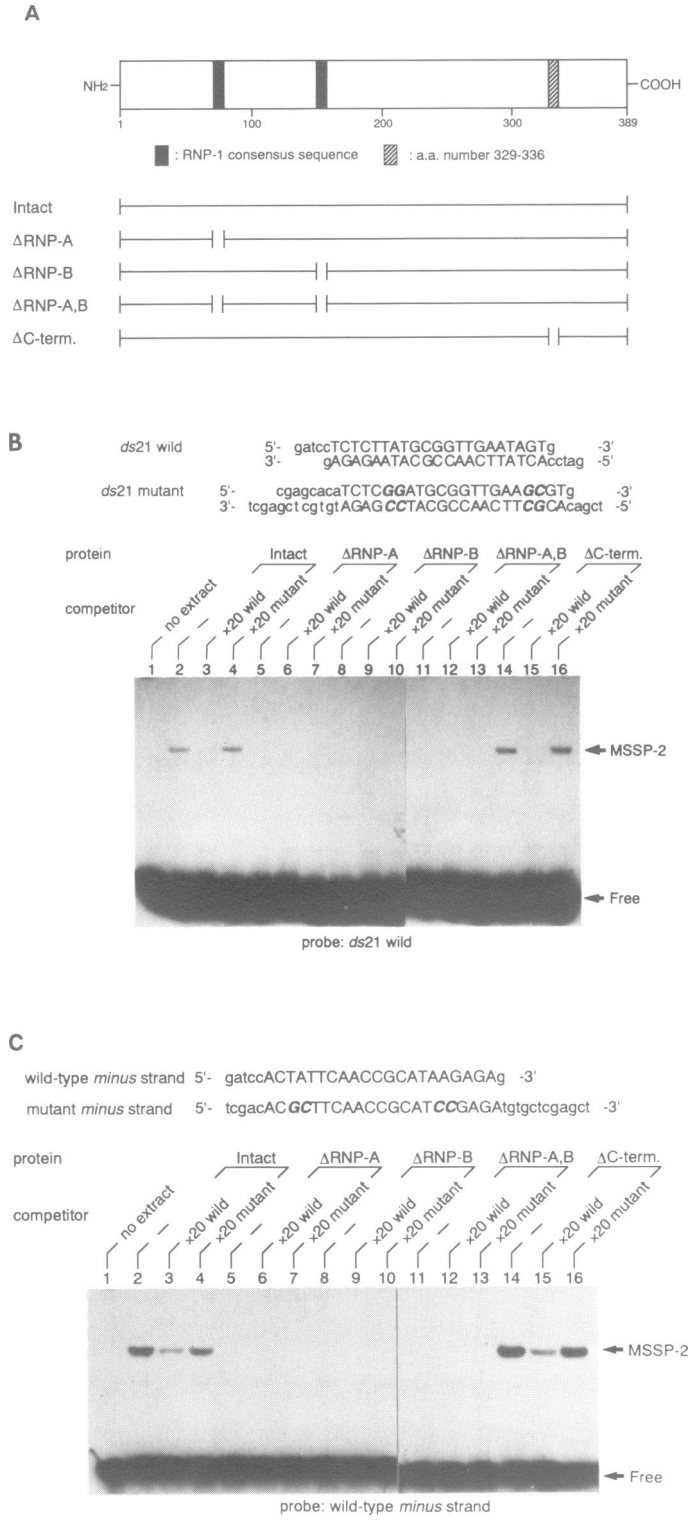

Figure 3. Determination of the domains in MSSP-2 required for DNA binding. (**A**) Schematic drawing of the motifs in MSSP-2, and the deletion mutants of MSSP-2. (**B** and **C**) Band shift assays using deletion mutants of MSSP-2−$(His)_6$ fusion protein purified from *E.coli* cells. Competition experiments were carried out as in Fig. 2. Nucleotide sequences of wild-type and mutant oligonucleotides used for probes and competitors are shown in the upper panels. Non-labelled competitor oligonucleotides were added to reaction mixtures at a 20 times molar excess of probe. Arrows 'MSSP-2' and 'Free' indicate the positions of the MSSP-2−DNA complex and free probe, respectively.

DNA binding specificity of MSSP-2

As previously reported, MSSP-1 binds to both double- and single-stranded DNAs in a sequence-specific manner, and the consensus sequence for recognition is CT(A/T)(A/T)T (8). To examine the binding activity of MSSP-2, MSSP-2−$(His)_6$ fusion protein was expressed in *E.coli*, purified on an Ni affinity column, and used for band shift assays (Fig. 2). The sequence of 21 base pairs present upstream from the human c-*myc* gene, originally identified as the binding sequence for MSSP, contains two copies of the recognition consensus which are invertedly present on either strand, and thus the 21 base pair sequence was divided into two segments, core A and core B (Fig. 2A). The bacterially expressed MSSP-2 bound to double-stranded probes of both cores A and B (Fig. 2B, lane 2). Various oligonucleotides containing serial mutations of two bases were used as competitors in binding reactions to determine the recognition target of MSSP-2. The results suggested that TCTT in core A or TATT in core B was

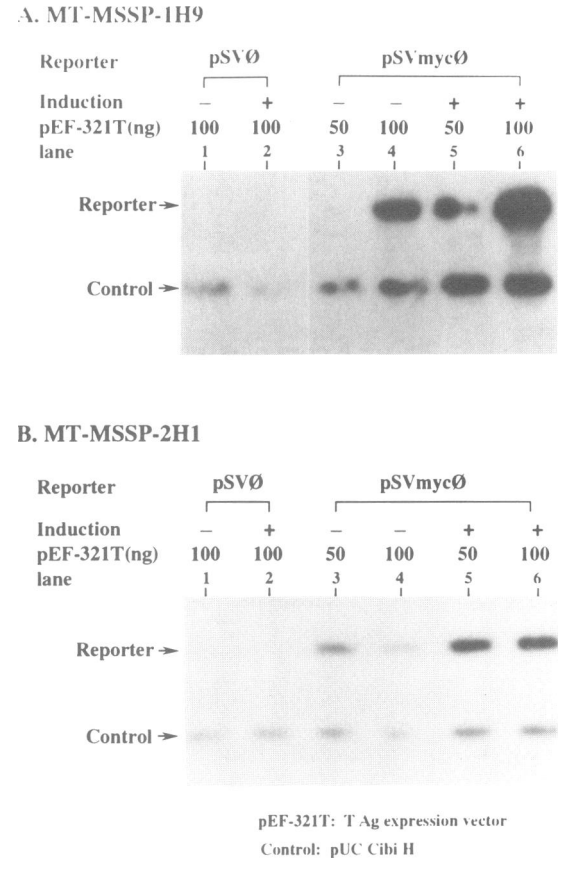

Figure 4. DNA replication activity of MSSP-2. Various cell lines which express MSSP-1 or MSSP-2 under the control of metallothionein promoter were established from HeLa cells, and the two lines 1H9 expressing MSSP-1 and 2H1 expressing MSSP-2 were used in this experiment. pSVmycØ, pUCcibiH (a control plasmid of small size, prepared in *dam*$^-$ strain of *E.coli*) (16), and pEF-321T (an expression vector for the SV40 T antigen) were co-transfected into 1H9 (**A**) or 2H1 (**B**). Forty eight hours after transfection, low molecular weight DNAs were extracted, digested with *Hin*dIII and *Dpn*I, and subjected to Southern blotting as described in Materials and Methods. Prior to the DNA extraction, the cells were cultured for 24 h in the presence (+) or absence (−) of 100 mM $ZnSO_4$ to induce MSSP-1 or -2. Arrows 'Reporter' and 'Control' indicate the positions of pSVmycØ and control pUCcibiH, respectively.

important for MSSP-2 binding. Similar results were obtained in the band shift assays with probes and competitors of single-stranded DNA (Fig. 2C). The consensus sequence for MSSP-2 binding was suggested to be T(C/A)TT in both single- and double-stranded forms.

DNA binding domains in MSSP-2

MSSP-1 and MSSP-2 commonly possess two copies of the RNA recognition motif, RNP-1, which may be responsible for the specific DNA binding activity of MSSP. To assess this possibility, deletion mutants of MSSP-2 lacking either or both RNP-1 motifs were constructed by use of PCR (termed ΔRNP-A, ΔRNP-B, and ΔRNP-A,B, respectively) (Fig. 3A). Another mutant carrying a deletion of similar size near the C-terminus (ΔC-term) was also constructed. The mutant proteins were expressed in *E.coli* as histidine fusion proteins and purified as wild-type MSSP-2−$(His)_6$, and their binding activities to DNA were examined (Fig. 3B and 3C). The results suggested that both of the RNP-1 motifs are required for binding of MSSP-2 either to single- or double-stranded DNA, while the segment near the C-terminus is not.

DNA replication activity of MSSP-2

We have reported that MSSP-1 stimulated DNA replication of a plasmid containing a variant of the SV40 origin in HeLa cells. In pSVmycØ, the AT-rich sequence in the intact SV40 origin was substituted by TCTCTTA, the binding sequence for MSSP present in the 21 base pairs upstream from the human c-*myc* gene (6, 8). Both the MSSP-1 and MSSP-2 cDNAs were linked to the metallothionein promoter, and the plasmids were co-transfected into HeLa cells with pLTR-hgr, a hygromycin B resistance gene expression vector. The transfected cells were cultured in the presence of hygromycin B, and several cell lines resistant to the drug were established. Southern blotting analyses of the cell lines revealed that they contained the intact form of the metallothionein promoter linked to the MSSP-1 or MSSP-2 cDNA. The expression of MSSP-1 or MSSP-2 at a high level in the cells was detected by Western blotting after the cells were cultured in the presence of 100 mM Zn^{2+} for 1 h (data not shown). Among the cell lines established, two lines, 1H9 and 2H1, which expressed MSSP-1 or MSSP-2, respectively, under the control of the metallothionein promoter, were used for further experiments. pSVmycØ was co-transfected into 1H9 or 2H1 cells together with pEF-321T expressing the SV40 T antigen, to sustain SV40 DNA replication, and pUCcibiH, a derivative of pUC19 prepared from *dam*$^-$ *E.coli* (16), as an internal control plasmid. Two days after transfection, low molecular weight DNAs were extracted, digested with *Hin*dIII and *Dpn*I, and analyzed by Southern blotting with a labelled pUC19 probe (Fig. 4). Both cells stimulated pSVmycØ replication by 5- to 10-fold in the presence of Zn^{2+}, while pSVØ lacking the TCTCTTA sequence did not replicate with or without Zn^{2+} in the medium. The results suggested that MSSP-2, like MSSP-1, stimulates DNA replication dependent on the TCTCTTA sequence.

In this manuscript, we have cloned the cDNA encoding MSSP-2, a second member of the MSSP family, and characterized the protein for DNA binding specificity and DNA replication activity. Despite the insertion of an extra 16 amino acids near the C-terminus, MSSP-2 recognized the same DNA sequence as MSSP-1 and similarly promoted DNA replication from the sequence. Furthermore, both proteins enhanced the cell transforming activity of c-*myc*/*ras* (data not shown). The results of genomic DNA cloning have revealed that MSSP-2 is derived from a gene different from that of MSSP-1: Two independent clones corresponding to MSSP-1 and 2, respectively, have been obtained; the gene encoding MSSP-1 contained no intron, while the clone for MSSP-2 consisted of more than 8 exons (Haigermoser, Iguchi-Ariga and Ariga, in preparation). MSSP-2 is therefore suggested not to be an artifact during cloning procedures, nor the product of alternative splicing of the MSSP-1 gene, although the structure of the protein is quite similar to that of MSSP-1. MSSP family proteins recognize the sequence overlapping, or close to, an origin of DNA replication/transcriptional regulatory element upstream from the c-*myc* gene in both single- and double-stranded forms. As shown in Fig. 1, the MSSP family includes Scr2 and Scr3 which complement the defect of cdc2 kinase in *S.pombe*. MSSP family proteins are thus suggested to be involved in the control of DNA replication and/or transcription, which may be coupled with the regulation of cell cycle progression.

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