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Induction of Apoptosis in HeLa Cells by MSSP, *c-myc* Binding Proteins

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MSSP (*c-myc* gene single strand binding proteins) were identified as protein factors binding to a putative replication origin/transcriptional enhancer sequence present upstream from the human *c-myc* gene, and two cDNAs encoding highly homologous proteins, MSSP-1 and MSSP-2, have been cloned. Scr2, independently cloned as a factor which complements the *cdc2* defective mutant of *Schizosaccharomyces pombe*, has turned out to be identical to MSSP-1. MSSP-1/Scr2 and MSSP-2 similarly stimulated the initiation of SV40 DNA replication, and thus were suggested to be involved in regulation of cell cycle movement, especially from the G₁ to S phase. Here, we examined the functions of MSSP in apoptosis. MSSP expression plasmids were transfected to human HeLa cells together with a β -galactosidase expression vector. After incubation in the presence of 2% calf serum, cells were stained with X-gal and morphologically apoptotic cells among the β -galactosidase-positive cells were counted. Both MSSP-1 and 2 induced apoptosis in a dose-dependent manner as in the control experiments with *c-myc* or adenovirus E1A. DNA fragmentation, a hallmark of apoptosis, was also observed in cells transfected with MSSP expression plasmids. The results of experiments using various deletion mutants of MSSP indicated that the region containing one of the two RNP consensus motifs, RNP1-B, is required for induction of apoptosis as well as specific DNA binding activity.

Key words MSSP; apoptosis; c-MYC

Apoptosis is one of the self-defense systems of cells and is thought to be regulated by series of genetic events. Various genes and their products have been found to induce, repress or associate with apoptotic events (see recent reviews, 1–14, references therein). Nuclear oncogene products including *c-MYC*,^{15–17)} *s-MYC*,¹⁸⁾ *p53*,^{19–21)} *JUN*,^{22–24)} *FOS*,²⁵⁾ *MYB*,²⁶⁾ or adenovirus E1A,^{27–31)} which progress or arrest the cell cycle, have been suggested to be correlated with apoptosis. Other proteins reported to be associated with the apoptotic process are E2F,^{32,33)} *Rb*,³⁴⁾ *cdc2* and *cdk2*,³⁵⁾ *PITSLRE* kinase,³⁶⁾ and cyclin A,³⁷⁾ which are involved in the regulation of cell-cycle movement from the G₁ to S phase. The mechanisms of involvement of the proteins in apoptotic processes, however, are still unknown. MSSP, *c-myc* gene single strand binding proteins, were identified as binding to a putative DNA replication origin and transcriptional enhancer sequence in the human *c-myc* gene forming a protein complex with *c-MYC*.³⁸⁾ Two cDNA clones encoding MSSP-1 and MSSP-2 have been obtained, and both function as DNA replication proteins.^{38,39)} Another group independently cloned human cDNAs coding for Scr2 and Scr3 which suppress the *cdc2* and *cdc13* mutations in *Schizosaccharomyces pombe*, and are thought to work downstream of *cdc2* and *cdc13*.⁴⁰⁾ Scr2 has turned out to be identical to MSSP-1. MSSP-1/Scr2 and MSSP-2 are, hence, believed to play a role in cell-cycle regulation as well as in DNA replication. Here, we examined the function of MSSP toward apoptosis, and found that MSSP-1 or 2 independently induced apoptosis in human HeLa cells at low serum concentrations, and that the RNP-1A domain of the proteins is necessary for apoptosis induction.

MATERIALS AND METHODS

Cell Culture, Transfection, Apoptosis Induction HeLa cells were cultured in Dulbecco's modified Eagle medium

supplemented with 10% calf serum. HL60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Various amounts of plasmid DNAs were transfected to HeLa cells about 60% confluent in a 6 cm dish using the calcium phosphate precipitation method⁴¹⁾ together with a β -galactosidase expression plasmid. At 16 h after transfection, the serum concentration in the medium was reduced to 2%. The cells were cultured for another 48 h and then stained with 0.1% X-gal in PBS overnight at 37 °C. Morphologically apoptotic or non-apoptotic cells were scored among the β -galactosidase-expressing cells. More than 200 cells were counted in total. For the fine observation of nuclei, the transfected cells were fixed with 1% glutaraldehyde for 30 min at room temperature, stained with Hoechst 33258 (Hoechst) and subjected to fluorescence microscopy.

Plasmids pEF-*c-myc*: Human *c-myc* cDNA spanning –34 from the initiation ATG (A is set as 1) to the C-terminus was introduced into the EcoRI site of pEF containing the elongation factor α promoter in pUC118.⁴²⁾ pCMV-E1A was kindly supplied by Eileen White. pEF-MSSP-1 and pEF-MSSP-2 have been described previously.³⁹⁾ Deletion mutants of MSSP-2, Δ RNP-A, Δ RNP-B, Δ RNP-A, B, and Δ RNP-C were constructed by using the polymerase chain reaction (PCR) in pGEM4Z as described previously. The XbaI-BamHI fragments containing the coding sequences of MSSP-2 with various deletions were inserted at the XbaI-BamHI site of pEF-X, a pEF variant where the EcoRI site was changed to XbaI site by linker insertion. The plasmids were designated as pEF-MSSP-2 Δ A, pEF-MSSP-2 Δ B, pEF-MSSP-2 Δ AB, and pEF-MSSP-2 Δ C.

Analysis of DNA Fragmentation Total cellular DNAs were extracted from transfected HeLa cells by the standard procedure.⁴³⁾ One μ g of DNA was labelled with ³²P-dCTP as described.⁴⁴⁾ The reaction mixture was 5 units Klenow DNA polymerase, 0.5 μ Ci ³²P-dCTP (3000 Ci/mmol), 10 mM Tris (pH 7.5) and 5 mM MgCl₂ in

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50 μ l, and incubation was carried out for 15 min, at room temperature. Labelled DNA was precipitated twice with ethanol, and one tenth of the sample volume was loaded on to a 1.4% agarose gel in tris-borate-EDTA (TBE) buffer. Gels were dried and autoradiographed.

RESULTS

Induction of Apoptosis in HeLa Cells by MSSP, *c-myc*, and E1A HeLa cells were transfected with various amounts of expression vectors of pEF-MSSP, pEF-*c-myc* or pCMV-E1A, based on the elongation factor (EF) or the cytomegalovirus (CMV) promoter, together with pCMV- β -gal, a β -galactosidase expression vector. At 16 h after transfection, the serum concentration in the culture medium was reduced to 2% and the cells were cultured for another 48 h. The transfected cells were stained with Hoechst 33258 and examined using a fluorescent microscope (Fig. 1). Typical apoptotic cells containing apoptotic bodies were observed among cells transfected with pEF-MSSP-1, pEF-MSSP-2, pEF-*c-myc* or pCMV-E1A, but not among cells transfected with pEF vector. To monitor the efficiency of apoptosis induction by transfected constructs, the cells were stained with X-gal. Approximately 5–10% cells were stained blue, showing they were β -galactosidase-positive. Morphologically apoptotic cells were easily distinguished from non-apoptotic ones under the microscope and counted (Fig. 2). All experiments were repeated at least three times and the average values are shown (Fig. 3). Among the cells transfected with pEF containing the elongation α promoter alone, approximately 8% exhibited apoptotic morphology in the presence of 2% serum. Introduction of *c-myc* or adenovirus E1A induced or stimulated apoptosis in HeLa cells in a dose-dependent manner as reported before. MSSP-1 and MSSP-2 also induced or stimulated

apoptosis, but to a lesser degree than *c-myc* and E1A. When the cells were transfected with the plasmids and cultured in medium containing 10% serum, no induction of apoptosis was observed. The results indicate that MSSP-1 and MSSP-2 have functions similar to *c-MYC* in terms of apoptosis induction.

DNA Fragmentation in Apoptotic Cells Induced by MSSP HeLa cells were transfected with expression vectors of *c-myc*, E1A, and MSSP-1 and cultured in the presence of 2% serum as above. Total cellular DNAs were then extracted by standard procedures using SDS and proteinase K followed by phenol extraction and were labelled with 32 P-dCTP and Klenow fragment as

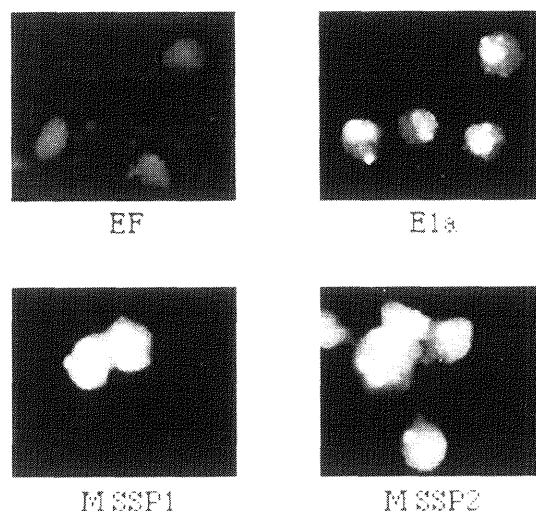


Fig. 1. Apoptotic Bodies Observed in the Cells Transfected with MSSP-Expression Plasmids

HeLa cells were transfected with 2 μ g expression vectors of MSSP-1 (MSSP1), MSSP-2 (MSSP2), adenovirus E1A (E1a) or the elongation factor (EF) promoter alone as described in Materials and Methods. The cells were stained with Hoechst 33258 and examined by fluorescence microscopy. Typical examples are shown.

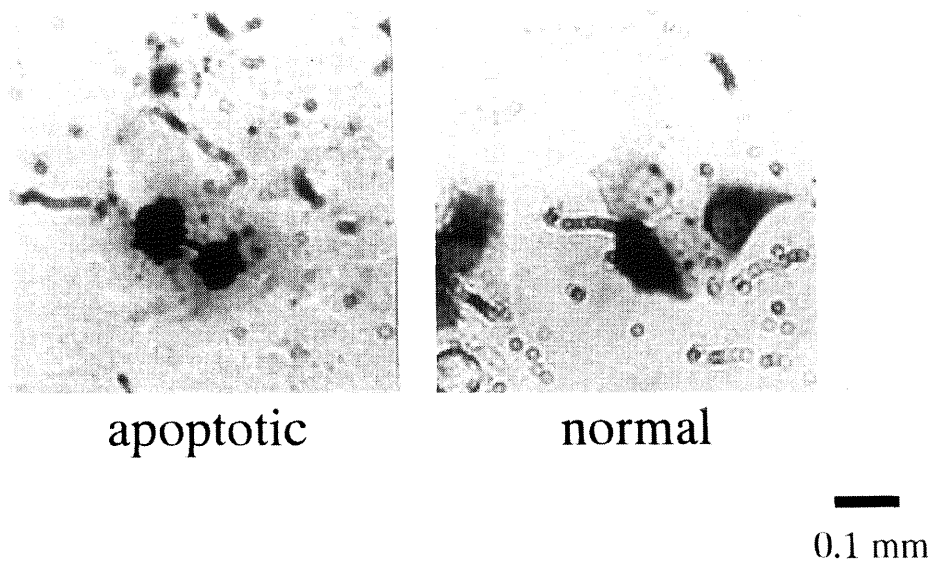


Fig. 2. Morphological Characteristics of Apoptotic Cells Induced by Transfection of MSSP

HeLa cells were transfected with 2 μ g expression vectors of *c-myc*, adenovirus E1A, MSSP-1, or MSSP-2, or the elongation factor (EF) promoter alone, together with pCMV- β -gal, an expression vector of β -galactosidase, as described in Materials and Methods. Cells were stained with X-gal and examined by microscopy. Typical examples are shown, apoptotic, apoptotic cells observed among the cells transfected with an MSSP-1 expression vector; normal; non-apoptotic cells observed among the cells transfected with a vector containing the EF promoter alone. Cells expressing the β -galactosidase look dark.

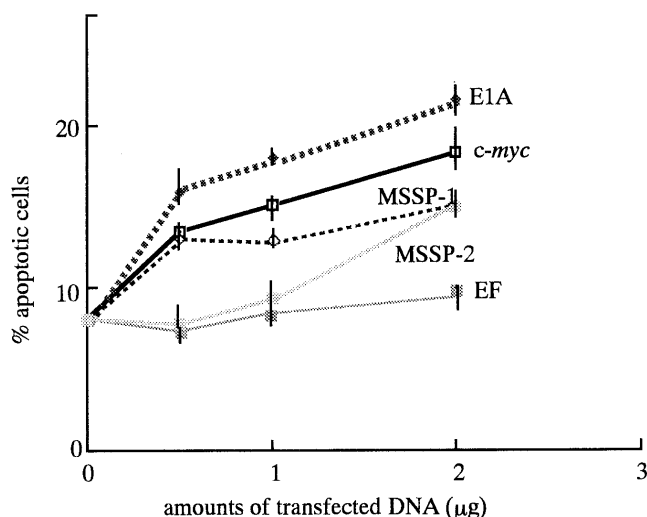


Fig. 3. Induction of Apoptosis by Several Genes in HeLa Cells

HeLa cells were transfected with expression vectors of *c-myc*, adenovirus E1A, MSSP-1, or MSSP-2, or the elongation factor (EF) promoter alone, together with pCMV- β -gal, an expression vector of β -galactosidase, as described in Materials and Methods. Cells were stained with X-gal and examined by microscopy. The percentage of apoptotic cells in the total cells counted is shown. Approximately 1000 cells were counted in total in each experiment.

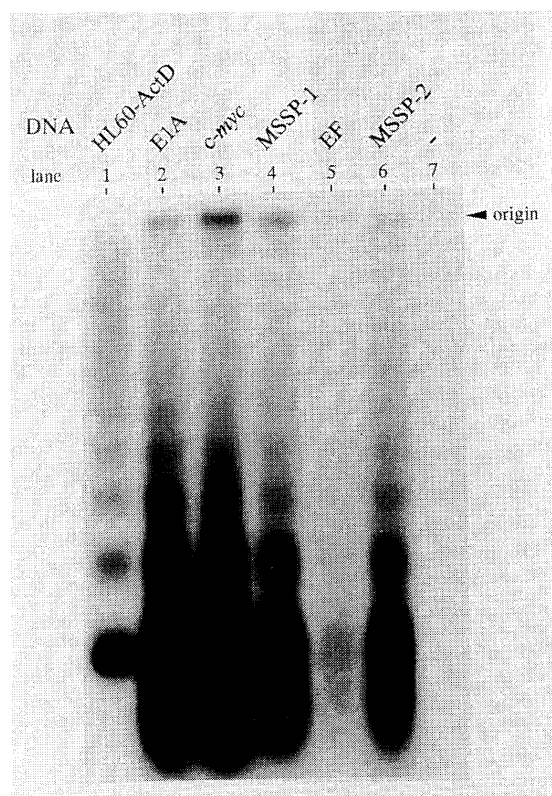


Fig. 4. DNA Fragmentation Induced by MSSP

HeLa cells were transfected with 2 μ g various plasmids as in Figs. 1 and 2, and total cellular DNAs were extracted. The DNAs were labelled with 32 P-dCTP and Klenow DNA polymerase, separated on a 1.2% agarose gel, and autoradiographed as described in Materials and Methods. As a control, 2×10^6 human HL60 cells were treated with 10 μ g/ml actinomycin D (ActD) for 4h, and the total DNAs were treated and analyzed in parallel as above.

described.⁴⁴) Autoradiography of the labelled DNAs separated on a 1.4% agarose gel indicated that internucleosomal DNA fragmentations occurred in the cells transfected with E1A, *c-myc*, and MSSP-1, similarly to that in human HL60 cells treated with 10 μ g/ml actino-

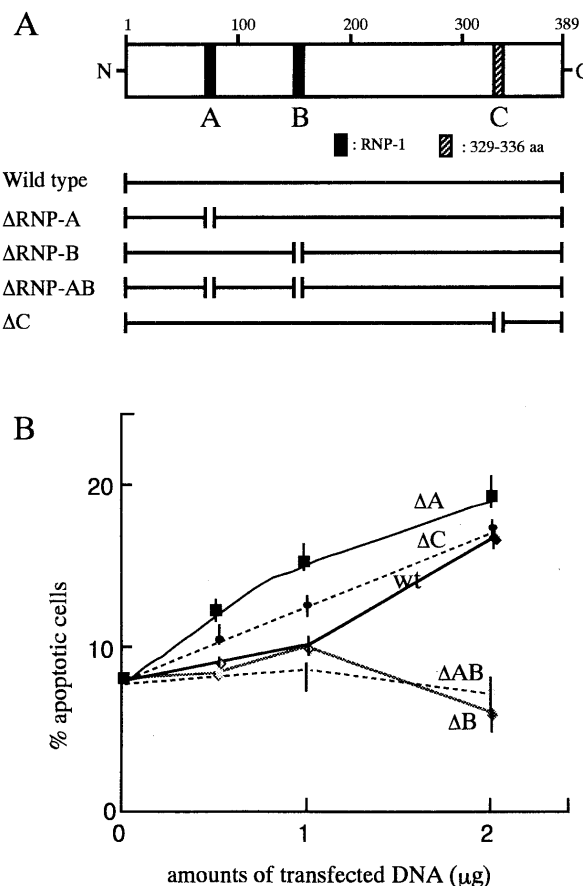


Fig. 5. Determination of the Domains of MSSP Necessary for Apoptosis Induction

A: Schematic drawing of the deletion mutants of MSSP-2. B: Apoptosis induction by deletion mutants of MSSP-2. HeLa cells were transfected with expression vectors for wild type and four deletion mutants of MSSP-2, and apoptotic cells were counted as in Fig. 3. Approximately 1000 cells were counted in each experiment.

mycin D where typical apoptotic DNA fragmentation is observed (Fig. 4). The intensities of the labelled fragments from the three DNA preparations were proportional to those of the apoptotic cells in Fig. 2. DNA fragmentation was scarcely observed in cells transfected with pEF as in non-transfected cells. MSSP-2 as well as MSSP-1 induced internucleosomal DNA fragmentation in HeLa cells. These results suggest that MSSP-1/Scr2 or MSSP-2 induces apoptosis in HeLa cells.

Determination of Domains of MSSP-2 Necessary for Apoptosis Induction Both MSSP-1 and MSSP-2 have two copies of RNP-1 consensus sequences (RNP1-A and RNP1-B), either of which is required for the sequence-specific DNA binding activity of the proteins.³⁹) As far as we could determine, MSSP-1/Scr2 and MSSP-2 were functionally indistinguishable with each other. We, therefore, used MSSP-2 for the deletion analyses of MSSP. Mutants of the MSSP-2 cDNA deleting RNP1-A (Δ RNP-A), RNP1-B (Δ RNP-B), both RNP1's (Δ RNP-AB), and the region at the C-terminus with a comparable number of amino acids (Δ C) were constructed using the polymerase chain reaction (PCR)³⁹) (Fig. 5A). The mutant cDNAs were linked to the elongation factor α promoter (EF) in pEF- Δ RNP-A, pEF- Δ RNP-B, pEF- Δ RNP-AB, and pEF- Δ C. Various amounts of expression vectors for the

wild-type or deletion mutants of MSSP-2 were transfected to HeLa cells together with pCMV- β -gal as above, and the ratio of apoptotic to non-apoptotic cells was examined by microscopy after X-gal staining. As shown in Fig. 5B, pEF- Δ RNP-A, pEF- Δ C and wild-type pEF-MSSP-2 similarly induced apoptosis in transfected cells. Transfection of pEF- Δ RNP-B or pEF- Δ RNP-AB, on the other hand, resulted in apoptosis induction to lesser degree than in the case of the wild-type plasmid. These results suggested that the RNP1-B sequence is required for the apoptosis inducing activity of MSSP.

DISCUSSION

MSSP were identified as the proteins which bind to a putative DNA replication origin/transcriptional enhancer in the human *c-myc* gene, and the cDNA for one of the proteins, MSSP-1, was cloned from a human cDNA library by the Southwestern method with a probe of single-stranded DNA of the *c-myc* replication origin/transcriptional enhancer.³⁸⁾ MSSP-2 was then cloned by DNA-DNA hybridization using MSSP-1 cDNA as a probe.³⁹⁾ Another group has independently cloned two cDNAs encoding human proteins, Scr2 and Scr3, which complement the defect of *cdc2* and *cdc13* in *Schizosaccharomyces pombe* and Scr2 has turned out to be identical to MSSP-1.⁴⁰⁾ MSSP-1/Scr2, MSSP-2, and Scr3 commonly possess two copies of RNP consensus sequences (RNP-1A and RNP-1B) and are considered to belong to the same protein family. The results of the complementation experiments in *S. pombe* suggest that Scr3 is involved in the pathway between *cdc13* and *cdc2*, and MSSP-1/Scr2 functions, *i.e.* downstream of *cdc2* during the G₁/S transition.⁴⁰⁾ This suggests that, in human cells, MSSP-1/Scr2 and MSSP-2 function downstream of *cdk2*, a mammalian homologue of *cdc2*. We have previously reported that MSSP-1/Scr2 and MSSP-2 stimulate initiation of DNA replication in the SV40 derivative in which the AT-rich sequence required for the initiation of viral DNA replication is substituted for the MSSP recognition sequence.⁴⁵⁾ MSSP-1/Scr2 and MSSP-2 may be regulatory factors of the cell cycle towards initiation of DNA replication.

In this report, MSSP were examined for apoptosis-inducing activity and the results suggest that MSSP-1 or MSSP-2 independently induces apoptosis in HeLa cells. The domain required for apoptosis-inducing activity of the protein was the region containing RNP1-B. Although various G₁/S checkpoint proteins including E2F,^{32,33)} Rb,³⁴⁾ *cdc2* and *cdk2*,³⁵⁾ PITSLRE kinase,³⁶⁾ and cyclin A³⁷⁾ have been reported to be associated with apoptosis induction, mechanisms for this induction by the proteins remain unclear. Induction of apoptosis in several cell lines by *c-myc* has been reported.¹⁵⁻¹⁷⁾ We found that the expression patterns of MSSP in various tissues are quite similar to those of *c-myc*, and that MSSP-1 and MSSP-2 form protein complexes with the N-terminal portion of *c-myc* protein (Ariga, Tajima, Taira, Saegusa, Izumi and Iguchi-Arigo, manuscript in preparation). It is, therefore, suggested that MSSP may enhance or suppress the functions of *c-MYC*, such as transactivation, cell transformation and apoptosis induction; in addition, MSSP

may independently induce apoptosis. When there is 10% serum in the culture medium, MSSP stimulated the cell transforming activity of *myc/ras* and promoted the anchorage independence of the transformed cells in soft agar. These events are considered to be carried out in association with *c-MYC*. The RNP1-A containing domain of the protein was required for formation of transformed cell foci in the culture medium, and the whole structure was necessary for anchorage-independent growth as well as for stimulating the initiation of DNA replication (Saegusa, Izumi, Taira, Iguchi-Arigo and Arigo, manuscript in preparation). As for apoptosis induction by MSSP, another RNP-1 motif of the protein, RNP1-B, has been shown to be required. Different requirements of the domains for different functions may reflect the participation of proteins other than *c-MYC*, which associate with MSSP, in respective functions.

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