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The RecA-like protein of *Schizosaccharomyces pombe*: its cellular level is induced by DNA-damaging agents

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RecA protein plays a central role in homologous recombination and DNA repair in *Escherichia coli* (*E. coli*). The function and structure of this protein are universal in prokaryotes and also conserved in eukaryotes such as yeast. The RecA-like protein with 74 kDa in size has already been identified and purified from a fission yeast *Schizosaccharomyces pombe* (*S. pombe*) (Lee, 1991). From this study it was revealed that the RecA-like protein of *S. pombe* was highly inducible to various DNA damaging agents and inhibitors of nucleotide pool synthesizing enzymes. The cellular level of the *S. pombe* RecA-like protein was markedly increased, upto 5- to 10-fold, by treatment with various DNA-damaing agents including ultraviolet (UV) light, methyl methanesulfonate (MMS), 4-nitroquinoline-1-oxide (4-NQO), and mitomycin-C (MMC), similar to *E. coli* RecA protein. Interestingly, the protein level was also increased by inhibitors of nucleotide pool forming enzymes such as methotrexate (MTX) and hydroxyurea (HU). The most effective doses for the inducibility of 4-NQO, MMS, UV, MMC, MTX, and HU were 0.2 μg/ml, 30 mM, 200 J/m^2, 0.4 μg/ml, 1 μg/ml, and 100 mM, respectively. The range of effective duration time for the inducibility of RecA-like protein was from 270 to 450 mins. These results suggest that the *S. pombe* RecA-like protein also plays an imortant role in cellular responses to DNA damage as in *E. coli* system.

KEY WORDS: *Schizosaccharomyces pombe*, DNA damage, RecA-like protein, Inducible repair, Immunoblotting

The product of *recA* gene from *Escherichia coli* (*E. coli*) is a multi-functional protein involved in homologous recombination, DNA repair, and the regulation of a complex cellular response to DNA damage (McEntee and Weinstock, 1979; Kenyon and Walker, 1980; Konforti and Davis, 1987; Story *et al.*, 1993). Exposure of *E. coli* to agents or conditions that damage DNA or interfere with DNA replication results in the induction of a diverse set of physiological responses referred to as the SOS responses (Kogama *et al.*, 1979; Kowalczykowski, 1991). A number of genes has been shown to be controlled by the SOS system, including *uvrA, uvrB, sulA, umuDC, recA, and recN* (Bagg *et al.*, 1981; Fogliano and Schendel, 1981; Casaregola *et al.*, 1982; Walker, 1984). Of these genes, *recA* gene plays a cental role in the control of the SOS system (Walker, 1984).

In the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), it has been identified that DMC1, Rad51, and Rad57 proteins are RecA-like ones related to the *E. coli* RecA protein (Resnick, 1987; Bishop *et al.*, 1992; Shinohara *et al.*, 1992; Story *et al.*, 1993). The *S. cerevisiae* RecA-like protein is inducible by DNA-damaging

agents or the condition of deficient nucleotide pool (Angulo *et al.*, 1985; Elledge and Davis, 1987 and 1989; Hurd *et al.*, 1987), similar to *E. coli* RecA protein. In addition, proteins that catalyze homologous sequence-dependent DNA strand transfer have also been identified and purified in the same yeast (Sugino *et al.*, 1988; Hamatake *et al.*, 1989).

Previously, the RecA-like protein with 74 kDa in size has been identified and purified (Lee, 1991) from higher yeast *Schizosaccharomyces pombe* (*S. pombe*). This study has been performed to investigate the inducible nature of *S. pombe* RecA-like protein against DNA-damaging agents or inhibitiors of nucleotide pool forming enzymes.

Materials and Methods

Culture of *S. pombe* cells

S. pombe (ura4-D18, *h⁻ ade6-704 ura4-D18 leu1-32*) cells were donated by Dr. M. Yanasida (Department of Biophysics, Tokyo University, Japan) and cultured in YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose, pH 5.5) at 30°C.

Enzymes and reagents

All culture supplies were purchased from Difco Laboratories. The protein A labelled with ^{125}I (spec. act., 30 mCi/mg total protein A) was obtained from Amersham International plc. Protease inhibitors were from Sigma Co. Methyl methanesulfonate (MMS), 4-nitroquinoline-1-oxide (4NQO), and mitomycin-C (MMC) were purchased from Aldrich Company Inc. and methotrexate (MTX) was from Sigma. Nitocellulose filter was obtained from Millipore.

Preparation of antisera against *E. coli* RecA protein

E. coli RecA protein was purchased from Sigma and RecA antisera were prepared as described by Lovett and Roberts (1985) with minor modifications.

Polyacrylamide gel electrophoresis and Western blot analysis

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Western blot analysis was performed by the method of Twobin *et al.* (1979). ^{125}I-protein A was used instead of second antibody and the incubation time was 1 hr, followed by two 15-min washes in phosphate-buffered saline (PBS)-Tween buffer (0.05% Tween-20 in PBS) and PBS. Filters were air-dried and exposed to X-ray film. The indensities of autoradiograms were measured with a Joyce-Loeble microdensitometer (Gateshead-on-Tyne, England).

UV-irradiation and chemical treatment

Cells grown to mid-exponential phase were harvested, washed with distilled water, and resuspended in 50 mM potassium phosphate buffer, pH 7.0. Cells were exposed to 254 nm UV-light from a mercury germicidal lamp at an incident dose rate of 1.42 J/m^2/sec, as determined by No. 65 radiometer (Yellow Spring Instrument Co., Yellow spring, Ohio, U.S.A.). Cells were treated with a desired dose of MMS, 4-NQO, MMC, HU, or MTX for an appropriate time.

Preparation of yeast crude cell extract

One gram of *S. pombe* cells was suspended in 1 ml of S buffer (1.1 mM Sorbitol, 20 mM KH_2PO_4, pH 6.5, 1 mM PMSF, 2 mM DTT, 10 mM $MgCl_2$) and incubated at 30°C for 10 mins. Cells were harvested by centrifugation at 5,000 rpm for 5 mins in a Sorvall SS-34 rotor and the pellet was resuspended in the same volume of S buffer containing 25 mM sodium citrate and 0.2 mg/ml of Novozyme 234. Cells were incubated at 30°C for 1 hr or more with gentle agitation. The resulting spheroplasts were frozen at −70°C, thawed at room temperature, and then resuspended in 1 ml of RIPA buffer (1 M NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM PMSF, 2 mM DTT, 14.3 mM 2-mercaptoethanol, 1% NP-40) containing various protease inhibitors (2 μg of leupeptin, 1 μg of pepstatin A, 2 μg of aprotinin and 2 μg of soybean trypsin inhibitor I

per ml). The sample was frozen again at −70°C and thawed at 30°C with vigorous vortexing. The lysate was placed on ice for 30 mins and centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 40 mins at 4°C. The supernatant was collected and saved at −70°C until used.

Results and Discussion

The specificity of the response to DNA-damaging agents can provide information on the nature of the sensory mechanisms in cells (Walker, 1984; Elledge and Davis, 1989). With this in mind, various agents were tested for their effects on the induction of RecA-like protein in *S. pombe*. All of the agents that damage DNA (4NQO, MMS, UV-light, and MMC) or block DNA synthesis (HU and MTX) resulted in the induction of RecA-like protein.

When *S. pombe* cells were treated with a various concentrations of 4-NQO (0-3 μg/ml) and MMS (0-30 mM) for 180 mins (Fig. 1), the cellular levels of RecA-like protein were increased to 5 to 6 fold (Fig. 1). Clear inducibility of the RecA-like protein was also evident in cells irradiated with UV-light (0-300 J/m^2) or treated with MMC (0-0. 5 μg/ml) for 180 mins (Fig. 2). By 200 J/m^2 of

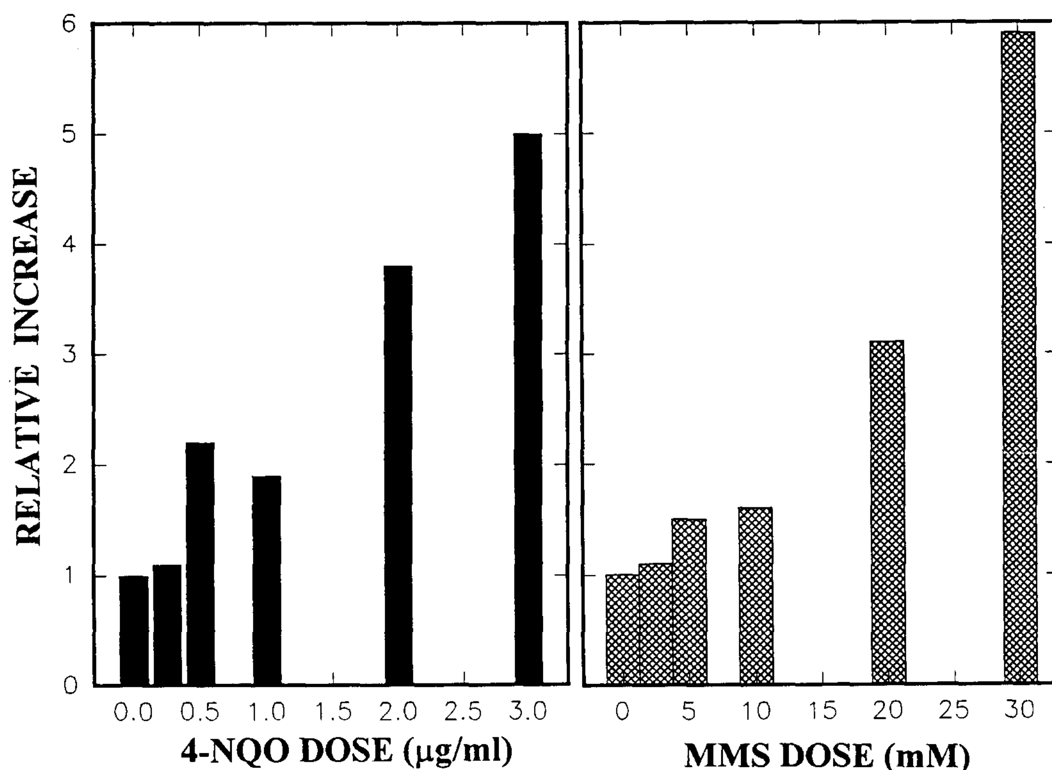

Fig. 1. Dose-dependent increase of RecA-like protein by 4-NQO and MMS. Exponentially growing *S. pombe* (ura4-D18) cells were treated with various concentrations of MMS (left pannel) or 4-NQO (right pannel) for 180 mins and cell extracts were prepared as described in Materials and Methods. An equal amount of proteins (60 μg/lane) was electrophoresed and blotted onto nitrocellulose filter. The filter was blocked for 1 hr with 3% non-fat milk, and then reacted with 1,000-fold diluted RecA anti-sera for 2 hrs. ^{125}I-protein A was used to react to IgG and the incubation time was 1 hr, followed by two 15-min washes in phosphate-buffered saline (PBS)-Tween buffer (0.05% Tween-20 in PBS) and PBS. Filters were air-dried and exposed to X-ray film. The indensities of autoradiograms were sepectrophotometically scanned with a microdensitometer. The relative increase values were expressed as fold-increases relative to control ones.

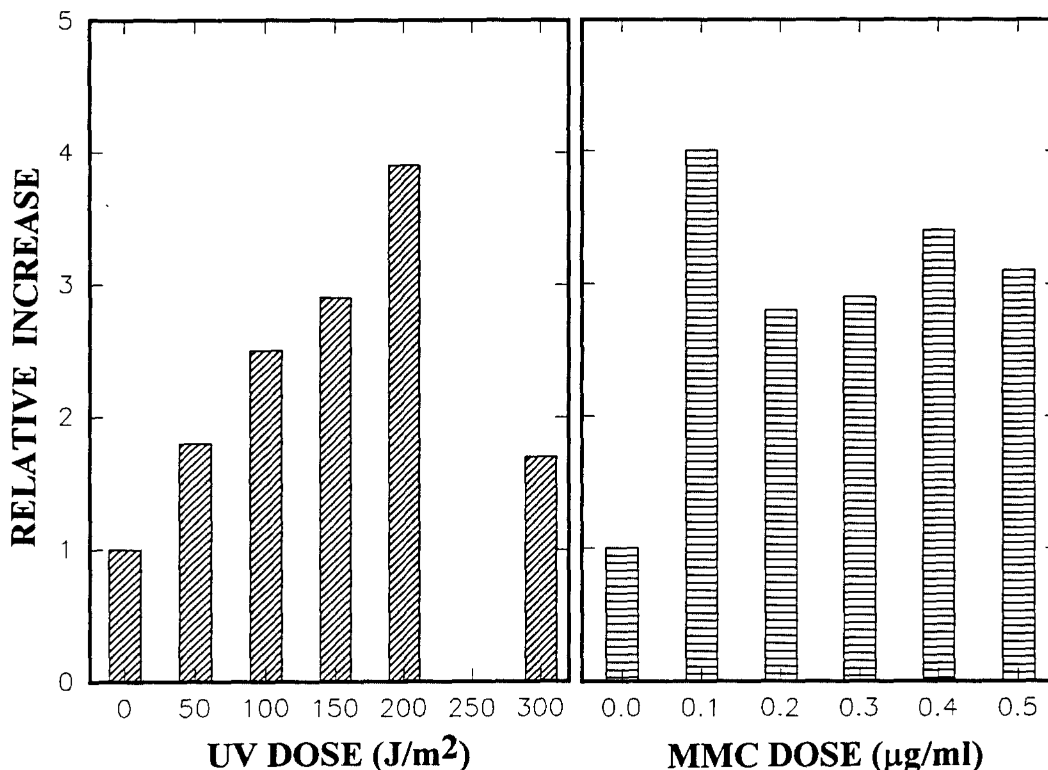

Fig. 2. Dose-dependent increase of RecA-like protein by UV-light and MMC. Exponentially growing cells were irradiated with various doses of UV-light (left pannel) or treated with various concentrations of MMS (right pannel) for 180 mins. The preparation of cell extracts and immunoblotting were carried out as described in Fig. 1.

UV-light (Fig. 2, left panel) or 0.1 μg/ml of MMC (Fig. 2, right panel), the levels of RecA-like protein were increased upto 3.9- and 3.4-folds, respectively. These results suggest that chromosomal damages induced by a variety of DNA-damaging agents can act as signal for the induction of *S. pombe* RecA-like protein, like in *E. coli* system (Bagg *et al.*, 1981; Smith and Wang, 1989).

Interestingly, different inducible nature of RecA-like protein was also demonstrated in *S. pombe* cells treated with two agents, e.g., HU and MTX that inhibit DNA synthesis by reducing the sizes of nucleotide pools (Elledge and Davis, 1989). As shown in Fig. 3, HU increased the cellular level of RecA-like protein to 3.1-fold at 100 mM (Fig. 3, left panel). Another inhibitor for DNA replication, MTX also increased the amount of RecA-like protein in *S. pombe* cells. One microgram per ml of MTX increased the protein level to 3.9-fold at 180 mins (Fig. 3, right panel). The concentrations higher than 100 mM of HU and 1 μg/ml of MTX were less effective because the high doses probably exert cytotoxicity under continuous presence of the drugs. From these data, it can be said that a depletion of deoxynucleotide triphosphates (dNTPs) by HU and MTX can make a signal for the induction of RecA-like protein probably through stalling the DNA replication. This idea could be supported by the notion that a stalled replication fork resulted from a depletion of dNTPs by HU and MTX remained as DNA strand breaks or gaps similar to DNA damages induced by direct DNA-damaging agents and these damage-like sites could generate an inducing signal for inducible repair system (Witkin, 1976; Walker, 1984; Elledge and Davis, 1989).

To determine the time points that the cellular level of RecA-like protein is induced to the maxium level by DNA-damaging agents or

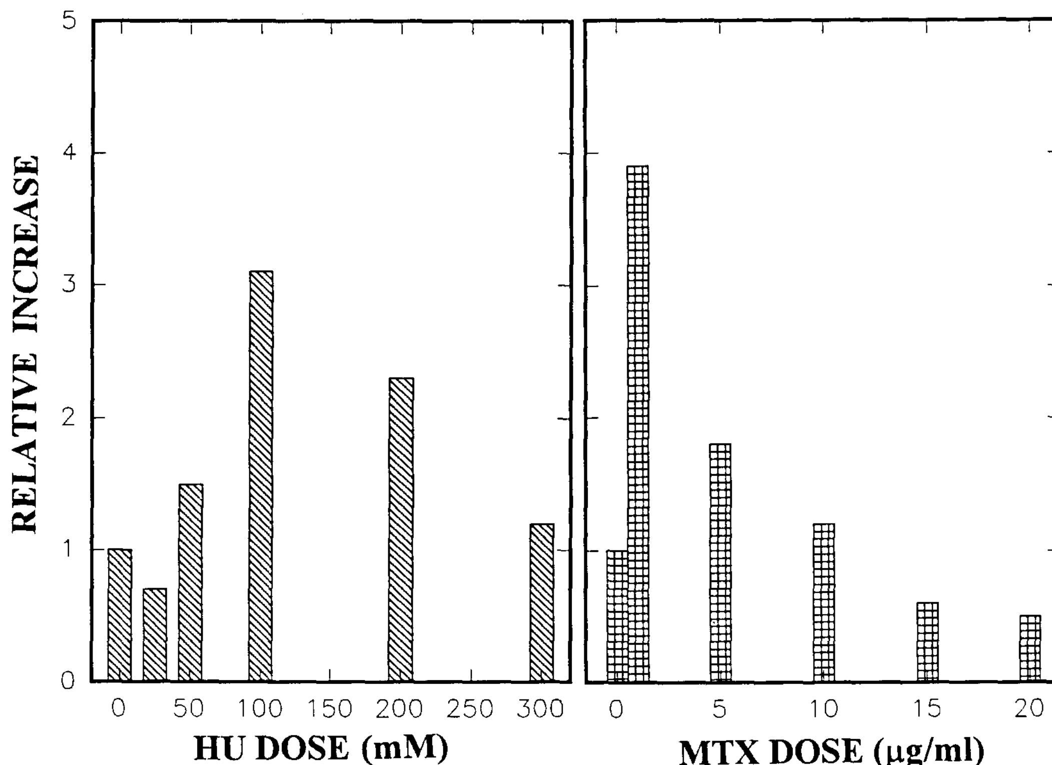

Fig. 3. Dose-dependent increase of RecA-like protein by HU and MTX. Exponentially growing cells were treated with various concentrations of HU (left pannel) or MTX (right pannel) as indicated for 180 mins. The preapration of cell extracts and immunoblotting were carried out as described in of Fig. 1.

inhibitors of nucleotide pool forming enzymes, time-dependency was checked (Fig. 4). When *S. pombe* cells were treated with 30 mM MMS for 0 to 450 mins as indicated in the figure, the RecA-like protein increased to maxium level at 180 mins and gradually decreased to below control level thereafter. It was supposed that this decrease maybe due to the cytotoxycity of MMS (Fig. 4, left panel). 4-NQO (3 μg/ml), UV-light (150 J/m^2), MMC (0.3 μg/ml), and MTX (1 μg/ml) continuously increased the cellular level of RecA-like protein for the periods tested (Fig. 3). In *S. pombe* cells incubated with 100 mM of HU, the RecA-like protein increased to the maximum level at 360 mins of incubation and thereafter dramatically decresed to control level.

The most effective doses and duration time points for inducibility of *S. pombe* RecA-like protein are summarized in Table 1. Although the effectiveneses depend on the dose and treatment time, among the agents assayed MTX was the most prominent inducer for RecA-like protein and the effective range of duration time for the inducibility was from 270 to 450 mins corresponding to 3-4 generation time for *S. pombe* cells.

In conclusion, RecA-like protein from *S. pombe* was greatly induced by a wide variety of agents that either damage DNA directly or induce stress by blocking DNA replication. Elledge and Davis (1987) have proposed two basic models accounting for specific induction of *S. cerevisiae* RNR2 protein, which is now known as Rad51 protein involved in the recombinational repair pathway (Shinohara *et al.*, 1992; Story *et al.*, 1993): (1) feedback regulation in response to depletion of the dNTP pools by repair processes or (2) direct induction in response to DNA damage.

As presented in this study, the first model is

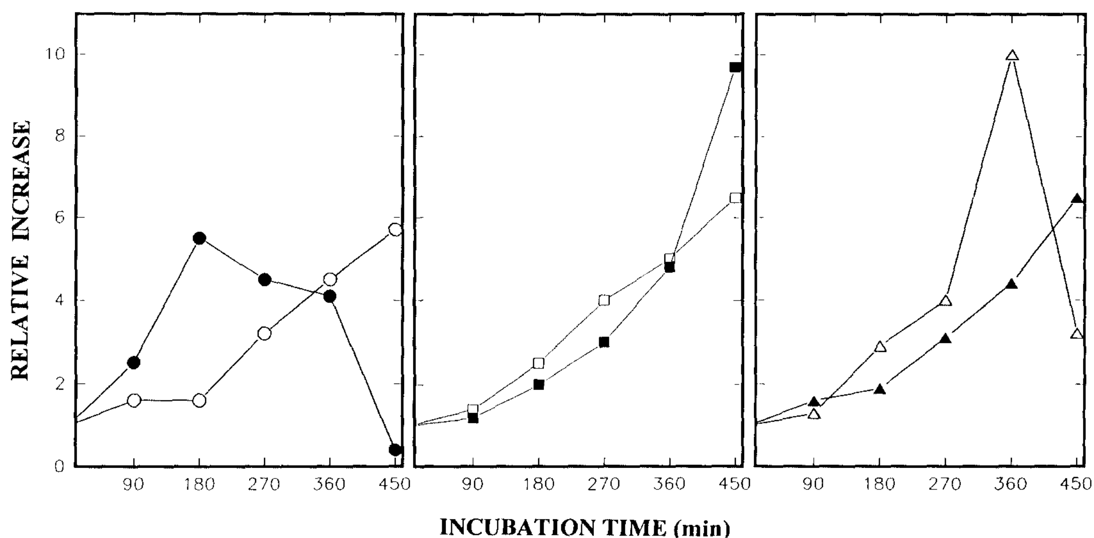

Fig. 4. Time course of increase in the cellular level of RecA-like protein in *S. pombe* cells. Left pannel: Cells were treated with 30 mM of MMS (● - ●) or 3 μg/ml of 4-NQO (○ - ○) for indicated times. Middle pannel: Cells were treated with 0.3 μg/ml of MMC (■ - ■) or irradiated with 150 J/m^2 of UV-light (□ - □). Right pannel: Cells were treated with 100 mM of HU (▲ - ▲) or 1 μg/ml of MTX (△ - △) for indicated times. At indicated time, cells were harvested and immunoblotting was carried out as described in Fig. 1.

Table 1. The most effective dose and duration time for inducibility of *S. pombe* RecA-like protein by DNA damaging agents and inhibitiors of nucleotide pool forming enzymes

Agent*	Dose[1]	Duration Time (min)[2]	Fold increased[3]
4-NQO	0.2 μg/ml	450	5.7
MMS	30.0 mM	270	5.5
UV	200.0 J/m^2	450**	6.5
MMC	0.4 μg/ml	450	9.7
MTX	1.0 μg/ml	360	10.1
HU	100.0 mM	450	6.5

*4-NQO, 4-nitroquinoline-1-oxide; MMS, methyl methanesulfonate; UV, ultraviolet-light; MMC, mitomycin-C; MTX, methorexate; HU, hydroxyurea. **Inubation time after UV-irradiation in YPD medium. [1]The most effective doses only were selected from the dose-dependent experiments (See Figs. 1, 2, and 3); [2]The most effective duration times only were chosen from the time-dependent experiments (See Fig. 4); [3]From the time-dependent experiments, the maximum values were selected.

supported by the induction by MTX (TTP starvation) and HU (depletion of all dNTPs). DNA damage appears to be the stimulus for induction of *S. pombe* RecA-like protein, but the precise nature of the inducing signal(s) remains to be further studied.

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DNA 상해요인에 의한 ***Schizosaccharomyces pombe*** RecA 유사 단백질의 유도생성에 관한 연구

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대장균의 RecA 단백질은 상동 재조합 및 DNA 회복에 핵심적인 역할을 하며, 그 구조와 기능이 원핵세포에서는 필수적이고 효모와 같은 진핵세포에서도 보존되어 있다. 본 연구실에서는 이미 74 kDa 크기의 RecA 유사 단백질을 *Schizosaccharomyces pombe* (*S. pombe*)로 부터 동정하여 순수분리한 바 있다(이, 1991). 본 연구에 의해 *S. pombe*의 RecA 유사 단백질이 여러가지 DNA 상해요인과 뉴클리오티드 생합성에 관여하는 효소의 저해제에 의해 유도생성됨이 확인되었다. RecA 유사 단백질은 대장균의 RecA 단백질처럼 자외선(UV), methyl methanesulfonate(MMS), 4-nitroquinoline-1-oxide(4-NQO) 및 mitomycin-C(MMC)와 같은 DNA 상해요인에 의해 최고 5-10배 유도생성되었으며, methotrexate(MTX)와 hydroxyurea(HU)와 같은 뉴클리오티드 생합성 저해제에 의해서도 크게 증가 하였다. 4-NQO, MMS, UV, MMC, MTX 및 HU의 가장 효과적인 선량 및 농도는 각각 0.2 μg/ml, 30 mM, 200 J/m^2, 0.4 μg/ml, 1 μg/ml 그리고 100 mM 등이었다. 가장 효과적인 처리시간 및 조사후 배양시간은 270-450분이었다. 이와같은 결과는 *S. pombe*의 RecA 유사 단백질이 대장균의 경우처럼 DNA 상해의 회복에 관여함을 시사하는 것이다.