BIOMEDICAL AND ENVIRONMENTAL SCIENCES **19**, 210-213 (2006)

Immediate-early Inducible Function in Upstream Region of *junB* Gene[1]

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Objective To analyze the upstream region of radiation-induced *junB* gene. **Methods** Four plasmids containing 250 bp, 590 bp, 900 bp and 1650 bp, and CAT reporter gene were constructed separately and introduced to L8704 cells. The cells were irradiated with 2 Gy X-rays and incubated at different intervals. Total RNA was extracted from the cells and fluctuation of the CAT mRNA level was assessed by the RNA ratio of CAT/β-actin measured by quantitative Northern blot hybridization. **Results** CAT mRNA expression containing 900 bp and 1560 bp *junB* promoter remarkably and rapidly increased, and reached its peak 30 min after 2 Gy X-ray irradiation. **Conclusions** 590~900 bp fragments located in the upstream region of *junB* gene play an important role in the early process of cells against radiation.

Key words: *junB* promoter; CAT reporter gene; Immediate-early response gene; Signal transduction

INTRODUCTION

Ionizing radiation stimulates various biological reactions in mammalian cells. When cells are irradiated, numerous mammalian genes are induced as radiation-response genes[1-2]. It has been shown that the mechanism of radiation-response genes involved in the recovery from radiation-damage in mammalian cells is not yet completely clear. However studies have shown that several cytokines can facilitate the recovery from such damages *via* activation of signal transduction systems.

We have previously found that mRNA for *junB* is expressed transiently within 30 min in C3H/He mice and 1 h in Balb/c mice after 3 Gy X-ray irradiation[3]. These findings imply that *junB* promoter, 2300 base pair, presents the immediate-early inducible function of X-rays. To analyze the unique mechanism against ionizing radiation, kinetic studies on the expression of upstream region of *junB* gene *in vitro* were performed.

MATERIALS AND METHODS

Irradiation

X-rays were generated at 200 kVp/20 mA and filtered through 0.5 mm-thick Cu and AI plates. The exposure rate was monitored with a Victreen Radio meter. Dose rates of 0.899 Gy/min were used for cultured cells.

Constructing 4 Plasmids Containing both junB Promoter Fragment and CAT Reporter Gene

Restriction enzyme HindⅢ was used to digest the pBSV11 plasmid and obtain the upstream region of *junB* gene, 2300 base pair fragments were inserted into the upstream region of TATA box, and a new plasmid pBSV11a was constructed as follows. Pst I, Kpn I, BamHI, and Xho Ⅰ enzymes carrying out the pBSV11a, 250, 590, 900, and 1560 base pair fragments containing TATA box were isolated and inserted into the upstream region of CAT reporter gene in pCAT(B) plasmid respectively, generating the pRSVC1, pRSVC2, pRSVC3, and pRSVC4 plasmids.

[1]This project was supported by Japanese Research Development Corporation (JRDC).

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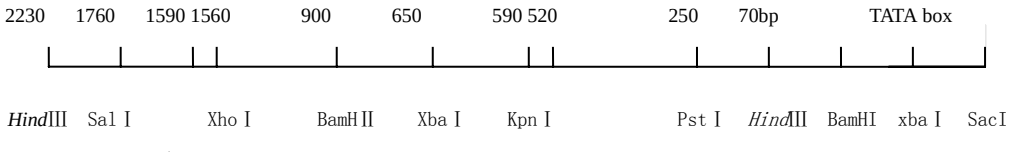
Biographical note of the first author: Hong WAN, female, born in 1963, professor, majoring in study on repair of injured central nervous system.

0895-3988/2006
CN 11-2816/Q



Four Plasmids Introduced to L8704 Cells and Cells Irradiation

Cell line L8704 is an adhesive macrophage-like leukemic cell line, established from C3H/He mice with radiation-induced leukemia[4]. L8704 cells were pre-cultured with MEM-α medium (Sigma) at 37℃ in 5% CO_2 for 16 h, transferred with pRSVC1, pRSVC2, pRSVC3, and pRSVC4 separately by liposome transduction, and continuously cultured for 5 h. The cells were irradiated at room temperature with 2.0 Gy X-rays and incubated at 37℃ in 5% CO_2 for 30, 60, 90, and 180 min. Immediately after incubation, the cells were chilled at 4℃, pelleted, and stored at -80℃. For positive control, addition of 12-o-tetradecanoyl phorbol acetate (TPA, Sigma, St. Louis, USA) at a final concentration of 83 nmol/L into the culture was replaced by irradiation.

RNA Isolation and Northern Blotting

RNA was extracted from cells using the quanidium/hot-phenol method[5], subjected to electrophoresis in 1% agarose gel after glyoxylation, and electro-transferred on a positively charged nylon membrane (IBI, Optiblot)[6]. The membrane was pro-hybridized with 50% formamide (Bethesda Reaearch Laboratories) containing 1% sodium dodecyl sulfate, 0.1% Ficoll 400 (Pharmacia), 0.1% bovine serum albumin (Sigma, fraction V), 0.1% polyvinyl pyrrolidone, 100 μg/mL sonicated salmon sperm DNA, 50 mmol/L Tris-HCl (pH 7.5), and 50 mmol/L sodium phosphate/5×SSC at 42℃ for 1 h. Hybridization was performed by incubation of the blot membrane with radio-labeled DNA probes for 16 h. To prepare the DNA probe, two probes, forward and reverse specific probes for CAT reporter gene and a template DNA for human β-actin gene were used. Radioactive DNA probes were prepared by the random-primer DNA labeling kit (Bethesda Research Laboratories) with template DNAs and ^{32}P-α-dCTP and diluted to a specific activity of about 10^8 cpm/μg DNA. After hybridization, the membrane was washed twice at 60℃ with 2×SSC containing 1% sodium dodecyl sulfate for 30 min and exposed to the imaging plate (Fuji Photo Film Co.).

Quantitative Northern Blotting

Quantitative auto-radiograms were obtained by 2-dimensional measurement of the imaging plate with the BAS2000 system (Fuji Photo Film Co.). Each sample was analyzed for mRNA expressions of forward and reverse CAT and β-actin on the same gels, and the ratio of CAT (forward-reverse)/ β-actin was calculated to represent the mRNA expression level of CAT. All experiments were repeated twice.

RESULTS

Constructing 4 Plasmids Containing Both junB Promoter Fragment and CAT Report Gene

Restriction enzymes Pst Ⅰ, Kpn I, BamHI, and Xho Ⅰ that digested the pBSV11a plasmid, 250 bp, 590 bp, 900 bp, and 1560 bp *junB* promoter fragments containing TATA box were obtained and inserted to the upstream region of pCAT (B) plasmid respectively. pRSV1, pRSV2, pRSV3, and pRSV4 plasmids were constructed (Fig. 1).

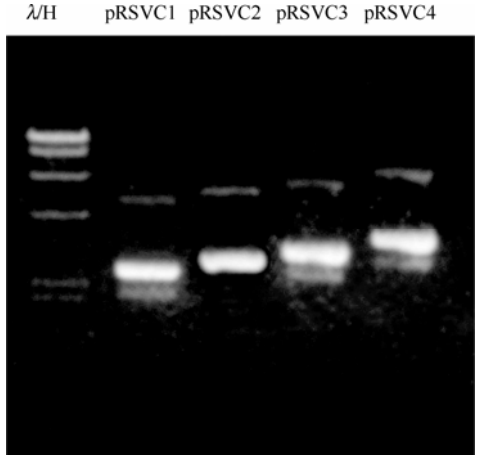


FIG.1. Plasmids pRSVC1, pRSVC2, pRSVC3 and pRSVC4 containing 250, 590, 900, and 1560 base pair fragments of *junB* promoter and CAT reporter gene.

CAT mRNA Expression in L8704 Cells After 2 Gy X-ray Irradiation

The auto-radiograms of Northern blot hybridization

were quantitatively analyzed. Relative level of CAT mRNA was determined by calculating the RNA ratio of CAT (forward-reverse) β-actin. For the positive control, when TPA was added, CAT mRNA expression was induced for 30 min and returned to the normal level 60 min after the addition (data not shown). Similar immediate-early induction was observed in the irradiation group. Level of CAT mRNA reached its peak within 30 min in pRSV3 group and reached its peak within 60 min by 2.0 Gy of X-ray in pRSV4 group (Fig. 2).

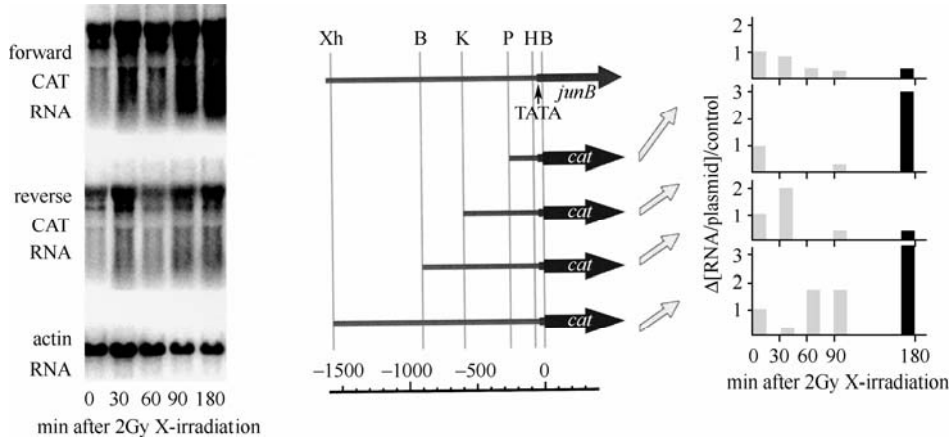


FIG. 2. Northern blot hybridization and analysis of immediate-early inducible function in the upstream region of *junB* gene.

DISCUSSION

The products of *c-jun* proto-oncogene and two related genes, *junB*, and *junD*, are components of transcription factor AP-1, that regulate the expression of a number of TPA-inducible genes by binding to TRE[7–8]. Both *c-jun* and *junB* are rapidly induced by a variety of extracellular stimuli such as serum, growth factors, phorbol esters (TPA), and activators of protein kinase A (PKA). It has been reported that the inverted repeat sequence positioned at -57 to -50 from the transcriptional initiation site of *junB* gene mediates induction of *junB* promoter by TPA and cAMP[9]. Signal transduction *via* the adenylate cyclase/cAMP system involves activation of cAMP-dependent protein kinase A (PKA). Activated PKA transduction message to cell nuclei as protein kinase C (PKC) reacts to TPA or receptor-linked phospholipids turnover. Activation of these kinases finally results in modulation of the activity of transcription factors such as NF-kappa B or members of the AP-1 gene family[10]. Ionizing radiation, as an extracellular stimulus, induces numerous cytokines including IL-1β and *junB* that have protective function against radiation[11], suggesting the emergence of the protection mechanism against radiation damage. Studies on the regulatory mechanism of *junB* genes are important to explore and control the endogenous mechanism for radiation protection.

In our previous studies, *junB* mRNA increased immediately after irradiation, and reached its peak within 30 min, indicating that *junB* gene plays an important role in the process of message transduction and presents the sensitive domain to X-ray irradiation. Conventional reporter assay is not suitable for analyzing molecular mechanisms underlying immediate-early activation, since the late-phase induction mainly contributes to accumulation of reporter gene products[12]. To reveal the immediate-early reaction surrounding the *junB* gene, we examined the promoter function after irradiation using modified reporter assay[13-14], and found that the *junB* promoter was induced by 2 Gy X-ray irradiation. Sequences between -900 and -590 were involved in the regulation of *junB* expression.

The target sequences for binding to X-ray specific protein in this study were longer than 300 nucleotides, larger than the retrotransposon activator[15] and typical binding motif of the nuclear factors. It is presumed that several nuclear components are bound to different sites in this 310 nucleotide region simultaneously. Several nuclear components are necessary to construct the binding complex with DNA. Determining the effect of 310 nucleotides on X-ray irradiation is essential for a further understanding of the mechanism of early response.

At present, it is still unclear why the expression of a gene for a cytokine as well as radio-protector has an immediate responsive phase after X-irradiation, although most of the genes are induced immediately

by X-ray encoding nuclear factors. Further cloning the 310 nucleotid and testing its sensitivity to X-rays is necessary.

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(*Received March* 25, 2005 *Accepted November* 11, 2005)