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UV Irradiation Upregulates Egr-1 Expression at Transcription Level

Ruo-Pan Huang,* Yan Fan, and Alton L. Boynton

Molecular Medicine, Northwest Hospital, Seattle, Washington 98125

Abstract UVC irradiation rapidly and strongly induces protein expression of the early growth response-1 gene (Egr-1) encoding a transcription factor which may have a protective function against UV damage. In this paper, we further investigate mechanisms responsible for such induction. We show that UVC irradiation also induced Egr-1 mRNA expression, increased transcription rate by nuclear run-on assay and stimulated Egr-1 promoter activity by CAT assay. The Egr-1 mRNA stability remained unchanged in UVC-treated cells. On the other hand, UVC irradiation slightly extended Egr-1 protein half-life. The induction of Egr-1 by UVC was observed in many different cell types. UVA and UVB also strongly induced Egr-1 expression. These results indicate that UVC regulates Egr-1 expression at transcription level. The induction pattern of Egr-1 by UV suggests the importance of Egr-1 in the UV response. J. Cell. Biochem. 73:227–236, 1999.

Key words: Egr-1; gene expression; transcription; uv irradiation

With the continuous reduction of ozone layer by environmental pollutants, human population is expected to be exposed to an increased doses of short wavelength of UV irradiation (UVC) [Prather, 1996]. A 2-h walk on a sunny afternoon at 2,000 m altitude afflicts the skin surface with a dose equivalent to 30 J/m^2 UVC [Herrlich and Rahmsdorf, 1994]. One of the consequences of exposure to UVC from thinner ozone layers is an increasing rate of skin cancer [Slaper et al., 1996]. Exposure to UV irradiation in mammalian cells triggers the UV response, which is characterized by the induction of many genes [Grether-Beck et al., 1997; Sachsenmaier et al., 1994]. These genes are associated with many different cellular processes including growth control, response to tissue injury, inflammation, protection function, signal transduction, and transcription factors. Among them, transcription factors such as AP-1 [Devary et al., 1992; Engelberg et al., 1994], NF-kB [Devary et al., 1993], p53 [Lu and Lane, 1993; Scharffetter-Kochanek et al., 1997] and Egr-1 [Huang and Adamson, 1995; Huang et al., 1998a; Huang et al., 1996] are receiving great attention. Recently, we demonstrated that Egr-1 protein level was rapidly and strongly induced by UVC irradiation [Huang and Adamson, 1995; Huang et al., 1998a]. The signal transduction pathway leading to induction of Egr-1 by UVC is involved in oxidative stress, EGFR, Ras and other serine/threonine and tyrosine kinases [Huang and Adamson, 1995; Huang et al., 1996]. The UVC-induced Egr-1 expression plays a certain role in the protection of cells against UVC damage [Huang and Adamson, 1995; Huang et al., 1998a].

Egr-1 is a member of the early growth response gene family, consisting of Egr-1, Egr-2, Egr-3, and Egr-4 [Liu et al., 1998]. They share close homology of their DNA-binding motif, a zinc finger domain. Another member (WT-1) that is more distantly related has been shown to underlie the developmental defect that leads to Wilm's tumor in the kidney. Egr-1 expression is induced in a large number of biological responses in many situations following a similar pattern to c-fos [Sukhatme et al., 1988]. Egr-1 induction is observed in differentiation of embryonal carcinoma cells [Darland et al., 1991; Edwards et al., 1991], lymphocyte [Maltzman et al., 1996; Nguyen et al., 1993], and osteroblasts [Suva et al., 1994; Suva et al., 1991].

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*Correspondence to: Ruo-Pan Huang, Molecular Medicine, Northwest Hospital, 120 Northgate Plaza Suite 230, Seattle WA 98125. E-mail: rphuang@nwlink.com or rhuang@nwhsea.org

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Expression of Egr-1 is also associated with excitatory stimuli in brain synapse [Worley et al., 1991], NMDA receptor activation [Worley et al., 1993], spatial learning performance [Fordyce et al., 1994] and song presentation [Mello et al., 1992; Mello and Ribeiro, 1998]. Furthermore DNA damage such as X-ray [Datta et al., 1993], UVC-radiation [Huang and Adamson, 1995; Huang et al., 1996], and H_2O_2 [Huang and Adamson, 1995] rapidly and transiently induces Egr-1 expression. These data indicate that Egr-1 is probably involved in a diversity of biological events. One possible biological function of Egr-1 is to protect cells from radiation damage [Hallahan et al., 1995; Huang et al., 1998a].

The precise mechanisms for the induction of Egr-1 by UV irradiation remain largely unknown. In this paper, we examined the regulatory mechanisms of UVC-induced Egr-1 expression. We present data to show that UVC regulates Egr-1 expression primarily at the transcription level. We also demonstrate that UVC induced Egr-1 expression in many different cell types and UVA and UVB strongly induced Egr-1 expression. Together, the expression patterns of Egr-1 by UV irradiation suggest the importance of Egr-1 in the UV response.

MATERIALS AND METHODS

UV Irradiation

UV Crosslinker model 2400 (Stratagene, La Jolla, CA) was used to irradiate cells as described before [Huang et al., 1996]. The energy of UV light was precisely controlled by the crosslinker. For UVC irradiation, 254 nm bulbs were used. For UVA and UVB irradiation, 365 nm bulbs and 312 nm bulbs were used respectively. All UV bulbs were purchased from Stratagene.

Cell Culture and Transfection

NIH3T3 cells [Huang and Adamson, 1995] were grown in DMEM containing 5% calf serum at 37°C and 5% CO_2. Transfection and CAT assay were performed as described previously [Huang et al., 1994]. NIH3T3 cells were seeded at 2×10^5 cells in 60 mm tissue culture dishes 20 hours before transfection. 8 μg of plasmids containing 1 μg of pEgr-1B950 CAT [Huang et al., 1994], 2 μg of Lac (β-galactosidase expression vector for control of transfection efficiency) and 5 μg of pUC8 carrier DNA were transfected into NIH3T3 by calcium phosphate precipitation procedure. Sixteen hours later, the medium was removed and cells were exposed to different doses of UVC. Cells were grown in fresh medium for another 24 hours and then harvested for chloramphenical acetyl transferase (CAT) assays. Signals were detected and analyzed using a phosphoimager system (Bio-Rad Laboratories, Hercules, CA).

Metabolic Labeling and Immunoprecipitation

Immunoprecipitation was performed as described elsewhere [Huang et al., 1998a]. Cells were metabolically-labeled by incubation of cells for 2 hours with 400 μCi of 35S-methione (met) and –cysteine (cys) (ICN, Irvine, CA) in met- and cys-free medium. Cells were lysated with RIPA buffer containing 20 mM Tris, pH7.5, 0.15 M NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate. Egr-1 in cell lysates was immunoprecipitated with rabbit anti-Egr-1 antibody. The antigen-antibody complexes were analyzed on 7.5% SDS PAGE gel. The signals were scanned and quantitated by densitometry (Ultroscan XL, LKB, Bromma).

Western Blotting Analysis

Western blotting was carried out as described [Huang et al., 1998b]. Essentially, equal amounts of protein were analyzed by SDS-PAGE. Proteins were transferred to polyvinyline difluoride membrane (Millipore Corp., Bedford, MA) The Egr-1 protein was detected according to ECL protocol (Amersham Corp, Aylesbury, UK) using 1:1000 dilution of a rabbit polyclonal Egr-1 antiserum.

Northern Blotting Analysis

Total RNA was isolated from cultured cells by the guanidine isothiocyanate RNA ZolB method (Cinna/Biotecx Laboratories, Houston TX). Northern blotting was performed as described [Huang et al., 1997]. Briefly, 30 μg of total RNA were denatured by formaldehyde and fractionated on an 1% agarose gel. The RNA was transferred to nitrocellulose filter. Hybridization was carried out with 32p-dCTP labeled Egr-1 cDNA (nucleotides 1–1217) which shares less homology among Egr-1 family at 42°C for overnight.

Nuclear Run-On

Nuclear run-on was performed as essentially described [Darland et al., 1991; Edwards et al.,

1991] with some modifications. Nuclei were prepared from five 100 mm dishes of NIH3T3 cells or cells that were exposed to UVC or stimulated by serum. Nascent RNA transcripts were labeled with 32p UTP (NEN, Boston, MA) and purified by phenol extraction and ethanol precipitation. Denatured linearized plasmids (10 µg) were fixed to nitrocellulose stripes using a slot blot manifold (Bio-Rad Laboratories). Plasmids used were a full length of Egr-1 cDNA clone (pCMV Egr-1), a full length of p53 (pC53-SN3) obtained from Dr. Bert Vogelstein at Johns Hopkins Oncology Center and bluescript as a control plasmid for nonspecific signal. Nitrocellulose stripes were prehybridizated for 16 hours in hybridization buffer (5xSSPE, 50% formamide). Hybridization was performed in fresh hybridization buffer with labeled RNA for 2–4 days at 42°C. Strips were washed and treated with RNase (10µg/ml) to remove background as described [Edwards et al., 1991; Wilson et al., 1992].

Immunostaining

The assay was carried out as described in [Huang et al., 1994]. Cells before or after UVC treatment were fixed in 100% methanol. Endogenous peroxidase activity was inactivated with 0.3% H_2O_2 in methanol. Nonspecific binding was blocked with 2% normal goat serum. Then the slides were incubated overnight at 4°C with anti-Egr-1 antiserum (1/100 dilution). Antigen-antibody complexes were detected by subsequent incubation with biotinylated goat anti-rabbit antibody at a 1:200 dilution and avidin-biotin complex conjugated to horseradish peroxidase with 3′-3′-diaminobenzidine-4-HCl (DAB) as the chromogen for visualization. The slides were then lightly counterstained with hematoxylin.

Subcellular Fractionation

NIH3T3 cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 3 volumes of Dignam A buffer containing 10 mM HEPES (pH7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, followed by incubation on ice for 15 min. Cells were then lysed by pushing through a 25 gauge needle 5 times. The cell lysates were centrifuged in an Eppendorf centrifuge at 14 k rpm for 30 seconds to pellet the nuclei. The disruption of cell membrane and intact of nuclei was monitored under a microscope. To lyse the nuclei, nuclei were resuspended in 1 volume of Dignam C buffer including 20 mM HEPES, pH7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM DTT, rotated at 4°C for 30 minutes and spun for 5 min at 14 k rpm in an Eppendorf centrifuge. The supernatant was then dialyzed against Dignam buffer D consisting of 20 mM HEPES pH7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT.

RESULTS

Egr-1 mRNA is Up-Regulated Upon UVC Irradiation Exposure at the Transcription Level

NIH3T3 cells in log-phase expressed a fairly low steady-state level of Egr-1 mRNA and this level was rapidly and greatly increased upon exposure to UVC irradiation (40 J/m^2) (Fig. 1). In Northern blot analysis, using a probe covering from 1 to 1217 bp, 10 min after UVC irradiation, Egr-1 mRNA level increased 3–4 folds. The maximal induction was observed between 30 and 60 min. After 1 hour, Egr-1 level started to decline and reached the baseline level at about 2 hours. As positive control, serum stimulation also rapidly induced Egr-1 transcript level at 30 min. To determine whether UVC signaling regulates Egr-1 expression through a transcription mechanism, nuclear run-on assay was performed. Nuclei were isolated from NIH3T3 cells with or without UVC irradiation or with

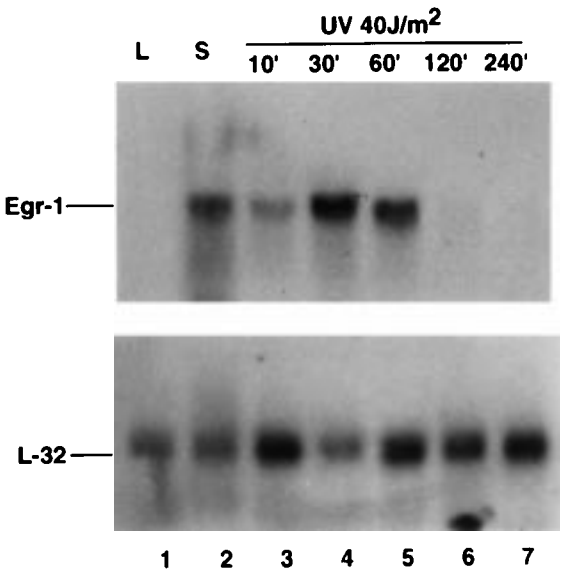

Fig. 1. Egr-1 mRNA is rapidly accumulated upon UVC irradiation. Total RNA from unirradiated cells (**lane 1**) or serum stimulated cells (**lane 2**) or UVC-treated cells (**lane 3–7**) was isolated and subjected to Northern blot analysis with Egr-1 (upper) or L-32 (lower) as probes.

serum stimulation. Fig. 2 shows that Egr-1 transcription rate rises about 5 folds upon UVC irradiation. A similar rise is also observed in serum stimulation. In contrast, UVC and serum did not increase transcription rate of p53. This is consistent with the early observation that UVC up-regulated p53 expression at the translation level [Maltzman and Czyzyk, 1984].

If induction of Egr-1 by UVC is regulated at the transcription level, such induction should not be abrogated in the presence of protein synthesis inhibitor. Indeed as shown in Fig. 3, Cycloheximide treatment did not diminish the magnitude of induced expression of Egr-1 in response to UVC irradiation. Cycloheximide treatment alone also rapidly induced Egr-1 expression as has been demonstrated in a number of immediate early growth response gene [Darland et al., 1991; Edwards et al., 1991]. There was no superinduction of Egr-1 expression in response to UVC irradiation as was observed in RA and serum stimulated fibroblast simultaneously treated with cycloheximide [Darland et al., 1991; Edwards et al., 1991]. These observations suggest that UVC-induced Egr-1 expression in NIH3T3 cells is independent of de novo protein synthesis but dependent on the preexisting trans-acting transcription factors.

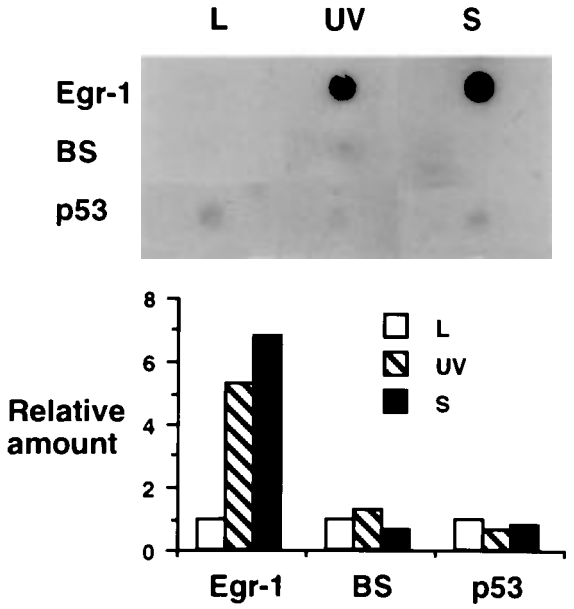

Fig. 2. Increase of Egr-1 transcription rate in response to UVC irradiation. Nuclei of NIH3T3 cells were isolated from log phase growing cells (L), UVC treated cells (40 J/m^2, 30 min, UV) and 20% serum-stimulated cells (30 min, S) and nuclear run-on transcription assays were performed with Egr-1 cDNA, Bluescript DNA (BS) and p53 cDNA. The lower panel is the quantitation of relative transcription rates determined by densitometry.

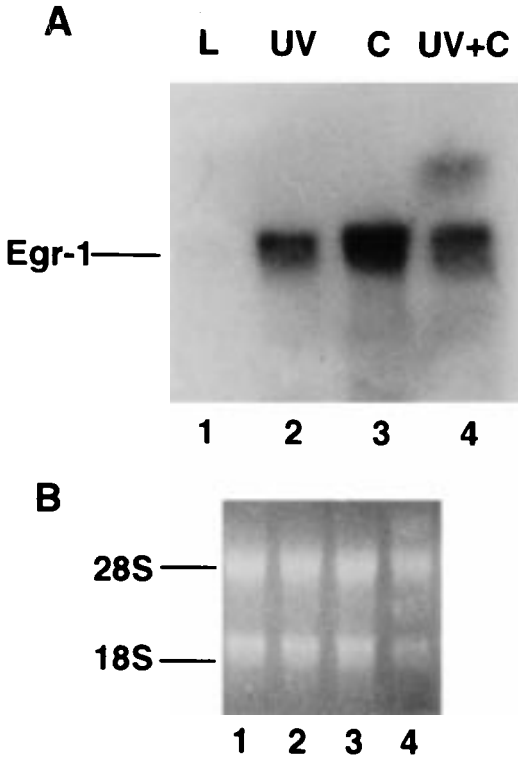

Fig. 3. Protein synthesis inhibitor fails to block the induction of Egr-1 by UVC. NIH3T3 cells in log phase (**lane 1**) were treated with either UVC (**lane 2**) or cycloheximide (**lane 3**) or both UVC and cycloheximide (**lane 4**) for 2 hours. Total RNA was then isolated and analyzed by Northern blot.

To further demonstrate that UVC regulates Egr-1 expression at the transcription level and to begin to identify a likely target for the signal pathway involved in the UVC response, CAT assays were performed. NIH3T3 cells were transfected with 950bp Egr-1 CAT reporter gene which contains a full Egr-1 5′ flanking region fused to the structure gene encoding for CAT. As shown in Fig. 4, CAT activity was highly stimulated after exposure of transfected cells to increasing doses of UV. The induction of CAT activity by UVC was dose-dependent. As control, UVC did not stimulate control vector activity.

Effect of UVC on Egr-1 Protein and Message Stability

The increase in accumulated Egr-1 transcripts after UVC treatment may also be explained by the increased stability of the message. The degradation rate of Egr-1 mRNA in response to UVC was measured using actinomycin D or α-aminitin to inhibit RNA synthesis. Log-phase or UV-stimulated (40 J/m^2, 30 min)

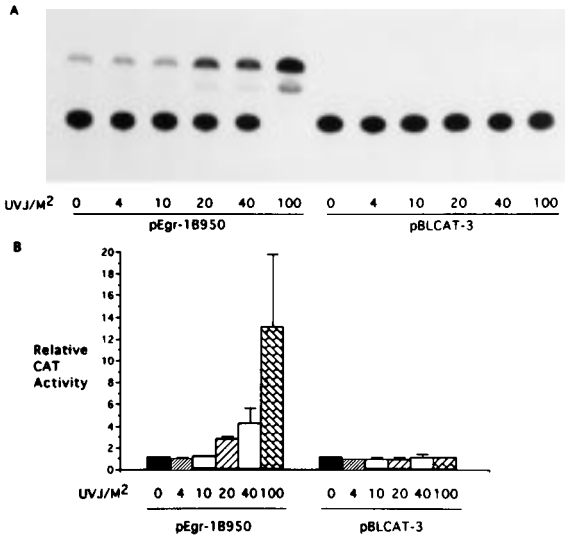

Fig. 4. UVC stimulates Egr-1 promoter activity. Transient transfection CAT assays were used to determine the Egr-1 full promoter pEgr-1B950 activity in response to different doses of UVC in NIH3T3 cells.

NIH3T3 cells were treated with actinomycin D (Fig. 5A) or α-aminitin (Fig. 5B). At different time points, cells were harvested for RNA extraction and determination of Egr-1 mRNA levels by Northern blotting analysis. Densitomitric scan of the resulting fluorographs (average of results in Fig. 5A and B) showed that the rates of degradation of mRNA in log phase growing or UVC-treated NIH3T3 cells were quite similar with a half life of about 40 min. Therefore the increase in Egr-1 mRNA in UV-treated cells most likely results from higher rates of transcription.

The changing levels of Egr-1 protein observed in response to UVC irradiation could also be due to Egr-1 protein stability. Thus, we measured the degradation rate of Egr-1 protein by incorporation of 35S-met and -cys into the culture for 2 hours before chasing the labeled products in the presence of excess cold methionine. At intervals up to 4 hours, the samples were analyzed for the presence of Egr-1 protein by immunoprecipitation assay. The results shown in Fig. 6 were averaged for three separate experiments. Egr-1 protein in NIH3T3 cells had a half life of about 1 hour, but in UVC-treated cells, the half-life of Egr-1 was about 1 hour 45 min. In serum-stimulated cells, the half-life was extended to 2 hours and 15 min. These studies demonstrated that Egr-1 protein is slightly more stable in UVC and serum-stimulated NIH3T3 cells and this difference may only have small impact, if any, on the accumulated levels of Egr-1 protein observed in response to UVC irradiation.

The Cellular Location of Egr-1 Protein in Response to UVC Irradiation

Translocation of transcription factors and other proteins in response to extracellular signals have been shown to be an important mechanism in the regulation of these protein activities [Kanemitsu et al., 1997]. Egr-1 protein has been reported to be located in nucleus. To examine whether UVC irradiation alters the cellular location of Egr-1 protein, histochemical staining of NIH3T3 cells before and after UVC irradiation was carried out. As shown in Fig. 7A, UVC irradiation strongly induces Egr-1 expression and Egr-1 is exclusively localized in nucleus. To further confirm this observation, nuclear extraction and cytoplasmic extraction prepared from log-phase growing cells, serum-stimulated cells and UVC irradiated cells were subjected to Western blotting analysis. As shown in Fig. 7B, Egr-1 protein was only detected in nuclear extracted fractions. Both histochemical and biochemical studies provide a strong evidence that Egr-1 protein accumulated in the nucleus in response to UVC irradiation.

Expression of Egr-1 in Response to UVA and UVB

Since exposure to UVA and UVB accounts for most human skin malignancies and UVC irradiation does not penetrate our atmosphere as well as UVA and UVB, we measured the induction of Egr-1 in response to UVA and UVB. As shown in Fig. 8, both UVA and UVB also strongly induced Egr-1 expression. Time course showed that induction of Egr-1 expression started as early as 10 min after UVA or UVB irradiation and reached the peak between 1 hour and 2 hours. Interestingly, even 4 J/m^2 of UVA and UVB strongly induced Egr-1 expression. Slow migrating species of Egr-1 were constantly observed in response to UVA, UVB and UVC irradiation compared to serum stimulation. Recently we demonstrated that these slow migrating species were phosphorylated forms of Egr-1 [Huang et al., 1998a].

The induction of Egr-1 by UVC appears to be a general phenomenon in many cell types. As shown in Table I, the induction of Egr-1 in response to UVC irradiation has been observed in various cell types including fibroblast, epithelial cell, nerve cells, melanoma cells and epi-

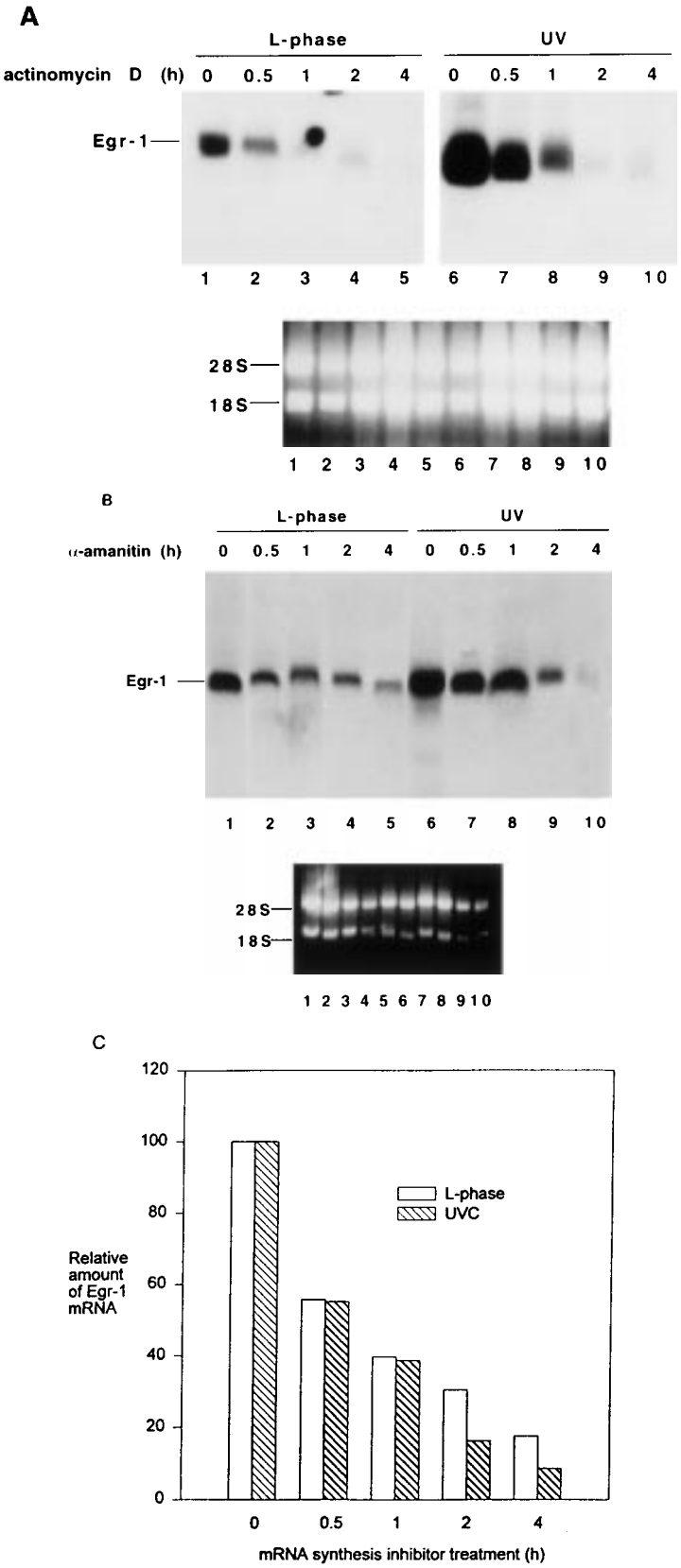

Fig. 5. Stability of Egr-1 mRNA in response to UVC. NIH3T3 cells in log phase (L) or UVC-irradiated (40 J/m^2) were treated with 10 μg/ml actinomycin (**A**) or 2 μg/ml α-amintin (**B**). RNA was then isolated at the time indicated and assayed by Northern blot using Egr-1 cDNA as probe. The average of quantitation of autofluographic signals from A and B is shown in **C**.

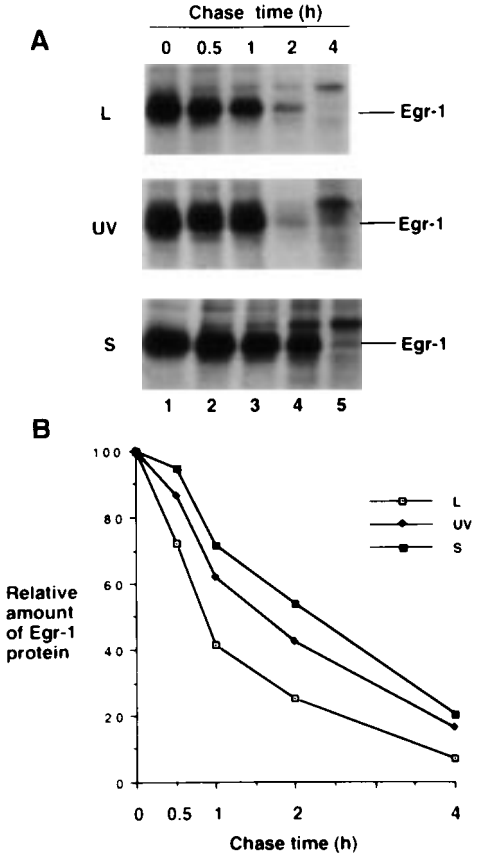

Fig. 6. Stability of Egr-1 protein. **A.** Labeled cells were harvested at times indicated after the addition of fresh medium. The cell lysates were immunoprecipitated with anti-Egr-1 serum. L, log phase growth; UVC, 40 J/m^2, 1 hour; S, 20% serum-stimulation. The experiments were repeated three times and only one representative result is shown. **B.** The results on autofluographs were optically measured. The levels of Egr-1 protein averaged from three experiments at each time point were plotted.

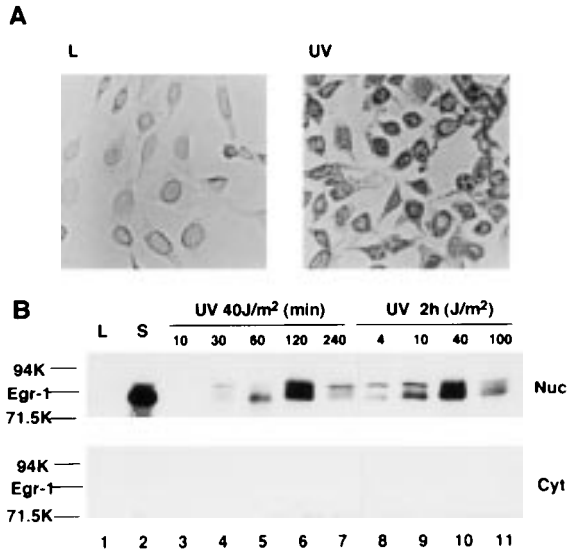

Fig. 7. Localization of Egr-1 protein. **A.** immunohistochemistry for Egr-1 protein stimulated with UVC irradiation (40 J/m^2, 2 h) were stained with affinity-purified rabbit anti-Egr-1 (4 μμg/ml) as described in Materials and Methods. Strong staining is detected in the nucleus after UVC exposure. As control, there is no staining of UVC-stimulated NIH3T3 by preimmune IgG at 5 μg/ml (data not shown). **B.** Western blot analysis of Egr-1 protein. NIH3T3 cells were treated by UVC irradiation as indicated or serum (20%, 1 h). The nuclear and cytoplasmic portions were prepared and subjected to Western blot using anti-Egr-1 antibody. Egr-1 protein is only detected in the nuclear fraction.

demic cells, in many species such as human, mouse and dog, and in different organs such as lung, brain, skin, mammary gland, bone, cervix, kidney and embryo. Among all of the cell lines we examined, only three cell lines (HT1080, T47D and ZR751) showed no response to UVC irradiation as well as TPA and serum stimulation. This lack of response is not due to the deletion of the Egr-1 gene since the Egr-1 gene is intact in Southern blotting analysis [Huang et al., 1997; our unpublished data]. Such cell lines provide a good system to study the function of Egr-1 and an opportunity to examine the regulation mechanism of Egr-1 by UV irradiation.

DISCUSSION

Previously we showed that UVC strongly induces Egr-1 protein level. In this communication, we investigated the possible mechanisms for such induction. By using Northern blotting, nuclear run-on and CAT assay, we show that induction of Egr-1 by UVC irradiation operated primarily at the transcription level. Additional level of regulation may also be somehow partially controlled at translation level since UVC irradiation slightly increased the Egr-1 protein stability. UV induces as many as 50 different gene expression. Most of them are regulated at transcription levels such c-jun and c-fos [Devary et al., 1992]. A few of UV-induced genes are controlled at translation levels exemplified by p53 [Maltzman and Czyzyk, 1984; Siliciano et al., 1997]. UV also enhances the activity of certain factors via post-modification mechanisms such as phosphorylation [Kapoor and Lozano, 1998; Siliciano et al., 1997]. Therefore, regulation of gene activity by UV irradiation is a complex process. The regulation of Egr-1 activity by UV irradiation may operate at these three different levels.

The induction pattern of Egr-1 by UV irradiation highlights the biological significance of Egr-1 in the UV response. Firstly, Egr-1 is earli-

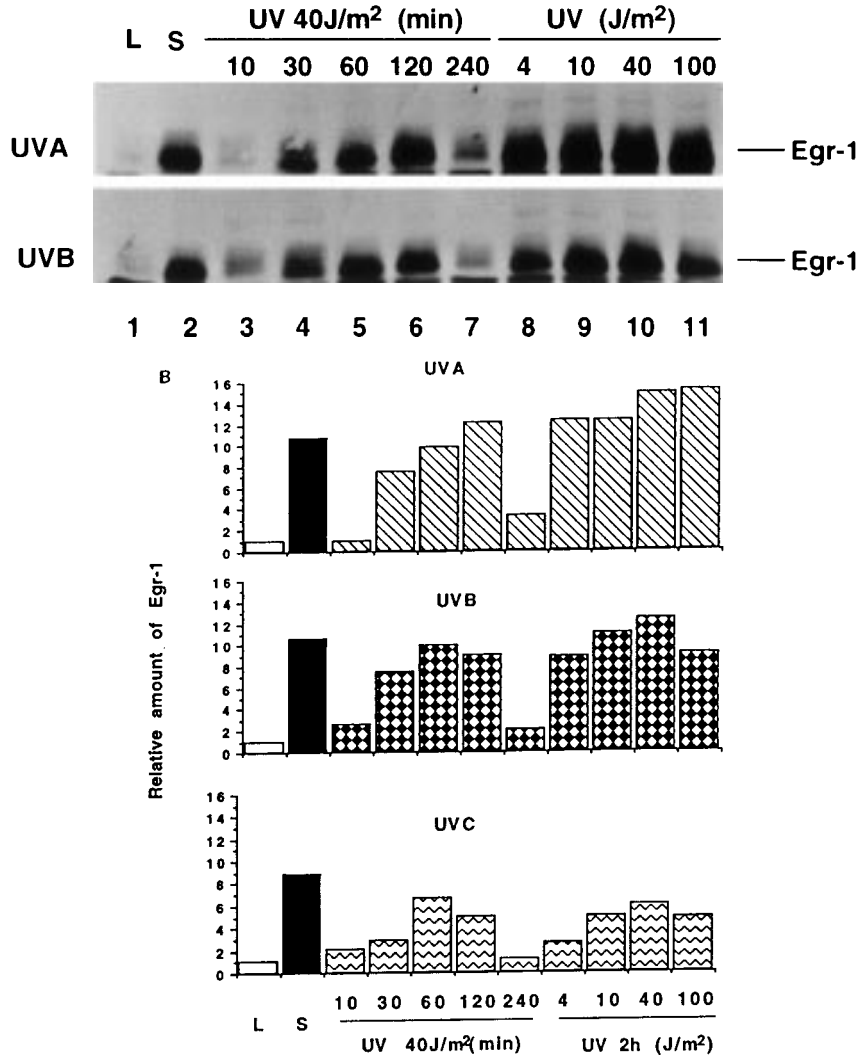

Fig. 8. Induction of Egr-1 expression by UVA and UVB. **A:** Log phase NIH3T3 cells (**lane 1**) were exposed to 40 J/m^2 of UVA or UVB and harvested for Western blotting at the time indicated (**lane 3 to 7**). From **lane 8** to **11**, cells were treated with different doses of UVA or UVB as shown and 2 h later were harvested for analysis by Western blot. **Lane 2** are cells stimulated with 20% FCS for 1 h (S). **B:** The results for UVA and UVB (from A) and for UVC (from Huang and Adamson, 1995) were quantitated by densitometry.

est and strongest response to UV in our system, suggesting that Egr-1 is a major primary response gene to UV. Secondly, UV-induced Egr-1 expression was observed in many different cell types, suggesting that UV-mediated Egr-1 expression is a highly conserved signal pathway. Thirdly, all three different UV sources, UVA, UVB and UVC strongly induced Egr-1 expression, suggesting that Egr-1 may have a broad and general role in the UV response. Indeed, our recent work indicated that Egr-1 plays a role in the protection of cells against UV damage [Huang and Adamson, 1995; Huang et al., 1998a]. It has been demonstrated that Egr-1 has a protective function in exposure to X-ray irradiation [Hallahan et al., 1995]. These observations suggest that Egr-1 may be an important gene in early evolutionary stages when cells need to maintain high population numbers in response to environmental damage.

The signal transduction pathway leading to induction of Egr-1 by UV irradiation is a complex process. We have shown that one of predominant signal pathway is use of pre-existing EGFR-mediated signal transduction pathway [Huang et al., 1996]. Since Egr-1 is rapidly, strongly and generally responsive to UV irradiation, analysis of mechanisms responsible for its activation should shed more light on this unique signaling pathway.

TABLE I. Induction of Egr-1 in Response to UVC[a]

Cell line	Type	Response
NIH3T3	Mouse embryo fibroblast	+
HC11	Mouse mammary epithelia	+
MDCK	Dog kidney epithelia	+
A431	Human epidermoid carcinoma	+
HF	Human lung fibroblast	+
MCF10A	Human mammary epithelia	+
184A1N4	Human mammary epithelia	+
MDA-MB-468	Human breast carcinoma	+
MDA-MB-231	Human breast carcinoma	+
BT20	Human breast carcinoma	+
MCF-7	Human breast carcinoma	+
ZR-75-1	Human breast carcinoma	−
T-47D	Human breast carcinoma	−
5637	Human bladder carcinoma	+
SAOS-2	Human osterosarcoma	+
Hela	Human cervix carcinoma	+
U251	Human glioblastoma	+
U-373MG	Human glioblastoma	+
T98G	Human glioblastoma	+
U-87MG	Human glioblastoma	+
IMR-32	Human neuroblastoma	+
WI-38	Human lung fibroblast	+
U937	Human lymphoma	+
HT1080	Human fibrosarcoma	−
IVCL-1	Human melanoma	+
T51B	Rat liver epithelia	+
TRMP	Rat liver epithelia	+

[a]Cells in log phase were treated with or without UVC irradiation (40 J/m^2). Egr-1 levels were then determined by Western blot.

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