Characterization of Expression of UV-Inducible Gene (UV100 and UV150) in Caenorhabditis elegans

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The present study intends to characterize the DNA damage-inducible responses in Caenorhabditis elegans. To study UV-inducible responses in C. elegans, two UV-inducible cDNA clones were isolated from C. elegans by using subtraction hybridization method. To investigate the expression of isolated genes, UV100 and UV150, the cellular levels of the transcript were determined by Northern blot analysis after UV-irradiation. The transcripts of isolated gene increased rapidly and reached maximum accumulation after UV-irradiation. Compared to the message levels of control, the levels of maximal increase were approximately 2 folds to UV-irradiation. These results implied that the effects of damaging agents are complex and different regulatory pathways exist for the induction of these genes. To study the function of UV100 and UV150 gene in response to UV irradiation, we carried out a RNAi experiment and investigated the UV sensivity. This result indicated that UV100 gene involved in stage-specific repair pathway or regulated by development.

Key words – C. elegans, UV100 and UV150 UV-inducible gene, RNAi, DNA Damage, DNA repair, cell viability.

Introduction

DNA can be damaged by a wide range of physical and chemical agents, both inside the cells and from the environment. This damage can result in the accumulation of mutation for increasing chances of survival. To overcome some of these deleterious effects, a variety of DNA repair systems evolved early. The most versatile of these is the nucleotide excision repair (NER) pathway that repairs a broad spectrum of lesions caused by agents such as UV irradiation and chemical mutagens. NER is found in all of the different kingdoms of life, including eubacteria, archaee, and eukaryotes ex-cept nematode Caenorhabditis elegans [C. elegans][6,9].

One of the most interesting aspects of cellular response to DNA damage may be the regulation of its activity. In E. coli, four major regulatory systems that control the expression of several genes induced by DNA damages of environmental stresses have been identified: the SOS response, the adaptive response to alkylation damage, the response to oxidative damage, and the heat-shock response[7,8,13]. The SOS response which plays multiple roles in DNA repair, recombination, and mutagenesis provides a molecular model of coordinate gene regulation. A number of DNA damage-inducible genes were recently identified and characterized in eukaryotes. However, available data do not indicate any obvious similarity to inducible responses in prokaryotes[1,2].

In higher eukaryotic cells, several cDNA clones showing the inducibility by damaging agents were isolated[2,5]. Although the functions of most genes are not yet defined, these studies elicited several interesting general insights. Firstly, multiple and diverse DNA-damaging agents can induce expression of several genes, and damage-inducible genes may be ubiquitous[14,16]. Secondly, constitutive expression and inducibility of genes can be influenced by the DNA repair capacity of cells. Thirdly, heat shock treatment induces the expression of some genes which are induced by DNA-damaging agents[12,15].

Among these genes, DIN1 was identified as a gene encoding a regulatory subunit of ribonucleotide reductase (RNR3). Many of yeast genes with known functions are also inducible by DNA damage. These include RNR2, a gene encoding the small subunit of ribonucleotide reductase[19,20]; CDC8 encodes thymidylate kinase ; UBI4, which encodes polyubiquitin ; POLI, which encodes DNA polymerase α; CDC9, the gene for DNA ligase[15,17]. Among these, RNR2 and CDC8 could play indirect roles in DNA repair by providing precursors for repair synthesis. The
CDC6, CDC9, and POL1 genes are also cell-cycle regulated. The enhanced expression of CDC9 following exposure to UV-irradiation has been demonstrated in non-cycling stationary phase cultures[11,18]. Therefore, the induction of this gene is the direct response to UV-irradiation, rather than simple synchronization of cell cycle. The UBI4 gene which is required for the degradation of proteins is increased in stationary phase and meiosis. The induction of this gene by DNA damaging agents suggests that some genes could be induced in response to aberrant proteins generated by the treatment of DNA damaging agents[28].

In recent years, most of the DNA repair studies employed disassociated cells, usually immortalized, in tissue culture. However, little is known about the effects of DNA damage on an intact organism and DNA damage processing that is related to development and aging. Since the free-living organism C. elegans emerged rapidly as an organism, to study many biological phenomena, particularly related to development, this organism may be a good model system to investigate developmental and tissuespecific DNA repair.

Several of the basic DNA repair pathways that are operative in C. elegans have been elucidated[9,25,26]. In addition, 9 radiation sensitive (rad) mutants show UV sensitivity, indicating C. elegans excision repair capacity[9]. Nonetheless, no genes of excision repair have been identified. To manifest DNA damage processing at the molecular, cellular, and organism levels in C. elegans, it is absolutely necessary to clone DNA repair genes and characterize their mutants. The present study intends to characterize the DNA damage-inducible responses in eukaryotic cells. To further understand the function of UV-inducible, C. elegans was used in this study as a model system for higher eukaryotes.

Materials and Methods

Strains, cell culture, and genetic methods

E. coli strain DH5α (F- endA1, hsdR17, r4, mK-), supE44, thi-1, recA1, gyrA96, relA1, lacZΔM15) and yeast c. elegans strain JY741 (b- ade6- M210 leu1-32 ura4-D18) was used for this study. E. coli strains were grown on LB media (1% tryptone, 0.5% sodium chloride, 0.5% yeast extract).

A hermaphrodite of the wild-type N2 Bristol strain was grown on NGM plates that feed with E. coli OP50 cells at 20°C[26]. Worms at mixed stages were collected by a washing buffer (200 mM Tris-HCl pH 7.5, 100 mM EDTA pH 8.0, 400 mM NaCl). They were precipitated by centrifugation at 2,500 rpm for 5 min. To separate eggs, the collected worms were lysed in 10 volume of a lysis solution (1% NaOCl and 0.5 M NaOH) for 5 min. The eggs were then collected from the lysates by centrifugation at 800 rpm for 2 min.

Standard molecular biology techniques were employed as described[23]. C. elegans chromosomal DNAs were prepared according to the methods of Cho[3,4].

RNA isolation and RT-PCR

Total RNA was isolated from mixed-stage worms of the wild-type N2 strain by using TRI reagent (MRC). The mRNA was further purified with an oligotex mRNA kit (Qiagen), according to the manufacturer’s instructions. RT-PCR was performed with approximately 4 μg of the obtained mRNA, oligo dT primer, and the following primer sets which specifically amplify the UV inducible gene: forward: 5’-TGG CGA TCT TAT TTA GCC TAA GTA CCC CCG CC3’, reverse: 5’-AGG TGC CGT AGA GCA CCC CTC AGT CGG GCC TGG AGT-3. The PCR products were digested by both HindIII and BglIII, then cloned into the pBlueScript SK vector (Stratagen) using blue/white colony selection.

Subtraction Hybridization

For subtraction hybridization, 15 μg of biotinylated DNA were mixed with 3μg of single stranded DNA from UV-induced cDNA library and resuspended in 1 X hybridization buffer(100 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1mM EDTA, 0.1% SDS, 1μg/ml poly(A)). The mixture was boiled for 1 minute and then incubated at 68°C for 36 hours. After hybridization, the biotinylated DNA was removed by the addition of streptavidin and extraction with phenol/chloroform. Remaining DNA was converted to double stranded DNA using T7 primer and klenow fragment. Ten μg of DNA solution was mixed with reaction buffer to final concentrations of 40mM potassium phosphate, pH 7.5, 6.6 mM MgCl2, 500μM dNTP, 20 ng T7 primer, 5 unit klenow fragment, 100 unit of T4 DNA ligase, and then incubated at 1 6°C for 6 hours. This mixture was used directly for transformation into E. coli cells.

DNA Sequencing

The insert contained in the plasmid, UV100 and UV150, as completely sequenced on both strands with a Bigdye
Northern en analysis

RNA samples were fractionated on a 1.2% formaldehyde-agarose gel, transferred to a Hybond N filter (Amersham Pharmacia Biotec), and hybridized with a [α-32P]dCTP nick-translated DNA probe (400 bp of the 5' end region of the isolated clone) in a Rapid-hybridization solution (Amersham) for 3 h at 68°C. The filter was washed in 2× SSC and 0.1% SDS at 40°C for 10 min, and finally in 0.2× SSC and 0.1% SDS for 5 min. Identical results were obtained with two independent worms and RNA preparations

Double-stranded RNA interference and measurement of UV sensitivity

To investigate the null phenotype of the isolated gene, RNAi was carried out using the method previously described[3,10]. For the preparation of double-stranded (ds) RNA, the plasmids that contained the cDNA were linearized with the BamHI or Sall restriction enzymes. The linearized plasmid DNA was extracted by phenol, precipitated by ethanol, and resuspended in DEPC-treated water. Antisense RNA was synthesized using the BamHI-digested plasmid DNA (0.5 µg), T7 RNA polymerase (5 units, Stratagene), ribonucleoside triphosphates (4 mM NTPs, 0.4 mM each), and RNase inhibitor (5 units; Fermentas, Japan) in a reaction buffer (40 mM Tris·HCl, pH 8.0, 8 mM MgCl2, 2 mM spermidine, 50 mM NaCl, 18 mM DTT) at 37°C for 2 h. Sense RNA was synthesized under the same reaction conditions as for antisense RNA, except for the use of Sall-treated DNA (0.5 µg) and T3 RNA polymerase (5 units). After the RNA synthesis, RQ1 RNase-free DNase (2 units, TaKaRa) was added to degrade the template DNA, then phenol (pH 4.5) extraction and ethanol precipitation followed. Antisense and sense RNAs were mixed in equivalent amounts. The RNA mixture (1 µg/µl) was microinjected into the intestine of N2 young adults. Microinjected worms were immediately placed on a NGM plate that was seeded with E. coli OP50 and transferred to a new plate 12 h after microinjection. In order to examine the effects of RNAi on C. elegans sensitivity to UV radiation, F0 worms were irradiated with a germicidal lamp (λ max = 254 nm) at doses of 25, 50, 100, and 200 J/m2[6,25,27]. Then eggs were laid. The hatching rate of eggs laid during the next 14 h was measured. Egg survival (F1 generation) was scored 3 d later. To further examine the effects of RNAi in the next generation, F2 eggs were collected from adult F1 progeny. The F2 eggs were irradiated with UV. The hatching rate and egg survival were scored as described in the F1 generation.

Results and Discussion

Isolation of UV inducible gene

DNA damage triggers complex cellular responses in E. coli that include induction of several genes involved in repair, recombination and mutagenesis. The SOS response is an example of a stress response, where an environmental stress condition activates transcription of a group of genes[18,24]. In these kinds of responses to environmental stress, heat shock response has been found and well characterized in both prokaryotic and eukaryotic organisms[12]. However, there is no direct evidence for an SOS-like response in eukaryotic cells. For the characterization of UV-inducible response in eukaryotic cells, UV-inducible genes were isolated from C. elegans cells by subtraction hybridization methods.

The isolation of UV-inducible genes from C. elegans was accomplished using subtraction hybridization method. For the induction of UV-inducible transcripts, C. elegans in young adult stage were irradiated with 50 J/m2 of UV-light and incubated for 24 hours. Poly(A)+ RNA isolation from UV-induced or normal cells were used for templates for cDNA synthesis. For the enrichment of UV-inducible cDNA fragments, subtraction hybridization was performed. About 10 µg of single stranded DNA obtained from normal RNA was hybridization with UV-induced RNA. The unhybridized UV-induced RNA was made cDNA and then ligated into TA cloning vector. After transformation into XL1 blue cells, about 20 clones were obtained as substracted library. To confirm UV-inducibility of these clones, dot blot analysis was performed (data not shown). Among these cDNA clone, two clones showed higher intensity with UV-induced. These isolated genes were designated as UV100 and UV150, respectively. To determine whether the inducibility of the isolated UV-inducible genes by UV-irradiation, total RNAs isolated from C. elegans cells were analyzed by Northern blotting using with in vitro transcribed RNA probes. To confirm UV-inducibility of the isolated gene, northern blot analysis was performed (Fig. 1). These results were indicated that the UV100 and UV150 genes were induced by UV-irradiation. ACTI gene was used as an internal control.
Fig. 1. Increase of UV100 and UV150 transcript levels by UV-irradiation. C. elegans cells were irradiated with 50 J/m² of UV-light and incubated at 24 hours. At the times indicated, total RNA was isolated and the transcript levels were determined by Northern blot analysis. ACT1 gene was used as an internal control.

Figure 2 shows the induction kinetics of UV100 and UV150 genes by UV-irradiation. The transcripts of UV100 and UV150 genes increased rapidly and reached maximum accumulation at UV-irradiation of 120 J/m². Compared to the message levels of control, the levels of maximal increase were approximately 2 folds to UV-irradiation. These results implied that the effects of damaging agents are complex and different regulatory pathways exist for the induction of these genes. These results imply that UV100 and UV150 gene products might be involved in specific cellular response such as DNA repair, recombination or mutagenesis.

Nucleotide Sequencing of isolated gene

C. elegans database for sequences that are homologous to yeast revealed that the C. elegans gene, K12Z6.3, is similar to these proteins. Although most of the predicted genes in C. elegans have already been transcribed[22,28], the C. elegans UV inducible gene has not yet been cloned. In this study, RT-PCR experiments yielded a single band of the expected size (data not shown) when amplified with the two primers and mRNA of the mixed stage of C. elegans. The cloned cDNA was completely sequenced (Fig. 3). The cloned gene contains 826 bp in length and this is identical to the ORF sequence of K12Z6.3. It encodes a predicted protein of 267 amino acids.

A comparison of the cloned gene sequences with other UV inducible gene, the isolated gene revealed 53% identity. The UV100 protein contains a coiled-coil structure and zinc binding site. These results revealed that isolated gene is a metallocprotein because of its zinc binding[22].

Effect of double-stranded RNA interference against UV

To study the function of the UV100 and UV150 gene in response to UV irradiation, we carried out a RNAi experiment and investigated the UV sensitivity. The dsRNA (1 μg/μl) was microinjected into the intestine of N2 young adults. The microinjected P0 worms were irradiated with a germicidal lamp (λmax = 254 nm) at doses of 25, 50, 100,
and 200 J/m² and eggs (F1 generation) were laid. The hatching rate was measured during the next 14 h after the eggs were laid. The survival rate of the F1 progeny was scored after 3-5 d. The hatching rate of F1 eggs from RNAi animals that were irradiated with UV was lower than those of F1 eggs from N2 animals (Fig. 4). The survival rate of the F1 progeny from the UV (50 J/m²)-irradiated RNAi animals was 34% lower than those of the F1 progeny of N2 animals. This difference was representative of various doses (Fig. 4). These results suggest that UV100 gene may function at egg development and worm growth in response to DNA damage. F2 eggs were collected from adult F1 progeny, then the F2 eggs were irradiated with UV light at doses of 25, 50, 100, and 200 J/m². The hatching rates of the F2 eggs and the survival rate of the F2 progeny was significantly lower that those of the unirradiated eggs and progeny. In general, the extent of the hatching and survival rates of the F2 generation is lower than those of the F1 generation. This may be due to the direct irradiation to eggs. Since the viability of the F2 progeny of the N2 animal strain at 200 J/m² is less than 4%, the measurement at 200 J/m² of the survival rate of the F2 progeny of the RNAi animals is considered insignificant.

A RNAi experiment is a powerful approach to show the null phenotype that concerns the specific gene. In this study, we investigated UV sensitivity by irradiation to the RNAi and wild-type N2 animal. Although no clear phenotype was observed in the RNAi of UV100 and UV150, significant embryonic and survival lethality were observed upon UV-irradiation. Since rad mutants of C. elegans show striking stage-specific variations in UV hypersensitivity[7], then it would be necessary to check UV sensitivity with C. elegans at different stages if UV100 and UV150 gene involved in stage-specific repair pathways or regulated by development. Even though RNAi seems to generate a loss-of-function temporarily, a UV100 mutant may provide a clear phenotypic feature of UV sensitivity.

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References

초록: Caenorhabditis elegans에서 분리한 유도유전자 (UV100과 UV150)의 발현 및 특성에 관한 연구

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본 연구는 DNA 상해유도기작을 규명하기 위하여 고마선충 (Caenorhabditis elegans) 으로부터 subtraction hybridization 방법을 이용하여 자외선 유도 유전자인 UV100과 UV150을 분리하고 그 유전자 구조와 발현양상을 조사하였다. 분리한 유전자의 발현양상을 Northern hybridization 방법으로 살펴본 결과 자외선 조사 후에 최대 2배 이상의 발현 증가를 나타내었다. 이 결과 이 미 발현한 다른 UV-inducible 유전자와 유사하게 UV100과 UV150 유전자는 자외선에 의해서만 발현이 증가될 수 있었다. 또한 분리한 유전자의 기능을 알기 위하여 RNAi 실험을 한 결과 분리한 자외선 유도유전자는 발생단계에 따라 다양한 DNA 회복기작을 나타내는 탈 수 있었다.