Components of DNA Damage Checkpoint Pathway Regulate UV Exposure–Dependent Alterations of Gene Expression of *FHIT* and *WWOX* at Chromosome Fragile Sites

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Abstract

Common chromosome fragile sites are highly recombinogenic and susceptible to deletions during the development of environmental carcinogen–induced epithelial tumors. Previous studies showed that not only genetic but also epigenetic alterations in cancerous cells are involved in inactivation of the genes *FHIT* and *WWOX* at chromosome fragile sites, reported to be potential tumor suppressor genes. Here we investigated the effect of UV light on the gene expression. After exposure to UV, the mRNA and protein of the two genes in murine embryonic fibroblasts (MEF) were unstable, apparently at the G_1-S phase of the cell cycle, which was consistent with nuclear run-on assay. A study of MEFs synchronized via a double thymidine block indicated that, after the exposure, the expression of Fhit and Wwox was reduced in E2f-1–deficient cells and markedly in wild-type cells, whereas the reduction was partially inhibited in Trp53-deficient cells; cells at the S phase seemed to be sensitive to exogenous *FHIT*, suggesting a role of the checkpoint at the G_1-S phase in the stability of gene expression and a possible involvement of *FHIT* function at the S phase. The transfection experiment showed that the UV-induced decrease in expression was partially inhibited by transfection of kinase-dead Atr (ataxia telangiectasia mutated and Rad3 related), which is a sensor of UV-induced damage. Taken together, the present study showed that UV-induced alterations of the fragile site gene expression are involved at least partially in the checkpoint function, suggesting the role in the process of carcinogenesis after exposure to UV.

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Introduction

Common chromosome fragile sites are specific regions susceptible to the formation of gaps or breaks under conditions of replication stress, such as exposure to the DNA polymerase α inhibitor, aphidicolin (1). Because common fragile sites overlap with, and are sometimes impossible to discriminate from, the genomic regions, which are deleted in tumors (2), it is hypothesized that the fragility may contribute to cancer development (reviewed in ref. 3). Whereas more than 70 regions are listed as fragile in the databases, just 20 regions represent 80% of the fragility; the genes *FHIT* and *WWOX* at *FRA3B* and *FRA16D* are outstanding (4, 5). Previous study showed that Atr [ataxia telangiectasia mutated (Atm) and Rad3 related], but not Atm, regulates the genomic stability of fragile sites, including *FRA3B* and *FRA16D* (5). ATR is a sensor molecule and activated *in vivo* by UV exposure, which stimulates Chk1 and activates p53 (6). Thus, the DNA damage response is supposed to play a role in the stability of fragile sites (5).

Whereas earlier studies showed that homozygous deletions contribute to losses of *FHIT* and *WWOX* (7, 8; reviewed in ref. 9), recent studies have revealed that epigenetic alterations can inhibit gene expression (10, 11; reviewed in ref. 12, 13). Because previous studies described *FHIT* and *WWOX* as candidates for tumor suppressor genes (3, 7, 14, 15), multiple alterations leading to the inactivation of fragile site genes may collaboratively contribute to tumor development. Although it can be hypothesized that epigenetic changes may underlie the early stages of carcinogenesis, the mechanism of reduction of endogenous *FHIT* and *WWOX* expression is not perfectly understood.

UV radiation in sunlight is the most prominent and ubiquitous physical carcinogen in the natural environment (16). Because a causative role of regular sun exposure in skin cancer was suspected by physicians in the late 19th century, numerous experiments have been done to show that increases in ambient UV loads are strongly expected to raise skin cancer

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incidences, and to identify UV radiation as the cause of gene mutations in skin carcinomas (16). UV radiation is highly genotoxic, causing DNA damage such as cyclobutane pyrimidine dimers and 6-4-photoproducts, which are usually repaired by nucleotide excision repair (reviewed in ref. 17). The relation between skin cancer and solar UV exposure is, however, still unclear, and so studies of novel mechanisms of UV-induced carcinogenesis are necessary to control skin cancer (16). In the view of a skin cancer condition, Fhit-deficient mice, after exposure to environmental carcinogens, develop tumors with a spectrum similar to that observed in the Muir-Torre syndrome, although a relationship between *FHIT* and the human syndrome remains to be elucidated (18), suggesting a rationale for further study of the possible involvement of fragile site genes in the carcinogenesis.

Here we assessed the effect of UV exposure on the expression of the fragile site genes Fhit and Wwox, and studied the mechanism involved. The present study showed a complex mechanism of the posttranscriptional and posttranslational modifications as well as transcriptional regulation, which are involved in the reduction of expression of the fragile site genes after exposure to UV. We showed data of (*a*) UV-induced alteration of the stability of mRNAs and proteins; (*b*) differential sensitivity of the alterations in synchronized cells; (*c*) transcriptional regulation of genes in cells at G_1-S phase; and (*d*) involvement of components of DNA damage checkpoint pathway. The present study shows that UV-induced alterations of expression of the fragile site genes are involved at least partially in the checkpoint function, suggesting that the alterations may accelerate an inactivation of the two tumor suppressor genes together with genomic alterations during the carcinogenesis.

Results

Expression of the Fragile Site Genes after Exposure to UV Radiation

Northern blot analysis was done to assess the expression of fragile site genes, Fhit and Wwox. The results showed that, after the UV exposure at 10 and 20 J/m^2, the expression of two genes was markedly reduced, whereas the transcription of p21 was induced and the alteration of actin was undetectable (Fig. 1A). To assess the amount of protein expression, immunoblot analysis was done. The result showed that the amounts of Fhit and Wwox proteins were reduced after UV exposure at 10 J/m^2, and even more at 20 J/m^2, whereas the phosphorylation of tumor suppressor p53 protein at Ser15 was increased, and the control band (*actin*) indicated that an almost equal amount of protein was loaded (Fig. 1B). The time course of protein expression after UV exposure showed that the expression of both Fhit and Wwox was reduced in a time-dependent manner compared with the control (Fig. 1C).

Cells treated with actinomycin D to inhibit *de novo* RNA synthesis were harvested at serial time points and assessed for mRNA after exposure to UV (Fig. 1D). Fhit and Wwox mRNAs were substantially decreased within hours. In comparison, actin mRNA level was essentially unchanged at time points of 8 and 16 hours and moderately decreased at 24 hours. We then studied whether Fhit and Wwox proteins might also be unstable after exposure to UV. To assess protein stability, cells with cycloheximide to inhibit *de novo* protein synthesis were harvested at serial time points following treatment and assessed for protein level (Fig. 1E). In accordance with reduced stability of mRNA, Fhit and Wwox protein levels were appreciably decreased within hours after exposure to UV. By contrast, levels of actin were virtually unchanged.

Study of Synchronized Cells

To assess the sensitivity to UV exposure of the expression at each phase of the cell cycle, a double thymidine block was done to synchronize the cell cycle at G_1-S (Fig. 2A, *0 hour*), followed by incubation without thymidine in the medium to allow the cell cycle to progress to S and G_2-M (*4 hours* and *8 hours + Noc*).

Flow cytometric analysis showed that, after the double thymidine block, a predominant fraction was accumulated at G_1-S, S, or G_2-M at each time point, although it was apparent in wild-type murine embryonic fibroblasts (MEF), compared with E2f-deficient and Trp53-deficient MEFs, suggesting the concept that the regulation of the cell cycle checkpoint may be altered in those deficient cells.

Cells at each phase of the cycle were exposed to UV and harvested 24 hours later to assess dead cells by flow cytometric analysis (Fig. 2B). Data showed that, after UV, wild-type cells at time 0 hour showed ~30% cell death, whereas after 4 and 8 hours showed ~20% cell death. UV-induced cell death was increased in E2f-deficient MEFs although it was reduced in Trp53-deficient MEFs. To assess whether the exogenous Fhit introduction might affect the cells, wild-type MEFs at time 0, 4, and 8 hours were infected with adenoviral *FHIT* and exposed to UV. Data showed that UV-induced dead cells were increased after Fhit introduction, but not control *GFP*, which was more significant when cells at time 4 hours (predominantly in S phase) were infected. The result suggests that cells at the S phase may be sensitive to Fhit introduction.

Genotype of E2f-deficient and Trp53-deficient MEFs was confirmed by genomic PCR (Fig. 2C). Immunoblot study with anti–phosphorylated p53 (Ser15) antisera showed the activation of p53 in wild-type (+/+) but not in Trp53-deficient (−/−) MEFs, whereas the phosphorylation was slightly detected in (+/−) cells (Fig. 2D).

Cells at each phase of the cycle were then exposed to UV and harvested 24 hours later to perform an immunoblot analysis with Fhit and Wwox antibodies (*Wt* in Fig. 3). The results showed that the amount of Wwox protein in three time points (0, 4, and 8 hours) was reduced after exposure to UV, whereas the control experiment before UV exposure [UV(−)] showed that Wwox was substantially expressed at each phase of the cell cycle. UV-induced reduction was more apparent at 0 hour after synchronization (Fig. 3) and at a higher dose of exposure (data not shown). A similar result was obtained in the study of Fhit. Taken together with Fig. 1, although the multiple mechanisms might be involved, the present data are compatible with the concept that the expression of those fragile site genes may be reduced after exposure to UV, at least partially due to regulation of the stability of mRNAs and proteins.

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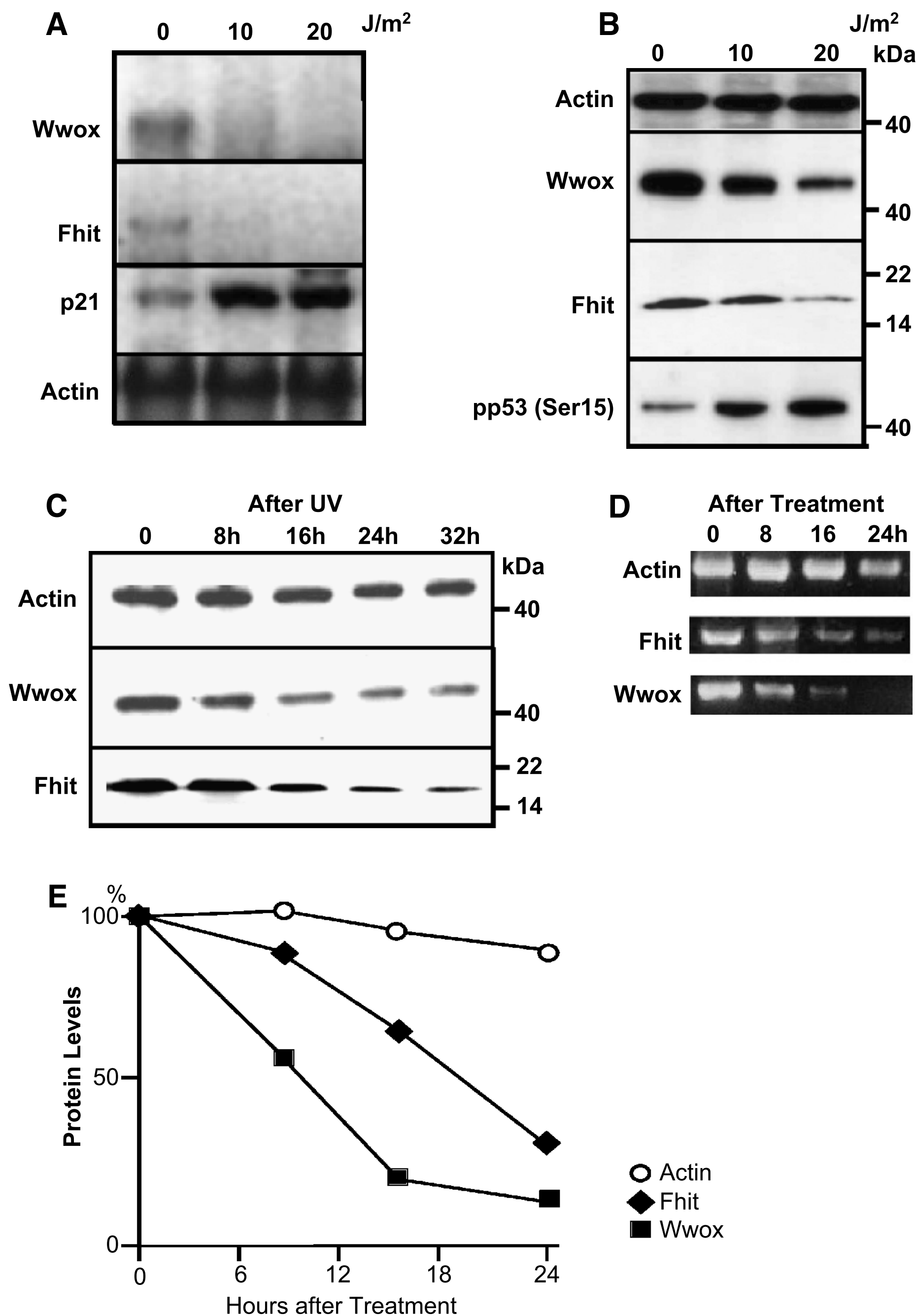

FIGURE 1. Gene expression after UV exposure. **A.** Northern blot. Twenty-four hours after MEF cells were exposed to UV as indicated in the figure, 3 μg of poly(A)$^+$ RNA were extracted, separated by electrophoresis, transferred to membranes, hybridized with radiolabeled DNA probes, and exposed to films. **B.** Immunoblot of cells 24 hours after UV exposure at 10 or 20 J/m^2. Cellular lysate (10 μg) was extracted from MEF after the exposure, separated by SDS-PAGE, transferred to membrane, probed with anti-Wwox or anti-Fhit antibodies, and reacted with secondary antibody in the enhanced chemiluminescence system. Actin staining was used as a control to show that almost equal amount was loaded. **C.** Immunoblot of cells was harvested at the time indicated after UV exposure at 20 J/m^2 and was analyzed as in **B. D.** Actinomycin D treatment. Two hours after culture in medium with actinomycin D (0.3 μg/mL), MEFs were exposed to UV at 20 J/m^2. Twenty-four hours after UV exposure, mRNA was extracted and subjected to reverse transcription-PCR. The amount of the product was loaded to gel and stained with ethidium bromide. **E.** Cycloheximide treatment. Two hours after culture in medium with cycloheximide (25 ng/mL), MEFs were exposed to UV at 20 J/m^2. Twenty-four hours after UV exposure, cell lysates were extracted and subjected to immunoblot as in **C**. The intensity of the bands corresponding to each protein was quantified by densitometry analysis and the values are expressed as percent of baselines (*bold lines*).

Regulation of the G_1-S Phase

Previously we have observed that the introduction of transcription factor E2f-1, which plays a role in regulation of the G_1-S transition, is involved in an increase of expression of *FHIT* and *WWOX* (19), although the regulation of endogenous gene expression remains to be elucidated. E2f-1 is regulated through phosphorylation of the retinoblastoma tumor suppressor gene (*RB*) under the control of the p53

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pathway, and the gene products are frequently altered in tumor cells (20). We studied the expression of Fhit and Wwox in E2f-1− or Trp53-deficient MEFs after synchronization and UV exposure (Fig. 3, *middle* and *right*). After UV exposure, the amount of Wwox protein in synchronized cells at each phase of the cell cycle (at each time point) was reduced in E2f-1−deficient cells and markedly in wild-type MEFs, whereas the reduction was partially inhibited in Trp53-deficient cells (Fig. 3). A similar result was obtained in the study of Fhit. The present study suggests that the reduction of Fhit and Wwox

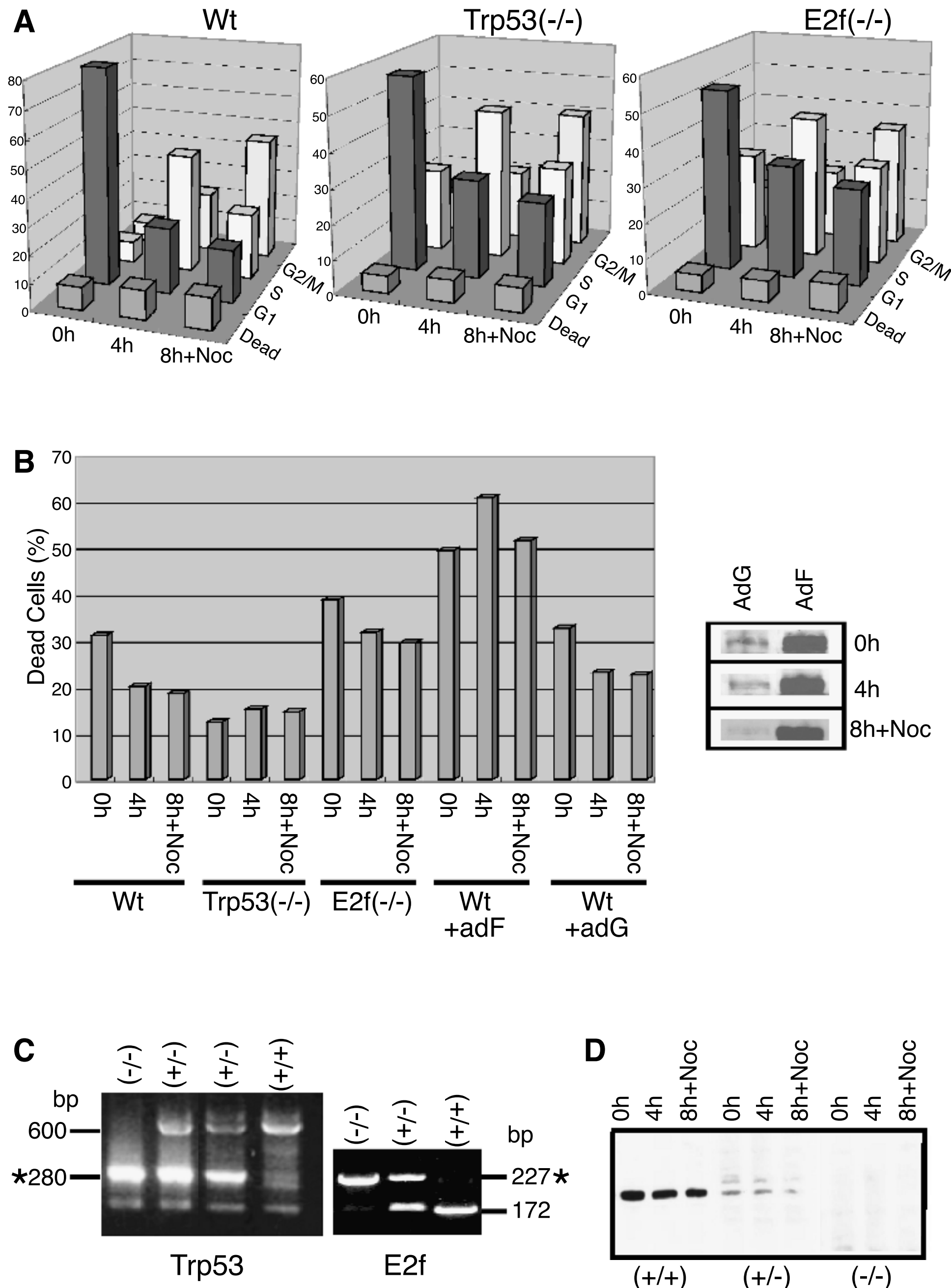

FIGURE 2. Cell cycle synchronization. **A.** Flow cytometric analysis. After wild-type, E2f-deficient and Trp53-deficient MEFs were treated by the double thymidine block as described in text. After growing without thymidine in the medium to release cells from the arrest, cells were harvested at specific times. *Noc,* culture with 40 μg/mL of nocodazole for 30 minutes. **B.** Dead cells (%) after exposure to UV at 20 J/m^2. Cells at each phase of the cycle were exposed to UV and harvested 24 hours later for flow cytometric analysis. *Right,* wild-type MEFs exposed to UV, 2 hours after infection with adenoviral *FHIT* (*adF*) or adenoviral *GFP* (*adG*). The gene expression was assessed by immunoblot with anti-Fhit antibody (*right*). **C.** Genotyping of MEF. PCR amplification was done with genomic DNA extracted from MEF cells according to the recommendation of the manufacturer (Jackson Laboratory). The product was separated in gel and stained. Asterisks show knockout allele. **D.** Phosphorylated p53 (Ser15) activation. Wild-type (+/+) and p53-deficient [(+/−) and (−/−)] MEF were treated with the double thymidine block, released, and harvested at specific times, similarly to **A**. Cell lysates were subjected to immunoblot analysis with anti−phosphorylated p53 (Ser15) antisera as described in text.

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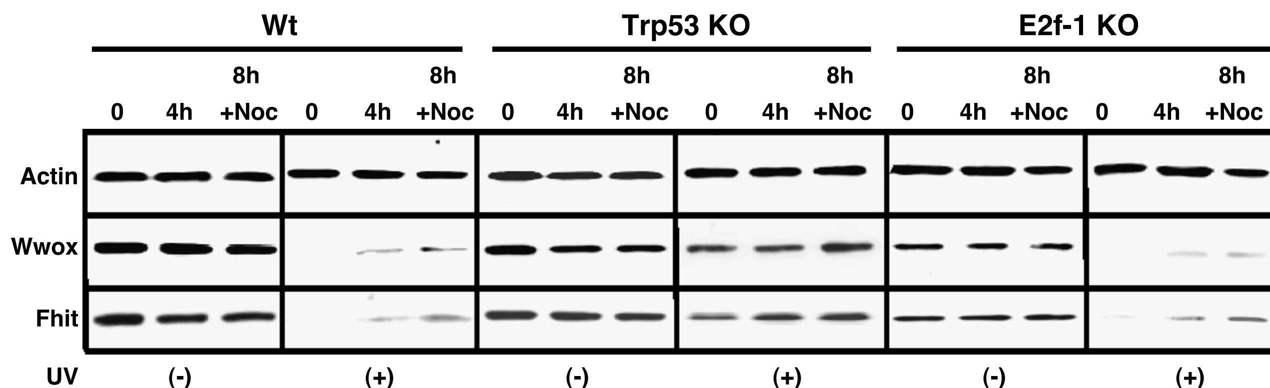

FIGURE 3. Effect of UV exposure on synchronized cells. Similarly to Fig. 2, after being synchronized via a double thymidine block at G_1-S, MEF cells were washed and incubated in fresh medium to release the arrest. Cells were then exposed to UV at 20 J/m^2 (*minus,* control without UV exposure), incubated for 24 hours, and subjected to an immunoblot analysis with antibodies against Wwox and Fhit. The control band of actin indicates that an almost equal amount of protein was loaded. *Wt,* wild-type; *Trp53 KO,* p53-deficient; *E2f-1 KO,* E2F-1–deficient MEF cells.

at the G_1-S phase after UV exposure may be dependent at least partially on the presence of p53, although data show the checkpoints of the S and M phases also might be involved. It is suggested that an acceleration of transcription of those fragile site genes at the G_1-S phase by E2f-1 could account for the reduction of proteins in E2f-1–deficient MEFs after exposure to UV.

Because an involvement of the G_1-S checkpoint was suggested, we studied further the regulation of the gene expression at this phase. To clarify whether the regulation of transcription at the G_1-S phase is altered by the UV exposure or not, the nuclear run-on assay was done using synchronized cells in G_1-S via a double thymidine block (Fig. 4). The results showed that the transcription of *FHIT* and *WWOX*

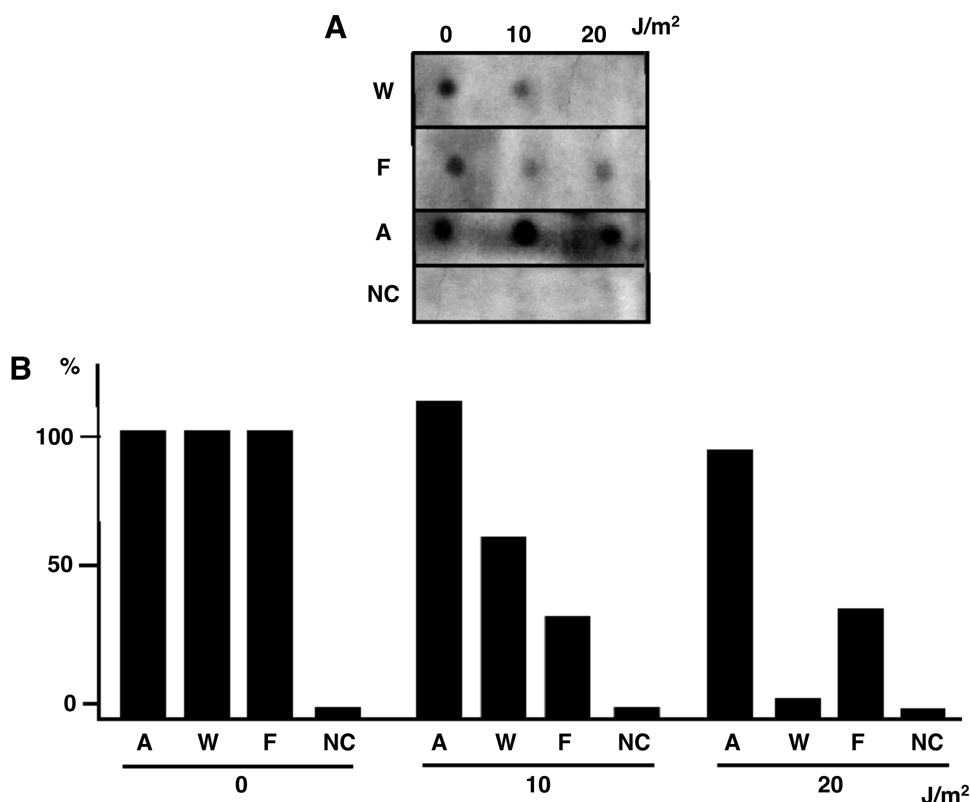

FIGURE 4. Nuclear run-on assay of synchronized 293 cells at G_1-S 24 hours after UV exposure. After the synchronizing of cells via a double thymidine block, nRNA was radiolabeled and hybridized to dot-blotted plasmids for *WWOX* (*W*), *FHIT* (*F*), and *ACTIN* (*A*) cDNAs. *NC,* negative control. The intensity of the spots corresponding to each protein (**A**) was quantified by densitometry, and the values were expressed as arbitrary units (**B**), where the data for each transcript at zero irradiation were expressed as 100% and negative control is 0%.

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was reduced in nuclei after exposure to UV at 10 J/m^2, the effect even more apparent at 20 J/m^2, whereas *ACTIN* expression was not altered, and the negative control reaction indicated the specificity of the experiment. The reduction was more apparent in *WWOX*. The data indicate that, after exposure to UV, the expression of the fragile site genes was reduced at least partially through the regulation of transcription of genes at G_1-S of the cell cycle, suggesting that components of the G_1-S checkpoint might be involved in gene regulation.

The Regulatory Effect of Wild-Type and Kinase-Dead Atr

A previous report showed that Atm and Atr are sensors for DNA damage leading to activation of the G_1-S checkpoint, although the signal transduction pathways exhibit cross-talk (reviewed in ref. 6). If the DNA damage is double-strand breaks caused by ionizing radiation or radiomimetic agents, Atm is activated and phosphorylates many target molecules, notably p53 and Chk2 (6). The phosphorylation of Chk2 initiates the G_1-S arrest by phosphorylating Cdc25A phosphatase (inactive form), followed by an accumulation of the phosphorylated (inactive) form of Cdk2, which is incapable of phosphorylating Cdc45 for initiating replication (6). If the DNA damage is due to UV light or UV-mimetic agents, the signal is sensed by Atr, Rad17-RFC, and the 9-1-1 complex, leading to phosphorylation of Chk1 by Atr, followed by phosphorylation of Cdc25A and G_1 arrest (6). Whether the initial arrest is caused by the Atm-Chk2-Cdc25A pathway or the Atr-Chk1-Cdc25A pathway, the rapid response is followed by a p53-mediated maintenance of G_1-S arrest, which becomes fully operational several hours after the detection of DNA damage (21). In the maintenance stage, Atm and Atr directly phosphorylate Ser15 of p53 (6). The present immunoblot analysis indicates that UV exposure resulted in phosphorylation of p53 at Ser15 (Fig. 1), which is consistent with the activation of the Atr/Atm signal transduction pathways at the G_1-S checkpoint.

We examined the involvement of the Atr pathway in the regulation of the UV-induced altered expression of Fhit and Wwox. The wild-type and kinase-dead mutant *ATR* plasmids were cotransfected with the G418-resistant plasmid, and then selection with G418 was carried out. Immunoblot analysis showed that the transgenes of both wild-type and kinase-dead mutant *ATR* vectors were overexpressed (Fig. 5A). After exposure to UV light, transfectants were subjected to immunoblotting (Fig. 5B). Result showed that UV exposure led to a reduction of Wwox and Fhit expression in wild-type transfectants and in the mock transfectants (*C*), whereas the reduction was inhibited by overexpression of kinase-dead mutant *ATR*. Phosphorylation of Chk1 at Ser345 was inhibited in the kinase-dead transfectants. It is suggested that the kinase activity of Atr plays a role in the alteration of Wwox and Fhit expression after UV exposure. Taken together with the report that Atr regulates the stability of chromosome fragility (5), the present study suggests that the kinase activity of Atr may contribute to both the regulation of genomic stability and the UV-induced alteration of the expression of fragile site genes, *FHIT* and *WWOX*.

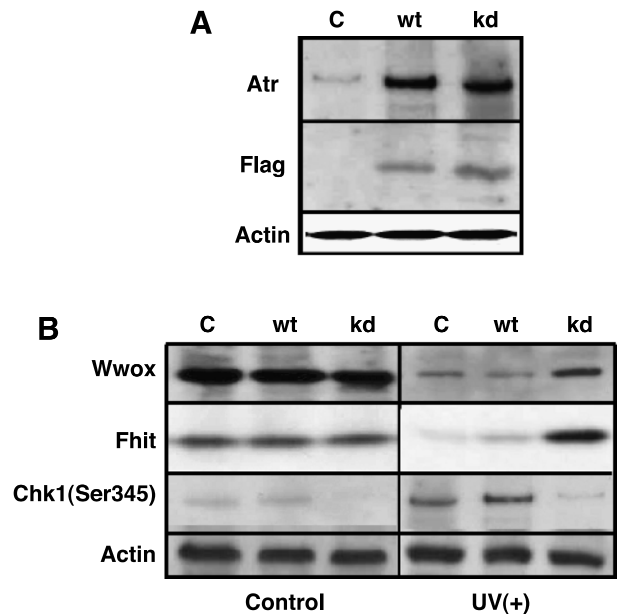

FIGURE 5. The regulatory effect of wild-type and kinase-dead ATR on Wwox and Fhit expression. Using human kidney embryonic 293 cells, plasmids for wild-type (*wt*) and kinase-dead (*kd*) Atr were cotransfected with G418-resistant vector for selection in G418 medium. **A.** Selected transfectants were harvested and subjected to immunoblot analysis with indicated antibodies. **B.** Transfectants were analyzed before (*Control*) and 24 hours after UV exposure at 20 J/m^2 by immunoblotting. *C,* mock transfection.

Discussion

Regulation of the Expression of the Fragile Site Genes

The present study showed that the exposure to UV resulted in a reduction of expression in the fragile site genes, *WWOX* and *FHIT*, through the complex mechanisms including the posttranscriptional and posttranslational modifications as well as transcriptional regulation. Data showed the involvement of the stability of mRNAs and proteins in UV-exposed cells, of the preferential sensitivity of UV-exposed cells at G_1-S phase of the cell cycle in alteration of gene expression, of the transcriptional regulation of genes at G_1-S phase after exposure to UV, as shown by run-on assay, and of the components of DNA damage checkpoint Atr pathway. Further biochemical experiments will be required to fully understand the mechanisms of UV-induced alterations of the fragile site genes.

UV-Induced Cell Death and Gene Expression

Previous studies have shown that E2f-1 is regulated at the G_1-S transition by the phosphorylation of the *RB* gene under the control of the Trp53 pathway (reviewed in ref. 20). E2f-1 plays a dual role, promoting cell cycle progression and stimulating programmed cell death. Forced E2F-1 expression induces quiescent cells to enter S phase, of which activity correlates with the ability of *E2F-1* to activate the transcription of molecules (22) such as cdc2 (23), cyclin E (24), cyclin A (25), and E2f-1 itself (26), whereas the ectopic expression of E2f-1 can induce apoptosis in several cell types (27, 28) through the transcriptional activation of proapoptotic molecules, Afaf-1 (29), caspases (30), and Fhit (19), or through

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alternative mechanisms involving Mdm2 (31), the bcl-2 family (32), and Wwox (19). Previous studies of E2f-1–deficient mice suggested E2f-1 to be a candidate for a tumor suppressor (33, 34).

The present study indicated that the expression of the Fhit and Wwox genes was reduced in E2f-1–deficient cells and markedly in wild-type cells compared with Trp53-deficient cells (Fig. 3). Considering the data of synchronized cells exposed to UV (Fig. 2B), it is possible that after exposure to UV, the remaining Fhit and Wwox proteins in Trp53-deficient cells might require the presence of p53 for the full execution of the proapoptotic function of the fragile site gene products. E2f-1, which can play dual roles, may function as a suppressor of an apoptosis pathway, initiated by DNA photoproducts (35); thus, E2f-1 deficiency seems to have resulted in an increase of cell death after exposure to UV. The observations are consistent with previous report (35).

Our data show that UV exposure up-regulated the phosphorylation of p53 at Ser15 and the expression of the p21 transcript, which are crucial regulators for cell cycle arrest and repair (20), whereas the exposure resulted in the down-regulation of the Fhit and Wwox expression. This supports the concept that the latter gene products are unlikely to be involved in the direct induction of cell cycle arrest after UV damage, but might be relevant to the induction of apoptosis to eliminate transformed cells, which could contribute to maintenance of the fidelity of genome.

A recent study indicated that Fhit-deficient normal and cancer cells are UV resistant (36). After UV doses, the rate of DNA synthesis in Fhit (−/−) cells decreased more rapidly and steeply than in (+/+) cells; UV-surviving Fhit (−/−) cells seem transformed and exhibit >5-fold increased mutation frequency. It is suggested that the increased mutation after exposure to UV may account for the susceptibility of Fhit-deficient cells *in vivo* to malignant transformation. Thus, after exposure to UV, the reduced expression of Fhit might contribute to an increase of mutation frequency. Interestingly, whereas the involvement of components of the DNA damage checkpoint pathway at G_1-S phase of the cell cycle in the reduction of expression level in the fragile site genes was shown, the experiment of adenoviral *FHIT* suggests that the proapoptotic Fhit protein might function in cells predominantly at the S phase. The results are compatible with the previous observation that *FHIT* inhibited or prevented the development of carcinogen-induced cancer through a mechanism involving apoptosis (37, 38).

Gene Expression and Genomic Stability of the Fragile Sites

The chromosome fragile sites are highly recombinogenic and susceptible to deletions in a large fraction of tumors, and *FHIT* and *WWOX* are potential tumor suppressors (3, 7-9, 14, 15). The additional alterations of transcriptional regulation may accelerate the inactivation of gene function at fragile sites. The present study suggests that the exposure to UV may contribute to the reduction of the expression of the tumor suppressor genes at fragile sites in the early stages of cancer, at least through damage-induced activation of checkpoints, leading to the inactivation of *FHIT* and *WWOX*. A previous report showed that Atr regulates the stability of chromosome fragile sites (5), and we showed here that the changes in the expression of the genes after UV exposure are involved in Atr activity. Atr is a sensor of UV-induced DNA damage (6), suggesting that the UV-induced regulation of transcription of those fragile sites genes occurs downstream of Atr, and also that Atr may be involved in both the regulation of genomic stability and gene expression at chromosome fragile sites. However, one may hypothesize that Atr is involved in UV-induced altered transcription via a different mechanism from the regulation of the genomic stability of fragile sites, considering the following observations: (*a*) Although the present data indicated that damage-induced reduction of gene expression was observed in cells synchronized at each phase of the cell cycle, the regulation of chromosome stability is likely involved predominantly in stalled replication forks (5, 39-41). (*b*) Although the present study links Atr with damage-induced alterations of the gene expression, it is unknown whether the UV exposure directly induced gaps or breaks at chromosome fragile sites. Nevertheless, both the altered expression and increased fragility would result in an increase in susceptibility to cancer; cancerous cells with one allele loss at chromosome fragile sites due to replication stress are likely more susceptible to inactivation of gene expression after exposure to carcinogens in the early stages of carcinogenesis. The present study provides a rationale for the further analysis of DNA damage checkpoints and the gene regulation of chromosome fragile sites in the early stages of cancer.[5]

Materials and Methods

Cell Culture

MEF cells and human kidney embryonic 293 cells were maintained in DMEM with 10% FCS. Immortalized MEF cells were obtained from wild-type, Trp53-deficient and E2f-1–deficient mice (Jackson Laboratory, Bar Harbor, ME) as previously described (19). Briefly, MEFs were separated from s.c. tissue of embryos at 13.5 postcoital days, grown in DMEM with 10% fetal bovine serum, and immortalized through SV40 large T transfection (19). Genotype was confirmed by PCR amplification with genomic DNA extracted from MEF cells, in the condition recommended by the manufacturer (Jackson Laboratory). DNA (100 ng) was subjected to PCR amplification in a tube (50 μL) with the following primers: for E2f-1, three primers, 5′-GGATATGATTCTTGGACTTCTTGG-3′ (oIMR0580), 5′-CTAAATCTGACCACCAAACGC-3′ (oIMR0581), and 5′-CAAGTGCCAgCGGGGCTGCTAAAg-3′ (oIMR0582); for Trp53, four primers, 5′-CTTGGGTGGA-GAGGCTATTC-3′ (oIMR0013), 5′-AGGTGAGATGACAG-GAGATC-3′ (oIMR0014), 5′-ATAGGTCGGCGGTTCAT-3′ (oIMR0336), and 5′-CCCGAGTATCTGGAAGACAG-3′ (oIMR0337).

[5] The study of the involvement of the Fhit gene in the ionizing radiation-activated ATR/CHK1 pathway, which also has DNA damage as a component, was recently published by Hu et al. (42).

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The plasmids pBJF-FLAG-*ATR*wt and pBJF-FLAG-*ATR*kd (kindly provided by Drs. S. Schreiber and K. Cimprich) were cotransfected with a G418-resistant pcDNA3 vector (BD Biosciences, San Jose, CA) at a molecular ratio of 50:1 using calcium phosphate precipitation (Promega, Tokyo, Japan) for selection in G418 medium (200 μg/mL). Selected transfectants were used for the analysis. Adenoviral *FHIT* and adenoviral *GFP* vectors (kindly provided by Dr. C.M. Croce) were infected to cells at a multiplicity of infection of 30.

For UV irradiation, 60% to 70% confluent monolayer cells were washed with PBS and irradiated with UVC emitted by germicidal lamps (GL-15; NIPPO lamp, Tokyo, Japan) predominantly emitting 254 nm. Irradiation dose was measured with a digital UVC densimeter (UCV-254, Custom, Tokyo, Japan). Control cells were taken into the UVC exposure source similarly to the irradiated cells but were not irradiated. Cells were examined at indicated time after exposure to UV.

Cell Cycle Study

Cell cycle was assessed by flow cytometry, as described (43). Briefly, 1×10^5 cells were fixed with 70% ethanol for 10 minutes, incubated with RNase A, and stained with propidium iodide. Cell viability and death were assessed by direct visualization of cell morphology, trypan blue exclusion, Hoescht 33342 vital staining, and flow-assisted cytometric analysis of cells with sub-G_1 DNA content. The methods showed a good general agreement. Viable cells were defined as those excluding trypan blue, with uncondensed chromatin or mitotic chromosomes as visualized by vital staining, and with G_1 or greater DNA content. A double thymidine block was done to synchronize the cell cycle at G_1-S as described (44). After growing without thymidine in the medium to release cells from the arrest, cells were harvested at specific times. The flow cytometric analysis after 4 hours in culture showed cells predominantly in S phase. Cells in M phase were harvested after 30 minutes in culture with 40 μg/mL of nocodazole in the medium, and after 8 hours without thymidine in the medium. Experiments were done at least thrice and representative data are shown. Cell lysates were subjected to an immunoblot analysis as described below.

RNA Study

Total RNA and poly(A)$^+$ RNA were extracted with a Qiagen kit (Tokyo, Japan). cDNAs were synthesized from 2 μg of poly(A)$^+$ RNA with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). For reverse transcription-PCR, PCR amplification was done from above cDNA with ExTaq DNA polymerase (Takara, Shiga, Japan) in the 50-μL reaction mixture using Applied Biosystems 2700. The following primers were used: for mouse Fhit (403-bp amplification), 5′-TGGTGAATAGGAAACCCGTTGTACCTG-3′ and 5′-TTGGGAATCGTTTGAGTTACTCTCAGG-3′; for mouse Wwox (425-bp amplification), 5′-CTACTTCTGTCTCCAGCATGGCAGCTC-3′ and 5′-TATTCCCGAATTTGCTCCAGTAACCAG-3′; and for mouse β-actin (474-bp amplification), 5′-ATTGAACATGGCATTGTTACCAACTGG-3′ and 5′-GGCCATCTCCTGCTCGAAGTCTAGAG-3′. PCR cycles were a cycle at 94°C for 1 minute, 35 cycles at 94°C for 8 seconds, 58 to 62°C for 10 seconds, and 72°C for 1 minute, followed by a cycle of 72°C for 7 minutes. For semiquantitative assessment, the number of amplification cycles was reduced to 26, in which the products were amplified linearly. As negative control, the reaction without reverse transcriptase was used. Products were fractionated on 1.5% agarose gels. To verify sequence, amplified fragments were cut from the gel and purified with Qiagen gel extraction kit. DNA sequencing was done using the RISA 384 capillary DNA sequencing system (Shimadzu, Kyoto, Japan).

For Northern blotting, 3 μg of poly(A)$^+$ RNA were transferred to a Nylon membrane (Amersham, Tokyo, Japan) and cross-linked under UV light. The above cDNAs and the peptide-coding region of p21 were amplified by reverse transcription-PCR, sequenced, and used as probes. After probes were radiolabeled by random primer extension, unincorporated nucleotides were removed by spin filtration on a G-50 column. Heat-denatured probes were added to filters in Perfect Hybridization buffer (Sigma, St. Louis, MO) at 5×10^6 to 6×10^6 cpm/mL. After incubation for 16 hours at 65°C, filters were washed in 0.2× SSC/0.1% SDS at 65°C for 40 minutes and exposed to X-ray film.

The nuclear run-on assay was done as described with minor modifications (45). After 5×10^6 cells were suspended in a lysis buffer containing 10 mmol/L Tris-HCl (pH 8), 40 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, and 0.02% NP40, nuclei were separated by centrifugation. nRNA was labeled with [^{32}P]UTP (Amersham) in a reaction buffer containing 20 mmol/L Tris-HCl (pH 8), 140 mmol/L KCl, 10 mmol/L $MgCl_2$, 1 mmol/L $MoCl_2$, 20% glycerol, 14 mmol/L β-mercaptoethanol, 10 mmol/L phosphocreatine, 100 μg/mL phosphocreatine kinase, and 1 mmol/L each of ATP, GTP, and CTP. The elongated RNAs were treated with DNase and proteinase K, followed by hybridization to dot-blotted plasmids of cDNAs.

Protein Study

Cells (1×10^6) were washed with cold PBS and suspended in protein lysis buffer. After assessment of concentration by BioRad kit (Tokyo, Japan), 20 μg of protein were used for SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membranes, probed with the following primary antisera, and detected with the secondary antisera in the enhanced chemiluminescence system (Amersham; ref. 45). The intensity of the bands corresponding to each protein was quantified by densitometry analysis (UN-SCAN-IT gel) and the values were expressed as arbitrary units. We used purified antisera against human Fhit (Zymed, South San Francisco, CA), mouse Fhit (BD Biosciences), Wwox (kindly provided by Dr. K. Huebner; ref. 11), p53 (BD Biosciences), phosphorylated p53 (Ser15; BD Biosciences), phosphorylated Chk1 (Ser345; BD Biosciences), Atm (Santa Cruz Biotechnology, Santa Cruz, CA), Atr (Santa Cruz Biotechnology), Flag (Sigma-Aldrich, St. Louis, MO), and actin (ICN, Irvine CA).

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Components of DNA Damage Checkpoint Pathway Regulate UV Exposure–Dependent Alterations of Gene Expression of *FHIT* and *WWOX* at Chromosome Fragile Sites

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