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p53-mediated cellular response to DNA damage in cells with replicative hepatitis B virus

(tumor suppressor gene/p21$^{CIP1/WAF1}$/DNA-damaging agent/viral carcinogenesis)

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ABSTRACT **Wild-type p53 acts as a tumor suppressor gene by protecting cells from deleterious effects of genotoxic agents through the induction of a G_1/S arrest or apoptosis as a response to DNA damage. Transforming proteins of several oncogenic DNA viruses inactivate tumor suppressor activity of p53 by blocking this cellular response. To test whether hepatitis B virus displays a similar effect, we studied the p53-mediated cellular response to DNA damage in 2215 hepatoma cells with replicative hepatitis B virus. We demonstrate that hepatitis B virus replication does not interfere with known cellular functions of p53 protein.**

Transforming proteins of several DNA tumor viruses specifically interact with p53 protein. The large T antigen of simian virus 40, the E1B 55-kDa protein of adenovirus, and the E6 protein of human papillomavirus all bind p53 and presumably alter its regulation of cellular proliferation (1–4). Since p53 is a tumor suppressor gene that is inactivated in most human tumors, its possible inactivation by certain viral proteins is considered to be one of the key mechanisms by which DNA tumor viruses are able to transform normal cells (5). The best known example of such a mechanism is the inactivation of p53 in cervical epithelial cells by the E6 protein of human papillomavirus types 16 and 18 (6). The p53 protein acts as a sequence-specific transcriptional regulator (7, 8). Its tumor suppressor role has been recently linked to its cell cycle checkpoint function during the cellular response to DNA damage (9, 10). E6 protein is able to abrogate p53-mediated transcriptional regulation (11). The expression of E6 protein also disrupts the p53-mediated cellular response to DNA damage (12).

Hepatitis B virus (HBV) is a small DNA virus implicated in the etiology of hepatocellular carcinoma (13). This virus that infects primarily hepatocytes is the major risk factor associated with hepatocellular carcinoma (14, 15). HBV has no acute transforming activity, but it has been found to be integrated into host genome in most HBV-related hepatocellular carcinomas (13, 16). Integrated viral DNA sequences might act in cis to modify host gene expression or encode viral proteins that may interfere with normal cellular functions either directly or indirectly. Accordingly, the inactivation of cellular proteins by viral proteins has been proposed as a potential mechanism of malignant transformation of hepatocytes by HBV (17). Recent studies have shown that the X protein of HBV (HBX) binds to cellular p53 (18, 19) and, *in vitro*, inhibits p53 sequence-specific DNA binding, p53 transcriptional activity, and its association with the transcriptional factor ERCC3. Based on these data, it has been suggested that HBV may affect a wide range of p53 functions (19). Since p53 plays a key role in maintenance of the integrity of the genome, such a mechanism of p53 inactivation would establish a direct link between HBV infection and the malignant transformation of hepatocytes. However, the conclusions of previous studies were mainly based on *in vitro* conditions that may not exactly reproduce the replicative viral production in the cellular context. We report here our studies on wild-type p53 activity in 2215 cells that reproduce all the conditions of HBV replication and show that HBV replication in these cells does not interfere with the known functions of p53 protein.

MATERIALS AND METHODS

Cell Cultures and Treatments. Human hepatoma cell lines HepG2, 2215, Hep3B, Mahlavu, PLC/PRF/5, SK-Hep-1, and HuH7 (see ref. 20) were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum. The 2215 cell line was obtained by Sells *et al.* (21) after transfection of HepG2 with a plasmid carrying two head-to-tail dimers of HBV in a head-to-tail orientation. The cell line produces high levels of HBe and HBs antigens. HBV-specific particles morphologically identical to infectious Dane particles are detected in conditioned growth medium of 2215 cells (21, 22). This cell line was grown in the presence of neomycin (0.5 mg/ml) to maintain integrated HBV sequences. However, all experiments were performed in neomycin-free medium in order to keep the same growth conditions with the HepG2 cell line. Radiation treatments were delivered by a high-energy x-irradiator (5 or 10 MV) at ≈3 Gy/min. Cells were treated with the chemotherapeutic agent doxorubicin (Adriamycin) at a concentration of 0.2 μg/ml for 0–48 h at 37°C. For UV treatments, cell culture medium was removed just before irradiation in a Spectrolinker XL1000 (Spectronics, Westbury, NY; peak emission at 254 nm), fresh medium was added, and cells were grown under standard culture conditions.

Western Blot Analysis. Cell lysates adjusted to an equal amount of proteins (100 μg) were harvested in sample loading buffer, separated by SDS/PAGE and transferred to a poly(vinylidene difluoride) Immobilon membrane. Immunodetection of p53 was performed with HR231 and PAb122 monoclonal antibodies (23, 24). p53 was detected with a murine peroxidase-conjugated antibody and enhanced-chemiluminescence detection reagent (Amersham).

Plasmids and Transfections. The vector pC53SN3 is a human wild-type p53 expression vector under the control of the strong cytomegalovirus promoter/enhancer (25). The plasmid pHVB-HTD allowing the expression of replicative forms of

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Abbreviations: HCC, human hepatocellular carcinoma; HBV, hepatitis B virus; HBX, HBV X protein.

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HBV was described by Blum *et al.* (kindly provided by H. Blum; ref. 26). The reporter plasmid pRGCΔFos lacZ contains two copies of the RGC-p53 binding fragment in head-to-head orientation upstream of a minimal *fos* promoter that controls expression of the bacterial *lacZ* gene to produce β-galactosidase protein (kindly provided by T. Frebourg; ref. 27). Cells were plated at 70% confluence in 100-mm^2 dishes and transfected with 10 μg of plasmid DNA as described (28). The β-galactosidase activity of transfected cells was analyzed, 72 h after DNA transfection, either by *in situ* staining or after cell lysis as described (27).

Northern Blot Analysis. Total cellular RNA was extracted by the guanidium isothiocyanate/phenol/chloroform extraction procedure as described (28). RNA samples (20 μg) were separated by electrophoresis through agarose gel and transferred to nylon membranes (Hybond-N; Amersham). Membranes were hybridized with a 561-bp ^{32}P-labeled *CIP1/WAF1* cDNA. The p21$^{CIP1/WAF1}$ cDNA was obtained from a normal lymphoblastoid cell line by PCR amplification performed by using primers 5′-CCGAGGATCCGAGGCACTCAGAGGAG-3′ and 5′-CGCGGATCCAGGACTGCAGGCTTCCT-3′ (29). The PCR product was cloned in the pCRII plasmid (Invitrogen).

Thymidine Incorporation. The proliferation of HepG2 and 2215 cell lines was evaluated by [^{3}H]thymidine incorporation assays. At appropriate times of culture, cells were incubated with 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine for 2 h at 37°C. After two washes, cells were solubilized and specific incorporation was evaluated with a liquid scintillation counter (Beckman). Each experiment was performed in triplicate.

RESULTS

p53 Response to DNA Damage in Human Hepatocellular Carcinoma (HCC) Cell Lines. p53 levels were studied in four different HCC cell lines (HepG2, SK-Hep-1, Huh-7, and Mahlavu) after exposure to genotoxic agents. As reported previously, HepG2 and SK-Hep-1 display wild-type sequences within the evolutionarily conserved region of p53 (20, 30). In HepG2 cells, two alleles differ from each other by a polymorphism at codon 72, which encodes alternatively for an arginine or a proline (data not shown). Consequently, p53 protein detected in this cell line migrates as a double band, each representing one allele (see Fig. 4). The Mahlavu cell line obtained from a Mozambican patient displays a homozygous mutation at codon 249 (AGG → AGT) and expresses the mutant p53-249ser protein (20, 31). The Huh-7 cell line also displays a homozygous mutation located at codon 220 (Phe → Cys; described in ref. 30). The p53 protein encoded by this mutant p53 allele displays an increased half-life, like many other mutant p53 proteins (20, 32). The four cell lines were first exposed to increasing doses of UV, and p53 protein levels were measured after 18 h of culture. As shown in Fig. 1*A*, two types of response were observed. There was a dose-dependent accumulation of p53 in HepG2 and SK-Hep-1 cells. The maximum was reached with a UV dose of 50 J/m^2. Thereafter, p53 levels decreased, probably as a result of nonspecific toxicity of the high UV dose. In contrast, under the same conditions, Huh-7 (data not shown) and Mahlavu cells (Fig. 1*A*) did not show a significant increase after exposure to UV. We next compared the response of HepG2 and Huh-7 cells to x-irradiation (Fig. 1*B*). After exposure to 10 Gy of x-rays, p53 protein levels increased in HepG2 cells, but there was no detectable change in Huh-7 cells. Taken together, these observations indicate that HCC cell lines with a wild-type p53 are able to respond to different types of DNA damage, whereas HCC cell lines with mutant p53 have lost this ability. HepG2 cells are well differentiated hepatoma cells, while SK-Hep-1 cells are undifferentiated. Thus, the HepG2 cell line appears to be a good model for the study of p53 function in hepatocytes.

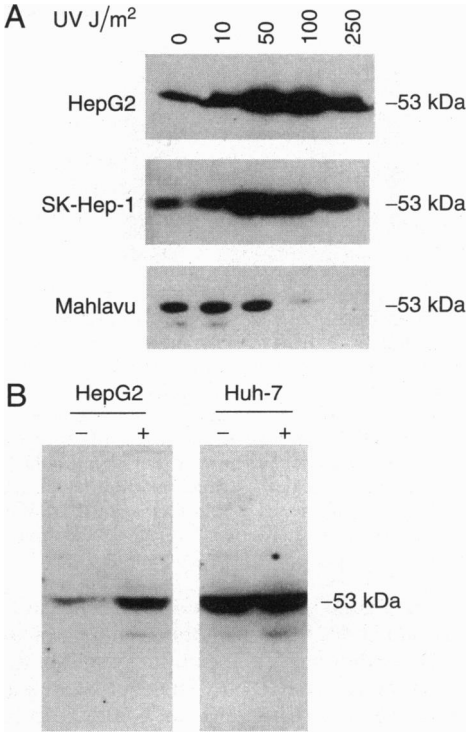

FIG. 1. p53 response to DNA damage in human HCC cell lines. (*A*) Dose-dependent accumulation of wild-type p53 in HepG2 and SK-Hep-1 cells after irradiation with UV-C. Note the absence of accumulation of mutant p53-249ser protein in the Mahlavu cell line. (*B*) Accumulation of p53 in HepG2 (wild-type p53) but not in Huh-7 (p53-220Cys) cells after irradiation with ionizing radiation at a dose of 10 Gy. Exponentially growing cells were irradiated with a UV-C lamp (peak emission at 254 nm) and tested for p53 protein by Western blotting (with HR231 monoclonal antibody) after 18 h of culture. Exposure to ionizing radiation was carried out with a high-energy x-irradiator (5 or 10 MV) at ≈3 Gy/min and cells were tested 3 h after treatment.

Several years ago, Sells *et al.* (21) developed an experimental model of HBV replication by introducing multiple tandem copies of HBV DNA into HepG2 cells. A clone named 2215 (described as 2.2.15) was selected on the basis of stable expression of HBs antigen. This clone was also shown to produce and secrete infectious viral particles into the culture medium. To study whether HBV replication is able to affect known cellular functions of wild-type p53 protein, we further analyzed 2215 cells.

Comparative Analysis of HBV and Cellular p53 Expression in HepG2 and 2215 Cells. The status of HBV was studied in 2215 cells and compared to parental HepG2 cells. As expected, clone 2215 used for this study secreted immunoreactive HBs antigen into the growth medium (data not shown). HBV-encoded transcripts were next studied by Northern blotting. As shown in Fig. 2, the 2215 clone expressed multiple HBV transcripts as well as characteristic forms of pregenomic RNA. The same pattern was observed after transfection in the hepatocarcinoma cell line Hep3B of a HBV expression vector allowing the production of replicative HBV particles (26).

We also studied the status of the p53 gene in 2215 cells. These cells do not display detectable aberration in exons 5–8 of the p53 gene, as reported previously (20). Human cancer cell lines that display the p53 mutation were shown to lose the wild-type p53 allele. As described above, the parental HepG2 cells display a polymorphism of p53 that causes the expression of two types of p53 polypeptides differing from each other by a difference of migration on SDS/polyacrylamide gels. Based on this characteristic, both HepG2 and 2215 cells express two

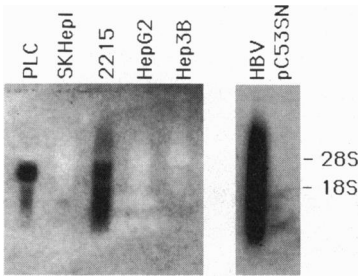

FIG. 2. HBV in HepG2 and 2215 cells. (*Left*) HBV-encoded transcripts were tested by Northern blot analysis of total RNA from different HCC cell lines. Note the presence of multiple transcripts and pregenomic RNA intermediates in 2215 cells as well as in Hep3B cells after transfection of a HBV expression vector. The major transcript detected in PLC/PRF/5 cells represents transcripts encoding surface antigen (33). This cell line does not produce replicating HBV. (*Right*) HBV-encoded transcripts tested by Northern blotting analysis of total RNA from a Hep3B cell line after transfection of a HBV expression vector (HBV) allowing production of replicative HBV particles or a p53 expression vector (pC53SN) used as control.

alleles of the p53 gene as manifested by the migration of p53 protein as a doublet on Western immunoblots (data not shown; see Fig. 4). To further analyze the status of p53 in these cells, a functional assay was carried out. The transcriptional activity of endogenous p53 was tested in HepG2 and 2215 cells after transfection of the plasmid pRGCΔFos lacZ by the phosphate calcium procedure. This reporter plasmid contains the RGC-p53 binding fragment upstream of a minimal *fos* promoter that controls expression of the bacterial *lacZ* gene to produce the β-galactosidase protein (27). Mutant forms of p53 found in different cancers are unable to drive the expression of β-galactosidase from this wild-type p53-responsive plasmid (27). In the presence of wild-type p53, there is a stimulation of β-galactosidase activity that can be revealed by an enzymatic reaction. As shown in Fig. 3, this functional assay demonstrates that endogenous p53 in both HepG2 and 2215 cells displays a similar wild-type activity. In contrast, endogenous mutant protein of Huh-7 cells is devoid of such transcriptional activity (data not shown).

Comparative Analysis of p53 Response to DNA Damage in HepG2 and 2215 Cells. Studies during the past 3 years have made it possible to link the cellular function(s) of wild-type p53 to the adaptive cellular response to DNA damage (9). Wild-type p53 protein participates in the cellular response to DNA damage induced by a variety of genotoxic agents such as ionizing radiation, DNA-alkylating agents, and chemotherapeutic drugs (9, 10). These agents create DNA lesions leading to DNA strand scissions either directly (e.g., ionizing radiation) or indirectly (e.g., topoisomerase inhibitors) during the repair process itself (34, 35). DNA strand breaks appear to be a necessary start signal for p53 activation. The protein gains increased stability by a posttranslational mechanism and accumulates in the nuclei of damaged cells (36, 37). Mutant forms of p53 do not display such an accumulation in response to DNA damage (9, 10). It has also been shown that E6 protein of human papillomaviruses disrupts the inducibility of wild-type p53 by genotoxic agents (12). To determine whether the induction of p53 by DNA-damaging agents is also disrupted in the presence of replicating HBV, we compared the responses of HepG2 and 2215 cells to ionizing radiation, doxorubicin, and UV light exposure. As shown in Fig. 4, all three DNA-damaging agents were able to induce p53 accumulation in HepG2 as well as in 2215 cells. The amplitude and the kinetics of p53 accumulation were similar in the two cell lines. Thus, it appears that the presence of replicating HBV in 2215 cells has no detectable effect on the activation of wild-type p53 after exposure to DNA-damaging agents.

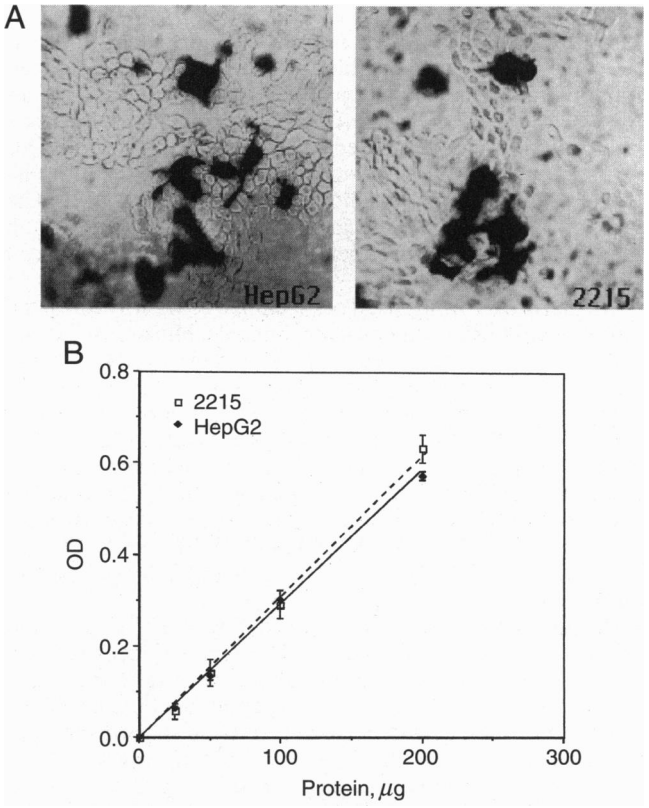

FIG. 3. Analysis of transcriptional activity of endogenous p53 in HepG2 and 2215 cells. (*A*) β-Galactosidase activity 72 h after transient transfection of the pRGCΔFosLacZ plasmid. Activity was analyzed by *in situ* staining. Dark cells demonstrate p53 transcriptional activity in transfected cells. (*B*) β-Galactosidase activity as measured on a spectrophotometer at 420 nm after cell lysis and enzymatic reaction performed with 25, 50, 100, and 200 μg of proteins. Four different transfections were carried out. For each of them, the transfection efficiency was determined. OD indicates calculated optical density for 1% efficiency. Vertical bars indicate SD.

The accumulation of p53 after exposure to genotoxic agents constitutes the initial step of the wild-type p53 participation in the cellular response to DNA damage. This accumulation of wild-type p53 then triggers induction of $p21^{CIP1/WAF1}$ expression (29). Cells with no wild-type p53 protein are unable to induce the accumulation of $p21^{CIP1/WAF1}$ after exposure to DNA-damaging agents (29, 38). The $p21^{CIP1/WAF1}$ protein has been independently identified as a component of almost all cyclin-dependent kinase (cdk) complexes (39, 40) as well as a potent inhibitor of G_1 cdks (41). $p21^{CIP1/WAF1}$ has also been shown to inhibit directly *in vitro* DNA replication by interaction with the proliferating cell nuclear antigen (42). Thus, p53 appears to participate in the response to DNA damage by inducing expression of $p21^{CIP1/WAF1}$, which blocks the entry of cells into the DNA replication phase of the cell cycle. Therefore, we were interested in determining whether HBV replication has any effect on the induction of $p21^{CIP1/WAF1}$ expression after p53 activation by DNA-damaging agents.

$p21^{CIP1/WAF1}$ Expression and DNA Synthesis in HepG2 and 2215 Cells After Exposure to Doxorubicin. We analyzed the transcript levels of $p21^{CIP1/WAF1}$ after activation of p53 by doxorubicin-induced DNA damage. Northern blot analysis showed that the expression of $p21^{CIP1/WAF1}$ was equally low in both HepG2 and 2215 cells. However, the expression of $p21^{CIP1/WAF1}$ was induced as early as 3 h after treatment of both cell lines with doxorubicin (0.2 μg/ml) (Fig. 5). Maximum induction was reached at 6 h and transcript levels remained high for at least 24 h, coinciding with the kinetics of p53 accumulation (see Fig. 4*B*). We observed no major difference

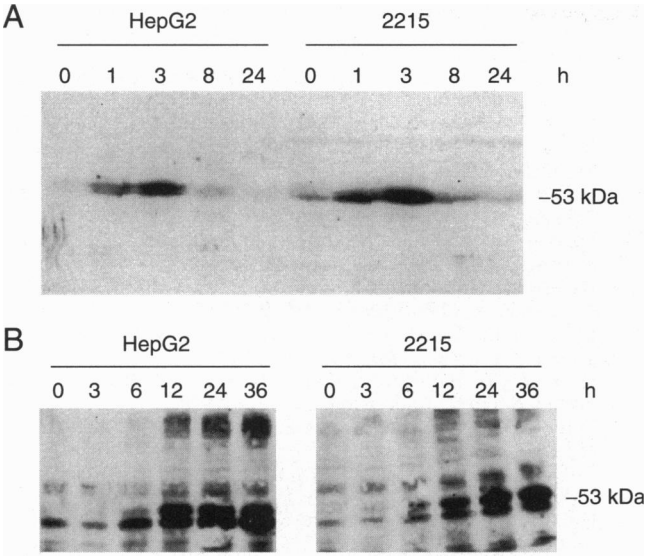

FIG. 4. Comparative analysis of p53 response to DNA damage in HepG2 and 2215 cells. (*A*) Induction of p53 levels in HepG2 (*Left*) and 2215 (*Right*) cells after irradiation with 6 Gy of ionizing radiation. (*B*) Progressive accumulation of wild-type p53 in both HepG2 (*Left*) and 2215 (*Right*) cells when exposed to doxorubicin (0.2 μg/ml). At the indicated times, cells were harvested for Western blot analysis (using the HR231 monoclonal antibody) and compared with a parallel culture of untreated cells (time 0).

between HepG2 and 2215 cells in terms of their ability to induce $p21^{CIP1/WAF1}$ levels in response to p53 activation.

Since $p21^{CIP1/WAF1}$ inhibits the entry of cells into S phase of DNA synthesis by inhibiting the activity of cyclin E/cdk2 complexes, we examined the ability of HepG2 and 2215 cells to synthesize DNA in parallel to the study of $p21^{CIP1/WAF1}$ expression. Fig. 5*B* shows that exposure of both cell lines to doxorubicin resulted in reversible inhibition of thymidine incorporation into DNA. The onset of this inhibition coincided with induction of $p21^{CIP1/WAF1}$ transcripts in these cells. Thus, in spite of higher basal levels of thymidine incorporation in HepG2 cells, there was no significant difference in inhibition of DNA synthesis between the two cell lines after induction of p53 and $p21^{CIP1/WAF1}$.

DISCUSSION

The p53 protein is a common target of transforming proteins encoded by several viruses, including simian virus 40, adenovirus, and papillomavirus. In noninfected cells, p53 acts as a transcriptional factor able to activate target genes through specific p53-binding sites (7, 8). This activity has recently been linked to the participation of p53 in a G_1 checkpoint control following DNA damage (43). Transforming proteins of oncogenic DNA viruses are able to block cellular functions of p53 by abrogating its transcriptional activity (12, 44). A similar mechanism has recently been proposed as a key event in the malignant transformation of hepatocytes by HBV (18, 19). However, the present data indicate that wild-type p53 activity is not affected in 2215 cells, which produce HBV particles. HBV DNA is carried in these cells as chromosomally integrated sequences and episomally as relaxed circular, covalently closed, and incomplete copies of the HBV genome (21). As shown in Fig. 2, HBV transcripts produced in 2215 cells are indistinguishable from those produced during natural infection. Our studies therefore indicate that p53 is able to transactivate a wild-type p53-responsive reporter gene during HBV replication. We have also shown that all known steps of p53 participation in the cellular response to DNA-damaging agents are not affected in 2215 cells. Indeed, alterations induced by three different genotoxic agents (ionizing radiation, doxorubicin, UV) are able to trigger p53 accumulation. More importantly, activated wild-type p53 is able to induce the expression of $p21^{CIP1/WAF1}$ with a concomitant G_1 arrest in these cells. $p21^{CIP1/WAF1}$ is an inhibitor of cyclin E–cdk2 complexes, whose activity is required for the entry of cells from G_1 into S phase (29, 38). Taken together, these observations indicate that the known cellular functions of p53 are not a primary target of HBV. Thus, HBV may differ from other known oncogenic DNA viruses studied to date.

Our findings appear to be in contradiction to recent reports by Feitelson *et al.* (18) and Wang *et al.* (19). According to those investigators, p53 and HBX form complexes both *in vivo* and *in vitro* (18). Consequently, experimentally, HBX inhibits sequence-specific DNA binding of p53, p53-mediated transcriptional activation, as well as *in vitro* association of p53 with ERCC3, a general transcription factor involved in nucleotide excision repair (19). However, there are some major differences between our experiments and those reported previously. First, we studied p53 during HBV replication in order to reproduce *in vivo* conditions of viral infection. Under these conditions, the expression of HBX transcripts is low or undetectable. In contrast, studies by Feitelson *et al.* (18) and Wang *et al.* (19) focused on the interaction between p53 and HBX, with HBX produced as a separate protein, in the absence of any other viral activity. Furthermore, the experimental conditions were different. We studied the activity of endogenous p53 protein in its normal cellular context; the other studies were conducted under *in vitro* or *in vivo* conditions with exogenous expression vectors with strong promoters that allow the production of very high quantities of both p53 and X

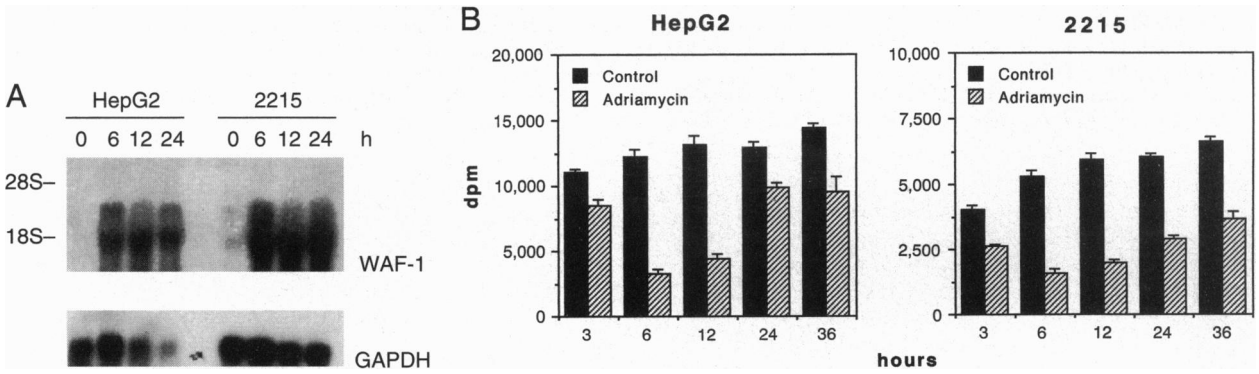

FIG. 5. Comparative analysis of p53-mediated cellular response to DNA damage in HepG2 and 2215 cells. (*A*) Similar induction of the expression of $p21^{CIP1/WAF1}$ was observed in both cell lines when exposed to doxorubicin (0.2 μg/ml). (*B*) Inhibition of DNA [^{3}H]thymidine incorporation into cellular DNA in both HepG2 and 2215 cells exposed to doxorubicin. Note that maximum inhibition of DNA synthesis (6 h) coincides with onset of induction of $p21^{CIP1/WAF1}$ expression.

transcripts at the cellular level. Under such conditions, some weak protein–protein interactions might be observed.

p53 gene mutations are frequent only in aflatoxin-related HCCs (31, 45, 46). In low-aflatoxin areas, mutation rates vary between 13% and 33% (for review, see refs. 47 and 48). This low frequency suggests that p53 might be inactivated by other mechanisms, such as functional inactivation of the protein. However, we observed no inhibition of p53 functions in cells with replicative HBV. Viral replication occurs during acute and chronic hepatitis. In HBV-related HCCs, the malignant cells display integrated forms instead of free replicating forms of the virus. In these cells, incomplete and rearranged forms of integrated viral DNA sequences may encode full-length or truncated forms of several viral proteins including HBV S protein and HBX. It will be important to determine whether such tumor-associated forms of HBV proteins are able to interact with p53 protein functions.

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