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Modulation of cell proliferation and gene expression by a p53–estrogen receptor hybrid protein

(tumor suppressor/oncogene/transcription)

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ABSTRACT **We report that p53her, a chimeric protein consisting of the complete human wild-type p53 and the human estrogen receptor hormone-binding domain, strongly suppresses proliferation and induces characteristic morphological changes in Saos-2 human osteosarcoma cells when induced by 17β-estradiol. In contrast, p53her constitutively transactivates a p53-responsive promoter in transfection assays, so that transactivation is not regulated by estradiol. However, coexpression of p53her and oncoprotein MDM-2, which associates with and presumably inactivates p53, results in suppression of p53her-mediated transactivation in the absence, but not the presence, of estradiol. Similarly, p53her induces expression of an endogenous MDM-2 transcript only in the presence of estradiol. These results suggest a correlation between the growth suppressor function of p53her and release of a transactivation block mediated by MDM-2.**

Wild-type (wt) nuclear phosphoprotein p53 is a tumor suppressor (1, 2). Overexpression of wt p53 in many tumor cells results in growth arrest (1). In normal cells, wt p53 overexpression and cell cycle arrest may result from DNA damage or from disturbances of nucleotide pools (3, 4). Here we show that a hybrid protein consisting of the human wt p53 and the human estrogen receptor hormone-binding domain suppresses cell proliferation and interacts with oncoprotein MDM-2 in a hormone-dependent manner. In contrast, its transcriptional transactivator activity is hormone-independent in transfection assays. We suggest that p53her will be a useful tool to further study the p53 tumor suppressor.

MATERIALS AND METHODS

DNA Constructions and Immunoprecipitation. To construct plasmid pSV53her, human wild-type p53 cDNA, linearized with *Eco*0109I at position 1300, and plasmid pHE14 (5), cut with *Bam*HI at a site corresponding to position 1068 in the human estrogen receptor cDNA, were linked through a 17-bp synthetic oligonucleotide (lowercase letters in Fig. 1). The hybrid sequence or the p53 cDNA was cloned into the *Eco*RI site of plasmid pKCR2 (6), generating pSV53her and pSV53, respectively. Plasmids pCmdm-2 and pCmdm-2as were constructed by inserting a 2.2-kb *Bam*HI fragment from vector MDM2-F.L.5 containing the complete human MDM-2 cDNA into the *Bam*HI site of vector pC53-SN3 (7) in the sense (pCmdm-2) or antisense (pCmdm-2as) orientation. Immunoprecipitations were performed as described (8).

Cell Studies, Transfections and RNA Analyses. Cells were stained for F-actin as described (9). For immunoperoxidase staining, we used antibody G59-12 (PharMingen; 0.5 mg/ml) diluted 1:500 and visualized by the Vectastain ABC kit (Vector Laboratories). For growth analysis, equal numbers of cells were plated in 35-mm culture dishes. After 48 hr (time 0), cells from representative dishes were counted with a Coulter ZM cell counter. 17β-Estradiol or tamoxifen (Sigma) was added as indicated. Saos-2 and Hep3B cells were transfected (10) and, after 48 hr, nonchromatographic chloramphenicol acetyltransferase (CAT) assays were performed (11). Northern analysis and flow cytometry were performed according to standard procedures (3, 11).

RESULTS

Expression of the p53–Estrogen Receptor Hybrid Protein p53her. We transfected Saos-2 human osteosarcoma cells, which are devoid of endogenous p53 (12), with plasmid pSV53her, expressing the p53–estrogen receptor hybrid protein, or control plasmids pSV53, expressing wt p53, and pHE14, expressing the estrogen receptor hormone-binding domain only. Antibody 1620 (13), which preferentially reacts with wt p53, precipitated a protein of the expected size (90 kDa) in cells transfected with pSV53her, and precipitation of this protein was independent of the presence of 2 μM 17β-estradiol (Fig. 1). Another protein, of 110 kDa, was precipitated in pSV53her-transfected cells with antibody 1620 but not with p53-antibody G59-12 (PharMingen). Independent of the presence of hormone, p53her had a short half-life (<30 min) in transfected cells. Similarly, immunoperoxidase staining of Saos-2 cells stably expressing p53her revealed that the hybrid protein was localized in the nucleus independently of the presence of hormone (data not shown).

We were unable to detect any endogenous estrogen receptor mRNA or elicit any response from a transfected CAT reporter plasmid carrying estrogen response elements in both parental Saos-2 cells and cells stably expressing p53her. Furthermore, growth and morphology of Saos-2 cells were unchanged in the presence of β-estradiol. Therefore, we conclude that Saos-2 cells do not express physiologically relevant levels of estrogen receptor which could interfere with the study of inducible p53her functions.

Hormone-Dependent Suppression of Cell Proliferation by p53her. We stably transfected Saos-2 cells with either pHE14 or pSV53her and isolated two individual cell clones expressing the estrogen receptor hormone-binding domain, designated S-her 2 and 6, and four cell clones transfected with pSV53her. Three of the latter clones, designated S-53her 1, 14, and 20, expressed approximately equal amounts of p53her while one, S-53her 17, expressed no detectable p53her by Northern analysis and immunoprecipitation. The growth of the six clones and of parental Saos-2 cells was analyzed in the presence or absence of 2 μM 17β-estradiol (Fig. 2 *A–D*). All cells grew exponentially in the absence of hormone. However, cells expressing p53her showed a doubling time of 70 hr compared with 40 hr for Saos-2 cells in estrogen-free me-

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Abbreviations: CAT, chloramphenicol acetyltransferase; wt, wild type.

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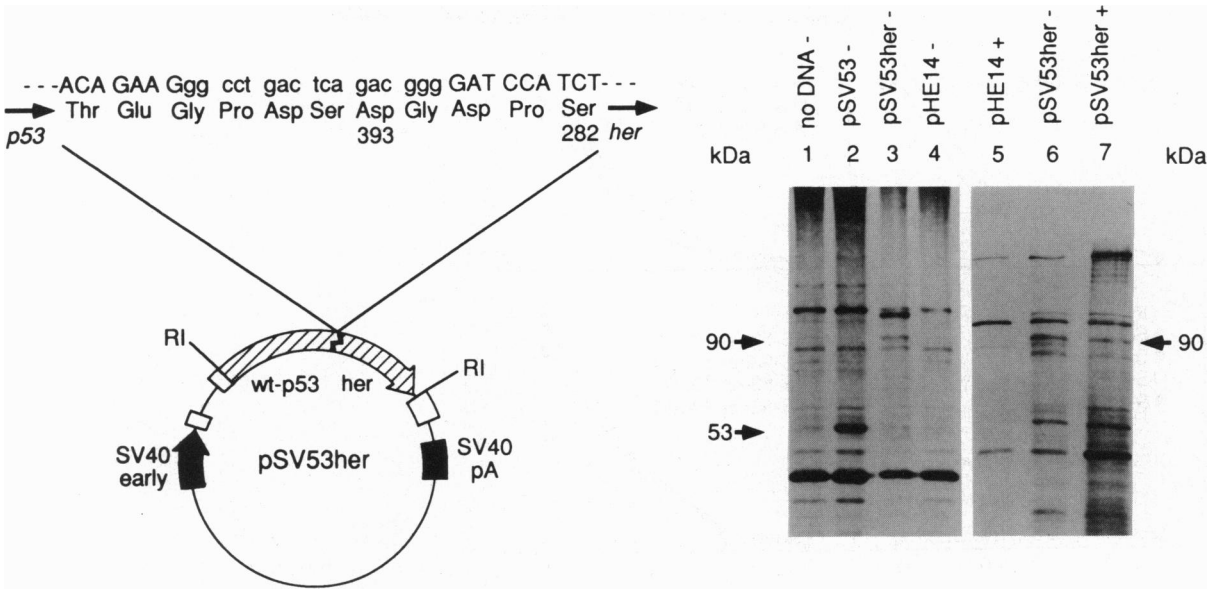

FIG. 1. Construction and expression of vector pSV53her, containing the full-length human p53 cDNA fused to the hormone-binding domain of the estrogen receptor gene (her). SV40 early and SV40 pA denote sequences from the simian virus 40 early promoter and polyadenylylation signal of plasmid pKCR2. Immunoprecipitations with antibody 1620 in the presence (+) or absence (−) of 2 μM 17β-estradiol revealed a 90-kDa signal in cells transfected with 10 μg of pSV53her (lanes 3, 6 and 7) and a 53-kDa signal in cells transfected with pSV53 (lane 2), the related plasmid that contains only the p53 gene. No p53-signal was found after transfection with pHE14 (lanes 4 and 5), containing only the estrogen receptor hormone-binding-domain sequences in pKCR2.

dium, suggesting a low level of activated p53her even in the absence of hormone. Strikingly, in the presence of β-estradiol, cells expressing the hybrid protein ceased to proliferate, whereas control cells were unaffected. Also, cells expressing p53her assumed an enlarged, flattened morphology during hormone exposure, resembling Saos-2 cells overexpressing the retinoblastoma tumor-suppressor protein (14). Most of these cells developed actin-containing microfilament bundles (Fig. 3), reminiscent of the reversion of the tumorigenic phenotype of transformed fibroblasts (9). After 4 days of hormone exposure, most p53her-expressing cells remained attached to the culture plate and incorporated [^{35}S]methionine, indicative of continued protein synthesis. Together, these results indicate that activated p53her suppresses cell proliferation and induces morphological changes in Saos-2 cells without causing acute cytotoxicity.

We next exposed p53her-expressing cells to 2 μM β-estradiol for 0.5–24 hr and examined their growth properties after removal of hormone (Fig. 2*E*). After 0.5–6 hr of hormone exposure, cell growth was not significantly different from that of cells not exposed to hormone. However, after temporary hormone exposure for 24 hr, cell proliferation remained suppressed for ≈4 days but resumed growth thereafter. Reexposure of these proliferating cells to hormone after 8 days in culture again resulted in suppression of proliferation (data not shown), demonstrating that the recovery was not due to selection of a subpopulation of cells unresponsive to hormone. Proliferation suppression was not reversible when the p53her-expressing cells were exposed to hormone for >2 days.

After exposure of S-53her cells for 24 hr to 0–0.2 μM 17β-estradiol and subsequent culture for 6 days in the absence of hormone, the cells resumed proliferation in a moderate but detectable dose-dependent manner (Fig. 2*F*). Reexposure at 6 days to 0.2 μM estradiol led again to a cessation of cell growth. However, continuous exposure to 0.2 μM hormone resulted in the appearance of individual colonies (Fig. 2*F*, days 6–12). Northern analysis of two such colonies failed to show detectable p53her RNA, consistent with the interpretation that p53her expression is required for cell growth suppression.

We then studied the proliferation of S-53her cells in the presence of the estradiol antagonist tamoxifen (15). Tamoxifen allows binding of the estrogen receptor to DNA but represses its ability to activate transcription. While 1 μM tamoxifen had no effect on the proliferation of S-her 6 control cells, it suppressed proliferation of S-53her 1 cells (Fig. 2*G*). This observation is similar to previous findings that tamoxifen, like β-estradiol, is capable of activating Myc–estrogen receptor hybrid proteins (16).

Analysis of the cell cycle of S-53her 1 cells after 24 hr of estradiol exposure revealed a relative decrease of 29% in cells in G_1 phase and a reciprocal increase in cells in S phase (Fig. 2*H*, arrow), whereas control cells were unaffected. These results suggest that p53her is capable of specifically influencing the cell cycle of Saos-2 cells. Although wt p53 seems to arrest many cell types at the G_1/S border (1), a similar increase in cells in S phase has been described for nontransformed murine C127 cells upon wt p53 expression (17).

Transcriptional Regulation by p53her. Wild-type, but not mutant, p53 binds to and regulates genes *in vivo* and *in vitro* (7, 18, 19). We therefore determined the effects of p53her on the expression of the CAT reporter gene from plasmids containing p53-responsive elements. Saos-2 and also Hep3B cells, a p53-negative human hepatoma cell line (20) more readily transfectable than Saos-2, were cotransfected with plasmids pPG_{13}-CAT or pMG_{15}-CAT and either plasmid pSV53, pHE14, or pSV53her. Plasmid pPG_{13}-CAT is responsive to wt p53, whereas pMG_{15}-CAT carries mutated sequences not responsive to p53 (7). As expected, pMG_{15}-CAT was not transactivated by either wt p53 or p53her. Surprisingly, pPG_{13}-CAT was transactivated by p53her both in the presence and in the absence of hormone (Fig. 4 *A* and *B*). Exposure to tamoxifen instead of 17β-estradiol resulted in a slight (≈2-fold) inducibility of the p53her-mediated transactivation (Fig. 4*C*). Similarly, we detected hormone-independent transactivation when S-53her cells were transfected with pPG_{13}-CAT (data not shown), indicating that transactivation was not an artifact due to high copy numbers of pSV53her in transiently transfected cells.

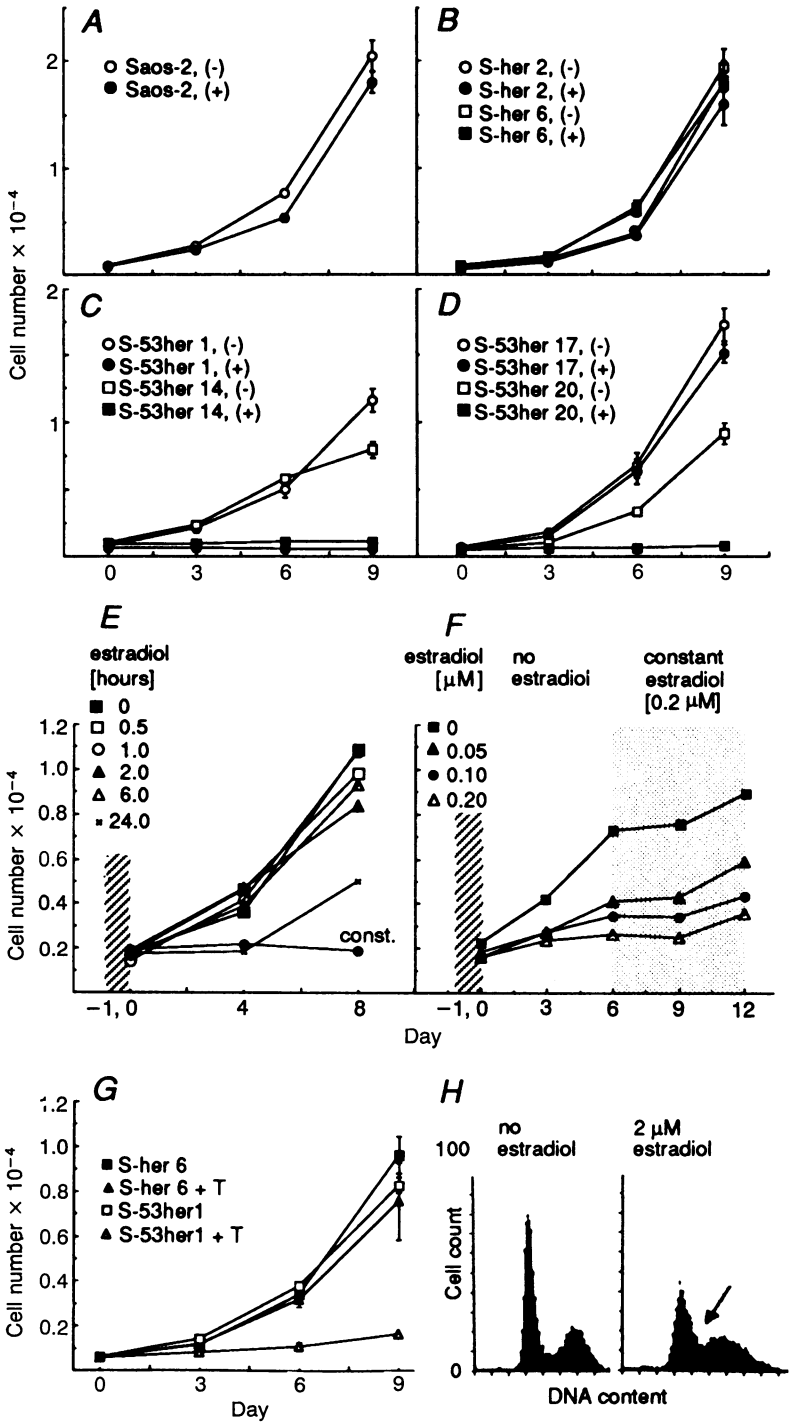

FIG. 2. Proliferation analysis of Saos-2 cells expressing p53her and control cells. (*A–D*) Parental Saos-2 cells, cells stably expressing pHE14 (S-her 2 and 6), and cells stably transfected with p53her (S-53her 1, 14, 17, and 20) were grown in the presence (+) or absence (−) of 2 μM 17β-estradiol. All p53her-transfected cells except S-53her 17 expressed p53her. (*E*) Reversibility of the estradiol effect on cell line S-53her 1 under transient versus constant (const.) exposure to 2 μM estradiol. The hatched area defines the time interval in which the cells were exposed to estradiol. (*F*) S-53her 1 cells were grown for 24 hr (hatched area) in the presence of various concentrations of estradiol. The hormone was then removed for 6 days. After 6 days in hormone-free culture medium, the cells were grown in the continuous presence of estradiol (days 6–12). (*G*) Proliferation of p53her 1 cells and control cells in the absence or presence (+T) of 1 μM tamoxifen. (*H*) S-53her 1 cells unexposed or exposed to estradiol for 24 hr were analyzed for their relative DNA content and counted by flow cytometry. Arrow points to an increase in the number of cells in S phase. Note the simultaneous decrease in cells in G_1 phase (left peak).

Oncoprotein MDM-2 binds to and represses wt p53 (21). To analyze the effect of MDM-2 on p53her, we transiently coexpressed pPG_{13}-CAT with effector plasmid pSV53, pSV53her, or pHE14 as well as with MDM-2 plasmids pCmdm-2 and pCmdm-2as. As expected, wt p53-induced transactivation of pPG_{13}-CAT was repressed by MDM-2 (Fig. 4*D*, bars 1–3). In contrast, expression of MDM-2 antisense message resulted in an increase of ≈30% in p53-induced transactivation (bar 4). Strikingly, p53her was also repressed by MDM-2 (bars 5 and 6), but the transactivation function was restored upon addition of β-estradiol (bar 7) or tamoxifen (bar 8), suggesting that MDM-2 conditionally interacts with p53her. We were unable to demonstrate direct physical interaction between the two proteins.

wt p53 increases the expression of endogenous MDM-2 (22). We therefore determined the amount of MDM-2 transcript in S-her 6 and S-53her 1 cells exposed for various times to β-estradiol (Fig. 5). For S-her 6, we found no detectable MDM-2 transcript in the presence or absence of hormone. In contrast, p53her expression significantly increased the amount of cellular MDM-2 transcript as early as 3 hr after induction and maximally at 6 hr after induction, but did so only in response to β-estradiol. This suggests that activated p53her, like wt p53, can induce MDM-2 expression.

DISCUSSION

Several of the properties of the hybrid protein p53her resemble those of wt p53, although some functions become hormone-inducible in the hybrid while others remain constitutively active and not inducible with estradiol. Only estradiol-

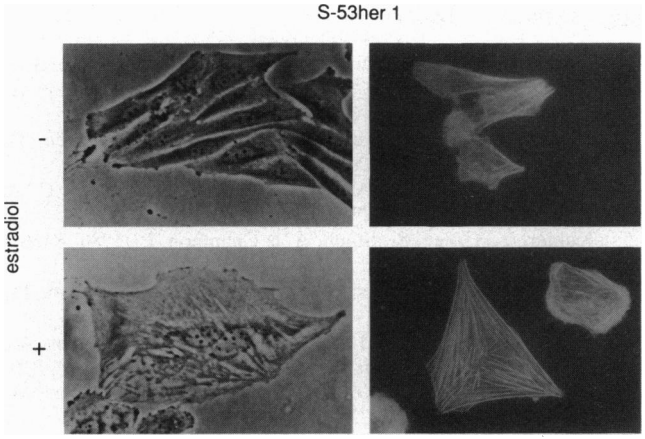

FIG. 3. Morphology in culture of S-53her 1 cells and distribution of actin-containing microfilament bundles in response to hormone treatment. Exponentially growing S-53her 1 cells were either mock-treated (*Upper*) or exposed to 2 μM 17β-estradiol (*Lower*) for 48 hr. The flat morphology and microfilament bundles were observed as early as 24 hr after contact with hormone. F-actin was stained with a fluorescent dye 7-nitrobenz-2-oxa-1,3 diazole-labeled phallacidin (NBD-phallacidin) (*Right*).

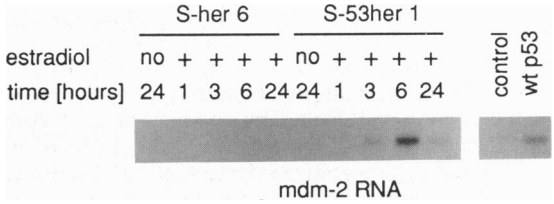

FIG. 5. Effect of p53her on the level of cellular MDM-2 gene transcript. Ten micrograms of total RNA from S-her 6 and S-53her 1 cells and from cells transiently transfected for 24 hr with either pSV53 (wt p53) or an irrelevant plasmid (control) (5 μg per 10^6 cells) was prepared and subjected to Northern blot analysis. Where indicated, cells were exposed to 2 μM 17β-estradiol for various times. We used the complete MDM-2 cDNA from plasmid MDM2-F.L.5 as hybridization probe.

activated, but not inactive, p53her suppresses proliferation and alters the morphology of Saos-2 cells similarly to wt p53 (23). The hybrid protein, like wt p53 (7), transactivates a p53-responsive promoter in transient transfections, but in contrast to the growth-suppressor activity, the transactivation function of p53her is not regulated by estradiol. Further, the transactivation function of both wt p53 (21) and p53her is suppressed by oncoprotein MDM-2, yet the suppression of p53her-mediated transactivation is overcome by estradiol. Finally, p53her, like wt p53 (22), induces MDM-2 gene expression, but does so only in the presence of β-estradiol. Together, these results suggest a correlation between the growth-suppressor function of activated p53her and release of a transactivation block mediated by MDM-2. It is possible, however, that the phenotypic changes in Saos-2 cells are caused by the combined actions of activated p53her and overexpressed MDM-2.

The transactivation function of p53her was constitutively active in transient transfections, whereas its growth-suppressor function in stably transfected S-53her cells was not. Thus, the regulation of potential cellular target genes by p53her via DNA motifs similar to the motif in pPG_{13}-CAT may be insufficient for, or uncoupled from, the function of cell proliferation suppression. This is supported by results from colony formation studies (unpublished data). In these studies as in the transactivation studies, we overexpressed p53her by transiently transfecting Saos-2 cells with plasmid

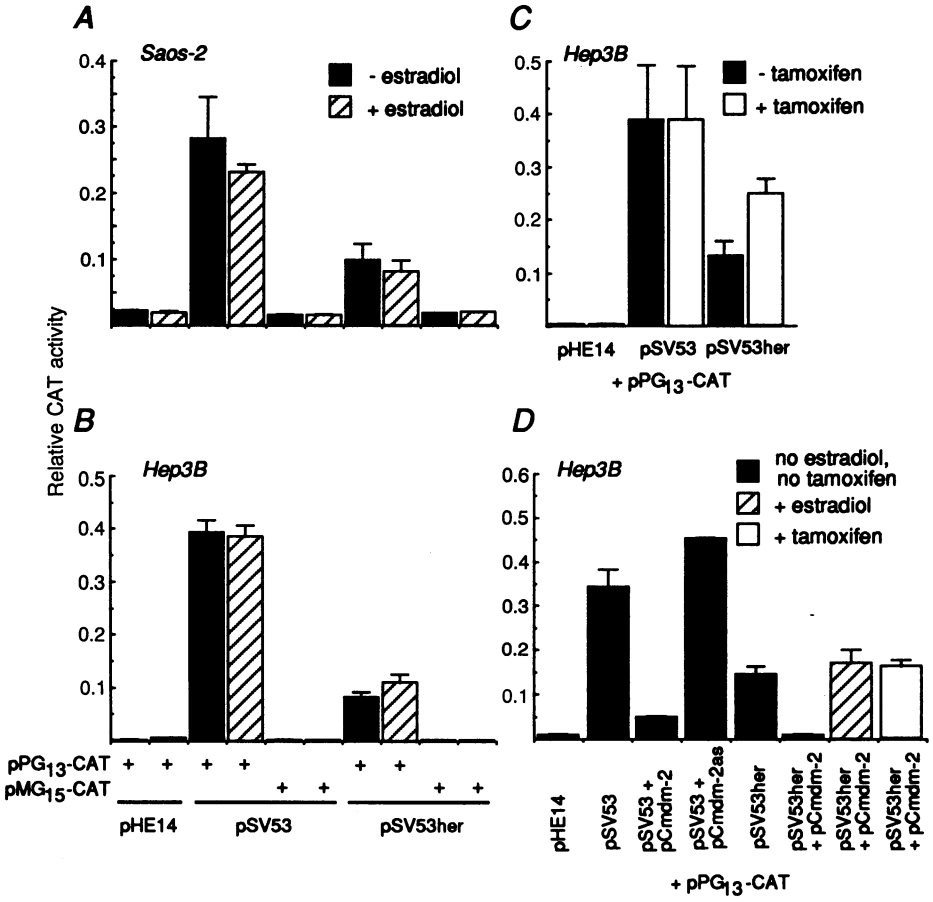

FIG. 4. Transcription transactivation mediated by p53her. Saos-2 (*A*) and Hep3B (*B–D*) cells were transiently co-transfected with 5 μg of plasmid pHE14, pSV53, pSV53her, pCmdm2, or pCmdm-2as and 10 μg of plasmid pPG_{13}-CAT or pMG_{15}-CAT per 10^6 cells, as indicated. Plasmid pCmdm-2 expresses the human MDM-2 oncoprotein, whereas plasmid pCmdm-2as expresses the MDM-2 cDNA in antisense orientation. Plasmid pPG_{13}-CAT contains wt p53-responsive promoter elements, whereas pMG_{15}-CAT contains mutated sequences that render the plasmid unresponsive to p53. CAT activity is expressed as activity relative to transfections with plasmid pSV2cat, in which the CAT reporter gene is driven by the simian virus 40 early promoter/enhancer. Cell extracts were prepared 36 hr after transfection. 17β-Estradiol (2 μM) or tamoxifen (1 μM) was added to the culture medium, where indicated, 16 hr before preparation of extracts. Bars represent standard deviations of the means from three experiments.

pSV53her, but unlike in the transactivation studies, we simultaneously cotransfected with the neomycin-resistance plasmid pSV2neo to confer resistance to the drug G418. Similarly, control plasmid pHE14 lacking p53 sequences was cotransfected with pSV2neo. The numbers of G418-resistant colonies formed by pSV53her-transfected cells were similar to those formed by control-transfected cells in the absence of hormone. In contrast, in the presence of hormone, the number of pSV53her-transfected colonies was significantly reduced. These results show that not only during low-level expression of p53her in stably transfected cells but also during p53her overexpression, under conditions that allowed p53her to transactivate a promoter in a hormone-independent manner, the growth-suppressor effect of p53her was dependent on the presence of β-estradiol. However, it is important to note that p53her transactivated the MDM-2 gene in the stable S-53her cell lines only in the presence of hormone. Therefore, it is possible that the p53-responsive DNA motif in the MDM-2 gene and perhaps in other cellular genes functions differently from the motif in pPG_{13}-CAT.

Hormone exposure of p53her-expressing Saos-2 cells for >2 days resulted in permanent arrest of cell growth. One reason for this may be that the cells were channeled into a differentiation pathway and had thereby lost their ability to reenter the cell cycle. Earlier studies have demonstrated that wt p53 expression indeed can induce partial differentiation of cells (24). We considered induction of apoptosis as another possible reason for the irreversibility of the phenotype (25). However, we were unable to demonstrate DNA degradation in the form of nucleosome ladders in these cells.

The finding that the estrogen receptor inhibitor tamoxifen, like estradiol, can arrest S-53her cell growth indicates that the inducible phenotypic changes in these cells are indeed the result of the action of p53her and not of some other cellular hormone-responsive factor. The activation of p53her by the estradiol antagonist tamoxifen is consistent with previous results on other hybrid proteins carrying the estrogen receptor hormone-binding domain (16). Since tamoxifen blocks the transactivation function of the hormone-binding domain (15), these results also suggest that this intrinsic transactivation function was not responsible for the effects of p53her on Saos-2 cells.

Our results and similar studies involving c-Myc, c-Fos and E1A (16, 26–28) make it clear that hormone-inducible hybrid molecules can serve as faithful models not only to study the functions of oncoproteins but also to dissect their pleiotropic effects in cells. We suggest that p53her can be a useful tool for further dissection of p53 functions.

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