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Transcriptional regulation of basic fibroblast growth factor gene by p53 in human glioblastoma and hepatocellular carcinoma cells

(mutant p53/tumor progression/brain tumor)

TETSUYA UEBA*†, TETSUYA NOSAKA†, JUN A. TAKAHASHI*, FUTOSHI SHIBATA‡, ROBERT Z. FLORKIEWICZ§, BERT VOGELSTEIN¶, YOSHIFUMI ODA*, HARUHIKO KIKUCHI*, AND MASAKAZU HATANAKA†‖

*Department of Neurosurgery, Faculty of Medicine, Kyoto University, Kyoto 606, Japan; †Institute for Virus Research, Kyoto University, Kyoto 606, Japan; ‡Faculty of Pharmaceutical Science, Toyama Medical and Pharmaceutical University, Toyama 930-10, Japan; §Departments of Biochemistry and of Molecular and Cellular Growth Biology, Whittier Institute for Diabetes and Endocrinology, La Jolla, CA 92037; and ¶Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD 21231

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ABSTRACT **Mutations of the p53 gene are found in various human cancers. The frequency of its mutation is reported to increase during tumor progression in most tumors. In human gliomas, mutations of the p53 gene are found in about one-third of the malignant forms and in few of the benign ones, indicating their possible involvement in tumor progression. On the other hand, we have recently shown that basic fibroblast growth factor (basic FGF) plays a crucial role in tumor progression as an autocrine growth factor in tissues of human gliomas. Therefore, we hypothesized that p53 might regulate the promoter activity of the basic FGF gene, which has several GC boxes and no typical TATA box. In this study, cotransfection assays using human glioblastoma and hepatocellular carcinoma cells and establishment of stable cell lines expressing mutant-type p53 were performed. The basic FGF gene promoter was demonstrated to be regulated by p53 at the transcriptional level and its basal core promoter was found to be responsive to p53. Expression of endogenous basic FGF was also demonstrated to be activated by mutant type p53. Wild-type p53 repressed gene expression of the basic FGF and its mutant activated it *in vitro*, implying one of the possible pathways in tumor progression.**

Basic fibroblast growth factor (FGF) is a mitogen and a morphogen for a wide range of neuroectoderm- and mesoderm-derived cells (1–7) as well as a potent angiogenic factor *in vivo* (3). Recently, the basic FGF transcripts have been reported to be abundantly expressed in >90% of human glioma tissues (8) and the basic FGF protein is reported to be produced *in vivo* (7–11). Elevated gene expression of its high-affinity receptors, such as FLG and BEK, has also been detected in these tissues (12, 13). Furthermore, a neutralizing antibody against human basic FGF inhibited both anchorage-dependent and anchorage-independent growth of human glioma cells (14). These results indicate that basic FGF is involved in automonous cell growth and tumorigenesis as an autocrine growth factor. In addition, the degree of malignancy in brain tumors was shown to be proportional to the expression level of basic FGF, suggesting that basic FGF plays a crucial role in tumor progression (7–11). A similar suggestion is true of human hepatocellular cell carcinomas (15–17). However, the mechanism of high expression of basic FGF in correlation with tumor malignant progression remains to be elucidated.

Recently, mutations of tumor suppressor gene p53 have been reported to be found frequently in various human cancers *in vivo* (18–22). In human gliomas, p53 mutations were found in about 30% of the malignant gliomas, such as anaplastic astrocytomas and glioblastomas, and in few of the benign forms (22). Similar results are also reported in human hepatocellular carcinomas (21). Furthermore, dominant clonal expansion of p53-mutant glioma cells has been reported (23). Taken together, their possible involvement in tumor progression is indicated. However, a target to which p53 links in tumor progression remains to be determined.

p53 is a nuclear phosphoprotein (24) and regulates various kinds of gene expression in the manner of sequence-specific DNA binding and/or direct protein–protein interaction (25–31). We speculated that p53 regulates the basic FGF promoter activity and that mutant-type p53 activates transcription of basic FGF, resulting in tumor progression.

We have cloned the 5′ flanking regulatory sequences of the human basic FGF gene. There are five GC boxes, which may represent Sp-1 binding sites, one potential AP-1 binding site, and no TATA box within the promoter region (32). Basic FGF represents the characteristics of a so-called "housekeeping gene." To test our speculation, cotransfection experiments have been carried out on the cells having no dominant negative mutant p53, using a bacterial chloramphenicol acetyltransferase (CAT) reporter construct driven by the human basic FGF promoter region and the human p53 expression vector of wild- or mutant-type ($143^{Val \rightarrow Ala}$) as an effector. A series of 5′ deletion constructs of the basic FGF promoter-CAT reporter was also cotransfected with p53 expression vectors to examine a p53 responsive element. As a control study, a bacterial CAT reporter construct driven by the human epidermal growth factor receptor (ER) promoter region was also transfected.

To make the data much more convincing, stable cell lines expressing mutant-type p53 were established and expression of endogenous basic FGF was examined.

MATERIALS AND METHODS

Cell Culture. A human glioblastoma cell line, U87MG (33), whose cell growth and tumorigenesis were reported to be inhibited by a neutralizing antibody against human basic FGF (14), and a human hepatocellular carcinoma cell line, SKHepG2 (34), were acquired from The Institute of Physical and Chemical Research (Japan). Cells were grown in Eagle's minimum essential medium (EMEM; U87MG cells) and Dulbecco's modified Eagle's medium (DMEM; SKHepG2 cells) supplemented with 10% heat-inactivated fetal calf serum and were incubated in humidified incubators under 5%

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Abbreviations: CAT, chloramphenicol acetyltransferase; FGF, fibroblast growth factor; ER, epidermal growth factor receptor; βGal, β-galactosidase.

‖To whom reprint requests should be addressed.

CO_2/95% air. Both cell lines do not express dominant negative mutant p53 and retain at least one allele of the p53 gene.

Plasmids. pC53-SN3 and pC53-SCX3 (35) contain human wild-type and mutant-type p53 cDNAs, respectively, under the transcriptional control of the human cytomegalovirus promoter. pC53-SCX3 encodes the mutant protein with a substitution from alanine to valine at position 143. pC53-S0 [identical to pCMV-Neo-Bam (35)] lacking the p53 coding region was constructed by *Bam*HI digestion of pC53-SN3 followed by self-ligation. Construction of pF2.0CAT, pFdCAT22/2, pFdCAT6N, and pBLCAT4 has been described (32). pFdCAT0.6 and pFdCAT+50 were constructed by digestion and blunt-ended ligation of pF2.0CAT at the sites of *Hin*dIII–*Acc* I and *Hin*dIII–*Bss*hII, respectively, using Klenow fragment of DNA polymerase I. Each construction is shown in Fig. 4. pBLERCAT was constructed by digestion and ligation of pBLCAT2 and pERCAT1 (36) at the sites of *Hin*dIII–*Xho* I. pRSV-βGal (37) was used as an internal control to normalize the transfection efficiency in the case of deletion mutants. pRAS-βGal (38) was used as an internal control for cotransfection experiments because the human Ha-*ras*1 gene was reported to be neither activated nor repressed by p53 (29). All plasmids were prepared by the Qiagen plasmid kit (Chatsworth, CA).

Transfection and CAT Assay. Cells (1×10^6) were plated in 10-cm dishes 1 day before transfections and were transfected by the calcium phosphate method with 5 μg of a series of 5′ deletion constructs, 5 μg of pRSV-βGal as an internal control, and 5 μg of sonicated salmon sperm DNA as a carrier. As a negative control, pBLCAT4 was also transfected in the same way. Mixtures of 5 μg of pC53-SN3, pC53-SCX3, or pC53-S0 as an effector plasmid, 5 μg of pF2.0CAT, a series of 5′ deleted constructs, or pBLERCAT as a reporter plasmid, and 5 μg of pRAS-βGal as an internal control were cotransfected into U87MG or SKHepG2 cells. Mixtures of 5 μg of pF2.0CAT, 5 μg of pRAS-βGal, and 5 μg of sonicated salmon sperm DNA as a carrier were also transfected into stable cell lines mentioned below. Forty hours posttransfection, cell lysates were extracted and assayed for CAT activity (39). All assays were normalized after measurement of the β-galactosidase (βGal) activities (40). The degree of CAT conversion was estimated by measuring the radioactivities of the ^{14}C-containing spots using a bioimage analyzer, BAS2000 (Fuji). All transfection experiments were repeated at least three times.

Stable Cell Lines. The U87MG cell line was transfected by the method mentioned above with 10 μg of pC53-SN3, pC53-SCX3, or pC53-S0, which were linearized by *Hin*dIII digestion, and 5 μg of sonicated salmon sperm DNA as a carrier and were cultured for 2 weeks under administration of 1.2 mg of geneticin per ml. Each subconfluent stable cell line was suspended in the buffer (50 mM Tris, pH 7.6/0.15 M NaCl/4 mM EDTA/1% Nonidet P-40/0.1% sodium deoxycholate/10 mM $Na_2P_2O_7$/10 mM NaF/2 mM $NaVO_3$/1 mM phenylmethylsulfonyl fluoride). Proteins were fractionated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and were transferred to the nylon membrane (Immobilon, Millipore). Constitutive expression of mutant p53 was detected by using an monoclonal antibody against p53, DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and subsequently incubating with alkaline phosphatase-conjugated anti-mouse antibody (Promega). As a positive control, the U251MG cell line, which retains mutant-type p53 (33), was examined.

RNA Preparation, RNase Protection Assay, and Northern Blot Analysis. For RNase protection assay of transient transcripts, SKHepG2 cells (1×10^6) plated in 10-cm dishes 1 day before transfection were transfected with 5 μg of pC53-SN3, pC53-SCX3, or pC53-S0, 5 μg of pF2.0CAT, and 5 μg of salmon sperm DNA as a carrier. Forty hours posttransfection, RNA was prepared by the acid guanidinium/phenol/chloroform method (41). A *Hin*dIII–*Eco*RI fragment (0.25 kb) of pSV2CAT (39) was subcloned into pBluescriptIISK+ (Stratagene) at the same sites. The resultant plasmid was linearized at an *Xho* I site and used as a template for a riboprobe spanning the 0.25-kb fragment of an initial part of the CAT coding region together with the neighboring sequences derived from pBluescript (see Fig. 3*b*). *In vitro* transcription by T3 RNA polymerase was performed using [α-^{32}P]UTP according to the manufacturer's protocol to obtain the antisense CAT riboprobe. Twenty micrograms of total RNA was hybridized with 5×10^5 cpm of the riboprobe at 45°C overnight, treated with RNase A and RNase T_1 at 30°C for 45 min, and electrophoresed in a 6% polyacrylamide/8 M urea denaturing TBE gel as described (42). Radioactivity of the 0.25-kb CAT transcript was measured by a bioimage analyzer, BAS2000 (Fuji). For Northern blot analysis, each stable cell line (2×10^6) was plated in 10-cm dishes 2 days before RNA preparation. Twelve hours after premedium change, RNA was prepared by the acid guanidinium/phenol/chloroform method. Twenty micrograms of total RNA was denatured with 2 M formaldehyde and electrophoresed onto a nylon membrane (Biodyne, Pall). Human basic FGF cDNA probe (8) was used for hybridization.

RESULTS

Wild-Type p53 Represses and Mutant-Type p53 Activates Activity of the Basic FGF Promoter. Using cotransfection assay, we examined whether or not p53 affects the activity of the basic FGF promoter and the ER promoter. In U87MG cells, no mutation of the p53 gene and an undetectable level of p53 protein expression were demonstrated by PCR single-strand conformation polymorphism and Western blot analyses, respectively (33). In SKHepG2 cells, heterozygosity of p53 gene was demonstrated by Southern blot analysis. Furthermore, pulse–chase analysis of p53 protein showed that

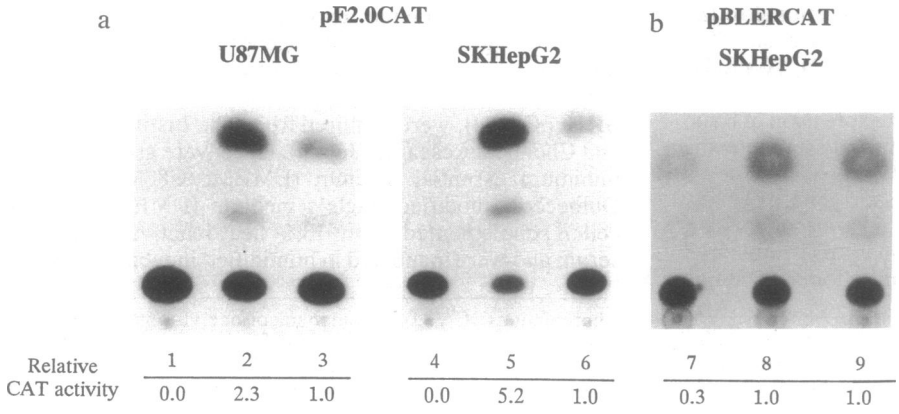

FIG. 1. (*a*) Effects of wild-type and mutant-type p53 expression on a basic FGF promoter-CAT construct. (*b*) Effects of wild-type and mutant-type p53 expression on an ER promoter-CAT construct. Wild-type p53 expressor, pC53-SN3 (lanes 1, 4, and 7), mutant-type p53 expressor, pC53-SCX3 (lanes 2, 5, and 8), or a vector lacking p53 sequences, pC53-S0 (lanes 3, 6, and 9), was cotransfected with pF2.0CAT reporter plasmid (lanes 1–6) or pBLERCAT (lanes 7–9) and pRAS-βGal into each cell line. CAT assays were carried out after normalization of the transfection efficiency by βGal activity. A typical pattern is shown.

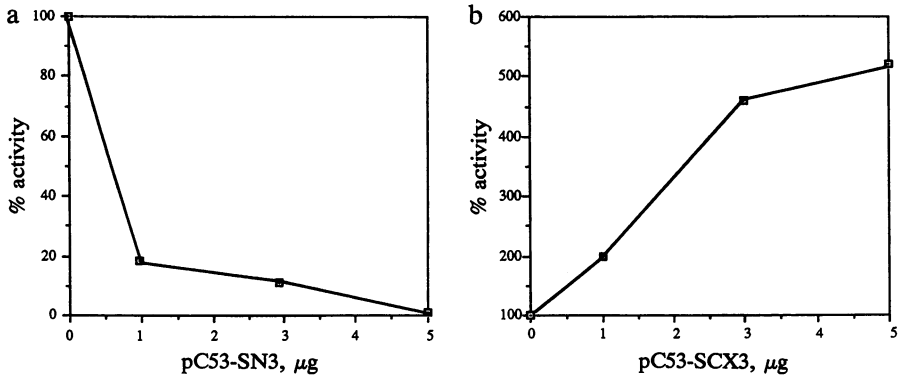

FIG. 2. Repression and activation of basic FGF promoter activity by p53 in a dose-dependent manner. SKHepG2 cells were transfected with 5 μg of pF2.0CAT and the indicated amount of p53 expression vectors (*a*, pC53-SN3; *b*, pC53-SCX3). The total amount of DNA was adjusted to 15 μg per 10-cm plate with added pC53-S0 DNA. CAT assays were performed as in Fig. 1 after measurement of βGal activity.

p53 protein in those cells is wild-type (34). These cell lines do not express dominant negative mutant p53. Fig. 1*a* shows that wild-type p53 completely represses the basic FGF promoter activity and mutant-type p53 activates it in both U87MG and SKHepG2 cell lines. Fig. 1*b* shows that wild-type p53 represses partially the ER promoter activity, but mutant-type p53 does not activate it in SKHepG2 cell lines. In U87MG cell line, the conversion rate of CAT activity was low but the same result was obtained (data not shown). The result that overexpression of mutant-type p53 does not activate the ER promoter activity in the cells retaining wild-type p53 suggests that p53 does not affect it. Its partial repression may be brought by nonspecific reaction induced by overexpression of wild-type p53. These assays were repeated at least three times and the results were reproducible. Normalization for CAT assay was performed by RAS-βGal activity. CAT assay using the equal amount of protein was also performed and the same results as the one normalized by the βGal activity were obtained (data not shown). Our data indicated that overexpression of mutant-type p53 overcame the function of their endogenous p53.

Repression and Activation of the Basic FGF Promoter Activity by p53 Constructs in a Dose-Dependent Manner. Relative CAT activities in Fig. 1 showed repression by almost 0% with the expression of the wild-type p53 and 2- to 5-fold activation with that of the mutant-type p53. Since CAT activity is an enzymatic activity, it may be augmented. To confirm the effects of the wild- or mutant-type p53, we examined the CAT activity with various doses of the effector plasmids. Five micrograms of pF2.0CAT, 5 μg of pRAS-βGal, and the indicated amount of effector plasmid, pC53-SN3 or pC53-SCX3, were introduced into SKHepG2 cells. The total amount of DNA was adjusted to 15 μg per 10-cm dish with expression vector lacking the p53 sequence. Forty hours posttransfection, cell lysates were extracted and assayed for CAT activity. All assays were normalized by the βGal activity. As shown in Fig. 2, dose-dependent repression or activation by wild- or mutant-type p53 was observed on the basic FGF promoter activity. Similar results were obtained in U87MG cells (data not shown). Taken together with Fig. 1, our findings indicate that repression and activation of the basic FGF promoter by p53 are not brought by a squelching effect in a limited amount of common transcription factors *in vitro*.

Regulation of the Basic FGF Gene by p53 at the RNA Level. We have tested the effect of p53 toward the basic FGF promoter by CAT assay. However, p53 is a cell-cycle regulator (43–45) and there might be the possibility of posttranslational modification of CAT expression in the cotransfection assay. Therefore, RNase protection analysis was performed (Fig. 3*a*). To detect the effect of p53 expression on the basic FGF promoter-CAT plasmid, and to avoid the hybridization with the endogenous basic FGF, a riboprobe derived from the CAT gene (Fig. 3*b*) was employed. Relative radioactivity of each 0.25-kb transcript was measured by a bioimage analyzer. The result was consistent with that of the CAT assay. These findings indicate that the basic FGF promoter activity is regulated by p53 at the transcriptional level.

Transcriptional Regulation by p53 at the Basal Core Promoter Machinery. To localize a p53 responsive element in the basic FGF promoter, we constructed various 5′ deletion constructs (Fig. 4*a*). Each construct was transfected into SKHepG2 cells. Relative CAT activity is demonstrated in Fig. 4*b*. pFdCAT0.6, which contains one AP-1 site and five GC boxes, showed a maximum CAT activity. pFdCAT6N showed about one-half of the activity of pFdCAT0.6. pFdCAT+50 had less activity than pBLCAT4. Similar results were observed in U87MG cells (data not shown). These findings suggest that a potent basal core promoter exists in the fragment between −20 and +50 relative to the transcription start site (+1) and that read-through transcription from the upstream cryptic promoter, if it exists, can be negligible.

Cotransfection experiments with each 5′ deleted construct and each effector plasmid were performed to search the fragment affected by p53 (Fig. 5). pFdCAT0.6 and pFdCAT22/2 were affected by p53. Repression in pFdCAT22/2 having no AP-1 site suggests the existence of another frag-

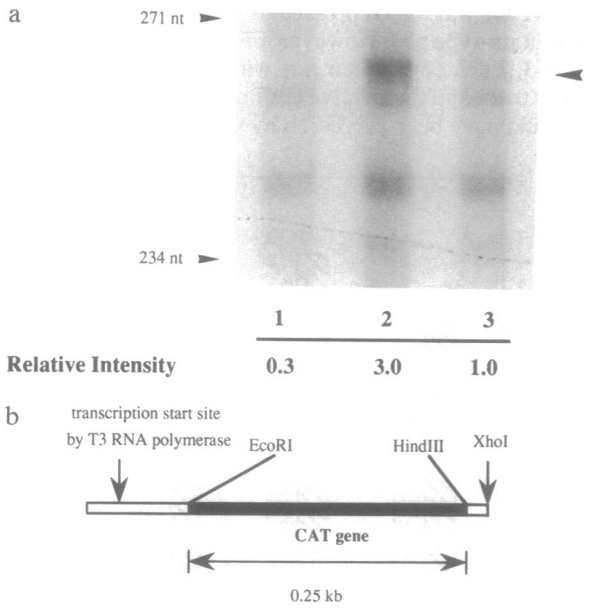

FIG. 3. (*a*) RNase protection analysis of the RNAs extracted from SKHepG2 cells. Cells were cotransfected with pF2.0CAT reporter plasmid and pC53-SN3 (lane 1), pC53-SCX3 (lane 2), or pC53-S0 (lane 3) as an effector plasmid. Positions of the end-labeled and denatured φX174 DNA that had been digested with *Hae* III are given on the left. RNA migrates slower than DNA in denaturing gels. A CAT transcript of 0.25 kb is indicated by the arrowhead on the right. Relative intensity of the 0.25-kb band is shown below the lane numbers. (*b*) Schematic presentation of the antisense riboprobe.

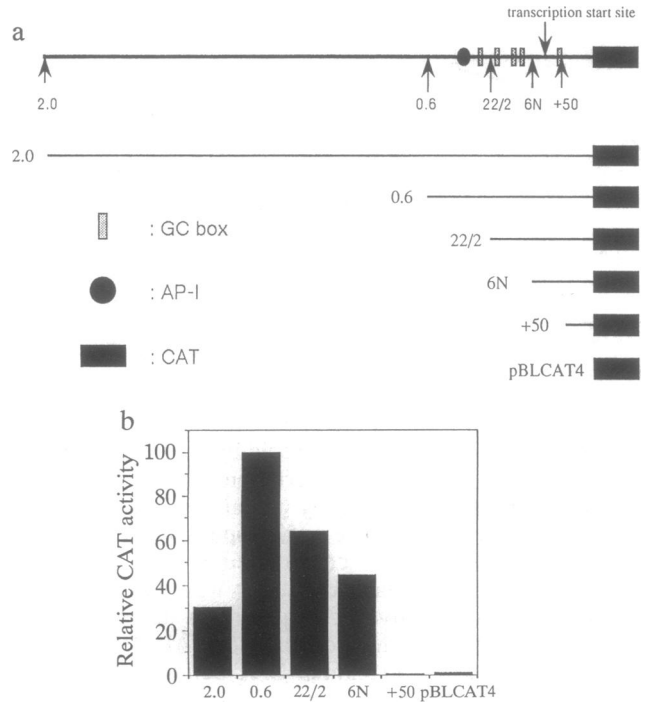

FIG. 4. (*a*) Schematic presentation of 5′ flanking basic FGF promoter-CAT and its 5′ deleted constructs. pF2.0CAT (2.0) and pFdCAT0.6 (0.6) have one AP-1 site and five GC boxes. pFdCAT22/2 (22/2) has four GC boxes. pFdCAT6N (6N) contains the transcription start site and one GC box. pFdCAT+50 (+50) was deleted about 50 bp downstream from the transcription start site and has no GC box. pBLCAT4 is a promoterless construct. (*b*) Relative CAT activity in SKHepG2 cells. Cells were transfected with 5 μg of each construct and pRSV-βGal. All assays were normalized by βGal activity.

ment affected by p53. AP-1 activity, which was reported to be repressed by p53 (25), is constituted by c-FOS and c-JUN. pF6NCAT was also repressed by wild-type p53 and activated by mutant-type p53. However, pFdCAT+50 was not affected by p53. Each conversion rate with wild- or mutant-type p53 was <0.2% and the relative CAT activities were nearly equal. It would have been repressed by wild-type p53 and activated

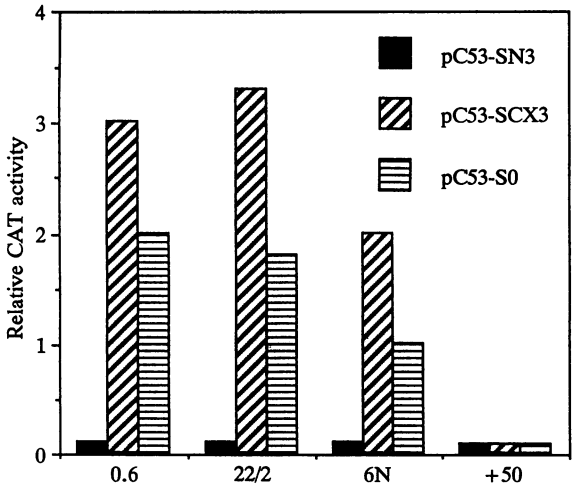

FIG. 5. Response of each 5′ deletion construct of the basic FGF promoter to p53 expression in U87MG cells. Five micrograms of pC53-SN3, pC53-SCX3, or pC53-S0 was introduced with each reporter plasmid (5 μg) and pRAS-βGal (5 μg) into the cells. Forty hours posttransfection, cell lysates were extracted and assayed for CAT activity. All assays were normalized by βGal activity. Similar results were obtained in SKHepG2 cells.

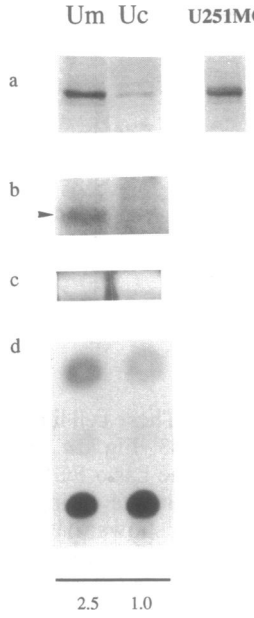

FIG. 6. Transcriptional activation of endogenous basic FGF gene in the stable cell line expressing mutant-type p53. (*a*) Western blot analysis of the mutant-type p53 protein in the Um and Uc stable cell lines and U251MG cell line as a positive control. (*b*) Northern blot analysis of total RNAs extracted from the Um or Uc stable cell line. Endogenous basic FGF transcript of 7.0 kb is indicated by the arrowhead on the left. (*c*) Ribosomal RNA (28 S). The same dose of total RNAs was applied. (*d*) CAT activity in the stable cell lines. A mixture of 5 μg of pF2.0CAT, 5 μg of pRAS-βGal as an internal control, and 5 μg of sonicated salmon sperm DNA as a carrier was transfected into the Um or Uc cell line. CAT assays were performed as in Fig. 1 after measurement of βGal activity. The numbers below indicate relative CAT activity.

by mutant-type p53 if putative read-through transcription had taken place. These findings suggest the transcriptional regulation of the basic FGF gene by p53 at the basal core promoter machinery.

Activation of Gene Expression of Endogenous Basic FGF by Mutant-Type p53. The results mentioned above were obtained by the transient assay. To make the data much more convincing, we established a stable cell line (Um) that constitutively expressed mutant-type p53. As a control, a cell line transfected pC53-S0 (Uc) was also established. We could not obtain the stable cell line expressing wild-type p53. The Um cell line did not overexpress mutant-type p53 protein compared with the U251MG cell line, which bears mutant-type p53 (33) (Fig. 6*a*).

By Northern blot analysis, endogenous basic FGF was detected more in the Um cell line than in the Uc cell line (Fig. 6*b*). CAT activity of pF2.0CAT was 2.5-fold activated in the Um cell line compared with that in the Uc cell line (Fig. 6*d*). These results in the stable cell line system reveal the transcriptional activation of endogenous basic FGF gene by mutant-type p53 corresponding to the results in the transient assay system.

DISCUSSION

Tumor suppressor gene p53 is a nuclear phosphoprotein (24) and a cell-cycle regulator (43–45). It regulates transcription of various genes (25–31). Previously, it was reported that p53 interacts with TATA binding protein (TBP) (46, 47), which is required for not only TATA-mediated but also TATA-less transcription (48). Recently, TATA-mediated but not TATA-less transcription has been demonstrated to be repressed by p53 (49). The promoters of the human c-Ha-*ras*1 gene (29, 30), the ER gene (29), which is associated with tumor progression in gliomas (50), and others (29, 30) have been reported to be resistant to repression by p53. This discrepancy seems to be dependent on the difference of TBP-associated factors.

Basic FGF promoter has five GC boxes, one AP1 site, and no TATA box (32). It represents the characteristics of a housekeeping gene. We demonstrate here that this TATA-less gene is regulated by p53 at the basal core promoter machinery.

It must be noted that several biological effects of p53 would be brought into play by the transcriptional regulation of the basic FGF promoter. The production of a growth factor and

its receptor by a cell that responds to the factor has been termed autocrine stimulation of proliferation. Several growth factors are thought to be involved in malignant transformation through autocrine stimulation of the growth (51–53). In gliomas, enormous amounts of basic FGF protein and its receptor protein are produced in tumor cells themselves (7–13), and a neutralizing antibody against human basic FGF inhibits cell growth and tumorigenesis of human glioma cell lines (14). These previous data suggest that basic FGF gives autonomous cell growth and tumorigenesis as an autocrine growth factor in gliomas and that it is associated with tumor progression (7–11). Basic FGF is also detected in hepatocellular carcinomas, but not in normal liver tissues, and is suggested to be an autocrine growth factor in hepatocellular carcinomas (15–17). In both tumors, basic FGF plays a crucial role in tumor growth and tumor progression. On the other hand, mutation or deletion of the p53 gene is also detected during tumor progression. Our finding that mutant-type p53 activates the basic FGF promoter activity, but not the ER promoter activity, demonstrates one of the possible pathways in tumor progression. Loss of normal function of p53 may bring the activation of basic FGF transcription, resulting in tumor progression.

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