Regulation of FGF-3 Gene Expression in Tumorigenic and Non-tumorigenic Clones of a Human Colon Carcinoma Cell Line*

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Catherine Galdemard, Hidehisa Yamagata‡, Olivier Brison, and Christian Lavialle§

From the Laboratoire de Génétique Oncologique, CNRS UMR 1599, Institut Gustave-Roussy, 94805 Villejuif, France

The FGF-3 gene is constitutively expressed in tumorigenic clones from the SW613-S human colon carcinoma cell line but is silent in non-tumorigenic clones. We have investigated the transcriptional mechanisms responsible for this differential expression. Mapping of DNase I-hypersensitive sites throughout the FGF-3 gene and the region extending 15 kilobases upstream disclosed differences in the patterns obtained between tumorigenic and non-tumorigenic cells. Transient expression assays carried out with a reporter gene driven by FGF-3 promoter fragments of various lengths (0.143 to 11 kilobases) did not reproduce the differential regulation of the resident gene between the two cell types. The same constructs did exhibit a differential activity in stable transfectants, suggesting the involvement of a chromatin-based mechanism in this regulation. Under these conditions, even the 143-base pair minimal promoter fragment was able to drive the differential expression of the reporter gene. During the course of these analyses, several transcriptional modulatory elements (mainly activators) were identified in the FGF-3 upstream region and were found to colocalize with DNase I-hypersensitive sites. Moreover, a putative new promoter was discovered 6 kilobases upstream of FGF-3. Altogether, these data provide a basis for the elucidation of the complex regulation of the human FGF-3 gene.

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† Supported by a Visiting Research fellowship from the Canon Foundation in Europe. Current address: Dept. of Geriatric Medicine, Ehime University School of Medicine, Shigenobu, Onsen-gun, Ehime Prefecture, Japan 791-0295.
‡ Supported by a Visiting Research fellowship from the Canon Foundation in Europe. Current address: Dept. of Geriatric Medicine, Ehime University School of Medicine, Shigenobu, Onsen-gun, Ehime Prefecture, Japan 791-0295.
§ To whom correspondence should be addressed: Laboratoire de Génétique Oncologique, CNRS UMR 1599, Institut Gustave-Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France. Tel.: 33 (0)1 42 11 49 60; Fax: 33 (0)1 42 11 52 61; E-mail: lavialle@igr.fr.

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EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The origins of the SW613-S human colon carcinoma cell line and cell culture conditions have been described previously (16–19). SW613-1DA1 is a tumorigenic clone and SW613-B3 and -2G1 are non-tumorigenic clones derived from the SW613-S cell line; 2G1mucP2Tu1 is a cell line established from a tumor induced in a nude mouse by inoculation of 2G1 cells stably transfected with human 10-kb FGF-3 expression vector (17). The EJ cell line, a kind gift of François Dautry (Institut de Recherche sur le Cancer, Villejuif, France), has been shown to be in fact the same as the T24 human bladder carcinoma cell line (21).

Preparation of Nuclei and DNase I Treatment—Nuclei were prepared essentially as described by Greenberg and Ziff (22). For each cell line, 10 Petri dishes (10 cm in diameter) were seeded at a density of 2 × 10^5 cells per dish in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 2 days in culture, cells were refed with fresh medium. Twenty-four hours later, cells (in log-phase growth) were scraped in their medium, pelleted at 500 × g for 5 min, washed twice with 20 ml of ice-cold phosphate-buffered saline (PBS), and centrifuged each time at 500 × g for 5 min at 4 °C. All further procedures until DNase I digestion were carried out on ice or at 4 °C. The cell pellet (usually 1 × 10^7) was resuspended in 5 ml of TM buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2), and 5 ml of TM buffer containing 1% Nonidet P-40 (w/v) were immediately added dropwise while gently vortexing the cell suspension. The tubes were kept on ice for 5 min (lysoscopy monitored by microscopy) and centrifuged at 500 × g for 5 min. The supernatant was discarded, and the nuclei pellet was washed once with TM buffer containing 0.5% Nonidet P-40 (v/v) and centrifuged at 500 × g. Washed nuclei were suspended in 2 ml of TM buffer, and the suspension was divided into 6 aliquots of 290 μl.

Isolation of DNA—Extraction of the DNA was performed by the “salting out” procedure described by Miller et al. (23), adapted to cultured cells. Cells from two to four 10-cm diameter Petri dishes (2–5 × 10^7) were harvested by scraping, pelleted at 500 × g for 5 min, resuspended in 10 ml of PBS, and transferred into a 15-ml polypropylene tube. After centrifugation and a second wash in 10 ml of PBS, the final cell pellet was suspended in 3 ml of TNE buffer (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 2 mM Na2EDTA). Extraction of the DNA was then carried out as described below for whole cells.

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Restriction Enzyme Digestion and Southern Blotting—Genomic DNA was digested with restriction enzymes and electrophoresed (10 μg of DNA per 7-mm-wide lane) in 0.7% agarose. A mixture of 1 μg of “1-kb DNA ladder” and 500 ng of HindIII-digested λ DNA (both from Life Technologies, Inc.), preheated at 65 °C for 5 min, was run in parallel in the left-most lane as a size marker. Prior to transfer, a photograph of the gel stained with ethidium bromide was taken with a graduated ruler placed next to the marker lane to measure precisely the migration distance of each fragment. Depurination and denaturation of the DNA were achieved by successively treating the gel twice for 10 min with 0.25 N HCl and twice for 15 min with 0.5 N NaOH, 1.5 M NaCl. The DNA was then transferred by capillary onto a neutral nylon membrane (Hybond N, Amersham Pharmacia Biotech) for 2–3 h, using the 0.5 N NaOH, 1.5 M NaCl solution. After a quick wash in 2× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium citrate, and 0.025% sodium azide), the membrane was air-dried, and the DNA was immobilized by UV irradiation.

DNA fragments to be used as probes were excised from recombinant plasmids by digestion with the appropriate enzyme(s), gel-purified, and radiolabeled by the random priming method (Multiprime DNA labeling system, Amersham Pharmacia Biotech) in the presence of [α-32P]dATP (3000 Ci/mmol) and T4 polynucleotide kinase. The corresponding positive control plasmids, pSVGCAT-C and pUSVCAT-C, containing the SV40 virus early promoter, have been reported (24, 25). FGF-3 promoter constructs were designed using fragments derived from the A3 recombinant plasmid (20). Fragments containing FGF-3 intron 1 sequences were derived from the insert of a recombinant plasmid that encompasses the whole FGF-3 gene (15). For the sake of simplicity, CAT constructs were called by the name of the promoter fragments that they contain, themselves designated by letters symbolizing the restriction sites at both ends, except for E2, E1, and E0. The E2 and E1 fragments were generated by polymerase chain reaction, whereas the E0 fragment was obtained by digestion with AscI and NciI; their boundaries are indicated in Fig. 3. The relative position of the promoter fragments present in each construct is shown below the maps of the FGF-3 locus in the diagrams presented in Figs. 4–6 and 9. All of the promoter fragments were inserted into pGCAT-C, except NcoB, BB, SB, E2, E1, E0, ANc-SB, ANc(as)-SB, SB-AK, HX, HX(as), NcX, and NcX(as), that were inserted into pUSCAT-C. Plasmid pUSCAT-C was preferentially used over pGCAT-C whenever small promoter fragments driving low CAT expression levels were tested. Indeed, this vector yields higher background values (25). The detailed protocol followed for the construction of each plasmid, as well as their map and full sequence, can be obtained directly from the authors. The construction of chimeric promoters containing activator elements from the SV40 virus early promoter (26) upstream of the FGF-3 minimal promoter E0 were performed as follows: three different fragments were excised from the pUSVCAT-C plasmid, (i) a 253-bp EcoRI-NcoI fragment containing the SV40 enhancer repeat sequence (26), (ii) a 763-bp SacI-SphI fragment containing only the 72-bp repeat, and (iii) a 91-bp SpnI-NcoI fragment containing only the GC boxes; each fragment was blunt-ended with the T4 DNA polymerase and inserted into the unique SpnI site (blunt-ended as well) of the E0 construct, immediately upstream of the E0 promoter fragment.

Cell Transfection and CAT Assay—Transient transfections were carried out using the calcium phosphate precipitation procedure described by Wigler et al. (27). Cells, plated at a density of 3 × 10^5 per 10-cm dish before the day of transfection, were transfected with 20 μg of plasmid DNA. After a 24-h incubation with the DNA-calcium phosphate precipitate, the medium was changed and cells were harvested 48 h later. Stable transfectants were generated by the same transfection method, using the CAT expression vector to be tested (10 μg) together with 1 μg of pSVTKneo (conferring resistance to the neomycin derivative G418, supplied by Life Technologies, Inc.) (28). The procedure for the establishment of four independent pools of G418-resistant clones and harvesting of the cells from each pool for the preparation of whole cell extracts and genomic DNA has been described (29). CAT assays and quantification of the results were carried out as previously reported (25, 29).

RNAse Protection Assay—Total cytoplasmic RNA was extracted according to a previously published procedure (16). To analyze the RNA of 12A1 cells stably transfected with the HX and BN constructs, two plasmids, pGHXC-PvPv1 and pGSNHc, respectively, were used as templates for the synthesis of antisense RNA probes. Plasmid pGHXC-PvPv1 was constructed by inserting into the Smal site of pGEM4, an
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**TABLE I**

Probes and restriction enzymes used in the mapping of DNase I-hypersensitive sites

| Probes* | Restriction enzymes | Mapped hypersensitive sitesb |
|---------|---------------------|-----------------------------|
| SB      | EcoRI              | HS1, HS2, HS3, HS4, HS5     |
| BK4     | XbaI               | HS3, HS5, HS6, HS7, HS8, HS9 |
| KpnI    |                     | HS8, HS9                   |
| S5/SK   | EcoRI              | HS8, HS9                   |
| HX      | HindIII            | HS1a-c, HS2a, b, HS3, HS4, HS5, HS6, HS7 |
| XhoI    |                     | HSu1, HSu2a, b, HSu3        |

*The position of each probe on a restriction map of the FGF-3 gene region is shown in Fig. 2.

**RESULTS**

DNase I-hypersensitive Sites—As a first approach to localize the sequences involved in the transcriptional regulation of the FGF-3 gene in cells of tumorigenic and non-tumorigenic clones from the SW613-S carcinoma cell line, we have mapped the DNase I-hypersensitive sites detectable in the chromatin of both cell types throughout the FGF-3 gene and the region extending 15 kb upstream. Nuclei prepared from cells of the tumorigenic clone SW613-12A1, the non-tumorigenic clone SW613-B3, or the EJ bladder carcinoma cell line were treated with increasing concentrations of DNase I. The EJ cell line was chosen as a negative control among a number of human tumor cell lines in which expression of the FGF-3 gene was never detected. Genomic DNA was then extracted, digested with various restriction enzymes, and analyzed by Southern blotting using small probes hybridizing close to one end of specific FGF-3 fragments. The seven combinations of enzymes and probes used in the different experiments are listed in Table I. These combinations were chosen to include overlaps so that all potential hypersensitive sites could be detected and to give redundancy allowing checks for internal consistency. An example of a Southern blot analysis is shown in Fig. 1, and a compilation of the results depicted as a map is shown in Fig. 2. On the basis of the map, many hypersensitive sites were detected in the FGF-3 gene region of EJ cells in agreement with the fact that these cells do not express the gene. In contrast, numerous sites were found in the chromatin of both B3 and 12A1 cells (Fig. 2). This was unexpected in the case of B3 cells since we have previously shown that the FGF-3 gene is silent in cells of non-tumorigenic clones (15). Some sites were detected in both cell types, either with the same (HSu3 and HS4) or with different intensities (HS1bc, HS2b, HS3, and HS6c). Several other sites were specifically found in tumorigenic 12A1 cells that express the gene as follows: HSu1, HSu2, HSu2a, HS5, HS7, HS8, HS9, and HS10. The HS5 site colocalized with the promoter of the gene. Finally, some sites were specific to the non-tumorigenic B3 cells as follows: the HS1a site, as well as the HS6 site which actually regroups a cluster of closely spaced sites (HS6a to HS6i) located in the 3′-half of the first exon and in the 5′-half of the first intron (see Fig. 2). With the exception of the HS6c site, all of the other HS6 sites were not detectable in the chromatin of 12A1 cells.

**FIG. 1.** DNase I-hypersensitive sites in the 5′ region and upstream sequence of the FGF-3 gene. DNA from DNase I-treated nuclei was digested with HindIII, electrophoresed, and blotted, and specific fragments were detected with probe HK (see Fig. 2). Cell lines are indicated at the top; EJ is a bladder carcinoma cell line, used as a control; B3 and 2G1 are non-tumorigenic clones; and 12A1 is a tumorigenic clone derived from the SW613-S cell line; 2G1myc2P7Tu is a cell line derived from a tumor induced in a nude mouse by 2G1 cells stably transfected with a c-MYC expression vector (see “Experimental Procedures”). The triangles symbolize the increase in DNase I concentration (0, 10, and 20 μg/ml), except for clone 2G1, whose nuclei were treated at a single DNase I concentration (15 μg/ml), as indicated by the square. Lane C contains deproteinized human DNA digested with HindIII, as a control for any spurious band that might result from cross-hybridization with the probe. The position of marker DNA fragments run on a parallel lane was determined on a photograph of the gel stained with ethidium bromide. The size (in kb) of some of them is indicated on the left. The arrows on the right point to the positions of the fragments generated by DNase I digestion at the indicated hypersensitive sites (HS) in the various cell lines. The bracket delimits the smear of unresolved bands generated by the HS6 cluster of hypersensitive sites in clones B3 and 2G1. The smear was actually resolved as a set of 9 bands using the probe/enzyme combination 5′-SK/KpnI (see Table I and Fig. 2 for a compilation of the results). Fragments generated by cleavage at the most sensitive sites are already detectable in the lanes containing DNA from control nuclei (not treated with DNase I) presumably due to the activity of endogenous nucleases during the 10-min incubation at 37 °C (see “Experimental Procedures”).

857-bp PvuII fragment excised from the HX construct and containing sequences from the FGF-3 upstream sequence (nucleotides 4835–5515; GenBank™ accession number X14445; XhoI or EcoRI, respectively, using phage T7 RNA polymerase (Promega Corp., Madison, WI), NcoI or PvuII, and linearized with SacI, I and PvuII, respectively, using phage T7 RNA polymerase (Promega Corp., Madison, WI), in the presence of (α-32P)UTP (800 Ci/mmol, NEN Life Science Products). RNase protection assays were performed as described (30).
cells and to compare the resulting pattern with those observed in B3 and 12A1 cells. An example of such an analysis, carried out with nuclei from 2G1mycP2Tu1 cells, is presented in Fig. 1, along with a nuclear sample prepared from cells of the non-tumorigenic 2G1 parental clone. The results obtained in this series of experiments showed that the pattern of hypersensitive sites found in 2G1 and 2G1mycP2Tu1 cells was indistinguishable from that detected in B3 and 12A1 cells, respectively (Fig. 1 and data not shown). Induction of the FGF-3 gene, following introduction of the c-MYC gene and in vivo selection, was therefore accompanied by a shift in the configuration of hypersensitive sites, from the pattern specific to the cells of non-tumorigenic clones to that characteristic of the cells from tumorigenic clones. Altogether, these results indicated that elements scattered throughout the whole FGF-3 gene region and up to 10 kb upstream of the gene (sites H5u1 and H5u2) could be involved in the regulation of its differential expression between tumorigenic and non-tumorigenic cells of the SW613-S cell line.

**Effects of FGF-3 Gene Upstream and Intron 1 Sequences on Promoter Activity in Transient Expression Assays**—Brookes et al. (14) had determined the sequence of the FGF-3 gene up to a BamH I site located about 500 bp upstream of the major initiation sites. An 840-bp BamHI fragment (BB) containing the promoter region (Fig. 3) was inserted upstream of the chloramphenicol acetyltransferase (CAT) reporter gene into the pUS-CAT-C vector. Non-tumorigenic B3 cells and tumorigenic 12A1 cells were transfected in parallel with this BB construct, and the CAT activity of cellular extracts prepared after 72 h was determined (Fig. 4). The pUSVCAT-C vector, containing the CAT gene under the control of the SV40 early promoter, was used as a control for transfection efficiency since we have previously shown that this promoter is equally efficient in both cell types (25). The BB fragment drove a low (~200-fold lower than the strong SV40 promoter), but measurable, expression of the CAT gene. However, this activity was comparable in 12A1 cells that express the resident FGF-3 gene and in B3 cells that do not. In an attempt to reproduce the differential expression of the resident gene, vectors containing increasing lengths of FGF-3 upstream sequences were constructed and assayed for CAT activity in both cell clones (fragments KB, HB, and BN in Fig. 4). All of them yielded analogous results as follows: a low, but non differential, activity. In contrast, no activity was detected in control EJ cells upon transient transfection with the HB promoter construct, although the activity of the pUSVCAT-C vector was of the same order of magnitude as that observed in B3 or 12A1 cells (results not shown).

We have shown above that a cluster of strong DNase I-hypersensitive sites was present in the first intron of the gene in non-tumorigenic but not in tumorigenic cells. We hypothesized that these sites might correspond to the binding of a repressor factor(s) responsible for the silencing of the gene in non-tumorigenic cells. Therefore, new vectors were constructed (ANc-SB and ANet(S)-SB in Fig. 5) in which an AccI-NcoI fragment containing the region of the first intron where the hypersensitive sites had been found was inserted in both orientations upstream of the promoter (SB fragment). In addition, the whole intron with its donor and acceptor sites was also placed down-stream.
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**Fig. 4.** Transient expression assays with FGF-3 promoter constructs containing various lengths of upstream sequences. The map at the top depicts the 12-kb region at the 5′ end of the FGF-3 gene; the box at right represents the first exon, and the shaded area shows the beginning of the open reading frame for the FGF-3 protein. Restriction sites are abbreviated as in Fig. 2 and in addition: Nc, NcoI, N, NaeI, Sf, SfI. Only the meaningful NcoI, NcoI, and SacI sites are indicated. The BamHI site in parentheses to the left corresponds to the cloning site of recombinant phage λD3 that was used to isolate promoter fragments (see “Experimental Procedures”; the position of this site coincides with that of the asterisk in Fig. 2). The restriction sites shown correspond to the ends of the promoter fragments (displayed as horizontal lines below the map) that drive the expression of the CAT gene in the various constructs tested. The 5′ and 3′ ends of the E0, E1, and E2 fragments are indicated on the sequence presented in Fig. 3. The histogram to the right shows the CAT activity assayed in extracts of 12A1 cells transiently transfected with the indicated constructs (gray bars). Only the EN, HB, KB, and BB constructs were assayed in B3 cells (dotted bars). Activity levels are compared with those yielded in both cell clones by the promoter-less vector pUSCAT-C (negative control) and by the strong SV40 early promoter-containing vector pUSVCAT-C (positive control). Extracts of cells transfected with pUSVCAT-C were diluted 100-fold before the assay. CAT activity is expressed as the percentage of conversion of chloramphenicol to its acetylated derivatives. Each value is the mean from at least two independent assays, each assay being carried out in triplicate for a given construct. Error bars indicate standard deviations.

**Fig. 5.** Lack of effect of intron sequences on FGF-3 promoter activity in transient expression assays. The map at the top shows the 5′-half of the FGF-3 gene, and the two boxes represent the first and second exons, and the shaded area represents the open reading frame for the FGF-3 protein. Restriction sites are abbreviated as in Fig. 4 with, in addition, A, Accl. Intronic fragment-containing constructs are schematized below the map. The Accl-NcoI (ANc) fragment containing the HS6 cluster of DNase I-hypersensitive sites identified in B3 cells was inserted in both orientations (arrows) upstream of the small SB promoter fragment. The Accl-KpnI (AK) fragment containing the whole intron 1 was inserted downstream of SB in its native configuration (the deletion of the sequence located between the BamHI and the AccI sites effectively removes the ATG initiation codon for the FGF-3 protein that would have interfered with the initiation of the synthesis of the CAT protein). The histogram to the right shows the CAT activity assayed in extracts of B3 and 12A1 cells transiently transfected with these three constructs. Activity levels are compared with those yielded in both cell clones by the SB construct and the pUSCAT-C control vector. CAT activity is expressed as described in the legend to Fig. 4.

**Fig. 6.** Transient expression assays disclose a promoter element located 6 kb upstream of the FGF-3 gene. The map at the top corresponds to the same region as that presented in Fig. 4. Only the meaningful NcoI, NcoI, and SacI sites are indicated. Selected fragments used in constructs designed to investigate the presence of a putative activator element between the HindIII and XhoI sites are depicted below the map together with the HB and SB fragments, already described in Fig. 4. The HindIII-XhoI (UX) fragment was inserted in both orientations (long arrows) in a CAT expression vector either upstream of the SN promoter fragment or alone. The subfragment NcoI-XhoI (NcX) at the 5′ end of HB was also inserted alone in both orientations (short arrows). The histogram to the right shows the CAT activity assayed in extracts of 12A1 cells transiently transfected with these constructs. Activity levels are compared with those yielded by the SB and HB constructs and the pUSCAT-C control vector. CAT activity is expressed as described in the legend to Fig. 4, except that, due to the high activity levels of some of the constructs in 12A1 cells, the corresponding extracts were diluted accordingly before the assay and the values obtained were multiplied by the dilution factor.

**FGF-3 gene promoter (SN fragment).** The presence of the HX fragment resulted in a 7.7-fold stimulation of CAT activity, but only when inserted in its native orientation. This was more suggestive of a promoter activity than of an enhancer. The HX fragment was therefore inserted alone into the CAT vector in either orientation (HX and HX(as)). The high level of CAT

stream of the promoter (SB-AK). All of these constructs yielded comparable activities when introduced into B3 and 12A1 cells (Fig. 5). We concluded that it was not possible to study the mechanisms responsible for the differential expression of the gene using transient expression assays, and we decided to perform this type of assay only in tumorigenic 12A1 cells, with the aim of unraveling regulatory DNA sequence elements that might be important for the activity of the promoter in these cells. Additional vectors were constructed and tested (EB, XB, SB2, SfB, NcB, SB, E2, E1, and E0 in Fig. 4). The E0 fragment was 143 bp long and encompassed the four major initiation sites (Fig. 3). From the results presented in Fig. 4, it appeared that the differences in activity of the various constructs were moderate. Drops in activity were noticed throughout several experiments between the HB and XB and between the SB and E2 constructs, suggesting that activator elements might be located between the HindIII and XhoI sites (HX fragment) and just upstream of the E2 fragment 5′ boundary, respectively. This second element was not investigated further, however, because its activity was no more demonstrable in the stable transfection experiments to be described below.

**A Promoter Activity in an NcoI-XhoI Fragment Located 6 kb Upstream of the FGF-3 Gene**—In order to confirm the presence of an activator element within the HX fragment, this fragment was inserted in either the sense or antisense orientation (HX-SN and HX(as)-SN, respectively, in Fig. 6) upstream of the
activity generated only by the HX sense construct did confirm that the HX fragment contains a strong promoter (Fig. 6). The location of this promoter was further refined to the 3' region of the HX fragment, within a 442-bp-long NcoI-Xhol (NcX) subfragment. Indeed, this subfragment was able to drive the expression of the CAT gene to the same level as the HX fragment (Fig. 6).

Stable 12A1 transfectants were derived using the HX and HX(as) constructs. The results (not shown) were comparable to those of the transient expression assays described above and indicated that the promoter located in the HX fragment was also very active when integrated into the chromatin of 12A1 cells. Four pools of stably transfected 12A1 cells carrying the HX plasmid were used to prepare total cytoplasmic RNAs in order to map the initiation site(s) in this promoter. The results of an RNase protection experiment performed with an antisense RNA probe prepared containing the upstream promoter fused to the CAT gene sequence up

**FIG. 8.** Nucleotide sequence of the 442-bp NcoI-Xhol (NcX) fragment containing the promoter located 6 kb upstream of the FGF-3 gene. Arrows indicate the position (±5 nucleotides) of the transcription start sites determined from the results of the RNase protection experiment presented in Fig. 7. The thick arrow corresponds to the major site. The thickly lined boxes outline the "initiator" element highly homologous to a CAP site consensus sequence, and thinly lined boxes indicate putative binding motifs for transcription factors that had been previously recognized upon analysis of this sequence (20). Homology to the binding motif consensus sequences in the reverse orientation is indicated by (c) for "complementary strand."

All of the vectors tested in the transient expression assays described above, as well as an additional construct (EvB), were cotransfected with each construct and with an expression vector conferring resistance to neomycin. Colonies of stably transfected cells, selected for their resistance to the neomycin analog G418, were pooled to minimize positional effects caused by integration at random sites within the genome. For each construct, four independent pools of G418-resistant clones were selected, and CAT activity was determined on aliquots of cellular extracts from each pool. Genomic DNA was also prepared from the cells of each pool and the copy number of the integrated plasmids was determined by Southern blotting. For the four different pools of cells transfected with a given construct, no clear correlation was found between the CAT activity level and the average number of integrated plasmid copies (data not shown). In fact, whenever the plasmid copy number differed significantly between four independent pools, the activity values did not vary accordingly. Therefore, CAT activity was not normalized to the number of integrated copies.

All of the vectors tested in the transient expression assays described above, as well as an additional construct (EvB), were used to generate stable transfectants (Fig. 9). The results first showed that a differential expression, reminiscent of that of the resident FGF-3 gene, could be obtained once these plasmids were integrated into the chromatin of B3 and 12A1 cells. In-
The activity was low (2.3%), of the same order of magnitude as that yielded by the neighboring promoter constructs (HB to EvB and KB to BB). One pool showed an intermediate CAT activity (11%). We interpreted these results as an indication that, for this construct, (i) the activity was highly sensitive to positional effects depending on the integration site within the cellular chromatin, and (ii) the number of independent clones in the pools at the time of analysis was probably not high enough to average the large variations in CAT activity between the individual clones.

As shown above, the SB, E2, E1, and E0 constructs yielded the same low, but detectable, CAT activity (0.7%), indicating that no essential modulatory element is present between the SacI site delimiting the 5’ end of SB and the E0 fragment 5’ boundary. In particular, none of the potential regulatory elements that can be recognized within the sequence of this region (a CARG box, a CAAT box, and an Sp1-binding site; see Fig. 3) appear to have a functional role. Finally, the smallest fragment tested, E0, retained some activity and will be hereafter considered as the minimal promoter. This activity was clearly measurable (above background) in 12A1 transfectants, but none could be detected in B3 transfectants. This result suggested that the minimal promoter itself had a differential activity in tumorigenic and non-tumorigenic cells of the SW613-S cell line.

To determine whether the accuracy of transcription initiation was preserved within the promoter region of the constructs stably integrated into the genome of 12A1 transfectants, RNase protection assays were carried out. Total cellular RNAs were extracted from the four pools of G418-resistant clones obtained after transfection with the BN construct, chosen for its ability to drive a high CAT expression level. The probe used in the protection assays was an antisense RNA encompassing the FGF-3 promoter fragment SN (SacI-NacI; see Fig. 3) and the 5’ end of the CAT gene (Fig. 10). This FGF-3-CAT chimeric probe was chosen so as to enable the RNAs transcribed from the BN construct to be distinguished from the endogenous FGF-3 mRNAs. With total RNA from control untransfected 12A1 cells, this probe yielded RNase-protected fragments of the size expected for the mRNAs initiated at the multiple known sites within the resident FGF-3 gene promoter (15). With the transc- fectant RNAs, a pattern of eight additional, more intense bands was found to be superimposed on that generated by the endogenous mRNAs. The size of six of these protected fragments was in good agreement with that expected from the known location of the initiation sites within the FGF-3 promoter, confirming that accurate initiation of transcription does occur in stably transfected cells. However, two extraneous bands were observed. The weak 330-nucleotide band does not correspond to a known initiation site but appears to have actually been missed in our previous analysis (15). The size of the longest fragment (570 nucleotides) is in agreement with a complete protection of the probe. It could actually correspond to the minor initiation site positioned upstream of the 5’ end of the probe (see Fig. 3).
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and CAT activities determined. The GC boxes (GC-E0) or the 72-bp repeats (72-E0) strongly stimulated the activity of the E0 promoter (140- and 330-fold, respectively) in 12A1 transfectants. In marked contrast, they had no effect in B3 transfectants in which CAT activity was still undetectable. When both elements were inserted upstream of the E0 promoter in their native configuration (72-GC-E0), the stimulation in 12A1 transfectants was comparable to that obtained with the 72-bp repeats alone (260-fold). In B3 transfectants, the presence of both types of SV40 elements did manage to induce the E0 promoter, but CAT activity still remained 10 times lower than that in 12A1 transfectants. The complete SV40 early promoter (pUSSVCAT-C) yielded a high and equivalent CAT activity in B3 and 12A1 transfectants. Altogether, these results indicate that the activity of the E0 minimal promoter is highly restricted in non-tumorigenic B3 cells, even when flanked by strong activator elements, which is not the case for the TATA box-containing SV40 minimal promoter.

**DISCUSSION**

**Regulatory Elements of the Human FGF-3 Gene**—As a first approach to localize regulatory elements of the human FGF-3 gene, we have mapped DNase I-hypersensitive sites in the neighborhood of the gene in both tumorigenic and non-tumorigenic SW613-S cells. DNase I-hypersensitive sites correspond to regions where the chromatin structure is loosened enabling transcriptional regulatory proteins to access their specific binding sequences. These sites are generally clustered in actively transcribed regions of the chromatin (33, 34). A number of hypersensitive sites were identified in tumorigenic 12A1 cells, consistent with the fact that the FGF-3 gene is transcriptionally active. Unexpectedly, such sites were also found in non-tumorigenic B3 cells where the gene is silent. In contrast, only a subset of very weak hypersensitive sites were detected in cells of the EJ bladder carcinoma cell line which does not express the gene either. This result suggests that, although B3 cells do not express FGF-3, they might have the potential to do so under appropriate circumstances. In agreement with this assumption, we have previously shown that FGF-3 expression is induced when B3 cells stably transfected with a c-MYC gene expression vector are grown in vivo, as tumors in nude mice (15). Similar observations have previously been made for other genes in other cell systems. For example, hypersensitive sites in the locus control region of the β-globin genes are present in cells of the erythroid lineage independently of the gene that is expressed at a given developmental stage but are absent in cells of the non-erythroid lineages that have never and will never express the gene (35, 36). Another unexpected result was

**FIG. 11. Effect of SV40 activator elements on the differential activity of the FGF-3 minimal promoter in B3 and 12A1 stable transfectants.** The left panel shows a diagrammatic representation of the promoter fragments that drive the expression of the CAT gene in the various constructs tested. The SV40 early promoter is depicted at the top with the 72-bp repeats represented as a pair of striped rectangles, the three GC boxes as open rectangles, and the minimal promoter as a thin line with its TATA box (small black rectangle) and initiation site (arrow). Next, the minimal FGF-3 promoter fragment, E0, is drawn as a thick line, with the position of the four major transcription start sites indicated by arrows. Three chimeric constructs containing the SV40 72-bp repeats, GC boxes, or both, ahead of the E0 promoter, are schematized below. The histogram to the right shows the CAT activity assayed in extracts of B3 or 12A1 cells stably transfected with the indicated constructs. CAT activity is expressed as in Fig. 4, except that, due to the high activity levels of some of the constructs, the corresponding extracts were diluted accordingly before the assay, and the values obtained were multiplied by the dilution factor. Each value is the mean from at least four independent pools of stably transfected cells. Error bars indicate standard deviations.

**FIG. 10. RNase mapping of the 5’ ends of the RNAs initiated within the promoter region of the BN construct stably integrated into the genome of 12A1 cells.** Aliquots (50 μg) of total cytoplasmic RNA from four pools of 12A1 cells stably transfected with the BN construct (lanes 1–4) were used in RNase protection experiments carried out with an antisense RNA probe prepared from the chimeric DNA fragment schematized in the lower panel. This fragment consists of the 419-bp SacI-NruI (SN; open box) fragment containing the FGF-3 promoter fused to the CAT gene sequence up to a PvuII (Pvu) restriction site (boxed box). The thick and thin arrows represent the major and minor start sites, respectively, that we have previously identified within the FGF-3 promoter (15). Aliquots (50 μg) of tRNA and of untransfected 12A1 cell RNA were used as controls. RNase-resistant fragments were fractionated on a 4% polyacrylamide-urea gel, along with a DNA size marker (lane M, 32P-end-labeled MspI-digested pBR322 DNA with fragment lengths in nucleotides indicated to the right). The upper band and the doublet visible in the control 12A1 lane result from the protection of the SN region upstream of the CAT sequence by the endogenous FGF-3 miRNAs and correspond to previously identified, minor initiation sites within the resident FGF-3 gene promoter. Bands corresponding to the major start sites were observed at the bottom of the gel, not shown here. The size of the superimposed bands specifically observed in all of the four pools of stable transfectants is indicated to the left. The asterisk points to a spurious band also visible in the tRNA control lane. The size of each of the RNase-protected fragments (thin horizontal lines in the lower panel) was used to position the 5’ end of each corresponding RNA species on the map of the chimeric DNA fragment used to prepare the probe.
the presence of a cluster of marked hypersensitive sites in the first intron of the gene, only in non-tumorigenic B3 cells. We hypothesized that this intronic cluster of hypersensitive sites might be due to binding a negative regulatory factor(s) implicated in the silencing of the gene in B3 cells. However, we could not provide any evidence for a repressor activity of intronic sequences on the FGF-3 gene promoter in transient expression assays. In addition, in vitro DNA footprinting experiments performed with DNA fragments derived from the region of the first intron where hypersensitive sites are clustered did not show any difference between the footprints generated with B3 or 12A1 cell extracts. Finally, the hypothesis of a silencer element in the first intron does not appear to be required, since we have observed that FGF-3 promoter constructs, devoid of intronic sequences, are fully repressed in stable transfectants derived from B3 cells. The significance of the intronic cluster of hypersensitive sites in non-tumorigenic cells remains unexplained at present.

The analysis of the effect on promoter activity of various fragment lengths from the FGF-3 gene upstream sequence in stable 12A1 transfectants allowed us to localize several regulatory elements as follows: two activator elements, one distal and one proximal to the promoter, and a new promoter element, located about 6 kb upstream of the gene. In addition, a fourth region with a peculiar modulatory activity was found to be located in the center of the upstream sequence, close to the SfiI site (see Fig. 9). The unusual behavior of the SfiI construct (either a high or a low activity level in four and three pools of stable transfectants, respectively) suggests that its activity is highly dependent on its site of integration into the cellular chromatin. The fact that this positional effect was no more seen with the next downstream fragment (KB) suggests the presence of a site-sensitive activator element located between the SfiI and KpnI sites (1460 bp). Since the effect was not seen either with the longer constructs (notably the next longer one, EvB), one has also to hypothesize that a squelching, negative regulatory element is present in the 530-bp-long region between the EcoRI and SfiI sites. Once the squelching element is deleted, the effects of the activator would become detectable but highly dependent on the integration site. In the next downstream fragments (KB, NcB, etc.) both the negative regulator and the site-sensitive activator would be removed, resulting in a low and steady activity.

It is noteworthy that, at this level of analysis, there was a good correlation between the position of the various regulatory elements that we have characterized and the location of several hypersensitive sites detected in 12A1 cells (compare Figs. 2 and 9). The most upstream activator element, tentatively located in the vicinity of the second EcoRI site, could correspond to the HsuII sites, whereas the proximal activator element identified between the SacI and the BamHI sites, 330–500 bp upstream of the major initiation sites, is compatible with the HS4 site. HS2 sites could reflect the presence of the putative repressive and site-sensitive activator elements (in the neighborhood of the SfiI site). HS1 sites, close to the XhoI site, coincide with the upstream promoter discovered in the NcX fragment. The HS5 site, undetectable in B3 cells, most probably corresponds to the FGF-3 gene promoter. In our series of experiments, no regulatory element could be associated with the strong HS3 site. However, preliminary evidence suggests that the KpnI-SacI fragment, encompassing this site, contain a negative regulatory element; deletion of this fragment from the HB construct raised its activity by about 5-fold (data not shown). Altogether, these data provide a first snapshot of a number of control elements involved in the complex regulation of the human FGF-3 gene. Refined localization and further characterization of each of these various regulatory elements are now required. Obviously, since we have limited our analysis to the region encompassing the gene and 11 kb of 5′ sequences, we cannot exclude the existence of additional regulatory elements farther upstream or downstream.

**How Many Promoters Drive the Expression of the Human FGF-3 Gene?**—We have shown by transient and stable expression assays that the 442-bp NcoI-XhoI fragment located 6 kb upstream of the initiation codon of the FGF-3 gene contains a strong promoter. Note that the activities of the HX and NcX constructs were much higher than that of the HB construct (Fig. 6). Identical results were obtained in stable 12A1 transfectants (data not shown). This observation indicates that the activity of the “NcX promoter” is strongly modulated by downstream sequences. This is in agreement with the possible presence of a negative regulatory element in the KpnI-SacI fragment (see above). We have preliminary evidence that this promoter is entirely contained within a 170-bp fragment and that several of the potential binding sites for transcription factors recognizable in the sequence of this region (Fig. 8) are essential for its activity.

Does the NcX promoter belong to the FGF-3 gene transcription unit? This question cannot be answered at this time since we have not been able to find any endogenous mRNAs originating from this promoter by Northern blot analysis, using as probes fragments encompassing the region located between the NcX promoter and the FGF-3 gene (data not shown). However, it is possible that the steady state level of such mRNAs may be too low for them to be detectable by this method. Another possibility might be that they comprise a very small leader exon spliced downstream to the body of the FGF-3 mRNA, making them difficult to unveil. Finally, this promoter could be specific of some cell types and/or developmental stages and not be active at the locus of the resident FGF-3 gene in SW613-S cells. Work is in progress to clarify this point.

Three promoters (P1, P2, and P3) have been previously identified at the 5′ end the mouse fgf-3 gene (37, 38) but only one for the human gene (14). This unique human promoter was shown to be the homolog of the murine P2 promoter. We have recently reported that a sequence stretch of the promoter discovered in the NcX fragment could be aligned with a region of the murine P1 promoter (20). The two sequences were 53% identical over 263 nucleotides. Moreover, the relative positions of the major and of several minor initiation sites were found to match closely in the aligned sequences. These observations had led us to suggest that the human promoter of the NcX fragment could be the homolog of the murine P1 promoter (20). A definitive conclusion will only be possible to draw once the structure of the transcription unit starting at this promoter has been fully elucidated.

**Is the Minimal Promoter Responsible for the Differential Expression of the FGF-3 Gene?**—The promoter of the human FGF-3 gene was previously mapped in an 838-bp BamHI fragment located 104 bp upstream of the ATG initiation codon (Figs. 3 and 5; see Refs. 14 and 15). Analysis of stable 12A1 transfectants has allowed us to show here that an activator element is located in the first 170 nucleotides (BamHI-SacI fragment) and that the minimal promoter is contained within the 143-bp-long E0 fragment (Fig. 3). Deletion of the various potential binding sites for transcription factors that are located between the SacI site and the 5′ boundary of the E0 fragment (noticeably a proximal Sp1 site) had no effect on the activity of the promoter, indicating that these sites are probably nonfunctional. Eukaryotic gene promoters have been divided into the

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2 B. Raynal and O. Brison, unpublished results.
following two classes: those containing a TATA box (sometimes also followed by an initiator element), and those devoid of a TATA box in which the position of the initiation sites is specified by initiator elements (39). The activity of TATA box-less promoters is frequently dependent on the presence of a proximal Sp1-binding site (40). Our results suggest an atypical structure for the TATA box-less FGF-3 promoter since neither initiator elements nor a functional Sp1 site are evident. It could belong to a new class of promoters described by Ince and Scotto (41), in which multiple initiation sites are followed by a specific sequence element that these authors have called MED-1 (Multiple start site Element Downstream-1). This sequence, which is located on average 110 bp downstream of the first initiation site, seems necessary for the positioning of the initiation sites and for efficient transcription. A canonical MED-1 element is present in the FGF-3 promoter, 100 bp downstream of the first major initiation site (Fig. 3). Its role, if any, in the activity of the promoter remains to be established.

When various promoter constructs were tested in transient transfection experiments, they displayed comparable levels of activity in both 12A1 and B3 cells, indicating that the differential expression of the resident FGF-3 gene could not be reproduced under these conditions. On the contrary, the FGF-3 promoter appeared to be completely inactive in transiently transfected cells from the control EJ cell line. These results imply that all of the factors necessary for the expression of the FGF-3 gene are present in both non-tumorigenic and tumorigenic SW613-S cells, whereas at least one of them must be missing in EJ cells. Thus, the differential expression of the gene between tumorigenic and non-tumorigenic SW613-S cells does not seem to be the direct consequence of a quantitative or qualitative difference in some transcription factor(s) essential for initiation of transcription on “free” plasmid DNA. In agreement with this conclusion, in vitro DNase I footprinting experiments performed with the E0 fragment did not show any difference between the footprints generated using either B3 or 12A1 cell extracts.2 In contrast, when the same constructs were stably integrated into the genome of the transfectants, a differential expression of the FGF-3 gene could alone be responsible for the differential expression of the gene. In non-tumorigenic cells, the promoter is apparently repressed through a chromatin-based mechanism. In tumorigenic cells, this repression seems alleviated, and several upstream elements modulate the activity of the promoter.

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