MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5423–5433 Vol. 15, No. 10
0270-7306/95/$04.00+0

G10BP, an E1A-Inducible Negative Regulator of Sp1, Represses Transcription of the Rat Fibronectin Gene

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Received 15 May 1995/Returned for modification 20 June 1995/Accepted 7 July 1995

Downregulation of the fibronectin (FN) gene in a rat 3Y1 derivative cell line, XhoC, transformed by the adenovirus E1A and E1B genes seems to be caused by the induction of a negative regulator, G10BP, which binds to three G-rich sequences in the promoter (T. Nakamura, T. Nakajima, S. Tsunoda, S. Nakada, K. Oda, H. Tsurui, and A. Wada, J. Virol. 66:6436–6450, 1992). These are the G_{10} stretch and two GC boxes consisting of the G_{10} stretch with one internal C residue insertion. The recognition sequences of G10BP and Sp1 (GGGCGG) overlap in these GC boxes. To analyze the mechanism of the downregulation, G10BP was purified by DNA affinity chromatography, and its molecular mass was estimated to be about 30 kDa. The promoter was modified by substituting the sequence GGGG with ATCC or CTTA in these G-rich sequences, leaving the Sp1 motif intact, and by replacing the Sp1 motif by the T stretch. Transcription of FN promoter-chloramphenicol acetyltransferase fusion genes carrying the base substitution in one or more of these G-rich sequences both in vivo and in vitro revealed that the base substitution in any G-rich sequence results in reduction of promoter activity, although the downstream GC box (GCd) plays a primary role. The addition of G10BP severely inhibited the activities of the FN promoters carrying the wild-type GCd in vitro, while the promoters carrying the mutant GCd were unaffected. The binding affinity of G10BP and Sp1 to each of the G-rich sequences, analyzed by gel shift assays, indicated that G10BP binds strongly to the GCd, moderately to the G_{10} stretch, and weakly to GCu, while Sp1 binds strongly to GCu, moderately to GCd, and weakly to the G_{10} stretch. Sp1 binding to GCd and the G_{10} stretch was inhibited by G10BP, while binding to GCu was unaffected. These results indicate that FN gene transcription is inhibited in XhoC cells primarily by exclusion of Sp1 binding to GCd by G10BP and that G10BP is a new class of Sp1 negative regulator.

Fibronectins (FNs) are large glycoproteins of the extracellular matrix and play a central role in cell adhesion, migration, wound healing, and tumor metastasis (24). FN binds to its receptor on the cell surface as dimers of similar subunits of about 250 kDa (24, 44), connecting intracellular actin filaments to the extracellular matrix at the receptor site (2, 23, 44), called focal contacts or adhesion plaques. The FN molecule consists of a series of globular domains which interact independently with components of the extracellular matrix, fibrin, glycosaminoglycans, collagen, and its receptor (22, 50), and the receptor binds indirectly to actin filaments through its cytoplasmic domain (4, 21).

Cell adhesion to the substratum is required for mammalian cells to proliferate in an in vitro culture system, while the loosening of cell adhesion is also required transiently for quiescent cells to enter the cell cycle after growth stimulation by extracellular proteases (3, 45) or by growth factors (19, 20). Most oncogenes exert their functions to disintegrate the structure of the extracellular matrix and cytoskeleton, and the transformed cells proliferate without requiring adhesion to the substratum. Platelet-derived growth factor or v-*sis* activates c-*src* by phosphorylation, which subsequently phosphorylates cell adhesion proteins, including the FN receptor, resulting in disorganization of the structures of focal contacts and actin filaments (19, 20). FN itself is a target of the oncogene products, and the level of FN expression is greatly reduced in avian sarcoma virus-transformed chicken embryo fibroblasts (13) and in primary rat kidney cells transformed by the EJ Ha-*ras* oncogene and the adenovirus E1A gene (28). However, the mechanism of downregulation of the FN gene has not yet been clarified.

We previously showed that the induction of E1A expression in quiescent rat 3Y1 cells, in which the adenovirus E1A gene linked to the hormone-inducible promoter was introduced, elicit the progression of the cell cycle through G_1 to S phase, concomitant with a steep decrease in the level of FN gene expression (18). The 3Y1 derivative cell line XhoC14, transformed by the adenovirus E1A and E1B genes, showed a drastic decrease in FN gene expression to an almost undetectable level (38). Analysis of transcription factors which interact with the rat FN promoter showed that an E1A-inducible negative factor (G10BP) binds to three G-rich sequences in the promoter (38, 39). One of the sequences, located at positions −239 to −230, consists of only G residues (G_{10} stretch), and the other two, located at positions −105 to −95 and −54 to −44, consist of the G_{10} stretch with one internal C residue insertion (GC boxes). The Sp1 motif GGGCGG therefore overlaps the recognition sequence of G10BP in both GC boxes. Substitutive mutations introduced into these G-rich sequences which convert the sequence GGGG to either ATCC or CTTA without impairing the Sp1 motif completely abolished the E1A sensitivity of the promoter, and the mutated promoter was no longer repressed by E1A (38, 39). G10BP may repress FN gene transcription by competing with Sp1.

In the present study, G10BP was purified by DNA affinity chromatography to near homogeneity, and its molecular mass was estimated to be approximately 30 kDa. The purified

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G10BP inhibited transcription of the FN promoter carrying the wild-type G-rich sequences but not the promoter carrying the mutated G-rich sequences in an in vitro transcription system. The competitive binding of G10BP and Sp1 to these G-rich sequences was analyzed by electrophoretic mobility shift assays (EMSAs). The mechanism of inhibition of Sp1-mediated transcription by G10BP was compared with those caused by the Sp1 inhibitors Sp1-I (6) and Sp3 (16), recently reported by other investigators.

MATERIALS AND METHODS

Cell culture. The 3Y1-B cell line clone 1-6 is a clonal line of Fischer rat embryo fibroblasts (32). A 3Y1 derivative cell line, g12-21, was established by transfecting 3Y1 cells with PM12SG, in which the adenovirus type 2 $E1A_{12S}$ cDNA (50) was placed downstream of the mouse mammalian tumor virus long terminal repeat (26). g12-21 cells express the $E1A_{12S}$ cDNA efficiently in response to dexamethasone (DEX). These cells were cultivated in Dulbecco's modified Eagle's minimal essential medium with 10% fetal calf serum (FCS) at 37°C. HeLa cells were cultivated similarly or in RPMI 1640 medium with 5% calf serum in suspension at 37°C.

Construction of FN promoter fusion plasmids. Plasmid pF414CAT was constructed by inserting the 564-bp *Dra*I-*Hin*dIII fragment containing the 5′-flanking region of the rat FN gene from positions −414 to +136 at the *Hin*dIII and *Bgl*II sites of pSV2CAT-XB after conversion of the *Dra*I site to a *Bgl*II recognition sequence (38). Plasmids containing substitutive mutations at the G_{10} stretch (−239 to −230) and/or two GC boxes (−105 to −95 and −54 to −44) in pF414CAT were constructed by site-specific mutagenesis by the method of Kunkel et al. (35). Plasmid pGST-RB was constructed by inserting the retinoblastoma (RB) gene cDNA at the *Eco*RI site of pGEX-3X. The 3.8-kb *Eco*RI fragment containing the RB gene cDNA was isolated from plasmid p4.9BT, obtained from T. P. Dryja.

Transient transfection and CAT assay. Subconfluent monolayer cultures of HeLa cells were transfected with 20 μg each of the FN promoter-chloramphenicol acetyltransferase (CAT) constructs by the calcium phosphate coprecipitation procedure of Chen and Okayama (5). Cell extracts were prepared after 48 h of transfection. CAT activity was assayed by the method of Gorman et al. (14). Four hundred micrograms of protein was used for each assay. The amount of [^{14}C]chloramphenicol converted to acetylated forms was determined by scanning the developed X-ray film with a densitometer.

Northern (RNA blot) hybridization. Northern blotting was performed with total cellular RNA prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (7). RNA was subjected to electrophoresis in 1% agarose gels in buffer containing 2.2 M formaldehyde, 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid, pH 7.0], 8 mM sodium acetate, and 1 mM EDTA and transferred to nylon membrane filters. Hybridization was carried out by adding 10^6 cpm of ^{32}P-labeled DNA probe per ml. The human FN cDNA was prepared by isolation of the *Eco*RI-*Bgl*II fragment from positions 5947 to 6679 from plasmid pFH1 (34), obtained from K. Umezawa. The murine β-actin cDNA probe from positions 1225 to 1892 was isolated from plasmid pSPMβA-3′ut (47), obtained from K. Tokunaga. ^{32}P labeling was carried out by the multiprime DNA labeling system (Amersham).

Purification of G10BP. Whole-cell extracts were prepared essentially by the method of Manley et al. (36). XhoC cells (approximately 3×10^9) were washed in phosphate-buffered saline (PBS) containing 0.5 mM $MgCl_2$ and suspended in 4 volumes of hypotonic buffer (10 mM Tris-hydrochloride [pH 7.9] at 4°C, 1 mM EDTA, 5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). After 20 min, the cells were homogenized, and 4 volumes of sucrose-glycerol solution (50 mM Tris-hydrochloride [pH 7.9] at 4°C, 10 mM $MgCl_2$, 25% [wt/vol] sucrose, 50% [vol/vol] glycerol, 2 mM DTT, 0.5 mM PMSF) was added. After gentle stirring, 1 volume of saturated $(NH_4)_2SO_4$ was added dropwise, and the homogenate was centrifuged at 53,000 rpm at 4°C for 3 h in a Hitachi RP65T rotor. To the supernatant was added solid $(NH_4)_2SO_4$ to a final concentration of 0.33 g/ml, and the suspension was centrifuged in a Hitachi RP65T rotor for 30 min. The precipitate, containing about 20 mg of proteins, was dissolved in 1 ml of D buffer (40 mM Tris-hydrochloride [pH 8.3], 6 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 20% [vol/vol] glycerol) containing 0.1 M KCl and dialyzed against the same buffer. The extract was loaded onto a 2-ml wheat germ agglutinin (WGA)-agarose column (Vector Laboratories) equilibrated with D buffer containing 0.1 M KCl to remove Sp1 (27). The flowthrough fraction was collected. The fraction was then applied to a 4-ml heparin-Sepharose CL-6B column (Pharmacia LKB Biotechnology), and the column was washed with 5 column volumes of D buffer containing 0.1 M KCl and with the same volume of D buffer containing 0.2 M KCl. Most of the G10BP was eluted with D buffer containing 0.4 M KCl. Approximately half of the eluate, containing about 2 mg of proteins, was loaded onto a 2-ml DNA affinity column made by coupling the 30-bp oligonucleotide containing the G_{10} stretch to CNBr-Sepharose 4B (Pharmacia LKB Biotechnology) at a concentration of 1 mg of oligonucleotide per 2 ml. The column was washed with 5 column volumes of D buffer containing 0.4 M KCl and with the same volume of D buffer containing 0.8 M KCl. G10BP was then eluted with D buffer containing 40% ethylene glycol, 1.5 M KCl, and 0.1% Nonidet P-40. The eluate was dialyzed against D buffer containing 0.1 M KCl and reapplied to the DNA affinity column. G10BP was similarly eluted and dialyzed and used as purified G10BP. The fractions containing G10BP were determined by EMSAs with the ^{32}P-labeled oligonucleotide containing the G_{10} stretch as stated below.

End labeling of oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. All oligonucleotides were purified by oligonucleotide purification cartridge columns (Applied Biosystems). The 24-bp oligonucleotides G_{10}, GCu, GCd, and Sp1, containing the G_{10} stretch, the upstream and downstream GC boxes in the rat FN promoter, and the high-affinity Sp1 recognition sequence, contain the following sequences: G10, (−245)ACCA AAGGGGGGGGGGAAGTTCTC(−222); GCu, (−111)GCAGGAGGGGCG GGGGGAGTCGGA(−88); GCd, (−59)CTCGTGGGGGGGCGGGAAGGG ACT(−36); and Sp1, TGGAGACCGGGGCGGGGAGAAGTA (9). The oligonucleotides G10^{mut}, GCu^{mut}, and GCd^{mut}, carrying base substitutions in the G_{10} stretch, the GCu, and the GCd, are shown in Fig. 1B. The oligonucleotides GCu-T and GCd-T, in which the Sp1 motif in GCu and GCd was replaced by the T stretch, are shown in Fig. 1C. For EMSAs, two complementary single-stranded oligonucleotides were annealed in 10 mM Tris-hydrochloride (pH 7.5) containing 1 mM EDTA and 0.2 M NaCl and end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The oligonucleotides were electrophoresed on a 15% nondenaturing polyacrylamide gel, and the double-stranded probe was isolated.

EMSAs. The HeLa cell extract was prepared from nuclei by the method of Dignam et al. (11). The nuclei prepared by Dounce homogenization in buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) was suspended in buffer C (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 0.42 M KCl, 1.5 mM $MgCl_2$, 25% [vol/vol] glycerol, 0.5 mM DTT, 0.5 mM PMSF) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant collected was dialyzed against D buffer containing 0.1 M KCl and used as the cell extract.

EMSAs were performed in a 20-μl reaction mixture containing binding buffer (37) (20 mM HEPES [pH 7.9], 0.1 M KCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 20% [vol/vol] glycerol), 1 μg of poly(dI-dC) · poly(dI-dC), 0.5 fmol (approximately 5×10^3 cpm) of ^{32}P-labeled oligonucleotide, and either G10BP or the HeLa cell extract or both at 0°C for 30 min. DNA-protein complexes were resolved by electrophoresis on 5% polyacrylamide gels at 4°C for 2.5 h at 250 V in TGE buffer (25 mM Tris-hydrochloride [pH 8.0], 192 mM glycine, 2 mM EDTA). For the supershift assay, G10BP or the nuclear extract was incubated with the antiserum at 0°C for 30 min prior to initiation of the binding reaction. The gels were dried and autoradiographed with an intensifying screen at −80°C.

Southwestern (protein-DNA) blotting. G10BP eluted from the DNA affinity column was concentrated by adsorption to G10 oligonucleotide-immobilized diol-silica beads as described previously (39). The beads were then boiled in Laemmli buffer (60 mM Tris-hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% [vol/vol] glycerol, 0.01% bromophenol blue) for 10 min and electrophoresed on a 17% polyacrylamide gel containing 0.1% SDS. The proteins were transferred to the nitrocellulose filter and denatured twice in 6 M guanidine hydrochloride in DNA-binding (DB) buffer (50 mM Tris-hydrochloride [pH 7.6] at 4°C, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM $ZnSO_4$) for 10 min. Renaturation of proteins was performed by treating the filter successively in DB buffer containing decreasing concentrations of guanidine hydrochloride from 6 to 0.1875 M at 4°C for 5 min each. The filter was then washed twice in DB buffer and blocked in 5% skim milk dissolved in DB buffer at 4°C for 30 min. The protein-DNA interaction was performed in 4 ml of DB buffer containing 0.25% skim milk, 80 μg of sonicated calf thymus DNA, 80 μg of poly(dI-dC) · poly(dI-dC), and ^{32}P-labeled G_{10} oligonucleotide (4×10^6 cpm) at 4°C for 1 h. The filter was then washed with DB buffer containing 0.25% skim milk. The filter was dried and autoradiographed.

In vitro transcription assay. The transcription reaction was performed with whole HeLa cell extract (45), prepared by the method of Manley et al. (36). The template DNA containing the FN promoter region from −414 to +136 fused to the 5′-end portion of the CAT gene was prepared from pF414CAT DNA by cleavage with *Bgl*II and *Pvu*II. The templates containing the substitutive mutation in the G-rich sequences in the FN promoter were similarly prepared from the various FN promoter-CAT constructs. The transcription reaction was performed in a 30-μl reaction mixture containing 15 mM HEPES buffer (pH 7.9), 0.1 M KCl, 6 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 15% (vol/vol) glycerol, 0.4 mM each of ATP, GTP, and CTP, 0.16 mM UTP, 185 kBq of [α-^{32}P]UTP, 5 U of RNase inhibitor, 20 μg of protein of the HeLa cell extract, 0 to 20 μl of G10BP, and 30 ng of the DNA template. The reaction was first initiated by preincubation of the DNA template with the HeLa cell extract and G10BP without nucleoside triphosphates (NTPs) and $MgCl_2$ at 0°C for 30 min. Transcription was then started by adding NTPs, $MgCl_2$, and RNase inhibitor and performed at 30°C for 1 h. The reaction was stopped with 170 μl of stop solution (0.3 M Tris-hydrochloride [pH 7.4] at 25°C, 0.3 M sodium acetate, 0.5% SDS, 10 μg of yeast tRNA per ml, 0.1 mg of proteinase K per ml). RNA transcripts were purified by phenol-chloroform extraction and ethanol precipitation. The tran-

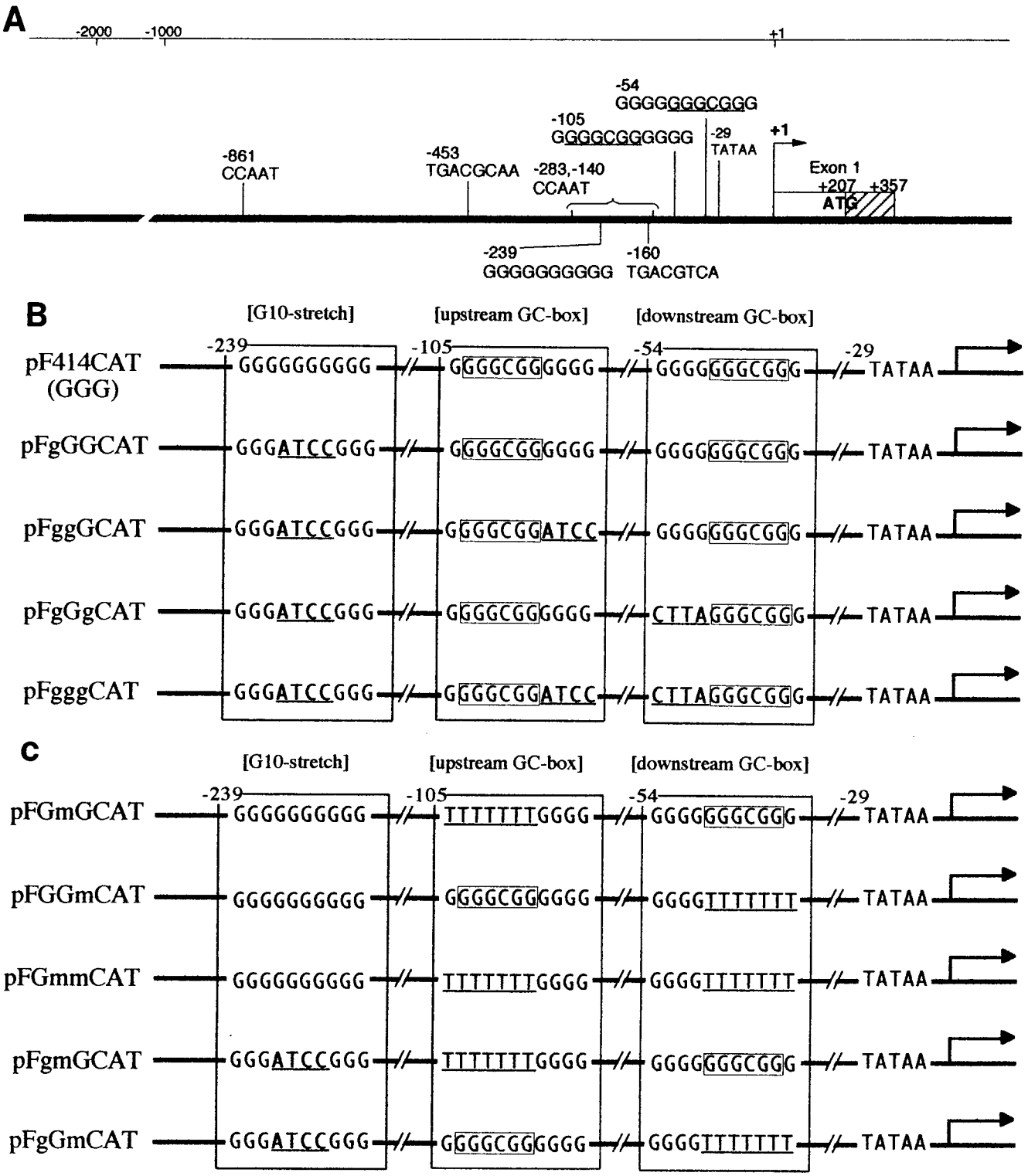

FIG. 1. Structures of the 5′-flanking region of the rat FN gene and FN promoter-CAT constructs. (A) The arrow designated +1 indicates the start site of transcription (40). The positions of the TATA box, two GC boxes, three CAT boxes, a cyclic AMP-responsive element (TGACGTCA), the G_{10} stretch, and the AP1-like motif are shown, together with the nucleotide numbers. The consensus sequence for Sp1 binding in the two GC boxes is underlined. (B) Chimeric CAT plasmids containing the FN promoter with substitution of the sequence GGGG with ATCC or CTTA in the G_{10} stretch and/or GC boxes linked 5′ to the CAT gene. The G-rich sequences in the plasmids are shown by three letters; the first letter represents the position of the G_{10} stretch, and the second and third letters represent the positions of the upstream and downstream GC boxes, respectively. Wild-type sequences are shown by a capital G, and substituted sequences are shown by a lowercase g. (C) FN promoter-CAT constructs in which the Sp1 motif in the upstream or downstream GC box or both was replaced by the T stretch. The T stretch introduced into the position of the Sp1 motif is shown by a lowercase m.

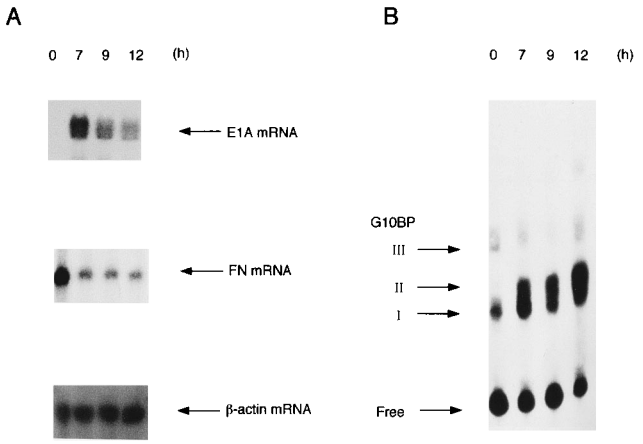

FIG. 2. E1A-dependent repression of the FN gene and induction of G10BP. (A) Northern blot analysis of E1A and FN mRNAs in quiescent g12-21 cells after induction of E1A expression. Quiescent g12-21 cells maintained in low-serum (0.5% FCS) medium were treated with 10^{-6} M DEX, and total cellular RNAs were prepared at the times indicated above each lane. Aliquots of 20 μg of RNA were used for Northern blotting. $E1A_{12S}$ cDNA, the *Eco*RI-*Bgl*II fragment (nt positions 5947 to 6679) of pFH1 (34), and the *Eco*RI-*Bam*HI fragment (nt positions 1225 to 1892) of the murine β-actin cDNA (47) were labeled with ^{32}P and used as probes for E1A, FN, and β-actin mRNAs, respectively. (B) Whole-cell extracts were prepared from quiescent g12-21 cells treated with DEX for 0, 7, 9, and 12 h as indicated above each lane. The 24-bp oligonucleotide from positions −111 to −88 containing the downstream GC box was ^{32}P end labeled, and aliquots of the oligonucleotide (5×10^{-3} cpm) were incubated with 4 μg of protein of each extract. The complexes formed were analyzed by EMSA and are indicated by the arrows.

scripts were analyzed by electrophoresis on denaturing 7 M urea–8% polyacrylamide gels, followed by autoradiography.

RESULTS

E1A-dependent repression of the FN gene and induction of G10BP. Transcription of the FN gene is highly elevated in rat 3Y1 cells when the cells become confluent and are maintained in low-serum (0.5% FCS) medium. The level, however, decreases steeply after growth stimulation by serum or by the induction of E1A expression (38). In a 3Y1 derivative cell line, XhoC, transformed by the adenovirus E1A and E1B genes, the level of FN mRNA decreased greatly to an almost undetectable level (39). The downregulation of the FN gene seems to be caused primarily by the induction of a negative factor, G10BP, by E1A or serum factors, which binds to three G-rich sequences in the FN promoter (Fig. 1A). These are the G_{10} stretch from positions −239 to −230 and two GC boxes consisting of the G_{10} stretch with one internal C residue insertion from positions −105 to −95 and −54 to −44. The consensus sequence for Sp1 binding, GGGCGG, therefore overlaps in these GC boxes. Substitution of the sequence GGGG with ATCC or CTTA in these G-rich sequences, leaving the Sp1 motif intact (Fig. 1B), completely abolished the E1A sensitivity of the promoter (38).

To demonstrate the E1A-dependent induction of G10BP and a concomitant decrease in the level of FN mRNA, the changes in the levels of G10BP and FN mRNA were analyzed by using a 3Y1 derivative cell line, g12-21, in which the expression of the $E1A_{12S}$ cDNA can be induced in response to DEX (26). Confluent monolayers of g12-21 cells were maintained in low-serum (0.5% FCS) medium for 48 h to synchronize the cells in the G_0 state, and $E1A_{12S}$ expression was induced by the addition of 10^{-6} M DEX. The levels of E1A and FN mRNA were analyzed by Northern blotting, and that of G10BP was analyzed by EMSA (Fig. 2). No detectable level of $E1A_{12S}$ mRNA was expressed in quiescent g12-21 cells in the absence of DEX. Expression of $E1A_{12S}$ mRNA was induced shortly after DEX treatment and reached its maximal level within 7 h. In contrast, the level of FN mRNA decreased to about 1/10 of the original level within 7 h and remained at a low level thereafter. The level was intermediate between those of quiescent 3Y1 cells and E1-transformed 3Y1 cells, as previously shown (39). No significant change was observed with β-actin mRNA (Fig. 2A). Under these conditions, DNA synthesis was induced within 8 h (26).

The induction of G10BP was analyzed by preparing cell extracts from quiescent g12-21 cells after treatment with DEX for 7, 9, and 12 h, which correspond to late G_1 phase, the G_1/S boundary, and S phase, respectively. These extracts were incubated with a ^{32}P-labeled 24-bp oligonucleotide containing the downstream GC box, and the complexes formed were analyzed by EMSA. The typical G10BP complexes I, II, and III were formed with all the extracts. The amounts of complexes I and II increased greatly, reaching a plateau within 7 h (Fig. 2B). The increase in the amount of G10BP is therefore correlated with the decrease in the level of FN mRNA.

Purification and characterization of G10BP from adenovirus E1-transformed 3Y1 cells (XhoC). To investigate the mechanism of inhibition of the FN promoter activity by G10BP and its binding specificity, G10BP was purified from XhoC cells. The cell extract was first passed through a WGA-agarose column to separate G10BP from Sp1, which binds to WGA-agarose through its carbohydrate moiety. The presence of G10BP in each reaction was determined by EMSAs. The flowthrough fraction containing G10BP was subsequently applied to a heparin-Sepharose column, and G10BP was eluted at 0.4 M KCl. This step was effective for elimination of most of the nuclease activity. The eluate was then loaded onto a DNA affinity column which contained Sepharose beads covalently attached to the double-stranded oligonucleotide containing the G_{10} stretch. G10BP was eluted with 40% ethylene glycol containing 1.5 M KCl and 0.1% Nonidet P-40. The extents of

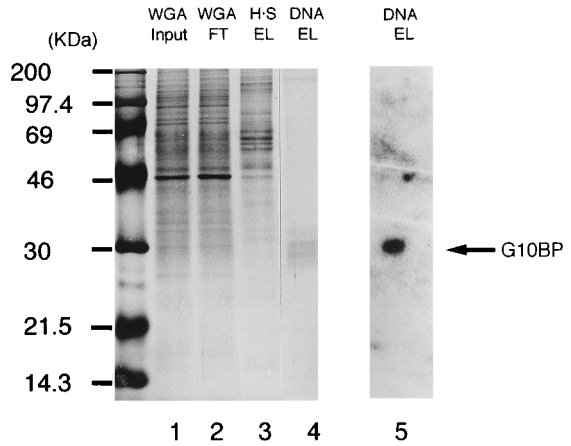

FIG. 3. Purification of G10BP from XhoC cells and estimation of its molecular weight. The fractions (1 μg of protein) obtained at each step of G10BP purification were electrophoresed on a 17% polyacrylamide gel and stained with 2D-silver stain II (Daiichi). Lane 1, $(NH_4)_2SO_4$-concentrated XhoC cell extract; lane 2, flowthrough fraction of a WGA-agarose column; lane 3, 0.4 M KCl eluate of a heparin-Sepharose column; lane 4, eluate (500 μl) of the DNA affinity column; lane 5, eluate (100 μl) of the DNA affinity column electrophoresed and blotted to the nitrocellulose filter. The nitrocellulose was probed with ^{32}P-end-labeled G10 oligonucleotide. The positions of size marker proteins are shown on the left.

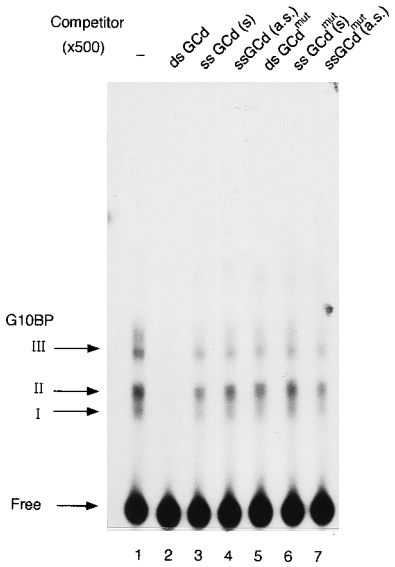

FIG. 4. Effect of single-stranded oligonucleotides on formation of G10BP complexes. Complex formation with the ^{32}P-labeled double-stranded 24-bp oligonucleotide containing the downstream GC box (0.5 fmol) and 0.4 ng of G10BP was performed in the presence of a 500-fold molar excess of the indicated unlabeled oligonucleotides as competitors: lane 1, no oligonucleotide; lane 2, the same double-stranded oligonucleotide; lane 3, the sense strand of the oligonucleotide; lane 4, the antisense strand of the oligonucleotide; lane 5, the double-stranded oligonucleotide carrying substitution of the sequence GGGG with CTTA in the downstream GC box (Fig. 1B); lane 6, the sense strand of the oligonucleotide carrying the base substitution; and lane 7, the antisense strand of the oligonucleotide carrying the base substitution. The complexes formed were analyzed by EMSA.

purification are shown in Fig. 3. Electrophoresis of the eluate from the DNA affinity column, followed by staining with 2D-silver stain II (Daiichi), revealed few protein bands (Fig. 3, lane 4). To determine which band corresponds to G10BP, the proteins were electrophoretically transferred to the nitrocellulose filter and probed with a ^{32}P-labeled oligonucleotide containing the G_{10} stretch. Autoradiography revealed that two protein bands migrating to the 30-kDa region bound to the G_{10} stretch (Fig. 3, lane 5). The upper band bound to the probe more strongly than did the lower band. These two proteins eluted from the gel were similarly electrophoresed and reprobed with the same labeled oligonucleotide. Autoradiography gave the same result. The presence of an excess of the unlabeled wild-type oligonucleotide, but not the oligonucleotide carrying the four-base substitution in the G_{10} stretch, completely abolished the binding of the labeled probe. The molecular mass of G10BP was estimated to be slightly greater than 30 kDa. The concentration of G10BP in the eluate was determined by comparing the density of bands with that of bovine serum albumin of known concentrations. The concentration was estimated to be 0.1 μg/ml. At least 2.6% of G10BP in the cell extract was recovered in the eluate from the DNA affinity column.

To confirm that the purified G10BP is a double-stranded-DNA-binding protein and not a single-stranded-DNA-binding protein, the formation of G10BP complexes with the ^{32}P-labeled 24-bp double-stranded oligonucleotide containing the downstream GC box and G10BP was inhibited by an excess of the sense or antisense single-stranded oligonucleotide containing the same sequence. As shown in Fig. 4, the formation of G10BP complexes I, II, and III was completely abolished by the presence of the unlabeled double-stranded oligonucleotide (dsGCd) but not by the sense or antisense single-stranded oligonucleotides [$ssGCd_{(s)}$ and $ssGCd_{(a.s)}$]. The amounts of the complexes formed were somewhat reduced, but the extents of reduction were similar to that caused by the double-stranded oligonucleotide carrying the four-base substitution in the GCd ($dsGCd^{mut}$). Neither the sense nor antisense single-stranded oligonucleotide carrying the same base substitution [$ssGCd^{mut}_{(s)}$ and $ssGCd^{mut}_{(a.s)}$] abolished complex formation. No complex was detected with purified G10BP and the ^{32}P-labeled $ssGCd_{(s)}$.

G10BP inhibits transcription of the FN gene in vitro. As shown in Fig. 1A, two of the G-rich sequences from −105 to −95 and from −54 to −44, termed the upstream and downstream GC boxes (GCu and GCd, respectively), contain one C residue in the G_{10} stretch, generating the consensus sequence GGGCGG for Sp1 binding. G10BP and Sp1 may compete with each other for binding to these overlapping sites. To investigate the inhibitory action of G10BP on the FN promoter, the effect of purified G10BP on FN promoters carrying base substitutions in the G-rich sequences was analyzed in vitro by using a HeLa cell extract which contains abundant Sp1 but not G10BP cross-reactive with the G-rich sequences in the rat FN promoter. The 704-bp fragment containing the FN promoter region from −414 to +136 linked to the 5′-end portion of the CAT gene was isolated by cleavage of pF414CAT with *Bgl*II and *Pvu*II. Fragments of the same length but containing the base substitution in the G-rich sequences were similarly prepared from the corresponding CAT constructs. The genotypes of the G-rich sequences in FN promoter-CAT constructs are shown as capital or lowercase G's. The first letter of GGG represents the G_{10} stretch, and the second and third letters represent the upstream and downstream GC boxes (GCu and GCd), respectively. The wild-type sequence is shown by a capital G, and substitution of the sequence GGGG with ATCC or CTTA is shown by a lowercase g (Fig. 1B). Base substitution of the Sp1 motif with the T stretch is shown by a lowercase m (Fig. 1C). Transcription of these templates (30 ng, 60 fmol) generated a transcript of 285 nucleotides (nt), as expected from the genetic map, starting from +1 and extending through the 5′ portion of the CAT gene (Fig. 1A). Transcription was performed with a constant amount of the HeLa cell extract, which contains approximately 5.0 ng (50 fmol) of Sp1 and increasing amounts of purified G10BP up to 2.0 ng (67 fmol), as shown in Fig. 5. When the template GGG prepared from pF414CAT was used, the level of the 285-nt transcript decreased with increasing amounts of G10BP and reached 1/10 of the original level at the highest concentration. Similar decreases but to slightly lesser extents were observed with the templates gGG and ggG, which contain the wild-type GCd. In contrast, transcription of the templates gGg and ggg, which contain the four-base substitution in the GCd, was almost unaffected by G10BP, although the basal levels of transcription were lowered. A similar pattern of inhibition was observed with the template GmG, carrying the wild-type GCd, but not with the templates GGm and Gmm, carrying the T stretch instead of the Sp1 motif in the GCd. These results indicate that G10BP inhibits promoter activity primarily through interaction with the GCd. We previously showed that the promoter activities of pF414(GGG)CAT, pFgGGCAT, and pFggGCAT were efficiently repressed in rat 3Y1 cells by cotransfection with the E1A expression plasmid, while the activities of pFgGgCAT and pFgggCAT, both carrying the base substitution in the GCd, were repressed to much lesser extents (39). The result is consistent with the results (Fig. 2 and 5) obtained in the present study.

Activities of rat FN promoters carrying substitutive mutations in the G-rich sequences in HeLa cells. To analyze the

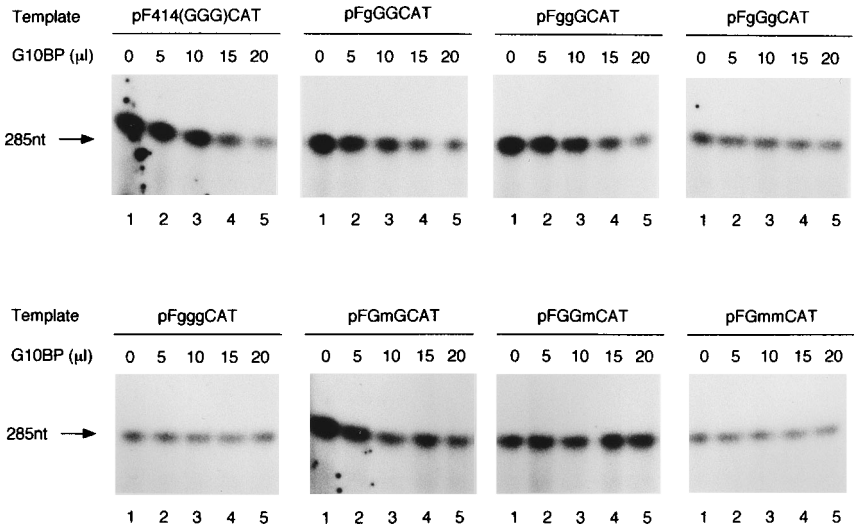

FIG. 5. Inhibition of FN promoter activity in vitro by G10BP. Thirty nanograms (60 fmol) of the 704-bp DNA templates prepared from pF414(GGG)CAT, pFgGGCAT, pFggGCAT, pFgGgCAT, pFgggCAT, pFGmGAT, pFGGmCAT, and pFGmmCAT (Fig. 1B and C) was transcribed in vitro in a HeLa cell extract containing approximately 5.0 ng of Sp1 (50 fmol) with increasing amounts of G10BP up to 2.0 ng (67 fmol). The concentration of G10BP in the purified preparation was about 0.1 μg/ml. The transcript was detected by autoradiography.

contribution of each G-rich sequence to the promoter activity of the rat FN gene and to compare the basal promoter activities expressed in the in vitro transcription system with those expressed in vivo, the promoter activities of various FN promoter-CAT constructs were analyzed in HeLa cells, which contain abundant Sp1 (12) but not G10BP. Subconfluent cultures of HeLa cells were transfected with derivatives of pF414 CAT carrying the base substitution in the G-rich sequences. CAT activities were assayed at 48 h posttransfection. A typical result (Fig. 6) indicates that base substitution in any G-rich sequence results in the reduction of promoter activity. The involvement of the G_{10} stretch in promoter activity was shown by the reduction in the activity of pFgGGCAT to about half of that with pFGGGCAT. The activities of pFgmGCAT and pFgGmCAT were also lower than those of pFGmGCAT and pFGGmCAT, respectively. This reduction is presumably due to the abolishment of Sp1 binding to the G_{10} stretch by introduction of the four-base substitution, as shown in the following section (Fig. 7B). The upstream GC box (GCu) also contributes to the promoter activity, since the activities of pFGmG CAT and pFgmGCAT were much lower than those of pFGG GCAT and pFgGGCAT, respectively. The replacement of the Sp1 motif by the T stretch completely abolished Sp1 binding (Fig. 7B). On the contrary, the activity of pFggGCAT was consistently higher than that of pFgGGCAT. This increase also seems to be caused by the alteration in the efficiency of Sp1 binding, since the binding affinity of Sp1 to the GCu even increased after introduction of the four-base substitution, as shown in the following section (Fig. 7B). The involvement of the downstream GC box (GCd) is evident. The activities of pFg GgCAT and pFgggCAT were much reduced compared with those of pFgGGCAT and pFggGCAT. A similar reduction was observed with pFGGmCAT and pFgGmCAT, in which the Sp1 motif in the GCd was eliminated by introduction of the T stretch. The activities of various FN promoters carrying the base substitution expressed in HeLa cells were nearly the same as those expressed in the in vitro transcription system in the absence of added G10BP. These results suggest that FN promoter activity is determined by the cooperative interaction of Sp1 with these G-rich sequences, although the GCd plays a primary role in promoter activation.

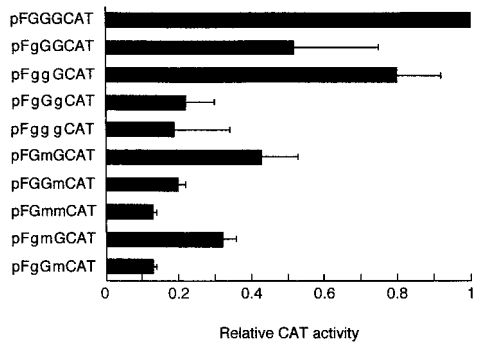

FIG. 6. Activities of FN promoters carrying the base substitution in the G-rich sequences in HeLa cells. Subconfluent monolayer cultures of HeLa cells were transfected with the various FN promoter-CAT constructs as indicated. CAT activities were assayed 48 h after transfection. The CAT activity expressed by pF414CAT was taken as 1, and the activities of other CAT constructs are shown as relative values. The values are averages of three independent experiments with standard deviations.

Binding affinities of G10BP and Sp1 to three G-rich sequences in the rat FN promoter. To analyze the binding affinities of G10BP and Sp1 to three G-rich sequences in the FN promoter, EMSAs were performed with the 24-bp oligonucleotides containing one of the G-rich sequence with or without the base substitution. The G10BP complex was formed with purified G10BP, and the Sp1 complex was formed with the HeLa cell extract, since the purified Sp1 sample often formed smear bands broadly distributed in the gel. Oligonucleotides containing the G_{10} stretch (G10) or the GCd formed typical complexes I, II, and III with G10BP (Fig. 7A). The oligonucleotide containing the GCu formed very few complexes, indicating that the binding affinity of G10BP to the upstream GC box is much weaker than those to the G_{10} stretch and the GCd. The oligonucleotides G10, GCu, and GCd carrying substitutions of the sequence GGGG with ATCC or CTTA ($G10^{mut}$, GCu^{mut}, and GCd^{mut}) were unable to form the complexes. The oligonucleotides GCu-T and GCd-T, in which the Sp1 motif

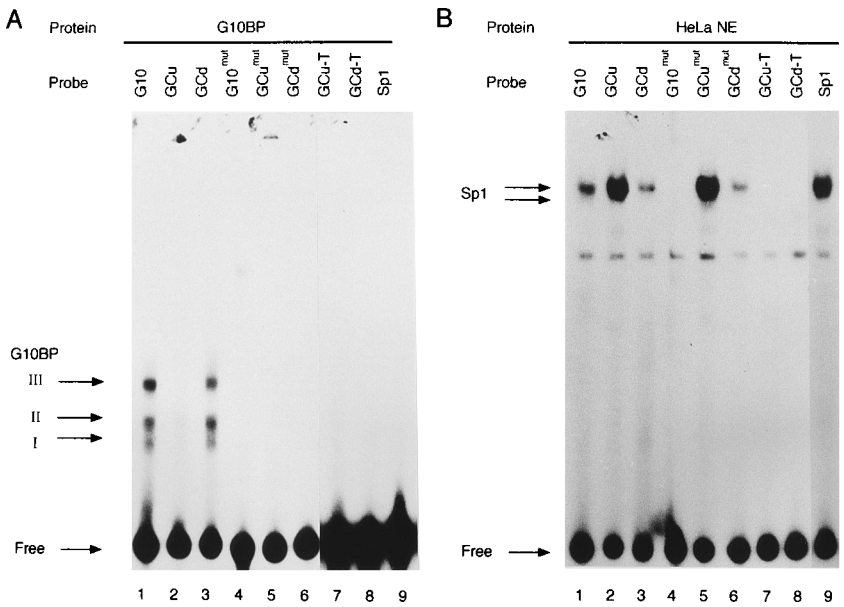

FIG. 7. Binding affinities of G10BP and Sp1 to the G_{10} stretch and the upstream and downstream GC boxes. The indicated 24-bp oligonucleotides (0.5 fmol) containing one of three G-rich sequences with or without base substitutions were used for binding assays. Substitution of the sequence GGGG with ATCC or CTTA (Fig. 1B) is represented by mut, and base substitution of the Sp1 motif with the T stretch (Fig. 1C) is represented by T. The following abbreviations are used: G10, the G_{10} stretch; GCu, the upstream GC box; GCd, the downstream GC box; $G10^{mut}$, the G_{10} stretch with the four-base substitution; GCu^{mut}, the upstream GC box with the four-base substitution; GCd^{mut}, the downstream GC box with the four-base substitution; GCu-T, the upstream GC box lacking the Sp1 motif; GCd-T, the downstream GC box lacking the Sp1 motif; and Sp1, the high-affinity Sp1 recognition sequence GGGGCGGGGC. These oligonucleotides were ^{32}P end labeled, and aliquots of the oligonucleotide having 5×10^3 cpm were incubated with 0.4 ng of G10BP (A) or 4 μg of protein of the HeLa cell extract (B). The complexes formed were analyzed by EMSAs and are indicated by the arrows.

was replaced by the T stretch, also did not form complexes. G10BP did not bind to the oligonucleotide Sp1, containing the high-affinity Sp1 recognition sequence GGGGCGGGGC.

In contrast to the binding of G10BP, Sp1 formed complexes predominantly with the GCu, and this binding affinity was even increased by the introduction of the four-base substitution (GCu^{mut}), which does not impair the Sp1 motif (Fig. 7B). The level of Sp1 complexes formed with the GCu^{mut} oligonucleotide was higher than that formed with the oligonucleotide Sp1. Less of the complexes were formed with the GCd and the G_{10}. Introduction of the four-base substitution into the G_{10} ($G10^{mut}$) completely abolished complex formation; however, introduction into the GCd did not affect the binding affinity significantly. Neither GCu-T nor GCd-T carrying the T stretch formed the complex. The higher promoter activity of pFggG CAT than of pFgGGCAT (Fig. 6) is likely due to the stronger binding affinity of Sp1 to GCu^{mut} than to the GCu. The fast-migrating band not shown by the arrow was not the Sp1 complex and could not be inhibited by the same unlabeled oligonucleotide.

Competitive binding of G10BP and Sp1 to the G-rich sequences in the rat FN promoter. Since the recognition sequences of G10BP and Sp1 overlap in three G-rich sequences, the preferential binding of G10BP to the G_{10} stretch and the GCd may prevent Sp1 from binding to these sites so as to inhibit FN gene transcription in XhoC cells. This possibility was assessed by the formation of complexes in the presence of both G10BP and Sp1, since the electrophoretic mobilities of complexes formed with these factors were quite different, reflecting their molecular weights. The complexes were formed with the 24-bp oligonucleotides (0.5 fmol) containing one of the G-rich sequence with or without the base substitution, a constant amount of the HeLa cell extract containing approximately 1 ng (10 fmol) of Sp1 (1), and increasing amounts of G10BP up to 1.2 ng (40 fmol) (Fig. 8). The competitive binding of G10BP and Sp1 was therefore performed in the presence of excess proteins. The amount of Sp1 complex formed with the G_{10} oligonucleotide (G10) decreased greatly along with the increase in G10BP added, and Sp1 complex formation was almost abolished at the highest concentration of G10BP. On the contrary, the amount of G10BP complexes increased along with the increase in G10BP added. Similar exclusion of Sp1 binding by G10BP was observed with the GCd. Sp1 complex formation with the G_{10} and the GCd was unaffected by the addition of increasing amounts of bovine serum albumin (data not shown). No exclusion was observed with the GCu, because Sp1 binds strongly to the GCu, while G10BP binds very weakly. GCu^{mut}, containing a substitution of the sequence GGGG with ATCC, also formed the complexes with Sp1 but not with G10BP. GCd^{mut} formed small amounts of the complexes with Sp1 but not with G10BP, consistent with the results shown in Fig. 7. The oligonucleotides GCu-T and GCd-T, carrying the T stretch instead of the Sp1 motif, formed neither the Sp1 nor the G10BP complexes. These results indicate that G10BP excludes the binding of Sp1 to the G_{10} stretch and the GCd, while the strong binding of Sp1 to the GCu is unaffected by G10BP.

Formation of G10BP and Sp1 complexes is unaffected by pRB. Besides its consensus sequence, GGGCGG, Sp1 binds to the RB control element (RCE) sequences GCCACCC, CCCACCC, and CCCACGC found in the human c-*fos*, c-*jun*, transforming growth factor β1, and c-*myc* promoters, and Sp1-mediated transcription of these promoters is modified positively or negatively by the RB gene product (pRB) in a cell type-dependent manner (27, 28, 38, 39). The stimulation of Sp1 binding to a c-*jun* Sp1 site by pRB has been suggested to occur by liberating Sp1 from an Sp1 negative regulator, Sp1-I (6). In this context, we first tested whether G10BP is able to bind to these RCEs by EMSAs with the 24-bp oligonucleotides

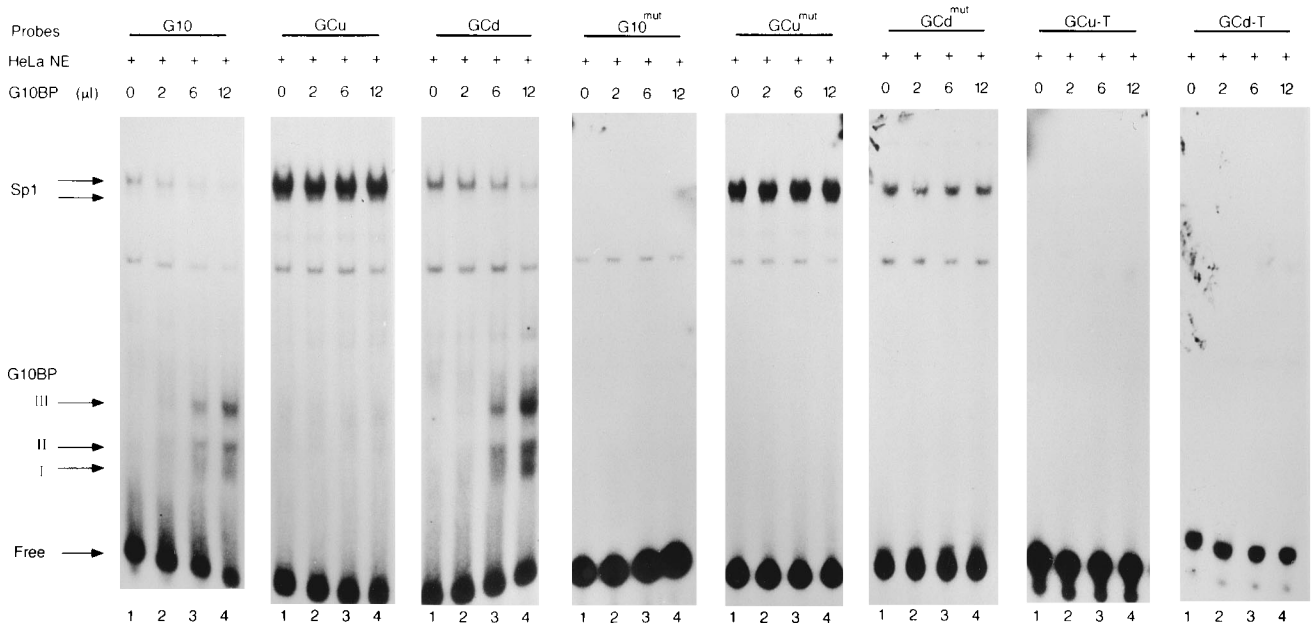

FIG. 8. Competitive DNA binding of G10BP and Sp1. The ^{32}P-labeled 24-bp oligonucleotides G10, GCu, GCd, G10^{mut}, GCu^{mut}, GCd^{mut}, GCu-T, and GCd-T (each at 0.5 fmol) were incubated with 4 μg of protein of the HeLa cell extract containing approximately 1 ng (10 fmol) of Sp1 (1) and increasing amounts of G10BP up to 12 μl, containing approximately 1.2 ng (40 fmol) of G10BP. The complexes formed were analyzed by EMSAs and are shown by the arrows. The amounts of G10BP added are indicated above each lane.

containing one of these elements. G10BP did not bind to any of these RCEs (data not shown). The effect of pRB on the formation of G10BP and Sp1 complexes was investigated by adding glutathione *S*-transferase-fused pRB (GST-pRB) to the binding mixture containing the GCd and either G10BP or the HeLa cell extract (Fig. 9). pRB did not alter the formation of G10BP and Sp1 complexes irrespective of the presence of anti-pRB antibody. Preincubation of G10BP with polyclonal anti-Sp1 antibody did not affect G10BP complex formation (Fig. 9A), indicating that G10BP is not a degradation product

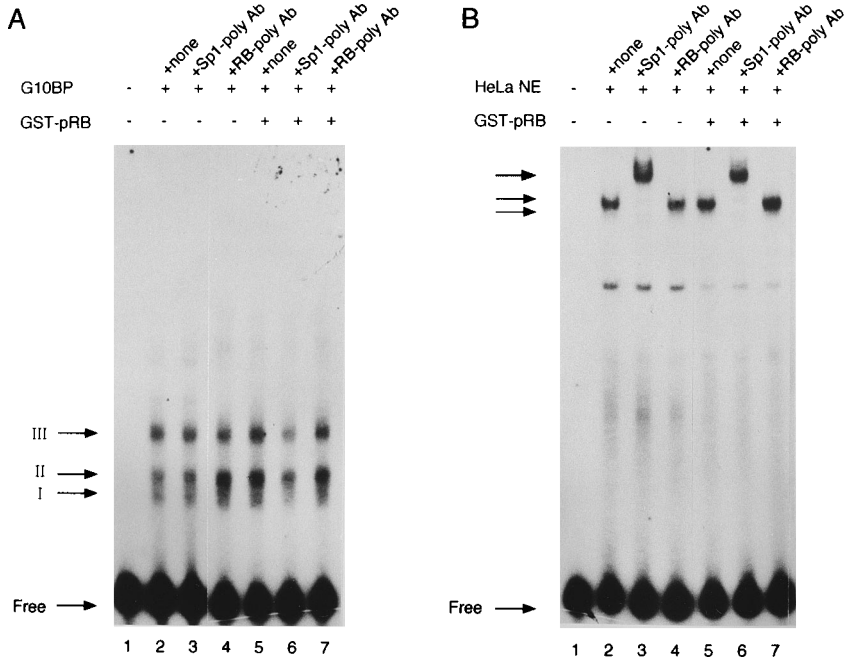

FIG. 9. Effect of pRB on the formation of G10BP and Sp1 complexes. (A) The ^{32}P-labeled GCd oligonucleotide (0.5 fmol) was incubated with 6 μl (0.6 ng) of G10BP with or without 2 μg of GST-pRB, as indicated above each lane. One microgram of polyclonal anti-Sp1 antibody (PEP2-X; Santa Cruz) was added to lanes 3 and 6, and 1 μg of polyclonal anti-pRB antibody was added to lanes 4 and 7. (B) The ^{32}P-labeled GCd oligonucleotide (0.5 fmol) was incubated with 4 μg of protein of the HeLa cell extract with or without 2 μg of GST-pRB, as indicated above each lane. One microgram of polyclonal anti-Sp1 antibody was added to lanes 3 and 6, and 1 μg of polyclonal anti-pRB antibody was added to lanes 4 and 7. The complexes formed were analyzed by EMSAs and are shown by the arrows.

of Sp1. Preincubation of the HeLa cell extract with anti-Sp1 antibody, however, resulted in supershift of the Sp1 complexes and concomitant reduction of the original complexes (Fig. 9B). This supershift was unaffected by the presence of either pRB or anti-pRB antibody. These results indicate that the inhibitory mechanism of Sp1 binding to the GCd by G10BP is different from that of Sp1 binding to the RCE element in the c-*jun* promoter by Sp1-I and that the inhibition is mediated by preferential binding of G10BP to the G_{10} stretch and the GCd.

	G10	GCu	GCd	$G10^{mut}$	GCu^{mut}	GCd^{mut}	GCu-T	GCd-T	Sp1
G10BP	++	±	+++	−	−	−	−	−	−
Sp1	++	+++	+	−	+++	±	−	−	+++

FIG. 10. Binding affinities of G10BP and Sp1 to the G_{10} stretch and the upstream and downstream GC boxes. The results shown in Fig. 7 were summarized. The symbols represent the following binding affinities: +++, high affinity; ++, moderate affinity; +, low affinity; ±, very low affinity; −, no affinity.

DISCUSSION

The level of FN gene expression is decreased greatly in a variety of transformed cells (13, 28). In the rat 3Y1 derivative cell lines transformed by various viral oncogenes, the degree of repression differs depending on the oncogene introduced but is well correlated with the level of G10BP induced in the cells (unpublished data), suggesting that G10BP plays a primary role in the repression of FN gene expression. G10BP was identified as a negative regulator of the transcription of the FN gene in adenovirus E1-transformed 3Y1 cells (XhoC), in which FN gene expression was almost completely repressed (39). To demonstrate the E1A-dependent induction of G10BP, the correlation between E1A expression and G10BP production was analyzed by using the g12-21 cell line, a derivative of rat 3Y1, in which the expression of $E1A_{12s}$ can be induced by the addition of DEX. Quiescent g12-21 cells expressed a high level of FN mRNA and low level of G10BP. After induction of $E1A_{12S}$, the level of FN mRNA decreased to about 1/10 of the original level, while that of G10BP, estimated by EMSA, increased nearly 10-fold (Fig. 2). This inverse correlation strongly suggests that the FN gene is repressed by $E1A_{12S}$ through induction of G10BP.

G10BP binds to three G-rich sequences in the promoter (Fig. 1); one is the G_{10} stretch located from positions −239 to −230, and the other two are the GC boxes consisting of the G_{10} stretch with one internal C residue insertion. These boxes are located from positions −105 to −95 and −54 to −44. The presence of a C residue in the G_{10} stretch generates the Sp1 consensus sequence GGGCGG. The overlapping of the recognition sequences of G10BP and Sp1 in these two GC boxes suggested that G10BP competes with Sp1 for their common DNA recognition sites.

To analyze the interaction of G10BP with the G-rich sequences, G10BP was purified from the XhoC cell extract by DNA affinity chromatography after elimination of Sp1 by a WGA-agarose column. Southwestern blotting of the purified G10BP fraction revealed that it was slightly larger than 30 kDa (Fig. 3). Transcriptional inhibition of the FN gene by G10BP was analyzed by adding the purified G10BP fraction to an in vitro transcription system with the HeLa cell extract, which contains abundant Sp1 (12) but not G10BP cross-reactive with the rat FN promoter. The 704-bp DNA fragments from various FN promoter-CAT constructs carrying the base substitution in G-rich sequences were used as templates. Transcription of the templates GGG, gGG, ggG, and GmG, all carrying the wild-type downstream GC box (the GCd), was inhibited by G10BP in a dose-dependent manner, while that of the templates gGg, ggg, GGm, and Gmm, all carrying the base-substituted GCd, was unaffected, although the basal levels of transcription were more or less reduced by base substitutions (Fig. 5). The results, together with those obtained in vivo (Fig. 6), suggest that the level of FN gene transcription is primarily regulated by the GCd and that G10BP inhibits transcription through interaction with the GCd. This result is consistent with our previous result, which showed that the promoter activities of the FN promoter-CAT constructs pF414(GGG)CAT, pFgGGCAT, and pFggG CAT, all carrying the wild-type GCd, were efficiently repressed in rat 3Y1 cells by cotransfection with the E1A expression plasmid, while the activities of the constructs pFgGgCAT and pFgggCAT were repressed to much lesser extents.

The contribution of each G-rich sequence to FN promoter activity was analyzed in vivo by transfection of HeLa cells, which contain abundant Sp1 (12), with various FN promoter-CAT constructs carrying the base substitution in these G-rich sequences (Fig. 6). The results indicated that all three G-rich sequences are more or less involved in promoter activation and that FN promoter activity is largely determined by the cooperative binding of Sp1 to these sites, although the GCd plays a primary role. The activities of various base-substituted promoters expressed in HeLa cells were nearly the same as those estimated in the in vitro transcription system in the absence of added G10BP.

The binding affinities of G10BP and Sp1 to each G-rich sequence were analyzed by EMSAs with the 24-bp oligonucleotides containing one G-rich sequence with or without base substitutions. The results obtained are summarized in Fig. 10. G10BP binds strongly to the G_{10} stretch, moderately to the GCd, and very weakly to the GCu. Base substitution in these sequences from GGGG to ATCC or CTTA or replacement of the Sp1 motif by the T stretch completely abolished the binding of G10BP. In contrast, Sp1 binds strongly to the GCu and moderately to the G_{10} and the GCd. Substitution of four G residues in the G_{10} stretch abolished Sp1 binding completely, and substitution adjacent to the Sp1 motif in the GCd reduced the binding affinity slightly. The substitution in the GCu even increased the binding affinity of Sp1. The result indicates that the binding affinities of G10BP and Sp1 vary considerably among these G-rich sequences and that the efficiency of Sp1 binding to these three G-rich sequences largely determines the promoter activity. The competition experiments for binding of G10BP and Sp1 to these G-rich sequences were performed with the HeLa cell extract containing approximately 1 ng of Sp1 (1) and increasing amounts of G10BP up to 1.2 ng. The results indicate that the exclusion of Sp1 binding by G10BP occurs at the G_{10} and the GCd, reflecting their strong binding affinities to these sites. The exclusion at the GCd seems to be critical for repression of the promoter activity, since the promoter activity of pF414(GGG)CAT was greatly reduced by G10BP in the in vitro transcription system when the promoter carrys the wild-type GCd, as stated above.

GC boxes are the most ubiquitous promoter element, and many transcription factors besides Sp1 interact with them (15, 17, 25, 33). Two negative factors, Sp3 and Sp1-I, that inhibit the binding of Sp1 to G-rich sequences have recently been reported (6, 16). Sp1 is a large glycoprotein with an apparent molecular mass of about 100 kDa (1). The DNA-binding domain is near the carboxyl terminus and has the zinc finger motif. The two glutamine-rich stretches located in the amino-terminal half and short sequences that flank the zinc fingers are required for optimal transcriptional activation (8, 29, 40). Sp3

has been found to be a member of the Sp1 family and has a structure similar to that of Sp1 (6, 15, 33). Sp3 has a unique property among several members and acts to repress Sp1-mediated transcription by competition for the Sp1 binding sites. G10BP, identified in the present paper, also competes with Sp1 for binding to their common recognition sites; however, G10BP is not a member of the Sp1 family, judging from its molecular weight and the lack of the carbohydrate moiety.

Besides GC boxes, Sp1 binds to the GT motif GGGTGTG GC with identical affinity (30). Sp1 also binds to the RCE GCCACC or CCACCC, and Sp1-mediated transcription from the RCE-containing promoters is modulated by pRB. Transcription of the c-*fos*, c-*myc*, and transforming growth factor β1 promoters, each containing the RCE, is regulated by pRB either positively or negatively, depending on the cell type (42, 43). The fourth promoter of the insulin-like growth factor II gene and the c-*jun* promoter have been shown to be stimulated by pRB (6, 31). The stimulation of the c-*jun* promoter seems to occur by binding of pRB to the Sp1 negative regulator Sp1-I, which liberates Sp1 from its complex with Sp1-I (6). The molecular mass of Sp1-I was estimated to be about 20 kDa. G10BP also differs from Sp1-I, judging from its molecular weight and its inability to bind to pRB. G10BP could not form the complex with the 24-bp oligonucleotides containing the RCE of either the c-*fos*, c-*myc*, or transforming growth factor β1 promoter (data not shown). G10BP is therefore a new class of Sp1 negative regulator.

Although G10BP does not bind to pRB, its induction seems to be negatively regulated by pRB in quiescent rat 3Y1 cells, since the E1A domain for binding to pRB is essential for E1A-mediated repression of the FN promoter (38). The oncogene products of E1A and T antigen antagonize the function of pRB by direct binding to it (10, 48) and may therefore have strong inducibility of G10BP. The molecular cloning of the G10BP cDNA and its regulatory region will clarify the function of G10BP and the mechanism of G10BP induction.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

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