Identification of a Novel Cis Element Required for Cell Density-dependent Down-regulation of Insulin-like Growth Factor-2 P3 Promoter Activity in CaCo2 Cells*

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The activity of the exogenous, full-length insulin-like growth factor-2 (IGF-2) P3 promoter is significantly up-regulated during the logarithmic growth phase but rapidly declines in confluent CaCo2 cells undergoing differentiation. Nuclear run-on assays confirmed cell density-dependent regulation of endogenous P3 promoter. To identify regulatory elements in the P3 promoter that may be required for regulating cell density-dependent transcriptional activity, we used the methods of promoter truncation, electrophoretic mobility shift assay, DNase footprinting, and mutation analysis. The relative activity of the full-length (−1229/+140) and truncated (−1090/+140) promoter was identical, being −19, 27, 7, and 3% of pSV-luc activity on days 3, 5, 7, and 9 of cell culture, respectively. However, truncation to −1048 resulted in complete loss of cell density-dependent down-regulation of P3 promoter activity on days 7 and 9, suggesting the presence of regulatory elements between −1091 and −1048 sequence. Further stepwise truncation to −515 did not change promoter activity. Truncation to −138/+140 resulted in complete loss of promoter activity, suggesting that the core promoter was within the −515−138 segment. A 14-base pair footprint (−1084/−1070) was identified by DNase footprinting within the distal −1091−1048 segment. Electrophoretic mobility shift assay with wild type and mutant probes confirmed the presence of a novel 7-base pair (CGAGGG) (−1084/−1078) cis element (P3-D); its mutation abolished binding. Functionality of P3-D cis element was confirmed by measuring the activity of core P3 promoter ligated to distal P3 segment containing either the mutant or wild type P3-D element. We have, therefore, identified a novel cis element, P3-D, that appears to play a critical role in regulating IGF-2 P3 promoter activity in a cell density/differentiation-dependent manner.

A large percent of colon cancers overexpress IGF-2 (1–5) and contain significant concentrations of IGF-1 receptor (5–7). Colon cancer-derived cell lines secrete unprocessed IGF-2, which is even more potent than the mature form (8) and is believed to contribute to autocrine growth of cancer cells (9). Since a significant percent of primary/metastatic human colon cancers express IGF-2 and IGF-I receptor (1, 3, 5, 10) and surrounding stromal/normal epithelial cells may also express IGFs (11, 12), IGFs are likely to be potent mitogenic factors for colon cancers in situ by autocrine, endocrine, and paracrine mechanisms. We and others have shown that IGF-2 is a potent autocrine growth factor for a significant percent of human colon cancer cells (4, 10). Evidence in literature strongly supports the contention that IGFs (via IGF-I receptor) play an important role not only in the proliferation/tumorigenicity of colon cancers but also in inhibiting differentiation of the cells (13–16). It is therefore important to understand the mechanisms that contribute to up-regulation of IGF-2 levels in colon cancers.

The IGF-2 gene consists of 9 exons, and the peptide is encoded by exons 7, 8, and 9 (17, 18). The gene is transcribed from four different promoters (P1-P4) (18). Multiple transcripts are synthesized as a result of alternative promoter usage and the splicing of the unique 5′-untranslated region to common coding exons (18). Enhanced levels of P3- and P4-driven IGF-2 mRNAs have been detected in many human tumors including colorectal cancers (1–4, 9, 10, 19). Increased levels of IGF-2 mRNA in neoplastic cells can be potentially accomplished by loss of imprinting, loss of heterozygosity, transcriptional activation, and/or loss of transcriptional suppression (reviewed in Ref. 20). With loss of imprinting or loss of heterozygosity (involving duplication of the active paternal allele), IGF transcripts are made from two copies instead of one, leading to −two times higher mRNA levels. However, the expression of IGF-2 mRNA is 10–40-fold higher in colon cancer cells compared with that in normal colonocytes (1–5). Although loss of imprinting can contribute to overexpression of IGF-2, the majority of overexpression in situ is now believed to be due to transcriptional up-regulation in several cancers (4, 13, 21, 22). Factors involved in transcriptional regulation of the IGF-2 gene in colon cancers are as yet unknown.

In previous studies we and others have used CaCo2 cells (a human colon cancer cell line) for investigating the role of the IGF system in growth and differentiation of colon cancer cells (10, 13, 15, 16, 19, 23, 24). CaCo2 cells spontaneously differentiate in culture at confluency (25) and are an ideal in vitro model for investigating cellular mechanisms involved in prolif-

β-Gal, β-galactosidase; bp, base pair(s); ds, double-stranded; EMSA, electrophoretic mobility shift assay; luc, luciferase; nt, nucleotide(s); WT, wild type;TES, N-tris(hydroxymethyl)-2-aminoethanesulfonic acid; FCS, fetal calf serum; PCR, polymerase chain reaction.

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‡The abbreviations used are: IGF-2, insulin-like growth factor-2;
eration and differentiation of colonic cells. IGF-2 mRNA levels are significantly up-regulated during logarithmic growth of CaCo2 cells (days 3–6) in culture followed by a steep decline in confluent cells undergoing rapid differentiation (days 7–10) (13, 23). We recently reported that P3- and P4-derived transcripts were significantly up-regulated during the proliferative phase of the cells (days 3–6 in culture) and declined rapidly in cells undergoing differentiation (days 7–10); P1- and P2-derived transcripts were not detected (19). Similarly, transcriptional activity of transiently transfected P3 and P4 promoters reached peak levels by days 4–6 and declined rapidly thereafter (19). At the present time, the regulatory element(s) within the P3 and P4 promoters, which is involved in the observed cell density-dependent regulation of the promoter activity, is unknown. Since a majority of colon cancers mainly express P3-derived transcripts, we further investigated the role of the endogenous P3 promoter in regulating IGF-2 mRNA levels, in a cell density-dependent/differentiation manner using the method of nuclear run-on assays. Additionally, for the first time, we have defined the promoter segment(s) and a putative novel cis element that may play a critical role in the cell density-dependent regulation of P3 promoter activity in CaCo2 cells using the methods of transient transfection with truncated promoter-reporter constructs, electrophoretic mobility shift assay (EMSA), and DNase footprinting.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The CaCo2 human colon cancer cell line was obtained from Dr. Jing Yu, Tufts School of Medicine, and cells were grown as monolayer cultures as previously described (13, 19). For all experimental purposes, exponentially growing cells were seeded at ~150,000 cells/35-mm cell culture dishes (Falcon, Fisher), and the cell culture medium was changed every second day with fresh growth medium containing 10% FCS.

**Nuclear Run-on Assay**—Nuclei were obtained, and assays were performed by a modification of previously described procedures (26). Briefly, 7–10 × 10⁶ CaCo2 cells, at the indicated time points, were washed twice in phosphate-buffered saline (4 °C). Cells were scraped and lysed in 2 ml of Nonidet P-40 buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40), and nuclei were harvested and washed with 100 µl (–1–2 × 10⁶ nuclei) of storage buffer (40% glycerol, 10 mM Tris, pH 7.5, 5 mM MgCl₂, 80 mM KCl, and 0.1 mM EDTA) and stored at ~80 °C. Thirty µl of nuclear run-on buffer (25 mM Tris, 12.5 mM MgCl₂, 750 mM KCI, 1.25 mM ATP, 1.25 mM CTP, 1.25 mM GTP) and 20 µl of [³²P]UTP (3000 Ci/mmol, ICN, Costa Mesa, CA) were added to 100 µl of nuclear suspension. The transcription runoff was allowed to proceed at 30 °C for 30 min and terminated with 100 µl (–1–3 µg) of proteinase K (100 µg/ml) and 25 µl of SET buffer (5% SDS, 50 mM EDTA, 100 mM Tris, pH 7.5) were added and incubated for 30 min at 37 °C. Subsequently, 550 µl of 4 µM guanidinium isothiocyanate and 90 µl of 3 M sodium acetate were added, and the mixture was extracted with phenol/chloroform/isooamyl alcohol (25:24:1). The aqueous phase was removed, and RNA was precipitated with 1 volume of isopropl alcohol. The pellet was washed 2 times in isopropl alcohol/isoamyl alcohol (25:24:1). The aqueous phase was removed, and RNA was precipitated with isopropl alcohol. The pellet was washed 2 times in 70% ethanol, dissolved in 100 µl of TES (10 mM Tris, pH 7.2, 1 mM EDTA, 0.1% SDS), and used for hybridization. A DNA slot or dot blot apparatus was used to blot equal concentrations of linearized plasmids (10 µg) onto nitrocellulose filters (Schleicher and Schuell). The DNA plasmids used included the negative control (Bluescript II SK+ vector without insert), the Bluescript II SK+ vector (containing human 18S cDNA used as a positive control for normalizing the hybridization signals), and a plasmid containing the human IGF-2 exon 5 DNA (to measure P3 promoter-driven transcriptional activity). The plasmid DNA were linearized by appropriate restriction endonucleases and denatured with 0.2 M NaCl. The pre-hybridization and hybridization solution consisted of 10 mM TES, 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt's, 0.25 mg/ml, Escherichia coli DNA. DNA blots were pre-hybridized in hybridization buffer for 3 h at 65 °C for 36 h. Hybridization was carried out at 65 °C for 36 h. Filters were washed in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) with 0.1% SDS for 10 min twice and then in 0.01× SSC with 0.1% SDS for 30 min at 60 °C before autoradiographic analysis. The nuclear run-on assay results were quantitated by densitometric analysis of the autoradiographic results using the UltraScan LX laser densitometer (Amersham Pharmacia Biotech). Relative levels of RNA on different days of cell culture were determined with the help of GelScan XL software (Amersham Pharmacia Biotech). RNA levels in each experimental blot are expressed as a ratio of the corresponding 18 S RNA blot.

**Human IGF-2 P3 Expression Vectors Used for Transfections**—The IGF-2 promoter-luc constructs used for transfection are presented diagrammatically in Fig. 1. In this figure, the length of human promoters P3 DNA fragment was subcloned into the BamHI and SalI sites of the luciferase reporter gene expression vector, pSla3 (18), resulting in pHUP3. Additional P3 clones containing the indicated truncated P3 fragments (Fig. 1) were derived from pHUP3 either by restriction enzyme digestions with the appropriate enzymes and filling in of the protruding ends by Klenow DNA polymerase (Fig. 1) or by PCR amplification of the indicated fragments (Fig. 1). For PCR amplification, six separate 5′ sense primers were used: (a) 5′-GGCGGCAGGAGGGCATCTGATTTG-3′ (1090/–1070); (b) 5′-GGCGGCGGAGGCGGCAATTGTTAGTTG-3′ (1048/–1012); (c) 5′-GGGCCGAGGGGCGGCAGCAGC-3′ (1066/–982); (d) 5′-CCTCCCTTGGTAGCTTGGAGGC-3′ (946/–923); (e) 5′-AGGCTGACCTCTATCCG-3′ (884/–864); and promoter 5′-TCTGGCCTGTATCCTTCTCC-3′ (139+/–115). The full-length HUP3-luc vector (linearized) was used as template for the PCR amplifications. The PCR reaction was performed in the presence of AmpliTaq DNA polymerase (PerkinElmer Life Science) as described previously (27). The PCR products were subcloned into PCR 2.1 cloning vector using a TA cloning kit (Invitrogen, San Diego, CA) as per protocols provided by the supplier. Positive clones (that were determined to have the appropriate orientation of the P3 promoter DNA fragment in relation to the KpnI site on the PCR 2.1 vector) were selected and checked by DNA sequencing. The inserts were released from PCR 2.1 vectors by XhoI and KpnI endonuclease digestion and religated into the same sites in promoterless luciferase reporter vector, pGL2-Basic (Promega, Madison, WI). The six new luciferase expression vectors, thus constructed, were termed HUP A1–HUP A6 luc vectors as shown in Fig. 1. In the orientation and the sequence of the inserts were confirmed by DNA sequencing using pGL primers one, 5′-GCTATCTTATCTGCTAATG-3′ (~10 bp upstream from multiple cloning site of pGL-2 basic vector), and two, 5′-TTCTAATGTTCGCTTCC-3′ (~20 bp downstream from multiple cloning site of pGL-2 basic vector).

**DNA Transfection of CaCo2 Cells**—CaCo2 cells in culture in 35-mm dishes (as described above under cell culture) were transfected with 5–10 µg of the indicated vector DNA on days 2–8 of cell culture by the calcium phosphate precipitation method, as described previously (18, 28, 29). Cells were cotransfected with the SV40 β-galactosidase expres-
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sion vector (pSV β-Gal vector, Promega) to normalize for transfection efficiency (19, 29). Duplicate dishes were transfected with 5 μg of luciferase expression vector (pGL2, Promega) under the transcriptional control of the SV40 promoter as a positive control (19, 29). Cells were grown in regular growth medium containing 10% FCS until 22 h before transfection. Growth medium was then exchanged for 10% FCS containing medium until the day of transfection. Additionally, before transfection, fresh growth medium plus 10% FCS was added. Post-transfection, the cells were washed with fresh medium plus 10% FCS and cultured for 24 h followed by cell lysis for purposes of measuring luciferase and β-Gal activities.

Luciferase and β-Gal Assays—Luciferase and β-Gal were measured in the cell lysates as described by us (29). Briefly, cells were washed twice with phosphate-buffered saline and lysed using the reporter lysis buffer (Promega) as per the protocols provided by the company. Luciferase activity was measured with the luciferase assay system (Promega) using 50 μl of cell extract and 100 μl of luciferase buffer at room temperature. Luciferase activity was measured within 15 s of adding the substrate, luciferin, with Berthold AutoLumat LB953 (Wallac/LKB, Gaithersburg, MD). The β-Gal assay was performed with the β-galactosidase enzyme assay system (Promega) as per the protocols provided by the company, as described by us (29). At the end of the reaction, the absorbance of the samples was read at 420 nm in a Umax kinetic microplate reader (Molecular Devices, Menlo Park, CA).

EMSAs were conducted by EMSA, with double-stranded (ds) DNA fragments that were obtained by restriction digestion of the full length HUP3-luc vector, as shown in Table I. The DNA fragments were purified by agarose gel electrophoresis and labeled with [α-32P]ATP by T4 polynucleotide kinase (Promega). The labeled DNA fragments were separated from unincorporated nucleotides by chromatography through a G50 spin column (Amersham Pharmacia Biotech). Nuclear extracts were prepared from CaCo2 cells on the indicated days of cell culture by methods described by Abmayr et al. (30). Protein concentrations were measured, and nuclear extracts were separated into aliquots and frozen at −70 °C. EMSA was performed essentially as described by Rietveld et al. (31). Briefly, EMSA was performed in a 20-μl binding reaction containing 5–10 μg of crude nuclear extract prepared from CaCo2 cells on different days of cell culture, 5 μl of DNA probe containing ~200,000 cpm, 1 μg of poly(dI-dC), 10 μM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05 mM EDTA, 1 mM MgCl2, 4% glycerol, and 1 mM dithiothreitol and incubated for 15 min at room temperature. For competition, a 100–200-fold excess of the unlabeled DNA probe was mixed with the radiolabeled probe before the addition of the protein extract. After incubating the samples for 15 min at room temperature, the DNA protein complexes were separated by electrophoresis in 5% nonequilibrium polyacrylamide gels in 0.5× Tris-boric acid-EDTA buffer. The gels were dried and subjected to autoradiography in the presence of intensifying screens at −80 °C. We additionally conducted EMSA with the indicated ds oligonucleotide probes using methods that were essentially similar to that described previously (31). The oligonucleotide probes used are detailed under "Experimental Procedures.”

RESULTS

Relative Levels of Endogenous P3-derived IGF-2 Transcripts in CaCo2 Cells on Days 3–11 of Cell Culture—To determine the levels of expression of endogenous P3-derived IGF-2 mRNAs in proliferating and differentiating CaCo2 cells, nuclear run-on transcription assays were conducted in the nuclei of CaCo2 cells on consecutive days of cell culture. In four separate experiments, RNA prepared from nuclei on the indicated days of cell culture were hybridized with various probes as described under "Experimental Procedures.”

Hybridization of nuclear RNA with the 18 S ribosomal DNA demonstrated a similar intensity in the signal on different days of cell culture, implying that equal amounts of RNA were used for the experiment (Fig. 2A). No specific signal other than background noise was obtained with the negative control DNA (Bluescript SK− vector without insert). However, IGF-2 tran-script was highly regulated and dependent on the differentiation status of the CaCo2 cells (Fig. 2A). A significant up-regulation in the relative abundance of IGF-2 transcripts was measured on day 5 (compared with day 3 and 4 values) when cells are in the proliferative phase. This was followed by a steep decline in the relative abundance of IGF-2 transcripts on days 6–7, when cells enter differentiation, and IGF-2 mRNA levels were minimal on days 8–11, the exponential phase of differentiation (Fig. 2A). Four independent experiments with nuclei from CaCo2 cells samples were performed using the IGF-2 and 18 S probes. Denstometric analysis of the autoradiographic data with the IGF-2 probe, normalized with the 18 S probe, is shown in bar graphs in Fig. 2B. Interestingly, the observed pattern of transcriptional activity for the endogenous IGF-2 gene is almost identical to the pattern of transcriptional activity measured with exogenous P3 promoter-reporter constructs as recently reported (19). The P3 promoter region has the earlier findings that reduction of IGF-2 mRNA levels in CaCo2 cells (13) reflect changes in the transcriptional activity of the exogenous (19) and endogenous (present study) IGF-2 gene caused by gradual progression of differentiation on consecutive days of cell culture.

Relative Contribution of the Distal, Middle, and Proximal Segments of the P3 Promoter in Cell Density-dependent Regulation of the P3 Promoter Activity—To determine if the promoter region responsible for the cell density-dependent regulation of transcription was located within the distal, middle, or proximal segment(s) of the P3 promoter, we used three IGF-2

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P3 promoter constructs in transient transfection assays. HUP3-Luc, the maximal promoter (−1229/+140), and two truncated promoter fragments, HBSP3-luc (−515/+140) and HAVP3-luc (−138/+140) (Fig. 1), were used in these studies. CaCo2 cells were transfected on days 3, 5, 7, and 9 of cell culture with one of the P3 promoter constructs and with a β-Gal expression vector to normalize for transfection efficiency as described under “Experimental Procedures.” Duplicate dishes were transiently transfected with DNA from the pSV-luc vector. The luc/β-Gal activity for each sample was determined, and the relative activity of the P3-luc vectors is described under “Experimental Procedures.” Duplicate dishes were transiently transfected with DNA from the pSV-luc vector. The luc/β-Gal activity for each sample was determined, and the relative activity of the P3-luc vectors is described under “Experimental Procedures.”

Nuclear run-on analysis of endogenous IGF-2 gene transcription in CaCo2 cells on days 3–11 of cell culture. A, representative autoradiographs from experiments I and II from a total of four experiments are shown. The probe used for measuring IGF-2 P3-derived transcripts is described under “Experimental Procedures.” As controls, 18 S ribosomal transcripts were measured simultaneously. P3-derived transcripts is described under “Experimental Procedures.”

Fig. 2. Nuclear run-on analysis of endogenous IGF-2 gene transcription in CaCo2 cells on days 3–11 of cell culture. A, representative autoradiographs from experiments I and II from a total of four experiments are shown. The probe used for measuring IGF-2 P3-derived transcripts is described under “Experimental Procedures.” As controls, 18 S ribosomal transcripts were measured simultaneously. B, the ratios of IGF-2/18 S mRNAs measured for all four experiments on different days of cell culture are shown as bar graphs. The data in the bar graphs from days 3–11 are presented as the mean ± S.D. of duplicate measurements from 2–4 separate experiments.

Fig. 3. Transcriptional activity of the full-length and truncated IGF-2 P3-luc vectors in CaCo2 cells. A, CaCo2 cells were cotransfected with DNA from the full-length (HUP3-luc) and truncated (HBSP3-luc and HAVP3-luc) vectors (Fig. 1) and with a β-Gal expression vector to normalize for transfection efficiency as described under “Experimental Procedures.” Duplicate dishes were transiently transfected with DNA from the pSV-luc vector. The luc/β-Gal activity for each sample was determined, and the relative activity of the P3-luc vectors is presented as a percent of the pSV-luc activity (arbitrarily assigned a 100% value) on the indicated days of cell culture. Each bar graph represents the mean ± S.E. of data obtained from three separate experiments. B, the fold increase in the activity of the HBSP3-luc vector compared with that of the full-length HUP3-luc vector (based on data presented in A) is presented as a ratio of HBSP3-luc/HUP3-luc activity on the indicated days of cell culture. a, b, and c indicate data from three separate experiments. The numbers on top of each bar graph represent the fold increase of the luc activity of construct HBSP3 compared with that of the HUP3-luc activity.}

abolished on days 7–10 of cell culture. The strong down-regulation of the transcriptional activity of HUP3, but not of HBSP3, suggested that the distal segment of the P3 promoter (−1229 to −515) contained a potent inhibitory element that is regulated in a cell density-dependent manner.

To further narrow down the location of the putative regulatory element(s) in the distal segment of P3 promoter, we initially measured transcriptional activity of three additional truncated constructs (HUP3-A1, HUP3-A5, and HUP3-A6) (Fig. 1) and compared the activity with that of HBSP3-luc and HAVP3-luc vectors. The results of a representative experiment (of a total of two similar experiments) are presented in Fig. 4. The transcriptional activity of HUP3-A1-luc construct was almost identical to that of the full-length HUP3-luc vector (Fig. 4), indicating that the extreme distal end of the P3 promoter (−1229 to −1091) does not contain the regulatory element(s). In contrast, further truncation to −889 (HUP3-A5-luc) resulted in a strong increase (up to 10-fold) of promoter activity, suggesting that the cell density-dependent regulation of P3 was lost on truncation of the segment between −1091 to −889. Further truncation of the distal segment to −712 (HUP3-A6-luc) showed no additional effects on transcriptional activity. These results suggested that the P3 promoter contained regulatory element(s) between −1091 and −889 (relative to the CAP site) that may be involved in silencing of the promoter by many fold. More importantly, the cell density-dependent regu-
studies as per methods described under “Experimental Procedures.” CaCo2 cells on different days of culture were cotransfected with the β-Gal expression vector, and the ratio of luc/β-Gal was determined at each time point for the various vectors. The luc/β-Gal values on day 5 with the HBS-luc vector were arbitrarily assigned a 100% value. All other values are presented as a percent of that measured with the HBS-luc vector on day 5. Data from two separate experiments are presented as bar graphs. Data represent the mean of 4–5 measurements from two separate experiments, wherein individual values varied by <10%.

![Figure 4](image)

**Figure 4.** Transcriptional activity of the truncated constructs of the IGF-2 P3 promoter in CaCo2 cells on days 3–9 of cell culture. Studies from two separate experiments, wherein individual values varied by <10%.

The distal segment of the P3 promoter was lost on removal of the distal segment between –1091 and –889 (as in HUP3-A5 vector). To further narrow down the location of the regulatory element, additional truncated constructs between –1091 and –889 were generated as shown in Fig. 1. Transient transfection studies with HUP3-A2-luc (–1048/+140), HUP3-A3-luc (–1066/+140), or HUP3-A4-luc (–946/+140) demonstrated that the luciferase activity of all these truncated constructs was identical to that shown in Fig. 4 for HUP3-A5-luc vector (–889/+140) on days 3–9 of culture, indicating that the putative distal regulatory element was between –1090 and –1048 sequence of the P3 promoter (data not shown). To further confirm the presence of putative DNA binding sites in the distal P3 promoter, we conducted DNase footprinting and EMSA studies as described below.

The Distal P3 Promoter Fragment Contains a Specific DNA-Protein Complex—We initially conducted EMSA with relatively large (100–400 bp) P3 promoter DNA fragments covering the entire distal to middle promoter (Table I). Using fragments 1, 3, and 4 as probes, similar patterns were observed for extracts from proliferating and post-confluent CaCo2 cells (data not shown). Only fragment 2 (–1090/–656) demonstrated significant differences in the binding pattern of nuclear proteins from proliferating (day 5) versus post-confluent (day 10) CaCo2 cells (Fig. 5A). A unique and specific DNA-protein complex (a) was present in proliferating cells (day 5) that was absent in post-confluent (day 10) cells.

Based on the above truncation and EMSA results, a DNA fragment (–1164/–871) containing the putative regulatory element was used as a radiolabeled probe for DNase1 footprinting experiments to confirm the presence of the binding site in this distal segment. A specific DNA footprint was obtained with nuclear proteins from proliferating day-5 samples but not from day-10 samples. Positive data with day-5 results are shown in Fig. 6. The 14-bp-nt sequence of the footprint was identified with the help of a 20-bp DNA ladder co-run with the samples as described under “Experimental Procedures” and is shown in Fig. 6.

![Figure 5](image)

**Figure 5.** Localization of a differentiation-dependent DNA-protein complex. A, EMSA of 32P-labeled IGF-2 P3 DNA fragment 2 (–1090/–656) (Table I) with nuclear proteins prepared from CaCo2 cells on either day 5 (lane 1) or day 10 (lane 2) of culture. Complex a is seen only in day-5 samples. All other complexes are common to day-5 and day-10 samples. Band e is free 32P-labeled probe. B, EMSA with 32P-labeled ds oligonucleotide probe (–1090/–1065) in the presence of nuclear proteins from CaCo2 cells on different days of culture in the presence or absence of excess unlabeled probe. Autoradiograph of EMSA with nuclear protein samples from days 5–14 (lanes 2–4) using the radiolabeled 25-bp oligonucleotide probe in the presence (lane 1) or absence (lanes 2–4) of a 100-fold excess of unlabeled homologous probe is shown. Lane 1, D5 sample in the presence of 100-fold excess of unlabeled ds-25 bp probe; lane 2, D5 (50 μg) nuclear protein; lane 3, D10 (50 μg) nuclear protein; lane 4, D14 (50 μg) nuclear protein. Complex a was prominent in D5 samples (lane 2), whereas it was significantly reduced in D10 samples (lane 3). Complex a was minimally observed in day 14 samples (lane 4). The binding of proteins in day-5 samples was specific and completely displaced by excess unlabeled probe, as shown in lane 1.

![Table I](image)

**Table I**

| DNA Probe Restriction sites Probe IGF-2 P3 position |
|-----------------|-----------------|-----------------|-----------------|
| HUP3-luc vector | HPA1/HINF1 | 257 | 1091/1115 |
| HUP3-A1-luc | HINF1 | 434 | 1090/656 |
| HUP3-A5-luc | HINF1 | 203 | 656/453 |
| HUP3-A6-luc | HINF1/Xba1 | 165 | 453/288 |

Since DNA footprinting studies also suggested the presence...
of a putative binding site within the −1090/−1065 segment of the P3 promoter, we focused on identifying the core binding element within this region by mutation analysis of the −1090/−1065 EMSA probe. Mutation of the 25-bp −1090/−1065 EMSA probe at the 5′ end (between −1090 and −1086) or at the 3′ end (between −1075 and −1065) had no effect on the binding of the mutant probes to day-5 nuclear proteins (data not shown). However, mutations between −1086 and −1075 had significant effects on the binding to day-5 samples (Fig. 7).

Binding of day-5 samples to mutants M-1 and M-4 was slightly reduced by 20%; binding was significantly decreased by 40 to 60% to mutants M2, M3, and M5. Since the mutations in M2, M3, and M5 overlapped within the region of −1084 and −1078, we mutated all 6 base pairs between this segment in mutant M6, which resulted in complete loss of binding (Fig. 7). The results with the mutant probes suggest that the core of the binding element is present within −1084 and −1078 as shown by the black bar above the WT sequence. The hatched bar below the WT sequence represents nt sequence of the DNA footprint, as shown in Fig. 6.

The characterization of the IGF-2 P3 promoter, a ds DNA fragment (−1164/−871) was generated and used in the DNase 1 footprinting assay, as described under "Experimental Procedures," in the presence of nuclear proteins from day-5 and day-10 CaCo2 cells. No footprint was visible with day-10 samples, whereas a specific footprint was visible with day-5 samples in two separate experiments. A representative autoradiograph of DNA footprint of a total of 2 autoradiographs from 2 experiments with day-5 samples is shown. A labeled DNA ladder was co-run with the DNase-treated samples as a size marker, and the nt sequence of the DNase-protected site was determined by Maxam and Gilbert G + A track method, as published previously (31, 35). A 14-bp region (−1084/−1070) with the indicated sequence was specifically protected from DNase 1 digestion (containing the putative P3-D binding site) in the presence of day-5 samples (lanes 1 and 2). Lanes 3 and 4, DNA samples run in the absence of nuclear proteins. The size of the DNA markers that were co-run with the samples is indicated on the right-hand side with arrows. The 5′/3′ direction of the DNA probe, used in the footprint assays, is indicated by the arrows facing up and down. On the left, the nt sequence of the DNA footprint is shown.

FIG. 6. DNA footprint of a distal fragment of IGF-2-P3 promoter. A ds DNA fragment (−1164/−871) was generated and used in the DNase 1 footprinting assay, as described under "Experimental Procedures," in the presence of nuclear proteins from day-5 and day-10 CaCo2 cells. No footprint was visible with day-10 samples, whereas a specific footprint was visible with day-5 samples in two separate experiments. A representative autoradiograph of DNA footprint of a total of 2 autoradiographs from 2 experiments with day-5 samples is shown. A labeled DNA ladder was co-run with the DNase-treated samples as a size marker, and the nt sequence of the DNase-protected site was determined by Maxam and Gilbert G + A track method, as published previously (31, 35). A 14-bp region (−1084/−1070) with the indicated sequence was specifically protected from DNase 1 digestion (containing the putative P3-D binding site) in the presence of day-5 samples (lanes 1 and 2). Lanes 3 and 4, DNA samples run in the absence of nuclear proteins. The size of the DNA markers that were co-run with the samples is indicated on the right-hand side with arrows. The 5′/3′ direction of the DNA probe, used in the footprint assays, is indicated by the arrows facing up and down. On the left, the nt sequence of the DNA footprint is shown.

Fig. 7. EMSA with WT and mutant (M1-M6) probes. EMSA was conducted with a 25-bp ds oligonucleotide probe (−1090/−1065) that had either a WT sequence or had mutated sequences (underlined and bold in M1-M6 probes) in the presence of nuclear samples from day-5 CaCo2 cells. An autoradiograph of the resulting EMSAs with WT or M1-M6 probes is shown in the top panel. A single band of oligos bound to day-5 nuclear proteins was seen with WT and mutant probes other than with the M6 probe; the pattern of binding was identical to that seen in Fig. 5B with the WT probe. The relative density of the WT band was arbitrarily assigned a 100% value, and the relative density of the bands in the presence of M1-M6 probes is presented as a percent of that measured with the WT probe and is shown as bar graphs. Lanes 1–7, relative density of binding to WT, M1, M2, M3, M4, M5, and M6 probes, respectively. The data are from a representative experiment of two similar experiments. That the binding to the probe was completely abolished on mutation of six nt (M6 probe) (lane 7) suggests the core of the binding element is present between −1084 and −1078 as shown by the black bar above the WT sequence. The hatched bar below the WT sequence represents nt sequence of the DNA footprint, as shown in Fig. 6.
The underlined sequences represent either the 25 base pairs containing the wild-type P3-D element or its mutant. The restriction sites at the ends are also underlined.

with the known cis elements for various transcriptional factors. The GAGGGC sequence may thus represent a novel cis element (arbitrarily named P3-D) that specifically and differentially binds nuclear proteins from proliferating CaCo2 cells.

To confirm a regulatory function of the identified regulatory element within the distal DNA segment of P3 promoter, we used either the WT or mutant P3-D HUPS-A6-luc vector in transfection studies, as described under “Experimental Procedures.” The results with the WT and mutant P3-D HUPS-A6-luc vectors is presented in Table II; the activity of the full-length HUPS-luc vector is presented for comparison. As can be seen from the table, the presence of the distal P3 promoter segment, containing the WT P3-D element, significantly reduced the transcriptional activity of the core HUPS-A6 promoter by ~80–90%, and the activity was similar to that of the full-length HUPS promoter. On the other hand, transcriptional activity of mutant P3-D HUPS-A6-luc vector, which was mutated at the P3-D element, was reduced only slightly compared with the activity of the parent HUPS-A6-luc vector. Importantly, transcriptional activity of the WT P3-D HUPS-A6 promoter mimicked the activity of the full-length HUPS vector and was similarly down-regulated in day-7 samples. These results confirm the functional significance of the P3-D element and once again suggest a negative role of the P3-D element in regulating the transcriptional activity of the P3 promoter in a cell density-dependent manner.

**DISCUSSION**

In a previous study, we could show by transient transfection studies that transcription of the P3 promoter of the IGF-2 gene is dependent on the growth phase of CaCo2 cells. Transcription was significantly up-regulated during logarithmic growth of the cells and rapidly declined in confluent cells undergoing differentiation (19). In the present study, we show that the transcriptional activity of the endogenous IGF-2 gene is identical to that of the exogenously transfected IGF-2 P3 promoter. By applying nuclear run-on assays at different stages of CaCo2 cell growth it was confirmed that the transcriptional activity of the endogenous IGF-2 P3 promoter was significantly up-regulated during the proliferative phase of cell growth (days 4–6) followed by a steep decline in transcriptional activity during the differentiation phase (days 7–11) (Fig. 2). This confirms our hypothesis that the endogenous IGF-2 levels in CaCo2 cells are regulated mainly at the transcriptional level in a cell density and differentiation-dependent manner.

Several functional regulatory elements are present in the proximal segment (~238/140) of the human IGF-2 P3 promoter (32). P3 contains a TATA-box and a CAAT-box sequence, and both are bound by their cognate factors. Furthermore, several Sp1 sites are present in the proximal promoter, which is very GC-rich. Other zinc finger transcription factors such as the early growth response proteins Egr-1 and Egr-2 can also bind to multiple sites in the proximal promoter, thereby activating transcription (20, 32). Interestingly, the Wilms’ tumor WT-1 gene product, a tumor suppressor protein, also recognizes the Egr binding sites in the proximal promoter and suppresses the transcriptional activity of the IGF-2 P3 promoter by binding to Egr-IWT-1 consensus sequences (33). The absence of WT-1 is associated with overexpression of IGF-2 in Wilms’ tumor (20). Other transcription factors that bind the proximal segment of the P3 promoter downstream of position ~288 include p53 (34), the P3–4 cis element-binding proteins (35, 36), and AP2 (22, 31) (reviewed in Ref. 37).

Transcription factors and cis elements that may be involved in the transcriptional regulation of the P3 promoter in human colon cancer cells have yet to be identified. In the current study we observed that the distal segment of the P3 promoter, upstream of nucleotide ~1048, was required for the cell density and differentiation-dependent regulation of IGF-2 transcription. Truncated promoter-luc constructs that lacked the distal segment 5’ of ~1048 did not reveal any differentiation-dependent down-regulation of the P3 promoter in CaCo2 cells. Additionally, down-regulation cannot be caused by the known tumor suppressor gene products because CaCo2 cells lack both wtP53 (38) and WT-1 proteins. Still the transcriptional activity of the P3 promoter was significantly down-regulated in a cell density-dependent manner. It thus appears unlikely that potential transcription factors identified to date that bind the proximal segment (~288/140) of the P3 promoter play a role in the density/differentiation-dependent down-regulation of the transcriptional activity of IGF-2 P3 promoter in CaCo2 cells. Instead, the results of the present study with transient transfection assays of truncated IGF-2 P3 promoter-luc vectors suggest the novel possibility that the distal segment of the P3 promoter contains cis elements that are involved in suppressing the transcriptional activity of IGF-2 P3 promoter in a cell density-dependent manner. An important finding was that the transcriptional activity of the truncated P3 promoter that lacks the distal segment (~1091 to ~1048) was enhanced by severalfold at all phases of cell growth. It thus appears likely that promoter sequences between ~1091 and ~1048 contain cis elements, which in the presence of specific transcription factors, confer regulated differentiation-dependent transcription activity to the promoter.

The promoter segments between ~1048 and ~515 appeared to lack any significant regulatory elements, since further tran-

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2 B. Dai, P. H. Wu, E. Holthuizen, and P. Singh, unpublished data.
of native transcription factors in colon cancer cells is speculated dependent manner in CaCo2 cells. Lack and/or overexpression of native transcription factors as determined with the help of the gene-

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