The antiproliferative activities of the USF proteins and the frequent loss of USF function in cancer cells suggest a role for these ubiquitous transcription factors in tumor suppression. However, the cellular targets that mediate the effects of USF on cellular proliferation and transformation remain uncharacterized. IGF2R, with multiple functions in both normal growth and cancer, was investigated here as a possible USF target in both nontumorigenic and tumorigenic breast cell lines. The 5′-flanking sequences of the human IGF2R gene contain multiple, highly conserved E boxes almost identical to the consensus USF DNA-binding sequence. These E boxes were found to be essential for IGF2R promoter activity in the nontumorigenic mammary epithelial cell line MCF-10A. USF1 and USF2 bound the IGF2R promoter in vitro, and both USF1 and USF2, but not c-Myc, were present within the IGF2R promoter-associated chromatin in vivo. Overexpressed USF2, but not USF1, transactivated the IGF2R promoter, and IGF2R mRNA was markedly decreased by expression of a USF-specific dominant negative mutant, identifying IGF2R as a USF target. IGF2R promoter-driven expression was USF-independent in both MCF-7 and MDA-MB-231 breast cancer cell lines, suggesting that a defect in USF function may contribute to down-regulation of IGF2R expression in cancer cells.

USF1 and USF2 are ubiquitously expressed basic helix-loop-helix-leucine zipper (bHLHZip) transcription factors that share nearly identical C-terminal bHLHZip motifs and bind as both homodimers and heterodimers to DNA sequences centered on CAC(G/A)TG. All USF dimers have identical affinities for the 12-bp consensus binding site, GGTCACGTGACC (1). The USF DNA binding specificity for the central 6 bp, but not the full 12-bp preference, is shared with several other bHLHZip transcription factors, including c-Myc (2–4).

Whereas c-Myc promotes cellular proliferation and transformation, overexpressed USF has been shown to suppress these processes (5). In focus formation assays, cotransfection of USF1 or USF2 prevented transformation of primary rat embryonic fibroblasts by c-Myc and activated Ras (5). USF1 blocked c-Myc-dependent but not E1A-dependent transformation, consistent with a mechanism in which USF1 competes with Myc for DNA-binding sites. In contrast to USF1, USF2 displayed more general growth suppression properties, inhibiting both c-Myc and E1A-mediated transformation and also blocking HeLa cell colony formation (5).

The ability of USF to antagonize the transforming potential of c-Myc together with the similar binding preferences of Myc and USF suggest that these factors share a subset of common targets, and indeed, c-Myc and USF bind to some common promoters in vivo (6, 7). The degree to which the DNA binding specificity bears on target selection and biological activity by USF and Myc is not known but is likely to depend on the promoter and cell context. USF1 and USF2 transcriptional and antiproliferative activities have been shown to be cell context-dependent. In cells derived from normal breast epithelium, both endogenous and exogenous USF1 and USF2 strongly activated transcription from artificial promoters containing USF-binding sites (8). In contrast, USF antiproliferative activities and transcriptional activity at artificial promoters were impaired in several tumorigenic cell lines. In Saos-2 osteosarcoma cells, USF failed to activate transcription, and exogenous USF did not inhibit cellular proliferation (9). Endogenous USF did not transactivate an artificial promoter in several breast cancer cell lines, even though the USF protein levels and in vitro DNA-binding properties were the same as in normal breast epithelial cells (8). In three of six breast cancer cell lines, both endogenous USF1 and USF2 were inactive, whereas in the other three cell lines, overexpressed USF1, but not USF2, activated the USF-responsive reporter. This latter situation parallels the case in HeLa cells, where overexpression of USF was required to observe transcriptional or antiproliferative activities (5, 10).

Besides the correspondence between loss of USF activity and tumorigenic status of the cells studied so far (8, 9), there is evidence for a role of USF in regulating growth in normal cells. In particular, mice lacking the Usf1 and Usf2 genes result in early embryonic lethality (11). Analysis of mammary tissue in postpartum Usf2−/− females revealed that USF2 plays a key role in normal mammary cell differentiation and lactation. Phosphorylation of USF1 by p38 kinase is a key step in the increased transcription of the tyrosinase gene in response to stress (12).

The role of USF in normal growth regulation, together with the loss of USF activity in cancer cells, suggests that USF activity loss is linked to tumorigenesis and that deregulation of
USF target genes in cancer cells contributes to the tumorigenic phenotype. USF has been implicated in the regulation of several genes involved in tumorigenesis, including cyclin B1 (13), Brca2 (14), adenomatous polyposis coli (15), and cathepsin D (16). Here we report a key role for USF in expression of the mannose 6-phosphate/insulin-like growth factor 2 receptor (IGF2R) gene in normal mammary epithelial cells. Three near-consensus USF sites within the promoter of the IGF2R gene indicated that it would be transcriptionally regulated by USF, and the growth suppressive function of the IGF2R gene product was consistent with a role for USF in maintaining its expression. The IGF2R gene encodes a 2491-amino acid multifunctional membrane receptor that plays a vital role during embryonic growth (17). IGF2R binds the growth factor IGF2 and is essential for promoter activity in liver cells (33). In hepatic transgenic experiments, most mice lacking IGF2R die shortly after birth, a phenotype rescued by targeted deletion of either IGF2R or its signaling receptor, IGF1R (17, 25). A number of studies have implicated IGF2R loss in carcinogenesis. Mutations have been identified in the IGF2R gene in many cancers (26–30), and tumor cell growth is modified in IGF2R expression (31, 32).

Outside of imprinting, the mechanisms controlling IGF2R levels and the contribution of transcriptional regulation to IGF2R expression are not well understood. Deletion analysis of the mouse Igf2r gene identified a 5′ region with two E boxes essential for promoter activity in liver cells (33). In hepatic stellate cells, Igf2r expression was down-regulated by a repressor binding at an E box of the mouse promoter (34). However, the identity of this repressor and other transcription factors of the IGF2R promoter are not known. Here we show that the USF proteins bind to multiple E boxes in the human Igf2r promoter. In addition, both A-USF and A-Max were shown to bind to multiple E boxes in the human IGF2R promoter and that these E boxes are essential for promoter activity. The role of USF at this promoter was investigated in a cell line with robust endogenous USF activity, the nontumorigenic human breast epithelial cell line MCF-10A. These experiments demonstrate that the human Igf2r gene is a target of USF2, but not of c-Myc. However, the IGF2R promoter is unresponsive to USF in two breast cancer cell lines, providing further evidence for disrupted USF transcriptional activity in cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MCF-10A cells were grown in monolayers at 37 °C and 5% CO2 in 50% Dulbecco’s modified Eagle’s medium, 50% F-12 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml insulin, 100 ng/ml cholaera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant human epidermal growth factor, and 1.05 mM CaCl2. MCF-7 and MDA-MB-231 cells were cultured in monolayers at 37 °C and 5% CO2 in 50% Dulbecco’s modified Eagle’s medium, 50% F-12 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Plasmids**—PRL-SV40 (Promega), pRSV-Luc, pMLLuc, pUSMLLuc, pSG424, and pRC/CMV566, and expression vectors for A-USF, A-Max, A-Myc, 10 μg/ml luciferase, were all transfected into the cells to give a final concentration of 10 μg/ml for each construct. The cells were cross-linked for 20 min by addition of formaldehyde to a final concentration of 1%. The cross-linking reaction was quenched by adding 100 mM glycine. The cross-linked cells were collected by centrifugation at 1000 × g for 5 min. The cell pellets were washed with phosphate-buffered saline, scraped from the dish, and then washed again in phosphate-buffered saline containing protease inhibitor mixture (10 μM/ml, Sigma P8340) and 10 μM phenylmethylsulfonyl fluoride. The resuspended cellular pellet was incubated on ice for 30–60 min in a solution of protease inhibitors (5 mM PIPES, pH 8.0, 0.05% KCl, 0.5% Nonidet P-40) with proteinase inhibitors and centrifuged for 20 min in a microfuge. The nuclear pellet was resuspended in nuclease-free buffer (50 mM Tris, pH 8, 10 mM EDTA, 1% SDS) with protease inhibitors and sonicated to obtain DNA fragments of 0.5–1 kb. After a spin of 14,000 rpm for 15 min at 4 °C, the chromatin extract supernatant was...
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FIG. 1. The IGF2R promoter contains multiple highly conserved E boxes and drives strong transcription. A, nucleotide sequences of the human (X53699; gi:1006660 (48)), opossum (AF344425; gi:18478894), and mouse (U26348; gi973191) IGF2R gene promoter regions are aligned. USF-type E boxes A–C are in boldface and a fourth E box (B’) is in italics. Vertical arrows mark the transcription start sites for the mouse promoter (35), and the transcription start site is shaded. The 183-bp promoter region used for footprinting and transfection experiments is underlined. B, MCF-10A cells were transfected with 1 μg of pABLCuc or, for comparison, the artificial reporters pMLLuc or pU3MLLuc. Luciferase measurements from a minimum of three independent transfections were averaged, with the S.D. represented by the error bar.

A treated with pre-blocked staphylococcus A cells (6) for 15 min at 4 °C and centrifuged again (14,000 rpm for 15 min). Anti-USP1 (C-20, sc229), anti-USP2 (N-18, sc861), or anti-Myc (N-262, sc764) antibodies (Santa Cruz Biotechnology) were added to this pre-cleared chromatin extract and incubated overnight at 4 °C with gentle rotation. A reaction with no added antibodies was carried out in parallel. Each reaction was incubated with 10 μl of pre-blocked staphylococcus A cells for 15 min at 4 °C followed by centrifugation at 14,000 rpm for 15 min and extensive washing of the pellet as described (7). Supernatant of the mock reaction served as input and was processed in parallel with the sera treated with pre-blocked staphylococcus A cells (6) for 15 min at 4 °C and centrifuged again (14,000 rpm for 15 min). Anti-USP1 (C-20, sc229), anti-USP2 (N-18, sc861), or anti-Myc (N-262, sc764) antibodies (Santa Cruz Biotechnology) were added to this pre-cleared chromatin extract and incubated overnight at 4 °C with gentle rotation. A reaction with no added antibodies was carried out in parallel. Each reaction was incubated with 10 μl of pre-blocked staphylococcus A cells for 15 min at 4 °C followed by centrifugation at 14,000 rpm for 15 min and extensive washing of the pellet as described (7). Supernatant of the mock reaction served as input and was processed in parallel with the

B

USF-binding Sites Are Essential for IGF2R Promoter Function in Human Breast Epithelial Cells—The proximal human IGF2R promoter contains three E boxes that are near-consensus matches for the optimal USF-binding site. A comparison of these three E boxes (boxes A–C, Fig. 1A) with the 12-bp consensus binding site for transcription factor USF (5’S-CCATCGACACAGCTTGC-3’) reveals that each contains not only the central Ca/C/T/GT motif shared by USF, Myc, and TFE3 bHLHZip family members (1, 2, 6, 41) but also flanking sequences that favor USF binding (1, 6, 7) (Table I). In particular, the T and A flanking residues (boldface, Table I) favor strong binding by USF but not c-Myc (1, 2, 6, 7). Also present in the proximal IGF2R promoter is a fourth E box (site B’) lacking the central USF motif and potential binding sites for SP1 family members or other transcription factors that recognize GC-rich sequences. All four E boxes are well conserved in the human, opossum, and mouse IGF2R promoters, including the precise spacing between E boxes B’ and B (Fig. 1A).

An earlier investigation of the mouse Igf2r promoter (33) revealed multiple transcription start sites (shown in Fig. 1A) and identified the E box containing proximal region of the promoter as the minimal region for driving transcription. Two E boxes corresponding to sites B and C (Fig. 1A) were found...
essential for strong promoter activity (33). We inserted a 183-bp segment of the human IGF2R promoter containing the three USF-type E boxes into a plasmid upstream of luciferase-encoding sequences. The luciferase activity of this reporter, pABCLuc, was measured after transfection into MCF-10A cells and compared with the pU3MLLuc reporter containing three copies of the adenovirus major late (ML) USF-binding site (Table I) upstream of the adenovirus ML TATA box and initiator elements (10). As observed previously in the MCF-10A cell line (8), pU3MLLuc was 24-fold more active than the corresponding pMLLuc reporter lacking the USF sites (Fig. 1B). However, luciferase expression driven by the human IGF2R promoter in pABC promoter at 21 times greater than that of the composite promoter in pU3MLLuc (Fig. 1B).

To determine whether the USF sites were important for activity of the human IGF2R promoter, reporter constructs with one or more mutated USF sites were constructed. Destruction of any one of these three E boxes did not significantly affect luciferase expression (Fig. 2), but mutation of the two most 5' or the two most 3' sites decreased activity to 10 and 28% of wild type, respectively. A mutant promoter leaving intact only the central USF-binding site, box B, retained ~60% of the activity of the wild-type promoter, indicating a more important role of this central USF-binding site. Finally, combined mutation of all three of the USF-type E boxes (pabcLuc reporter) eliminated this central USF-binding site. To determine whether exogenous USF1 or USF2 could enhance IGF2R promoter activity, expression vectors for these factors were cotransfected with the pABC promoter in MCF-10A cells (Fig. 4A). Both the USF proteins were overexpressed (Fig. 4B) and capable of binding to an oligonucleotide containing a USF-binding site as determined by electrophoretic mobility shift assay of cell lysates (data not shown). Overexpressed USF2, but not USF1, significantly enhanced expression driven by the IGF2R reporter. Although the stimulatory effect of USF2 was highly reproducible, the extent of stimulation ranged from 3- to 4-fold in some experiments up to more than 15-fold in others (compare Fig. 4, A and C). These results were consistent with involvement of USF2 in IGF2R gene transcription. In contrast, overexpressed USF1 reproducibly failed to stimulate activity of the pABC promoter in MCF-10A cells (Fig. 4A). To rule out squelching as an explanation for the inactivity of overexpressed USF1, a titration experiment was carried out. Independently of its expression level, USF1 failed to substantially increase luciferase activity when cotransfected with the WT IGF2R reporter gene, whereas exogenous USF2 transactivated the same reporter in a dose-dependent manner (Fig. 4C).

USF1 and USF2 have different transcriptional activation domains in their divergent N-terminal regions but share an activation domain, the USR, located adjacent to the DNA-binding motif in both proteins (10). In HeLa cells, the USR was found to be necessary and sufficient for activation of the adenovirus ML promoter by USF (10). The inability of USF1 to strongly activate the WT pABC promoter suggested that the USR might be dispensable for transcriptional activation of IGF2R by USF2. Consistent with this possibility, overexpression of a USF2 mutant lacking the USR, U2ΔUSR, stimulated IGF2R promoter activity to the same extent as wild-type USF2 (Fig. 4A). Together, these results suggest that recruitment of USF2-specific coactivators is essential for activity of the IGF2R promoter.

The bHLH zinc transcription factor c-Myc, as a heterodimer

| Site              | Sequence          |
|-------------------|-------------------|
| Human IGF1IR      |                   |
| Site A            | GTTCACTGAGCC      |
| Site B            | GTTCACTGGAGC      |
| Site C            | GTTCACTGGAGC      |
| Site D            | GTTCACTGGAGC      |
| Adenovirus ML     | GTTCACTGGAGC      |
| USF consensus     | GTTCACTGGAGC      |
| Myc/Max consensus | CACGTGG           |

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| Site              | Sequence          |
|-------------------|-------------------|
| Human IGF1IR      |                   |
| Site A            | GTTCACTGAGCC      |
| Site B            | GTTCACTGGAGC      |
| Site C            | GTTCACTGGAGC      |
| Site D            | GTTCACTGGAGC      |
| Adenovirus ML     | GTTCACTGGAGC      |
| USF consensus     | GTTCACTGGAGC      |
| Myc/Max consensus | CACGTGG           |

Table I

Sequence comparison of the human IGF2R promoter E boxes to the USF- and Myc-consensus binding sites and the adenovirus ML E box that binds both USF and c-Myc.
with Max, activates transcription of its target genes by binding to a 6-bp core DNA element that is shared with USF-binding sites. However, different sequences flanking the central bases are favored by Myc/Max and USF dimers (1, 6, 7) (Table I).

More importantly, USF and Myc have distinct biological roles, although their different activities appear to involve both common and exclusive targets. The growth-suppressive role of IGF2R makes it a candidate USF target perhaps not shared with Myc. Moreover, the E boxes essential for the IGF2R promoter activity (Fig. 2) are favorable for USF but not c-Myc binding. Consistent with these premises, overexpressed c-Myc failed to stimulate luciferase activity driven by the WT IGF2R promoter in MCF-10A cells (Fig. 4A).

**Endogenous USF Activates an IGF2R Reporter in MCF-10A Cells**—To determine whether not only exogenous but also endogenous USF can activate the WT IGF2R reporter in mammary epithelial cells, expression vectors for altered forms of USF2 that act as dominant suppressive mutants were transfected into MCF-10A cells along with the pABCLuc reporter. Overexpressed USF2 failed to decrease pABCLuc reporter activity by only about 40%, whereas overexpressed USF2ΔB was slightly stimulatory (data not shown). The ineffectiveness of these mutants is un-

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**Fig. 2.** The three USF-type E boxes are essential for IGF2R promoter activity in MCF-10A cells. *A,* schematic representation of the promoter constructs. Substitution mutations (designated by lowercase letters), replacing the central 6 bp of the USF sites by the sequence AGATCT, were introduced into the pABCLuc reporter. For the pCBALuc reporter, the 183-bp promoter fragment was inserted in the reverse orientation upstream of *Renilla* luciferase encoding sequences and compared with the corresponding forward orientation IGF2R *Renilla* reporter. *B,* firefly or *Renilla* luciferase measurements from a minimum of three independent transfections were averaged and normalized to WT promoter activity.

**Fig. 3.** USF binds to the IGF2R promoter. DNase I footprinting was carried out with purified bacterially expressed recombinant USF1 (rUSF1) (A), USF2 (rUSF2) (B), or MCF-10A nuclear extract (NE) (C) and a probe spanning 183 bp of the human IGF2R promoter (see Fig. 1). *1st lane,* binding buffer only; *2nd and 3rd lanes,* increasing amounts of USF or nuclear extract. Lanes G, G-specific chemical sequencing reaction of the probe. The probe sequence with the locations of the 8-bp core USF-binding sites in boldface letters and the protected regions indicated are shown for recombinant USF1 and USF2 (D) and for MCF-10A nuclear extract (E). DNase I-hypersensitive sites are marked by vertical arrows, with the size reflecting the extent of cleavage enhancement.
understandable if the stability of endogenous USF complexes on promoter DNA is comparable with or greater than the stability of the heterodimers formed between native USFs and USF2\textsubscript{N} or USF2\textsubscript{B}. The slight stimulatory effect of USF2\textsubscript{B} can also be explained if USF recruitment to the \textit{IGF2R} promoter is triggered both by specific DNA contacts and by specific contacts with other DNA-bound proteins.

To more effectively prevent binding of endogenous USF1 and USF2 to the \textit{IGF2R} promoter, a more potent dominant negative mutant of USF, A-USF (9, 42), was employed. A-USF was derived from USF1 by deleting all N-terminal activation domains and substituting acidic amino acids for the stretch of basic amino acids that normally contacts DNA in the native USF dimers. Because A-USF retains the intact HLHZip domain, it is able to dimerize with endogenous USF1 and USF2 and prevent USF binding to DNA. Dimers of the native USFs with A-USF are more stable than the DNA-bound native dimers (42), so USF is removed from its target DNA with only modest overexpression of A-USF (9). When A-USF was introduced into MCF-10A cells, pABCLuc reporter activity was markedly suppressed, indicating an important contribution of endogenous USF to expression driven by the \textit{IGF2R} promoter (Fig. 5A). The corresponding dominant negative mutant for Myc, A-Max, in which the basic DNA binding domain of the Myc dimerization partner Max is replaced by acidic amino acids, was employed to interfere with any endogenous Myc transactivation of the \textit{IGF2R} promoter. Although A-Max was expressed to similar levels as A-USF (Fig. 5B), it had only a minimal effect on the \textit{IGF2R} reporter activity (Fig. 5A). This result, consistent with the failure of overexpressed c-Myc to transactivate (Fig. 4), further established the specificity of the \textit{IGF2R} promoter activation by USF.

**Prominent Role of USF in the Control of Endogenous IGF2R Expression**—The strong USF dependence of transfected IGF2R reporters suggested that the endogenous \textit{IGF2R} promoter in MCF-10A cells was also under the control of USF. To investigate this possibility, we next tested whether USF was bound to the cellular \textit{IGF2R} promoter \textit{in vivo}. Proteins in MCF-10A cells were cross-linked, and chromatin was sheared and immunoprecipitated by antibodies against USF1, USF2, or c-Myc. As illustrated in Fig. 6A, the \textit{IGF2R} promoter encompassing the USF sites was amplified by PCR of DNA coprecipitated by either USF1 or USF2 antibodies, demonstrating that both USF family members interacted with the \textit{IGF2R} promoter \textit{in vivo}. Negative controls for this analysis included PCR of the topoisomerase II promoter and the CDK4 exon 8 regions, both of which lacked USF-binding sites and did not coprecipitate with the USF-containing chromatin (data not shown). A region of somerase II promoter and the CDK4 exon 8 regions, both of which lacked USF-binding sites and did not coprecipitate with the USF-containing chromatin (data not shown). A region of

**FIG. 4.** USF2 transactivates the IGF2R promoter in nontumorigenic breast epithelial cells. A, relative values of luciferase reporter activities in MCF-10A cells. Cells were transfected with 1 \(\mu\)g of pABCLuc and 1 \(\mu\)g of expression vectors for USF1, USF2, USF2\textsubscript{USR}, c-Myc, or the control vector, pSG424, as indicated. pRLSV40, a plasmid containing the SV40 promoter driving Renilla luciferase expression, was added as an internal control for each transfection, and each experimental firefly luciferase measurement was normalized to the Renilla luciferase value. B, representative Western blots for whole cell MCF-10A extracts in the presence or absence of expression vectors for USF1, USF2, or c-Myc. C, relative luciferase activities for increasing quantities of overexpressed USF1 or USF2 in cotransfection experiments with the pABCLuc reporter.

**FIG. 5.** Transcriptional activity of endogenous USF in MCF-10A cells at the IGF2R promoter. A, MCF-10A cells were transfected with pABCLuc and either 0.01, 0.04, or 0.10 \(\mu\)g of expression vectors for A-USF, A-Max, or the corresponding empty expression vector, as indicated. Three or more independent sets of transfections were carried out for each plasmid amount. Luciferase activities for each experiment were normalized to the empty vector readings (plotted as 1.0) and then averaged. Renilla luciferase expression from 0.02 \(\mu\)g of transfected pRLSV40 served as an internal control. B, representative Western blot of whole cell lysates showing relative expression levels of A-USF and A-Max in MCF-10A cells transfected with 0.010, 0.04, and 0.1 \(\mu\)g of expression vector for each construct. The epitope-tagged A-USF and A-Max were detected with an antibody against HA.
the hTERT second intron, which contains approximately 40 USF-type E boxes (43), provided an even more effective control. In contrast to USF interaction with the IGF2R promoter, no USF1 or USF2 was detectable by ChIP within the hTERT intronic region, suggesting that USF binds to only a subset of its cognate sites within the genome (Fig. 6A).

ChIP with antibodies specific to c-Myc did not precipitate detectable amounts of the IGF2R promoter in MCF-10A cells, suggesting that c-Myc was not present at the IGF2R promoter. As a positive control, a known c-Myc target, CDK4, was employed (44, Fig. 6B). c-Myc was present on the CDK4 promoter to the small extent expected in nontumorigenic cells (8). ChIP with c-Myc antibodies was repeated in the MCF-7 breast cancer cell line, where c-Myc is more abundant (8), to rule out a problem of detection sensitivity. This analysis clearly demonstrated the presence of c-Myc at the CDK4 promoter. In contrast, the IGF2R promoter was not bound by c-Myc in MCF-7 cells (Fig. 6B), indicating that IGF2R was not a direct target of c-Myc in either of the mammary epithelial cell lines tested. However, both USF1 and USF2 were found associated with the IGF2R promoter chromatin in MCF-7 as well as in MCF-10A cells (Fig. 6A).

The presence of USF at the cellular IGF2R promoter, along with the effects of wild-type and mutant USF proteins on IGF2R reporter activity, pointed to the IGF2R gene as a direct target of USF2. To demonstrate directly that endogenous USF indeed transactivated the cellular IGF2R gene, we determined the consequences of expressing the A-USF dominant negative mutant on endogenous levels of IGF2R mRNA in MCF-10A cells. Cells were transfected with either A-USF or the corresponding empty vector, together with a selectable cell surface marker, truncated CD4. The CD4-expressing cells were then separated from nontransfected cells on a column of magnetic beads linked to CD4 antibodies (Fig. 7). A Western blot confirmed strong A-USF expression and enrichment in the positively selected transfected cells (data not shown). The relative levels of IGF2R messages in vector- versus A-USF-transfected cells were determined by quantitative RT-PCR of total RNA (Fig. 7B). When compared with a reference gene, RPLP0, whose message levels were the same in cells transfected by either A-USF or empty vector, the MCF-10A IGF2R RNA level decreased by ~5-fold in the presence of A-USF. A similar decrease in IGF2R/RPLP0 message ratio was determined using RNA isolated from cells separated by fluorescence-activated cell sorting on the basis of green fluorescence after cotransfection of A-USF with a green fluorescent protein expression vector (data not shown). These results demonstrated a prominent role of endogenous USF in controlling transcription of the IGF2R gene in MCF-10A cells.

USF Does Not Transactivate the IGF2R Promoter in Breast Cancer Cell Lines—Transcriptional activity of USF at the USF-responsive composite promoter in pU3MLLuc was earlier found to be impaired in a variety of cell lines derived from human tumors (8). However, USF transcriptional activities in normal versus breast cancer cells have not been examined at the cellular promoter clearly identified as a USF target. We tested the ability of USF to transactivate the IGF2R promoter in two different breast cancer cell lines, MCF-7 and MDA-MB-231, where both overexpressed USF1 and USF2 failed to activate the artificial pU3MLLuc reporter (8). In contrast to USF2 transcriptional activity in MCF-10A (Fig. 4), overexpressed USF2 did not activate the IGF2R-driven pABCLuc reporter in MCF-7 cells (Fig. 8A). Under these conditions, USF1 overexpression had only a slight stimulatory effect, whereas c-Myc
overexpression was slightly inhibitory. Western analysis confirmed that all three transcription factors were well expressed in MCF-7 cells (Fig. 8B). The failure of exogenous USF2 to transactivate, also observed for the MDA-MB-231 cell line (data not shown), suggests that differences in the abundance or activity of essential USF2 cofactors account for the transcriptional inactivity of USF2 in breast cancer cell lines.

A comparison of the activities of promoter mutants revealed that IGF2R promoter activity in MCF-7 cells was dependent on the USF-type E boxes (Fig. 2A and Fig. 8A). However, the relative contributions of the different E boxes to overall promoter activity were significantly different in MCF-7 cells than in MCF-10A cells (compare Fig. 8C with Fig. 2B). In particular, site A was important for promoter activity in MCF7 cells (Fig. 8C, 1st lane). The differences in E box contributions to activity for the two cell lines were not reflected by patterns of proteins bound to the wild-type IGF2R promoter fragment in electrophoretic mobility shift assays. In both MCF7 and MCF-10A cells, proteins from nuclear extract displayed identical binding patterns at the IGF2R promoter (data not shown).

In contrast to the absence of USF transcriptional activity, ChIP in MCF-7 cells revealed that both endogenous USF1 and USF2 were bound to the native chromosomal IGF2R promoter (Fig. 6). To determine whether endogenous USF contributed to the activity of the IGF2R promoter in MCF7 and MDA-MB-231 cells, the dominant negative mutant, A-USF, or the corresponding empty vector, was cotransfected with the pABCLuc reporter (Fig. 9). A-USF was well expressed in both cell lines (Fig. 9C) but did not affect IGF2R reporter gene activity, indicating that, despite its presence on the gene, endogenous USF in these breast cancer cell lines was...
unable to activate the IGF2R promoter. Thus, the previously observed loss of USF transcriptional function in cancer cells also holds for true cellular targets, including IGF2R. A-Max overexpression in the same breast cancer cell lines only slightly affected IGF2R-driven transcription (Fig. 9, A and B), similar to the small effect observed in MCF-10A cells (Fig. 5A). This result was consistent with the absence of bound c-Myc on the IGF2R promoter in MCF7 cells in vivo (Fig. 6B) and demonstrated that the IGF2R promoter was not a target of c-Myc even in these breast cancer cell lines with both impaired USF function and high c-Myc levels.

**DISCUSSION**

We have investigated expression driven by the proximal human IGF2R promoter in normal and tumorigenic breast epithelial cells. Expression of a dominant negative mutant specific to USF family members, A-USF, suppressed IGF2R promoter activity in MCF-10A cells (Fig. 5) and markedly decreased IGF2R message levels (Fig. 7), demonstrating that this promoter was activated by endogenous USF and that the cellular IGF2R gene in MCF-10A cells was under USF control. Together with ChiP showing both USF1 and USF2 bound to the IGF2R promoter in vivo (Fig. 6), these results conclusively demonstrate a prominent role for USF in maintaining IGF2R expression.

The strength of the IGF2R promoter relative to composite pU3MLLuc promoter (Fig. 1) and the extensive DNase I footprints in MCF-10A nuclear extract (Fig. 3) are consistent with the idea that USF2 activates in the context of a highly stable multiprotein-DNA complex. Some of the interactions within the promoter-transcription factor complexes can be disrupted without abolishing all promoter activity. For example, the USF2 deletion mutant USF2ΔB transactivated the IGF2R promoter, even though it lacks the USF2 DNA-binding domain.

The question of whether USF2 alone or both USF1 and USF2 activates IGF2R transcription in MCF-10A cells remains unanswered. Both proteins were present at the IGF2R promoter in vivo as revealed by ChiP (Fig. 6), and USF1/USF2 heterodimers are the predominant species in extracts from most cell types, including the human breast cell lines investigated here (8). In MCF-10A cells, overexpressed USF2, but not USF1, transactivated the IGF2R promoter. This selectivity stems from more than DNA binding specificity, because USF1 and USF2 binding affinities for consensus and nonconsensus sites are identical in vitro (data not shown). Because the dominant negative mutant A-USF does not distinguish the contributions from USF1 and USF2, we cannot rule out limiting USF1-specific coactivators in MCF-10A cells as explaining differences in exogenous USF2 versus USF1 activation of the IGF2R promoter (Fig. 4). Nevertheless, robust activation by a USF2 mutant lacking the USR, the only defined activation domain shared by both USFs (10), suggested that the IGF2R gene was a target only of USF2, and not USF1. A second activation domain mapped in HeLa cells is encoded by USF2 exon 5, a region with little homology to USF1. However, our preliminary results indicate that the exon 5-encoded region of USF2 is also not responsible for USF2 activity in MCF-10A cells and that the USF2 activation domains are dependent on cell context (data not shown).

A role for USF2 activation domains alone is not inconsistent with the binding of heterodimers at the IGF2R promoter. We postulate that, within the promoter complex, USF2 activation domains are oriented to interact with coactivators, and USF1 is located away from the activation surface and plays a different role, for example, in anchoring the complex (Fig. 10). It is noteworthy that USF2 displays a stronger antiproliferative activity than USF1 (5), and an asymmetrical cross-regulation of the Usf1 and Usf2 genes limits the concentration of USF2 homodimers in proliferating cells (35). Thus, USF2 may specifically target genes that down-regulate growth, such as IGF2R.

In contrast to the effect of A-USF, the corresponding dominant negative mutant for the c-Myc protein, A-Max, had little effect on IGF2R promoter activity in any of the cell lines tested, and c-Myc was not bound to the IGF2R promoter in MCF-10A cells or even in MCF-7 cancer cells (Fig. 6), where Myc is overexpressed (8). The absence of Myc binding is consistent with the full 12-bp sequence of the IGF2R E boxes, which are optimal for USF but not c-Myc binding. The small degree of repression in cancer cell lines observed on expression of A-Max (Figs. 5 and 9) may be indirect or could result from transactivation in a small subpopulation of cells. Clearly, for the predominant population of asynchronous breast epithelial cells, USF is a critical transcriptional activator of the IGF2R gene, whereas c-Myc is not.

Despite the absence of USF activity, MCF7 cells retained E box-dependent IGF2R promoter activity. Given that USF and c-Myc do not transactivate the IGF2R promoter in MCF7 cells, an additional transcription factor is responsible for MCF-7 activity. Mutations of sites A and B had large effects, suggesting that in MCF-7 cells, a factor other than USF bound to and transactivated through either the core E box motif or shoulder of sites A and B (Fig. 8C). In contrast, mutation of site C had no effect on promoter activity in MCF-7 cells, indicating that site C may be a binding site for the (inactive) USF observed by ChiP (Fig. 6). Taken together, the results are consistent with occupation of IGF2R promoter in all three breast cell lines by similar sets of DNA binding factors (Fig. 10) and suggest that the drastic differences in activities among cell lines result from

**FIG. 10. Schematic representation of the transcription factors bound to the IGF2R promoter in MCF-10A versus MCF-7 cell lines, illustrating a model for different utilization of the USF-type E boxes in these cell lines.** Because USF1 and USF2 do not behave identically at the IGF2R promoter in MCF-10A cells, USF1/USF2 heterodimers are shown in a specific orientation with USF2 interacting with coactivator(s). The small circles represent DNA-bound transcription factors other than USF, such as Sp1, that bind to the IGF2R promoter. The arrow represents the major transcription start site. GTFs, general transcription factors. A, USF activates transcription in MCF-10A cells. All three USF-type E boxes may not be occupied by USF in MCF-10A cells, and the MCF-7-active-factor may also be present at the promoter in MCF-10A cells (not shown). B, USF is inactive but bound to the IGF2R promoter in MCF-7 cells at site C. An MCF-7-active-factor acts through site A in MCF-7 cells. Site B in MCF-7 cells may be bound by USF, the MCF-7 active factor, or another unknown factor.
the variations of USF coactivators or corepressors. Investigations to date clearly indicate the presence of several different underlying mechanisms of transcriptional inactivation of USF for different cancer cell lines (8).\(^3\) Note that any USF posttranslational modifications in these breast cell lines, although likely, are not revealed by differences in mobility of USFs on SDS-PAGE (8) or the ability of USF to bind DNA (8).

Previous studies of USF transcriptional impairment in cancer cells employed artificial promoters. We have now demonstrated loss of USF transcriptional activity in cancer cell lines at a cellular promoter that is clearly a USF target. Precise levels of IGF2R must be maintained by organisms, as even 2-fold differences in this protein affect growth (45). There is also much evidence to suggest that a decrease in IGF2R protein function contributes to uncontrolled cellular growth in cancer. A significant decrease in IGF2R protein levels has been noted for invasive breast cancers relative to normal adjacent tissue (46). Analysis of breast tumor samples indicated that IGF2R allelic alterations may be an early event in tumor formation (29), and a 2-fold differences in this protein affect growth (45). There is significant decrease in IGF2R protein levels has been noted for different cancer cell lines (8).\(^3\) Note that any USF posttranslational variations of USF coactivators or corepressors. Investigations to date clearly indicate the presence of several different USFs on SDS-PAGE (8) or the ability of USF to bind DNA (8).

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