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# Molecular Markers of Endometrial Epithelial Cell Mitogenesis Mediated by the Sp/Krüppel-Like Factor BTEB1

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## ABSTRACT

**Basic transcription element binding (BTEB1) protein is one of at least 20 Sp/KLF family members that function as transcriptional activators or repressors by binding to GC/GT-rich sequences within target genes to influence cellular homeostasis in mammals. Previously, we demonstrated that increased expression of BTEB1 in a human endometrial epithelial cell line Hec-1-A resulted in serum dependent-enhanced proliferation, which was accompanied by heightened expression of cell cycle- and growth-associated genes. In the present study, we examined the mechanism underlying the altered proliferative potential associated with BTEB1 by the identification of additional BTEB1 downstream gene targets and by the demonstration of BTEB1 transactivation of promoters for a number of growth-associated genes. Using mRNA differential display in the analysis of RNA populations from Hec-1-A sublines with high (4S, 9S) and low (2As, 3As) BTEB1 cellular content, we identified 10 distinct differentially expressed transcripts, nine of which had higher levels in S than in As sublines. The expression levels of two of these cDNAs, Axl receptor tyrosine kinase and mitosin, whose encoded products are implicated in cellular proliferation, were modestly induced by serum, albeit in a BTEB1-independent manner. Moreover, insulin-like growth factor-I, a mitogen present in serum, had no significant effect on their expression in either subline. In transient reporter assays, the basal activities of the Axl gene promoter and those for two other growth-regulatory genes, namely p21$^{WAF1}$ and IGFBP-2, were increased by serum and were significantly higher in 4S than in 2As lines. However, while BTEB1 and its ubiquitous family member Sp1 increased basal p21$^{WAF1}$ and IGFBP-2 transcription when added as expression constructs in the parental Hec-1-A cell line, only Sp1 activated Axl transcription, despite the presence in all three gene promoters of GC-enriched regions that presumably can bind BTEB1 and Sp1 with similar affinities. To elucidate intracellular signaling pathways that might involve BTEB1, inhibitors of specific kinase-dependent transducers were used in transient transfection assays involving the *IGFBP-2* gene promoter in 4S and 2As sublines. While inhibitors of the MAPK, PI-3K, and PKA pathways elicited similar effects on the *IGFBP-2* gene promoter activity, irrespective of cellular BTEB1 content, that for JNK had a more pronounced effect on Hec-1-A sublines exhibiting higher BTEB1 expression levels. Taken together, the results suggest that BTEB1 mediates the expression of growth-associated genes through direct and indirect transactivation mechanisms, one of which may involve the participation of a JNK family member.**

## INTRODUCTION

THE Sp/KRÜPPEL-LIKE FAMILY (KLF) of transcription factors consist of at least 20 members that function as transcriptional activators, repressors, or both by binding primarily to GC/GT-rich sequences within target gene promoters through three highly homologous $C_2H_2$ zinc finger motifs (Philipsen and Suske, 1999; Turner and Crossley, 1999; Black *et al.*, 2001). The ubiquitous distribution of GC box sequences in 5′-regulatory regions of multiple eukaryotic genes involved in growth, differentiation, or apoptosis (Jensen *et al.*, 1997; Cook *et al.*, 1998) suggests that these nuclear proteins likely regulate mam-

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malian homeostasis at a magnitude considerably greater than first thought. The functional redundancy of KLF members has not been substantially elucidated, although data from studies of the knockout phenotypes for a number of these genes in mice suggest these proteins to have distinct functions (Supp *et al.*, 1996; Marin *et al.*, 1997; Wani *et al.*, 1998; Bouwman *et al.*, 2000). However, specific family members are known to antagonize each other's activities *in vitro* by competing for binding to the same site to modify the transactivation of discrete sets of promoters (Hagen *et al.*, 1994; Birnbaum *et al.*, 1995; Kaczynski *et al.*, 2001). Moreover, they can directly interact in common with other promoter-specific transcription factors and transcriptional coactivators or corepressors, depending on cellular context (Owen *et al.*, 1996; Galvagni *et al.*, 2001; Kaczynski *et al.*, 2001; Zhang JS *et al.*, 2001; Zhang D *et al.*, 2002) as well as influence each other's levels of expression (Song *et al.*, 2001). Thus, it is likely that the signaling pathways mediated by some or all of these proteins are functionally associated, although the nature and extent of these interactions remain largely unknown.

The elucidation of the molecular mechanisms underlying cell proliferation is important for understanding normal growth processes and oncogenesis. The uterine endometrial epithelium represents an excellent paradigm for many of these investigations because *in vivo*, it is subject to numerous extracellular stimuli, allowing for the highly orchestrated cycle of proliferative and differentiative processes that underlie the physiologic changes associated with diverse reproductive events. A subset of KLF family members, most notably Sp1 and Sp3, have been linked to distinct biologic processes particularly occurring during pregnancy, based on studies that document the direct regulatory effects of progesterone on Sp1 (stimulatory) or Sp3 (inhibitory) gene expression (Krikun *et al.*, 2000); the direct interaction of Sp1 and related proteins with the nuclear receptors for estrogen and progesterone (Owen *et al.*, 1998; Stoner *et al.*, 2000; Zhang D *et al.*, 2002); and the control by these nuclear proteins of pregnancy-associated endometrial epithelial-specific gene transcription (Dennig *et al.*, 1995; Simmen *et al.*, 2000). Likewise, the loss of normal cellular brakes underlying proliferation, leading to endometrial hyperplasia and hence tumorigenesis, likely involves KLF members, because, in addition to the numerous studies establishing their function as major transactivators of growth-associated genes (Zhu *et al.*, 2000; Feng *et al.*, 2000), altered cellular expression of distinct family members has been associated with modified growth status (see review by Black *et al.*, 2001). Consistent with this, the hypermethylation of CpG islands within the promoter region of the gene for the growth-regulatory progesterone receptor-B (PR-B) isoform, perhaps resulting from diminished GC-box binding activity, has been correlated with endometrial carcinoma (Sasaki *et al.*, 2001).

We have recently shown that forced expression of the 244-amino acid KLF member basic transcription element binding (BTEB1) protein in a human endometrial epithelial cell line that normally exhibits low levels of this protein (Simmen *et al.*, 1999) resulted in enhanced cellular DNA synthesis under serum conditions, indicative of increased growth rates (Zhang XL *et al.*, 2001). Further, we showed in the same study that this increased growth response with BTEB1 was associated with the enhanced expression of numerous cell cycle-associated and growth-related genes. The present study focused on the elucidation of the potential mechanisms underlying BTEB1-mediated induction of growth response by the isolation and identification of additional BTEB1 downstream genes and by the demonstration that some, but not all, transactivation properties of BTEB1 are directly mediated at the level of gene promoter. Further, we provide initial evidence to indicate that the signaling pathway of BTEB1 may involve in part the participation of a JNK member.

## MATERIALS AND METHODS

### *Expression and reporter constructs*

The 983-bp fragment corresponding to human Axl receptor tyrosine kinase promoter was cloned by PCR from human genomic DNA (Promega Biotech) using specific primers (forward: 5′-GACAAGCTTCTATTCTTATTCCCATTTT A-3′; reverse: 5′-TATAAGCTTGGTGCCAAACTTCCTCAGAA-3′) based on the published sequence of human *Axl* 5′ regulatory and promoter sequences (Schulz *et al.*, 1993). This fragment was subcloned into the *Hin*dIII site of a luciferase gene-containing plasmid (Luciferase Basic; Promega). Sequence analysis verified the correct orientation and nucleotide sequence of the cloned fragment. The pIGFBP-2-Luc reporter construct (−1397-BP2) contained 1397 bp of 5′ regulatory sequences (+1 = translation initiation codon) of the porcine *IGFBP-2* gene (Badinga *et al.*, 1998). The human $p21^{WAF1}$ promoter constructs (p21 and p21 Sma) were generous gifts of Dr. Xiao-Fan Wang (Duke University Medical Center) and contained ~2.4 kb and 111 bp, respectively, of p21 5′ regulatory sequences. The expression constructs for human Sp1 (pCMV-Sp1) and rat BTEB1 (pCDNA-BTEB1) were kindly provided by Drs. Robert Tjian (University of California) and Hiroaki Imataka (McGill University), respectively.

### *Cell culture and transient transfections*

The human endometrial carcinoma cell line Hec-1-A and derived clonal lines stably expressing sense (4S, 9S sublines) and antisense (2As, 3As sublines) BTEB1 constructs were cultured in McCoy's 5A medium containing 10% FBS (GIBCO/BRL) as previously described (Zhang XL *et al.*, 2001). Cells were transfected with 5 $\mu$g of reporter construct DNA, 0.5 $\mu$g of expression construct, or both or with corresponding empty vector on reaching 70% confluence, in the presence and absence of 10% FBS using either polybrene or LipofectAMINE reagent (GIBCO/BRL) as previously described (Simmen *et al.*, 1999, 2000). At 48 h post-transfection, cell lysates were collected, and luciferase activity was determined using the Luciferase Reporter kit (Promega). The results were normalized to cellular extract protein for each sample and are presented as least square means ± SEM. All transfections were done in triplicate plates per experiment and were repeated three to six times.

### *Northern and Western blot analyses*

The parental Hec-1-A and derived BTEB1 sense and antisense sublines were cultured as described above. At confluence, $1 \times 10^6$ cells were replated in the same medium in the

presence or absence of 10% FBS and either further incubated to reach confluence or harvested at various time points as specified for each experiment. RNA isolation and Northern blot analysis were performed as described previously (Zhang XL *et al.*, 2001). The membranes were hybridized with $^{32}$P-labeled DNA fragments generated from differentially expressed clones of 4S and 2As mRNAs (see below). Resultant hybridization signals, when indicated, were quantified using the Alpha Imager 2000 Documentation System (Alpha Innotech Co.). Western blot analysis was carried out on nuclear extracts, whole cell extracts, or conditioned medium prepared from BTEB1 sense and antisense lines following previously described protocols (Gonzalez *et al.*, 1995). Proteins were visualized with Ponceau S (Sigma) after membrane transfer to confirm equal protein loading among samples. Commercially available antibodies raised against human mitosin (GeneTex) and recombinant human Sp1 (Santa Cruz Biotechnology) or rabbit anti-recombinant porcine IGFBP-2 antibody generated in our laboratories (Badinga *et al.*, 1999) were used in overnight incubations at 4°C with the indicated membranes. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL) detection kit (Amersham/Pharmacia Biotech).

### *mRNA differential display RT-PCR*

Differential display was performed following the procedures initially described by Liang and Pardee (1992) and subsequently adapted in our laboratories (Green *et al.*, 1996). Briefly, total RNA was pooled from different passages of confluent BTEB1 sense (4S) or antisense (2As) Hec-1-A cell lines grown in McCoy's 5A medium containing 10% FBS and isolated using Tri-Zol reagent (GIBCO/BRL). RNA (5 $\mu$g) from each cell line was DNAse treated, and reversed transcribed using anchored oligo(dT) primer sets (0.2 $\mu$M) provided in the HIERO-GLYPH™ kit (Genomyx Corp.) and SuperScript™ II RT enzyme. The resultant cDNA was amplified in the presence of [$\alpha$-$^{33}$P]-dATP (250 $\mu$Ci) with six arbitrary 5′ primers and ten anchored 3′ primers for four PCR cycles at 46°C, followed by 25 PCR cycles at 60°C. The denaturation (92°C, 15 sec) and extension (72°C, 2 min) conditions were similar for both annealing temperatures, and the final extension step was carried out at 72°C for 7 min. The PCR products were separated on 6% polyacrylamide denaturing gels in Tris-borate-EDTA buffer using the GenomyxLR™ DNA Sequencing System (Genomyx Corp.) and visualized by autoradiography. Putative differentially expressed cDNA fragments were excised from the gels and reamplified by PCR using the same primer combinations as those initially used for their generation. The PCR fragments were subcloned into pCRII-TOPO™ vector, and their nucleotide sequences were determined. The resultant sequences were compared with those reported in the GenBank database by using the BLAST algorithm (National Center for Biotechnology Information).

### *Statistical analysis*

Comparisons between groups were analyzed using predicted differences (pdiff) of the least squares means following the General Linear Models Procedure of the Statistical Analysis System (SAS, 1988). Means were considered significantly different at $P < 0.05$.

TABLE 1. SUMMARY OF ddRT-PCR CLONES

| *No. clones* | *Clone ID* | *Gene symbol* | *Gene name* | *Match length (%)* | *Match region (bp)* | *Accession no.* |
|---|---|---|---|---|---|---|
| 1 | 1-3-2 | | Unnamed protein | 552/555(99) | 1936-2490 | AL 157482<br>AK 027052 |
| 1 | 1-3-5 | LINE 1 element | LINE 1 putative p150 protein | 538/549(97) | 3210-3758 | U 93563 |
| 1 | 2-11 | ALU repeat | 7SL RNA | 175/175(100) | 129210-129384 | AL 109613 |
| 2[a] | 6-20 | CENP-F | Centromere protein F; mitosin | 416/416(100) | 828-1243 | XM_001418 |
| 3[a] | 2-2 | Ax1 | Ax1 receptor tyrosine kinase | 709/714(99) | 4069-4782 | NM_021913<br>NM_001699 |
| 1 | 2-14 | | Unnamed protein | 540/543(99) | 5-547 | AF 222023 |
| 1 | 3-3 | RPL14 | Ribosomal protein L14 | 260/260(100) | 463-722 | NM_003973 |
| 1 | 2-10 | | | No match | | |
| 3[b] | 2-7 | | | No match | | |
| 1 | 2-13 | | | No match | | |

[a]As deduced from DNA sequences.
[b]As judged from identical northern blot patterns.

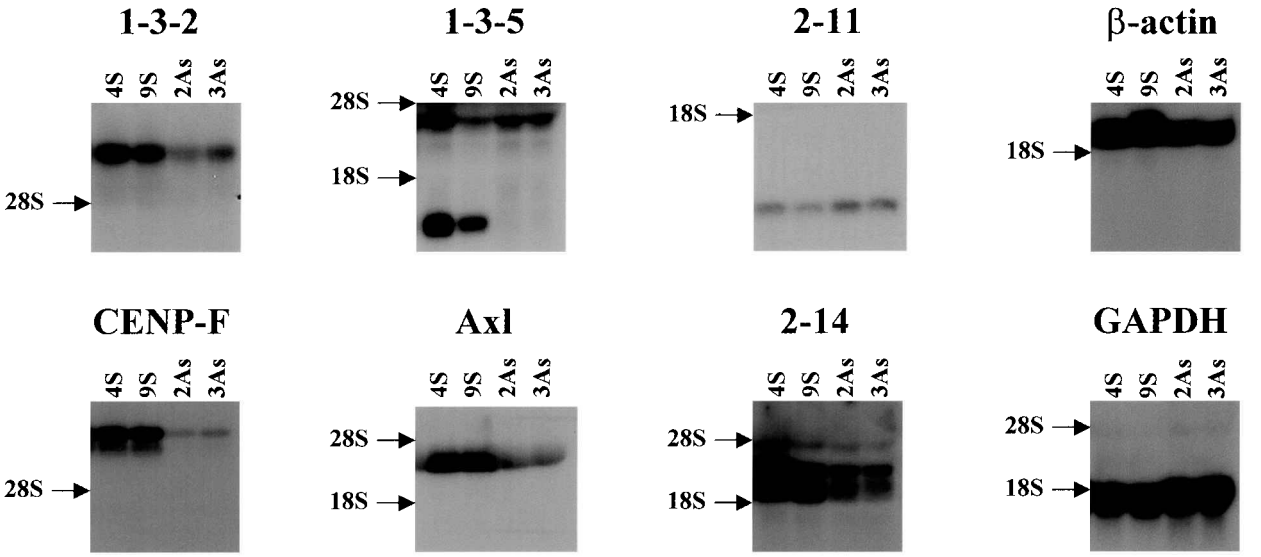


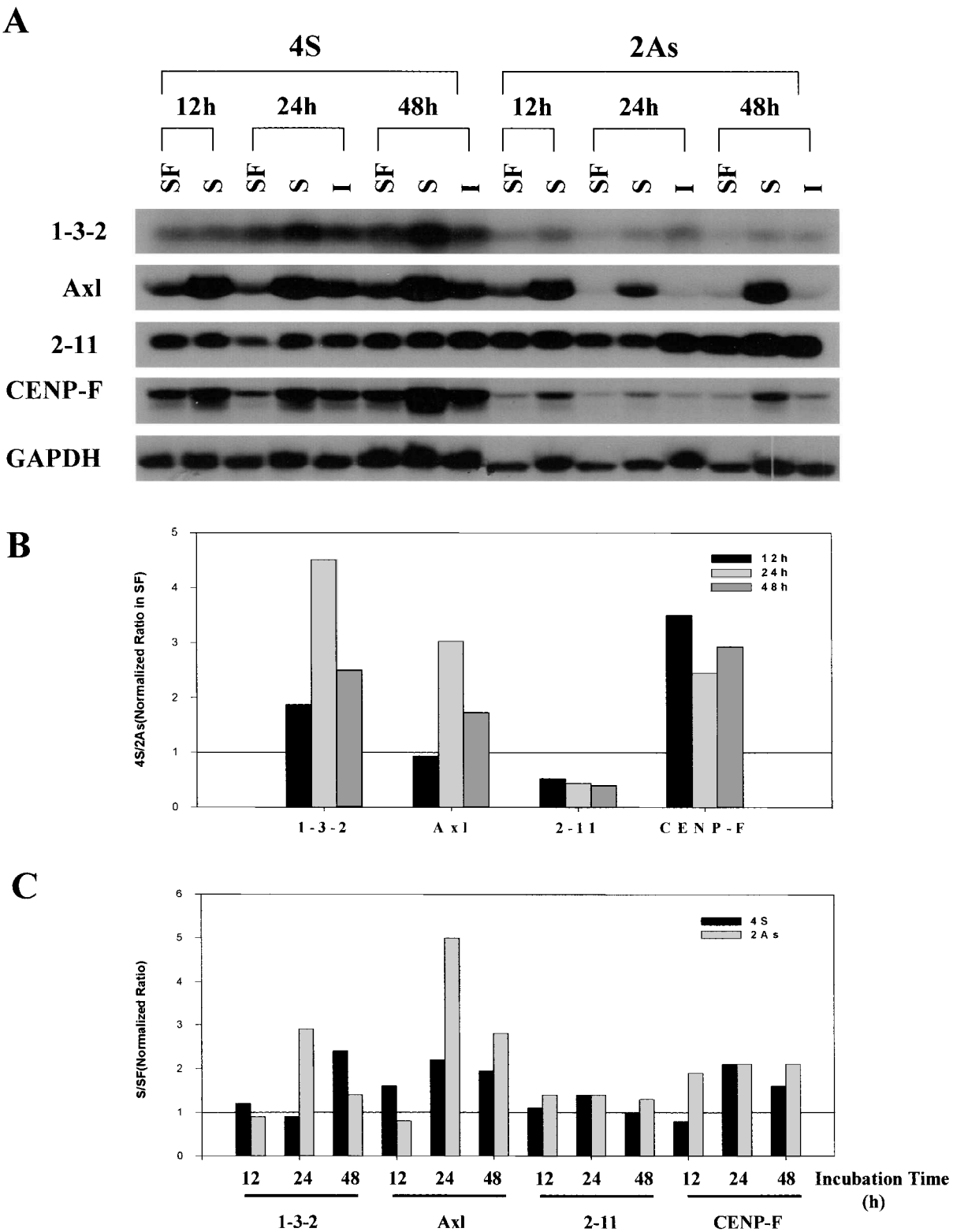

**FIG. 1.** Identification of differentially expressed mRNAs. Clones isolated by mRNA differential display analysis of RNAs prepared from high (4S, 9S) and low (2As, 3As) BTEB1-expressing human endometrial epithelial sublines (see Table 1) were used as probes in Northern blot analysis of total RNA prepared from these cells. Each lane contained 30 $\mu$g of total RNA.

## RESULTS

### *Identification of differentially expressed genes*

The mRNA differential display analysis of high (4S) and low (2As) BTEB1-expressing Hec-1-A cell lines utilized a total of 10 anchored and 6 arbitrary primers, respectively, to give a total of 60 different PCR combinations, which in theory, represent approximately 35% of the mRNA population. Examination of the resultant autoradiograms yielded 15 cDNA fragments that were readily visually distinguishable as being differentially expressed. These fragments were excised from gels, reamplified, and subcloned into the pCRII-TOPO vector. The resultant cDNA insert for each plasmid DNA was then used as a hybridization probe in Northern blots containing total RNAs prepared from high (4S, 9S) and low (2As, 3As) BTEB1-expressing lines. Those that demonstrated differential expression by this analysis were subjected to nucleotide sequencing. Table 1 summarizes the identities of the cDNA clones based on reported DNA sequences in GenBank; of these 15 clones, 10 represent distinct mRNAs. Clone 6-20 encodes human mitosin (also known as centromere protein F, CENP-F), a 3113-amino acid protein whose expression throughout the S, $G_2$, and M, but not $G_1$ phases, of the cell cycle is suggestive of an important role in mitogenesis (Rattner *et al.*, 1993; Liao *et al.*, 1995; Zhu *et al.*, 1995; Clark *et al.*, 1997). Clone 2-2 encodes Axl receptor tyrosine kinase, a membrane-bound protein involved in cell proliferation as well as in mediating cell aggregation (O'Bryan *et al.*, 1991; Craven *et al.*, 1995; Crosier and Crosier, 1997; Berclaz *et al.*, 2001). Clone 1-3-2 is identical in sequence (99%) to the 3′ UTR of a human cDNA that encodes a hypothetical membrane protein of 495 amino acids and which exhibits fibronectin type III domains found in a variety of proteins, including tyrosine kinases, protein tyrosine phosphatases, fibronectin, and class I cytokine receptors (GenBank database). Clone 2-11 appears to be a member of the *Alu* family of repetitive DNA sequences, which is a component of the signal recognition particle involved in protein biosynthesis (Leinwand *et al.*, 1982; Ullu and Tschudi, 1984). The ORF of Clone 1-3-5 is greater than 95% identical in sequence to that of human long interspersed nuclear elements (LINEs),

**FIG. 2.** Effects of serum and insulin-like growth factor-I on the expression of distinct BTEB1-regulated genes. **(A)** The human endometrial epithelial sublines 4S (high BTEB1) and 2As (low BTEB1) were seeded at the same density in serum-containing medium for 24 h and transferred to serum-free medium for an additional 24 h to synchronize the cells. Cells were then cultured in serum-containing (S) or serum-free (SF) medium or in SF medium with added recombinant human IGF-I (100 ng/ml; I) for the indicated times. Total cellular RNA was isolated by the TriZol method and analyzed (30 $\mu$g/lane) for expression of specific genes using the probes denoted on the left. **(B)** The intensities of the hybridization signals obtained for each gene in the 4S and 2As sublines grown in serum-free (SF) medium for the indicated times (12, 24, 48 h) (see panel A) were determined by densitometric scanning and normalized to those of corresponding GAPDH signals. The graph represents the normalized ratios of the hybridization intensities for each gene in 4S over 2As sublines. **(C)** The analysis of the hybridization signals obtained for each gene in the 4S and 2As sublines grown in serum-containing (S) or SF medium for the indicated incubation times (12, 24, 48 h) followed those for panel B. The graph represents the normalized ratios of the hybridization intensities for each gene in S over SF. The lines across the graphs indicate a ratio of 1.


A
4S
2As
12h
24h
48h
SF
S
I
1-3-2
Axl
2-11
CENP-F
GAPDH
B
4S/2As(Normalized Ratio in SF)
12h
24h
48h
1-3-2
Axl
2-11
CENP-F
C
S/SF(Normalized Ratio)
4S
2As
12
24
48
Incubation Time (h)
1-3-2
Axl
2-11
CENP-F

which belong to the family of Kpn I repetitive DNAs and which are transcribed by RNA polymerase III (Shafit-Zagardo *et al.*, 1983; Smit, 1996). Clone 2-14 showed a match only with the partial sequence of a cDNA clone isolated from an SV-40-transformed human fetal gastric epithelial cell line. Clone 3-3 is 100% identical to a region encoding the ribosomal protein L14, while three other clones (2-10, 2-7, and 2-13) had no match with any sequences in GenBank.

Representative Northern blots using six of these clones as hybridization probes are shown in Figure 1; these blots were subsequently reprobed with $\beta$-actin and glyceraldehyde3-phosphate dehydrogenase (GAPDH) to normalize for RNA loading and integrity among samples. Clones 1-3-2, 1-3-5, 6-20 (CENP-F; also mitosin), 2-2 (Axl), and 2-14 displayed elevated transcript levels in Hec-1-A cell lines with increased BTEB1 cellular content (4S, 9S > 2As, 3As); by contrast, the transcript levels for clone 2-11 were higher in lines with lower cellular levels of BTEB1.

### *Effect of serum on levels of differentially expressed mRNAs*

The proliferative potential of higher BTEB1-expressing sublines was previously demonstrated to differ from that of lower BTEB1-expressing lines in the presence, but not in the absence, of serum (Zhang XL *et al.*, 2001). To examine whether the expression levels of the isolated differentially expressed clones (Table 1, Fig. 1) may be associated with the distinct responses to serum of cells with higher BTEB1 content, the mRNA levels for several of these clones were examined in 4S and 2As lines grown in either serum-containing or serum-free medium. In serum-free medium, the expression levels of the transcripts for 1-3-2, Axl, and CENP-F (mitosin) were higher, while those of 2-11 were lower, in 4S than in 2As lines (Fig. 2A, B), confirming their differential expression as a function of BTEB1 cellular levels. Serum had a modest inductive effect (approximately twofold when averaged over a 48-h period) on the expression of 1-3-2, *Axl*, and *CENP-F* genes in both 4S and 2As sublines but had no effect on 2-11 expression in either line (Fig. 2A, C). Insulin-like growth factor-I (IGF-I; I), a known mitogenic component of serum, had no significant effect on the expression of these genes, irrespective of BTEB1 cellular content. For all analyses, hybridization signals were normalized to those of GAPDH.

### *Gene expression of BTEB1-related family members*

The possibility that altered cellular expression of BTEB1 could result in parallel changes in the expression of other BTEB family members was examined in high (4S, 9S) and low (2As, 3As) BTEB1-expressing lines grown in serum-containing medium. In these studies, the expression of Sp1 and BTEB2 genes and of Sp1 protein was analyzed by Northern and Western blots, respectively, using total RNAs or nuclear extracts prepared from these cells (Fig. 3). The BTEB2 mRNA levels were higher (threefold to fourfold) in 4S and 9S than in 2As and 3As lines. In contrast, Sp1 transcript, as well as protein, levels were not different among these lines. The analysis of the same nuclear extracts for mitosin (CENP-F), whose gene expression was greater in sense than in antisense sublines (see Fig. 1) demonstrated increased levels of this protein in high BTEB1-expressing cells, suggestive of the specificity of the observed effects for Sp1.

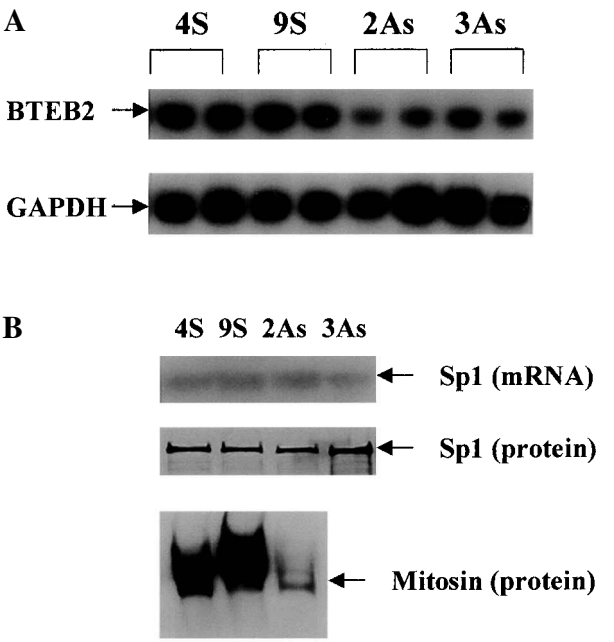



**FIG. 3.** Expression of BTEB2 and Sp1 as a function of BTEB1 cellular content. **(A)** Total RNA isolated from high (4S, 9S) and low (2As, 3As) BTEB1-expressing human endometrial epithelial sublines grown in serum-containing medium was analyzed for expression of *BTEB2* and *GAPDH* by Northern blot analysis. Each lane contained 30 $\mu$g of total RNA per sample. Two distinct RNA preparations representing different passage numbers for each subline were analyzed. **(B)** High (4S, 9S) and low (2As, 3As) BTEB1-expressing sublines of Hec-1-A were grown in serum-containing medium and analyzed for expression of Sp1 mRNA or immunoreactive Sp1 and mitosin, respectively. Total RNA blots contained 30 $\mu$g per sample. Nuclear extracts were prepared from isolated cells, and equal amounts of protein (50 $\mu$g for Sp1, 100 $\mu$g for mitosin) were subjected to Western blot analysis as described in Materials and Methods.

### *Regulation by BTEB1 of Axl receptor gene expression*

The characterization of *Axl* (clone 2-2) as a potential BTEB1-regulated gene was further examined at the level of transcription. For these studies, the 5′ flanking and promoter region (983 bp) of the human *Axl* gene, which contains GC-rich regions known to confer transcriptional activation by binding Sp family members including BTEB1 when present in other gene promoters (Imataka *et al.*, 1992; Sogawa *et al.*,1993), was linked upstream of the luciferase reporter gene. In the first experiment, the promoter activity of the Axl-Luc reporter construct was evaluated upon transient transfection in high (4S) and low (2As) BTEB1-expressing lines, in the presence and absence of serum in the medium. In the second experiment, the parental Hec-1-A cell line was

cotransfected with the Axl-Luc reporter construct and the expression vectors for Sp1, BTEB1, and their combination. The results of both experiments are shown in Figure 4. The promoter activity of *Axl* was higher in 4S than in 2As lines, irrespective of the presence of serum components in the medium (Fig. 4A). Moreover, serum increased *Axl* promoter activity in both lines. Cotransfection of the reporter construct and expression constructs for BTEB1 and/or Sp1 in the parental Hec-1-A cell line, which has a low level of endogenous BTEB1 expression (Simmen *et al.*, 1999), was used to evaluate whether the difference in receptor promoter activity in 4S vs. 2As sublines was a direct consequence of and specific to BTEB1. In both serum-containing and serum-free medium, BTEB1 had no effect on the basal *Axl* promoter activity (Fig. 4B). By contrast, Sp1 significantly increased ($P < 0.05$) the transcriptional activity of the *Axl* gene from basal levels. This effect of Sp1 was augmented by BTEB1, but only in the presence of serum.

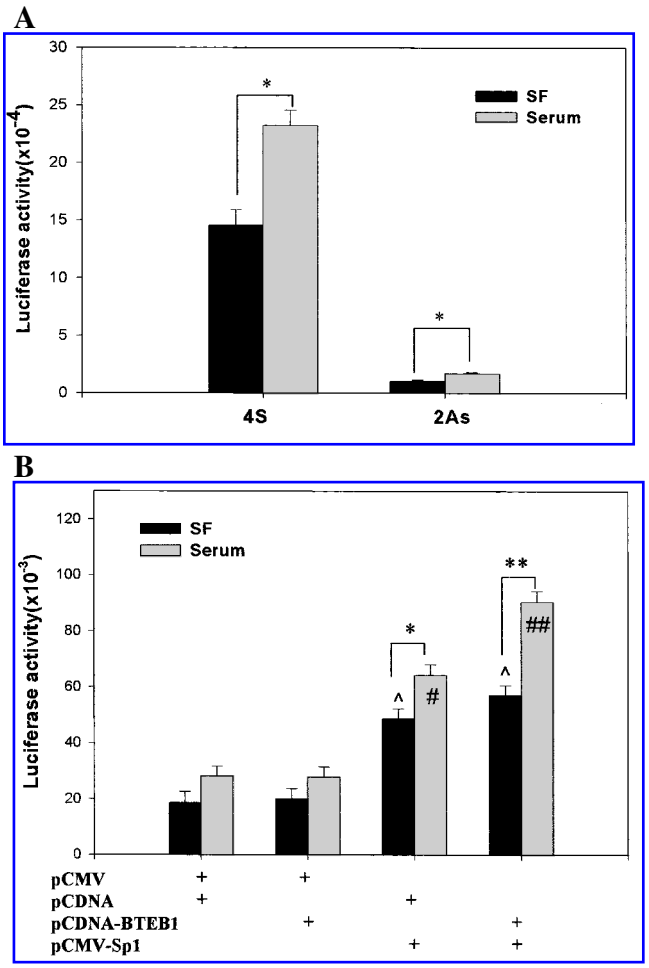



**FIG. 4.** Transactivation of Axl promoter activity by BTEB1 and Sp1. **(A)** The 4S and 2As sublines of the human endometrial epithelial cell line Hec-1-A were transfected with the Axl-Luc reporter construct in serum-containing (Serum) or serum-free (SF) medium as described in Materials and Methods. $*P < 0.0001$. **(B)** The parental Hec-1-A cell line was cotransfected with the Axl-Luc reporter construct and expression constructs for BTEB1, Sp1, their combination, or corresponding empty vectors in serum-containing (Serum) and serum-free (SF) medium, as described in Materials and Methods. Serum vs. SF: $*P < 0.005$, $**P < 0.0001$; SF, expression vector vs. control vector: $\wedge P < 0.0001$; Serum, expression vector vs. control vector: # or ##$P < 0.0001$. Each column represents least squares means ± SEM from three independent experiments, with each experiment performed in triplicate.

### *Transactivation by BTEB1 of other growth-associated genes*

The lack of currently published sequences for the promoter and 5′ regulatory region of mitosin/*CENP-F*, another BTEB1-induced gene identified in the present study, precluded further studies on its transcriptional activation by BTEB1. Hence, we examined the effects of BTEB1 on the activity of the promoter for two other genes namely, the cell cycle-associated Cdk inhibitor $p21^{WAF1}$ and *IGFBP-2*. The selection of these genes was based on previous observations from our laboratories demonstrating increased expression of the p21 gene in higher BTEB1-expressing Hec-1-A sublines (Zhang XL *et al.*, 2001) and a mitogenic role for IGFBP-2 protein in uterine endometrial epithelial cells (Badinga *et al.*, 1999). Similar to that observed for *Axl*, the promoter activity of the reporter gene construct containing 2.4 kb of the human p21 gene 5′ regulatory region was higher in 4S than in 2As lines. The inclusion of serum increased p21 promoter activity in both lines (Fig. 5A). In parental Hec-1-A cells in the presence of serum (Fig. 5B, C), the longer construct (p21; 2.4 kb) exhibited higher basal promoter activity than the shorter construct (p21Sma; 111 bp). In contrast to that observed for the *Axl* receptor promoter, cotransfected BTEB1 (pCDNA-BTEB1) expression vector increased transcriptional activity from both p21 promoter constructs, although this effect was more pronounced for the longer construct (Fig. 5B). Cotransfected Sp1 expression vector exhibited an effect similar to that of BTEB1 (Fig. 5C).

The transactivation of *IGFBP-2* by BTEB1 was examined at the levels of protein and promoter activity (Fig. 6). Results indicated that Hec-1-A sublines with increased BTEB1 expression had higher levels of secreted (CM; 9S > 2As) and cellular (WCE; 4S, 9S > 2As, 3As) immunoreactive IGFBP-2 (Fig. 6A). On a per-protein basis, however, Hec-1-A expression of IGFBP-2 was considerably lower than that of pregnant pig endometrium (days 30 and 60 of pregnancy), the positive control for these studies (Song *et al.*, 1996). Luciferase-reporter transcription from the *IGFBP-2* promoter appeared to be increased also by BTEB1 (Fig. 6B, C). In particular, basal (SF) Luc activity was higher in 4S than in 2As lines, and this activity was further stimulated by serum. In the parental line, cotransfection of BTEB1 expression vector increased the activity of the IGFBP-2 promoter (Fig. 6C). Sp1 had a greater effect on *IGFBP-2* promoter activity than did BTEB1, irrespective of serum conditions, and their combination had no additive or synergistic outcome (Fig. 6C).

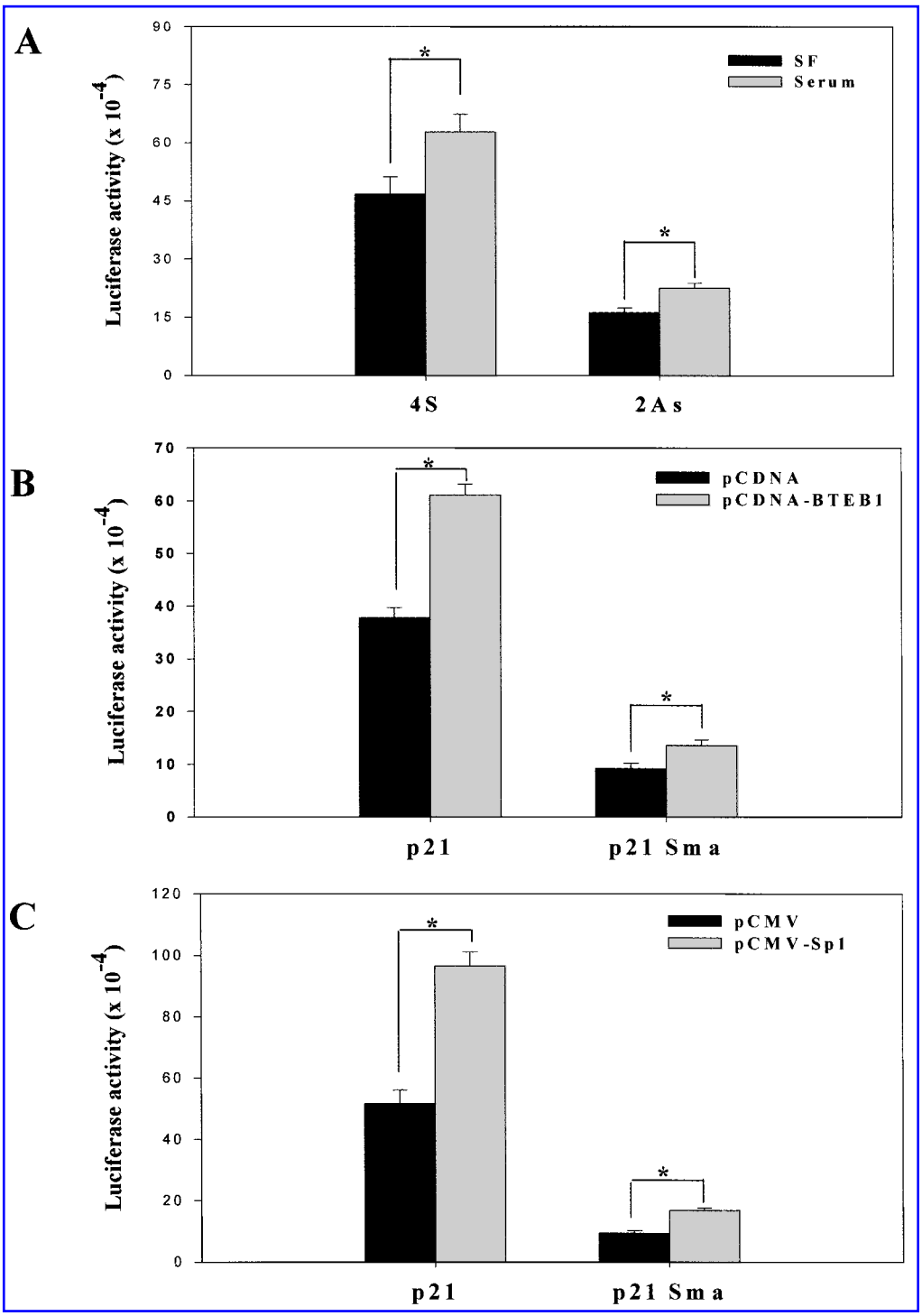



**FIG. 5.** Transactivation of p21 promoter activity by BTEB1 and Sp1. **(A)** The 4S and 2As sublines of the human endometrial epithelial cell line Hec-1-A were transfected with the p21-Luc reporter construct in serum-containing (Serum) or serum-free (SF) medium as described in Materials and Methods. *$P < 0.05$. **(B, C)** The parental Hec-1-A cell line was cotransfected with the long (p21) and short (p21 Sma) forms of the p21-Luc reporter constructs and expression constructs for either BTEB1 **(B)** or Sp1 **(C)** or corresponding empty vectors as described in Materials and Methods. *$P < 0.05$. Each column represents least squares means ± SEM from three independent experiments, with each experiment performed in triplicate.

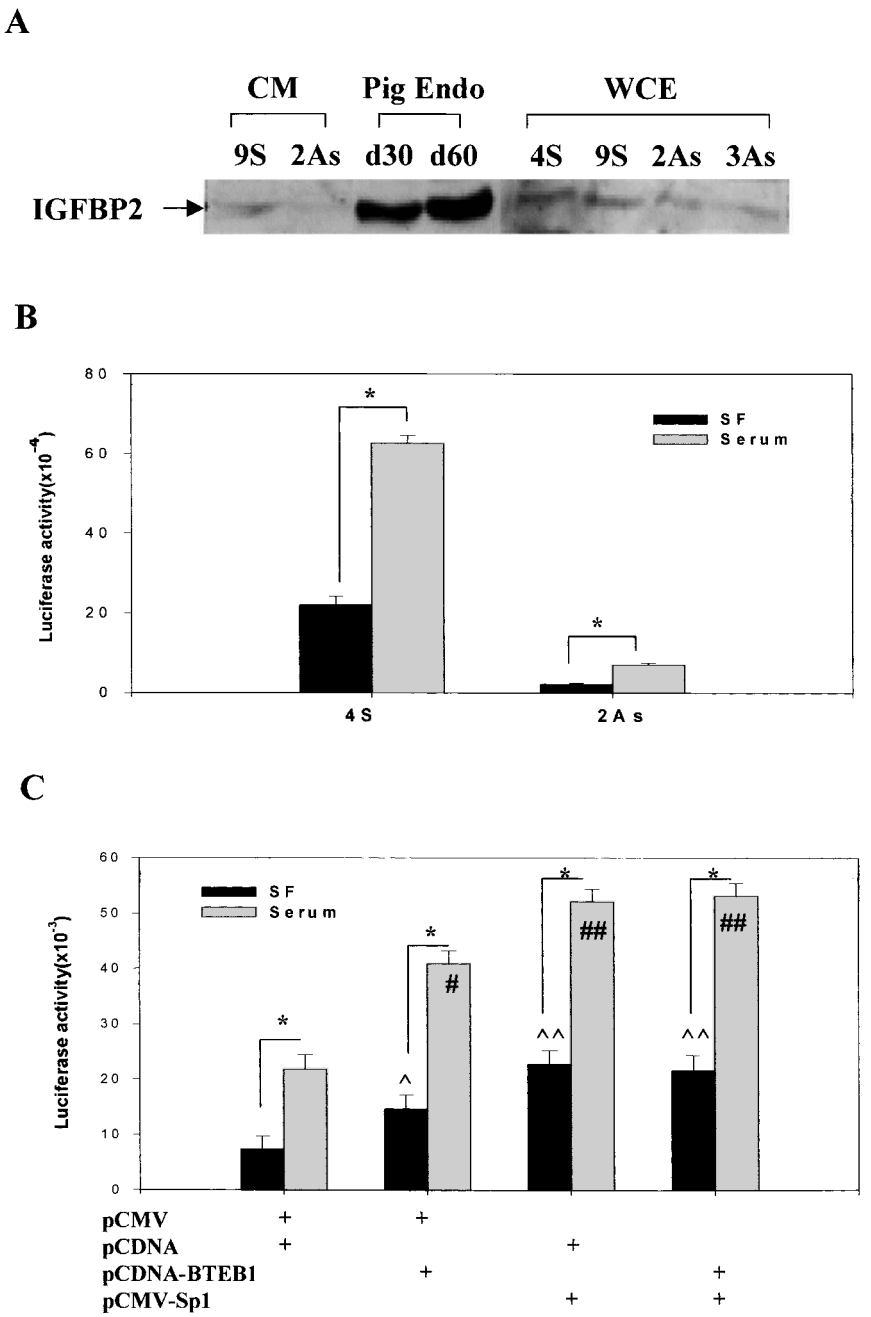



**FIG. 6.** Effect of BTEB1 on IGFBP-2 gene expression. **(A)** Immunoreactive IGFBP-2 in medium conditioned by Hec-1-A sublines exhibiting high (9S) and low (2As) BTEB1 cellular content (CM) or in whole extracts prepared from high (4S, 9S) or low (2As, 3As) BTEB1-expressing cells (WCE) were detected by Western blot analysis using a specific antibody raised against recombinant porcine IGFBP-2 in rabbits. Tissue extracts prepared from pig endometrium at pregnancy days 30 and 60 (d30, d60) were used as positive controls. Protein amounts loaded for each lane are CM, 250 $\mu$g; pig endometrial extracts, 50 $\mu$g; WCE, 100 $\mu$g. **(B)** The 4S and 2As sublines of the human endometrial epithelial cell line Hec-1-A were transfected with the IGFBP-2-Luc reporter construct in serum-containing (Serum) or serum-free (SF) medium as described in Materials and Methods. $*P < 0.0001$. **(C)** The parental Hec-1-A cell line was cotransfected with the IGFBP-2-Luc reporter construct and expression constructs for BTEB1, Sp1, their combination, or corresponding empty vectors in serum-containing (Serum) and serum-free (SF) medium, as described in Materials and Methods. Serum vs. SF: $*P < 0.0001$; SF, expression vector vs. control vector: $\wedge P < 0.05$, $\wedge\wedge P < 0.005$; Serum, expression vector vs. control vector: $\#P < 0.001$; $\#\#P < 0.0001$. Superscripts with different designations within each group indicate significant differences ($P < 0.05$). Each column represents least squares means ± SEM from at least three independent experiments, with each experiment performed in triplicate.

*Signaling pathways for BTEB1 transactivation of* IGFBP-2 *gene*

To begin to define the signaling pathway(s) by which BTEB1 mediates the transactivation of growth-related uterine epithelial-associated genes in concert with serum-derived components, several signal transduction inhibitors were added to serum-containing medium during transient transfection experiments. Specific inhibitors of the ras-raf-MAPK (PD98059; 2 $\mu$M; Cell Signaling Technology), PI-3 kinase (wortmannin; 2.5 nM; Upstate Biotechnology), protein kinase A (H89; 10 $\mu$M; Sigma), and c-jun-N-terminal kinase (JNK) (curcumin; 10 $\mu$M; Sigma) pathways were examined for their abilities to alter the activity of the *IGFBP-2* gene promoter in high (4S) and low (2As) BTEB1-expressing Hec-1-A sublines. Results in Figure 7A and B demonstrate that the addition of PD98509 increased, while PI-3 kinase inhibition by wortmannin addition had no effect on, the transactivation of *IGFBP-2* in both sublines. Inhibitors of the PKA and JNK pathways decreased *IGFBP-2* promoter activity in both lines. However, while the JNK inhibitor effectively abolished *IGFBP-2* promoter activity in the 4S line, the 2As subline had 20% of its basal (serum alone) activity remaining after treatment with the inhibitor. The latter observation was confirmed in additional clonal lines (9S and 3As), where inhibition of basal *IGFBP-2* gene promoter activity by curcumin was decreased to a greater extent in 9S (4.3-fold) than in 3As (2-fold) sublines (Fig. 7C).

## DISCUSSION

The present study characterized potential mechanism(s) by which BTEB1, a member of the BTEB subfamily of Sp/Krüppel-like factors, mediates increased proliferation in endometrial epithelial cells. In a previous study (Zhang XL *et al.*, 2001), we demonstrated that human endometrial carcinoma Hec-1-A sublines (4S, 9S) engineered to express higher BTEB1 levels exhibited increased proliferative responses to serum, concomitant with enhanced expression of a number of cell cycle- and growth-associated genes, relative to the parental cell line or sublines with diminished BTEB1 expression. Here, we show that: (1) higher cellular BTEB1 content is associated with increased expression of mitosin and Axl receptor tyrosine kinase, two markers of proliferation correlated with tumorigenesis and malignancy in multiple tissues and cell lines; (2) BTEB1 directly transactivates p21 and *IGFBP-2* promoters, but not the *Axl* promoter, even though the 5′ regulatory sequences of all three genes contain GC-rich regions; and (3) the serum-mediated signaling pathway associated with increased IGFBP-2 expression may involve the participation of a JNK family member acting in concert with BTEB1. Together, these results suggest that BTEB1 mediates cell proliferation through regulation of growth-associated gene expression that may involve in part the JNK intracellular signaling cascade.

As an unbiased approach to identifying genes that are correlated with distinct cellular BTEB1 expression, the methodology of mRNA differential display was used in the present study. Although this technique has a limited sensitivity, as evidenced by our inability to detect differentially expressed genes previously identified to be upregulated by BTEB1 using the candidate gene approach (Zhang XL *et al.*, 2001), it nevertheless enabled the isolation of a number of potentially interesting genes whose increased expression is associated with higher cellular proliferative potential. In particular, the increased expression of mitosin (CENP-F) and of Axl in Hec-1-A sublines with higher cellular BTEB1 levels is consistent with a role for BTEB1 in cell proliferation. Mitosin, identified previously as centromere protein F (Rattner *et al.*, 1993) is a component of centrosomes and is involved in chromosome segregation during mitosis. Axl, on the other hand, is a member of a subfamily of receptor tyrosine kinases that includes Sky and Mer (O'Bryan *et al.*, 1991; Nagata *et al.*, 1996) and is implicated in cellular proliferation (Berclaz *et al.*, 2001) via binding of its cytoplasmic domain to distinct substrates, including phospholipase C$\gamma$1, GRB2, and phosphatidylinositol 3′-kinase subunits p85$\alpha$ and p85$\beta$ known to mediate growth factor signaling (Braunger *et al.*, 1997). Our previous findings that BTEB1 increased transcript levels for Cdk2 (Zhang XL *et al.*, 2001), the activity of which is requisite for proper replication of centrosomes during mitosis (Hinchcliffe *et al.*, 1999) may account for the increased expression of mitosin in higher BTEB1-expressing endometrial cells. The mechanism underlying BTEB1 induction of *Axl* gene expression is likely distinct from that of mitosin, however, since Axl, unlike mitosin, is a membrane-bound protein and is not anticipated to directly influence cellular division. Indeed, the significantly higher activity of the *Axl* promoter in sublines with increased BTEB1 expression suggests transcriptional control, although this was not confirmed in cotransfection experiments using the parental Hec-1-A cell line and a BTEB1 expression construct (see Fig. 4A, B). Because the *Axl* promoter region evaluated in the present studies contained multiple GC/GT-rich regions (Schulz *et al.*, 1993) that bind Sp1, as shown by its transactivation in the presence of the Sp1 expression construct, the lack of a direct BTEB1 effect may reflect a DNA binding-independent mechanism for BTEB1 activation of *Ax1* transcription. Alternatively, the latter could also be attributed to the absence of an important cofactor(s) in the parental Hec-1-A line and the 2As subline, but which is present in the 4S subline, or to a lack of a cofactor binding site within the *Ax1* promoter region that is requisite for BTEB1 transactivation. Although additional studies will be necessary to clarify these possibilities, it is worth noting the increased BTEB2, but not correspondingly Sp1, transcript levels in 4S relative to 2As sublines. Because BTEB2 has an affinity for GC/GT box sequences similar to Sp1 and BTEB1 (Sogawa *et al.*, 1993), BTEB1 upregulation of *BTEB2* gene expression may account for the observed increased *Axl* transcription in 4S vs. 2As lines, suggestive of an indirect BTEB1 effect. In this regard, it has been shown that transactivation of the transforming growth factor-$\beta$1 gene promoter by the KLF member Zf9 (also known as KLF6) requires the actions of other family members. The use of a BTEB2 expression construct in transient transfection studies with the *Axl* promoter-reporter construct would be required to further evaluate this possibility.

In contrast to Axl receptor tyrosine kinase genes encoding p21 and IGFBP-2, two regulatory proteins involved in endometrial epithelial cell growth, appeared to be directly transactivated by BTEB1. In this regard, the p21 and *IGFBP-2* promoter constructs utilized in these studies contained GC-rich regions that likely bind BTEB1 and Sp1 with similar affinities.

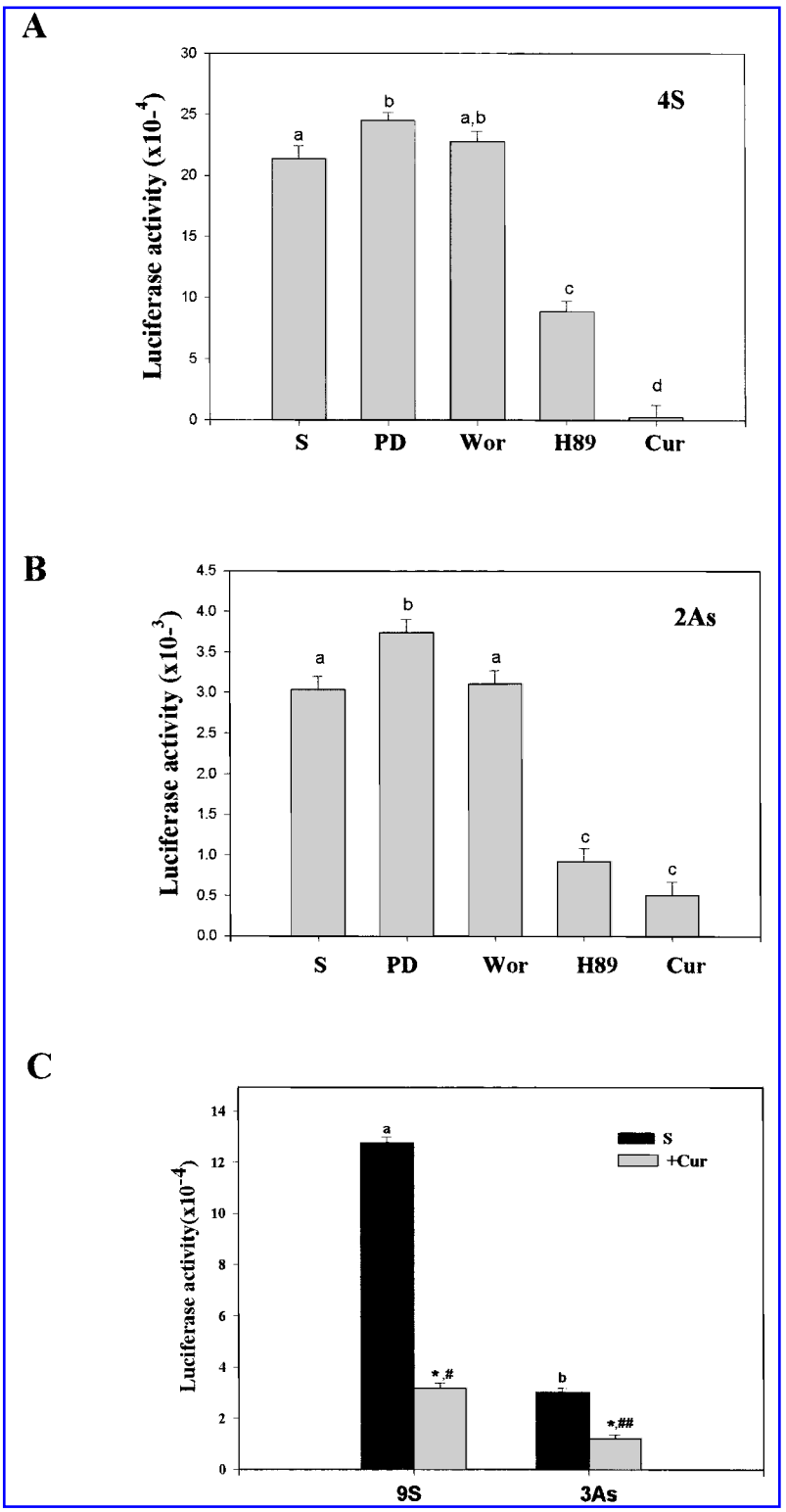



**FIG. 7.** Effects of specific kinase inhibitors on IGFBP-2 promoter activity as a function of BTEB1 cellular content. High (4S, panel **A**) and low (2As, panel **B**) BTEB1-expressing cell lines were transiently transfected with the IGFBP-2-Luc reporter construct in serum-containing medium (S) alone or with added inhibitors for the MAPK (PD, PD98059), PI3-K (Wor, wortmannin), PKA (H89), or JNK (Cur, curcumin) signal transduction pathways, following conditions described in the text. Superscripts with different letters denote significant differences ($P < 0.05$) from each other. Each column represents least squares means ± SEM from three independent experiments, with each experiment performed in triplicate. **(C)** The effect of JNK inhibitor curcumin (Cur) on IGFBP-2 promoter activity in additional clonal lines expressing high (9S) and low (3As) BTEB1. Each column represents least squares means ± SEM from two independent experiments, with each experiment performed in triplicate. Superscripts with different designations differ ($P < 0.05$). 9S vs. 3As in serum (S), a vs. b; Serum (S) vs. serum + curcumin (Cur), * for 9S and 3As; 9S vs. 3As in serum + cur, $^{\#}$ vs. $^{\#\#}$.

Interestingly, it appears that for p21, BTEB1, or Sp1 transactivation was not exclusively mediated by the multiple (total of four) Sp1 sites (Datto *et al.*, 1995) present in both the long and short reporter constructs; this suggests the contribution of additional GC-boxes or distinct regulatory elements that bind other, yet unknown, nuclear factors, in Sp1 or BTEB1-mediated transcriptional control. Similar to that observed for the regulation of the gene encoding the uterine endometrial protein uteroferrin (Simmen *et al.*, 2000), the effects of Sp1 and BTEB1 shown here for the p21 promoter are consistent with these proteins' exclusion of each other's binding to recognition sequences within target gene promoters. Together, these results suggest that BTEB1, and by analogy Sp1, may require promoter context-dependent interactions with other proteins for full transcriptional activity. Previous studies reported by this laboratory (Zhang D *et al.*, 2002) and others (Lu *et al.*, 2000; Kaczynski *et al.*, 2001) demonstrating physical interactions of Krüppel-like family members with numerous transcription factors and transcriptional coactivators at distinct promoters support this proposition.

Our previous findings that BTEB1-overexpressing Hec-1-A sublines exhibited higher proliferative potential in serum-containing, but not in serum-deprived, medium (Zhang XL *et al.*, 2001), suggested functional interactions between BTEB1 and serum-associated components in the molecular processes underlying cell proliferation. Results provided here are consistent with this model. We showed that the expression of a number of identified BTEB1 downstream genes are regulated by serum at distinct levels, which include mRNA (1-3-2, Axl, CENP-F, 2-11), protein (IGFBP-2), and gene promoter activity (p21, IGFBP-2, Axl). Further, we showed that the JNK intracellular signaling cascade(s) may interact with BTEB1 for control of expression of a growth-associated gene (*IGFBP-2*) by demonstrating that a BTEB1-dependent effect in transcriptional responses to inhibitors of kinase-associated signaling cascades can only be ascribed to that involving curcumin, a JNK inhibitor. This functional difference in cellular response not only suggests a role for BTEB1 as a downstream signaling component of a growth-regulatory factor(s) that utilizes JNK transduction pathway, but also provides additional corroboration for the participation of BTEB1 in altering cellular sensitivity to growth-regulatory signals. In previous reports, we showed that high (4S) and low (2As) BTEB1-expressing Hec-1-A sublines had distinct responses to transforming growth factor-$\beta$1 (Zhang XL *et al.*, 2001) and progesterone (Zhang D *et al.*, 2002). Nevertheless, there are clearly other JNK-mediated pathways that are independent of BTEB1, and additional studies will be required to further delineate the components specifically involved in BTEB1-mediated regulation of JNK targets.

In summary, our study showing BTEB1 induction of the expression of distinct genes with functions related to growth regulation, which occurs in part through transcriptional control of gene promoter activity and which involves, to some extent, growth-signaling transduction pathways utilized by JNK, is in line with a central role for BTEB1 in endometrial cell proliferation. Thus, the elucidation of the precise gene networks involving BTEB1 in the signaling of growth modulators may lead to the development of novel molecular strategies for addressing the control of abnormal growth of reproductive and nonreproductive tissues.

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