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The Bax Inhibitor-1 Gene Is Differentially Regulated in Adult Testis and Developing Lung by Two Alternative TATA-less Promoters

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We identified Bax inhibitor-1, BI-1, as a developmentally regulated gene product in perinatal lung using suppressive subtractive hybridization. BI-1 is a novel suppressor of apoptosis that was previously cloned as testis-enhanced gene transcript (TEGT). However, sequence analysis of lung BI-1 revealed unique nucleotides starting 29 bases upstream of the ATG initiation codon and extending to the 5′ end of lung-derived BI-1 cDNA compared to the original transcript from the testis. Cloning and sequencing of the upstream region of the BI-1 gene revealed that these unique sequences originated from two alternative first exons, located in tandem and separated by ~600 bases. Neither was preceded by a TATA box in the usual position, and S1 nuclease mapping at each exon 1 revealed multiple transcription start points with a major site being overlapped by a consensus initiator element. Promoter activity from each region was documented by transient transfection analysis *in vitro* using DNA sequences ligated to a reporter gene. The proximal promoter, P1, may exhibit cell type-specific differences in fibroblasts versus epithelia, whereas the distal promoter, P2, may exhibit species-specific differences in rat versus human cells. RT-PCR analysis for expression in adult tissues using exon 1-specific 5′ primers and common 3′ primers revealed that P1 is tissue-specific; P2 is ubiquitously active. The developmental regulation of BI-1 in the late fetal and early postnatal lung is specific for P2, indicating that these two TATA-less promoters are differentially regulated in adult testis and developing lung. Since Bax inhibitor-1 functions as a suppressor of apoptosis, its expression could provide a survival advantage for select cell populations during the peak period of apoptosis that occurs at birth.

INTRODUCTION

A dramatic reorganization occurs as the lung is transformed from a organ of secretion to one of gas exchange near the end of gestation. The developmental program that orchestrates this process is initiated in the fetus and, in most mammals, extends into the postnatal period. While the structural and cellular features of this transition have been well described, the genes involved remain poorly defined. Hence we used suppression subtractive hybridization (Diatchenko *et al.*, 1996) to identify developmentally regulated genes in the perinatal lung and found the testis-enhanced gene transcript, TEGT, to be up-regulated in the perinatal period. The function of this gene was unknown, until recently, when it was shown to be identical with a newly defined mammalian suppressor of apoptosis termed Bax inhibitor-1, BI-1 (Xu and Reed, 1998). Recent studies have also shown that a period of intense cellular apoptosis occurs in the perinatal lung, particularly in mesenchymal cells (Kresch *et al.*, 1998). We noted that lung BI-1 cDNA differed from the TEGT sequence in the literature and decided to characterize the rat BI-1 gene further. Herein we identify two tandem BI-1 promoters, P1 and P2, map their respective transcription start points, and demonstrate their differential activity *in vitro*. Both appear to be $TATA^{-}Inr^{+}$ promoters, but the proximal one, P1, has tissue-specific activity *in vivo*. The distal promoter, P2, is active in a ubiquitous fashion but developmentally regulated in the lung during the perinatal period. We speculate that this gene may play a key role in lung cell survival at birth.

MATERIALS AND METHODS

Materials and probes. Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) and QIAamp Tissue Kit (Qiagen, Germany) were used according to the manufacturer's protocol to isolate total RNA and genomic DNA, respectively. Electrophoresis-grade agarose was from International Biotechnologies, Inc. (New Haven, CT), and DNA standards were from BRL. X-OMAT film for radiography was from The Eastman Kodak Co. (Rochester, NY). [α-^{32}P]CTP, 800 Ci/mmol, was from ICN (Irvine, CA). The pGL3-basic vector and pGL3-promoter vector were obtained from Promega (Madison, WI). Plasmid constructs were prepared, and the β-galactosidase assay was performed as previously described (Joyce-Brady *et al.*, 1996). The

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF118564.

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substrate buffer for the luciferase assay was obtained from Boehringer Mannheim. The PCR primers for β-actin are exactly as described in Joyce-Brady *et al.* (1994). These were used to monitor mRNA integrity during PCR and to generate an actin cDNA probe to do the same for Northern experiments. Primer synthesis and nucleotide sequencing were performed at the DNA–Protein Core facility of Boston University School of Medicine.

Isolation and characterization of rat lung BI-1 clones. The suppression subtractive hybridization analysis of prenatal and perinatal lung will be described in detail elsewhere. Briefly, this was carried out exactly as described by Diatchenko *et al.* (1996). We used poly(A)$^+$ mRNA from 1.5-day-old lung as the tester and 20-day fetal lung as the driver. PCR products were cloned into the TA vector (InVitrogen, San Diego, CA), sequenced, and analyzed using the BLASTN program at the National Center for Biotechnology Information (Altschul *et al.*, 1990). We identified three independent clones of rat BI-1.

5′ RACE and genomic cloning. The 5′ end of our lung-derived BI-1 transcript was cloned by 5′ RACE using the Marathon cDNA Amplification Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA). Poly(A)$^+$ RNA was isolated from the lung, reverse transcribed, and amplified in a primary PCR with adaptor primer 1 (AP-1) and BI-1 primer BI-2, CAGGGCAGAGAGCAGGCCAGCCTG, followed by a secondary reaction with AP1 and BI-6, GCTGCTGTGTGGAGGGAGTTATGTGG, and the products were cloned and sequenced. Upstream BI-1 genomic DNA was amplified in two sequential PCRs with BI-5, GAGGGCATCAAAGTTGATCTTCCG, as the 3′ primer in each reaction and BI-12, CAGAGCACATCCGGTTTTAGCCG, and BI-10, GCGGGGAAGCTCCGAAGAGGTGGGC, as the 5′ primers for primary and secondary PCR, respectively, followed by cloning and sequencing. Additional upstream genomic DNA was amplified by using the GenomeWalker Kit according to the manufacturer's directions (Clontech) with adaptor primer-1 (AP-1) and BI-1 primer BI-13, CGGAGCAGACTTTGGGGCTCACC, in the primary PCR and adaptor primer-2 (AP-2) and BI-16, ACCTCGGCCTCTACCGTTGGCTTC, in the secondary PCR. A second genomic walk was performed using AP-1 and BI-20, ACTGAAATCACAAGTACAGAATG, and then AP-2 and BI-19, CACAAGTACAGAATGGGCCCCAG, for primary and secondary PCR, respectively. Products were then cloned and sequenced.

S1 nuclease mapping analysis. Mapping of the 5′ end of the testis-derived BI-1 cDNA onto the gene was performed according to manufacturer's instructions using the S1 nuclease protection assay kit from Ambion (Austin, TX). Poly(A)$^+$ obtained from rat testis, 1 or 3 μg, was incubated with a purified 256-bp ^{32}P-labeled, single-stranded probe (1.5×10^5 cpm) encompassing 81 bp of cDNA and 175 bp of 5′ flanking sequence. After S1 nuclease hydrolysis, protected fragments were resolved on a 6% acrylamide/7 M urea gel and visualized with autoradiography together with a sequence ladder generated by using the oligonucleotide BI-S1A, CCACAGGTAACCAATCAGATC, which also served to generate the single-strand probe.

A similar strategy was used to map the 5′ end of the lung-derived BI-1 cDNA onto the gene. Here the probe was 197 bp in length and encompassed 58 bp of cDNA sequence and 139 bp of 5′ flanking sequence. The primer used to generate the probe was BI-S1B, CGCTTCTTAGCCCACCTCTTCGG, and poly(A)$^+$ RNA was isolated from the fetal rat lung.

Plasmid construction, cell cultures, and transfection assays. Several BI-1–luciferase plasmids were constructed by subcloning BI-1 genomic sequences into the promoterless pGL3 vector (Promega). Four P1 promoter constructs were prepared from −1630, −1128, −567, and −255 bp upstream of the major transcription start point, tsp, and all extended to +66 downstream. Likewise, five P2 promoter constructs were prepared from −1395, −1028, −526, −255, and −146, and all extended to +41. BI-1 promoter activity was assessed *in vitro* using rat embryonic fibroblasts, Rat-1, fetal rat alveolar epithelial cells, 20-3 (Levine *et al.*, 1998), and human HeLa 3S cells. Rat-1 cells were the generous gift of Dr. Xiang Yu from The Pulmonary Center at Boston University School of Medicine, and 20-3 cells were an equally generous gift of Dr. Roy Levine at Cornell Veterinary School (Ithaca, NY). All cells were maintained with MEM supplemented with 10% FCS. Transfection was performed by electroporation using subconfluent cells. These were suspended at $2–6 \times 10^6$ cells/ml in serum-free RPMI medium with 10 mM dextrose and 0.1 mM dithiothreitol, combined with 15 μg of plasmid DNA (13 μg BI-1-luciferase, 2 μg of CMV-β-galactosidase) in a 400-μl volume in a 0.4-cm gene pulser cuvette (Bio-Rad) and exposed to a single-voltage pulse at 300 V, 960 μF at room temperature using a Bio-Rad Gene pulser transfection apparatus (Graham and Van Der Eb, 1973). The cells were incubated for 10 min at 23°C before being plated and were then harvested and assayed for reporter gene activity 40–48 h later. Each transfection experiment was repeated at least three times. In all transfection experiments the promoterless-luciferase construct, pGL3-Basic, was examined as a negative control, and the SV40 promoter-luciferase construct, pGL3-SV40, was examined as the positive control. The luciferase assay was performed according to the manufacturer's guidelines. Cotransfection with a CMV-β-galactosidase construct was used to correct for transfection efficiency. This assay was performed as described in Joyce-Brady *et al.* (1996).

RNA analysis. The Northern blot method is described in detail in Oakes *et al.* (1997). Briefly, total RNA was isolated from various tissues, quantitated, electrophoresed on a 1.0% agarose gel, transferred to a HyBond membrane, and cross-linked with a Stratagene UV cross-linker. The membrane was prehybridized, incubated with [α-^{32}P]CTP radiolabeled probe, washed, and dried. Absolute counts were measured on a Packard Instrument Image Analyzer (Downer's Grove, IL), and then the filter was exposed to Kodak X-OMAT film. The absolute counts were used to determine relative levels of mRNA expression. The RT-PCR is described in detail in Joyce-Brady *et al.* (1994). PCR primers were selected for each PCR, which was performed for 30 cycles on a MJ Research thermal cycler. PCR products were isolated by agarose gel electrophoresis, eluted, cloned, and sequenced. The primers for P1-derived BI-1 cDNA were AGGTGAGAAGCCCCGAGCTTGGCC and GCTGCTGTGTGGAGGGAGTTATGTGG for the 5′ and 3′ ends, respectively. The primers for P2 were TCCGAAGAGGTGGGCTAAGAAGCG and CAGGGCAGAGAGCAGGCCAGCCTG. A 676-bp cDNA probe directed against nucleotides within the coding region was generated by PCR using GGAGCCATGAATATATTTGATCGG and GCCAAGATCAGCATGAGCTTGCTG as the 5′ and 3′ primers, respectively.

Statistics. Transfection results for P1 and P2 in each cell line were analyzed for differences by nonparametric analysis of variance using the Kruskal–Wallis test. Pairwise comparison was performed with the Mann–Whitney *U* test using a Bonferroni correction for multiple comparisons. Differences that generated a $P < 0.05$ were considered significant.

Nucleotide sequence accession number. The GenBank accession number for the rat Bax inhibitor-1 genomic sequence is AF118564.

RESULTS

Lung BI-1 cDNA Has a Novel 5′ End

The coding region of our lung-derived BI-1 transcript matched exactly with that of the original testis-derived transcript (Walter *et al.*, 1994). This identity continued until 29 bases upstream of the ATG initiation codon, where the two diverged (see Fig. 1). We used 5′ RACE to clone any remaining 5′ sequences from lung BI-1 cDNA and found a total of 55 unique bases that extended to the very 5′ end. Hence a novel BI-1 transcript is expressed in the lung compared to that of the testis. The unique sequences in the 5′ end are shown for each transcript in Fig. 2. The lung sequences extend from 1434 to 1489 and the testis sequences from 2055 to 2139.

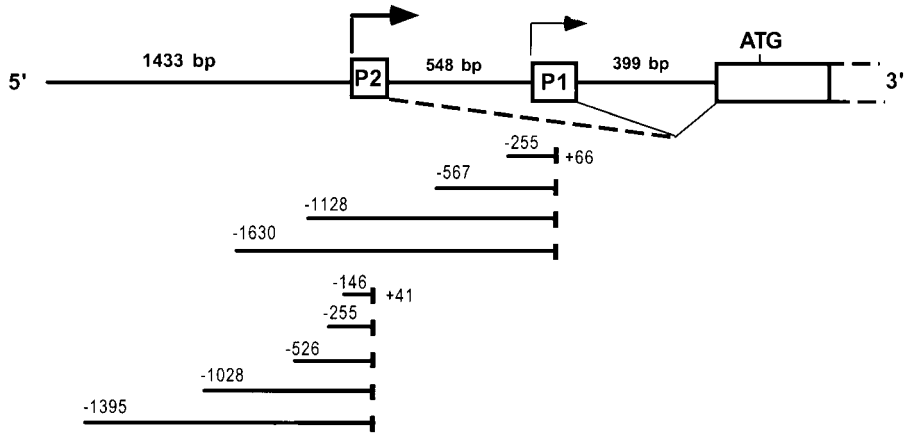

FIG. 1. BI-1 gene organization. Schematic view of the rat BI-1 gene and the location of upstream DNA sequences utilized for identification of promoter activity.

Sequence and Transcription Start Point Analysis of the 5′ Region

The heterogeneity at the 5′ end of these two BI-1 mRNA transcripts suggested the presence of alternative first exons in this gene. To examine this, we analyzed the 5′ end of the BI-1 gene using a genomic DNA template and a sequence-specific PCR primer from the unique regions of lung-derived versus testis-derived BI-1 mRNA as the 5′ primer together with a 3′ primer from the common domain as described under Materials and Methods. The PCR product obtained with the former primer was ~1 kb while that with the latter primer was only ~0.5 kb. When the ~1-kb product was cloned and sequenced, the unique sequences from the 5′ UT of testis-derived TEGT were located ~500 bp downstream of our lung-derived sequences. The 5′ boundary for each alternative intron 1 was delineated by the highly conserved dinucleotides GT and the common 3′ intron boundary by a CAG trinucleotide (Fig. 2).

An additional 1.4 kb of upstream genomic BI-1 DNA was also cloned and sequenced via two consecutive rounds of genomic walking for a total of ~2.5 kb as shown in Fig. 2. There was no identifiable TATA element at the usual position in either region of DNA although P2 did contain a CAAT box (1346–1349). Therefore, we mapped each transcription initiation site by S1 nuclease assay as shown in Fig. 3. A major transcription start point, tsp, for the testis-derived transcript was located 18 bp upstream of the most 5′ nucleotide in the published cDNA but multiple other minor sites were also evident, spanning at least six consecutive nucleotides. Sequences surrounding a major tsp at adenine 2037 conform to a consensus initiator element and putative binding sites for SP1 (1863–1873, 1907–1917), NF-κB (2019–2029, ATF (1992–2001), USF (1859–1871), and CAAT-CP2 (1869–1880) are identifiable within 180 bp upstream.

A major tsp for the lung-derived transcript agreed quite well with the 5′ RACE analysis of the cDNA. This was another adenine residue at position 1435. Minor sites at the preceding two nucleotides were also present but here transcription initiation was limited to these three consecutive nucleotides. Sequences overlapping this tsp also conformed to an initiator element, and putative binding sites for Sp1 (1361–1371, 1373–1383), AP2 (1337–1346, 1345–1354, 1357–1367, 1384–1394), NF-κB (1378–1387), and CAAT-NF1 (1261–1275) were identifiable within 180 bp upstream. Neither promoter had identifiable AP1, AP3, or C/EBP sites but, commensurate with a role in apoptosis, 15 putative p53 sites as well as sites for the Myb family and the Wilms tumor suppressor gene, WT-1, are identifiable within upstream BI-1 DNA.

Identification of Alternative Promoter Activity

To assess the ability of the putative alternative exon 1 sequences to drive reporter gene expression *in vitro*, a series of heminested deletion constructs were prepared for P1 and P2 in the promoter-less pGL3-Basic vector. Transient tranfections were performed in Rat-1 ($n = 3$), 20-3 ($n = 6$), and HeLa ($n = 4$) cells. Activity is expressed relative to the control pGL3-promoter vector under the control of SV40 sequences, all as described under Materials and Methods and shown in Fig. 4. The promoterless pGL3 Basic vector was the negative control and exhibited little activity (data not shown).

All four P1 promoter constructs generated luciferase activity in each cell line tested *in vitro*. The patterns were similar in rat and human epithelial cells but differed slightly in rat fibroblasts. Luciferase activity was lowest in human cells and, although not directly evident on the graph, highest in rat fibroblasts when adjusted for the relative protein content needed for the assay. Compared to the longest P1 construct, −1630 to +66, the deletion of P1 sequences down to −1128 to +66 and −567 to +66 failed to alter activity in any dramatic way. Exclusion of all sequences in common with the P2 region in the −567 to +66 construct clearly indicates that P1 exhibits P2-independent promoter activity. In addition, deletion of additional sequences down to −255 to +66 uniformly reduced activity to approximately fivefold in all cells tested. The elimina-

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aaacttcaga tccaaatcta gaatgctagt tgcttccccc acatctcttc 50
accaccatca ccacctccct cctctttcct cctcctcctc cctgtctggt 100
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cctgtttctc catggtttct ctgggtattt ctagctgttc tggaacttgc 200
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tgggtagact tccagtgcca acccccggac actcAGAGCA CATCCGGTTT 1450
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gctaggatcg ggcaagcctg gagtgaggga tactgacgga gcaagagcgg 1550
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tcgggttcct ccgcctcgcc tgggtgagcc gtcgggtttg gggacttgag 1950
gtaggcccgg ttaggcagac tgccctgtca cggcgggcta ggtgatgtca 2000

cacttctctg tgacacgcga gattccttag ttacttAGAA GCCAACGGTA 2050
GAGGCCGAGG TGAGAAGCCC CGAGCTTGGC CTGGCCCCCC CGCCCCACGG 2100
AGCAGCCCCA CTCTGATCTG ATTGGTTACC TGTGGACAGg tgaggtggcc 2150
ttgattgctt gatcctgaac cctgggtgag ccccaaagtc tgctccggaa 2200
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TGAACAGACT TGGAGCCATG AATATATTTG ATCGGAAGAT CAACTTTGAT 2600
GCCCTC 2606
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FIG. 2. BI-1 genomic sequence. A total of 2.6 kb of genomic sequence in shown. Capital letters at 1434 to 1489 and 2037 to 2139 denote sequences for the two alternative first exons derived from P2 and P1, respectively. Capital letters from 2539 to 2606 denote the 5′ region of common exon 2 and the boldface ATG marks the translation initiation site. Arrows identify major transcription start points and dots additional sites. Solid, dashed, and broken underlines are putative transcription factor binding sites for *Myb*, p53, and WT-1, respectively.

tion of binding sites for ubiquitous factors such as AP2 (1683–1693), NF-κB (1700–1709), and Sp1 (1637–1647) may have led to the reduction in reporter gene activity.

All five of the P2 constructs also generated luciferase activity in each cell line tested. In common with P1, activity was lowest in the human epithelial cells and highest in rat fibroblasts. Sequential deletion of sequences down to −255 to +41 also failed to alter activity in any dramatic fashion when compared to the longest construct from −1395 to +41. However, the patterns of activity with P2 sequences in the rat-derived cells were more similar than that from the human cells, and deletion of additional sequences down to −146 to +41 failed to reduce activity in any circumstance. Hence these limited P2 sequences alone can sustain reporter gene expression in all of the cell lines tested, and potential binding sites for ubiquitous factors such as Sp1, AP2, and NF-κB are found here.

Statistically, the activities generated by the four promoter constructs of P1 and the five promoter constructs of P2 varied significantly in all the cell lines tested (overall ANOVA $P < 0.05$). Pairwise comparisons reached statistical significance only in the 20-3 cells where the P1 −1128 to +66 construct has significantly higher activity and the −255 to +66 construct had significantly lower activity than all other P1 constructs ($P < 0.05$). The −1630 to +66 construct and the −567 to +66 construct were not different from each other but were different from the −1128 to +66 and the −225 to +66 constructs. Similar analysis for the P2 constructs

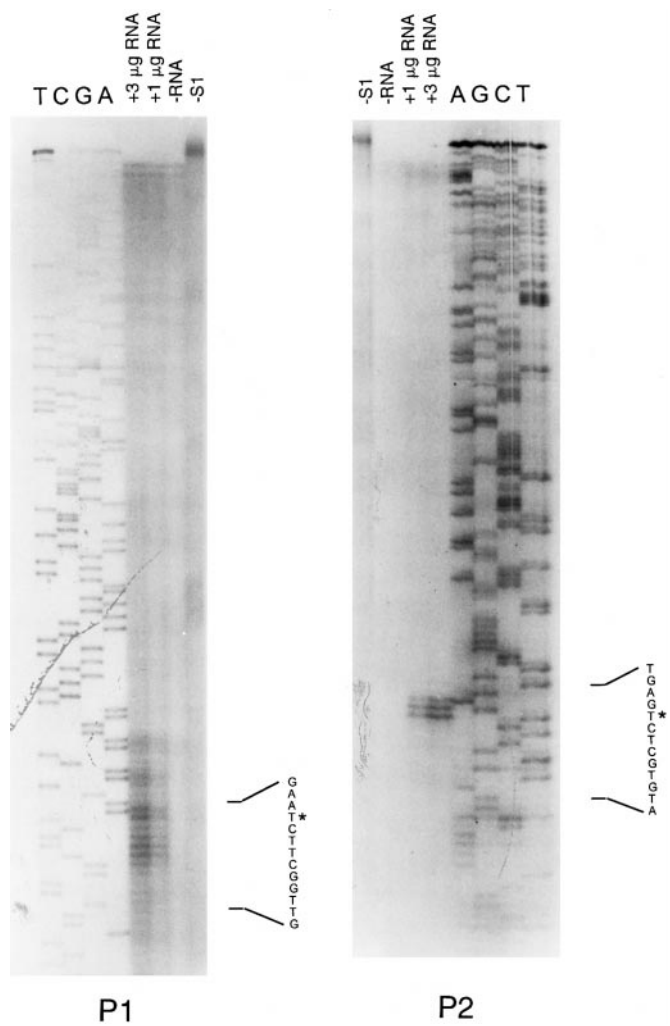

FIG. 3. Mapping of transcription initiation sites. S1 nuclease analysis is shown at left for P1 and at right for P2. In each case, probe is shown in the absence of enzyme (−S1), in the absence of RNA (−RNA), and in the presence of 1 or 3 µg of RNA along with a sequencing ladder as described under Materials and Methods. Protected sequences from the complementary strand are shown to the right and asterisk denotes +1.

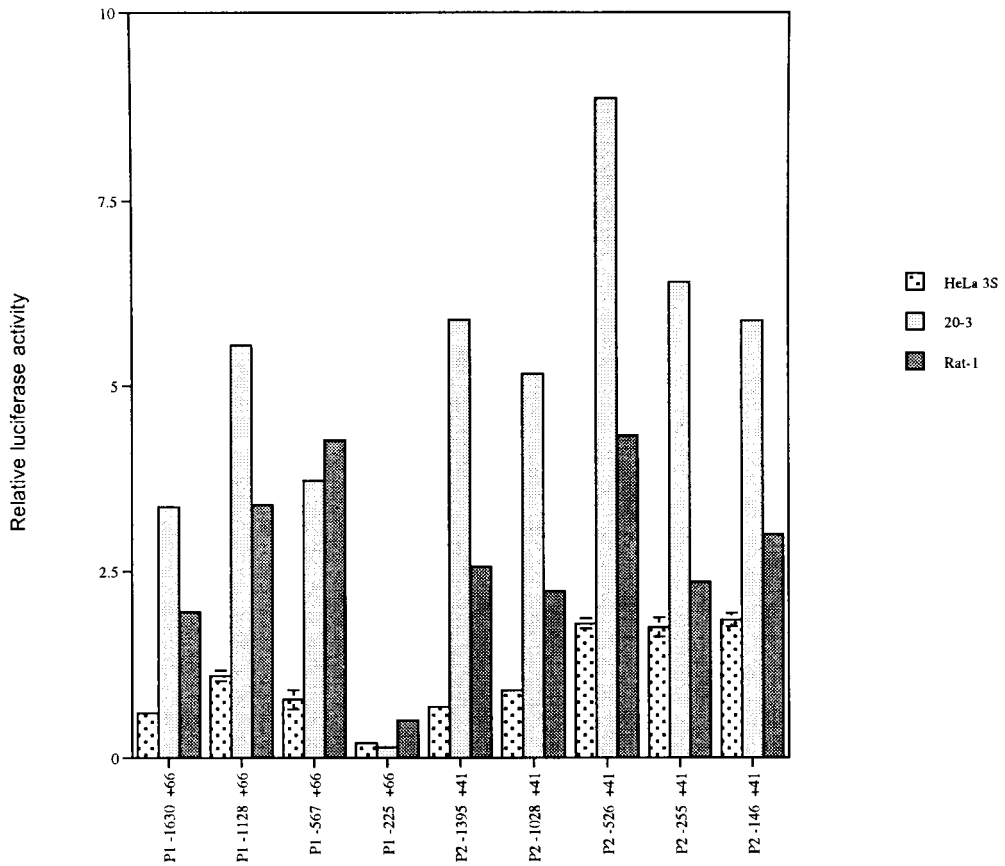

FIG. 4. Alternative BI-1 promoter activity *in vitro.* Transient transfection analyses were performed and analyzed as described under Materials and Methods. Abscissa shows various P1 and P2 constructs tested in three different cell lines, and ordinate shows activity normalized to that obtained with SV40-driven reporter gene.

revealed that the activity of the −526 to +41 construct was significantly greater ($P < 0.05$) than the other four constructs but the these four constructs did not differ from one another.

Tissue-Specific Expression of BI-1 P1

To determine whether these promoters are active in a tissue-specific fashion *in vivo,* we analyzed RNA obtained from several organs for BI-1 P1 and P2 expression by RT-PCR using exon 1-specific 5′ primers as described under Materials and Methods. BI-1 P1 was undetectable in RNA derived from any tissue except that of the testis. In contrast, BI-1 P2 was detected in lung, heart, liver, kidney, spleen, and testis. Hence, BI-1 P1 activity is highly tissue-specific while BI-1 P2 appears to be active in a ubiquitous fashion. P1 and P2 are both active in the testis (Fig. 5).

Lung Developmental Regulation of BI-1 P2

We used RT-PCR as described above to examine the pattern of BI-1 mRNA expression in fetal lung. We found that BI-1 P2 alone is expressed in the 20-day gestation fetal lung. We then analyzed total lung RNA at several different times during gestation for BI-1 mRNA expression by Northern analysis to confirm that rat BI-1 is developmentally regulated in lung as shown in Fig. 6. Two BI-1 mRNA transcripts of distinct sizes are evident in the upper panel, and this difference in transcript size in lung likely results from differential usage of polyadenylation signals as described previously (Walter *et al.*, 1994). One is ~2.8 kb and the other ~1 kb but the larger transcript is more abundant than the smaller one. β-actin was used as a control of mRNA integrity (Fig. 6, lower panel). Using the absolute counts obtained from the Image analyzer, the 2.8-kb BI-1 mRNA is induced threefold, compared to the actin signal, between 18 days of gestation and the time of birth. Changes in the smaller transcript are less evident, in part due to the lower level of expression. The larger transcript remains up-regulated over the first 24 h after birth but at postnatal day 7, it has declined to the level seen in fetal lung at 21 days of gestation. These transcripts remain detectable in RNA from the late postnatal (20 days of age) and adult lung, and the abundance is similar to that at postnatal day 7. Hence BI-1 exhibits lung developmental regulation during the perinatal period.

DISCUSSION

BI-1 was originally cloned as testis-enhanced gene transcript, TEGT, a novel, conserved, single-copy gene in the rat. The gene was developmentally regulated in

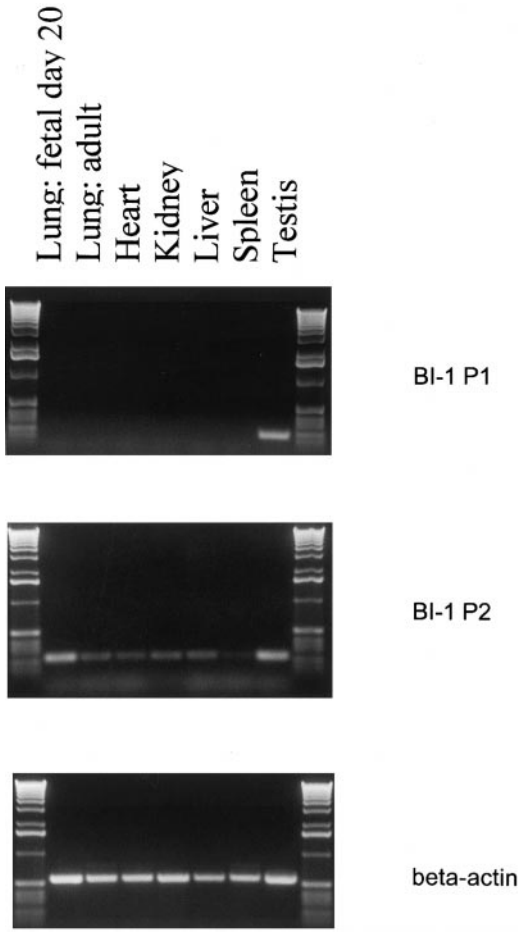

FIG. 5. Tissue-specificity of BI-1 P1 and lung developmental regulation of P2. RT-PCR analysis was performed as described under Materials and Methods. P1 (**top**) and P2 (**middle**) derived BI-1 mRNA expression was examined in fetal and adult lung plus five other tissues from adult rat. β-actin (**bottom**) expression served as a control for mRNA integrity.

the testis, and transcript abundance was greatest in the postmeiotic spermatocyte. Two cDNA transcripts of different sizes were identified, and these were thought to differ only at the 3′ end based on the usage of alternative polyadenylation signals. TEGT transcripts were also evident in many extratesticular tissues but the presence of distinct nucleotides at the 5′ ends was not readily apparent since the difference is so small in relation to the size of the intact transcripts. These original studies also showed that the encoded gene product exhibited weak homology with two bacterial proteins, which, taken together, suggested that this gene product was widely expressed in adult rat tissues and highly conserved in evolution. The gene was localized to rat chromosome 7, and homologs were identified in mouse and human and mapped to chromosomes 15 and 12q12–q13, respectively. TEGT's function was unknown (Walter *et al.*, 1994, 1995) until the recent identification of Bax inhibitor-1.

The BI-1 gene was identified through a functional screening assay in yeast cells designed to select for human cDNA that could inhibit Bax-induced apoptosis. Hence BI-1 is a new mammalian suppressor of apoptosis. While BI-1 inhibits cell death to a similar extent as Bcl-2, it is not a universal suppressor as it fails to block Fas-induced apoptosis. Furthermore, the exact mechanism by which BI-1 suppresses apoptosis is not yet fully understood because BI-1 protein does not appear to interact directly with Bax, a mediator of apoptosis, but rather with Bcl-2, an anti-apoptotic protein. Although the biological significance of BI-1 as an apoptotic suppressor gene remains to be defined, this gene is identical to TEGT (Xu and Reed, 1998).

Our laboratory has had a long-standing interest in lung development. As such, we used subtractive suppression hybridization as a tool to identify developmentally regulated genes in perinatal rat lung, and one of the genes recovered was BI-1. The recent functional identification of BI-1 together with the ontogeny of apoptosis in perinatal lung led us to characterize lung-derived BI-1 cDNA further. We have now shown that the lung and the testis transcripts are derived from two alternative promoters in this single-copy rat gene. The transcript from the testis originates from a proximal promoter, P1, which is located ~400 bp upstream of the translation initiation site. In contrast, our novel lung-derived transcript originates from a more distal promoter, P2, which is located 602 bp upstream of P1. These two promoters generate alternative first exons that splice into a common site of exon 2 using highly conserved sequences at the intron boundaries. Exon 2 encodes the shared sequences in the 5′ untranslated region of the two cDNAs and the initial coding domain, and we did not identify any alternative spliced products within this region. Neither P1 nor P2 contains identifiable TATA elements within 100 bp of the transcription start points. Rather each transcription start point is overlapped by a consensus initiator element (PyPyA + 1 NT/APyPy), and several Sp1 and AP2

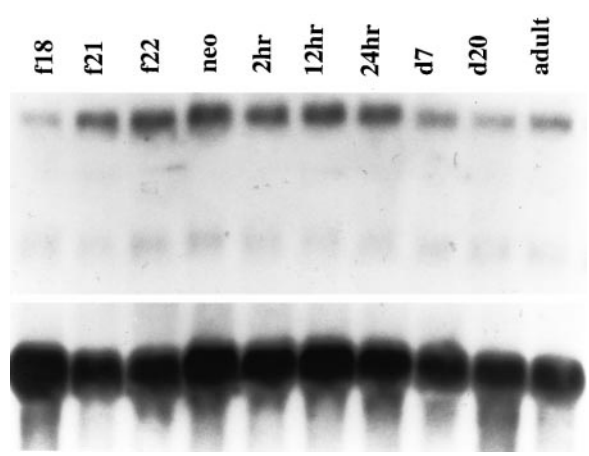

FIG. 6. Lung developmental regulation of BI-1 mRNA. Northern blot analysis was performed as described under Materials and Methods. Time points are fetal days 18, 21, and 22 (**f18, f21, f22**), newly born (**neo**), 2, 12, and 24 h after birth (**2 hr, 12 hr** and **24 hr**), 7 days old (**d7**), 20 days old (**d20**), and adult. (**Top**) 2.8- and 1-kb signals obtained with the BI-1 probe. (**Bottom**) β-actin signal as a marker of mRNA integrity.

consensus sites reside upstream. In each case the initiator elements fulfill the critical criteria of A at +1, T/A at +3, and a pyrimidine at −1. Only three of the nucleotides surrounding the tsp are pyrimidine residues but these need not be present in all four sites (Smale, 1997). Hence both promoters appear to be $TATA^{-}Inr^{+}$ but P2 does contain a CAAT box.

Dual promoters also regulate the mouse *bcl*-2 gene. Here the proximal promoter exhibits a restricted pattern of activity in a subpopulation of B cells while the distal promoter is utilized in all other B cells (Smith *et al.*, 1998). The factors that mediate this differential activity remain undefined. Only one promoter has been defined thus far for the human *bax* gene but this promoter is a target for p53-mediated transcriptional activation (Miyashita and Reed, 1995). In contrast, the *bcl*-2 promoter contains a p53-dependent negative response element (Miyashita *et al.*, 1994). Clearly these two genes, which function in apoptosis as opposing factors, are also differentially regulated by p53. At least 15 sites for p53 binding are identifiable in the BI-1 promoter but further studies will be required to determine whether they function to repress BI-1 activity. In addition, the BI-1 promoter contains putative transcription factor binding sites for the *Myb* family and the Wilms tumor suppressor gene, which function to activate and repress the bcl-2 gene during myeloid and renal development, respectively (Hewitt *et al.*, 1995; Frampton *et al.*, 1996; Taylor *et al.*, 1996).

Our transfection studies have clearly demonstrated that BI-1 P1 and P2 are functional promoters *in vitro.* All of the promoter constructs exhibited more activity in rat-derived cells than in human cells. However, the pattern of activity generated by the various P1 constructs appears more similar between epithelial cells, regardless of species, than in fibroblasts, suggesting that P1 activity could be regulated by cell type-specific factors. In contrast, the pattern for the various P2 constructs appears more similar for rat-derived cells, regardless of cell type, than for human cells, suggesting that P2 could be regulated by species-specific factors. Further studies will be required to address these differences.

Despite the activity of both promoters *in vitro,* the results of our RT-PCR analysis *in vivo* showed that P1 is tissue-specific in the adult and that P2 is developmentally regulated in the lung. The difference in the activity of the P1 promoter *in vitro* and *in vivo* could result from an altered DNA methylation (Kuramasu *et al.*, 1998) but this remains to be determined. Our results have extended the findings of previous studies because we have now shown that both BI-1 promoters are active in the testis. This could reflect either developmental stage-specific or cell-specific regulation of these promoters in testicular cells.

BI-1 P2 is developmentally regulated in perinatal lung but the mechanism is not yet known. The up-regulation of BI-1 mRNA expression in the late fetal lung could involve a change in gene transcription at the distal P2 promoter or a change in BI-1 mRNA stability. Many genes are activated at a transcriptional level in the late fetal lung. Activation of BI-1 P2 could be mediated in a hormone-dependent fashion by either of two glucocorticoid response elements located within upstream DNA or in a hormone-independent fashion via a developmentally regulated change at the transcription start site mediated by the initiator (Smale and Baltimore, 1989). The down-regulation of BI-1 expression in early postnatal lung could reflect an actual change in cellular BI-1 mRNA levels or a change in the cell populations that express BI-1 mRNA.

The biological role of BI-1 expression in perinatal lung also remains to be defined. The degree of apoptosis appears to rise dramatically around birth (Kresch *et al.*, 1998), and this process may play a critical role in eliminating cells from the perinatal lung, particularly mesenchyme. A gene such as BI-1, then, could function in an equally critical role to select for cell survival. Our preliminary *in situ* hybridization studies reveal that BI-1 expression is accentuated throughout the epithelium of the developing lung where little apoptosis occurs. Hence further study of BI-1 gene expression in these cells may provide new insight into the biology of lung epithelial cells during the perinatal period and potentially during repair following lung injury and during lung carcinogenesis.

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