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DNA Binding Proteins That Amplify Surfactant Protein B Gene Expression: Isolation and Characterization

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We identified and characterized two proteins that bind the promoter of surfactant protein B (SP-B) and affect its expression. Proteins A2 and B were identified and their cDNAs cloned and sequenced. Both were novel. They bound a 212-bp functional promoter region at an NF1 site, located between -184 and -198. Effects of these DNAbp on SP-B promoter activity were studied by contransfecting a reporter construct of this 212-bp sequence + luciferase, together with expression constructs for A2 and B into H441 cells. Alone, A2 and B expression elicited modest but statistically significant increases in SP-B promoter activity. When dexamethasone was added, B further increased SP-B promoter activity. For SP-B, basal expression and glucocorticoid responsiveness may involve a number of hitherto unknown gene activators.

Pulmonary surfactant is a combination of proteins and phospholipids produced by type II pneumocytes. Surfactant establishes and maintains low intraalveolar surface tension. Of the several surfactant proteins, surfactant proteins B (SP-B) and C (SP-C) participate most directly in surface activity. A mixture of surfactant phospholipids, plus SP-B or SP-C recapitulates the surface activity of natural surfactant (1).

The mechanisms that regulate surfactant production and secretion are not well understood. In culture, surfactant proteins are expressed at a basal rate, which may be affected by pharmacologic, physical and physiologic factors (2). In particular, glucocorticoids enhance surfactant secretion, and accelerate lung maturation (3,4), and upregulate SP-B expression (5,6). Bohinski and coworkers recently described the control of human SP-B gene expression, defining the SP-B

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promoter regions that drive reporter gene expression. They reported SP-B promoter binding site for five lung-specific DNA binding activities (7).

We report here two novel DNA binding proteins (DNAbp), A2 and B that influence expression of the rabbit SP-B gene. Both increase SP-B promoter activity in the absence of added steroids. In addition, protein B increases SP-B promoter activity when dexamethasone is added.

METHODS

Proteins A2 and B. An adult human lung cDNA library, cloned into the Eco RI site of λgt11, was prepared (8). was screened using probes for A2 and B. A2 and B were identified as DNA binding activities from human lung that bound an area of the SP-B gene that we and others had found to have promoter activity (Luzi, *et al.*, submitted for publication, 10). By virtue of these characteristics, and by partially characterizing their sequences and DNA binding activity, we isolated and cloned cDNAs for proteins A2 and B (Luzi, *et al.*, in preparation). *E. coli* strains Y1090 and Y1089 were used as host cells. The cDNAs for proteins A2 and B were then subclone as Eco RI fragments into pGEM3 for sequencing.

DNA labeling. Gel-purified DNAs were isolated and labeled by random priming (8). For other purposes, insert identification, etc., DNA fragments were end labeled with ^{32}P-ATP using polynucleotide kinase or the Klenow fragment of DNA polymerase (8), and purified using G50 Sephadex (Pharmacia).

Sequencing using pGEM3. cDNAs for proteins A2 and B were cloned into pGEM3 and sequenced (8) using Sequenase (USB). SP6 and T7 primers were used initially. Specific synthetic oligonucleotides were subsequently used as continuing primers. Both strands were completely sequenced. DNAs were also subjected to automatic sequencing using Applied Biosystems 373A DNA Sequencing System (Jefferson Cancer Institute).

The sequence of the rabbit SP-B gene was reported to GenBank and assigned accession number L11572. The human cDNA clones were reported to GenBank and given accession numbers L10403 (A2 cDNA) and L10404 (B cDNA).

Sequence analysis. Analysis and assembly of DNA sequences was facilitated by DNA Inspector IIe (Textco, Inc., Lebanon, NH). Comparison of DNA sequences to those reported to GenBank was done with GCG Software (Jefferson Cancer Institute), using the algorithm of Needleman and Wunsch is used to identify and define the degree of homology (9).

Lysogeny. DNAbp encoded by A2 and B cDNAs were prepared in order to study their function. Lysogenic λgt11 phage infection was induced in Y1089 cells (10). Lysogens containing the λgt11 recombinant phage were purified, and recombinant phage-encoded proteins prepared according to published protocols (10), after treatment of the lysogenic colonies with 10 mM IPTG Protein extracts were aliquoted immediately, quick-frozen in liquid nitrogen and stored at -70° C. These preparations were used to analyze DNA binding in gel-shift assays.

DNAs used. All locations and fragment sizes refer to the rabbit SP-B genomic DNA sequence, and are relative to the *translational* start site (the first "A" of ATG). A restriction map of this portion of the SP-B gene is shown in Fig. 1.

Gel shift analyses. Protein-DNA binding reactions were performed according to standard protocols (10). End-labeled double stranded DNA fragments and 5-8 μl of protein extracts from Y1089 strain *E. coli* lysogenically infected with the several cDNA-containing phage, + 1 μg/μl poly (dI-dC) in an incubation buffer (16) were incubated for 40 min at RT, electrophoresed on 8% acrylamide gels with 1x TRIS-Glycine buffer + 2.5% glycerol, and visualized by autoradiography.

Plasmid construct p212luc. The Bam HI-Dpn I fragment was gel-purified, and cloned into the Hind III site of pGL2 (Promega) using Hind III linkers. Correct orientation was ascertained by DNA sequencing. This fragment is 5' to the SP-B transcriptional initiation site and includes TATAA. The resulting plasmid is called p212luc.

Expression of recombinant DNAbp. Recombinant DNA binding proteins were expressed by cloning them as Eco RI fragments into the Eco RI site of the expression vector pKC4 (kind gift of Dr. J. Whitsett). Expression of genes in the cloning cassette is controlled by SV40 early promoter. Insert orientations were ascertained by DNA sequencing. The resulting plasmids in which proteins A2 and B cDNAs were cloned into pKC4 were pKC4A2 and pKC4B respectively.

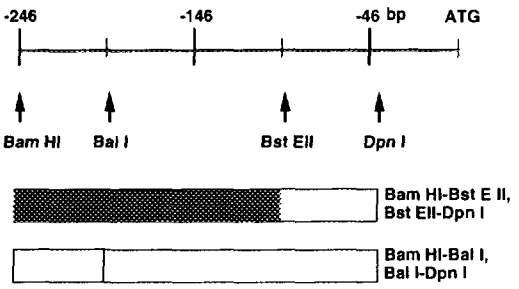

Figure 1. Partial Restriction Map of 5' portion of rabbit SP-B gene. The 246 bp from the first translated ATG of rabbit SP-B gene upstream to the Bam HI site at -246 are shown here, highlighting the restriction sites used to generate both SP-B genomic fragments for gel shift assays and the SPB212 reporter construct. In this illustration, the schematic bars under the map points indicate which restriction subfragments were bound by A2 and B (dark bar) and which were not (light bar). (Refer to Fig. 2)

Luciferase assay and effects of DNAbp on reporter gene expression. Luciferase was measured according to instructions provided with pGL2 plasmid (Promega). Briefly, H441 cells (ATCC) were grown in RPMI1640 + 10% FCS (Hyclone). They were transfected in suspension using the $CaCl_2$ method (16) and harvested 3 days later. To study the effects of A2 and B on SP-B promoter activity, pKC4A2 or pKC4B was cotransfected into H441 cells with two other plasmids (see above). For a 25 cm^2 culture dish of H441 cells, 4 μg of control plasmid, pCMVβ, and 8 μg of reporter plasmid, p212luc, were used. In addition, 16 μg of the pKC4A2 or pKC4B were used. When the effects of dexamethasone (dex) were studied, the final concentration of dex was 1 μM. After cell lysis with Reporter Lysis Buffer, the Luciferase Assay System was used according to manufacturer's instructions (Promega). A luminometer (Chem-Glow, Aminco, Inc.) was used to assay luciferase activity. Light output is expressed in arbitrary units, background (buffer-only blank) subtracted. Transfection efficiency was ascertained by cotransfection with pCMVβ (Clontech). Luciferase activity was normalized to β-galactosidase levels.

RESULTS

Structural analysis of proteins A2 and B DNA. On screening of phage DNA for cDNA clones that encoded proteins A2 and B (Luzi, *et al.*, in preparation), we identified and plaque-purified a unique phage clone containing A2 cDNA and one containing B cDNA. Phage DNA was digested with Eco RI to liberate inserts. The A2 insert was 1.3 kb, and that for B was 1.2 kb Table 1 describes the features of A2 and B open reading frames (orfs). These proteins show some amino acid similarity to steroid receptor proteins (Table 2). However, in part because of their small size, the numerical values assigned these homologies are relatively low (opt <60) (Table 3).

Characterization of the DNA-binding activities of these proteins. DNA binding properties of the SP-B DNAbp were analyzed by gel shift assay. Recombinant λgt11 phage containing A2 and B cDNAs were used to express the encoded DNAbp. The ability of these proteins to bind SP-B gene, and the binding locations were studied by gel shift analysis.

By gel retardation analysis, we found that both A2 and B to bound the SP-B gene through recognition of a 212 bp Bam HI-Dpn I fragment (SPB212, -253 to -42 bp). Subfragments of

Table 1. Characteristics of A2 and B cDNAs and open reading frames

cDNA	length (bp)	orf size (kDa)	orf size (aa)
A2	1336	11.1	106
B	1266	9.3	83

Characteristics of the 2 cloned, sequenced cDNAs whose protein products bind the SP-B upstream region are shown.

SPB212 were then tested in the same way (Fig. 2). Subfragments of SPB212 containing the Bal site at -194 were bound by both proteins. However, digestion of the 212 bp SP-B genomic DNA fragment with Bal I (at -194) virtually eliminated the ability of both A2 and B to bind either of the resulting daughter genomic DNA fragments. Control protein extracts were prepared using the same clones of *E. coli*, infected lysogenically with each individual recombinant phage, but cultured without IPTG. Protein mixtures from such uninduced cultures often yielded slight gel shifts that were considerably weaker than those obtained with protein preparations from cultures stimulated with IPTG (Fig. 2).

Effects of A2 and B on SP-B promoter activity. Using a reporter construct (p212luc) containing SPB212 cloned upstream from luciferase in pGL2luc (Promega) we found that the 212 bp upstream SP-B genomic fragment bound by A2 and B showed promoter activity (Luzi, *et al.*, submitted). The functional significance of A2 and B binding to SP-B DNA was thus addressed. To express the DNAbp, A2 and B were cloned into pKC4 (to yield pKC4A2 and pKC4B). pKC4A2 or pKC4B was cotransfected with p212luc and pCMVβ into H441 cells (see **Methods**), a Clara cell tumor used to express SP-B (7). A2 and B transcripts were expressed in this system (not shown).

In cultures lacking dexamethasone expression of A2 and B increased reporter gene activity to 1.7x and 1.85x (respectively) (Fig. 3). These increases were statistically significant when compared to p212luc alone: for A2, P = 0.01; for B, P = 0.02. Adding dex to cultures receiving pKC4B increased luciferase activity to 1.6x that seen with p212luc + dex alone (P = 0.04). This

Table 2. Amino acid sequences for orfs A2 and B

orf	Amino Acid Sequence
A	MPPCSCARSL CALQVLLLTV LGSSTNGQTK RNIGKRKCRD LFLAPVAASA IPVSGRKTGL VLLGLESAII TTNPSGGVTE RGHFTFAGHS AGHFTKALQI IFTMAL
B	MILKHHFNPV FPLLTKTNSG IKGTLHLGLK CLSLLPVLSP PFRSDRTTQS PTLVRLILLL PFFSRNPSLD LRNSTSHLKV TVH

The open reading frame sequences for A2 and B, as deduced from cDNA sequencing.

Table 3. Homology of A2 and B to steroid receptors and other proteins

orf	Homologous Proteins	Degree, Location of Homology
A2	human glucocorticoid receptor	48% similarity, 22% identity (aa39-199)
	human estrogen receptor	49% similarity, 22% identity (aa426-590)
	human mineralocorticoid receptor	40% similarity, 20% identity (aa148-266)
	human progesterone receptor	42% similarity, 21% identity (aa99-246)
	S. cerevisiae mitochondrial HSP70	35% similarity over 27 aa
B	human glucocorticoid receptor	41% similarity, 22% identity (aa351-450)
	human estrogen receptor	49% similarity, 22% identity (aa408-508)
	human mineralocorticoid receptor	51% similarity, 23% identity (aa777-898)
	human progesterone receptor	48% similarity, 18% identity (aa690-787)
	Drosophila sevenless protein	33% similarity over 35 aa

Protein homologies were determined from orf sequences of A2 and B GCG software, using the algorithm of Needleman and Wunsch (15). Amino acid position designations refer to portions on the several steroid receptor proteins that are homologous to the orf indicated. All homologies include gaps.

value (pKC4B + p212luc + dex) was not significantly different from that seen when H441 cells received p212luc + pKC4B – dex ($P = 0.06$). Dex did not alter luciferase activity in H441 cells transfected with pKC4A2 or with the positive control plasmid, pGL2luc(SV40).

DISCUSSION

Our studies were undertaken to identify DNA binding proteins that might affect expression of the surfactant protein B gene. Based on data from analysis of DNA binding activities A2 and B that were found in the lung, a lung cDNA library was screened to identify cDNA clones encoding A2 and B proteins that bound SP-B genomic DNA. By gel shift analysis, we found that both of these proteins recognized a small part of that SP-B gene, located at a Bal I site 194 bp upstream from the translational start site. Though these proteins bound the SP-B gene specifically at that Bal I site, their ability to bind other genes is not known and is under study.

This portion of the rabbit SP-B gene has promoter activity. In H441 cells, SPB212 (-248 to -41 promotes expression of a reporter gene efficiently. These data confirm in part the observation of Bohinski *et al.*, who found promoter activity in the region from -80 to -218 of the human SP-B gene (7) (corresponding, with gaps, to rabbit sequences -98 to -227).

It is not yet clear whether proteins A2 and B represent one or more previously undescribed classes of DNA binding proteins, since the structural basis for their DNA binding activity remains unclear. However, whatever their mechanism of DNA binding, A2 and B increased luciferase activity in the absence of dex to slightly less than twice controls. These increases, though not large, were reproducible and were statistically significant.

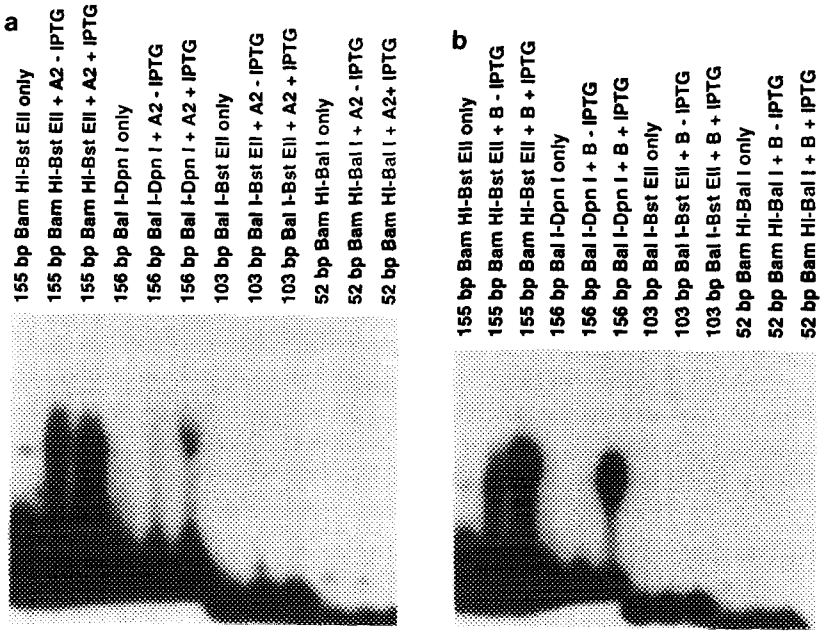

Figure 2. Gel shift analyses of clones A2 and B. Lysogenically infected Y1089 strain *E. coli* were prepared for both recombinant phage. Protein production was induced by adding IPTG to these cultures and protein preparations made from these lysogenically infected bacteria. These protein preparations were used in gel shift assays to identify the DNA binding site(s) of the several encoded recombinant proteins. (a) Localization of SP-B DNA binding by A2. (b) Localization of SP-B DNA binding by B. The individual analyses of DNA binding activities of subfragments of SPB212 by protein preparations from Y1089 cells infected lysogenically with A2 and B cultured with or without IPTG.

When protein B + dex were added to cultures receiving p212luc, luciferase activity increased significantly above that seen with p212luc + dex alone, but was not significantly greater than that seen in H441 cells transfected with p212luc + pKC4B without dex. The ability of protein B, to augment or mediate steroid responsiveness must thus be considered slight at this point. However, dex added to lung explant cultures increases SP-B expression to only 4x basal secretion levels (11). Protein B, which in our system increases dex-stimulated promoter activity almost two-fold, may therefore be one of several factors involved in this effect, which has not yet been found to involve classical glucocorticoid response mechanisms. Therefore, although the magnitude of the increased luciferase activity that we observe is not large in comparison to studies that use different experimental approaches, it is highly reproducible, statistically significant, and–most importantly–generally comparable to the increase in SP-B transcription seen in steroid-treated lungs (11).

Generally the effects of steroids on gene expression are thought to involve direct interaction between steroid receptor-steroid complexes and a responsive DNA element to which the lig-and-receptor complex binds, usually (but not always) to stimulate transcription (12,13). Our

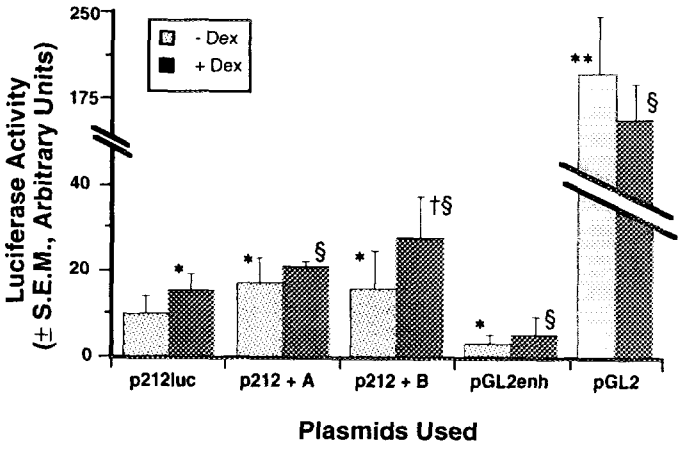

Fig. 3. Function of the 212 bp SP-B upstream region and responsiveness to dexamethasone. *H441 cells were cotransfected with a combination of plasmids and* cultured with or without 1 μM dexamethasone. All received pCMVβ as a control for transfection efficiency. Some cultures received p212luc, in which SPB212 was cloned 5' to the luciferase gene in pGL2enh, which contains an SV40 enhancer and luciferase. pGL2enh was used as a negative control. pGL2luc(SV40), in which luciferase expression was controlled by the SV40 early promoter, was our positive control. Expression of luciferase was measured 3 days after transfection using a luminometer, was corrected to reflect β-galactosidase expression (transfection efficiency), and is expressed here in arbitrary units, as the mean ± S.E.M. of 4 independent experiments.

*, $P<0.05$ compared to promoter activity p212luc alone.

**, $P<<0.01$ compared to promoter activity of p212luc alone.

†, $P<0.05$ compared to promoter activity of p212luc + dexamethasone.

§, $P>0.05$ compared to promoter activity of same plasmid combination, without added dex.

findings, and other recently reported data suggest that responses of some genes to added steroids may involve additional mediators (14,15). Proteins SWI1, SWI2 and SWI3 are required for steroid receptor-steroid complexes to activate transcription in yeast (15). Recently reported human (hbrm) and murine homologues of SWI2 potentiate glucocorticoid responses in cultured cells to about 4x those observed without transfected hbrm (16).

Regulation of SP-B gene expression is critical to several pulmonary diseases, including neonatal and adult respiratory distress syndromes. The identification and characterization of proteins that mediate and amplify SP-B expression may provide a molecular physiologic framework for new approaches to the therapy of these diseases.

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