RESEARCH COMMUNICATION

Translation *in vivo* of 5′ untranslated-region splice variants of human surfactant protein-A

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Transcripts of human SP-A genes, SP-A1 and SP-A2, undergo alternative splicing of 5′ untranslated-region exons. We reverse-transcribed and amplified free cytoplasmic and polysome-bound RNA and showed that (a) all splice variants of both genes are translated *in vivo*, (b) the relative translatability of splice variants can differ among individuals, and (c) the relative levels of different SP-A splice variants differ among individuals.

INTRODUCTION

Surfactant-association protein A (SP-A) is the major lung-specific protein present in pulmonary surfactant, the lipoprotein complex that decreases surface tension at the alveolar air/liquid interface. SP-A has important functions at a number of points in the 'life cycle' of surfactant [1–3], and also appears to play a role in local host defence in the lung [4]. In humans, SP-A is encoded by two functional genes, SP-A1 [5] and SP-A2 [6], both of which are expressed [7]. Both SP-A1 and SP-A2 exhibit a high degree of complexity at the level of the gene [8–12]. The complexity includes alternative splicing of 5′ untranslated (5′ UT) exons [8,12]. Studies *in vitro* showed that the major 5′ UT splice variants of the mRNA of each SP-A gene, and some of the minor variants, can be translated into protein [8]. The goal of the work reported here was to determine whether all or some of the SP-A splice variants are translated into protein in the intact lung.

EXPERIMENTAL

Tissue acquisition

Human lung tissue used for total RNA extraction and polysome fractionation was discarded surgical tissue. These specimens were 'normal', as assessed by gross inspection by the pathologist.

Polysome fractionation

The polysome fractionation procedure used was a modification of that described by Vary and colleagues [13]. Fresh lung tissue (0.5–1.0 g) was homogenized in 4 vol. of homogenization buffer, by using two or three brief bursts with a Polytron (Brinkman Instruments, Westbury, NY, U.S.A.) controlled by a rheostat set at 30% of maximum speed [homogenization buffer was (in mM): 25 Hepes, pH 7.5, 250 KCl, 1 magnesium acetate, 1 dithiothreitol, 250 sucrose, 0.1 EDTA]. The homogenate was centrifuged in a Beckman JA-17 rotor at 630 *g* ($r_{av.}$ 90 mm) at 2 °C for 2 min. A $\frac{1}{9}$ volume of 10% Triton X-100/10% deoxycholate was added to the supernatant and mixed. The homogenate was then centrifuged at 10100 *g* ($r_{av.}$ 90 mm) at 2 °C for 10 min. Linear 0.6–2.0 M sucrose density gradients were formed in buffer containing (in mM) 250 KCl, 2.5 magnesium acetate, 25 Tris, pH 7.7, 5 β-mercaptoethanol. Approx. 700 μl of supernatant was layered on top of each gradient, and the gradients were centrifuged at 175000 *g* ($r_{av.}$ 110.2 mm) in a Beckman SW41Ti rotor at 4 °C for 3.5 h. The gradients were fractionated with an Isco Density Gradient Fractionator (Lincoln, NE, U.S.A.). The A_{254} of the gradient was monitored during fractionation, and polysomal and non-polysomal fractions of interest were pooled. To each pooled fraction, guanidine thiocyanate, sodium citrate and β-mercaptoethanol (final concns. 4 M, 0.25 M and 4.1% respectively) were added. The samples were stored frozen at −70 °C.

Isolation of RNA and reverse-transcription (RT) PCR

RNA was isolated from pooled gradient fractions essentially as described by Ausubel and colleagues for single-step RNA isolation from cultured cells or tissues [14]. Total RNA was isolated from frozen human lung tissue as previously described [15].

RT was carried out in a total volume of 25 μl with MMLV reverse transcriptase (Gibco/BRL, Gaithersburg, MD, U.S.A.). For RT of total lung RNA, 1 μg of RNA was incubated with 15 ng of primer at 70 °C for 10 min. The reaction mixture was incubated at room temperature for 15 min with buffer provided by the vendor of the reverse transcriptase, plus 1 mM of each dNTP, 10 mM dithiothreitol and 0.5 μl of RNase block. MMLV reverse transcriptase (0.75 μl, 150 units) was added and the reaction mixture incubated at 46 °C for 1 h. The reaction was terminated by heating at 95 °C for 5 min. For RNA isolated from polysome gradients, 1 μg of RNA from the 'free' fraction and 2 μg of RNA from the 'bound' fraction (most of RNA in the bound fraction is RNA) were incubated with 25 ng of primer. PCR was carried out by using 1 μl of RT reaction mixture as DNA template in a 50 μl reaction volume. The reaction mixture contained buffer supplied by the polymerase vendor, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 150 ng of each primer, 1 unit of AmpliTaq polymerase (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). For radiolabelled PCR reactions, 0.8 pmol of ^{32}P-5′-end-labelled primer was added to the reaction mixture. Cycling conditions were: 94 °C, 30 s; 57 °C, 30 s; 72 °C, 30 s; for 40 cycles plus a 10 min extension at 72 °C. RT-PCR amplification products were separated on either non-denaturing 12% polyacrylamide gels or 6% polyacrylamide/6 M urea sequencing

Abbreviations used: SP-A, pulmonary-surfactant-associated protein A; 5′ UT, 5′ untranslated region; RT, reverse transcription.

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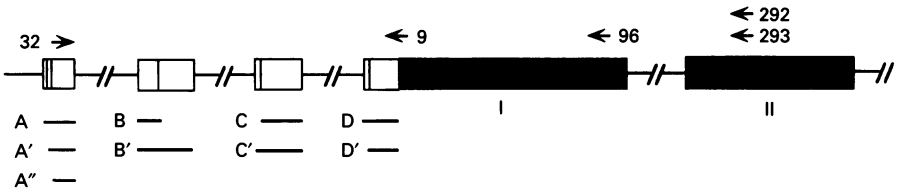

Figure 1 Genomic structure of the 5′ UT region of human SP-A

White blocks (A, B, C, D) are 5′ UT exons. Bars beneath the exons illustrate length variants of the exons. The 5′ ends of exons A, B, C are exaggerated to illustrate the size differences. The black blocks represent the first and second coding exons (I and II). The position and orientation of oligonucleotide primers used for RT and amplification in the studies described in the text are shown above the exons.

gels, and were revealed by staining with ethidium bromide or by autoradiography of the dried gel, respectively. SP-A splice variant standards were amplified by using the above conditions and cDNA clones as template. Sequences and positions of primers were (numbering of White et al. [5]): 96, 5′-TCCT-TTGACACCATCTC-3′, antisense (nucleotides 1185–1201); 292, 5′-CCATTATTCCCAGGAGGACATGGTG-3′, antisense (1555–1579); 293, 5′-CCATCATTTCCAGGAGGACATGG-CA-3′, antisense (1555–1579); 32, 5′-CTGGAGGCTCTGTG-TGTGGG-3′, sense (181–200); 9, 5′-GGCACAGCCACATG-GCTCTG-3′, antisense (1039–1058).

RESULTS AND DISCUSSION

We [8] and others [12] recently showed that the 5′ UT exons of human SP-A transcripts undergo alternative splicing. A number of different splice patterns were described and named as shown in Figures 1 and 2. These patterns vary in frequency among transcripts, some occurring frequently (AD′, ABD, ABD′) and others less commonly (ACD′, AB′D, AB′D). The most common patterns are AD′ for SP-A1, and ABD and ABD′ for SP-A2. ACD′ and AD′ transcripts occur only for SP-A1, and ABD and ABD′ transcripts occur primarily for SP-A2 [8,12]. An ABD′ cDNA for SP-A1 was described in one previous paper [7], but has not been reported subsequently, suggesting that it is a rare transcript. Most of the alternatively spliced variants were translated into protein *in vitro* [8]. In this paper we describe experiments designed to determine whether all the variants are translated *in vivo*. We considered an mRNA to be translated *in vivo* if it was bound to ribosomes.

Before isolating RNAs that are actively translated *in vivo*, it was necessary to develop a simple way of identifying and analysing the distribution of SP-A 5′ UT splice variants. We used RT-PCR with the primers shown in Figure 1. Primers 96, 292 and 293 were used for RT. Primer 96 reverse-transcribes SP-A RNA from both SP-A1 and SP-A2 genes, whereas primers 293 and 292 are specific for SP-A1 and SP-A2 respectively. Primers 32 and 9, common to both SP-A genes, were used to amplify cDNAs. Primer 32 is in 5′ UT exon A and primer 9 overlies the junction of 5′ UT exon D and coding exon I. Total lung RNA was reverse-transcribed by using primer 96 and then amplified with primers 32 and 9, along with cDNAs of known SP-A splice variants for use as standards. Amplification products were separated on a non-denaturing 12% polyacrylamide gel and stained with ethidium bromide (Figure 2). Lanes 1–6 contain the splice variant standards as indicated [8]. The RT-PCR products of total lung RNA are in lane 7. AD′, ABD, ABD′ and AB′D′ variants can be identified by comparison with the splice standards. Two prominent bands (just above the ABD and

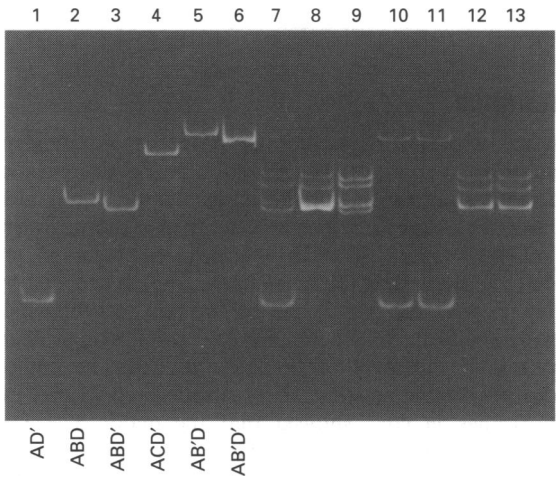

Figure 2 Human SP-A 5′ UT splice variants via RT-PCR

Splice variants are separated on a non-denaturing 12% polyacrylamide gel and stained with ethidium bromide. Lanes 1–6 contain splice variant standards AD′, ABD, ABD′, ACD′, AB′D and AB′D′ respectively. Lane 7 contains RT-PCR products of total lung RNA using primer 96 that reverse-transcribes both SP-A1 and SP-A2 mRNA. Lanes 8 and 9 demonstrate ABD/ABD′ heteroduplex formation as discussed in the text. Lanes 10–13 illustrate gene-specific RT from total lung RNA of two different individuals. Lanes 10 and 11 show RT-PCR products after RT of total lung RNA using SP-A1-specific primer 293. Lanes 12 and 13 show RT-PCR products of RT of total lung RNA from the same individuals, using SP-A2-specific primer 292.

ABD′ variants) that do not appear to correspond to any of the splice standards are also present. These bands may represent as yet undescribed splice variants, or they may be heteroduplexes formed by hybridization of closely related splice variants. To test the latter possibility, (a) ABD and ABD′ cDNAs were mixed and amplified with primers 32 and 9 (lane 8), and (b) ABD and ABD′ cDNAs were separately amplified and the amplification products were mixed, denatured by heating to 95 °C, and allowed to re-anneal at room temperature (lane 9). Both experiments demonstrate that the additional bands in question are heteroduplexes formed from ABD and ABD′ variants.

The specificity of RT from primers 293 (SP-A1) and 292 (SP-A2) is shown in Figure 2, lanes 10–13. Total lung RNA from two individuals was reverse-transcribed by using primer 293 (lanes 10, 11) and primer 292 (lanes 12, 13) and then amplified as described above. The results confirm the earlier observations [8,12] that AD′ transcripts come from SP-A1 and ABD and ABD′ from SP-A2. It also appears that, in the two individuals

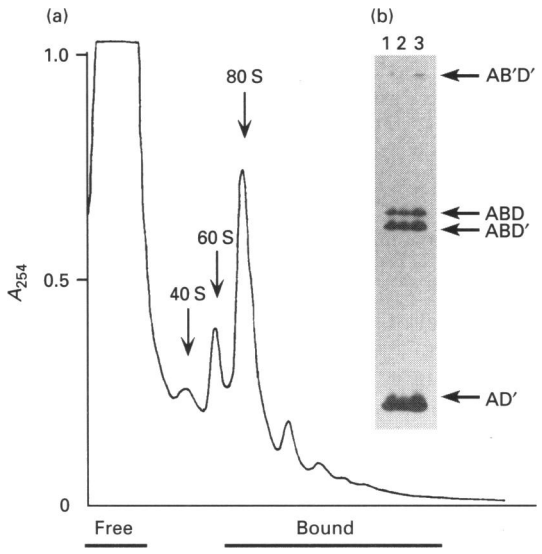

Figure 3 Translation *in vivo* of SP-A 5′ UT splice variants

(**a**) Polysome profile from human lung tissue. Polysomes were separated on sucrose density gradients, and the gradients were fractionated as described in the Experimental section. The 80 S monomer peak and free 40 S and 60 S ribosomal-subunit peaks are indicated. Material from the gradient before and after the free subunit peaks was pooled as shown (Free and Bound fractions respectively). (**b**) RT-PCR products from RNA isolated from the 'free' and 'bound' gradient fractions. This panel shows an autoradiography of RT-PCR products separated on a sequencing gel. Lane 2 contains products from 'free' mRNA; lane 3 contains products from 'bound' mRNA; lane 1 contains RT-PCR products, using primer 96 for the RT, from total lung RNA from the same individual whose lung tissue was used for the polysome fractionation.

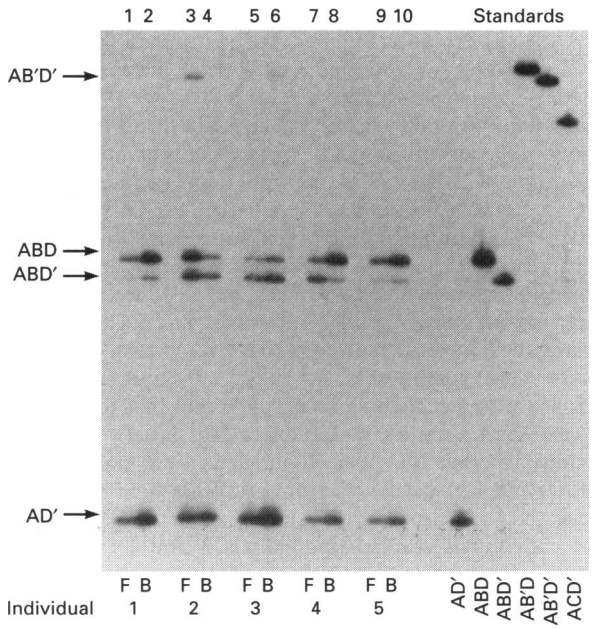

Figure 4 Relative levels and translatability of SP-A 5′ UT splice variants among individuals

RT-PCR, using primer 96 for the RT, was carried out as described in the Experimental section on RNA isolated from 'free' and 'bound' fractions of polysome gradients derived from lung tissue of five unrelated individuals. An autoradiograph of RT-PCR products separated on a sequencing gel is shown. Lanes marked F contain products from 'free' RNA. Lanes marked B contain products from 'bound' RNA. Six splice variant standards, AD′, ABD, ABD′, AB′D, AB′D′ and ACD′, are shown on the right.

studied here, AB′D′ transcripts are from SP-A1. Two minor splice patterns, ACD′ and AB′D, which were previously described [8,12], are not evident in the samples examined here and may not be discernible at the level of resolution obtained from ethidium bromide staining. For subsequent studies to determine which SP-A splice variants are translated *in vivo*, we adapted the RT-PCR procedure for use with radiolabelled primer 9 and separated amplification products on a sequencing gel.

To obtain mRNAs that are translated *in vivo*, we isolated ribosome-bound RNA. The mRNA that is actively translated into protein is bound to one or more ribosomes, forming polysomes, whereas mRNA that is not being translated is not associated with polysomes, but is present in the cytoplasm in a ribonucleoprotein complex. Polysomes were isolated by subcellular fractionation of lung post-mitochondrial supernatant on a sucrose density gradient. Untranslated mRNA remains at the top of the gradient, and free ribosomal subunits and polysomes are distributed throughout the gradient according to their relative densities. The A_{254} was continuously monitored during fractionation of the gradient. Figure 3(a) is a polysome profile obtained from human lung tissue. The 40 S (small subunit) and 60 S (large subunit) free ribosomal-subunit peaks and the 80 S peak are indicated. The 80 S ribosome complex contains mRNA bound to a single ribosome. Smaller polysome peaks containing two, three or more ribosomes can also be identified. Polysomes are extremely fragile, and are susceptible to random enzymic and mechanical disruption, breaking the mRNA strand between ribosomes. The shearing action of the Polytron during homogenization of the lung tissue caused significant polysome fracture, resulting in a shift of the polysome profile into smaller units and a large 80 S monomer peak. However, the 5′ UT of human SP-A is 50–90 nucleotides long, and would usually have a single ribosome bound to it at the translation initiation codon [16]. As a result, the 5′ UT of the SP-A transcripts remain intact, despite fracture of the mRNA molecule further downstream. The gradient fractions that contain 'free' and 'polysome-bound' RNA were pooled as indicated by the bars in Figure 3(a). RNA was isolated from each of these fractions. RT-PCR, using primer 96 (common to both genes) for the RT and primers 32 and 9 for PCR, was used to display the 5′ ends of translated (bound) and untranslated (free) SP-A mRNA.

The results of RT-PCR of free and polysome-bound RNA from one individual are shown in Figure 3(b) (free, lane 2; bound, lane 3). RT-PCR was also carried out with total lung RNA from the same individual (lane 1) to ensure that the splice variants observed in the fractionated RNA reflect those present in total lung RNA. Comparison of the splice variants and their relative levels in total RNA (lane 1) with those in RNA isolated from polysome gradients (lanes 2 and 3) shows that RNA isolated from the gradients does indeed reflect the splice-variant population of total RNA in that individual. All the splice variants present in the free RNA fraction (lane 2) are also present in the polysome-bound fraction (lane 3), suggesting that all splice variants are translated *in vivo*.

Although the above finding suggests, by virtue of their binding to ribosomes, that all the detectable SP-A splice variants can be translated *in vivo*, it is not known whether all of them produce functional SP-A molecules. Based on the sequence of the 5′ UT of the different splice variants, the deduced protein products differ for some minor variants [8,12]. Because these transcripts comprise only a small subpopulation of the SP-A mRNA (Figures 3 and 4), the physiological significance of their translation is

unclear. However, the relative amounts of the minor transcripts vary among individuals (Figure 4); thus it is possible that, under some conditions, or in some individuals, increased aberrant SP-A protein is synthesized.

Individual variability in the ABD/ABD′ ratio was reported previously [8]. To confirm and extend this observation, and also to determine whether additional individual variability exists at the level of translation, we isolated free and polysome-bound RNA from five unrelated individuals and performed RT-PCR. To ensure that the RT-PCR was reproducible and accurately reflected the types and relative levels of the splice variants, RT was carried out twice for all RNA samples, and each RT reaction was amplified at least twice. The relative intensities of splice variants were comparable for both RT and amplification reactions for a particular sample, suggesting that the variability that we observe among samples reflects individual variability. To determine whether the tissue homogenization, polysome fractionation and RNA recovery from gradients were reproducible, we repeated these procedures, using frozen lung tissue from two of the individuals studied (individuals 3 and 5). Though the recovery of polysomes was decreased, the results of the subsequent RT-PCR were similar to those obtained from fresh tissue. The results of RT-PCR from the five individuals are shown in Figure 4. A number of observations can be made. (1) The ABD/ABD′ ratio varies among these individuals. For example, the amounts of ABD and ABD′ transcripts are about equal for individual 2, but for individual 5 there is more ABD than ABD′. (2) Information about the relative translatability of different splice variants within an individual can be inferred by comparison of the free and bound lanes for the individual. For example, different relative amounts of a particular splice variant in the free and bound RNA fractions suggests that the variant is more or less 'translatable' than the other splice variants. An example is described for individual 4. The relative amounts of both the ABD and ABD′ variants differ in the free and bound fractions. The ABD/ABD′ ratio is higher in the bound fraction than in the free fraction, suggesting the ABD is more translatable than ABD′. However, this is not the case for individual 2, where ABD and ABD′ appear to be translated equally. (3) The levels of the minor splice variants vary among individuals. The AB′D′ splice variant is represented in detectable amounts in some individuals (2 and 3), but is barely detectable, or undetectable, in others (1, 4 and 5). The ACD′ variant can be discerned in some individuals (2, 3 and 4) only after long exposure of the autoradiographs (results not shown). From these observations we conclude that both the relative levels of different SP-A splice variants and their translatability can differ among individuals. Variability in the relative level of alternatively spliced transcripts and in transcript translatability has also been reported for rat insulin-like growth factor 1 (IGF-1) [17,18]. Hypophysectomy and growth-hormone treatment increased and decreased, respectively, the levels of some IGF-1 splice variants compared with their levels in sham-operated animals [18]. The relative translatability of IGF-1 splice variants also differed [17,18]. Neither the physiological significance nor the molecular mechanisms of the variability are known.

Hormones and developmental stage can affect the relative levels of SP-A1 and SP-A2 mRNA [19]. The findings reported here suggest that SP-A expression is also regulated at the level of translation. Both SP-A genes are expressed *in vivo* [7], producing SP-A products that vary in at least four amino acids. Functional SP-A is a large, ordered, multimeric complex of 18 SP-A monomers [20,21], and it has been suggested that both SP-A1 and SP-A2 gene products, in the ratio of two SP-A1 to one SP-A2, are needed to form the functional complex [22]. If this is indeed the case, conditions that alter the relative translatability of SP-A1 and SP-A2 transcripts could have important implications for SP-A function, and, by extension, for surfactant function. A number of studies have demonstrated an association of the level of SP-A with respiratory distress syndrome in newborns [23–26] and in adult respiratory distress syndrome [27,28]. The association of SP-A with severe human disease makes the elucidation of mechanisms involved in SP-A expression an important goal of surfactant research.

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