Abstract. The mouse UGRP gene family consists of two genes, Ugrp1 and Ugrp2. In this study, the genomic structure and expression patterns of Ugrp2 and its alternative spliced form were characterized. The authentic Ugrp2 gene has three exons and two introns, similar to the Ugrp1 gene, which produces a secreted protein. The Ugrp2 variant uses a sequence located between authentic exons 1 and 2, resulting in a cytoplasmic form due to a termination codon within the inserted sequence. Both mouse and human UGRP2 mRNAs are expressed in lung. In the case of human, the mRNA is expressed at the highest level in trachea, followed by salivary gland at a level similar to lung. Weak expression was also found in fetal lung and mammary gland. Ugrp2 was mapped by fluorescence in situ hybridization to mouse chromosome 11A5–B1 and human chromosome 5q35. These regions are known to be homologous. Interspecific mouse backcross mapping was also performed to obtain further detailed localization of mouse Ugrp1 and Ugrp2.
Bonini, 1997; Cookson and Moffatt, 2000; Ober and Moffatt, 2000). The Ugrp1 gene is composed of three exons and two introns, and alternative splicing produces two additional transcripts that either partially or completely retain the intron 2 sequence (Niimi et al., 2001). RT-PCR analysis revealed that these two splicing variants are expressed at very low levels. Promoter analysis demonstrated that a homeodomain transcription factor T/EBP (thyroid-specific enhancer-binding protein)/NKX2.1, also called TTF1 (thyroid transcription factor 1) binds two sites between –182 and –120 bp of the Ugrp1 gene promoter, and activates transcription of the gene (Niimi et al., 2001).

Ugrp1 is a homodimeric secretory protein of approximately 10 kDa and is mainly expressed in the lung and trachea (Niimi et al., 2001). The protein properties and the site of tissue-specific expression resemble those of UG/CCSP (Niimi et al., 2001), which is believed to function as an anti-inflammatory agent (Broeckaert and Bernard, 2000; Mukherjee and Chilton, 2000). Previous studies using a Th2 cytokine-based antigen model demonstrated decreased expression of Ugrp1 and Ugrp2 in inflamed mouse lungs, suggesting that Ugrp1 and Ugrp2 may also play a role in lung inflammation (Niimi et al., 2001). In fact, our recent studies demonstrated that the human UGRP1 gene is an asthma susceptibility gene (Niimi et al., 2002).

In this study, the mouse Ugrp2 gene was characterized by determination of gene structure, the presence of alternative splicing, expression pattern, and chromosomal localization by FISH and interspecies mouse backcross mapping.

Materials and methods

Cloning and DNA sequencing

The mouse Ugrp2 gene was identified by searching the GenBank nucleotide sequence databases including expressed sequence tags (ESTs) for sequences similar to the mouse Ugrp1 gene using the BLAST program (National Center for Biotechnology Information, Bethesda, MD). An EST clone A391046 containing an apparent entire coding sequence that exhibited a nucleic acid sequence similar to the Ugrp1 gene, was designated Ugrp2. A partial cDNA sequence of the Ugrp2 (EST A391046) was obtained by RT-PCR using mouse adult lung RNA as a template. This was then used to isolate a full-length cDNA by screening a mouse lung cDNA library in the ZAPII vector (Stratagene, La Jolla, CA). Hybridization was carried out at 65 °C in 6× SSC, 0.5% SDS, 5× Denhardt’s, 0.1 mg/ml of denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2× SSC containing 0.1% SDS at room temperature for 10 min and twice with 0.1× SSC containing 0.1% SDS at 55 °C for 20 min, followed by exposure to X-ray film at –80 °C.

For reverse transcription of mRNA, 2 µg of total RNA was pretreated with DNase I, incubated for 10 min at 70 °C, and chilled on ice. The reactions were carried out in a final volume of 20 µl containing RNA, 4 µl of 5× first strand synthesis buffer (Invitrogen Life Technologies, Carlsbad, CA), 1 µl of a mixture of four dNTPs (2.5 mM each), 2 µl of 0.1 M dithiothreitol (DTT) and 100 ng of random primers. After incubation at 37 °C for 2 min, 200 units of SuperScript II reverse transcriptase (Life Technologies) was added and the incubation continued for 60 min at 37 °C. Single stranded cDNAs in 0.1 µl of the reaction mixture were amplified by PCR using AmpliTaq DNA polymerase (PE Applied Biosystems) under the following conditions; denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, for 30 or 25 cycles when tissue RNAs or plasmids were used as template, respectively. The oligonucleotide primers used for RT-PCR were as follows (see Fig. 1): P1: 5'-GAGACTCTTCTACCTAGAAG-3' (nt 37–57), P2: 5'-CTCCTGGTGAACACTCTCGG-3' (nt 408–389).

Fluorescence in situ hybridization

Mouse and human UGRP2 probes of entire BAC clone genomic DNAs labeled with biotin or digoxigenin were used for fluorescence in situ hybridization (FISH) of chromosomes derived from methotrexate-synchronized normal peripheral lymphocytes and from mouse spleen cultures, respectively. The conditions of hybridization, detection of fluorescence signals, digital image acquisition, processing and analysis, direct localization of signals on banded chromosomes were carried out as previously described (Popeceu et al., 1994; Zimonjic et al., 1995). To confirm the identity of mouse chromosomes, preparations were rehybridized with mouse chromosome painting probes (Cambio) and previously observed labeled metaphases were re-corded.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × M. spretus) F1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N2 mice were used to map the Ugrp1 and Ugrp2 loci (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The Ugrp1 probe, an ~500-bp fragment of mouse cDNA, was labeled with [α-32P]dCTP using a random primed labeling kit (Strategene); washing was done at a final stringency of 1.0× SSC, 0.1% SDS, 65 °C. A fragment of 7.9 kb was detected in EcoRV digested C57BL/6J DNA and a fragment of 6.8 kb was detected in EcoRV digested M. spretus DNA. The Ugrp2 probe, an ~500-bp fragment of mouse cDNA, detected an 11.0-kb EcoRI fragment in C57BL/6J DNA and a 14.0-kb EcoRI fragment in M. spretus DNA. The presence or absence of the M. spretus-specific fragments was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to Ugrp1 including Nr3c1, Mec, and Lmnb1 has been reported previously (Justice et al., 1992); those linked to Ugrp2 include Sox30 and H13 (McKenzie et al., 1993; Osaki et al., 1999). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.
Fig. 1. (A) Nucleotide and deduced amino acid sequences of the mouse Ugrp2 cDNA. The number of the nucleotide sequence is indicated in the right margin. Exon 1b sequence is boxed. Two alternative initiating methionines are circled. The putative signal peptide sequence in the type A polypeptide is underlined. The termination codon is marked with an asterisk and the polyadenylation signal is shown in boldface. (B) Structure of mouse Ugrp2. Organization of exons and introns, and how the two types of transcripts were produced are illustrated. Shaded boxes represent exons and the positions of translation initiation and termination codons defining ORF are indicated. A thin jagged line shows sequences that are spliced out in mature mRNAs. The allele encoding UGRP2 type A is designated as Ugrp2*1 and that encoding UGRP2 type B as Ugrp2*2. Arrows indicate the positions of primers used for RT-PCR analysis.

Results and discussion

Cloning of a novel Ugrp1-related cDNA

The mouse Expressed Sequence Tags database (dbEST) was searched for sequences similar to the mouse Ugrp1 cDNA using the BLAST algorithm software. An extensive set of sequences was identified that had an entire or a partial open reading frame (ORF) encoding a protein exhibiting significant similarity to UGRP1. A clone (GenBank Accession No. AI391046) that contained an entire ORF sequence was designated as mouse Ugrp2. RT-PCR was performed to obtain a Ugrp2 partial cDNA clone using mouse adult lung RNAs as template and two regions of the EST sequence as primers, which was then used to isolate a full-length mouse Ugrp2 cDNA by screening a mouse adult lung cDNA library. Eight clones with positive hybridization signal were identified in 1 × 10⁶ recombinant phage. Among them, seven appeared to contain an entire ORF and the 5′- and 3′-noncoding region of Ugrp2. Interestingly, one clone contained an additional 105-bp sequence inserted within the coding region. A stop and a start codon were present at positions 15 and 81 of the inserted sequence, respectively. As a result, this cDNA encodes a UGRP2 variant with a shorter amino acid sequence. We refer to the two Ugrp2 cDNAs as types A and B, encoding of 104 and 94 amino acids, respectively (Fig. 1A). Computer analysis revealed that the first 21 residues of the UGRP2 type A polypeptide may function as a signal sequence for targeting the protein to a secretory pathway.

The UGRP2 type B polypeptide has nine unique amino acids at the N-terminus that replace the first 19 amino acids of the type A polypeptide, suggesting that the type B polypeptide is likely to be a cytoplasmic protein.

Characteristics of the Ugrp2 gene and the encoded polypeptide

In order to analyze the Ugrp2 gene structure, a mouse BAC genomic library was screened using a full-length cDNA as a probe. The mouse Ugrp2 gene encoding type A polypeptide is composed of three exons and two introns, and thus resembles the structure of the Ugrp1 gene (Fig. 1B) (Niimi et al., 2001). The exon/intron splice sites are well conserved between Ugrp1 and Ugrp2, suggesting that they constitute a gene family and share a common ancestral gene. The 105-bp insertion sequence (exon 1b) was found 300 bp downstream of the authentic exon 1, named exon 1a, indicating that UGRP2 type A and B polypeptides are encoded by splicing variation of a transcript from the same gene. Based on the gene nomenclature system, the type A is encoded by the Ugrp2*1 allele, and the type B by the Ugrp2*2 allele. The presence of splicing variants has also been reported for the Ugrp1 gene (Niimi et al., 2001). Thus, the Ugrp1 gene encodes three types of transcripts generated through alternative splicing, which in addition to normal splicing, either partially or entirely retains the second intron sequence.
The mouse UGRP1 type A, the most abundant form, and UGRP2 type A polypeptides exhibit 47% similarity in amino acid sequences (Fig. 2A). Similarity is especially high in the C-terminal one-third of the polypeptide sequences. In the middle, many hydrophobic amino acid residues are present in the UGRP2 polypeptide, which do not align with any of the UGRP1 amino acid residues. This results in UGRP2 being more hydrophobic than UGRP1 (Fig. 2B).

Since a high level of Ugrp2 expression was observed in mouse lung (see below) and the Ugrp1 gene promoter is known to be transactivated by a homeodomain transcription factor T/ebp/NKX2.1 (Niimi et al., 2001), the mouse Ugrp2 gene promoter was examined to determine whether it is regulated by the same transcription factor. Sequence analysis using TF (transcription factor) Search (Heinemeyer et al., 1998) revealed that several T/ebp/NKX2.1 binding consensus sites are present within 400 bp of the mouse Ugrp2 gene promoter. However, no transactivation was observed when a co-transfection experiment was performed using Ugrp2 gene promoter-luciferase reporter constructs and a T/ebp/NKX2.1 expression plasmid (data not shown). Expression of lung-specific genes such as surfactant proteins-A (Bruno et al., 1995; Bruno et al., 2000), -B (Bohinski et al., 1994; Yan et al., 1995), and -C (Kelly et al., 1996), and UG/CCSP (Sawaya et al., 1993; Ray et al., 1996; Braun and Suske, 1998) has been demonstrated to be controlled by a combination of transcription factors including T/ebp/NKX2.1, HNF-3a and b, and GATA-6 (also see Mendelson, 2000; Costa et al., 2001). Further analysis of the mouse Ugrp2 gene promoter is required to understand the regulation of lung-specific expression of this gene.

Expression of Ugrp2 transcripts
Ugrp2 expression was examined by Northern blot analysis in adult mouse tissues. A single 0.6-kb transcript was detected only in the lung among all tissues examined (Fig. 3A). Human multiple tissue expression arrays were next examined for the expression of UGRP2 using RNA dot blot analysis (Fig. 3B). The strongest signal was obtained with trachea, followed by salivary gland and lung. Mammary gland and fetal lung also had a weak, but clear positive signal. Contrary to this, the expression of Ugrp2 was not detected in mouse salivary gland and mammary gland as determined by RT-PCR (data not shown).

In order to confirm the presence of two types of transcripts, RT-PCR was performed using mouse embryonic and adult lung mRNAs as templates (Fig. 3C). Initially, PCR reactions were carried out using exon 1a (P1) and 2 (P2)-specific primer pair (see Fig. 1B) and an individual type of cDNA clone as template. These produced fragments of 268 and 372 bp that correspond to type A and B transcripts, respectively. Both RNAs

Fig. 2. Alignment of amino acid sequences and Kyte-Doolittle hydrophobicity plots of UGRP family proteins. (A) Mouse UGRP2 type A amino acid sequence is aligned with that of mouse UGRP1 type A. The identical and conserved residues are shown in black and shaded boxes, respectively. (B) Analysis of UGRP family protein structures by Kyte-Doolittle hydrophobicity plot.

Fig. 3. Expression of Ugrp2 transcripts. (A) Northern blot analysis of adult mouse tissues probed with 32P-labeled mouse Ugrp2 cDNA. (B) Dot blot analysis of human tissues probed with 32P-labeled human UGRP2 cDNA. RNA sources are as follows; A1: whole brain, A2: amygdala, A3: caudate nucleus, A4: cerebellum, A5: cerebral cortex, A6: frontal lobe, A7: hippocampus, A8: medulla oblongata, B1: occipital lobe, B2: putamen, B3: substantia nigra, B4: temporal lobe, B5: thalamus, B6: subthalamic nucleus, B7: spinal cord, C1: heart, C2: aorta, C3: skeletal muscle, C4: colon, C5: bladder, C6: uterus, C7: prostate, C8: stomach, D1: testis, D2: ovary, D3: pancreas, D4: pituitary gland, D5: adrenal gland, D6: thyroid gland, D7: salivary gland, D8: mammary gland, E1: kidney, E2: liver, E3: small intestine, E4: spleen, E5: thymus, E6: peripheral leukocyte, E7: lymph node, E8: bone marrow, F1: appendix, F2: lung, F3: trachea, F4: placenta, G1: fetal brain, G2: fetal heart, G3: fetal kidney, G4: fetal liver, G5: fetal spleen, G6: fetal thymus, and G7: fetal lung. (C) RT-PCR for the expression of two types of transcript. The positions of primers are indicated in Fig.1B. RT-PCR was carried out using RNAs prepared from lungs of E12.5 to E18.5 mouse embryos and adult. The product sizes are shown on the right. The results obtained with type A and B cDNA clones as a control template are indicated as type A and B, respectively. One-kb DNA ladder (Invitrogen Life Technology) was used as a size marker (M) and the size is indicated on the left margin.
Fig. 4. FISH analyses for Ugrp2 chromosomal localization. Human (A) and mouse (B) chromosome spreads after hybridization with species-specific biotin-labeled Ugrp2 gene probes are shown. Both chromosome 5 in human and chromosome 11 in mouse exhibit symmetrical fluorescent signals at regions 5q35 and 11A5–B1, respectively, as depicted by enhanced DAPI-induced chromosome banding.

Fig. 5. Diagram of gene localization in human chromosome 5q31 → q35. Genes listed include those that may be involved in inflammation. They are shown in the order of which they are localized from those close to the centromere (top) to those close to the telomere (bottom). Abbreviation for genes; IL3: interleukin 3, CSF2: colony stimulating factor 2, IRF1: interferon regulatory factor 1, IL5: interleukin 5, IL13: interleukin 13, IL4: interleukin 4, IL9: interleukin 9, EGR1: early growth response 1, CD14: CD14 antigen, FGF1: fibroblast growth factor 1, NR3C1: glucocorticoid receptor 1, ADRB2: adrenergic receptor β 2, IL17B: interleukin 17B, CSF1R: colony stimulating factor 1 receptor, PDGFR: platelet-derived growth factor receptor, CD74: CD74 antigen, IL12B: interleukin 12B, LCP2: lymphocyte cytosolic protein 2, HRH2: histamine receptor, LCT: leukotrien C4 synthase. The number shown to the right of the gene is bp (M: mega) starting from the tip of the telomere of 5p used in the Celera Discovery System. Triangle indicates direction of gene. The mouse chromosome number for each gene is shown on the far right. This figure was generated through the use of the Celera Discovery System and Celera’s associated databases.

Prepared from mouse adult and embryo lungs demonstrated the expression of both types of transcripts although the band corresponding to the type B transcript was faint, suggesting that the type B transcript seems to be expressed at low levels. The expression of both type A and B transcripts was detected around E16.5, which increased toward the end of gestation and stayed at a similar level thereafter. Although this type of RT-PCR is not quantitative, the ratio in the expression levels between types A and B appears to be similar regardless of developmental stages. This is somewhat different from what was found for the three types of Ugrp1 transcripts, in which type A is most abundant and two other forms were barely detected. These data suggest that the two forms of UGRP2 may possess distinct functions.

Previously, it was demonstrated that the expression of Ugrp1 decreased in lungs of the TH2 cytokine-based antigen mouse model (Niimi et al., 2001). The expression of Ugrp2 appeared also to be decreased in these lungs although the level of reduction was not as large as that for Ugrp1 (Niimi et al.,...
2001). These results suggested that UGRP1 and 2 may be involved in TH2 cytokine-based inflammation. In fact, our recent results demonstrated that human UGRP1 is involved in asthma (Niimi et al., 2002). It would be interesting to examine the role of the two forms of UGRP2 in lung inflammation. Further, the function of UGRP2 in mammary gland and salivary gland remains to be understood. In this regard it was recently reported that HIN-1, which is identical to UGRP2 is a candidate tumor suppressor gene in mammary gland (Krop et al., 2001).

Chromosomal localization of Ugrp2 gene by FISH

The human and mouse UGRP2/UGRP2 genes were mapped by FISH using chromosomes prepared from normal human peripheral leukocytes and mouse spleen cultures. In human cells, symmetrical fluorescent signals on sister chromatids were observed at the telomeric region of the long arm of chromosome 5 in 25 out of 30 metaphases. The probe had high specificity for this site as a symmetrical signal was not observed on other chromosomes. In ten metaphases with good resolution DAPI G-like banding the FISH signal was localized on chromosome 5 at region q35 where we assign the UGRP2 gene (Fig. 4A). Similarly, mouse Ugrp2 probe showed label specificity for a single medium size chromosome identified by banding and chromosome painting as chromosome 11 and precisely localized at region 11A5–B1 (Fig. 4B). Region 5q35 in human is homologous with region 11A5–B1 in mouse (Searle et al., 1989; DeBry and Seldin, 1996).

Mouse Ugrp1 gene was previously mapped to chromosome 18C–D (Niimi et al., 2001), a region that is homologous to human chromosome 5q31→q32 where the human UGRP1 gene was assigned (Niimi et al., 2002). Human chromosome 5q31→q35 has homologous regions in three mouse chromosomes; chromosomes 11, 13, and 18 (Searle et al., 1989; DeBry and Seldin, 1996). Thus, the UGRP1 and UGRP2 genes in human are located on chromosome 5q31→q35 whereas they are split into two mouse chromosomes 18 and 11, respectively (Fig. 5).

Human chromosome 5q31→q34 has been reported to contain one of the asthma susceptibility genes (Postma et al., 1995; Ruffilli and Bonini, 1997; Cookson and Moffatt, 2000; Ober and Moffatt, 2000). In addition to the UGRP1 gene, this region contains numerous gene candidates that may potentially be involved in the airway inflammation associated with atopic asthma, including a number of proinflammatory cytokines such as interleukin (IL)-3, 4, 5, 9, and 13, granulocyte macrophage colony-stimulating factor (CSF), and the β2-adrenergic receptor (ADRB2). Relative locations of these genes to the human UGRP1 and UGRP2 genes were determined using human genomic sequence databases (Fig. 5). The distance between UGRP1 and UGRP2 was calculated to be approximately 30 Mbp. Thus, it may be possible that the expression of UGRP1 and/or UGRP2 is directly or indirectly regulated by one of these cytokines, which affects the inflammatory status of the lung involved in asthma. Further experiments are required to determine whether this is the case.
Fig. 6. Murine chromosomal location of **Ugrp1** and **Ugrp2**. The segregation patterns of **Ugrp1** (A) and **Ugrp2** (B) and their flanking genes in 144 and 112 backcross animals, respectively, that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. Partial chromosome 18 and 11 linkage maps showing the location of **Ugrp1** (A) and **Ugrp2** (B) in relation to linked genes are shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the GDB (http://www.gdb.org/).

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