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Mouse ARF-Related Protein 1: Genomic Organization and Analysis of Its Promoter

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ARF-related protein 1 (ARFRP1) is a membrane-associated GTPase which inhibits the ARF/Sec7-dependent activation of phospholipase D. We have recently shown that deletion of *Arfrp1* in mice results in increased apoptosis of mesodermal cells during gastrulation, leading to early embryonic lethality. Here we describe the organization of the *Arfrp1* gene and of its promoter region. The *Arfrp1* gene spans approximately 7 kb and contains 8 exons. The proximal 5′-flanking regions of mouse and human *ARFRP1* lack a TATA box and a CAAT box, are highly GC-rich and contain potential transcription factor binding sites. Interestingly, sequence analysis of human *ARFRP1* showed its 5′-flanking region contains the first exon of another gene (DJ583P15.3 in the ensembl data base; www.ensembl.org) on the opposite strand. Promoter analysis revealed that the intergenic region between both genes (54 bp) exhibits bidirectional promoter activity. However, deletion analysis demonstrated that transcription of both genes is regulated by different *cis*-elements. Mutational analysis and electrophoretic mobility shift assays indicated that two short cRel- and cEts1-like elements in the 5′-flanking region of *Arfrp1* (−76 to −53 and −45 to −23) are critical for the regulation of *Arfrp1* expression.

ADP-ribosylation factors (ARFs) are GTP-binding proteins that are involved in multiple steps of membrane trafficking and regulation phospholipase D (PLD) (1–3). ARFRP1 (ADP-ribosylation factor related protein 1) is a membrane associated 25-kDa GTPase with remote similarity to ARF and ARF-like proteins (33% and 39% identical amino acids to ARF1 and ARF-like 3, respectively). ARFRP1 contains all characteristic sequence motifs involved in nucleotide binding and GTP hydrolysis. Guanine nucleotide exchange of recombinant ARFRP1 is slow, but GTPase activity is high in the absence of an activating protein. ARFRP1 is predominantly located in the plasma membrane and absent in the cytosol (4), whereas ARF and ARF-like proteins shuttle between membranes and the cytosol, depending on the bound nucleotide (5). Previous studies have suggested that ARFRP1 is involved in a pathway inhibiting the ARF-controlled activity of PLD (6). ARFRP1 binds the ARF-specific nucleotide-exchange factor Sec7-1/cytohesin in a GTP-dependent manner and inhibits the ARF/Sec7 dependent activation of PLD. Furthermore, we have recently shown that deletion of *Arfrp1* results in embryonic lethality caused by apoptosis of epithelial cells during gastrulation (7).

In the present study, we describe the genomic organization of *Arfrp1* and the characterization of the *Arfrp1* promoter. In order to define the region responsible for the regulation of the expression of *Arfrp1*, we used reporter constructs obtained by sequential deletion and by site-directed mutagenesis. From these studies, we conclude that a putative cRel binding site at −58 bp to −54 bp, and a putative cEts1 binding site at −34 bp to −30 bp are critical for expression of the *Arfrp1* gene. In addition, we demonstrate that the short, 5′-flanking region of human and mouse *Arfrp1* (49 and 54 bp, respectively) harbors the promoter of the adjacent gene which is regulated by other *cis*-elements in a head-to-head orientation.

MATERIAL AND METHODS

Library screening and DNA sequencing. Genomic clones of *Arfrp1* were isolated by screening a 129 SvJ mouse genomic library (Lambda FIX II Vector; Stratagene, La Jolla, CA) with a cDNA probe derived from the rat *Arfrp1* cDNA. Two clones were isolated and were sequenced after fragmentation by sonication or digestion with restriction enzymes and subcloning into pUC18 and pGEM-5Zf(+). Sequencing was performed in both directions by the method of Sanger (Thermo-sequenase fluorescent labeled primer cycle sequencing kit; Amersham Life Science, Little Chalfont, Bucks, UK) with the aid of an automated sequencer (LI-COR, Lincoln, NE).

Reporter plasmid construction. Deletions of the intergenic region of *Arfrp1* and its 5′-flanking gene were generated by exonuclease digestion in both directions. Site-directed mutagenesis of the core sequences of the NFκB, cRel and both cEts1 binding sites was performed by the overlap extension procedure as described by Vallejo *et al.* (8) using the following oligonucleotides: NFκB-site: 5′-CTC CGC

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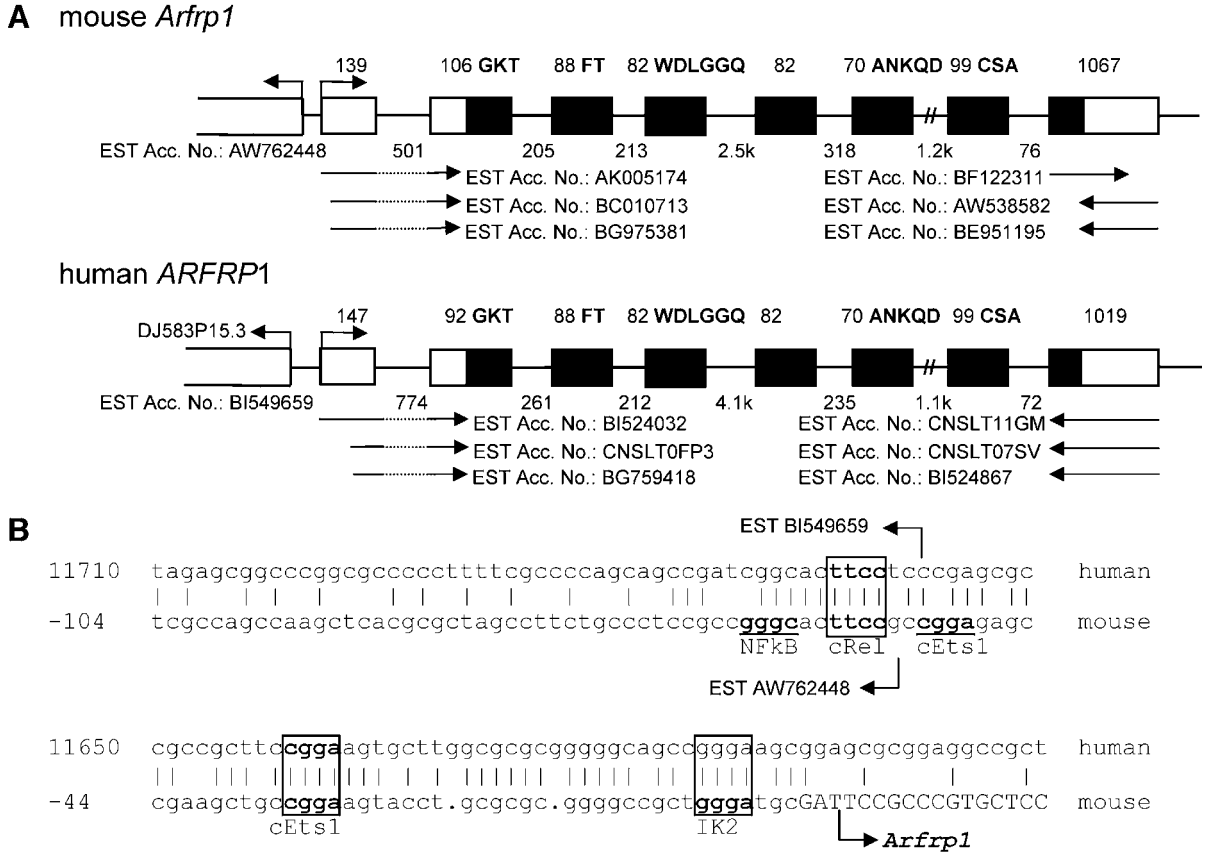

FIG. 1. Organization of the mouse and human *ARFRP1* gene and alignment of their 5′-flanking region. (A) Schematic representation of the exon–intron organization of the mouse and human *ARFRP1*. Transcription initiation start sites of *ARFRP1* and its neighboring gene DJ583P15.3 (ensembl data base) are indicated. Boxes represent exons as identified by comparison with cDNA. The coding regions are drawn as shaded boxes. Localizations of sequence motifs that are essential for GTP-binding (PM1-3, G1-3) on the exons are indicated. (B) Comparison of nucleotide sequences of the 5′-flanking regions of mouse and human *ARFRP1*. Numbering of the nucleotides of the human gene corresponds with that of the clone RP4-583P15 (Accession No. 003215); numbering of the mouse gene starts with the transcription initiation site. Putative *cis*-elements as identified with the MATINSPECTOR program are boxed.

CAT ATA CTT CCG CC-3′ and 5′-GGC GGA AGT ATA TGG CGG AG-3′; cRel-site: 5′-CGG GCA CAA TAG CCG GAG AG-3′ and 5′-CTC TCC GGC TAT TGT GCC CG-3′; the first cEts1-site: 5′-GAA GCT GCA ATT ATT AGT ACC TG-3′ and 5′-CAG GTA CTA ATT GCA GCT TC-3′; the second Ets1-site: 5′-GCA CTT CCG CAT TCG AGC CG-3′ and 5′-CGG CTC GAA TGC GGA AGT GC-3′. All constructs were fused to luciferase cDNA in the vector pGL3-basic (Promega, Madison, WI).

Promoter analysis. Reporter gene constructs were transfected into COS-7 cells with the aid of FuGene 6-Reagent (Boehringer Mannheim GmbH, Mannheim, Germany). 24 h after transfection, cells were lysed, and luciferase activity was analyzed with a kit from Promega (Luciferase Assay System) according to the instructions of the manufacturer. Transfection efficiency was determined by co-transfection of a plasmid harboring lacZ, and data were normalized to β-galactosidase activity.

Electromobility shift assays (EMSA). Nuclear extracts of COS-7 cells were prepared as described by Sadowski *et al.* (9). Oligonucleotides (oligo 1: 5′-(−83) TATCCTTCTGCCCTCCGCCGGGCACTTC-CGCCGGAG (−44)-3′; oligo 2: 5′-(−41) AGCTGCCGGAAGTACCT-GCGCGCGGGG (−9)-3′) were end labeled by incubating 3 pmol of the double stranded DNA with [^{32}P]dTTP and the Klenow fragment of polymerase I. 9.5 μg of nuclear extracts were used for EMSA as described by Garner and Revzin (10).

RESULTS

Organization of the mouse and human ARFRP1 gene. The organization of the mouse *Arfrp1* gene (Accession No. AJ413952) is illustrated in Fig. 1A. The gene spans approximately 7 kb and consists of 8 exons. The localization of the exons was identified by comparison of the genomic sequence with the cDNA of human (Accession No. X91504) and rat ARFRP1 (Accession No. X78603). The transcription start was defined by the longest 5′-EST (Accession No. AK005174) which was identical with the bases 1–896 of the mouse cDNA. Two other ESTs (Accession Nos. BC01713 and BG975381) started at base 13. The genomic clone terminated in exon 8 at base 6198. An additional search of EST databases was obtained with the 3′-flanking sequence of the *Arfrp1* clone and led to 3 overlapping ESTs. Since two ESTs comprise a polyadenylation signal the length of exon 8 was estimated as 1067 bp (Fig. 1A). The translation start of *ARFRP1* was mapped to exon 2, the translation stop to exon 8.

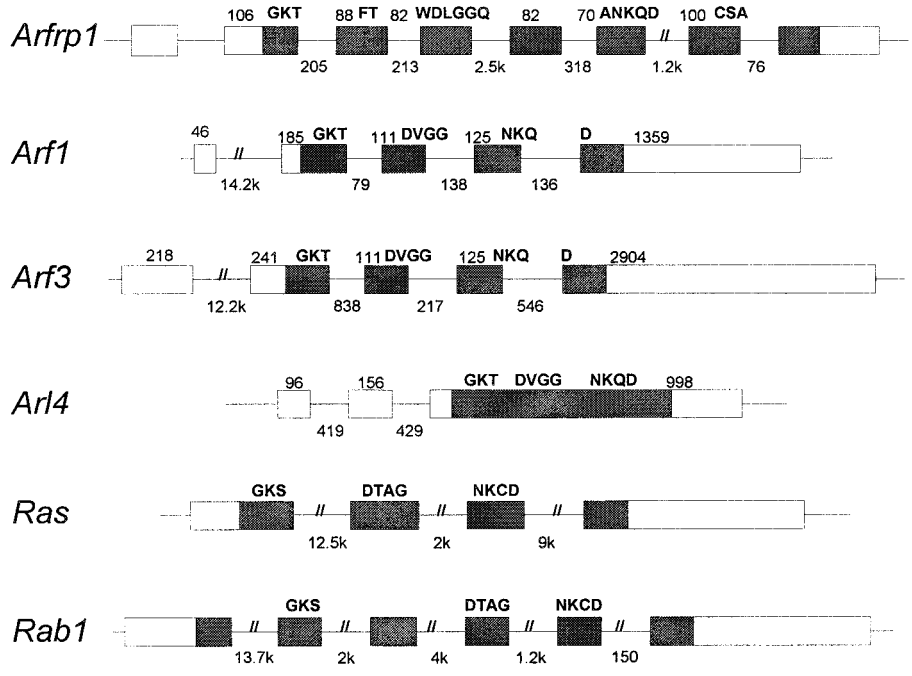

FIG. 2. Comparison of the *ARFRP1* gene with genes of other Ras-related GTPases. The genomic organization of the GTPases was obtained from references (*Arf1,* 22; *Arf3,* 23; *Arl4,* 11; *Rab1,* 24; *Ki-Ras,* 25). Boxes represent exons, filled boxes denote coding regions. The sizes of the exons and introns are given above and below the structures, respectively. The main GTP-binding motifs PM1 (GKT or GKS), PM3 (DVGG or DTAG) and G2 (NKQD or NKCD) are indicated above the structures at their approximate positions within the coding region.

In order to compare the human and mouse *ARFRP1* gene, the exon-intron borders of the human *ARFRP1* were mapped by a comparison of the genomic sequence as identified in a data base search (clone RP4-583P15; Accession No. 003215) with the ARFRP1 cDNA. As is illustrated in Fig. 1A, mouse and human *ARFRP1* are nearly identical with regard to exon number and size, and highly similar with regard to the intron size.

A data base search with the 5′ sequence of the genomic clone revealed that the first exon of another gene (EST Accession No. AW762448) is located on the opposite strand of *Arfrp1* only 54 bp upstream of the transcription start of the GTPase (Fig. 1B). EST AW762448 encodes a mRNA weakly similar to T34154, a hypothetical protein (C33H517) of *Caenorhabditis elegans.* Interestingly, the head-to-head arrangement of the two genes is conserved in the human genomic sequence. The transcription start of the neighboring gene DJ583P15.3 (Accession No. Q9H401, mapped to chromosome 20q13.3) is located 49 bp upstream of the human *ARFRP1* gene. This observation suggests that the promoter of *ARFRP1* is very short, and confined to the 49 bp segment between the transcription starts of *ARFRP1* and its 5′-flanking gene. Alignment of the human EST BI549659 and the mouse EST AW76448 indicated that the UTR of both cDNAs (bases 1–139 and bases 1–266, respectively) exhibit no similarity, whereas the coding region exhibited 83% identity.

Comparison of the genomic organization of Arfrp1 with that of other GTPases. Figure 2 illustrates a comparison of the genomic organization of *Arfrp1* with that of other Ras-related GTPases. This comparison indicates that the organization of the *Arfrp1* gene differs significantly from that of other members of the ARF family and of other Ras-related GTPases with regard to the number of its exons. *Arf1* and *Arf3* are very similar and consist of 5 exons. *Arl4* is unusual in that its complete coding region is located on a single exon; this gene has probably evolved by retroposition of another *Arf* or *Arf-like* gene (11).

Analysis of the promoters of Arfrp1 and its 5′ neighboring gene. The results of the sequence analysis led us to assume that the very short 5′-flanking region of *Arfrp1* (50 bp) serves as a bi-directional control region which regulates the transcription of two genes in head-to-head orientation. In order to identify regulatory elements in this region, several deletion constructs were fused to the luciferase gene and expressed in COS-7 cells in order to analyze their promoter activity. Figure 3A illustrates that two parts of the sequence were identified that appear to harbor essential promoter elements for the *Arfrp1* promoter: Deletion of the bases −76 to −53 and of −45 to −23 lead to a marked decrease in luciferase activity (Fig. 3A).

In a parallel set of experiments, deletions of the intergenic region fused to the luciferase gene in the

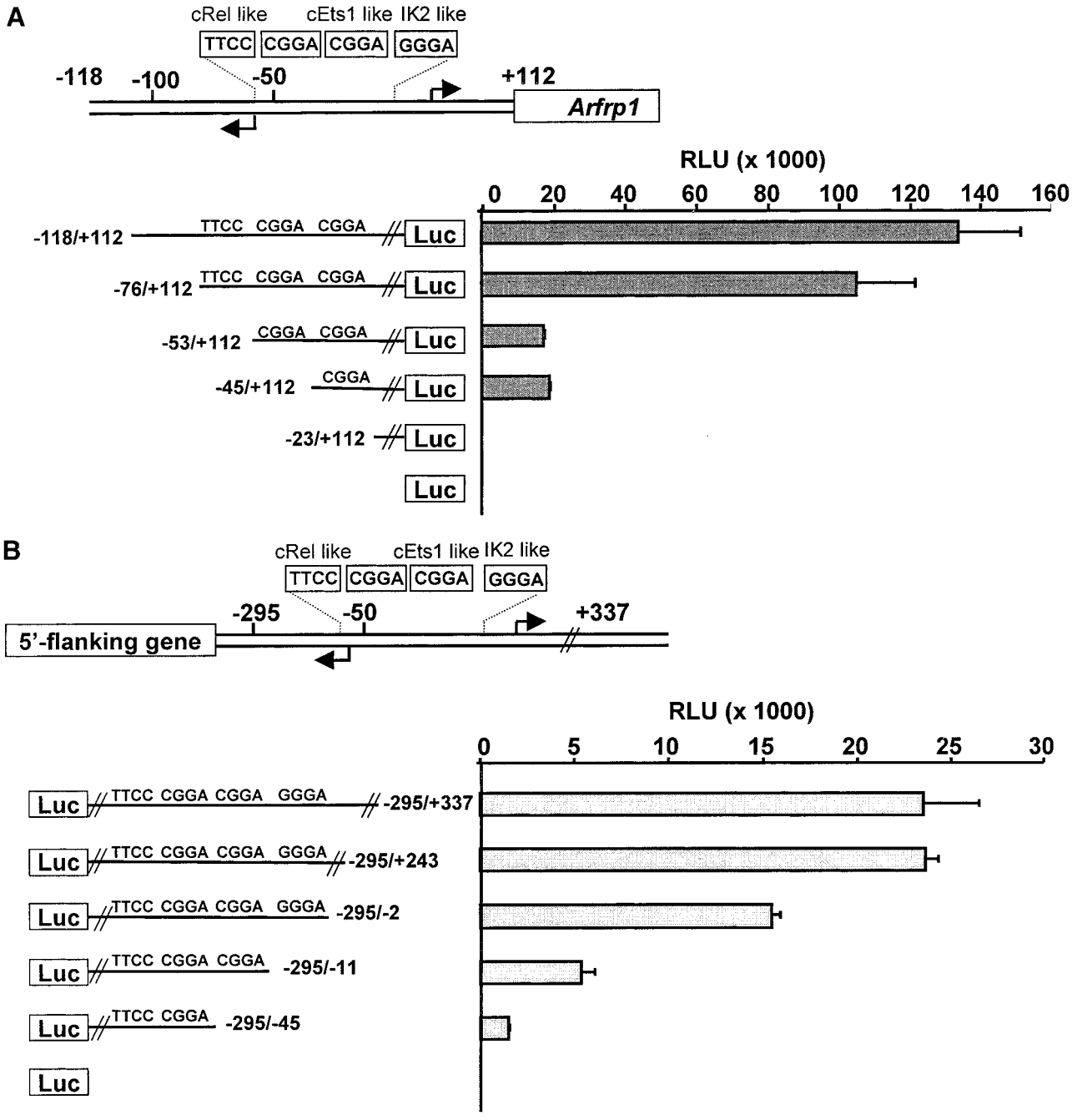

FIG. 3. Promoter activity of the 5′-flanking regions of the *Arfrp1* and its 5′-flanking gene. (A) Deletions of the 5′-flanking region of *Arfrp1* (A) or its 5′-flanking gene (B) were fused with luciferase cDNA, and constructs were expressed in COS-7 cells. Luciferase activity was assayed and results were corrected for transfection efficiency. Data represent means ± SEM of triplicate samples from a representative experiment which was repeated three times.

opposite direction were analyzed for promoter activity. Deletion of the bases +243 to −2 decreased luciferase activity by about 30%; deletion of the following 9 bp (construct −295/−45) disrupting the core sequence of a putative IK2 binding site resulted in an additional marked reduction of the promoter activity. After deletion of the bases −11 to −45 (construct −295/−45), only 10% of the maximal luciferase activity was detected (Fig. 3B). In this construct the first cEts1 site which seems to play a role for the *Arfrp1* promoter was deleted. These results suggest that the expression of *Arfrp1* and its neighboring gene (encoding for a protein similar to T34154 hypothetical protein C33H517 of *C. elegans*) is controlled predominantly by other regulatory elements than the *Arfrp1* promoter.

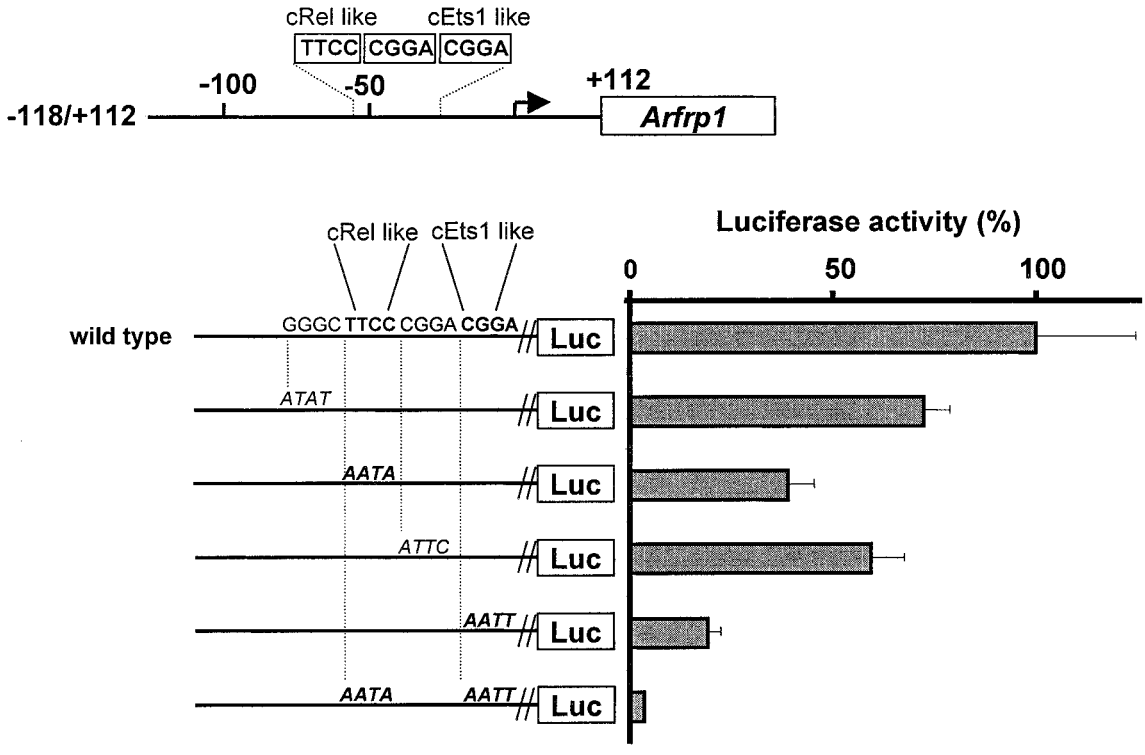

FIG. 4. Identification of putative transcription factor binding sites by analysis of promoter mutants. Core sequences of binding sites of transcription factors NFκB, cRel and cEts1 were mutated and expressed as luciferase fusion constructs in COS-7 cells. Luciferase activity was assayed, and results were corrected for transfection efficiency and normalized for the activity of the construct −118/+112. Data represent means ± SEM of triplicate samples from a representative experiment which was repeated three times.

The alignment of the nucleotide sequences of the 5′-flanking regions of mouse and human *ARFRP1* (Fig. 1B) shows that the two regions are highly homologous in the area between −65 to +1. A computer-based search for binding sites of transcription factors (MATINSPECTOR) identified putative binding sites for NFκB, cRel and cEts1 within the 5′-flanking region between bases −76 and −23 of the *Arfrp1* gene (Fig. 1B). Two of these sites were essential for full promoter activity: the putative binding site of cRel (TTCC) which is eliminated by deletion of the bases −76 to −53, and the first binding site of cEts1 which is removed by deletion of the bases −45 to −23 (CGGA; Fig. 3A).

Contribution of the putative cRel and cEts1 binding sites to the promoter activity of the Arfrp1 gene. In order to define whether the putative binding sites of NfκB, cRel and cEts1 contribute to the *Arfrp1* promoter activity, each core sequence was mutated in the construct −118 to +112 and expressed as luciferase fusion protein in COS-7 cells. Luciferase activity was markedly reduced after mutation of the core sequences of cRel (TTCC/AATA) and of the first cEts1 site (CGGA/AATT). Promoter activity was essentially abolished, when both binding sites were mutated (Fig. 4), suggesting that transcription factors binding to these sites are involved in the regulation of *Arfrp1* expression.

Electrophoretic mobility shift assay analysis with the putative cRel and cEts elements of the mouse Arfrp1 promoter. For further characterization of the putative regulatory elements, electrophoretic shift assay (EMSA) analysis were performed. ^{32}P-labeled oligonucleotides including the putative binding sites of cRel (oligo 1: bases −82 to −46) or to cEts1 (oligo 2: bases −40 to −13) were incubated with or without nuclear extracts from COS-7 cells. A shift was detected after the addition of nuclear extracts indicating that nuclear proteins bind to the labeled oligonucleotides. Complex formation was inhibited specifically by adding excess amounts of unlabeled cRel or cEts1 consensus oligonucleotides (Fig. 5A). In accordance with the reporter assays, no shift was detected with oligonucleotides containing the mutation of the core sequence of cRel or cEts1 (Fig. 5B). This observation leads to the conclusion that the 5′-flanking sequence between bases −58 and −54 and between bases −34 and −30 are essential for the binding of regulatory elements.

DISCUSSION

Because of its essential role in embryogenesis, ARFRP1 appears to control an important, specific signaling pathway involved in cellular differentiation (7).

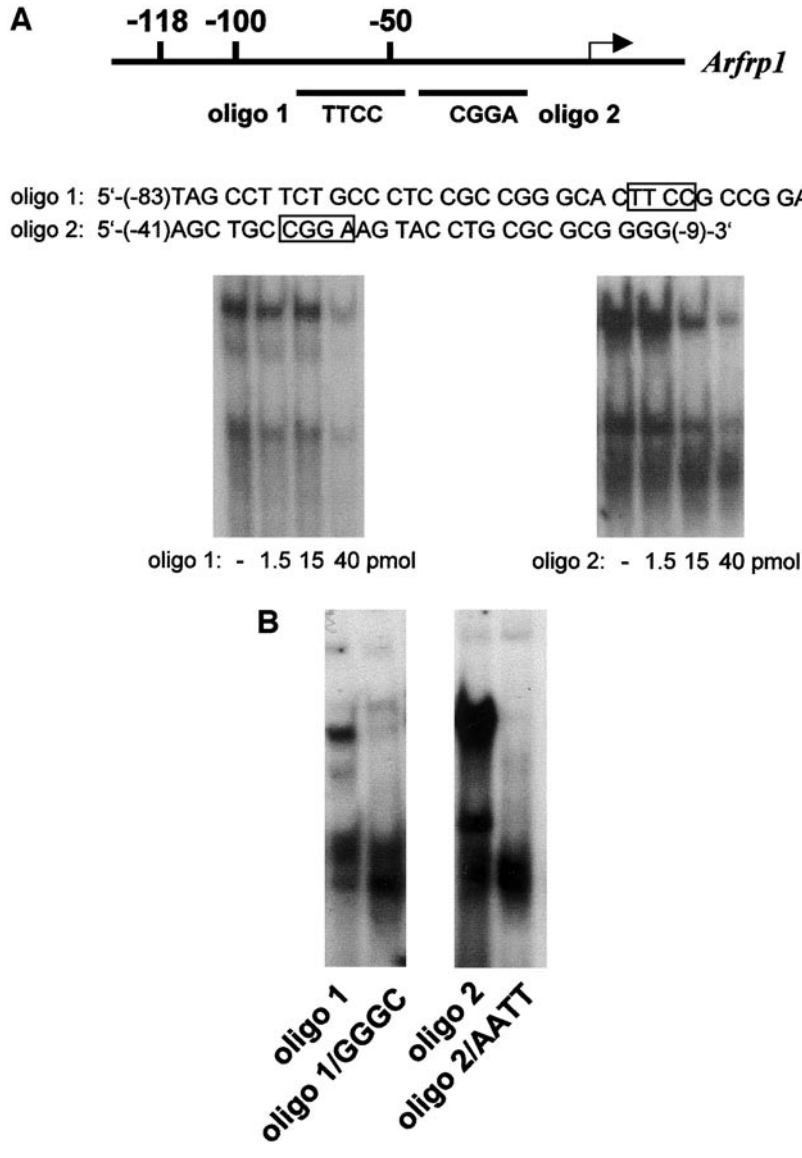

FIG. 5. EMSA analysis with the cRel and cEts elements of the *Arfrp1* promoter. Nuclear extracts from COS-7 cells were incubated with radiolabeled oligonucleotides corresponding with the cRel (oligo 1) or the cEts (oligo 2) consensus sequence, and were separated by non-denaturating gel electrophoresis. (A) Competition of DNA binding by unlabeled nucleotides. Competition analysis was performed in the presence of 1.5, 15, or 40 pmol of unlabeled oligonucleotides containing the cRel consensus sequence (left panel) or the cEts consensus sequence (right panel). (B) Effects of mutations of cRel and cEts1-like elements on the formation of DNA-protein complexes. EMSAs were performed with oligos 1 and 2 and with oligonucleotides corresponding with the inactivating mutations (see Fig. 4).

Thus, it is surprising that according to the present data the expression of *Arfrp1* is controlled by a DNA segment of only 54 bp. The promoter of *Arfrp1* is TATA and CAAT-less, but contains a GC-rich region upstream of the transcription initiation site. In addition, we were able to demonstrate the presence of two *cis*-acting, cRel and cEts-like elements which appear to control the specific expression of the gene in tissues and during embryogenesis.

The critical importance of the cRel and cEts1-like elements for the regulation of *Arfrp1* expression is shown here by mutational analysis. This finding is compatible with the pattern of expression of *Arfrp1* in tissues (thymus, liver, kidney, testis) (4) and during embryonic development (7). cRel is a member of the Rel/nuclear factor (NF) κB superfamily that comprises four additional DNA binding proteins (p50, p52, RelA and RelB). These ubiquitously expressed transcription factors have been implicated in the regulation of a wide variety of genes. They associate in homo- and heterodimers (12), and control expression of genes involved in immune and inflammatory responses as well as in basic cellular functions such as adhesion, proliferation, and apoptosis (13–15). The pro- or antiapop-

totic as well as the pro- or antiproliferative functions of cRel depend on the cell type or the cellular context. In HeLa cells, overexpression of cRel protects against apoptosis induced by TNFα (16). In contrast, high levels of cRel mRNA were found in apoptotic cells of avian embryo, and overexpression of cRel in bone marrow cells induced massive cell death (17).

The Ets family is a large family of transcription factors that are involved in the regulation of cellular proliferation, differentiation, migration, apoptosis and cell-cell interaction. The expression of *Arfrp1* in thymus and kidney of adult animals could be controlled by cEts1, since cEts1 has been shown to present in these tissues (18). However, the regulation of *Arfrp1* expression during embryonic development appears to be independent of cEts1 since deletion of cEts1 in mice, in contrast to that of *Arfrp1* (7), did not result in embryonic lethality (19).

The head-to-head organization of *Arfrp1* and its neighboring gene in a very short distance is unusual. Thus, we tested the possibility that they might have related regulation mechanisms, and demonstrated that the intergenic region exhibits promoter activity in both directions. However, the deletion analysis suggested that the two promoters are predominantly controlled by different regulatory elements. Corresponding with this observation, the pattern of expression of both genes differs. ARFRP1 was mainly detected in liver, kidney, thymus, and testis (4). The 5′-flanking gene, in contrast, appears to be expressed ubiquitously, because mRNA (ESTs) was detected in brain, eye, liver, lung, mammary gland, testis, and colon.

There are at least two other examples of a head-to-head orientation of two gene in a short intergenic distance. The human *UFD1L* and *CDC45L* genes are located on chromosome 22q11, and are separated by a 884 bp segment (20). UFD1L is a human homologue of the *Saccharomyces cerevisiae* protein UFD1 which is involved in ubiquitin-directed protein degradation. CDC42L binds to a pre-replication complex on the DNA replication origin during the G1 phase and recruits DNA polymerase α. Igaki *et al.* (20) demonstrated that the promoters of these genes do not overlap. A second example is the head-to-head organization of *Dlad* and *Uox* which has also been described to be conserved in the human genomic sequence (21). *Dlad* encodes a DNA endonuclease, and *Uox* encodes urate oxidase. The authors demonstrated that the intergenic region between *Dlad* and *Uox* (803 bp) exhibited promoter activity in both directions, and suggested that the expression of *Dlad* and *Uox* is coordinated by common regulatory mechanisms.

In summary, the present data demonstrate that a very short DNA segment (54 bp) controls the expression of both *Arfrp1* and a head-to-head oriented, 5′-neighboring gene. However, these genes are regulated independently by different *cis*-acting elements. Furthermore, the data suggest that transcription factors of the cRel and the cEts family are involved in the regulation of *Arfrp1* expression.

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