

Mammalian Homologues of the *Drosophila melanogaster flightless I* Gene Involved in Early Development

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Abstract: The many obvious differences between simple and more complex multicellular organisms mask the extraordinarily high degree of conservation of the basic life processes and the proteins involved in them. The evolutionary conservation of many essential genes allows knowledge gained from mutational studies in simpler organisms to be used to identify the corresponding genes in mammals. The function of mammalian homologues can then be investigated by gene targeting in mice. Early development in the fruitfly *Drosophila melanogaster* is far more accessible to investigation than corresponding mammalian processes and many interesting genes involved in early development in this organism have been identified and isolated. Embryos carrying null alleles of the *Drosophila melanogaster flightless I (fliI)* gene exhibit only partial cellularization and gastrulation fails. Weak alleles of *fliI* result in adult flies lacking flight ability and exhibiting abnormal indirect flight muscle ultrastructure. The *fliI* gene was positionally cloned and the predicted protein of 1256 amino acids found to contain leucine-rich-repeat (LRR) and gelsolin-like domains. Homologues of *fliI* have subsequently been cloned from *C. elegans*, human (*FLII*) and mouse (*Fliih*) via PCR. *FLII* maps to 17p11.2, into a region affected in Smith-Magenis syndrome and in childhood primitive neuroectodermal tumours. The gelsolin domain interacts with G-actin in a Ca²⁺-independent manner, and has F-actin binding and severing activities. FLAP1 and FLAP2, novel ligands for the LRR domain detected in the yeast two-hybrid system, are derived from related mammalian genes. FLAP2 has been independently reported as an HIV-1 TAR RNA binding protein and as a transcriptional repressor. The LRR has been shown to interact with Ras both in vitro, and in vivo in yeast. Genomics-based studies on *fliI* and its homologues have opened up their involvement in a novel pathway of cytoskeletal regulation which appears to play an essential role in early development.

GENERAL INTRODUCTION

Mutational studies in organisms such as bacteria, yeasts and lower invertebrates have identified many genes involved in essential processes. Extensive studies with key model organisms including in particular the fruitfly *Drosophila melanogaster* have uncovered numerous genes which, when mutated, result either in alterations in the course of development or in the arrest of development. It is now clear that, because of the high degree of conservation of basic cellular and developmental processes, many genes in lower organisms have homologues in mammals which encode proteins with significant structural similarity at the amino acid sequence level. We were interested in using sequence

information on *Drosophila* genes involved in developmental and neurobiological processes to uncover novel mammalian genes that would be expected to participate in functionally similar processes to those occurring in *Drosophila*. We planned to use gene targeting in mice to study the role of these novel genes in mammals.

A group of positionally cloned genes from the *Drosophila* X chromosome [1-4] was selected for study as all of these genes were novel, offering the potential for the uncovering of corresponding novel mammalian genes. Several of the genes with known phenotypes were originally selected for cloning and the remainder were transcription units of unknown function that were cloned incidentally. We have identified mammalian homologues of all of the group of 12 genes initially selected for study. The genes with known phenotype include *flightless I (fliI)* [3], *sluggish A (slgA)* [2] and *small optic lobes (sol)* [1] and we have initially focused on the study of mammalian homologues

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of these three genes. The main techniques used to identify homologues have been bioinformatics-based methods and PCR-based methods which depend on significant amino acid sequence identity between the encoded proteins. Most of the homologues have been identified by the bioinformatics-based methods, by using the ever-expanding sequence databases.

FLIGHTLESS I MUTATIONS

Mutations in *flightless I* were isolated by Homyk and Sheppard [5], Koana and Hotta [6] and Deak et al. [7] as X chromosome-linked viable mutations that impaired flight ability. Earlier, Lifschytz and Falk [8] had discovered the X chromosome-linked *W-2* lethal complementation group. Subsequently, it was shown that *flightless I* and several other mutations all mapped to the *W-2* complementation group and were, therefore, all alleles of a gene which could be mutated to lethality [9, 10].

Ultrastructural analysis of viable mutants has revealed ultrastructural defects in the indirect flight muscles [6, 7, 10], but the direct flight muscles appear unaffected [6]. The myofibrils in the indirect flight muscles appear frayed and disorganized, with abnormal or even absent Z-bands. Striated bundles also occur and may be aggregates of thin filament and possibly Z-band material.

In the case of severe *fliI* alleles, mutant embryos lacking maternal product do not cellularize completely and gastrulation fails. In a recent detailed germline clone study of the defects in cellularization and gastrulation in *fliI* mutant embryos, [11] it was found that there are a number of ultrastructural defects in these embryos at this stage. Although the nuclei migrate normally to the periphery, their cortical positioning is slightly less regular than in the wildtype and some even drift towards the centre of the egg. The advancing cleavage furrows are distinctly disordered relative to the wildtype. The ends of the advancing cleavage furrows in wild-type embryos terminate in a tear-drop-shaped area of the furrow canals. In mutant embryos, these structures are not seen, and the ends of the furrow canals appear collapsed. The mutant furrow canals are also associated with small membrane vesicles not found in the wild-type.

In mutant embryos, gastrulation commences even though a significant fraction of the peripheral cytoplasm remains open towards the interior of the

blastoderm. Visualization of the actin cytoskeleton with fluorophore-labelled phalloidin reveals that actin is distributed irregularly along the cleavage furrow membranes. As is the case with the cleavage furrow membranes themselves, the actin cytoskeleton in the mutant embryos reaches to varying depths. Actin staining is localized to the interior region of the cellularizing blastoderm in the wild-type, but reaches into the cellularizing region of *fliI* mutant embryos [11]. These observations support the concept that *fliI* is closely involved with the actin cytoskeleton, at least at this stage of development. Previous observations indicate that the actin cytoskeleton is involved in the cellularization process [12].

DROSOPHILA AND C. ELEGANS GENES

The *Drosophila fliI* genomic region was cloned by chromosome walking from entry points generated by chromosome microdissection and cloning [3]. Northern analysis with genomic probes was used to detect candidate transcription units for *fliI*. Mutant flies carrying a viable *fliI* allele regained flight ability when a wild-type *fliI* transgene on a P-element vector was present and viability was restored to flies that were homozygously deficient for *fliI*. The 5 kb transcription unit encoded by the 10.2 kb *XhoI* fragment that carried the *fliI* gene, as demonstrated by the transgenic rescue, was then cloned by screening cDNA libraries. The cDNA sequence consisted of 4672 bp plus a polyA tail. The encoded *fliI* protein consists 1256 amino acid residues, with the N-terminal region containing a leucine-rich-repeat (LRR) sequence. The C-terminal region shows sequence homology to the actin binding protein gelsolin [13] and other members of the gelsolin family. Analysis of two viable mutant alleles of *fliI* revealed single point mutations leading to conservative amino acid substitutions in the first subdomain of the gelsolin-like region of the protein [14]. Analysis of the lethal allele *l(1)D44* revealed a 400 bp deletion centred on the translation initiation site, indicating that this is highly likely to be a null mutation. Another lethal allele appeared complex, with a 5.4 kb mobile element insertion in *fliI* and a substantial deficiency of more proximal sequences [14].

Identification of *fliI* LRR-related sequences encoded by a *C. elegans* genomic cosmid enabled the isolation of full-length *C. elegans* cDNA [3]. Complete sequence analysis of this 4529 bp clone revealed an encoded *fliI* protein homologue of 1257 amino acids, also consisting of an LRR

domain and a gelsolin-like domain. Goshima et al. [15] have also isolated *C. elegans* cDNAs for the *fliI* homologue based on the similarity between sequences encoded on the same *C. elegans* genomic cosmid and the LRR region of yeast adenylate cyclase and have subsequently studied the functional properties of the encoded protein in some detail (see below). They note that the *C. elegans* gene is most abundantly expressed during the embryonic stages of development of *C. elegans*. The *C. elegans* gene consists of 14 exons split by 13 introns. The first intron splits the 5' untranslated region. It is of interest that the *C. elegans fliI*-homologous gene contains the previously identified *sup-5* gene (GenBank X54122), encoding tRNA^{Trp}, located in intron 9 [3]. The complete genomic *fliI* sequence from the Oregon R strain of *D. melanogaster* was determined in Canberra, and revealed the presence of four exons and three introns, with the first intron of the *Drosophila* gene corresponding exactly in position to intron two of the *C. elegans* gene [3]. G. de Couet and coworkers [14] also determined the *Drosophila fliI* genomic sequence in Hawaii for the Canton S strain.

HUMAN AND MOUSE HOMOLOGUES OF *FLII*

The *Drosophila fliI* protein showed 49% identity (69% similarity) to the *C. elegans* protein. Degenerate oligonucleotide primers corresponding to regions of highly conserved amino acid sequence in the two proteins were used in nested PCR reactions with human genomic DNA and cDNA. One combination of primers gave bands encoding a putative human homologue of the *fliI* protein (~60% identity at the amino acid sequence level). These primers also allowed the identification of a mouse homologue of *fliI*. The Human Gene Nomenclature Committee has assigned the name *FLII* to the human gene, and the MGD Mouse Nomenclature Committee has assigned the name *Fliih* to the mouse gene. Using the human PCR fragments as probes, an essentially full-length *FLII* cDNA was isolated. The encoded human protein of 1269 amino acids is 58% identical (74% similarity) to the *Drosophila fliI* protein [3].

THE HUMAN *FLII* AND MOUSE *FLIIH* GENES

Southern analysis of human genomic DNA with *FLII* cDNA probes showed that *FLII* is present as a single copy in the haploid human genome, and no other related sequences were detectable under

conditions of reduced hybridization stringency [16]. Using FISH analysis, *FLII* was localised to a single chromosomal location [17]. Northern analysis of polyA⁺ mRNA showed that the gene is expressed in all of the tissues tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) [16]. Skeletal muscle and to a lesser extent, heart and lung, showed an elevation of *FLII* mRNA over the relatively uniform level in the remaining tissues. The levels of *FLII* mRNA in all tissues tested appear to parallel the expression levels of actin. Human gelsolin mRNA has a rather different tissue distribution [18]. The high level of *FLII* mRNA in skeletal muscle is of interest in view of the muscle phenotype of the viable alleles in *Drosophila*. One interesting possibility is that homozygous mutations in the human *FLII* gene analogous to the viable *Drosophila* mutations could result in a human genetic muscular disorder.

Initial FISH mapping with the *FLII* cDNA probe showed that *FLII* maps to 17p11.2, a region also containing Smith-Magenis syndrome (SMS) deletions. SMS is a human chromosomal microdeletion syndrome characterized by mental retardation and a range of physical, developmental and behavioural abnormalities [19, 20]. The *FLII* cDNA was used to isolate three human genomic cosmid clones from a chromosome 17-specific cosmid library, and these cosmids were used in FISH mapping experiments to map the position of *FLII* in relation to SMS deletions. FISH mapping with a mixture of the three cosmids showed that *FLII* mapped to 17p11.2, into the critical region deleted in all SMS patients tested. These results were confirmed by Southern analysis of DNA from somatic cell hybrid cell lines containing a range of 17p abnormalities including SMS deletions [17].

Using the three *FLII* cosmids as a probe, the *FLII* gene was shown to be deleted in a clinical case where cytogenetic findings were previously equivocal [21]. Analysis by flow cytometry suggested that the deletion in this case was less than 2 Mb. The general utility of these *FLII*-related probes in cases of this type was indicated. SMS deletions ranging from less than 2 Mb up to 9-10 Mb are known, with no apparent correlation between the size of the deletion and the severity of symptoms [22]. The most common types of SMS deletion appear to be generated by recombination between long flanking repeated sequences termed *SMS-REPD*, *SMS-REPM* and *SMS-REPP* [23]. *FLII* lies between *SMS-REPD* and *SMS-REPM*. Recent evidence suggests that the size of the minimal SMS deletion interval may be as small as

700 kb [24]. The corresponding mouse genomic region on chromosome 11 has recently been cloned and characterized [25]. In the mouse, a large segment containing *Fliih* and a number of other genes, and corresponding to the human region between *SMS-REPD* and *SMS-REPM* appears to be substantially conserved in gene order although this group of genes has the opposite orientation with respect to the centromere to the human region on chromosome 17. A second group of genes between *SMS-REPM* and *SMS-REPP* in human is inverted in order in the mouse with respect to the first group. The nature of these rearrangements of the mouse region corresponding to the human SMS region is being considered in experiments aimed at generating a mouse model of SMS via chromosome engineering [25]. As it appears that the corresponding genes on the non-deleted chromosome are normal, it seems likely that haploinsufficiency of one or a small number of genes in the SMS interval is responsible for SMS in humans. However, it is also possible that mutations of the remaining allele of a gene or genes deleted in SMS may play some role in the generation of SMS phenotypic effects. A number of other genes have been shown to map into the SMS interval, including *LLGL* [23, 24, 26-28] the human homologue of the *D. melanogaster lethal(2) giant larvae (l(2)gl)* gene [29].

Childhood primitive neuroectodermal tumours (PNETs) are the commonest childhood brain tumours. Recent work indicates that chromosomal breakpoints involved in the hemizygous loss of 17p commonly involved in PNETs cluster at 17p11.2 in an area which overlaps the SMS critical region [24, 30, 31]. The possible location of previously unidentified tumour suppressor genes in this general region is further indicated by additional evidence beyond the scope of this review. Gelsolin itself is also implicated as a candidate tumour suppressor gene [13, 32, 33]. Indirect evidence suggests that other gelsolin-related genes may be tumour suppressors [34] *FLII* and *LLGL* are therefore candidate genes for involvement in the biology of PNETs and possibly other tumours, as well as SMS.

In order to determine the structure of the human *FLII* gene, the complete gene was isolated. Cosmids spanning the gene were isolated from gridded libraries, and a restriction map of 70 kb of genomic DNA in the vicinity of *FLII* was constructed. Cosmid c5C2, which spanned the complete *FLII* gene was chosen for detailed analysis. A 13.7 kb *NotI* fragment containing most of the gene was fully sequenced on both strands,

and additional sequencing was done on an immediately adjacent 9 kb *NotI* fragment, yielding the complete structure of the human *FLII* gene [16]. One end of the 9 kb *NotI* fragment contained part of the final exon of *FLII*. The other end matched part of the *LLGL* cDNA sequence, with the results indicating that the *FLII* and *LLGL* genes are close together, but are transcribed in the opposite orientation. As mentioned above, *LLGL* is a human homologue of the *Drosophila lethal(2) giant larvae* gene, one of the first tumour suppressor genes to be identified and cloned from any organism [29]. The mouse homologue *Lglh* was cloned as a potential target gene for the homeobox gene *Hox-C8* [26] More recently, *LLGL*, the human homologue of *l(2)gl* was also cloned [27, 28].

Five cosmids from a gridded chromosome 17 cosmid library hybridized to both the *FLII* cDNA probe and an *LLGL* cDNA probe, confirming the close proximity of these genes [16]. A search of the sequence databases with the *FLII* cDNA 3' end sequence revealed a short, strong match to the 3' end (in the opposite orientation) of the cDNA for mouse *Lglh*. The available cDNA sequences for *LLGL* are truncated at the 3' end so it was not possible immediately to compare the 3' end of *FLII* cDNA with that of human *LLGL* cDNA. However, analysis of 2.5 kb of sequence extending 3' from within the mouse *Fliih* gene, which was also being sequenced at that time, showed that it corresponded exactly to the 3' end of *Lglh* cDNA, allowing for several introns. This formally established that mouse *Fliih* and *Lglh* overlap.

Sequence corresponding to the complete 3' end of *LLGL* cDNA was required to verify that human *FLII* and *LLGL* also overlap. We used PCR to amplify an *LLGL* cDNA fragment which overlapped an EST cDNA clone carrying the complete 3' end of *LLGL*. Comparison of the 3' end of the human *FLII* cDNA and genomic sequence with this 3' end sequence for *LLGL* cDNA confirmed that the 3' ends of the human *FLII* and *LLGL* transcripts overlap. The region of overlap is highly conserved between human and mouse [16].

The overlap region contains at least two functional polyA signals for *FLII*, both of which are the variant ATTAAA signal rather than the more usual AATAAA. The majority of human *FLII* EST clones are polyadenylated using the most 5' polyA signal. Mouse brain *Fliih* cDNAs are also polyadenylated using the corresponding signal. A few *FLII* EST clones use a more 3'

polyA signal. A third conserved ATTTAA sequence is located just downstream and possibly may also serve as a minor polyA signal for *FLII/Fliih*. It is unclear whether the use in the *FLII* gene of multiple copies of the weaker, variant ATTTAA polyA signal has regulatory significance, and how signal/site selection is governed. The *C. elegans* *flii*-related gene uses the variant poly(A) signal AATAAT, whereas the *Drosophila* *flii* gene has a single copy of the canonical ATTTAA sequence. A single conserved AATAAT polyA signal for *LLGL* is also present in the overlap region but on the opposite strand. An additional potential polyA signal for *LLGL/Llglh* consisting of the variant ATTTAA sequence is also present in the overlap region, but it is not known whether this signal is functional.

While the significance of the overlap of these genes is unclear, one consequence is that mutations in this region could affect expression of both genes in mammals. In *Drosophila*, *l(2)gl* maps to 21A [35], whereas *flii* maps to 19F [10]. In *C. elegans*, a homologue of *l(2)gl* is present, mapping to the X chromosome, whereas the *flii* homologue maps to chromosome III. [36]. Therefore it is clear that it is not necessary for the genes to be overlapping, or even on the same chromosome, at least in some eukaryotic organisms.

In the context of the overlap of the genes in humans and mice, it is interesting that the two genes may both be involved with the actin-based cytoskeleton. *FLII* and its homologues encode members of the gelsolin family of actin-binding proteins, and a subdomain of the *FLII* protein itself has been shown to bind G actin in a 1:1 stoichiometry [37], although it does not sever F actin filaments as has been shown for the cognate subdomain of gelsolin. In *Drosophila*, the *flii* protein may be required for the correct distribution of actin during cellularization [11]. The *Drosophila* *l(2)gl* protein has been shown to be a component of the cytoskeleton and to interact with nonmuscle myosin II [38, 39]. Moreover, it has been shown that the *l(2)gl* protein is required for epithelial cell shape changes during development, and it was concluded that it probably plays a role in gastrulation [40]. In the yeast system, recent evidence suggests that yeast lethal(2) giant larvae homologues are involved with other functions such as exocytosis [41] and cation homeostasis [42] although strong evidence of their involvement with the cytoskeleton has also been adduced [43]. Evidence has been presented indicating that the human *LLGL* protein is a component of the

cytoskeleton and interacts with nonmuscle myosin II [27].

FLII GENE FAMILY EVOLUTION

The human *FLII* gene contains 30 exons split by 29 introns and spans 14 kb of genomic DNA [16]. Intron 1 of *FLII* corresponds exactly in position to intron 1 of *Drosophila* and intron 2 of *C. elegans*. This intron is located in the N-terminal region of the LRR domain, and is the only intron conserved in position between all three homologues. This intron is highly likely to predate the divergence of the lineages that led to *C. elegans*, *Drosophila* and human. The third intron of *Drosophila* is conserved in position in human *FLII*, and two other introns are conserved in position between *C. elegans* and human. These latter two introns are also likely to predate the divergence of the lineages that led to *C. elegans*, *Drosophila* and human. A number of introns are conserved between various gelsolin family members, and at least some may have been present prior to the final gene duplication event that gave rise to the dimeric family members. Some may also have been present, prior to the ancestral triplication and may, therefore, be very ancient. Determination of the genomic sequences of further members of the gelsolin gene family from various species may help verify this. Information from intron positions is contributing to our understanding of gelsolin family evolution [16, 44].

The mammalian genome contains genes encoding a number of different gelsolin family members with readily detectable sequence homology. These include gelsolin itself [13], villin [45], Cap G [46], the *FLII* protein, adseverin or scinderin [47], supervillin [48, 49] and advillin [50]. At the sequence level, gelsolin contains two large related domains, and both of these contain evidence of a triplication [51]. The 3D structures of portions of gelsolin and related proteins indicates that each of the triplicated units folds into a discrete subdomain. Recent evidence has established that the related proteins destrin and cofilin, while having essentially no primary sequence homology to gelsolin-related proteins, have tertiary structures closely related to the individual triplicated subdomains of gelsolin, villin and severin [52].

Comparison of particular gelsolin family members between human and *Drosophila* indicates that the *FLII* proteins are much more

strongly conserved than either gelsolin or villin. The gelsolin-like domains of the human and *Drosophila* FLII proteins are 59% identical (75% similarity). In comparison, human and *Drosophila* cytoplasmic gelsolins are 40% identical (58% similarity). The gelsolin-like domain of human villin is only 29% identical (50% similarity) to the gelsolin-like domain of the *Drosophila* quail protein [53], a villin homologue.

Detailed phylogenetic analysis of FLII proteins and other members of the gelsolin gene family indicates that the mammalian capping protein Cap G has arisen by deletion of segment 2 from a dimeric precursor closely related to the gelsolin/adseverin branch of the family [16, 44]. Therefore, it does not represent the precursor molecule from which the duplication was originally generated, as had earlier been thought, but arose subsequent to the duplication. It is possible that the fungal proteins severin and fragmin may have evolved from the hypothetical precursor of the duplication event.

INTERACTION WITH ACTIN

The gelsolin domain of the fliI-related proteins has been shown to exhibit a Ca^{2+} -independent G actin binding activity as well as F actin binding and severing activities [15, 54]. Liu and Yin [54] produced ^{35}S -labelled human FLII protein and its LRR domain by in vitro transcription/translation. Binding of the labelled proteins to actin-Sepharose resin was then tested, and evidence supporting specific binding of FLII, but not the FLII LRR, was obtained. These results indicate that the observed actin binding was mediated via the gelsolin-like domain of FLII. Goshima and coworkers [15] noticed that amino acids involved in Ca^{2+} binding in gelsolin are not conserved in the *C. elegans* fliI homologue. Recombinant *C. elegans* fliI protein was shown to bind to monomeric-actin-agarose in a Ca^{2+} -independent manner. The recombinant *C. elegans* fliI gelsolin domain was shown to sediment with F-actin on ultracentrifugation, indicating that fliI binds to F-actin as well as G-actin. At higher levels of the *C. elegans* fliI gelsolin domain, both actin and fliI were found in the supernatant, suggesting that fliI may have an F-actin severing activity. Electron microscopic studies on the effect of the *C. elegans* fliI gelsolin domain on the polymerization state of actin confirmed the F-actin severing activity.

LIGANDS FOR THE LRR DOMAIN

FLAP

Liu and Yin [54] were the first to clone a novel protein that interacts with the fliI LRR. They called this protein FLAP or FLAP1 (FLII LRR associated protein). Other workers have subsequently cloned similar molecules, but have given them different names (see below). However, we suggest that the name FLAP has the advantage of priority of discovery as well as being a simple, clear acronym (**FLII LRR associated protein**). Liu and Yin [54] cloned FLAP via the yeast two-hybrid system, using the human FLII LRR as bait. No interaction between FLAP and the FLII gelsolin-like domain was detected in the two-hybrid system. Following the transfection of cultured human embryonic kidney cells with appropriate expression plasmids, immunoprecipitation of expressed epitope-tagged FLII LRR led to specific co-precipitation of FLAP. These results further supported the interaction of the FLII LRR with FLAP. The original human FLAP clone contained an insert of 0.65 kb, with a translation initiation codon but no translation termination codon. On database searching, two mouse EST clones were found to match *FLAP*, although no other significant homologies were detected. One of these contained two insertions relative to *FLAP* and the authors speculated that there could be multiple splice variants or multiple genes. Northern analysis of human polyA⁺ mRNA from various tissues revealed at least four hybridizing bands of 2.7, 2.9, 3.3 and 5.1 kb present in varying ratios in all of the tissues examined. *FLAP* was most strongly expressed in skeletal muscle, in the form of the 3.3 kb mRNA. It was also verified that *FLII* was most strongly expressed in skeletal muscle, as shown previously [16]. The human *FLAP* cDNA was used to clone longer mouse cDNAs. The longest open reading frame obtained encoded a protein of 628 amino acids (MW 71 kDa). The mouse cDNA cloning provided further evidence for splicing variants as had also been found for the human *FLAP*. The mouse protein was dubbed FLAP-1. We suggest Flap1 for the mouse protein and *Flap1* for the mouse gene, consistent with mouse genetic nomenclature rules. Similarly FLAP1 for the human protein and *FLAP1* for the human gene would be consistent with human nomenclature rules.

Liu and Yin [54] then used the yeast two-hybrid system to show that Flap1 interacts with the FLII LRR to an extent comparable with the original

short human FLAP clone. Immunoprecipitation experiments on material expressed in cultured human embryonic kidney cells were used to show that mouse Flap1 interacts with the human FLII LRR. The Flap protein that was coimmunoprecipitated with the epitope-tagged FLII LRR had an apparent MW of 85 kDa compared with the predicted size of 71 kDa. An anti-human FLAP polyclonal antibody was used to confirm the identity of the 85 kDa band as Flap1. One possibility is that this protein, which is particularly rich in acidic (18.3% Asp, Glu) and basic (18.2% Lys, Arg, His) residues, binds SDS anomalously, affecting its migration, and hence apparent MW, in SDS gels. Another possibility is that Flap1 is posttranslationally modified, e.g., via glycosylation.

A molecule closely related to FLAP was cloned by Wilson et al. [55] as a double-stranded RNA binding protein. The clone was obtained by screening a Chinese hamster ovary cell line cDNA expression library with a radiolabelled RNA probe based on the HIV-1 TAR element. The encoded molecule was called TRIP (TAR RNA interacting protein). Identification of a corresponding human EST clone enabled isolation of a full-length human *TRIP* cDNA. Database searches indicated a close relationship between TRIP and the FLII LRR associated protein FLAP. The human *TRIP* cDNA encodes a protein of 784 amino acids with a MW of 86 kDa. The human and partial hamster sequences were expressed as fusion proteins in *E. coli* and the human cDNA was also expressed in cultured mammalian cells using a CMV expression vector. Both exhibited significantly higher apparent MWs on SDS gel electrophoresis than the calculated values, as observed for FLAP [54]. Detailed experiments confirmed the binding of TRIP to TAR RNA (K_d 14 nM) and localized the binding to a lysine-rich sequence of 20 amino acids. Once the relationship between FLAP and TRIP was realized, the binding of TRIP to the FLII LRR was tested. The FLII LRR was expressed in radiolabelled form by *in vitro* transcription/translation and shown to bind to a GST-TRIP fusion protein immobilized on glutathione agarose beads. The intracellular localization of TRIP was examined in monkey COS-7 cells using a TRIP-green-fluorescent-protein (GFP) fusion mammalian expression vector. The TRIP-GFP fusion protein was expressed throughout the cytoplasm but excluded from the nucleus.

Reed et al. [56] cloned a gene they termed *GCF2*. The gene was cloned using the 5'-end

fragment from a previously obtained clone for GC-binding factor (GCF), a repressor for the epidermal growth factor receptor (*EGFR*) gene. This 5'-end fragment of *GCF* was shown to specifically detect a 4.3 kb mRNA on Northern blots that was not detected by probes based on the remainder of the *GCF* cDNA. The cloned *GCF2* cDNA shows a short, centrally-located region of homology with the 5'-end of *GCF*. Sequence comparisons have now established that *GCF2* is derived from the same gene as *TRIP*. Like FLAP and TRIP, the *GCF2* protein shows an anomalously high apparent MW on SDS gel electrophoresis. Extensive studies appear to demonstrate specific binding of *GCF2* to the *EGFR* promoter via DNA footprinting as well as repression of *EGFR* expression in cotransfection experiments, abolished by mutation of the binding site [56]. Nerve growth factor treatment of PC12 cells leads to a decrease in *EGFR* numbers correlating with an increase in *GCF2*, suggestive of an *in vivo* role for the observed *in vitro* repression of *EGFR* expression by *GCF2* [57]. A recent study [58] demonstrates the ability of *GCF2* to repress expression of a reporter gene driven by the platelet-derived growth factor A chain gene promoter. *GCF2* was shown by electrophoretic mobility shift assays to bind to a site within a GC-rich promoter region and to compete for binding to this region with positive regulatory factors such as Sp1, Egr-1 and Sp3 [58]. Expression of *GCF2* from a transfected plasmid was shown to repress PDGF A chain gene expression *in vivo* in two vascular cell types. In smooth muscle cells, addition of the PDGF A-chain dimer protein reversed the growth inhibition resulting from transfection of a *GCF2* expression plasmid [58]. Overall, these results support the hypothesis that *GCF2* is a negative regulator of transcription that exerts its effects via binding to specific DNA sequences in target genes.

Fong and de Couet [59] have also cloned human proteins interacting with the human FLII LRR using the yeast two-hybrid system. They named these LRRFIP1 (LRR FLI interacting protein 1) and LRRFIP2 (LRR FLI interacting protein 2). The *LRRFIP2* transcript appears to be derived from the same gene as that from which *FLAP1* transcripts are derived. Similarly, *LRRFIP1* appears to be derived from the same gene as *TRIP*. Consistent with the discussion of the nomenclature of these genes above, we propose that the two genes be named *FLAP1* and *FLAP2*. *FLAP1* would encompass *LRRFIP2*, and *FLAP2* would encompass *TRIP* and *LRRFIP1*. It is of interest that what may be a processed

pseudogene for *FLAP* is present on the X chromosome. We detected this in a human genomic DNA sequence from the X chromosome (GenBank Accession No. AC004074) by database searching. Since it is an open question at present as to whether this gene has any functionality, we propose the name *FLAPL* ("FLAP-like") for this gene, consistent with human nomenclature rules.

The *C. elegans* genome appears to encode at least one homologue of FLAP. The N-terminus of this FLAP homologue is encoded by cosmid F57B9 (reading frame F57B9.7) and the C-terminus on the overlapping cosmid T07E3 (reading frame T07E3.7). Fong and de Couet [59] detected the relatively short region of homology between FLAP and a reading frame encoded by cosmid F57B9.7 and suggested that the mammalian proteins are effectively multimers of a "primordial interaction motif" present in F57B9.7. However it appears, as noted above, that a larger FLAP-like molecule is encoded by the overlapping cosmids F57B9 and T07E3. Complete analysis of this gene may require analysis of full-length cDNAs, as the level of homology with FLAP appears too low to successfully determine all of the *C. elegans* exons based on homology alone. This low level of homology is intriguing, as the LRR domains of the human and *C. elegans* *flightless* homologues exhibit strong homology (54% identity and 73% similarity at the amino acid sequence level). The LRR domain also interacts with Ras, which exhibits a much higher level of conservation between *C. elegans* and human (e.g., 41% identity, 57% similarity for the *C. elegans* Ras2 and human N-ras proteins). While it is clear that FLAP1 (LRRFIP2) and FLAP2 (TRIP, LRRFIP1, GCF2) bind to FLII under certain circumstances, it will be important to establish the *in vivo* functional role of this interaction. Further studies on the transcriptional regulation function and RNA-binding functions of FLAP2, and possibly FLAP1 if it also possesses similar activities, may also be required to help clarify whether these proteins fulfil multiple functions *in vivo*.

Ras

One of the most closely related LRR sequences to that present in the *fliI*-related proteins is that of yeast adenylyate cyclase [44, 60]. The yeast adenylyate cyclase LRR has been shown to directly interact with the Ras protein [61], suggesting a similar role for the FLII LRR. The LRR region of FLII is also more closely related to the LRRs of the other Ras-binding proteins than to other LRR proteins. Detailed analysis of FLII protein LRRs in

comparison with a number of known LRRs in various proteins including yeast adenylyate cyclase and other Ras-binding proteins led to the conclusion that the LRRs of FLII proteins may be involved in interaction with a member of the Ras family of proteins [44, 60].

Recently, strong evidence has been presented that confirms an interaction between Ras and the *C. elegans* *fliI* homologue [15]. The N-terminal 770 amino acids of the *C. elegans* protein containing the LRR domain were synthesized in epitope-tagged form by *in vitro* transcription/translation. Ha-Ras was expressed as a GST fusion protein and immobilized on glutathione agarose. Incubation of the *C. elegans* *fliI* fragment with the immobilized Ha-Ras followed by elution with glutathione and detection by Western blotting enabled demonstration of specific binding of the *C. elegans* *fliI* fragment to the immobilized Ha-Ras. Moreover, the *C. elegans* *fliI* fragment did not associate with GST fusion proteins for Rac1, RhoA, Cdc42 and RalA, emphasizing the specificity of the interaction with Ras. The binding of Ras to the *C. elegans* *fliI* homologue was also demonstrated by showing *in vitro* inhibition of Ras-dependent yeast adenylyate cyclase activity by a GST fusion protein containing the complete *C. elegans* homologue. A GST fusion protein lacking the LRR domain and GST itself were ineffective. The full-length fusion protein had no effect on Mn²⁺-dependent (Ras-independent) adenylyate cyclase activity, indicating that the inhibitory activity is mediated via Ras and is not a direct effect on adenylyate cyclase. Detailed analysis using this system established that the *K_d* value for binding of the *C. elegans* protein to Ras2 was 11 nM.

The association was also demonstrated *in vivo* in yeast. Yeast cells carrying an activated *RAS2* gene (*RAS2^{Val-19}*) exhibit a heat-shock-sensitive phenotype known to be suppressed by expression of Ras-binding proteins. Expression of the full-length *C. elegans* *fliI* protein or a corresponding GST fusion protein was shown to suppress this heat-shock-sensitive phenotype, whereas a form lacking the LRR, or GST itself, were ineffective. Previous attempts to confirm the predicted interaction between FLII and Ras (and Ras-related proteins such as Rho, Rac and CDC42) were unsuccessful [54]. One possibility is that while Ras may be an LRR ligand for *C. elegans* *fliI*, this is not the case for human FLII. The ligand for FLII could conceivably be an untested Ras-related protein, for example. Alternatively [15], the human FLII LRR expressed by *in vitro*

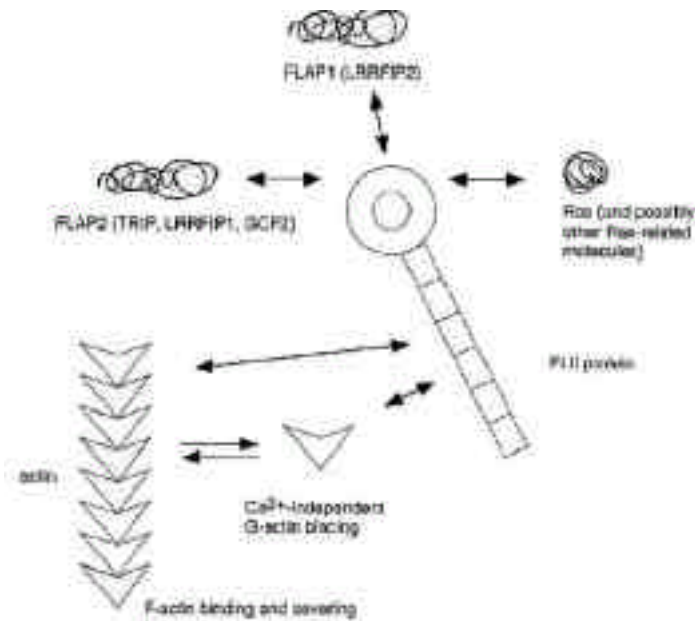


Fig. (1). Schematic representation of the FLII-related proteins and their interactions. See text for details. The LRR domain of the FLII protein is represented by a ring and the gelsolin-like domain by a linear arrangement of 6 joined, open boxes representing the repetitive units that make up this region. The LRR domain is shown as interacting with Ras, and with FLAP1 (also known as LRRFIP2) and FLAP2 (also known as TRIP, LRRFIP1 and GCF2). The interactions of the gelsolin-like domain of FLII with F- and G-actin are also shown.

transcription translation, as used by Liu and Yin [54] for binding assays, may not be correctly folded. In support of this, Goshima et al. [15] report that the *C. elegans* fliI LRR is expressed in an insoluble form in *E. coli*, yeast and insect Sf9 cells. Goshima et al. [15] also argue that the failure to detect FLII LRR-Ras interactions in the yeast two hybrid system is not significant, as the well-characterized interaction between Ras and the yeast adenylate cyclase LRR is only barely detectable in the two-hybrid system. Given the high degree of primary amino acid sequence conservation in fliI and its homologues, and in particular, in the LRR domain, as noted above, it seems very likely that Ras or a closely related molecule is a ligand for the *D. melanogaster*, mouse and human proteins as well as for the *C. elegans* protein. A summary of the experimentally observed interactions of the fliI and related proteins is presented in Fig. (1).

A role for the FLII protein in modulation of the cytoskeleton by Ras-related signal transduction pathways can readily be envisaged. There is a significant amount of indirect evidence supporting a link between Ras-related pathways and regulation of the cytoskeleton. In addition, mammalian systems contain more than 50 Ras-related GTPases [62] and some of these, including Cdc42, Rac and Rho, are directly implicated in cytoskeletal organization and morphogenetic

processes. The *Drosophila* homologues of *CDC42* and *Rac* are involved in muscle development at the myoblast fusion stage, and in neuronal development [63].

In summary, studies on the *D. melanogaster* flightless 1 gene and the subsequent genomics-based discovery of homologues of this novel gene in organisms ranging from the nematode *C. elegans* to mouse and human, have led to increased understanding of the nature and evolution of this conserved gelsolin-related gene. Bioinformatics-based methods led to the prediction that the fliI protein and its homologues interact with the central signalling molecule Ras. Recent work with the *C. elegans* homologue has confirmed this prediction. This may be the first example of the successful prediction of the ligand for an LRR protein based solely on sequence classification. Further examples will surely follow. The interaction of the gelsolin-related domain of fliI with actin has been demonstrated with both the human and in more detail with the *C. elegans* proteins. The recent targeted inactivation of the mouse *Fliih* gene (unpublished data)¹ via homologous recombination in embryonic stem

¹ Campbell, H.D., Young, I.G. and Matthaei, K.I., in preparation.

cells should shed important light on the role of this gene in mammalian development.

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REFERENCES

- [1] Delaney, S.J.; Hayward, D.C.; Barleben, F.; Fischbach, K.F. and Miklos, G.L. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7214-7218.
- [2] Hayward, D.C.; Delaney, S.J.; Campbell, H.D.; Ghysen, A.; Benzer, S.; Kasprzak, A.B.; Cotsell, J.N.; Young, I.G. and Miklos G.L. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 2979-2983.
- [3] Campbell, H.D.; Schimansky, T.; Claudianos, C.; Ozsarac, N.; Kasprzak, A.B.; Cotsell, J.N.; Young, I.G.; de Couet, H.G. and Miklos, G.L.G. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 11386-11390.
- [4] Maleszka, R.; de Couet, H.G. and Miklos, G.L. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 3731-3736.
- [5] Homyk, T.; Jr. and Sheppard, D.E. (1977) *Genetics*, **87**, 95-104.
- [6] Koana, T. and Hotta, Y. (1978) *J. Embryol. Exp. Morphol.*, **45**, 123-143.
- [7] Deak, I.I.; Bellamy, P.R.; Bienz, M.; Dubuis, Y.; Fenner, E.; Gollin, M.; Rähmi, A.; Ramp, T.; Reinhardt, C.A. and Cotton, B. (1980) *J. Embryol. Exp. Morphol.*, **69**, 61-81.
- [8] Lifschytz, E. and Falk, R. (1969) *Mutat. Res.* **8**, 147-155.
- [9] Perrimon, N.; Smouse, D. and Miklos, G.L. (1989) *Genetics*, **121**, 313-331.
- [10] Miklos, G.L.G. and de Couet, H.G. (1990) *J. Neurogenet.*, **6**, 133-151.
- [11] Straub, K.L.; Stella, M.C. and Leptin, M. (1996) *J. Cell Sci.*, **109**, 263-270.
- [12] Schejter, E.D. and Wieschaus, E. (1993) *Annu. Rev. Cell Biol.* **9**, 67-99.
- [13] Kwiatkowski, D.J. (1999) *Curr. Opin. Cell Biol.*, **11**, 103-108.
- [14] de Couet, H.G.; Fong, K.S.; Weeds, A.G.; McLaughlin, P.J. and Miklos, G.L. (1995) *Genetics*, **141**, 1049-1059.
- [15] Goshima, M.; Kariya, K.-i.; Yamawaki-Kataoka, Y.; Okada, T.; Shibatohe, M.; Shima, F.; Fujimoto, E. and Kataoka, T. (1999) *Biochem. Biophys. Res. Commun.*, **257**, 111-116.
- [16] Campbell, H.D.; Fountain, S.; Young, I.G.; Claudianos, C.; Hoheisel, J.D.; Chen, K.-S. and Lupski, J.R. (1997) *Genomics*, **42**, 46-54.
- [17] Chen, K.-S.; Gunaratne, P.H.; Hoheisel, J.D.; Young, I.G.; Miklos, G.L.G.; Greenberg, F.; Shaffer, L.G.; Campbell, H.D. and Lupski, J.R. (1995) *Am. J. Hum. Genet.*, **56**, 175-182.
- [18] Paunio, T.; Kangas, H.; Kiuru, S.; Palo, J.; Peltonen, L. and Syvanen, A.C. (1997) *FEBS Lett.*, **406**, 49-55.
- [19] Smith, A.C.; McGavran, L.; Robinson, J.; Waldstein, G.; Macfarlane, J.; Zonona, J.; Reiss, J.; Lahr, M.; Allen, L. and Magenis, E. (1986) *Am. J. Med. Genet.*, **24**, 393-414.
- [20] Greenberg, F.; Lewis, R.A.; Potocki, L.; Glaze, D.; Parke, J.; Killian, J.; Murphy, M.A.; Williamson, D.; Brown, F.; Dutton, R.; McCluggage, C.; Friedman, E.; Sulek, M. and Lupski, J.R. (1996) *Am. J. Med. Genet.*, **62**, 247-254.
- [21] Juyal, R.C.; Greenberg, F.; Mengden, G.A.; Lupski, J.R.; Trask, B.J.; van den Engh, G.; Lindsay, E.A.; Christy, H.; Chen, K.S.; Baldini, A.; Shaffer, L.G. and Patel, P.I. (1995) *Am. J. Med. Genet.*, **58**, 286-291.
- [22] Trask, B.J.; Mefford, H.; van den Engh, G.; Massa, H.F.; Juyal, R.C.; Potocki, L.; Finucane, B.; Abuelo, D.N.; Witt, D.R.; Magenis, E.; Baldini, A.; Greenberg, F.; Lupski, J.R. and Patel, P.I. (1996) *Hum. Genet.*, **98**, 710-718.
- [23] Chen, K.-S.; Manian, P.; Koeuth, T.; Potocki, L.; Zhao, Q.; Chinault, A.C.; Lee, C.C. and Lupski, J.R. (1997) *Nature Genet.*; **17**, 154-163.
- [24] Seranski, P.; Heiss, N.S.; Dhorne-Pollet, S.; Radelof, U.; Korn, B.; Hennig, S.; Backes, E.; Schmidt, S.; Wiemann, S.; Schwarz, C.E.; Lehrach, H. and Poustka, A. (1999) *Genomics*, **56**, 1-11.
- [25] Probst, F.J.; Chen, K.-S.; Zhao, Q.; Wang, A.; Friedman, T.B.; Lupski, J.R. and Camper, S.A. (1999) *Genomics*, **55**, 348-352.
- [26] Tomotsune, D.; Shoji, H.; Wakamatsu, Y.; Kondoh, H. and Takahashi, N. (1993) *Nature*, **365**, 69-72.

- [27] Strand, D.; Unger, S.; Corvi, R.; Hartenstein, K.; Schenkel, H.; Kalmes, A.; Merdes, G.; Neumann, B.; Krieg-Schneider, F.; Coy, J.F.; Poustka, A.; Schwab, M. and Mechler, B.M. (1995) *Oncogene*, **11**, 291-301.
- [28] Koyama, K.; Fukushima, Y.; Inazawa, J.; Tomotsune, D.; Takahashi, N. and Nakamura, Y. (1996) *Cytogenet. Cell Genet.*, **72**, 78-82.
- [29] Baek, K.H. (1999) *Mutat. Res.*, **436**, 131-136.
- [30] Scheurlen, W.G.; Seranski, P.; Mincheva, A.; Kühl, J.; Sörensen, N.; Krauss, J.; Lichter, P.; Poustka, A. and Wilgenbus, K.K. (1997) *Genes Chromosomes Cancer*, **18**, 50-58.
- [31] Wilgenbus, K.K.; Seranski, P.; Brown, A.; Leuchs, B.; Mincheva, A.; Lichter, P. and Poustka, A. (1997) *Genomics*, **42**, 1-10.
- [32] Tanaka, M.; Sazawa, A.; Shinohara, N.; Kobayashi, Y.; Fujioka, Y.; Koyanagi, T. and Kuzumaki, N. (1999) *Cancer Gene Ther.*, **6**, 482-487.
- [33] Asch, H.L.; Head, K.; Dong, Y.; Natoli, F.; Winston, J.S.; Connolly, J.L. and Asch, B.B. (1996) *Cancer Res.*; **56**, 4841-4845.
- [34] Ishikawa, S.; Kai, M.; Tamari, M.; Takei, Y.; Takeuchi, K.; Bandou, H.; Yamane, Y.; Ogawa, M. and Nakamura, Y. (1997) *DNA Res.*, **4**, 35-43.
- [35] Mechler, B.M.; McGinnis, W. and Gehring, W.J. (1985) *EMBO J.*, **4**, 1551-1557.
- [36] Sulston, J.; Du, Z.; Thomas, K.; Wilson, R.; Hillier, L.; Staden, R.; Halloran, N.; Green, P.; Thierry-Mieg, J.; Qiu, L.; Dear, S.; Coulson, A.; Craxton, M.; Durbin, R.; Berks, M.; Metzstein, M.; Hawkins, T.; Ainscough, R. and Waterston, R. (1992) *Nature*, **356**, 37-41.
- [37] Orloff, G.J.; Allen, P.G.; Miklos, G.L.G.; Young, I.G.; Campbell, H.D. and Kwiatkowski, D.J. (1995) *Mol. Biol. Cell*, **6**, 139.
- [38] Strand, D.; Raska, I. and Mechler, B.M. (1994) *J. Cell Biol.*, **127**, 1345-1360.
- [39] Kalmes, A.; Merdes, G.; Neumann, B.; Strand, D. and Mechler, B.M. (1996) *J. Cell. Sci.*, **109**, 1359-1368.
- [40] Manfrulli, P.; Arquier, N.; Hanratty, W.P. and Sémériva, M. (1996) *Development*, **122**, 2283-2294.
- [41] Lehman, K.; Rossi, G.; Adamo, J.E. and Brennwald, P. (1999) *J. Cell. Biol.*, **146**, 125-140.
- [42] Larsson, K.; Bohl, F.; Sjostrom, I.; Akhtar, N.; Strand, D.; Mechler, B.M.; Grabowski, R. and Adler, L. (1998) *J. Biol. Chem.*, **273**, 33610-33618.
- [43] Kagami, M.; Toh-e, A. and Matsui, Y. (1998) *Genetics*, **149**, 1717-1727.
- [44] Claudianos, C. and Campbell, H.D. (1995) *Mol. Biol. Evol.*, **12**, 405-414.
- [45] Pringault, E.; Robine, S. and Louvard, D. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10811-10815.
- [46] Mishra, V.S.; Henske, E.P.; Kwiatkowski, D.J. and Southwick, F.S. (1994) *Genomics*, **23**, 560-565.
- [47] Nakamura, S.; Sakurai, T. and Nonomura, Y. (1994) *J. Biol. Chem.*, **269**, 5890-5896.
- [48] Pestonjamas, K.N.; Pope, R.K.; Wulfkühle, J.D. and Luna, E.J. (1997) *J. Cell Biol.*, **139**, 1255-1269.
- [49] Pope, R.K.; Pestonjamas, K.N.; Smith, K.P.; Wulfkühle, J.D.; Strassel, C.P.; Lawrence, J.B. and Luna, E.J. (1998) *Genomics*, **52**, 342-351.
- [50] Marks, P.W.; Arai, M.; Bandura, J.L. and Kwiatkowski, D.J. (1998) *J. Cell Sci.*, **111**, 2129-2136.
- [51] Way M. and Weeds, A. (1988) *J. Mol. Biol.*, **203**, 1127-1133.
- [52] Hatanaka, H.; Ogura, K.; Moriyama, K.; Ichikawa, S.; Yahara, I. and Inagaki, F. (1996) *Cell*, **85**, 1047-1055.
- [53] Mahajan-Miklos, S. and Cooley, L. (1994) *Cell*, **78**, 291-301.
- [54] Liu, Y.T. and Yin, H.L. (1998) *J. Biol. Chem.*, **273**, 7920-7927.
- [55] Wilson, S.A.; Brown, E.C.; Kingsman, A.J. and Kingsman, S.M. (1998) *Nucleic Acids Res.*, **26**, 3460-3467.
- [56] Reed, A.L.; Yamazaki, H.; Kaufman, J.D.; Rubinstein, Y.; Murphy, B. and Johnson, A.C. (1998) *J. Biol. Chem.*, **273**, 21594-21602.
- [57] Shibutani, M.; Lazarovici, P.; Johnson, A.C.; Katagiri, Y. and Guroff, G. (1998) *J. Biol. Chem.*, **273**, 6878-6884.
- [58] Khachigian, L.M.; Santiago, F.S.; Rafty, L.A.; Chan, O.L.; Delbridge, G.J.; Bobik, A.; Collins, T. and Johnson, A.C. (1999) *Circ. Res.*, **84**, 1258-1267.
- [59] Fong, K.S. and de Couet, H.G. (1999) *Genomics*, **58**, 146-157.

- [60] Buchanan, S.G. and Gay, N.J. (1996) *Prog. Biophys. Mol. Biol.*, **65**, 1-44.
- [61] Minato, T.; Wang, J.; Akasaka, K.; Okada, T.; Suzuki, N. and Kataoka T. (1994) *J. Biol. Chem.*, **269**, 20845-20851.
- [62] Macara, I.G.; Lounsbury, K.M.; Richards, S.A.; McKiernan, C. and Bar-Sagi, D. (1996) *FASEB J.*, **10**, 625-630.
- [63] Luo, L.; Liao, Y.J.; Jan, L.Y. and Jan, Y.N. (1994) *Genes Dev.*, **8**, 1787-1802.