The EMBO Journal vol.8 no.10 pp.2983 – 2988, 1989

An antisense transcript from the *Xenopus laevis* bFGF gene coding for an evolutionarily conserved 24 kd protein

Rüdiger Volk, Manfred Köster, Annette Pöting, Ludger Hartmann and Walter Knöchel

Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, 1000 Berlin 33, FRG

Communicated by A.Graessmann

Screening of a *Xenopus laevis* oocyte cDNA library with a rat basic fibroblast growth factor (bFGF) cDNA led to the isolation of a 1.35 kb sequence containing exon III of the bFGF gene. Reverse complementary listing of this sequence revealed a polyadenylated transcript with an open reading frame coding for an unknown protein of mol. wt 24 292 daltons. The coding part of bFGF exon III is located in this putative mRNA in opposite direction within the 3′ untranslated region. By hybridization studies on transcription orientation with single-stranded probes it could be proven that this transcript actually represents an antisense transcript to part of the *Xenopus* bFGF gene. Sequence organization on corresponding genomic fragments revealed that it is processed from a larger precursor by splicing mechanisms. Sequence comparison with elongated transcripts from the bFGF gene in human hepatoma has shown that the gene coding for the antisense mRNA is evolutionarily conserved.

Key words: evolutionary conservation/fibroblast growth factor/genes/*Xenopus laevis*

Introduction

Numerous genes encoding different types of growth factors are transcribed during vertebrate embryogenesis (for reviews see Mercola and Stiles, 1988; Knöchel and Tiedemann, 1989), but until today only in a few cases has a successful approach towards the elucidation of the biological function of the corresponding proteins in early development been achieved. To this end the basic fibroblast growth factor (bFGF) is particularly interesting because this protein has been shown to induce the formation of mesoderm-derived tissues in ectodermal explants or in total embryos of different amphibian species (*Xenopus laevis, Ambystoma mexicanum, Triturus alpestris*) (Slack *et al.*, 1987; Kimelman and Kirschner, 1987; Knöchel *et al.*, 1987; Grunz *et al.*, 1988; Knöchel, unpublished data). Since this factor is actually expressed in early embryogenesis (Kimelman *et al.*, 1988; Slack and Isaacs, 1989), it is reasonable to assume that bFGF, among other substances, participates as a natural inducer in the process of mesoderm formation. Other proteins which have been characterized as active inducers of mesoderm formation in amphibia are the vegetalizing factor (Born *et al.*, 1985), the XTC factor (Smith, 1987; Smith *et al.*, 1988), TGF-β1 (Knöchel *et al.*, 1987) and TGF-β2 (Rosa *et al.*, 1988).

bFGF mRNA, which is present in *Xenopus* oocytes and in the early embryo, has been cloned and sequenced (Kimelman *et al.*, 1988). The bFGF coding sequence which comprises 155 triplets is located at the 5′ region of a 4.2 kb transcript. Initially, partial sequence analysis (623 nucleotides) of a smaller transcript of 1 kb has been reported (Kimelman and Kirschner, 1987). This sequence contains only that part of the coding region of the *Xenopus* bFGF gene which, on the basis of its homology to the human bFGF gene (Abraham *et al.*, 1986a), codes for exon III and, additionally, some intron like sequences. It was thought to represent an unspliced fragment of the bFGF pre-mRNA. In the present work we have cloned this smaller transcript and sequenced it completely. In contrast to the previous suggestion it turned out to be an antisense transcript to part of the bFGF gene and to encode a hitherto unknown protein.

Results

Since the *Xenopus* bFGF gene seems to be involved in such a fundamental developmental process as mesoderm induction, it is of great interest to analyse the structure and temporal transcription of this gene in order to understand its transcriptional control mechanisms. Screening of a *X. laevis* genomic DNA library with synthetic oligonucleotides derived from the three exons of the human bFGF gene (Abraham *et al.*, 1986a) led to the isolation of six different clones with positive autoradiographic signals (exon I: 3; exon II: 1; exon III: 2). The identity of these sequences was confirmed by hybridization to a rat bFGF cDNA clone (Kurokawa *et al.*, 1988). Partial sequence analysis of restriction fragments carrying the exon sequences revealed complete homology to the recently reported cDNA sequence (Kimelman *et al.*, 1988; deposited at GenBank under accession number M21092), but one exon I containing fragment showed a divergence at 15 out of 178 coding nucleotides (data not shown). Therefore, we also find evidence for the existence of at least two of such genes in *X. laevis*, as already mentioned by Kimelman *et al.* (1988). However, this is not surprising, because *X. laevis* represents a tetraploid species, in which a genome duplication event occurred about 50 million years ago (Bisbee *et al.*, 1977; Knöchel *et al.*, 1986). The question of whether both of these genes are transcribed cannot yet be answered, since the coding sequences are very similar and until now only one type of cDNA has been analysed.

We next analysed transcription of the bFGF genes by Northern blotting of oocyte and embryonic RNA with subsequent hybridization to cloned rat bFGF cDNA (Kurokawa *et al.*, 1988) (see Figure 1, left side). Two autoradiographic signals can be detected in all stages investigated (~1.35 kb and 4.2 kb). This supports the previous findings by Kimelman *et al.* (1988). However, we did not observe another reported transcript (2.1 kb), which might have been due to using a different probe in our

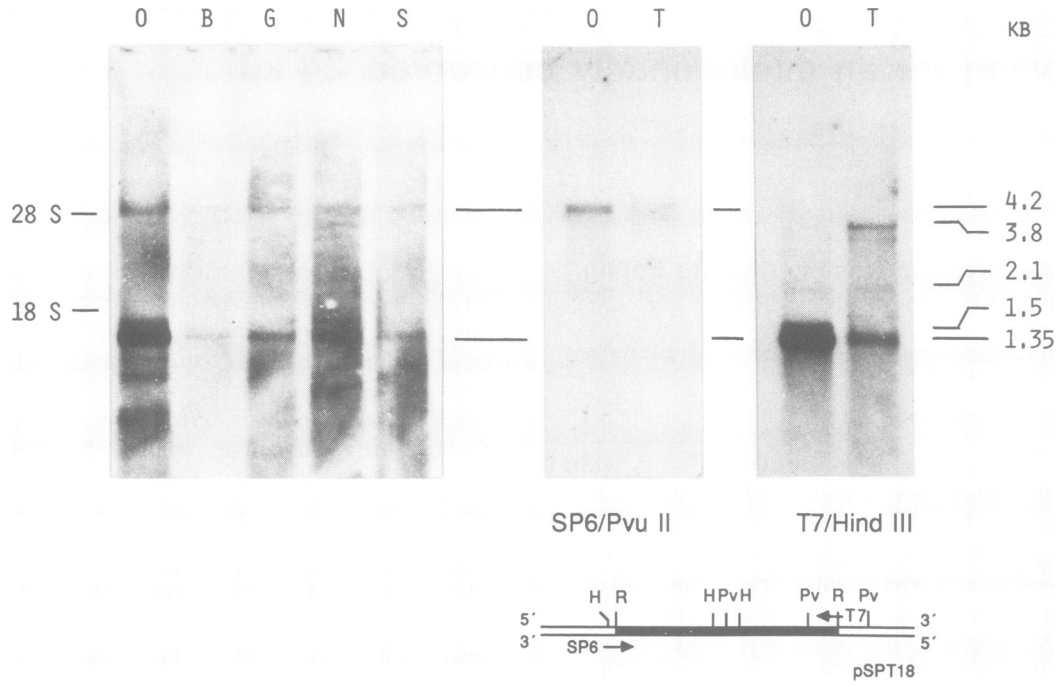


Fig. 1. RNA transcribed from the bFGF gene in *Xenopus* embryogenesis. **Left side**: 5 μg/lane polyadenylated RNA (O, oocyte; B, blastula; G, gastrula; N, neurula; S, somite segregation) was glyoxylated and run on an agarose gel. After Northern blotting to a positively charged nylon membrane (Gene Screen Plus) (NEN) the blot was hybridized to an oligonucleotide primed [^{32}P]dCTP-labelled rat bFGF cDNA probe. **Right side**: 2.5 μg/lane polyadenylated RNA from oocytes (O) and tadpoles (T) was run and blotted as above. SP6/*Pvu*II: hybridization to [^{32}P]CTP-labelled RNA being transcribed by SP6 polymerase from the recombinant pSPT18 clone after cleavage with *Pvu*II. T7/*Hin*dIII: hybridization to [^{32}P]CTP-labelled RNA transcribed by T7 polymerase from the same plasmid after cleavage with *Hin*dIII. Positions of rRNAs, run in a separate lane, are indicated. Transcripts of different sizes (kb) are referred to in the text. The diagram illustrates transcription orientation from the recombinant pSPT18 clone. The upper DNA strand corresponds to the noncoding strand of the antisense transcript as shown in Figure 2 (R, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II).

hybridization experiment. The strongest signal is always due to the 1.35 kb RNA. Since an already published partial cDNA sequence analysis (623 bp) of the smaller transcript revealed an unspliced sequence containing the third exon of the *Xenopus* bFGF gene (Kimelman and Kirschner, 1987), we asked whether the generation of this RNA could alternatively be due to transcription of the noncoding DNA strand resulting in an antisense transcript to bFGF mRNA. To investigate this hypothesis and for further sequence analysis we had to isolate the 1.35 kb RNA from a cDNA library. Screening of an ovary cDNA library in λ gt11 with rat bFGF cDNA led to the isolation of eight recombinant clones containing the 1.35 kb sequence. Single-stranded hybridization probes were obtained by subcloning one of these sequences in the transcription vector pSPT 18, by linearization of the recombinant plasmid with *Hin*dIII or with *Pvu*II and by transcription with T7 or SP6 RNA polymerase, respectively. The T7 transcript correlates with a sense RNA to the bFGF gene and the SP6 transcript is equivalent to a bFGF mRNA antisense transcript. Hybridization of these probes with polyadenylated RNA from oocytes and tadpoles renders clear evidence that the 1.3 kb transcript does only hybridize to the bFGF sense probe and must therefore represent an antisense transcript to the bFGF gene (Figure 1). Consequently, it can be expected that the bFGF antisense probe only hybridizes to the 4.2 kb bFGF mRNA and not to the 1.35 kb transcript. This is actually shown in Figure 1. In the case of the sense probe we detected additional signals at 1.5 kb and 3.8 kb and a very weak signal at 2.1 kb. Although the nature of these transcripts is not known, the latter possibly corresponds to the 2.1 kb transcript reported previously (Kimelman *et al.*, 1988). The 3.8 kb RNA is highly enriched in tadpole RNA as compared to oocyte RNA, thereby suggesting that it is generated either from another related gene or by different transcription/processing mechanisms from the same transcription unit.

Having established that the 1.35 kb RNA represents a bFGF antisense transcript we have subcloned and sequenced overlapping restriction fragments in M13mp8/mp9 using the dideoxy chain termination method. The sequence comprises 1325 nucleotides and, in antisense direction to the bFGF exon III, contains a poly(A) addition signal and a poly(A) tail of another 14 nucleotides at the 3′ end (Figure 2). The reverse complement of the 3′ half fully corresponds to the recently published sequence (Kimelman *et al.*, 1988) but differs in a few positions from the previously published sequence (Kimelman and Kirschner 1987) (indicated in Figure 2). The RNA contains an open reading frame coding for a protein composed of 217 amino acids and a mol. wt of 24 292 daltons. The reverse complement of the bFGF exon III translated sequence is located within the 3′ untranslated region of this antisense RNA. Apart from the homology to published bFGF cDNA sequences of human, bovine and rat (Abraham *et al.*, 1986a,b; Kurokawa *et al.*, 1988), a comparison of the nucleotide as well as of the protein sequence with the EMBL data banks revealed no significant matches.

To investigate the organization of the corresponding gene, we sequenced a subcloned fragment of 5.5 kb from a recombinant λ phage (genomic DNA library in Charon 24A) which carries part of intron II, exon III and 3′ flanking sequence of the *Xenopus* bFGF gene. The sequence

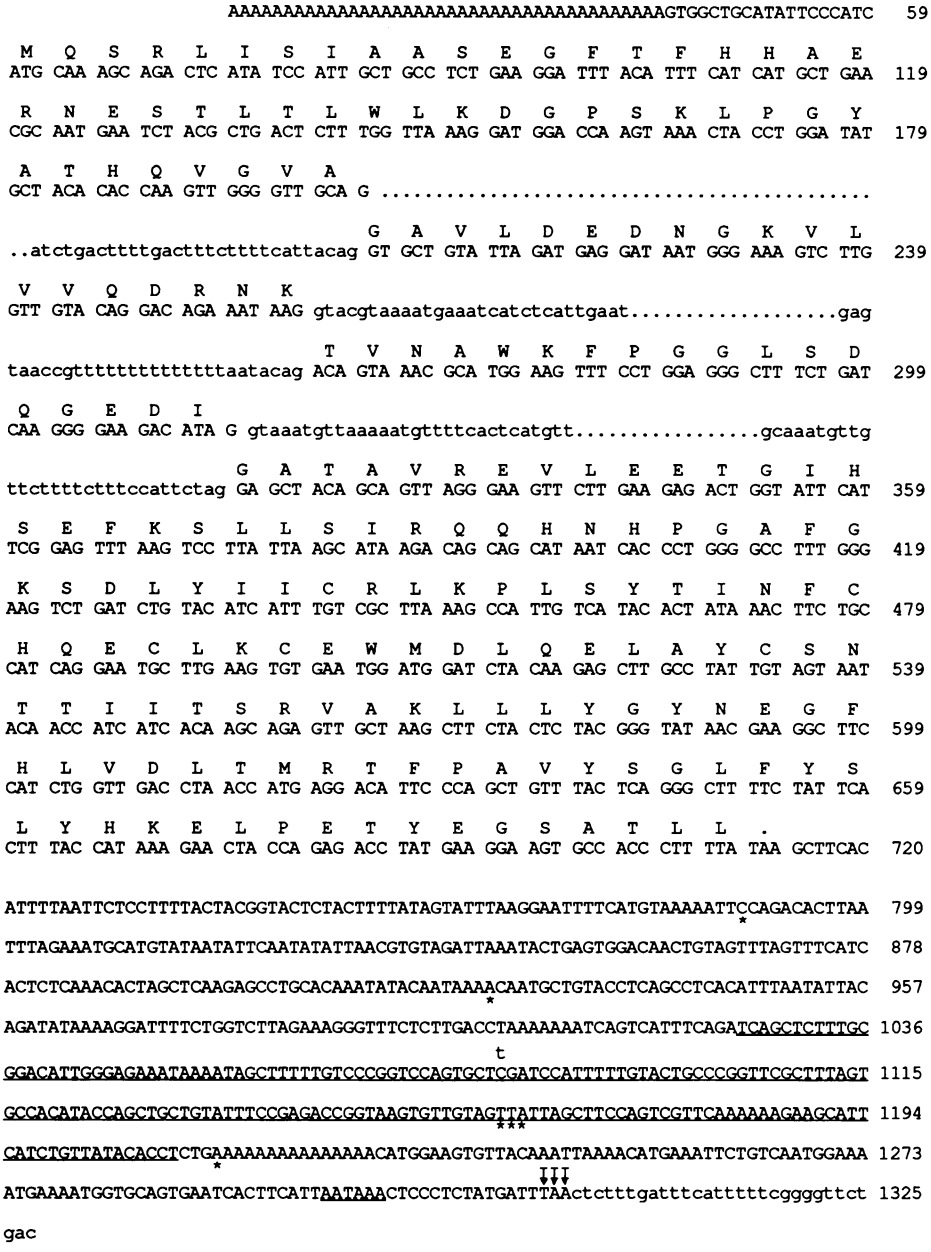


Fig. 2. Nucleotide and derived amino acid sequence of the antisense cDNA. The sequence of the antisense cDNA is shown in the 5′ to 3′ direction and the nucleotides (large letters) are numbered. Amino acids are given on top. Small letters denote beginnings or ends of the intervening sequences and the 3′ sequence beyond the polyadenylation site of the noncoding DNA strand. At position 1082 the genomic sequence differs by a C/T transition from the cDNA sequence shown. The polyadenylation signal is underlined and three possible polyadenylation sites are indicated by arrows. The cDNA clone contains another 14 A nucleotides and thus comprises 1339 nucleotides. The sequence from position 712 (*Hind*III site) to position 1210 (beginning of bFGF exon III) fully corresponds to the recently described bFGF cDNA sequence (Kimelman *et al.*, 1988; GenBank accession number M21092). Asterisks denote differences to a previously published partial sequence (Kimelman and Kirschner, 1987). The 3′ untranslated region contains the reverse complementary sequence of the coding region of bFGF exon III (underlined).

corresponds over a stretch of ~1000 nucleotides to that of the antisense transcript in reverse complementary orientation starting from the poly(A) addition site, located in intron II of the bFGF gene, to nucleotide position 315. Although there is one nucleotide exchange within the coding region of bFGF exon III (indicated in Figure 2), it is reasonable to assume that the antisense mRNA is transcribed from this genomic locus (the exchange represents a silent mutation; cDNA and genomic libraries originated from different animals). At position 315 we found in the genomic sequence a canonical exon/intron boundary. The 5.5 kb restriction fragment contained another two short exons coding for 18 and 19 amino acids, respectively, separated by 1 kb and 2.8 kb intervening sequences, but neither sequence nor hybridization studies from this and other fragments of the recombinant λ clone led to the identification of the 5′ part of the antisense transcript. Therefore another screening of the genomic library will be necessary to isolate the 5′ region and the promoter sequence of the antisense gene. From the results presented in this work we conclude that the gene for the antisense transcript is organized into at least four exons, which are separated by rather large introns. The genomic organization and the overlapping parts of the bFGF gene and the antisense gene are illustrated in Figure 3. There is

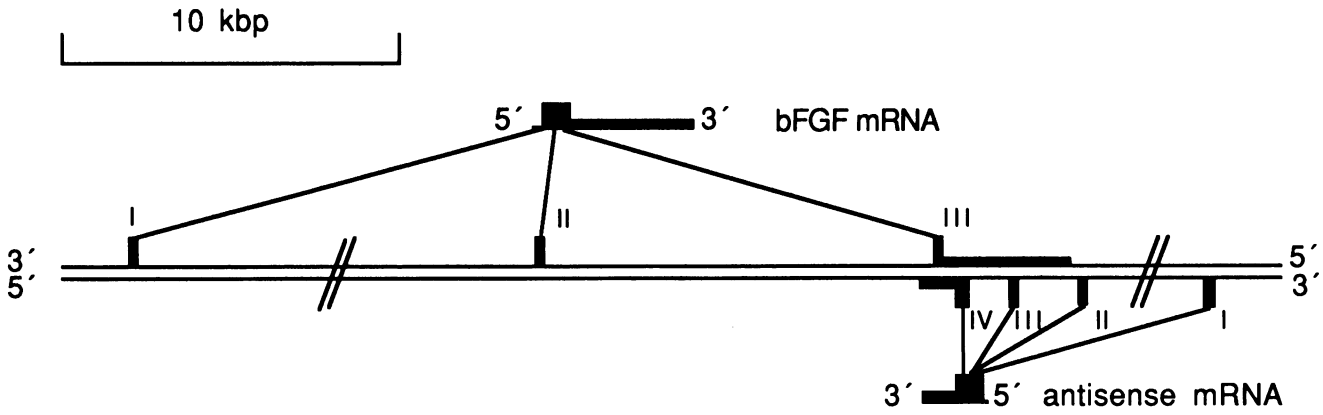
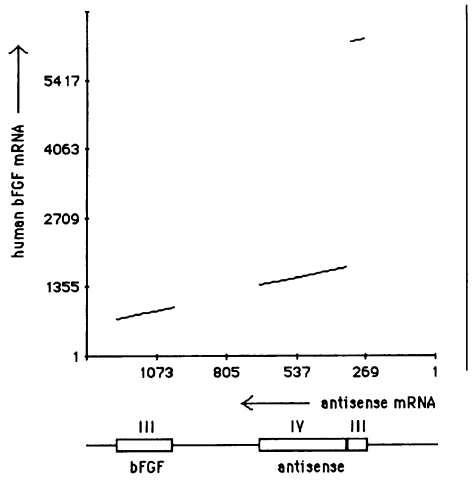


Fig. 3. Genomic organization and opposite transcription of bFGF gene locus. The diagram displays genomic organization of the *Xenopus* bFGF gene (black) and the antisense gene (shaded). Exons are indicated by Roman numerals. The untranslated regions of the last exons are shown as smaller boxes. Overlapping recombinant λ clones for bFGF exons II and III but not for exon I have been isolated. Exon I of the antisense gene has not yet been identified on genomic DNA.




```
human:  LKNMWKFPGG LSEPEEDIGD TAVREVFEET GIKSEFRSVL SIRQQHTNPG
          |*|||||| ||*  ||||  ||||||*||| ||*|||*|*| ||||||  ||
X.laev: TVNAWKFPGG LSDQGEDIGA TAVREVLEET GIHSEFKSLL SIRQQHNHPG   117

        AFGKSDMYII CRLKPYSFTI NFCQEECLRC EWMDLNDLAK TENTTPITSR
        ||||||*||| ||||| |*|| |||  |||*| |||||**||    ||| ||||
        AFGKSDLYII CRLKPLSYTI NFCHQECLKC EWMDLQELAY CSNTTIITSR   167

        VARLLLYGYR EGFDKIDLTV EELPAVYTGL FYKLYHKELP ENYKTMKGID
        ||*|||||  |||  *|||*  *||||*|| || |||||||  | |    *
        VAKLLLYGYN EGFHLVDLTM RTFPAVYSGL FYSLYHKELP ETYEGSATLL   217
```

Fig. 4. Comparison of *Xenopus* with human DNA sequences.
Upper: MacMolly (Softgene, Berlin) matrix plot of human hepatoma derived bFGF cDNA (Prats *et al.*, 1989) and reverse complement of *Xenopus* antisense cDNA (window size: 50; mismatches allowed: 17). Individual exons are identified along with the x-coordinate.
Lower: comparison of the human and *Xenopus* putative antisense proteins (exons III and IV). Complete homology is marked by (|) and homology due to conservative amino acid exchanges is marked by (*).

a cross overlap between the 3′ untranslated sequence of the bFGF gene and the last two exons of the antisense transcript and, vice versa, the 3′ untranslated sequence of the antisense mRNA and the translated region of exon III of the bFGF gene.

A recently published bFGF cDNA sequence derived from human hepatoma (Prats *et al.*, 1989) comprises 6.75 kb, the bFGF coding portion located at the 5′ region of this sequence. Thus the sequence extends very far in the 3′ direction. We have checked by computer analysis whether this sequence contains any homologies to the *Xenopus* antisense mRNA or its transcription unit which is located 3′ to the bFGF gene. A matrix plot of human bFGF mRNA with the reverse complement of *Xenopus* antisense mRNA is shown in Figure 4. Besides the overlap of bFGF exons III there is a striking homology between exons III and IV of the antisense transcript with two nucleotide stretches of the human bFGF mRNA which are not contiguous but separated by a long sequence being absent from the antisense mRNA. This reflects the sequence organization of the antisense transcription unit with a large intron between these two exons. Computer comparison of the human bFGF mRNA to the antisense genomic sequence did not reveal any further matches being located within this and the next intervening sequence. This implies that the bFGF transcript does not contain the remaining exons I and II, but we would predict their existence when further sequencing the human DNA in the 3′ direction. A more detailed inspection of the matches revealed conserved exon/intron boundaries also in the case of the human sequence and a termination signal at the same location as in the *Xenopus* sequence. We conclude that an analogous transcription unit is present in human DNA and that the gene linkage between the bFGF gene and the antisense gene has been conserved during evolution. Based upon the homology between the two species, we have deduced the amino acid sequence encoded by the putative human antisense gene and compared it to the *Xenopus* sequence (Figure 4). Exons III and IV encode 150 amino acids, 106 of which are strictly identical and 17 of which represent conservative amino acid replacement. Based upon a comparison of these two exons from human and *Xenopus* DNA there is an overall homology of 82%.

Discussion

The results presented in this study clearly show that the *X.laevis* bFGF gene is partially transcribed into an antisense mRNA. This is in contrast to previous suggestions (Kimelman and Kirschner, 1987), according to which this RNA should represent an unspliced fragment of bFGF pre-mRNA. The antisense transcript is a typical polymerase II transcript by means of processing mechanisms including the elimination of introns, open reading frame and a polyadenylation signal/poly(A) tail at the 3′ end. It is

interesting to note that the intron sequences and the splice sites are differentially used for bFGF and antisense mRNAs. The intervening sequences II and III of the antisense gene form part of the 3′ untranslated sequence of bFGF mRNA and, vice versa, intron II of the bFGF gene forms part of the untranslated sequence of the antisense transcript. The presence of a poly(A) stretch at the 5′ end of this RNA is rather curious. While we cannot rule out at the moment a cloning artefact, sequencing of the corresponding genomic fragment will answer the question whether it is transcribed or added due to cloning procedures. In this context it is noteworthy that the RNA band at 1.35 kb is preceded by a slightly larger band of 1.5 kb. It is possible that the different migration of these two RNAs might be due to varying lengths of 3′ and 5′ poly(A) stretches. The protein encoded by the antisense transcript comprises 217 amino acids and has a mol. wt of 24 292 daltons. It is unrelated to the bFGF sequence and there is no significant homology to any other protein sequenced so far.

There is strong evidence that the bFGF and antisense transcripts are generated from the same genetic locus. The nucleotide sequence homology noted above makes the assumption that they are transcribed from different but corresponding genes of a tetraploid species like *X.laevis* rather unlikely [nucleotide divergence between these genes is on average 5–10% (Knöchel *et al.*, 1986)]. While we cannot rule out that after fertilization the bFGF and antisense transcripts are generated from two zygotic alleles, transcription in the oocyte would argue against this. Although the transcription of both DNA strands of an eukaryotic genetic locus is an unusual event, in a few cases it has already been reported (Bentley and Groudine, 1986; Henikoff *et al.*, 1986; Nepveu and Marcu, 1986; Spencer *et al.*, 1986; Williams and Fried, 1986; Adelman *et al.*, 1987; Miyajima *et al.*, 1989). This finding is intriguing and raises a lot of interesting questions. First, it is not clear whether transcription from opposite strands occurs at the same time or not. If it does, what does the three-dimensional structure of a double-stranded DNA look like with two polymerases on complementary strands running in opposite directions? If it does not, what kind of mechanisms regulate the time clock of transcription of the two genes at different time intervals in oogenesis or during the cell cycle? Secondly, the presence of two reverse complementary RNA molecules leading to a double-stranded RNA may have dramatic effects on RNA processing, nuclear/cytoplasmic export and on the translation of both RNA molecules. Although an unwinding activity for double-stranded RNA, which leads to structural alterations by means of adenosine to inosine conversions, has recently been described (Bass and Weintraub, 1988; Wagner *et al.*, 1989), a detailed molecular analysis of all the possible implications remains to be performed.

Northern analysis has shown that bFGF and antisense mRNAs are both present in the oocyte and in early embryos. Whether they are located in different cellular compartments or, later in embryogenesis, in different cells remains to be elucidated. Moreover, we do not know at the moment whether the antisense transcript is actually translated into the corresponding protein, but the high degree of evolutionary conservation strongly suggests that this protein is actually expressed and that it should be aligned to an important biological function. Gene linkage and coding sequences have been conserved from *Xenopus* to mammals. Although the putative antisense protein seems not to be related to bFGF one should bear in mind that opposite transcription from the same genetic locus may also lead to functionally related proteins (Miyajima *et al.*, 1989). But this speculation can only be tested when the antisense protein has been identified and isolated.

In view of the suggested function of the *Xenopus* bFGF gene as a mesoderm inducer, the regulatory mechanisms involved in the expression of this gene gain an outstanding importance for an understanding of the formation of germ layers in early embryogenesis. The generation of antisense transcripts may influence the expression of this gene post-transcriptionally. Alternatively, the antisense protein itself may have an important biological function. Therefore, further experiments on the translation of endogenous and injected messages, on the distribution of sense and antisense transcripts in oocytes and early embryos as well as on the location of corresponding proteins will be essential for a better understanding of the regulation and the function of the *Xenopus* bFGF and antisense genes.

Materials and methods

Screening of genomic and cDNA libraries

In initial studies we screened a *X.laevis* genomic DNA library in Charon 24A (kindly provided by Dr G.Spohr, Geneva) with mixed oligonucleotide probes derived from the three exons of the human bFGF gene (Abraham *et al.*, 1986a); in later studies we used a rat bFGF cDNA clone (Kurokawa *et al.*, 1988) (a kind gift of Dr K.Igarashi, Takeda Chemical Ind. Ltd, Osaka, Japan). Hybridization and washing conditions used in the case of oligonucleotides and of cDNA probes were as previously described (Köster *et al.*, 1988). Antisense transcript containing cDNA clones were isolated from a *X.laevis* ovary λ gt11 library (kindly provided by Dr J.Kleinschmidt, Heidelberg) using a 568 bp *Nco*I–*Eco*RI fragment of the rat bFGF cDNA clone as labelled probe. Out of eight clones with positive autoradiographic signals we selected one for further analysis.

Isolation of RNA and Northern blotting

RNA from oocytes and embryos at different developmental stages was prepared by the guanidinium isothiocyanate/hot phenol method (Maniatis *et al.*, 1982). Polyadenylated RNA was selected by two consecutive runs on oligo(dT)–cellulose columns. Glyoxylated RNA was run on 1.2% agarose gels. After transfer to Gene Screen Plus membranes (NEN) hybridization was performed in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA and 1% BSA either at 58°C (using as probe a random oligonucleotide primed [^{32}P]dCTP-labelled rat bFGF cDNA) or at 68°C (using [^{32}P]CTP-labelled RNAs transcribed from the recombinant pSPT18 clone by SP6 or T7 polymerase). After final washing in 0.04 M sodium phosphate (pH 7.2), 1% SDS and 1 mM EDTA at 5°C below hybridization temperatures the membranes were subjected to autoradiography at −70°C for 48 h.

Nucleotide sequence analysis

DNA fragments were subcloned in M13mp8/mp9 vectors (Messing and Vieira, 1982) and sequenced using the universal primer or synthetic internal primers according to the dideoxy chain termination method (Sanger *et al.*, 1977). Sequence data were analysed with a commercially available software package (MacMolly; Softgene, Berlin).

In vitro transcription

A commercially available kit (Boehringer, FRG) was used in this experiment. The 1.3 kb cDNA sequence was cloned into the *Eco*RI site of transcription vector pSPT18. After control of cloning orientation by digestion with different restriction enzymes and gel analysis of resulting fragments the recombinant plasmid was linearized with *Hin*dIII or *Pvu*II and transcribed in the presence of ^{32}P-labelled CTP by T7 or SP6 RNA polymerase, respectively. After removal of DNA by DNase digestion the RNA was purified by ethanol precipitation.

Acknowledgements

The authors greatly appreciate the skilful technical assistance of G.Bollmus, I.Cichocka and I.Ziglowski. We would like to thank Dr G.Spohr, Geneva, for the genomic library, Dr J.Kleinschmidt, Heidelberg, for the oocyte cDNA library and Dr K.Igarashi, Osaka, for the rat bFGF cDNA clone. This investigation was aided by grants from the Deutsche Forschungsgemeinschaft (Kn 200/3-1) and by Fonds der Chemischen Industrie.

References

Abraham,J.A., Whang,J.L., Tumolo,A., Mergia,A., Friedmann,J., Gospodarowicz,D. and Fiddes,J.C. (1986a) *EMBO J.*, **5**, 2523–2528.

Abraham,J.A., Mergia,A., Whang,J.L., Tumolo,A., Friedman,J., Hjerrild,K.A., Gospodarowicz,D. and Fiddes,J.C. (1986b) *Science*, **233**, 545–548.

Adelman,J.P., Bond,C.T., Douglass,J. and Herbert,E. (1987) *Science*, **235**, 1514–1517.

Bass,B.L. and Weintraub,H. (1988) *Cell*, **55**, 1089–1098.

Bentley,D.L. and Groudine,M. (1986) *Nature*, **321**, 702–706.

Bisbee,C.A., Baker,M.A., Wilson,A.C., Hadji-Azimi,I. and Fischberg,M. (1977) *Science*, **195**, 785–787.

Born,J., Hoppe,P., Schwarz,W., Tiedemann,H., Tiedemann,H. and Wittmann-Liebold,B. (1985) *Biol. Chem., Hoppe-Seyler*, **366**, 729–735.

Grunz,H., McKeehan,W.L., Knöchel,W., Born,J., Tiedemann,H. and Tiedemann,H. (1988) *Cell Diff.*, **22**, 183–190.

Henikoff,S., Keene,M.A., Fechtel,K. and Fristrom,J.W.(1986) *Cell*, **44**, 33–42.

Kimelman,D. and Kirschner,M. (1987) *Cell*, **51**, 869–877.

Kimelman,D., Abraham,J.A., Haaparanta,T., Palisi,T.M. and Kirschner, M.W. (1988) *Science*, **242**, 1053–1056.

Knöchel,W., Korge,E., Basner,A. and Meyerhof,W. (1986) *J. Mol. Evol.*, **23**, 211–223.

Knöchel,W., Born,J., Hoppe,P., Loppnow-Blinde,B., Tiedemann,H., Tiedemann,H., McKeehan,W.L. and Grunz,H. (1987) *Naturwissenschaften*, **74**, 604–606.

Knöchel,W. and Tiedemann,H. (1989) *Cell Diff.*, **26**, 163–171.

Köster,M., Pieler,T., Pöting,A. and Knöchel,W. (1988) *EMBO J.*, **7**, 1735–1741.

Kurokawa,T., Seno,M. and Igarashi,K. (1988) *Nucleic Acids Res.*, **16**, 5201.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mercola,M. and Stiles,C.D. (1988) *Development*, **102**, 451–460.

Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269–276.

Miyajima,N., Horiuchi,R., Shibuya,Y., Fukushige,S., Matsubara,K., Toyoshima,K. and Yamamoto,T. (1989) *Cell*, **57**, 31–39.

Nepveu,A. and Marcu,K.B. (1986) *EMBO J.*, **5**, 2859–2865.

Prats,H., Kaghad,M., Prats,A.C., Klagsbrun,M., Lelias,J.M., Liauzun,P., Chalon,P., Tauber,J.P., Amalric,F., Smith,J.A. and Caput,D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1836–1840.

Rosa,F., Roberts,A.B., Danielpour,D., Dart,L.L., Sporn,M. and Dawid,I.B. (1988) *Science*, **239**, 783–785.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5436–5467.

Slack,J.M.W. and Isaacs,H.V. (1989) *Development*, **105**, 147–153.

Slack,J.M.W., Darlington,B.G., Heath,J.K. and Godsave,S.F. (1987) *Nature*, **326**, 197–200.

Smith,J.C. (1987) *Development*, **99**, 3–14.

Smith,J.C., Yaqoob,M. and Symes,K. (1988) *Development*, **103**, 591–600.

Spencer,C.A., Gietz,R.D. and Hodgetts,R.B. (1986) *Nature*, **322**, 279–281.

Wagner,R.W., Smith,J.E., Cooperman,B.S. and Nishikura,K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2647–2651.

Williams,T. and Fried,M. (1986) *Nature*, **322**, 275–279.

Received on June 20, 1989