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Characterization of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene

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Abstract

Comparison of nucleotide sequence data of the 5′ region of a *fes/fps* viral oncogene with those of the v-*fes/fps* homologous regions of man and cat revealed the position of the 3′ portion of an as yet unidentified c-*fes/fps* exon. Comparative Southern blot and heteroduplex analysis of human and feline DNA immediately upstream of the v-*fes/fps* homologous regions showed extensive but discontinuous homology over a 9 kbp DNA stretch, which we have designated as *fur*. Northern blot analysis of mRNA from KG-1 myeloid cells with *fes/fps*- or *fur*-specific probes revealed a 3.0 kb *fes/fps* and a 4.5 kb *fur* transcript. Analysis of a number of tissues of an adult Wistar Lewis rat for the presence of *fur* transcripts revealed its differential expression pattern. An 0.95 kbp *fes/fps*-related and a 2.2 kbp *fur*-related cDNA recombinant clone were isolated from an oligo(dT)-primed KG-1 cDNA library. Comparative nucleotide sequence analysis of the *fes/fps* cDNA and its human genomic counterpart indicated that the cDNA contained genetic sequences that were identical to and colinear with exon 15–19 and, furthermore, that the poly(A) addition signal near the 3′ end of exon 19 was functional. Similar analysis of the 2.2 kbp *fur* cDNA indicated that the poly(A) addition signal of the *fur* transcript was in close proximity of the newly discovered *fes/fps* exon. The region in between contained a CATT sequence but no 'TATA' box. The *fur* transcript was characterized by a long noncoding region at its 3′ end.

Introduction

Proto-oncogenes can be defined as cellular genes which under normal physiological conditions are thought to exhibit proliferative and developmental functions (13, 26, 27) but which exhibit transforming activity upon transduction by a retrovirus (3, 4, 10). From data gathered to date, the consensus has emerged that retroviral mobilization of the transforming potential of proto-oncogenes involves alterations in regulation of their expression, alteration of their primary gene product, or both (5).

Transduced c-*fes/fps* proto-oncogene sequences of cat and chicken are present in the genomes of a number of independent feline and avian sarcoma virus isolates (4, 12, 18, 32). Mobilization of its transforming potential was in all these cases characterized by fusion of c-*fes/fps* proto-oncogene sequences to retroviral *gag* gene sequences.

The function of the c-*fes/fps* proto-oncogene is not yet known but the proto-oncogene might be of particular interest in hematopoietic proliferation since expression seems mainly restricted to hematopoietic tissue. Expression of the c-*fes/fps* proto-oncogene has been found in normal and leukemic myeloid cells and cell lines of a number of species (2, 9, 22, 23, 31). As its main translational product, a tyrosine-specific protein kinase of about 92000 mol. wt. has been described. The c-*fes/fps* mRNA species that have been reported varied in size between 2.6 and 3.2 kb (19, 29, 31). However,

the genetic organization of the *fes/fps* proto-oncogene is not yet completely defined (11, 14, 19, 28, 35, 37). In previous studies, we have described the isolation and genetic organization of c-*fes/fps* proto-oncogene sequences of man (28) and cat (37) and their malignant potential was established by fusing them to *gag* gene sequences of feline leukemia virus origin (33, 37). Recently, we have derived the topographical distribution of almost all human v-*fes/fps* homologous sequences from the complete nucleotide sequence of a human 13 kbp *Eco*RI/*Eco*RI restriction fragment (28). A sequence homologous to 12 bp at the 5′ end of the v-*fes* viral oncogene of the Gardner-Arnstein strain of feline sarcoma virus (GA-FeSV) (16) and 140 bp at the 5′ end of v-*fps* of Fujinami sarcoma virus (FSV) (19) were still missing. In the present study, we have identified the position of the missing 12 bp in the human and feline proto-oncogene. We also present characteristics of the 5′ and 3′ end of the human c-*fes/fps* transcription unit. Furthermore, we describe the identification and characterization of a new transcription unit, designated *fur*, in the immediately upstream region of the v-*fes/fps* homologous sequences.

Materials and methods

Cell line and cosmid clones

The permanent KG-1 human cell line, which was established from bone marrow cells of a patient with erythroleukemia that developed into acute myelogenous leukemia (21), was kindly provided by Dr. H. P. Koeffler. Isolation of the human v-*fes/fps* cellular homolog was described previously (14). Subclones in pSP64 of a 13 kbp *Eco*RI/*Eco*RI DNA fragment, which contains all v-*fes/fps* homologous sequences, and a 9.2 kbp *Hpa*I/*Eco*RI DNA fragment flanking the former at its 5′ end were described (28). The feline v-*fes/fps* cellular homolog was isolated from a feline cosmid library (37).

DNA probes and hybridization

Probes were isolated and labeled as described before (36). Hybridization experiments on nitrocellulose membranes were performed as described previously (36) and for hybridizations on nylon membranes (Hybond N, Amersham) the method of Church and Gilbert (7) was used. The Nylon membranes were dehybridized by incubation in 5 mM Tris-HCl (pH 8.0), 2 mM EDTA and 0.1 × Denhardt's solution (1 × Denhardt's solution contains 0.02% (w/v) bovine serum albumin, 0.02% (w/v) polyvinylpyrrolidone and 0.02% (w/v) ficoll) at 65 °C for 2 h. If necessary, the procedure was repeated for up to three times. Dehybridization was always checked by autoradiography. Blots could be used for up to six times without significant loss of signal.

Heteroduplex analysis

Electron microscopic analysis of duplexes between the 9.2 kbp human *Hpa*I/*Eco*RI DNA fragment and the 14 kbp feline *Eco*RI/*Eco*RI DNA fragment was performed as described by Davis *et al.* (8). Grids were examined in a Zeiss EM109 electron microscope at 40 kV. Electron micrographs were taken at a magnification of 6000 ×. Measurements were made on 25 heteroduplex structures.

mRNA isolation and Northern blot analysis

Total cellular RNA was isolated using the lithium-urea procedure described by Auffray and Rougeon (1). Ten μg of oligo(dT)-cellulose purified mRNA was glyoxalated and size fractionated on 1.0% agarose gels and transferred to Hybond-N (procedure as recommended by Amersham).

Construction and screening of cDNA library

An oligo(dT) primed cDNA library was constructed in λgt11 as described by Huynh *et al.* (20). The cDNA reaction was modified according to Gubler and Hoffman (15). About 250000 plaques obtained upon infection of *E. coli* Y1090 (20) were screened as described by Hanahan and Meselson (17).

DNA sequence analysis

DNA fragments were inserted into the polylinker region of M13mp8–11 (24). All of the DNA sequences were determined by the dideoxy-sequencing method (30). All parts of the reported

DNA sequence were obtained from both strands of the cloned DNA. The gel readings were recorded, edited, and compared using the Staden programs (34).

Results and discussion

Comparison of human and feline DNA immediately upstream of the v-fes/fps homologous regions

The topography of most of the human v-*fes/fps* homologous genetic sequences has recently been described. Upon nucleotide sequence analysis of a 13 kbp *Eco*RI/*Eco*RI DNA fragment, they appeared to be split up in at least 19 exon segments and, from these, exons 2–19 were characterized (28). However, genetic sequences at the 5′ end of v-*fps* of FSV or v-*fes* of GA-FeSV remained unaccounted for. In KG-1 cells, which were shown to exhibit a high level of expression of the *fes/fps* proto-oncogene (9), no such transcripts could be detected in a Northern blot analysis with the 1 kbp DNA region upstream of exon 2 as a probe (data not shown). In an attempt to identify the missing sequences in the human genome and to investigate whether the region immediately upstream of the v-*fes/fps* homologous sequences contained additional sequences that were not represented in any of the known v-*fes/fps* isolates but did belong to the c-

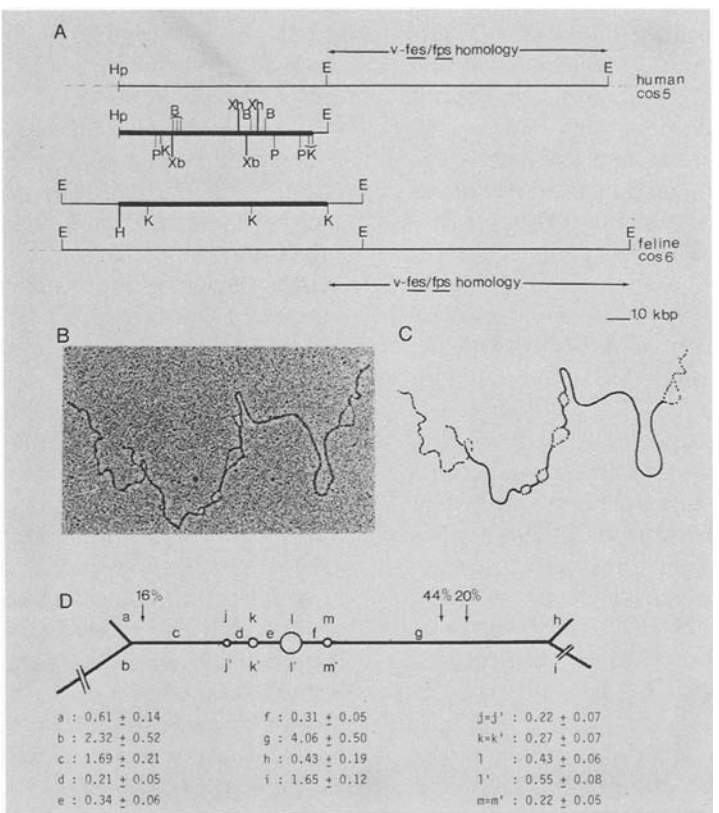

Fig. 1. Comparison of human and feline DNA upstream of the v-*fes/fps* homologous region. (A) A schematic representation of the v-*fes/fps* cellular homolog and its upstream region in man and cat. The v-*fes/fps* homologous regions indicated in the figure are described elsewhere in more detail (28, 37). The upstream regions are depicted in more detail in the middle of this part of the figure. The heavy bars represent the DNA regions in which man and cat share common genetic sequences. B, *Bam*HI; E,*Eco*RI; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; Xb, *Xba*I; Xh, *Xho*I. (B) Electron micrograph of a heteroduplex between the 9.2 kbp human *Hpa*I/*Eco*RI and 14 kbp feline *Eco*RI/*Eco*RI DNA fragment. (C) Interpretive drawing of heteroduplex shown in part B. (D) Schematic representation of the results of measurements on 25 heteroduplexes. The loops that are shown were observed in all heteroduplexes. Arrows indicate the positions and frequencies of loops found in a part of the duplex structures.

fes/fps proto-oncogene, we compared human DNA upstream of the v-*fes/fps* homologous region (9.2 kbp *Hpa*I/*Eco*RI DNA fragment) with a 14 kbp *Eco*RI/*Eco*RI DNA fragment, representing the corresponding feline region, by Southern blot analysis. The 9.2 kbp *Hpa*I/*Eco*RI human DNA fragment (Fig. 1A) was digested with restriction endonuclease *Eco*RI, *Kpn*I, *Pst*I, *Xba*I, and *Xho*I or various combinations of these in double digestions, and hybridizations were performed under conditions of high stringency with the 14 kbp *Eco*RI/*Eco*RI feline DNA fragment (Fig. 1A) as a molecular probe. It appeared that an extensive and strong homology existed between the two fragments (data not shown). In a reciprocal experiment, similar restriction endonuclease digestions of the feline 14 kbp *Eco*RI/*Eco*RI DNA fragment and hybridization analysis with the human 9.2 kbp *Hpa*I/*Eco*RI DNA fragment as a probe revealed similar results (data not shown). As is schematically indicated in Figure 1A, homology between the two fragments started almost immediately upstream of the v-*fes/fps* homologous regions and extended over a DNA region of about 9 kbp. We propose to designate this *fes/fps* upstream region as *fur*.

To further characterize the *fur* region in man and cat, heteroduplex analysis was performed with the human 9.2 kbp *Hpa*I/*Eco*RI and the feline 14 kbp *Eco*RI/*Eco*RI DNA fragments (Fig. 1B and 1C). This experiment confirmed the observations of the Southern blot experiments and, furthermore, revealed that the homologous genetic sequences were interspersed with at least four non-homologous regions. In a number of the 25 heteroduplex structures that were analyzed, three regions of apparently reduced homology were observed. Their positions and frequencies are indicated by arrows in Figure 1D.

Human DNA upstream of the v-fes/fps homologous region is transcriptionally active

To establish whether DNA immediately upstream of the v-*fes/fps* homologous region was a part of the proto-oncogene, we studied c-*fes/fps* transcription in the human KG-1 cell line, which was derived from a patient suffering from acute myelogenous leukemia (21). RNA was isolated from these cells, poly(A) selected, size fractionated on a 1% agarose gel and blotted onto Hybond-N (Amersham) as described under Materials and methods. Expression of c-*fes/fps* proto-oncogene sequences could readily be detected with the S_r v-*fes*-specific probe (11), as a 3.0 kb transcript (Fig. 2A, lane 1). Subsequently, hybridization of the same Northern blot with a number of probes, each representing a different part of the 9.2 kbp *Hpa*I/*Eco*RI human *fur* region (see lower part of Fig. 2A) revealed a 4.5 kb transcript (Fig. 2A, lane 2, 3 and 4). The autoradiogram in Figure 2A, lane 2, shows a 4.5 kb transcript and a descending smear of radioactivity. This could be due to the presence of repetitive sequences in the probe that was used (2.35 kbp *Hpa*I/*Bam*HI DNA fragment). From these Northern blot experiments we concluded that human (and also feline, data not shown) *fur* constituted a transcription unit for a 4.5 kb mRNA that was different from the c-*fes/fps* transcript detected with the S_r probe. The fact that probe 3 also strongly hybridized with the 4.5 kb *fur* transcript indicated that newly discovered *fur* sequences could be located in close proximity of the 5′ v-*fes/fps* homologous genetic sequences (see also Fig. 1A).

Analysis of *fur* expression was also studied in a number of tissues of an adult Wistar Lewis rat (Fig. 2B). These tissues included brain, heart muscle, kidney, lung, testis and thymus. High levels of the 4.5 kb *fur* transcript were found in brain, kidney and thymus. No detectable levels of *fur* transcripts were present in heart muscle, lung and testis. These data indicate a differential expression pattern of the *fur* transcription unit. To get more insight in the genetic organization the *fur* and *fes/fps* transcription units, nucleotide sequence data of the transcripts and their genomic counterparts should be compared.

Molecular cloning and DNA sequence analysis of human c-fes/fps- and fur-related cDNA

To isolate c-*fes/fps*- and *fur*-related cDNA, we constructed an oligo(dT) primed cDNA library in λgt11 using KG-1 mRNA as template. Upon screening of 2.5×10^5 plaques with the v-*fes*-specific S_r probe (11), we isolated a cDNA clone containing a c-*fes/fps*-specific insert of 0.95 kbp. The same library was also screened with a human *fur*-specific probe (probe 3, see lower part of Fig.

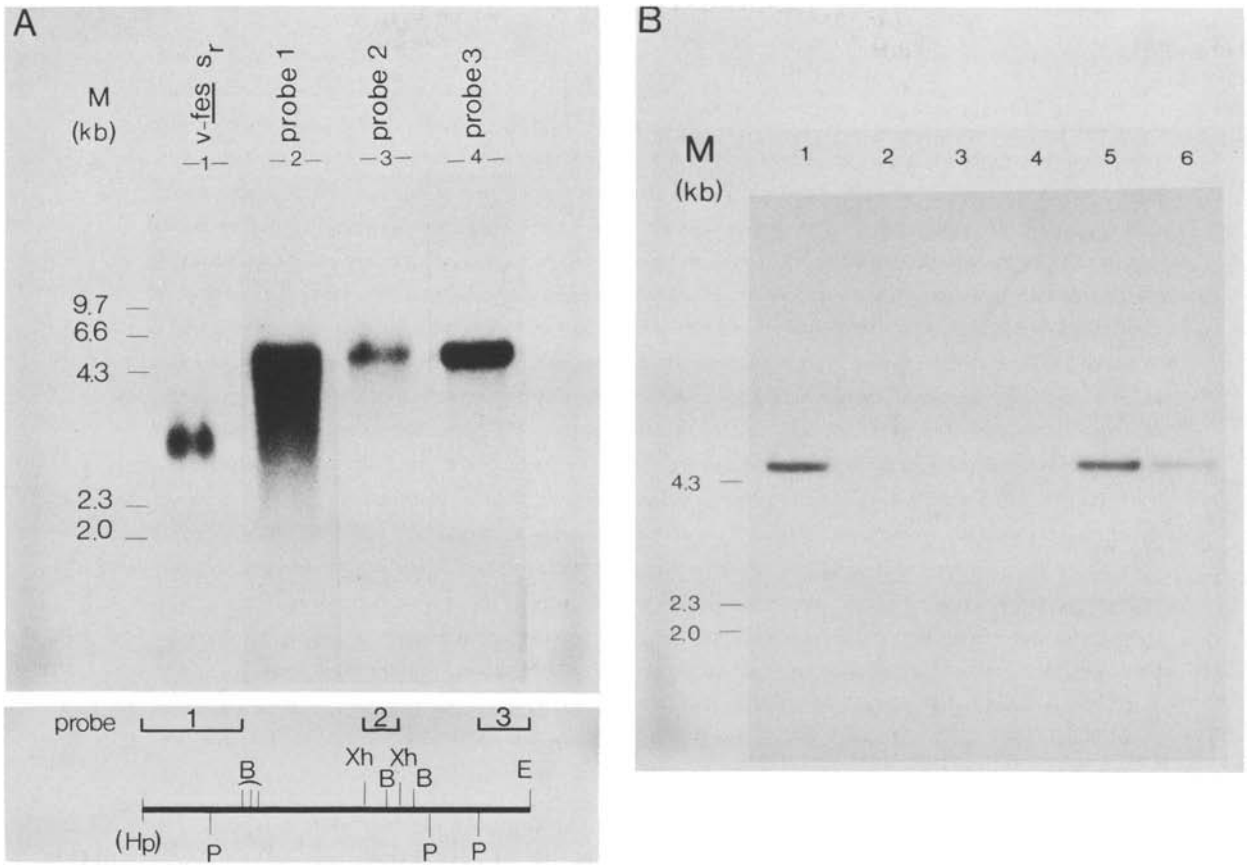

Fig. 2. (A) Expression of the human *fes/fps* proto-oncogene and its immediately upstream region in KG-1 cells. Poly(A)-selected mRNA isolated from KG-1 cells was glyoxylated, size fractioned by electrophoresis using a 1% agarose gel, immobilized on Hybond-N and subjected to hybridization analysis as described under Materials and methods. DNA probes included v-*fes*-S_r (lane 1), probe 1 (lane 2), probe 2 (lane 3), and probe 3 (lane 4). Probe 1, 2, and 3 are defined in the lower part of the figure. As molecular weight markers λ DNA digested with restriction endonuclease *Hin*dIII was used. B, *Bam*HI; Hp, *Hpa*I; P, *Pst*I; Xh, *Xho*I. (B) Expression of the c-*fes/fps* upstream region in a number of rat tissues. Poly(A)-selected mRNA was isolated from thymus (lane 1), lung (lane 2), heart muscle (lane 3), testis (lane 4), kidney (lane 5), or brain (lane 6) of an adult Wistar Lewis rat and subjected to Northern blot analysis. As molecular probe, a mixture of probe 2 and 3 was used. As molecular weight markers λDNA digested with restriction endonuclease *Hin*dIII was used.

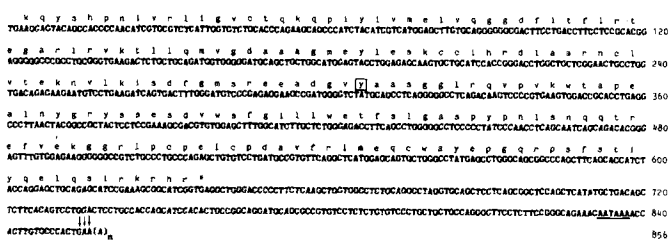

Fig. 3. Nucleotide sequence of the 0.95 kbp *fes/fps*-related cDNA and its predicted amino acid sequence in the convential one letter code. The consensus sequence for the polyadenylation signal is underlined. The poly(A)-stretch of the cDNA clone is indicated by $(A)_n$. Arrows indicate the positions of the three possible poly(A) addition sites. Open box (□), possible phosphoacceptor tyrosine. Asterisk (*), stop codon.

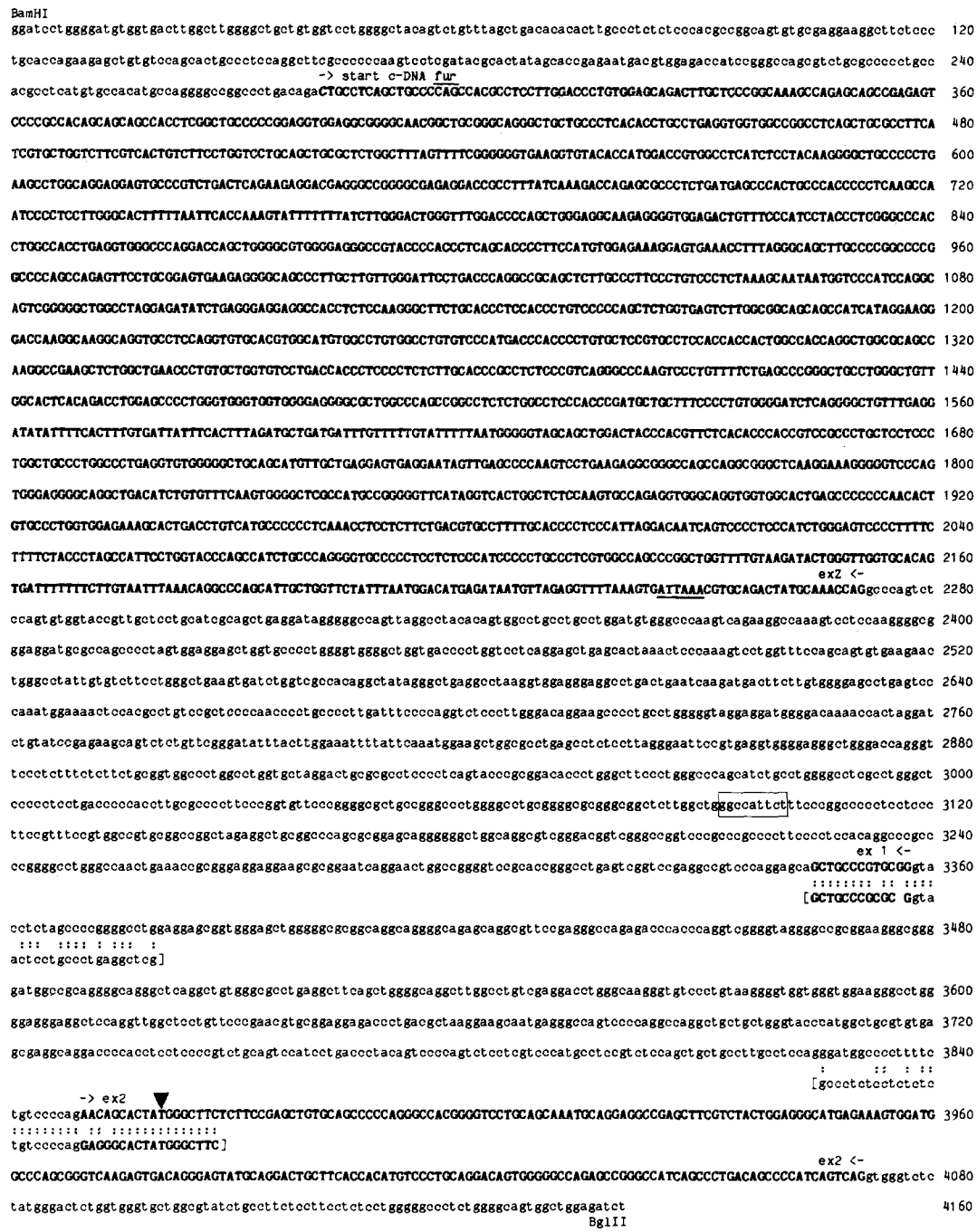

Fig. 4. Nucleotide sequence of the 5′ portion of the human v-*fes/fps* cellular homolog and its immediately upstream region. The nucleotide sequence of the 4.16 kbp *Bam*HI/*Bgl*II human DNA fragment includes *fur* exon Z and c-*fes/fps* exons 1 and 2. Established exon sequences are represented by capitals. Established exon borders are indicated by arrows. Relevant nucleotide sequence data of feline genomic DNA are given between square brackets in positions below the corresponding human genomic sequence data. The consensus sequence for the polyadenylation signal of the *fur* transcription unit is underlined. A potential CATT box (□) is represented in a box. Triangle (▼), putative start codon (28).

2). This resulted in the isolation of a cDNA clone with a 2.2 kbp *fur*-specific insert. Both cDNA inserts were subcloned in M13mp10 for DNA sequence analysis.

Previously, we have determined the topography of the exon sequences in the human c-*fes/fps* proto-oncogene on the basis of homology with v-*fes/fps* (28). The 3′ border was tentatively placed at a position at about 200 bp downstream from a stopcodon in exon 19 where a potential polyadenylation signal was present. The availability of nucleotide sequence data of the 0.95 kbp human *fes/fps*-related cDNA (Fig. 3) and previously published sequence data of genomic *fes/fps* DNA (28) enabled a comparative analysis. The 5′ end of the cDNA clone appeared to start 5 nucleotides downstream of the 5′ end of exon 15 and at the 3′ end, the cDNA clone contained a poly(A)-stretch representing the poly(A)-tail. Therefore, the 3′ border of the *fes/fps* transcript in KG-1 cells could be determined rather precisely. The data confirmed the previous suggestion by Roebroek *et al.* (28) that the poly(A) addition signal at a position about 200 bp downstream from the stopcodon in exon 19 was functional. Furthermore it could be established that the poly(A) addition site was 17, 18 or 19 bp downstream from the polyadenylation signal. There were no differences found between the nucleotide sequence of the human c-*fes/fps*-related cDNA described here and the corresponding genomic region of the human *fes/fps* proto-oncogene. The exon/intron arrangement from exon 15 to 19, as proposed by Roebroek *et al.* (28), also proved to be correct.

Although the c-*fes/fps*-related cDNA was only one third of the observed mRNA length, some additional characteristics of the c-*fes/fps* transcription unit could be derived from analysis of the *fur* transcription unit. Comparative analysis of nucleotide sequence data of the 2.2 kbp *fur*-related cDNA and its corresponding and flanking region in genomic DNA revealed that the nucleotide sequence of the cDNA insert was identical to that of the genomic DNA fragment (Fig. 4). These sequences are indicated by capitals in Figure 4. At its 3′ end, the *fur* cDNA had a poly(A)-stretch marking the end of the *fur* transcription unit. At precisely 21 bp upstream of the poly(A) addition site, there was an ATTAAA consensus sequence of a poly(A) addition signal (38). Comparative sequence analysis further indicated that the 2.2 kbp *fur* cDNA was contained in a single exon, which we have designated 'Z'. To predict a potential *fur* translation product, we compared the three reading frames in exon Z. In all three reading frames numerous stop codons were present. The first stop codon in each of the three different reading frames was encountered within the first 420 bp from the 5′ end of the cDNA clone. We therefore concluded that the *fur* mRNA is characterized by a long noncoding region at its 3′ end.

The 3′ end of the *fur* transcription unit was found in close proximity of the v-*fes/fps* homologous region in man and cat. However, it should be noted that in front of exon 2 of c-*fes/fps,* 12 bp at the 5′ end of v-*fes* of Ga-FeSV (16) and 140 bp at the 5′ end of v-*fps* of FSV (19) were unaccounted for (28). In case of the 140 bp of v-*fps*, we realised that in human *c-fes/fps* possibly no sequences homologous to these 140 bp could be identified because of divergence of non-coding exon sequences. In case of the 12 bp of v-*fes*, it could not be excluded that homologous sequences were not recognized because comparison was limited to a DNA stretch of only 12 nucleotides. In order to resolve this matter, we have sequenced the feline c-*fes/fps* proto-oncogene region that contained exon 2 and about 1 kbp of upstream flanking sequences (data not shown). These sequence data supported the boundary assignment of human exon 2 as suggested by Roebroek *et al.* (28). The homology between the genetic sequences in exon 2 of man and cat was about 85%. Upstream of exon 2, the homology was about 65%. In this region in the feline DNA, a stretch of 12 bp was found, which was identical to the 12 bp at the 5′ end of the feline v-*fes* oncogene. In the human DNA, an almost identical stretch was found at a similar position. From these bases, 11 were shared by the viral oncogene and the feline proto-oncogene. An insertion of one nucleotide was observed in the human DNA stretch. The 3′ end of both the feline and the human DNA stretch (see Fig. 4) was followed by a sequence characteristic for a splice donor site (25). These data suggested that these stretches overlapped the 3′ region of a new human and feline c-*fes/fps* exon. The 3′ boundary of exon 1 and the 5′ end of exon 2 in human and feline DNA are compared in Figure 4. The 5′ boundary of exon 1 remains to be established but, based upon the size of the polyadenylated mRNA detected in KG-1 cells, one can roughly esti-

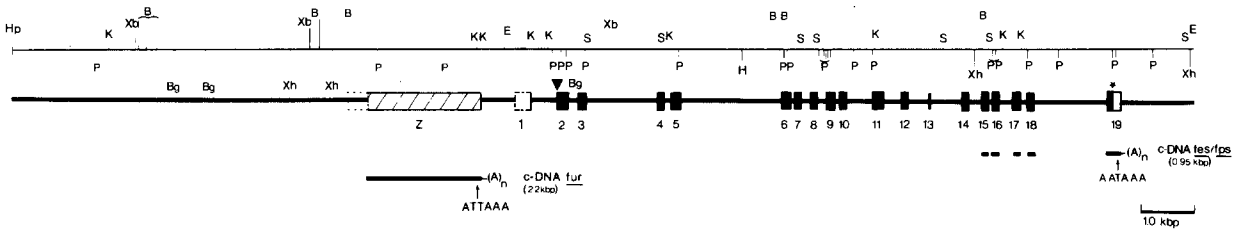

Fig. 5. Schematic representation of the topography of the human v-*fes/fps* cellular homolog and its immediately upstream region. A schematic restriction map of a 22 kbp human DNA region containing the v-*fes/fps* cellular homolog and its flanking sequences is depicted in the upper part of the figure. The numbered black boxes represent c-*fes/fps* exons described before. The open box representing exon 1 is indicated with a dashed line since its 5′ border is not yet defined. The triangle (▼) above exon 2 indicates the position of the putative start codon (28). The asterisk (*) above exon 19 indicates the position of a TGA stop codon. The non coding region of exon 19 is indicated with an open box. The hatched box, indicated with a 'Z', represents sequences of the 3′ region of the *fur* transcription unit. In the lower part of the figure, the positions of the genetic sequences in the cDNA clones are given. The positions of consensus sequences for polyadenylation signals are indicated with arrows.

mate the first c-*fes/fps* exon as about 200 nucleotides.

The location of the 3′ end of the *fur* transcription unit in relative close proximity of *fes/fps* exon 1 (see Fig. 5) raised also the question as to whether the promotor of the *fes/fps* transcription unit was located between *fur* exon Z and *fes/fps* exon 1. Searching for sequences typical for promotor regions revealed a sequence that is in good agreement with a CATT box (6), namely the GGCCATTCT sequence at position 3093–3101 in Figure 4. Limited sequence data available from feline c-*fes/fps* indicated that the GGCCATTCT sequence was also present at a similar position in the feline DNA. However, no sequences resembling the consensus sequence of a TATA box (6) could be identified. The potential CATT box would be in an appropriate position relative to the 5′ end of the transcription unit to accommodate a c-*fes/fps* transcript of 3.0 kb as found in KG-1 cells. More data are required to locate the 5′ end of the proto-oncogene and to identify the DNA sequences that control its expression. The observation that in man and cat the linkage of the *fur* and *fes/fps* transcription units has been conserved during evolution is interesting because it could have functional implications and, therefore, be instrumental in elucidating regulation of expression of c-*fes/fps*.

Acknowledgements

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