GENOMICS 18, 223–229 (1993)

ZNF75: Isolation of a cDNA Clone of the KRAB Zinc Finger Gene Subfamily Mapped in YACs 1 Mb Telomeric of HPRT

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Received February 16, 1993; revised June 28, 1993

We have previously mapped a zinc finger genomic motif (ZNF75) to the Xq26 cytogenetic band by using a hybrid panel. Here, we report the isolation of the transcribed counterpart in a cDNA clone and its further localization. The cDNA clone, from a lung fibroblast library, is assembled from three exons, including a 289 amino acid (AA) long open reading frame containing a recently described motif, the Kruppel-associated box, 42 AA long, in exon 2. By comparison with other reported members of the subfamily, the exon–intron boundaries also appear to be very well conserved. Further analysis allowed us to map this gene 1 Mb downstream from the HPRT gene in the published YAC contig that extends across Xq26. Two other motifs, 87 and 78% homologous to ZNF75 at the amino acid level, were identified by PCR on total human DNA, but map outside Xq24–qter.

INTRODUCTION

Several hundred zinc fingers (ZF) containing genes of the Kruppel type are present in the human genome (Bellefroid *et al.*, 1989). Clones coding for ZF motifs of the C2H2 type have been isolated from genomic or cDNA libraries by hybridization with ZF probes or degenerate oligonucleotides representing the link between two consecutive fingers or by PCR with degenerate primers (Bellefroid *et al.*, 1989, 1991; Bray *et al.*, 1991; Huebner *et al.*, 1991; Pellegrino and Berg, 1991; Thiesen, 1990; Hoovers *et al.*, 1992). Although almost 100 human ZF genes have been found (McAlpine *et al.*, 1991), for the majority of the reported clones only incomplete information is available, since they often represent partial clones characterized with regard only to the sequence of the zinc finger-containing fragments. Their relationship with the genomic counterpart has rarely been investigated, and exact localization has been determined only for some, usually at megabase resolution (McAlpine *et al.*, 1991). In addition, some of the sequence material obtained by PCR could pertain to the same gene, since the amplification procedure can result in amplification of only a portion of the ZF segments that are known to be present in long arrays.

Of several ZF clones that have been detected in the human genome, some have been located to the same chromosomal region by *in situ* or hybrid panel techniques (Huebner *et al.*, 1991; Hoovers *et al.*, 1992; Lichter *et al.*, 1992; Rousseau-Merck *et al.*, 1992). This suggests that ZF genes may be clustered. However, as pointed out by some authors, since the accuracy of mapping by *in situ* or by hybrid panels is in the range of several megabases, the exact meaning of these findings is still unclear (Lichter *et al.*, 1992). Here, we report further studies on ZNF75, a ZF-containing genomic clone previously mapped to Xq26 by *in situ* and hybrid panel techniques (Villa *et al.*, 1992).

MATERIALS AND METHODS

YAC identification. The genomic X3000.11 YAC library (provided by D. Schlessinger, St. Louis, MO) carrying the Xq24–qter region as the only human component (Abidi *et al.*, 1990) was screened with the ^{32}P random-labeled 0.5-kb *Hin*dIII–*Eco*RI fragment that includes the zinc finger region of ZNF75 sequence (Villa *et al.*, 1992). Hybridization, washing, and identification of positive gridded clones were performed as described in Little *et al.* (1989). YAC DNA minipreparation, digestion, and fingerprinting analysis were performed as in Porta *et al.* (1993) for *Alu* and LI interspersed repeats and as in Zucchi and Schlessinger (1992) for the pTR5 and LF1 moderate repeats.

cDNA library screening. A human lung fibroblast cDNA library (Stratagene) in lambda ZapII vector was screened with the 0.5-kb *Eco*RI–*Hin*dIII fragment (Villa *et al.*, 1992). DNA probe was labeled to a specific activity of 2×10^9 cpm/μg by random oligonucleotide priming (Feinberg and Vogelstein, 1983). Hybridizations were carried out at 65°C in 5× SSPE, 10× Denhardt's solution, 2× SDS, and denatured salmon sperm DNA (100 μg/ml). Filters were washed in 2× SSPE, 1% SDS and 1× SSPE, 1% SDS at 65°C for 15 min and autoradiographed at −70°C with intensifying screens for 48 h.

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0888-7543/93 $5.00

DNA sequencing and sequence analysis. Lambda ZapII-positive clones were transformed in pBSSKII, and DNA was prepared and sequenced by the dideoxynucleotide chain termination method using the Sequenase kit (Sequenase, USB). Nucleotide and amino acid sequence analyses were performed with the GCG software package running on a VAX 3600 computer. Homology searches were performed on GenBank release 73.0 (9/92), Pir-Protein release 34.0 (8/92), and Swiss Protein release 23.0 (7/92).

Probes and PCR. The probe (EH0.5) utilized to screen cDNA libraries was a 0.5-kb genomic *Eco*RI/*Hin*dIII fragment containing part of the zinc finger motif and part of the 3′ UT region (Villa *et al.*, 1992). PCR amplification was performed with the following reaction components: 5–50 ng of purified genomic DNA template, 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 0.001% gelatin, 2 m*M* $MgCl_2$, 200 μM dNTP, 20 pmol of primers, 2.5 U of *Taq* polymerase, and water to 50 μl. A Perkin–Elmer thermal cycler was used for 30 cycles of reactions under conditions of denaturing at 94°C for 1 min annealing at 55°C for 1 min, and extension at 72°C for 1 min.

The primers that were used for the polymerase chain reaction on genomic DNA (underlined in Fig. 1) were D8C6 forward, 5′-gtgacaggaggtttagatgga-3′; and D8C6 reverse, 5′-cccttcttctgtggagtttc-3′. For reverse-transcribed PCR (RT-PCR) the following primer was used together with D8C6 reverse: zf2, 5′-aaaagaatcaaacactggaa. PCR products were subcloned in TA cloning vector (Invitrogen).

RESULTS

Isolation of a cDNA for ZNF75

To identify cDNAs transcribed from the ZNF75 locus, we screened a human lung fibroblast cDNA library by filter hybridization, as described under Materials and Methods. One 937-bp-long clone was identified and sequenced. We found a 289 amino acid (AA) long ORF starting at nucleotide 22 with an ATG codon and ending at nucleotide 889. The complete sequence of this clone is shown in Fig. 1.

By comparing genomic and cDNA sequences, we were able to define the exon–intron structure of this clone. The cDNA is assembled from three exons, the third of which exactly matches the previously published genomic sequence (Villa *et al.*, 1992). Starting from the methionine codon at position 22, 70 additional AAs are present; 11 are coded by the first exon, 42 by the second, and 16 by the third. The 54th codon is split by the exon–intron boundary after the first nucleotide. There is only one discrepancy with the previously described genomic sequence: a G to A transition is present in cDNA at position 792 (position 1207 in Fig. 1), at the third nucleotide of codon 257, which does not change the amino acid. This silent mutation could be due to a polymorphism or could represent a mutation present in the X3000.11 hybrid DNA from which the genomic clone was derived, since this hybrid underwent radiation treatment to decrease the size of the human component (Nussbaum *et al.*, 1986).

As shown in Fig. 1, two exon–intron junctions were identified; the first is at position 54 and the second at position 181 of the cDNA sequence. The sequence of the first intron is also reported in Fig. 1, whereas the second intron (about 3 kb) has been only partially sequenced and is not included. The general structure of the cDNA clone is also depicted at the bottom of Fig. 2 (see legend).

A search for homologies in databases demonstrated that the amino acid sequence coded by exon 2 (AA 12 to 53) recognizes a motif present in several ZF coding genes in a region that is different from the ZF itself (Fig. 2). This motif has been referred to as KRAB-A (Kruppel-associated box A) and it is present exclusively in the N-terminal region of a subset of ZF genes. Not only the AA sequence but also the length of the motif and its exon–intron structure are strictly conserved. In fact, in the majority of the cases reported, both the number of amino acids coded for this exon and the positions of the splice sites are maintained, since the latter are located just before the first AA of this motif and after the first nucleotide of the 43rd AA.

Most of the sequences reported in Fig. 2 have been previously identified as containing the KRAB motif (Bellefroid *et al.*, 1991; Rosati *et al.*, 1991; Costantinou-Deltas, 1992). New homologies with human ZF genes were obtained by searching protein databases. Interestingly, in screening nucleotide databases we detected two additional significant scores, one with an EST from random cDNA sequencing of cDNA brain library (Venter group) and one in the 5′ untranslated region of a human ZF cDNA. In this latter case (HSPHPLK), the frame is interrupted by a stop codon, but the homology still persists after it. This could represent an example of KRAB motif inactivation by single-point mutation.

As can be seen in Fig. 2, the KRAB-A motif is preceded by short stretches of amino acids in all cases. This strongly suggests that our cDNA includes all or almost all of the potential coding sequence of ZNF75. We have also analyzed the 300 nucleotides of sequence just 5′ of the genomic counterpart of the beginning of the cDNA clone; an additional in-frame ATG codon is present 21 nucleotides upstream from the beginning of the cDNA, and an in-frame stop codon is present 56 nucleotides upstream of that. At the 3′ end, the cDNA sequence includes a TGA codon at position 889 and extends 46 nucleotides in the 3′ untranslated (UT) region, not reaching the two putative poly(A) signals present in the genomic sequence; it is conceivable that this cDNA includes all the coding segments at the COOH terminus but not the whole 3′ UT region. Therefore, although our cDNA could lack a few amino acids at the 5′ end, it is clear from the whole set of data that it should contain most of the coding portion of the ZNF75 transcript. This conclusion is strengthened by the fact that further screening of the library gave two more clones of the same size. This conclusion does not exclude the possibility that an alternative longer transcript exists for ZNF75, as has been shown to occur with other ZF genes. In this regard, it must be mentioned that we were unable to detect reproducible transcript signals in Northern blots of poly(A) RNA from human tissues. This suggests that ZNF75 is not ubiquitously expressed gene or that it is expressed at very low levels. However, we were able to detect the ex-

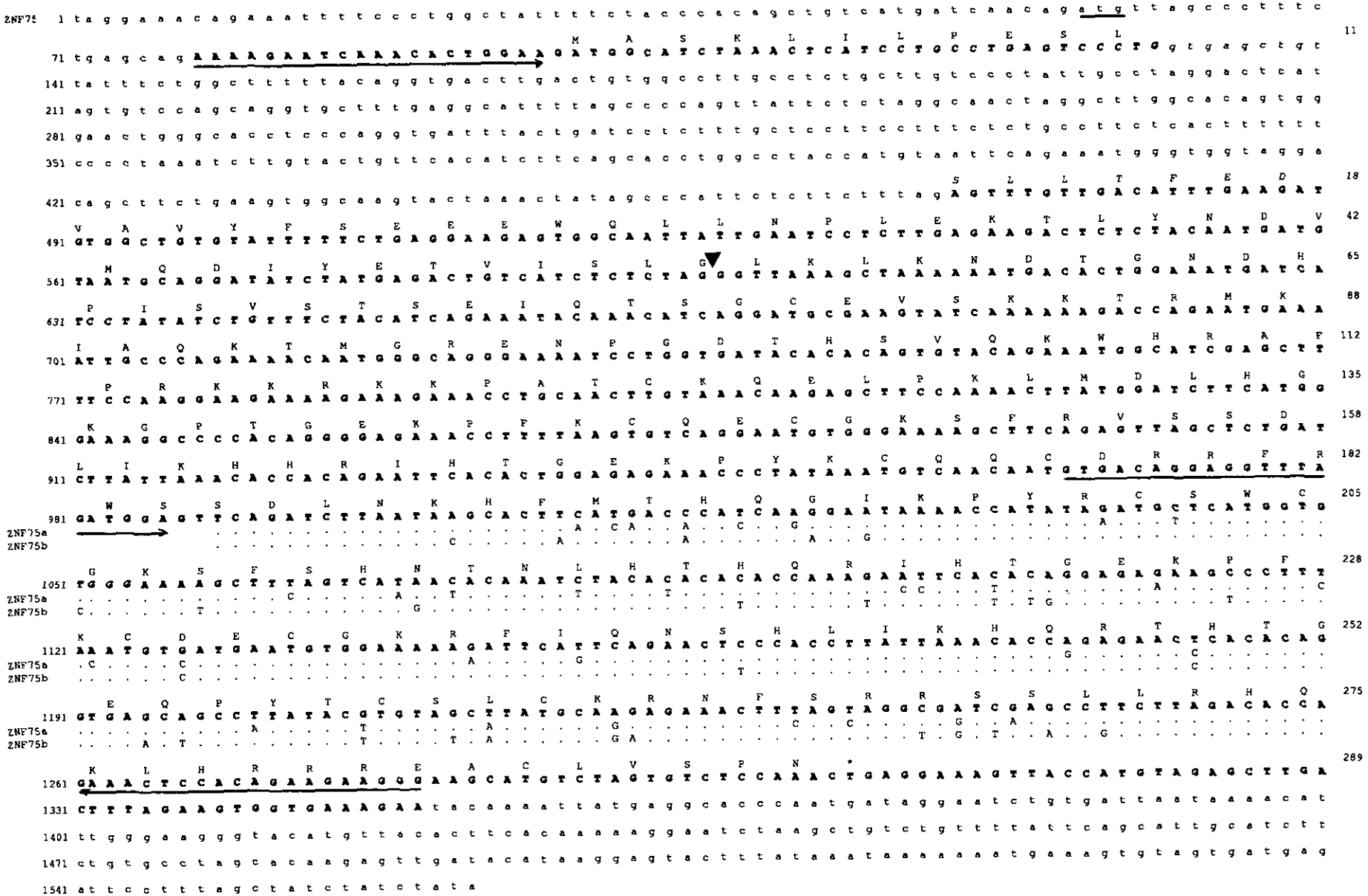

FIG. 1. Nucleotide and deduced amino acid sequences of the genomic and cDNA ZNF75 and its homologues ZNF75a and ZNF75b. The lower case letters indicate the first intron region, while the capital boldface letters represent the coding region. The arrowhead shows the location of the second intron (not sequenced). The dots, present in the homologous ZNF75a and ZNF75b, indicate an identical nucleotide. The DNA sequence is numbered on the left. Numbers on the right define the positions of amino acids. The asterisk shows an in-frame termination codon. The primers (D8C6F–D8C6R) used for the PCR on genomic DNA are underlined. The G at position 1207 is an A in the genomic sequence of D8C6 cosmid (Villa *et al.* 1992).

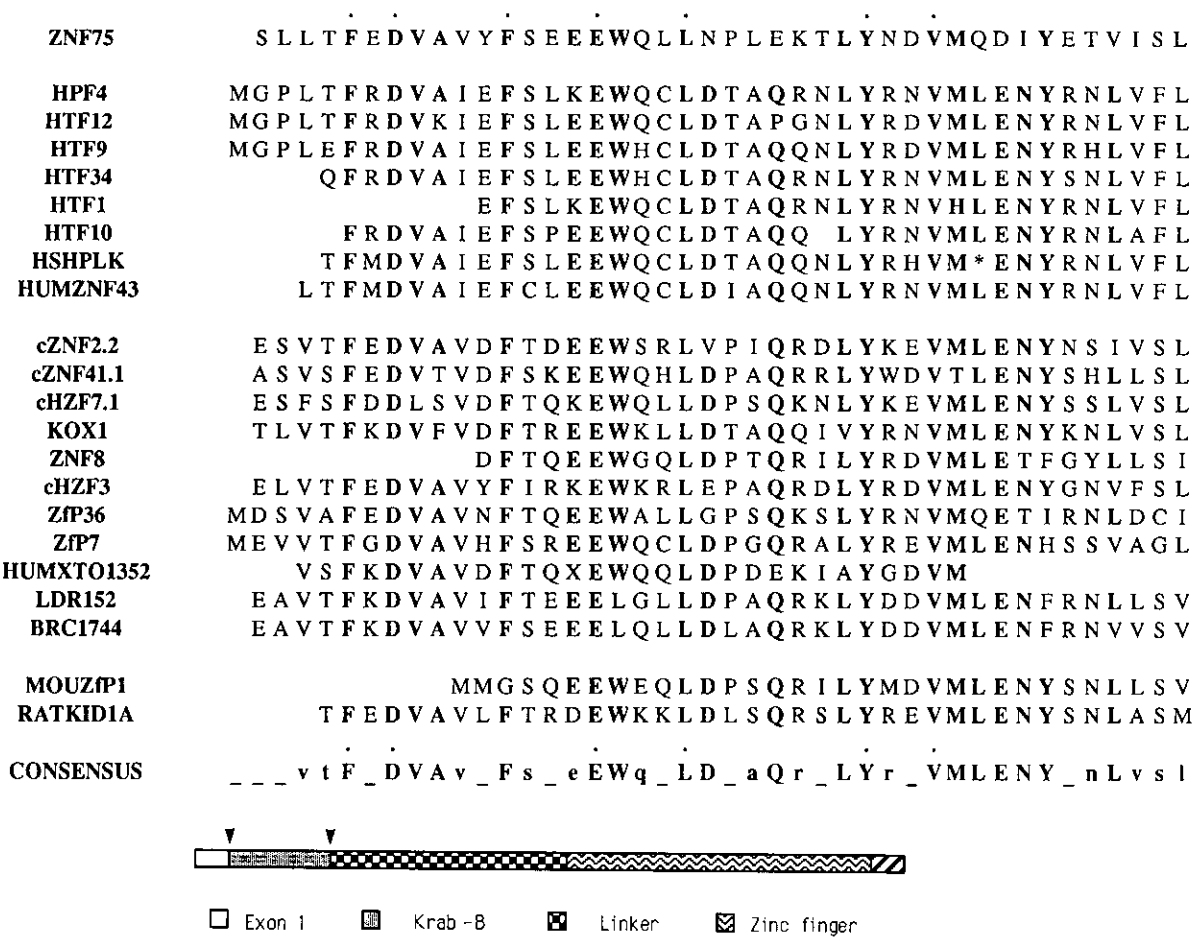

FIG. 2. Amino acid sequence of the ZNF75 KRAB-A motif and of its homologous clones from databases. Most of the first group (from HPF4 to HUMZNF43) have been described in Bellefroid *et al.* (1991). Most of the second group (from cZNF2.2 to BCR1744) have been reported in Rosati *et al.* (1991) and in Costantinou-Deltas *et al.* (1992). Two rodent sequences are included at the bottom. Columns with boldface letters and a dot represent 100% conserved amino acids. Boldfaced letters without a dot indicate conservation between 75 and 100%. Lowercase letters indicate amino acids conserved between 50 and 75%. Dashes indicate amino acids conserved less than 75%. A diagram of the ZNF75 cDNA coding region is shown at the bottom; the arrowheads indicate the positions of the two introns. The KRAB motif is contained in the second exon, while both the linker and the zinc finger regions are contained in the third exon.

pected amplification product in U937 and HT29 cell lines (but not in K562 and one melanoma cell line) with the RT-PCR technique by using a combination of two primers, one in the first exon and the other in the third.

Fine Mapping of ZNF75

To identify YACs containing the ZNF75 genomic fragment, grids derived from a specific YAC library for the Xq24–qter portion were screened with probe EH0.5 (see Materials and Methods for details). The screening of the X3000 YAC library with ZNF75 probe led to the identification of four hybridization-positive clones from about 3 genome equivalents (150 Mb): YWXD290, YWXD382, YWXD614, and YWXD893. The clones identify a set of YACs assembled by their content of the repetitive element pTR5 in 10.6 and 0.5-kb common fragments (Zucchi and Schlessinger, 1992) and also assembled by probe content in a YAC contig across Xq26 (Little *et al.*, 1992).

To confirm these results, we amplified the YACs with a primer pair that gives a 0.3-kb product on cosmid D8C6 (Villa *et al.*, 1992) and human genomic DNA. The same amplification products were seen with all the positive YACs but not with the negative control YAC (Fig. 3B). No additional bands that could have been derived from other highly homologous ZF genes were apparent with this primer pair. However, due to the high number of ZF genes in human genome, there is the possibility that this PCR product could be the result of the amplification of ZF genes different from ZNF75. For this reason we cloned and sequenced two different clones of this PCR product; both were identical to ZNF75 (data not shown), indicating that this gene indeed maps to these YAC clones. These results were also confirmed by Southern blot of digested YAC DNA hybridized to EH0.5 probe (Fig. 3B). The collection of YACs has been analyzed in detail by Schlessinger's group in St. Louis and several contigs have been assembled and published. All EH0.5-positive YACs were overlapping clones map-

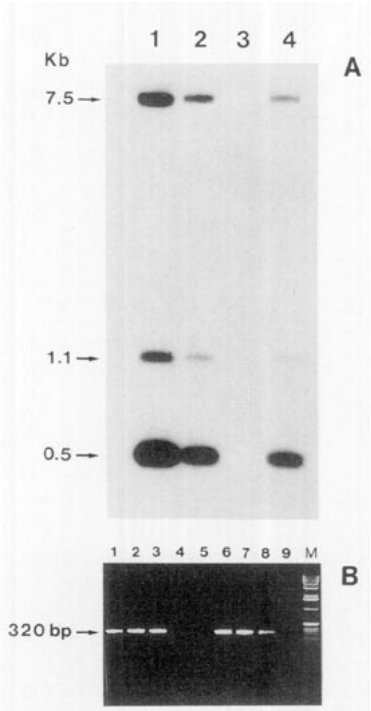

FIG. 3. (**A**) Hybridization analysis of the radiolabeled ZNF75 cDNA of the *Taq*I-cleaved DNA (1 μg/lane) from YWXD290 (lane **1**), YWXD382 (lane **2**), control negative YAC (lane **3**), and YWXD614 (lane **4**). (**B**) PCR amplification with primers D8C6F–D8C6R on the YACs and genomic DNA. Lane **1**, YWXD290; lane **2**, YWXD382; lane **3**, YWX614; lane **4**, control negative YAC; lane **5**, control negative YAC; lane **6**, YWX893; lane **7**, D8C6 cosmid; lane **8**, human genomic DNA; lane **9**, blank control; lane **M**, 1-kb ladder (BRL).

ping to Xq26 in an 8-Mb contig covering a region between HPRT and Factor IX (Little *et al.*, 1992). They are grouped in a region about 1 Mb downstream from the HPRT locus. These results are in agreement with our previous mapping of ZNF75 to Xq26 by *in situ* and hybrid panel analyses and suggest, that no other ZF gene with high homology to ZNF75 maps to Xq24–qter, although the possibility that more distantly related Kruppel genes are present in this region cannot be excluded.

Identification of Two Highly Homologous ZF Motifs Not Mapping on Xq24–qter

During the search for other ZF clones that are highly homologous to ZNF75 and that could derive from the same chromosomal region by gene duplication, we amplified total genomic DNA by two primers designed in less-conserved portions of the zinc finger motifs. The amplification product of primers D8C6F and D8C6R was cloned in pTA1000, and some clones were sequenced. Whereas previously detected homologies with ZNF75 zinc finger motifs were at most 65%, these new ZF motifs had homologies of 88 and 89% at the nucleotide level (Fig. 1) and of 87 and 78% at the amino acid level, respectively (Fig. 4). Hybridization to a panel of DNAs containing whole human genomic DNA, hybrid X.3000.11, and other controls demonstrated in both cases a specific signal in human genomic DNA that is absent in X3000.11 DNA (data not shown). This demonstrates that these homologous ZF genes do not map to the same chromosomal region as ZNF75.

At the moment, we cannot speculate whether these homologous genes are indeed expressed genes or pseudogenes. In the approximately 300 bp determined, a single ORF containing all the zinc finger motifs was conserved in both ZNF75a and ZNF75b. However, ZNF75b, which diverged the most from ZNF75, has some changes that might suggest that it could be a relict, that is, a Y substitution for the conserved H at position 33 and an R substitution of a conserved L at position 87 (Fig. 4). All the generally conserved positions in C2H2 motifs (the two cysteines, the two histidines, the phenylalanine, and the lysine in the loop) are conserved in ZNF75a. We also examined the position in the loop that has recently been identified as important for DNA binding in Sp1, Zif268, and Krox20 (Pavletich and Pabo, 1991; Nardelli *et al.*, 1992; Jacobs *et al.*, 1992; Krivacki *et al.*, 1992). Taking the first cysteine as position 1, in each ZF the relevant positions are 10, 13, and 16. Position 10 is conserved in five of the six instances determined, but is notably different in the first determined finger of ZNF75a. Position 13 is always conserved, whereas position 16 is different in one case each in ZNF75a and ZNF75b.

DISCUSSION

The cDNA clone represents a transcript from the ZNF75 locus. In addition to the zinc finger motifs present in the 3′ portion of the gene, it shows also a conserved motif in the 5′ region. This motif, recently described by three groups, can be divided into two regions that appear to be coded by two distinct exons, one coding for 42 AA and the other coding for 33 AA (Bellefroid *et al.*, 1991; Rosati *et al.*, 1991; Constantinou-Deltas *et al.*, 1992). These submotifs can be present in the same cDNA or can be alternatively spliced. ZNF75 cDNA presents only the KRAB-A motif, with an exact conservation of the splice sites. From the few data reported in the literature on the exon–intron structure of these genes, this appears to be a general feature of the KRAB subfamily. Thus, the general pattern of this subfamily is a short first exon followed by the KRAB-A and/or -B coding exon(s), a linker between these motifs and the zinc finger motifs that is not very conserved, and an array of contiguous zinc finger motifs followed by few AAs before the stop codon. It could be that this family represents the most ancient prototype of zinc finger-containing genes, which duplicated as a whole, maintaining in approximately one-third of the cases the same general structure with the same exon–intron boundaries.

In a previous study, we localized a ZF motif (ZNF75) in Xq26 by using a hybrid panel (Villa *et al.*, 1992). Here, the localization is established in the published 8-Mb YAC contig spanning the Xq26 region containing the HPRT and FIX genes. The distance between HPRT and

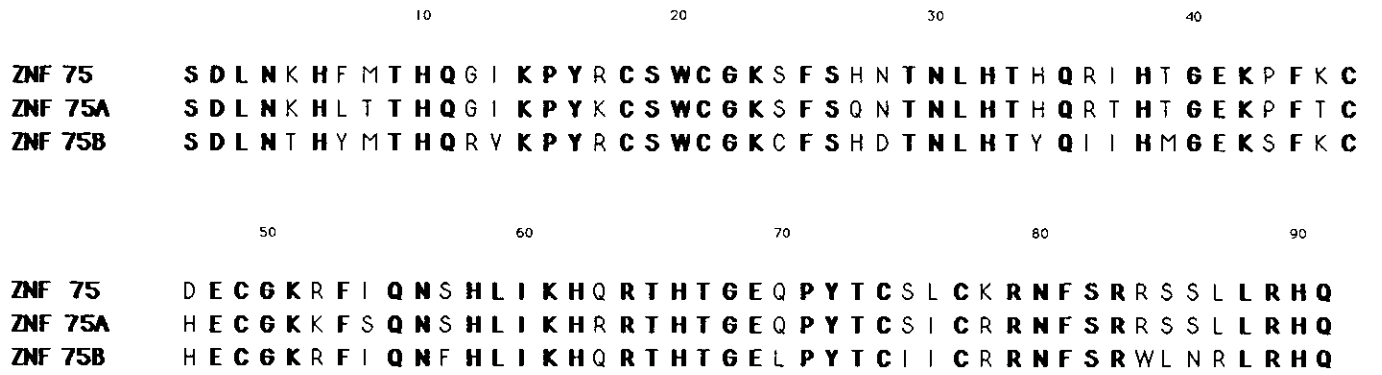

FIG. 4. Amino acid comparison of ZNF75 cDNA and its homologues ZNF75a and ZNF75b. The boldface capital letters indicate amino acids conserved in all three clones. The sequences are numbered above.

FIX is estimated at about 4 Mb, and our results place ZNF75 about 1 Mb telomeric to the HPRT gene. To our knowledge, this is the only gene mapped thus far between HPRT and FIX.

ZNF75 was found in YACs that overlapped both in their content of the middle-repetitive element pTR5 and in assembly into a contig that extends from Xq26.1 to q27.1. The availability of the large contig helps in detecting any clustering of ZF genes. Many ZF genes have been mapped to neighboring chromosomal locations by *in situ* hybridization or by hybridization to hybrid panels (summarized in McAlpine, *et al.* 1991). Others appear to be clustered in smaller intervals (Crossley and Little, 1991). Clustering could, however, result solely from the large number of ZF motifs in the genome. In this case, we tested for association with two ZF genes highly homologous to ZNF75, but neither mapped to the Xq24–qter region. Together with the fact that hybridization of ZNF75 to the clones from the Xq26 contig detected no additional signal, this finding argues against any local cluster, although more divergent ZF motifs might still be present and as yet undetected.

ACKNOWLEDGMENTS

We thank Professor R. Dulbecco, Dr. G. Evans (Salk Institute, San Diego, CA) and Dr. D. Schlessinger for encouragement and Dr. P. Little (Imperial College, London) for the gift of the oligonucleotide probe. We are indebted to Dr. D. Schlessinger for the gift of the X3000.11 YAC library. This work was supported in part by grants from P. F. Ingegneria Genetica (to P.V.), P. F. Biotecnologie e Biostrumentazoine (to C.P.), ACRO (to A.V.), Telethon (to P.V.), and the EEC Human Genome Project (to P.V.).

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