A mitochondrial elongation factor-like protein is over-expressed in tumours and differentially expressed in normal tissues

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Abstract The tissue-specific expression of an antigen (P43) ubiquitously expressed at high levels in a variety of tumours of human and animal origin was investigated using a monoclonal antibody to P43. Whereas low amounts of P43 are expressed in the spleen, skeletal muscle and pancreas, P43 is abundantly produced in the liver and in other tissues such as the kidney, heart and brain which have high levels of oxidative metabolism. Interestingly, a related protein of higher molecular weight is abundantly expressed in the lung and in amounts which were higher than those observed with other tissues. The human cDNA for P43 was isolated from a human liver cDNA library and mapped to chromosome 16 between p11.2 and 12 and also to a position near the centromere on the long arm of chromosome 17. The deduced amino acid sequence of P43 is remarkably similar to that of *E. coli* EF-Tu and the mitochondrial EF-Tu of *S. cerevisiae* with the structurally and functionally important amino acids of EF-Tu being completely conserved in P43. A comparison of the distribution of P43 and a mitochondrial protein Hsp 60 among different cellular fractions indicated a likely mitochondrial localisation for P43. Taken together these results suggest that P43 is a human mitochondrial elongation factor.

Key words: Mitochondrial; Elongation factor; Human; EF-Tu homolog; Expression

1. Introduction

The aberrant expression of many important regulatory proteins is observed in tumour cells and the identification and study of the genes of these products has greatly increased our understanding of regulatory processes in normal and malignant cells. One novel approach taken to identify such proteins involved the screening of monoclonal antibodies raised against total human hepatoma cell proteins for antibodies which detected tumour specific antigens. This work led to identification of an antigen (P43) which was ubiquitously expressed at high levels in a variety of tumours derived from humans and other animals [1]. The protein sequence of three tryptic peptides of P43 revealed 50–70% homology to different domains of the eukaryotic-elongation factor EF-1a and also to the prokaryotic factor EF-Tu suggesting that P43 might be a member of the GTPase superfamily of proteins which carry out diverse cell functions.

We have now investigated the expression levels of P43 in different tissues and describe the isolation and characterisation of the cDNA for P43. The evidence presented suggests that P43 may be a human mitochondrial elongation factor.

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2. Materials and methods

2.1. Preparation of cell and mitochondrial protein extracts

Cultured cells and mouse tissues were harvested in PBS containing 10 mM EDTA and lysed by dounce homogenisation in RIPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% NP40, 10 mM NaHPO4, 10 mM EDTA, pH 7.5) to which a mixture of protease inhibitors was added at the following concentrations (0.2 mM PMSF, 1.5 μg/ml aprotinin, 0.5 mg/ml leupeptin 1 mg/ml pepstatin, and 0.5 μg/ml E-64). The viscous lysates were then sonicated for 20 s and centrifuged to remove the cellular debris and the cleared extracts stored at −20°C. Mitochondria were isolated from HeLa cells by gentle homogenisation in 10 volumes of homogenisation buffer (50 mM sucrose, 200 mM sorbitol, 1 mM EDTA, 5 mM Tris-HCl, pH 7.5) in a dounce homogeniser. Unlysed cells and nuclei were removed by two successive centifugation steps at $1,000 \times g$ for 10 min. The mitochondria were then recovered from the supernatant by centifugation at $10,000 \times g$ for 20 min and washed three times in homogenisation buffer. Crude mitochondrial protein extracts were prepared by lysing the mitochondrial pellets in homogenisation buffer containing 0.5% NP-40. Proportional amounts of the mitochondrial protein extracts and supernatant fractions from the isolation and washing steps were mixed with 2 × SDS-PAGE sample buffer [2] and boiled for 10 min before electrophoresis in denaturing polyacrylamide gels.

2.2. Immunoblotting

Proteins were separated by electrophoresis in 12.5% SDS polyacrylamide gels and electroblotted onto nitrocellulose [3]. The transfer of protein was checked by reversibly staining the filter with Ponceau S, after which P43 was detected by the use of an affinity purified monoclonal antibody (MAb 68), horse-radish peroxidase (HRP) conjugated rabbit anti-mouse immunoglobulins and the Amersham enhanced chemiluminescence (ECL) detection system.

2.3. Construction of the cDNA expression library

Total cellular RNA was prepared from cells of the human hepatoma cell line PLC/PRF/5 (ECACC 85061113) harvested from ten culture dishes (10 cm diameter) at 50% confluence using the single step extraction method of Chomozynski and Sacchi [4]. Poly(A)$^+$ RNA was isolated from purified total RNA using pre-packed spun columns containing oligo(dT)-cellulose (Pharmacia LKB) according to the manufacturers recommendations. Purified RNAs were analysed by electrophoresis in agarose gels containing 2% formaldehyde [5]. A cDNA library was prepared from 5 μg of purified poly(A)$^+$ RNA using a lambda ZAP-cDNA synthesis kit and packaged in vitro using Gigapack II gold packaging extract (Stratagene Ltd). Each 100 ng of packaged cDNA yielded approximately 3×10^6 recombinant bacteriophages.

2.4. Screening of the cDNA library

In order to design oligonucleotide probes for screening the cDNA library the p43 protein was purified by affinity chromatography using the monoclonal antibody and three peptide amino acid sequences partially determined [1]. Based on the longest peptide sequence two long (54 nt) 'guessmer' oligonucleotides (sense and antisense) were synthesised by taking into consideration the frequency of codon usage in human genes [6]. The oligonucleotides (which overlapped by 18 nt at their 3′ ends) were annealed together and radiolabeled by incubation with Klenow enzyme in the presence ^{32}P-radiolabeled dCTP and dATP and unlabelled dGTP and dTTP using standard conditions [5]. A second probe based on a shorter peptide sequence was synthesised as a

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mixture of oligonucleotides reflecting all codon combinations and incorporated a 20 nt long T-tail for radiolabelling with [α-^{32}P]dATP by the hairpin extension method [7]. About 6×10^5 recombinant plaques were screened by plaque hybridisation as described [8,9]. Hybridised filters were washed at 42°C in 0.2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate) containing 0.1% SDS and exposed to X-ray film for 2–3 days at −70°C using intensifying screens. Forty positive plaques were identified from duplicate filters and twenty of these plaque purified by standard procedures [5].

2.5. Subcloning and DNA sequencing

Plasmid Bluescript containing the cDNA inserts were obtained by in vivo excision and recircularisation of phagemid DNA from the lambda ZAP according to the procedure recommended by the manufacturer (Stratagene Ltd). Restriction endonuclease mapping of the cDNA inserts and the subcloning of DNA fragments for sequencing was performed using standard procedures [5]. Denatured plasmid DNA was sequenced by the dideoxy chain-termination method of Sanger et al. [10] using commercially available kits. The restriction endonuclease map and sequencing strategy for P43 are shown below. The coding region is indicated by an open bar and arrows indicate the extent and direction of the sequence determination. A database of the cDNA sequence was compiled using the Staden software package [11]. Comparisons with known nucleic and amino acid sequences were carried out using sequence analysis programs for the VAX [12].

2.6. Transfection and expression of P43 in Chang liver cells

Cultured Chang liver cells (line CCL-13) were transfected with 15 μg of plasmid pCMVP43 containing the full-length cDNA for P43 downstream of the CMV promoter using the standard calcium phosphate procedure [13]. A duplicate series of transfections with plasmid pCH110 (Pharmacia) expressing β-galactosidase indicated that 5–10% of the cells had taken up plasmid DNA. Total protein extracts were prepared from cells 48 h after transfection and assayed for P43 expression by immunoblotting with MAb68 as described above.

2.7. Southern and Northern analysis

Purified genomic DNA (10 μg/track) was digested with restriction enzymes using the conditions recommended by the manufacturer and separated by agarose gel electrophoresis [5]. Phage lambda DNA digested with *Hin*dIII were included as molecular size markers. The DNA fragments were transferred to nitrocellulose filters by the method of Southern [14]. Northern blots of cellular mRNA were prepared by standard procedures [5]. Filters were hybridised at 42°C with a randomly primed [^{32}P]dCTP labelled probe in 5 × SSC containing 50% formamide, 10% dextran sulphate, 5 × Denhardts solution and 0.05% sodium pyrophosphate. The filters were washed in 0.2 × SSC, 0.1% SDS at 65°C and autoradiographed at −70°C for 1–2 days with the use of intensifying screens.

2.8. Fluorescence in situ hybridisation

Whole plasmid DNA was biotinylated by nick translation [15]. Metaphase chromosome spreads were prepared from a normal human male lymphoblastoid cell line following a standard methotrexate block and BrdU release protocol. Fluorescence in situ hybridisation was performed essentially as described by Fan et al. [16] except that proteinase K digestion was carried out prior to denaturation in 70% formamide in 2 × SSC at 65°C for 2 min. Forty nanograms of biotinylated probe and 5 μg of sonicated placental DNA were mixed with 15 μl of hybridisation buffer and denatured at 65°C for 10 min and applied to prewarmed slides for hybridisation overnight at 42°C. The biotinylated probe was detected by incubation with avidin-FITC (4 μg/ml in 5% non fat milk, 0.05% Tween 20, 4 × SSC), and the signal amplified by successive incubations with biotinylated anti-avidin antibody and avidin FITC respectively [17]. The slides were finally mounted in antifade (Citifluor AF1) containing a mixture of propidium iodide and DAPI [16] and analysed using a Nikon epifluorescence microscope and MRC Lasersharp 600 confocal imaging system (Bio-Rad).

3. Results

3.1. Differential expression of P43 in normal tissues

The monoclonal antibody MAb68, raised to human P43, detects a protein of the same size in woodchuck, mouse and fish (*Xiphophorus*) cells suggesting that the antigen is highly conserved. We have used MAb68 to compare the relative amounts of P43 antigen in total cell protein extracts from different mouse tissues. Western blots of approximately equal amounts of total cell proteins isolated from different mouse tissues show that while relatively low amounts of P43 are expressed in the spleen, muscle and pancreas, P43 is abundantly produced in the liver, kidney, heart and brain tissue (Fig. 1). Most striking was the detection of a protein of apparently higher molecular weight (50 kDa) which was highly expressed in the lung and also detected in trace amounts in the liver and kidney (Fig. 1). Relatively low amounts of the 43 kDa antigen were also detected in protein extracts from lung tissue. To check that the results observed were not due to differences in the amounts of protein loaded, which might be caused by the high level expression of certain tissue-specific proteins, identical protein gels were also analysed by Coomassie blue staining (data not shown). Similar results were obtained using tissues from 6 individual animals indicating that the pattern of tissue expression shown in Fig. 1 is reproducible and representative.

3.2. Isolation and nucleotide sequence analysis of the cDNA for p43

A lambda ZAP bacteriophage cDNA library of the human hepatoma cell line PLC/PRF/5 was screened with oligonucleotide probes based on the amino acid sequences derived from three peptide fragments of affinity purified P43 as described in section 2. Forty positive clones were identified from about 6×10^5 plaques and 10 of these were isolated for further analy-

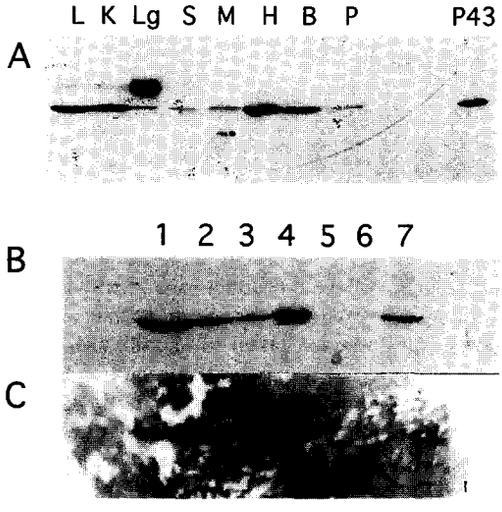

Fig. 1. (A) Western blot analysis of the P43 expression in different mouse tissues. Approximately equal amounts of total cell protein were loaded in each track as follows; L, liver; K, kidney; Lg, lung; S, spleen; M, muscle (gastrocnemus); H, heart; B, brain; P, pancreas. Protein extracts from the human HeLa cell line were loaded in track labelled P43. Proteins recovered at different stages during the isolation of mitochondria from intact HeLa cells were analysed by immunoblotting using (B) the monoclonal antibody to P43 and (C) antisera to Hsp 60. The final volume of each fraction was kept constant and equal volumes were loaded in each track in order to analyse the relative proportion of P43 and Hsp 60 in the different fractions. Tracks: 1, unlysed cells and nuclei; 2, first wash of nuclear pellet; 3, second wash of nuclear pellet; 4, mitochondria fraction; 5, first wash of the mitochondria; 6, second wash of mitochondria; 7, cytoplasmic fraction.

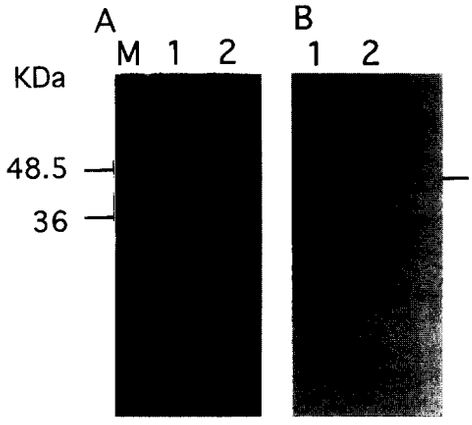

Fig. 2. Expression of P43 in Chang liver cells (CCL-13). (A) Coomassie brilliant blue stained gel of total protein extracts showing that similar amounts of protein were loaded in tracks 1 and 2. (B) Western blot analysis of P43 expression. Tracks: 1, protein extract from untransfected cells; 2, protein extract from cells transfected with pCMVP43.

sis. The sequencing of the ends of these cDNAs revealed that they were of all homologous and clone pBS43.5, having one of the longest cDNA inserts, was completely sequenced. Cultures of clone pBS43.5 produced a cDNA fusion protein upon induction with IPTG which was specifically detected by MAb68 (data not shown).

In order to obtain further evidence that the cDNA we have isolated encodes the P43 gene Chang liver cells (line CCL 13) were transfected with 15 μg of a plasmid expressing the full-length cDNA under control of the CMV promoter. Immunoblotting with the monoclonal antibody MAb68 showed that the expression of P43 was substantially increased in transfected cells (Fig. 2).

Analysis of the complete nucleotide sequence of the cloned cDNA revealed one long open reading frame (ORF) encoding the three tryptic peptide fragments of P43 which had been previously sequenced (Fig. 3). No start codon could be found in clone pBS43.5 but by aligning the overlapping N-terminal sequences of a second clone pBS43.7, with that of pBS43.5 it was possible to identify two putative start codons (only 6 nt apart and in the same reading frame) which would initiate translation of the longest ORF. As N-terminal sequence analysis of the protein was not possible it is not known which of these two closely spaced start codons is used to initiate translation of P43. For the purposes of this paper we have used the longest predicted ORF for analysis of the nucleotide and amino acids sequences of P43. This open reading frame of 1356 nt would code for a polypeptide of 452 amino acids with a predicted molecular weight of 49.5 kDa.

3.3. The predicted amino acid sequence of the P43 protein

A computer assisted comparison of the deduced amino acid sequence of P43 with that of proteins available in the database revealed a striking homology to the translation elongation factors found in bacteria and eukaryotic mitochondria. The protein sequences of P43, the *S. cerevisiae* mitochondrial elongation factor mEF-Tu and *E. coli* EF-Tu are compared in Fig. 3. While the first 43 amino acids of the yeast mEF-Tu share little homology with EF-Tu of *E. coli* the remaining protein sequence is identical at 62.2% of residues. The extended N-terminal sequence of the yeast EFTu has been attributed to the presence of a N-terminal signal sequence required for translocation of the protein into the yeast mitochondria. Similarly, the first region of homology between P43 and EF-Tu of *E. coli* begins at the arginine residues at positions 56 and 7 respectively. The *E. coli* EF-Tu sequence is identical to P43 at 54.3% of positions with a high proportion of the other corresponding residues having similar biochemical properties (Fig. 4). A computer assisted alignment of the yeast mEF-Tu and P43 amino acid sequences revealed that 56.1% of the yeast residues were identical to P43 and that most of the remaining amino acids were conserved with respect to their biochemical properties (Fig. 4). Although the N-terminal amino acid sequences of P43 and yeast mEF-tu are less well conserved than other regions of the proteins they display similar features. Both N-terminal sequences encode predominantly hydrophobic amino acids and a moderate number of positively charged lysine and arginine residues but no negatively charged amino acids (Fig. 4). These features are typical of the targeting signals found on other proteins imported into mitochondria [18]. This fact and the overall level of homology of the P43 protein sequence to that of *E. coli* EF-Tu and yeast mEF-Tu strongly suggests that P43 is a human mitochondrial elongation factor. This hypothesis is supported by the finding that the functionally important amino acids of EF-Tu, as revealed in a recent study of the crystal structure [19], are conserved in P43 (Fig. 4).

EFTu becomes activated when it exchanges GDP for GTP resulting in a conformational change in the protein and the formation of a cleft between two domains of the protein which has been proposed as the binding site for amino acyl-tRNA [19]. The alignment of the amino acid sequences of P43 with bacterial and mitochondrial elongation factors shows that the amino acids which bind GTP and also undergo a conformational change in response to GTP binding are spatially conserved within these proteins (functionally conserved sequences boxed in Fig. 4). Once the ternary complex of EF-Tu, GTP and the aminoacyl-tRNA are bound to the A site of the ribosome, GTP hydrolysis occurs leading to release of the EF-Tu GDP from the protein synthesis machinery. It is thought that binding of EFTu to the ribosome triggers hydrolysis of GTP thereby promoting the release of EFTu-GDP from the ribosome so that it can participate in another exchange reaction. All of the amino acids implicated in the intrinsic and effector mediated mechanism of GTP hydrolysis are also conserved in P43 (see Fig. 4 and legend).

As the activity of some elongation factors is regulated by phosphorylation and dephosphorylation we scanned the P43 amino acid sequence for potential consensus phosphorylation sites using the Motifs program of the Genetics Computer Group Software package. This analysis revealed two potential protein kinase C (PKC) phosphorylation sites at amino acid positions 345 and 423 and four putative caesin kinase II (CKII) phosphorylation sites at amino acid positions 304, 360, 423, and 443. The PKC site at position 345 and the CKII phosphorylation sites at amino acid positions 304 and 360 are spatially conserved within the yeast and *E. coli* EF-Tu protein sequences (Fig. 4).

3.4. Mitochondrial location of P43

As the predicted amino acid sequence of P43 showed highest homology to elongation factors of mitochondrial and bacterial

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                                                                -91 GGCACGAGCCGGAAGT  -76

-75 GCCTTCCAGCCGCACGCCCTTCCTCTTCTGTGAGCTCGGGCTCCTGGTCCCAAGTGGGGAATTACCGGCGCGAGT   -1

  1 ATGACCACAATGGCGGCCGCCACCCTGCTGCGCGCGACGCCCCACTTCAGCGGTCTCGCCGCCGGCCGGACCTTC   75
  1 MetThrThrMetAlaAlaAlaThrLeuLeuArgAlaThrProHisPheSerGlyLeuAlaAlaGlyArgThrPhe   25

 76 CTGCTGCAGGGTCTGTTGCGGCTGCTGAAAGCCCCGGCATTGCCTCTCTTGTGCCGCGGCCTGGCCGTGGAGGCC  150
 26 LeuLeuGlnGlyLeuLeuArgLeuLeuLysAlaProAlaLeuProLeuLeuCysArgGlyLeuAlaValGluAla   50

151 AAGAAGACTTACGTGCGCGACAAGCCACATGTGAATGTGGGTACCATTGGCCATGTGGACCACGGGAAGACCACG  225
 51 LysLysThrTyrValArgAspLysProHisValAsnValGlyThrIleGlyHisValAspHisGlyLysThrThr   75
                      peptide A
226 CTGACTGCAGCCATCACGAAGATTCTAGCTGAGGGAGGTGGGGCTAAGTTCAAGAAGTACGAGGAGATTGACAAT  300
 76 LeuThrAlaAlaIleThrLysIleLeuAlaGluGlyGlyGlyAlaLysPheLysLysTyrGluGluIleAspAsn  100

301 GCCCCGGAGGAGCGAGCTCGGGGTATCACCATCAATGCGGCTCATGTGGAGTATAGCACTGCCGCCCGCCACTAC  375
101 AlaProGluGluArgAlaArgGlyIleThrIleAsnAlaAlaHisValGluTyrSerThrAlaAlaArgHisTyr  125

376 GCCCACACAGACTGCCCGGGTCATGCAGATTATGTTAAGAATATGATCACAGGCACTGCACCCCTCGACGGCTGC  450
126 AlaHisThrAspCysProGlyHisAlaAspTyrValLysAsnMetIleThrGlyThrAlaProLeuAspGlyCys  150

451 ATCCTGGTGGTAGCAGCCAATGACGGCCCCATGCCCCAGACCCGAGAGCACTTATTACTGGCCAGACAGATTGGG  525
151 IleLeuValValAlaAlaAsnAspGlyProMetProGlnThrArgGluHisLeuLeuLeuAlaArgGlnIleGly  175

526 GTGGAGCATGTGGTGGTGTATGTGAACAAGGCTGACGCTGTCCAGGACTCTGAGATGGTGGAACTGGAGATCCGG  600
176 ValGluHisValValValTyrValAsnLysAlaAspAlaValGlnAspSerGluMetValGluLeuGluIleArg  200

601 GAGCTGCTCACCGAGTTTGGCTATAAAGGGGAGGAGACCCCAGTCATCGTAGGCTCTGCTCTCTGTGCCCTTGAG  675
201 GluLeuLeuThrGluPheGlyTyrLysGlyGluGluThrProValIleValGlySerAlaLeuCysAlaLeuGlu  225

676 GGTCGGGACCCTGAGTTAGGCCTGAAGTCTGTGCAGAAGCTACTGGATGCTGTGGACACTTACATCCCAGTGCCC  750
226 GlyArgAspProGluLeuGlyLeuLysSerValGlnLysLeuLeuAspAlaValAspThrTyrIleProValPro  250
                                              peptide B
751 GCCCGGGACCTGGAGAAGCCTTTCCTGCTGCCTGTGGAGGCGGTGTACTCCGTCCCTGGCCGTGGCACCGTGGTG  825
251 AlaArgAspLeuGluLysProPheLeuLeuProValGluAlaValTyrSerValProGlyArgGlyThrValVal  275

826 ACAGGTACACTAGAGCGTGGCATTTTAAAGAAGGGAGACGAGTGTGAGCTCCTAGGACATAGCAAGAACATCCGC  900
276 ThrGlyThrLeuGluArgGlyIleLeuLysLysGlyAspGluCysGluLeuLeuGlyHisSerLysAsnIleArg  300

901 ACTGTGGTGACAGGCATTGAGATGTTCCACAAGAGCCTGGAGAGGGCCGAGGCCGGAGATAACCTCGGGGCCCTG  975
301 ThrValValThrGlyIleGluMetPheHisLysSerLeuGluArgAlaGluAlaGlyAspAsnLeuGlyAlaLeu  325

976 GTCCGAGGCTTGAAGCGGGAGGACTTGCGGCGGGGCCTGGTCATGGTCAAGCCAGGTTCCATCAAGCCCCACCAG 1050
326 ValArgGlyLeuLysArgGluAspLeuArgArgGlyLeuValMetValLysProGlySerIleLysProHisGln  350

1051 AAGGTGGAGGCCCAGGTTTACATCCTCAGCAAGGAGGAAGGTGGCCGCCACAAGCCCTTTGTGTCCCACTTCATG 1125
 326 LysValGluAlaGlnValTyrIleLeuSerLysGluGluGlyGlyArgHisLysProPheValSerHisPheMet  375
               peptide C
1126 CCTGTCATGTTCTCCCTGACTTGGGACATGGCCTGTCGGATTATCCTGCCCCCAGAGAAGGAGCTTGCCATGCCC 1200
 376 ProValMetPheSerLeuThrTrpAspMetAlaCysArgIleIleLeuProProGluLysGluLeuAlaMetPro  400

1201 GGGGAGGACCTGAAGTTCAACCTAATCTTGCGGCAGCCAATGATCTTAGAGAAAGGCCAGCGTTTCACCCTGCGA 1275
 401 GlyGluAspLeuLysPheAsnLeuIleLeuArgGlnProMetIleLeuGluLysGlyGlnArgPheThrLeuArg  425

1276 GATGGCAACCGGACTATTGGCACCGGTCTAGTCACCAACACGCTGGCCATGACTGAGGAGGAGAAGAATATCAAA 1350
 426 AspGlyAsnArgThrIleGlyThrGlyLeuValThrAsnThrLeuAlaMetThrGluGluGluLysAsnIleLys  452

1351 TGGGGTTGAGTGTGCAGATCTCTGCTCAGCTTTCCTTGCGTTTAAGGCCTGCCCTAGCCAGGGCTCCCTCCTGCT 1425
 451 TrpGly 452

1426 TCCAGTACCCTCTCATGGCATAGGCTGCAACCCAGCAGAGGGCAGCTAGATGGACATTTCCCCTGCTCGGAAGGG 1500

1501 TTGGCCTGCCTGGCTGGGGAGGTCAGTAAACTTTGAATAGTAAGCCAAAAAAA 1553
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Fig. 3. Nucleotide sequence and predicted amino acid sequence of the P43 gene product. The first four methionine residues are underlined and the predicted amino acid sequence of P43 is numbered from first of the two possible ATG translation initiation codons. The coding sequence of 1356 nucleotides would encode for a protein of 452 amino acids with a predicted molecular weight of approximately 49.5 kDa. The three tryptic peptides of P43 which had been sequenced are underlined. The N terminal sequences derived from the overlapping cDNA clone pBS43.7 start at position −91 and end at position +15.

origin we investigated whether P43 was mitochondrially located by cellular fractionation and immunoblotting. The results of immunoblotting with proteins recovered at different stages during the isolation of mitochondria from intact HeLa cells is shown in Fig. 1B. As expected P43 was detected in the pellet of unlysed cells and nuclei that were recovered after gentle lysis by Dounce homogenisation. P43 could be completely recovered from the low speed supernatant by centrifugation at 10,000 × *g* for 20 min indicating that the antigen is most probably associated with the pellet of mitochondria. The distribution of Hsp 60, a protein associated with the inner and outer membrane of mitochondria, among the cellular fractions of HeLa cells was similar to P43, providing further evidence for the mitochondrial localisation of P43 (Fig. 1C).

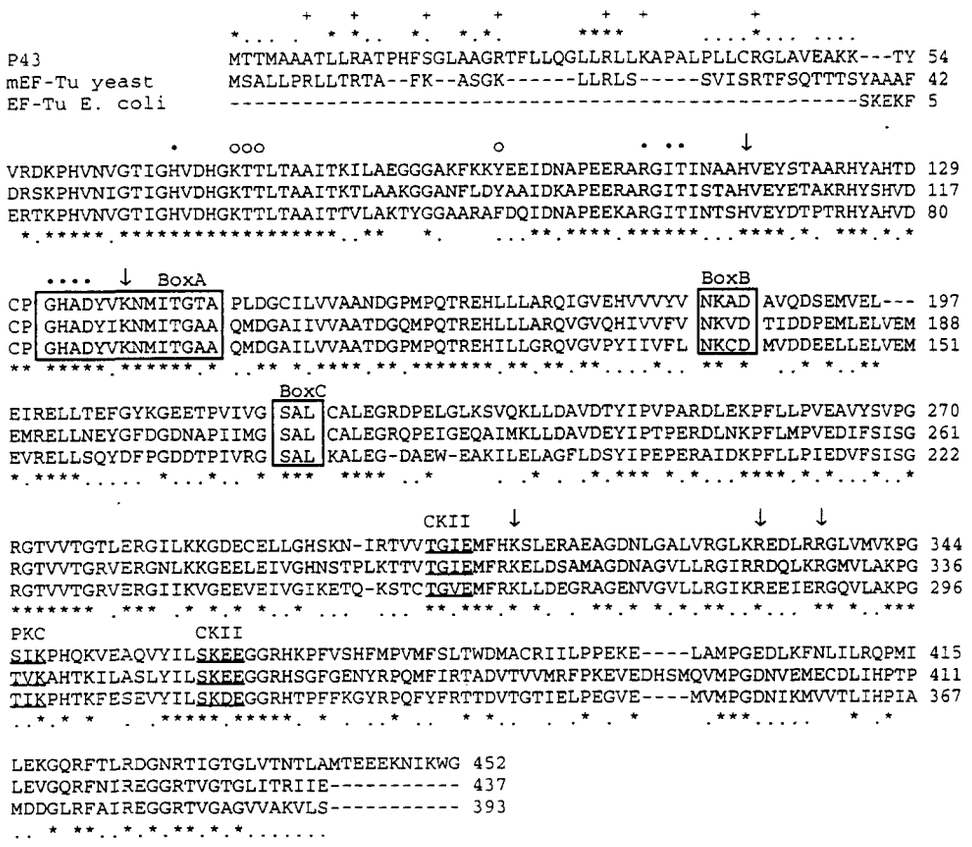

Fig. 4. Comparison of amino acid sequences in P43, the *S. cerevisae* mitochondrial elongation factor (mEF-Tu) and the EF-Tu of *E. coli*. The alignment was optimised by computer analysis. *, identical amino acids in all three proteins; •, amino acids with similar biochemical properties. Box A: sequences which mediate the conformational change in response to nucleotide exchange. Boxes B and C: conserved sequences involved in guanine ring binding. ○, amino acids involved in nucleotide binding; ●, amino acids involved in GTPase mechanism; ↓, charged residues which may be involved in binding of the amino acyl-tRNA. For the N-terminal signal sequences only amino acid residues with a positive charge are indicated by +. Potential phosphorylation sites for PCK and CKII which are conserved in all three proteins are underlined.

3.5. Southern and Northern blot analysis

A Southern blot of *Hin*dIII digested DNAs from three different hepatoma cell lines was hybridised with a ^{32}P-labelled *Kpn*I fragment of the p43 cDNA. Two DNA fragments of approximately 18 kb and 4.0 kb in size were detected in each track (Fig. 5A).

A Northern blot of mRNA from the hepatoma cell line PLC/PRF/5, a normal liver cell line CCL13 and the HeLa tumour cell line was hybridised with the same probe as that used for Southern blot hybridization. Fig. 5b shows that a single transcript of approximately 2.2 kb in size was detected in all three cell lines. Based on the length of the cDNA and the predicted start site for the P43 ORF, the mRNA transcript would therefore contain approximately 650 nt of 5′ non-coding sequence. The results show that the steady state levels of $mRNA_{P43}$ were approximately the same in both tumour (HeLa and PLC/PRF/5) and normal (CCL13) cells lines. This was not due to differences in the amounts of mRNA loaded as approximately the same levels of a β-actin control transcript were detected in each cell line (not shown). However, these tumour cell lines have previously been shown to express substantially higher amounts of P43 antigen than the normal liver cell line by Western blotting [1]. The reasons for the differences in mRNA and protein levels are not known but warrant further investigation as they may indicate differences in the protein stability.

3.6. Chromosomal mapping of the p43 gene by in situ hybridization

Metaphase spreads of chromosomes from a normal male lymphoblastoid cell line were hybridised to a biotinylated probe prepared from pBS43.5 and detected by fluorescence in-situ hybridization using avidin FITC. A signal was reproducibly detected on chromosome 16 between p11.2 and 12 and also near the centromere on the long arm of chromosome 17 indicating that similar sequences must be present at two loci (Fig. 6). While a signal was detected on both chromatids of metaphase chromosome 16 this was not the case for chromosome 17. However, this might be due to the constriction of the chromosome at the centromeric region.

4. Discussion

The cDNA for p43 was isolated by the screening of a human liver cDNA library with oligonucleotide probes based on the sequences of tryptic peptides of affinity purified P43. Analysis of the nucleotide sequences of two overlapping clones revealed one long ORF of 1356 bp, a 197 bp 3′-untranslated region plus

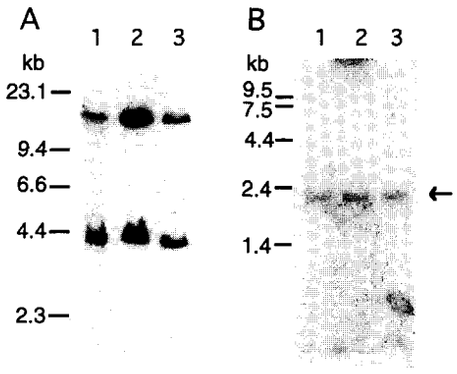

Fig. 5. Southern and Northern hybridisation analysis. (A) Southern blot of *Hin*dIII digested genomic DNA from three cultured hepatoma cell lines as follows; lane 1, primary hepatoma CB8017; lane 2, Huh7; lane 3, PLC/PRF/5 (Alexander cells). (B) Northern blot analysis of poly(A)$^+$ RNA isolated from cultured cell lines as follows; lane 1, PLC/PRF/5 (Alexander cells); lane 2, normal liver line CCL13; lane 3, HeLa. Hybridisation to a control probe (β actin) showed that approximately equal amounts of mRNA were loaded in each track (not shown).

poly(A)$^+$ tail and a 5′-untranslated region. This is compatible with the size of the mRNA transcript (2.2 kb) detected in Northern blots. Evidence that the cDNA we have isolated encodes the antigen detected by the MAb68 is provided by the fact that the peptide sequences derived from the antibody affinity purified protein are encoded within the ORF predicted for P43. The product of the cDNA when produced in *E. coli* as a fusion protein to *lacZ* specifically reacts with MAb68. Additionally, we have shown by immunoblotting that an increased level of P43 is detected in liver cells transfected with a plasmid expressing the cDNA we have isolated and characterised.

Fluorescence in situ hybridisation with the cDNA encoding P43 was used to localize the gene to chromosome 16 between p11.2 and 12 and also to a position near the centromere on the long arm of chromosome 17. Future studies are aimed at establishing whether both loci encode proteins with similar properties and functions or whether a pseudogene is located at one of the loci.

The deduced amino acid sequence of P43 is remarkably similar to that of *E. coli* EF-Tu and the mitochondrial EF-Tu of *S. cerevisiae*. Furthermore, we have shown that the amino acid sequences proposed to be important for nucleotide binding and the mechanisms of intrinsic and effector-enhanced GTPase activity of EF-Tu from *Thermus thermophilus* are completely conserved in P43. Several potential sites for phosphorylation by PCK and CKII were found in P43, some which were also conserved in the yeast mEF-Tu and the EF-Tu of *E. coli*. As these sites occur commonly in proteins and are not necessarily phosphorylated their significance would need to be further investigated, distributed.

The N-terminal sequences of P43 share features in common with the N-terminal sequences of *S. cerevisiae* mEF-Tu and other nuclear-encoded mitochondrial proteins which become processed during transport into the mitochondria. The processing of P43 in this way would account for the discrepancy between the predicted molecular weight of 49.5 kDa and the 43 kDa sized protein detected on Western blots. A protein corresponding to the predicted size of the unprocessed precursor is faintly detected in Western blots of protein extracts from cells transfected with a cDNA expression plasmid (Fig. 2). This may have arisen as a consequence of the over-expression of P43 in transfected cells leading to incomplete processing of the mitochondrial targeting signal. Further evidence for the mitochondrial localisation of P43 comes from the finding that the mitochondria associated proteins Hsp 60 and P43 are similarly distributed among the subcellular fractions of HeLa cells.

Previous studies showed that the expression of P43 was only readily detected in tumour cell lines and not normal liver tissues or cell lines by immunoblotting [1]. However, in contrast to these previous results we have found that P43 is in fact expressed in normal tissues but at markedly different levels. This may be due to the fact that the enhanced chemiluminescence detection system used here is more sensitive and highlights a need to re-examine the levels of P43 in normal and transformed cells of different tissue origin.

The finding that P43 is most likely a human mitochondrial elongation factor and that its expression is elevated in transformed cells led us to investigate the possible tissue specific differences in the levels of expression of P43. Relatively high levels of P43 were detected in mouse heart, brain, liver, and kidney whereas expression was substantially lower in the spleen, skeletal muscle, lung and pancreas. The relatively high

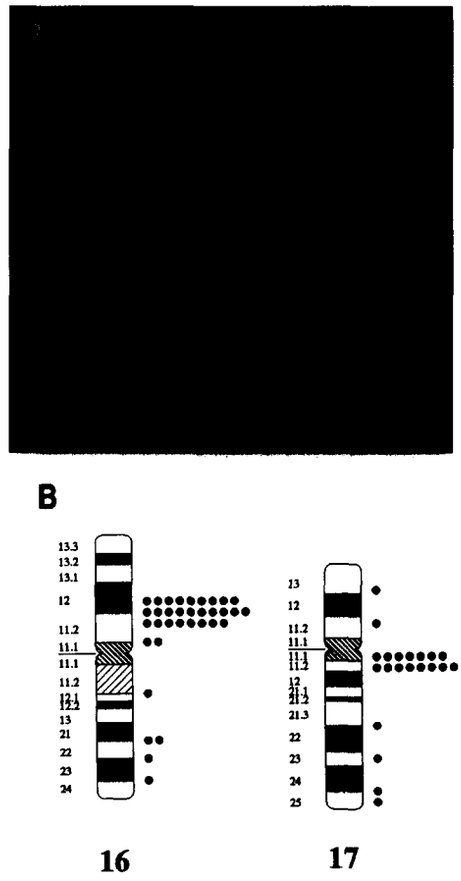

Fig. 6. (A) Fluorescence in situ hybridisation of P43 probe to metaphase chromosomes prepared from a normal male lymphoblastoid cell line. Localisation to chromosome 16 and 17 is indicated. (B) Frequency distribution of hybridisation signals detected on chromosomes 16 and 17.

level of P43 expression in the heart, brain and kidney would correlate with its potential role as a mitochondrial elongation factor as these tissues have intrinsically high levels of oxidative metabolism. Interestingly, a related protein with higher molecular weight (approximately 50 kDa) was abundantly expressed in the lung and in amounts which were higher than those observed with other tissues. Although this protein might simply be a tissue specific protein which cross-reacts with the antibody it warrants further investigation as it might be a related protein with a different function or a modified form of P43 found only in the lung. In this respect it is interesting to note that eukaryotic protein elongation factors are increasingly being shown to be involved in functions other than protein synthesis, such as organisation of the mitotic apparatus [20], developmental regulation [21,22], aging [23], signal transduction [24] and transformation [25].

The increased expression of the human cytoplasmic elongation factor EF-1a in adenocarcinomas has also been recently reported [26]. Moreover constitutive expression of EF-1a increases the susceptibility of several fibroblast cell lines to transformation [25]. The expression of other eukaryotic proteins involved in protein translation such as eIF-4E and eIF-2α has also been shown to increase in response to growth induction by the cellular oncogene c-*myc* [27]. An increase in steady state levels of mitochondrial transcripts and nuclear genes encoding mitochondrial proteins is also found in hepatoma cells [28]. Taken together the results suggest that components of the protein translation machinery may play an important role in the regulation of cell growth.

Mitochondria are thought to have arose by the invasion of aerobic or anaerobic photosynthetic bacteria into ancestral protokaryotic cells [29] and several components of the mitochondrial translation machinery, characterised so far in yeast, share similarities with their prokaryotic counterparts. Despite these similarities previous attempts to clone mammalian mitochondrial translation initiation and elongation factors by approaches based on their expected homology have been unsuccessful. The only mitochondrial elongation factor to be cloned previously (the rat mtEF-G) was identified because of its fortuitous homology to a domain of the growth hormone receptor [30]. The cloning of the first human mitochondrial elongation factor should prove useful for investigating protein synthesis in normal mammalian mitochondria as well as its role in diseases attributed to mitochondrial dysfunction. We are currently attempting to investigate the possible effects of increased P43 expression on cell growth, mitochondrial physiology, and transformation.

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