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Apis mellifera cytoplasmic elongation factor 1α (EF-1α) is closely related to *Drosophila melanogaster* EF-1α

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Using low stringency hybridisation with a *Drosophila melanogaster* EF-1α gene fragment we have isolated a genomic DNA clone encoding elongation factor 1α (EF-1α) from *Apis mellifera*. The hybridising *Apis mellifera* sequence could be delineated to two small *Eco*RI fragments that were also revealed by genomic Southern hybridisation. By comparison with the corresponding *Drosophila melanogaster* data the complete translational reading frame has been deduced. It is interrupted by two intervening sequences of 220 and about 790 nucleotides. Comparison with known eucaryotic EF-1α sequences further confirms that certain amino acid sequences seem to be invariable within the EF-1α protein family.

Protein synthesis; Elongation factor; Translation; Genomic sequence; *Apis mellifera*; *Drosophila melanogaster*

1. INTRODUCTION

Translation of genetic information into protein is a highly conserved process. This holds true specifically for the chain of interacting events that take place at the ribosome. It is, therefore, not surprising that many of the factors that take part in translation have allowed very little variation of their amino acid sequence during evolution. The elongation of the amino acid chain involves a series of protein components that are known as elongation factor 1 and elongation factor 2 complexes [1]. Elongation factor 1 consists of the three proteins EF-1α, -β and -γ. EF-1α catalyses the transport of aminoacyl-tRNA to the 80 S ribosome. A prerequisite for EF-1α activity is its activation by GTP, a process that is thought to be catalysed by the EF-1β,γ complex. A growing number of elongation factor 1α sequences of different origin is now available due to the possibility of screening by crosshybridisation between species. We have recently described two independent genes of *Drosophila melanogaster* that encode two different elongation factors 1α [2]. They follow an individual pattern of expression during development and were named EF-1α,F1 and EF-1α,F2.

Here, we describe the cloning and sequence analysis of an EF-1α gene from *Apis mellifera*. Because of the evolutionary distance of 250 million years between *Drosophila* and *Apis* the analysed sequence allows an interesting comparison between distantly related insects. Moreover, it completes the knowledge about those amino acid sequences that stay invariable which is indicative of their structural and functional importance. The determined sequence revealed a translational reading frame that is closely related to *both Drosophila* genes, EF-1α,F1 and EF-1α,F2.

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The nucleotide sequences presented here have been submitted to the EMBL/GenBank database under the accession numbers: X52884 and X52885

2. MATERIALS AND METHODS

2.1. *General methods*

Genomic *Apis mellifera* DNA was prepared as described by Walldorf et al. [3] and Blin and Stafford [4]. Restriction endonuclease digestions, gel electrophoresis of DNA fragments, library screening, isolation of phage λ, restriction site mapping and subcloning in plasmid DNA vectors was performed according to Maniatis et al. [5] and references therein.

2.2. *Library construction*

Genomic *Apis mellifera* DNA was partially digested with the restriction enzyme *Sau*3A. After size selection using NaCl density gradient centrifugation DNA of 15–20 kb in length was cloned in the *Bam*HI site of the EMBL4 phage λ vector [6].

2.3. *Screening and hybridisation conditions*

1.5×10^5 phages corresponding to 5 genomic equivalents were screened under low stringency hybridisation conditions at 37°C. The hybridisation buffer consisted of 43% deionized formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM Na-citrate), 4 × Denhardts solution [7], 0.1% SDS, 0.1% Na-pyrophosphate, 20 μg/ml tRNA and 50 μg/ml heparin. The filters were washed twice for 30 min each at 50°C in 2 × SSC.

2.4. *DNA sequence determination*

Phage M13 subcloned restriction fragments were sequenced using the chain termination method of Sanger et al. [8].

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3. RESULTS AND DISCUSSION

Intending to clone an EF-1α gene from *Apis* we first performed genomic Southern hybridisations to obtain information whether a crosshybridisation approach with *Drosophila* EF-1α sequences is feasible. We used a 0.6 kb *Bam*HI-*Pst*I fragment of the *Drosophila* EF-1α,F1 gene that covers the coding sequence from amino acid 52 to 251 and, thus, contains a portion of the most conserved part of EF-1α [2]. Fig. 1 shows a Southern blot of *Eco*RI-digested *Apis* DNA in parallel with a control lane of *Eco*RI-digested *Drosophila* DNA. Since the *Drosophila* *Bam*HI-*Pst*I probe contains an internal *Eco*RI site, two fragments appear in the *Drosophila* DNA lane after hybridisation. Two positive signals also appear in the *Apis* DNA. We interpret this result as an indication that at least one, possibly two genes are present in the *Apis* genome that seem to be closely related to the *Drosophila* gene because of the strong hybridisation signal. The additionally observed faint band in the high molecular weight range might eventually result from weaker crosshybridisation with sequences that also contain the highly conserved GTP-binding domain which is present in the EF-1α probe and other translation factors [14]. However, we cannot completely rule out the possibility that this signal indicates an additional elongation factor 1α of *Apis* which shows only little sequence conservation.

In order to isolate an EF-1α gene of *Apis* we screened a library of cloned genomic DNA fragments with the same *Drosophila* probe as used for Southern hybridisation. From several positive signals one phage, bef-1, was plaque purified, the DNA was isolated and mapped (Fig. 2). The recombinant contained neighbouring *Eco*RI fragments of 1.0 kb and 1.1 kb, the same size that had been observed in the genomic Southern analysis (Fig. 1), favoring the conclusion that a single copy of the *Drosophila* EF-1α homologue is present in *Apis*. These two fragments were subsequently cloned in pUC18, mapped and smaller subfragments were then cloned in phage M13 and used for sequence determination according to Sanger et al. [8]. The DNA sequence between the *Cla*I and *Hin*dIII sites (see expanded map region in Fig. 2) revealed 77% sequence conservation when compared to the EF-1α,F1 *Drosophila* reading frame. The determined sequence is shown in Fig. 3. Surprisingly, a nucleotide sequence conservation of 77% is also revealed when compared to the second *Drosophila* elongation factor gene, EF-1α,F2 that by itself shares 78% homology with the EF-1α,F1 gene. Because of this high degree of sequence conservation we were able to assign the translation start and stop signal within the *Apis* sequence to nucleotide 365 and 2121, respectively. The interruption of the reading frame by two introns, like in the *Drosophila* EF-1α,F2 gene, is also corroborated from sequence comparison.

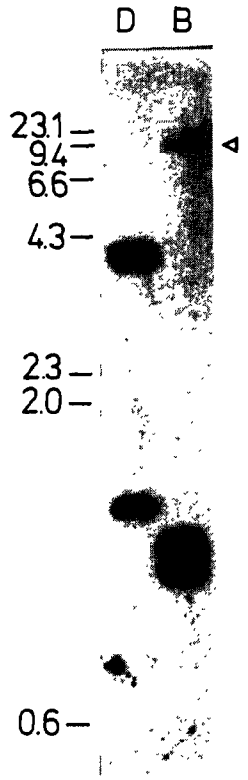

Fig. 1. Genomic Southern hybridisation with a *Drosophila melanogaster* EF-1α gene fragment as probe. (Left lane) *Drosophila melanogaster* strain CantonS DNA (D). (Right lane) *Apis mellifera* DNA (B), both digested with *Eco*RI. The size marker is deduced from *Hin*dIII digested phage λ DNA that has been run in parallel. The open triangle indicates the position of weak hybridisation.

Within the *Apis* intron sequence – intron two has only partly been sequenced – no reasonable homology with the *Drosophila* genes is found. We conclude from sequence comparison with the *Drosophila* genes and several other EF-1α sequences [9–17] (Fig. 4) that the identified *Apis* reading frame comprises a complete EF-1α protein sequence. It consists of 461 amino acids and has a calculated molecular mass of 50.5 kDa. Since our interest was mainly focussed on the amino acid sequence of EF-1α we made no further attempts to obtain additional nucleic acid sequence information.

We observe 49 and 44 amino acid differences between the *Apis* elongation factor and *Drosophila* EF-1α,F1 and EF-1α,F2, respectively. Nineteen of the exchanges between *Apis* and either one of the *Drosophila* sequences occur at identical positions. On the other hand, 19 *Drosophila* EF-1α,F1 sequence changes are present at positions where EF-1α,F2 and *Apis* are unchanged. Conversely, 14 times the EF-1α,F2 amino acid sequence changed leaving EF-1α,F1 and *Apis* identical. In 11 positions all 3 sequences are different (Fig. 4). In summary, about the same number of changes happened comparing each of the amino acid or nucleotide sequences with the two remaining ones.

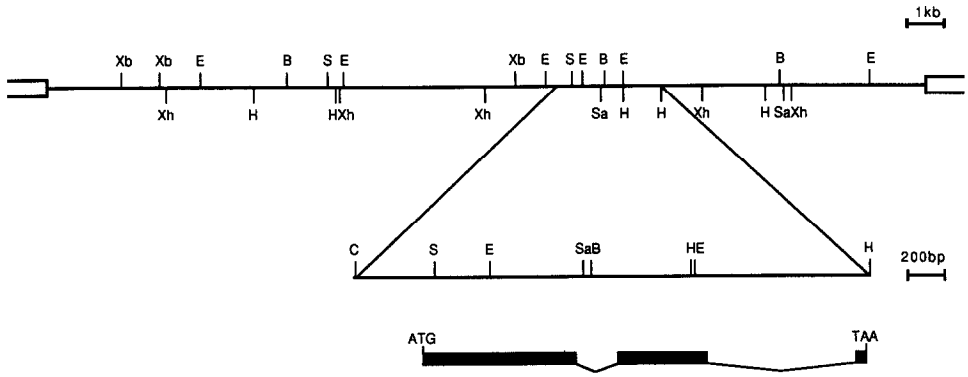

Fig. 2. Structure of the *Apis mellifera* EF-1α gene locus as contained in phage bef-1. Restriction enzyme abbreviations are: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sa, *Sac*I; Xb, *Xba*I; Xh, *Xho*I. The EF-1α gene region is expanded 5-fold between the *Cla*I and the *Hin*dIII site. The additional *Cla*I sites are not shown. The putative gene structure of the region that gives rise to the amino acid reading frame as revealed by sequence comparison with the *Drosophila melanogaster* EF-1α genes is depicted. Filled boxes confine the open reading frame, lines represent intron sequences.

Two introns interrupt the reading frame in the genomic *Apis* sequence as well as in the *Drosophila* EF-1α,F2 sequence. However, only the position of the first intron is identical in *Apis* and *Drosophila* EF-1α,F2. In contrast, the EF-1α,F1 coding sequence is free of introns. Taken together, these observations lead us to conclude that *Drosophila* EF-1α,F2 and the *Apis* gene evolved from a common ancestor. The addition of the second intron at different positions in the *Apis* and the *Drosophila* EF-1α gene may then be the result of a more recent insertion event.

Whether *Apis* also contains a second *cytoplasmic* elongation factor 1α gene copy is still an open question. We consider this as less likely because of the insignificance of the hybridization to the high molecular weight fragment (Fig. 1). However, *nuclear* encoded mitochondrial elongation factors that are more related to the procaryotic gene type exhibit a low degree of sequence conservation to cytoplasmic EF-1α genes and this might result in a weak crosshybridisation signal [13].

Our aim was to obtain additional information on the

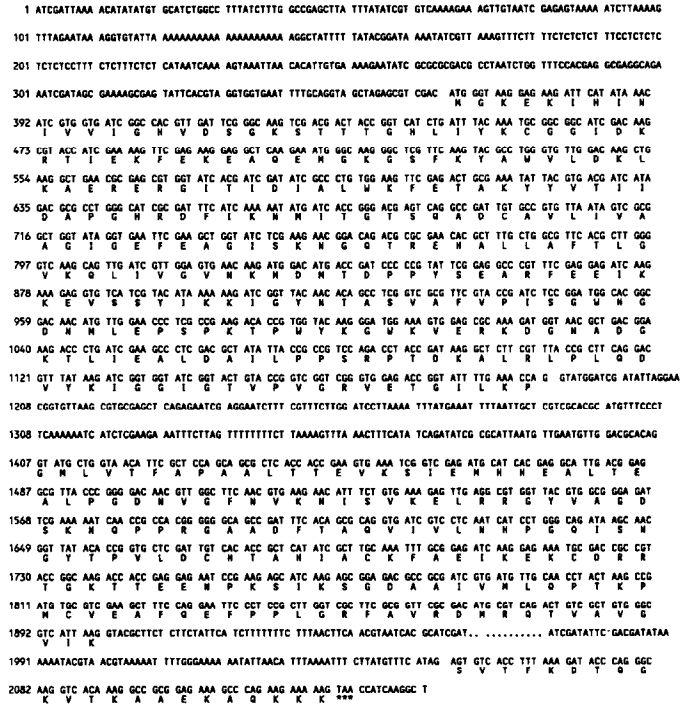

Fig. 3. Sequence of the *Apis mellifera* EF-1α gene. The amino acid reading frame as delineated by the putative translation start and stop codons is denoted by asterisks. Within the reading frame area intron sequences appear above the gaps of the deduced amino acid sequence. About 700 nucleotides of intron two indicated by a number of dots at position 2048 were not determined.

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          10        20        30        40        50        60        70        80        90       100
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  1 MGKEKSHINVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYQVTVIDAPGHRDFIK yeast I
  1 MGKEKSHINVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYQVTVIDAPGHRDFIK yeast II
  1 MGKEKTHVNVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEEFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYNVTVIDAPGHRDFIK Mucor racemosus I
  1 MGKEKTHVNVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYNVTVIDAPGHRDFIK Mucor racemosus II
  1 MGKEKTHVNVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYNVTVIDAPGHRDFIK Mucor racemosus III
  1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGSIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGITIDIALWKFETAKYYVTIIDAPGHRDFIK Artemia salina
  1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGITIDIALWKFETAKYYVTIIDAPGHRDFIK D. melanogaster F1
  1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGITIDIALWKFETSKYYVTIIDAPGHRDFIK D. melanogaster F2
  1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGITIDIALWKFETAKYYVTIIDAPGHRDFIK Apis mellifera
  1 MGKEKTHIKIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVLDKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIK Xenopus laevis
  1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVLDKLKAERERGITIDISQRKFETSKYYVTIIESPGHRDFIK mouse
  1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVLDKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIK human
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101 NMITGTSQADCAILIIAGGVGEFEAGISKDGQTREHALLAFTLGVRQLIVAVNKMDSVK--WDESRFQEIVKETSNFIKKVGYNPKTVPFVPISGWNGDN yeast I
101 NMITGTSQADCAILIIAGGVGEFEAGISKDGQTREHALLAFTLGVRQLIVAVNKMDSVK--WDESRFQEIVKETSNFIKKVGYNPKTVPFVPISGWNGDN yeast II
101 NMITGTSQADCAILIIAGGTGEFEAGISKDGQTREHALLAFTLGFRQLIVAINKMDTTK--WSQDRYNEIVKEVSGFIKKIGFNPKSVPFVPISGWHGDN Mucor racemosus I
101 NMITGTSQADCAILIIAGGTGEFEAGISKDGQTREHALLAFTLGFRQLIVAINKMDTTK--WSQDRYNEIVKEVSGFIKKIGFNPKSVPFVPISGWHGDN Mucor racemosus II
101 NMITGTSQADCAILIIAGGTGEFEAGISKDGQTREHALLAFTLGVRQLIVAINKMDTTK--WSQDRYNEIVKEVSGFIKKIGFNPKSVPFVPISGWHGDN Mucor racemosus III
101 NMITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLVVKQLIVGVNKMDSTEPPFSEARFEEIKKEVSAYIKKIDYNPAAVAFVPISGWHGDN Artemia salina
101 NMITGTSQADCAVQIDAAGTGEFEAGISKNDQTREHALLAFTLGVKQLIVGVNKMDSSEPPYSEARYEEIKKEVSSYIKKVGYNPAAVAFVPISGWHGDN D. melanogaster F1
101 NMITGTSQADCAVLIDAAGTGEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDSTEPPYSEARYEEIKKEVSSYIKKIGYNPASVAFVPISGWHGDN D. melanogaster F2
101 NMITGTSQADCAVLIVAAGIGEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDMTDPPYSEARFEEIKKEVSSYIKKIGYNTASVAFVPISGWHGDN Apis mellifera
101 NMITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLGVKQLIVGINKMDSTEPPYSQKRYEEIVKEVSTYIKKIGYNPDTVAFVPISGWNGDN Xenopus laevis
101 NMITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPYSQKRYEEIVKEVSTYIKKIGYNPDTVAFVPISGWNGDN mouse
101 NMITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPYSQKRYEEIVKEVSTYIKKIGYNPDTVAFVPISGWNGDN human
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199 MIEATTNAPWYKGWEKETKAGVVKGKTLLEAIDAIEQPSRPTDKPLRWPLQDVYKIGGIGTVPVGRVETGVIKPGMVVTFAPAGVTTEVKSVEMHHEQLE yeast I
199 MIEATTNAPWYKGWEKETKAGVVKGKTLLEAIDAIEQPSRPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVIKPGMVVTFAPAGVTTEVKSVEMHHEQLE yeast II
199 MLDESTNMPWFKGWNKETKAGSKTGKTLLEAIDAIEPPVRPSDKPLRLPLQDVYKIGGIGTVPVGRVETGTIKAGMVVNFAPAAVTTEVKSVEMHHETLT Mucor racemosus I
199 MLDESTNMPWFKGWNKETKAGSKTGKTLLEAIDAIEPPVRPSDKPLRLPLQDVYKIGGIGTVPVGRVETGTIKAGMVVNFAPAAVTTEVKSVEMHHETLT Mucor racemosus II
199 MLDESTNMPWFKGWNKETKAGSKTGKTLLEAIDAIEPPVRPSDKPLRLPLQDVYKIGGIGTVPVGRVETGTIKAGMVVNFAPAAVTTEVKSVEMHHETLT Mucor racemosus III
201 MLEASDRLPWYKGWNIERKEGKADGKTLLDALDAILPPSRPTEKPLRLPLQDVYKIGGIGTVPVGRVETGIIKPGMIVTFAPANITTEVKSVEMHHESLE Artemia salina
201 MLEPSTNMPWFKGWEVGRKEGNADGKTLVDALDAILPPARPTDKALRLPLQDVYKIGGIGTVPVGRVETGVLKPGTVVVFAPANITTEVKSVEMHHEALQ D. melanogaster F1
201 MLEPSEKMPWFKGWSVERKEGKAEGKCLIDALDAILPPQRPTDKPLRLPLQDVYKIGGIGTVPVGRVETGLLKPGMVVNFAPVNLVTEVKSVEMHHEALT D. melanogaster F2
201 MLEPSPKTPWYKGWKVERKDGNADGKTLIEALDAILPPSRPTDKALRLPLQDVYKIGGIGTVPVGRVETGILKPGMLVTFAPAALTTEVKSVEMHHEALT Apis mellifera
201 MLEPSPNMPWFKGWKITRKEGSGSGTTLLEALDCILPPSRPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVIKPGMVVTFAPVNVTTEVKSVEMHHEALT Xenopus laevis
201 MLEPSANMPWFKGWKVTRKDGSAVAPTLLEALDCILPP-RPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPVNVTTEVKSVEMHHEALS mouse
201 MLEPSANMPWFKGWKVTRKDGNASGTTLLEALDCILPPTRPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPVNVTTEVKSVEMHHEALS human
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299 QGVPGDNVGFNVKNVSVKEIRRGNVCGDAKNDPPKGCASFNATVIVLNHPGQISAGYSPVLDCHTAHIACRFDELLEKNDRRSGKKLEDHPKFLKSGDAA yeast I
299 QGVPGDNVGFNVKNVSVKEIRRGNVCGDAKNDPPKGCASFNATVIVLNHPGQISAGYSPVLDCHTAHIACRFDELLEKNDRRSGKKLEDHPKFLKSGDAA yeast II
299 EGLPGDNVGFNVKNVSVKDIRRGNVCSDSKNDPAKESASFTAQVIILNHPGQISAGYAPVLDCHTAHIACKFSELIEKIDRRSGKKMEDSPKFVKSGDSA Mucor racemosus I
299 EGLPGDNVGFNVKNVSVKDIRRGNVCSDSKNDPAKESASFTAQVIILNHPGQISAGYAPVLDCHTAHIACKFSELIEKIDRRSGKKMEDSPKFVKSGDSA Mucor racemosus II
299 EGLPGDNVGFNVKNVSVKDIRRGNVCSDSKNDPAKESASFTAQVIILNHPGQISAGYAPVLDCHTAHIACKFSELIEKIDRRSGKKMEDSPKFVKSGDSA Mucor racemosus III
301 QASPGDNVGFNVKNVSVKELRRGYVASDSKNNPARGSQDFFAQVIVLNHPGQISNGYTPVLDCHTAHIACKFAEIKEKCDRRTGKTTEAEPKFIKSGDAA Artemia salina
301 EAVPGDNVGFNVKNVSVKELRRGYVAGDSKANPPKGAADFTAQVIVLNHPGQIANGYTPVLDCHTAHIACKFAEILEKVDRRSGKTTEENPKFIKSGDAA D. melanogaster F1
301 EAMPGDNVGFNVKNVSVKELRRGYVAGDSKNNPPRGAADFTAQVIVLNHPGQIANGYTPVLDCHTAHIACKFSEIKEKYDRRTGGTTEDGPKAIKSGDAA D. melanogaster F2
300 EALPGDNVGFNVKNISVKELRRGYVAGDSKNQPPRGAADFTAQVIVLNHPGQISNGYTPVLDCHTAHIACKFAEIKEKCDRRTGKTTEENPKSIKSGDAA Apis mellifera
301 EAVPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAGSFTAQVIILNHPGQIGAGYAPVLDCHTAHIACKFAELKEKIDRRSGKKLEDNPKFLKSGDAA Xenopus laevis
300 EALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPVLDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSGDAA mouse
301 EALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPVLDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSGDAA human
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399 LVKFVPSKPMCVEAFSEYPPLGRFAVRDMRQTVAVGVIKSVDKTEK-AAKVTKAAQKAAK-K*                                  yeast I
399 LVKFVPSKPMCVEAFSEYPPLGRFAVRDMRQTVAVGVIKSVDKTEK-AAKVTKAAQKAAK-K*                                  yeast II
399 IVKMVPSKPMCVEAYTDYPPLGRFAVRDMRQTVAVGVIKAVEKVDK-AGKVTKAAAKASK-K*                                  Mucor racemosus I
399 IVKMVPSKPMCVEAYTDYPPLGRFAVRDMRQTVAVGVIKAVEKVDK-AGKVTKAAAKASK-K*                                  Mucor racemosus II
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399 IVKMVPSKPMCVEAYTDYPPLGRFAVRDMRQTVAVGVIKAVEKVDK-AGKVTKAAAKASK-K*   Mucor racemosus III
401 MITLVPSKPLCVEAFSDFPPLGRFAVRDMRQTVAVGVIKSVNFKDPTAGKVTKAAEKAGK-KK*  Artemia salina
401 IVNLVPSKPLCVEAFQEFPPLGRFAVRDMRQTVAVGVIKAVNFKDASGGKVTKAAEKATKGKK*  D. melanogaster F1
401 IIVLVPSKPLCVESFQEFPPLGRFAVRDMRQTVAVGVIKSVNFKETTSGKVTKAAEKAQK-KK*  D. melanogaster F2
397 IVMLQPTKPMCVEAFQEFPPLGRFAVRDMRQTVAVGVIKSVTFKDT-QGKVTKAAEKAQK-KK*  Apis mellifera
401 IVDMIPGKPMCVESFSDYPPLGRFAVRDMRQTVAVGVIKAVEKKAAGSGKVTKSAQKAAKTK*   Xenopus leavis
400 IVDMVPGKPMCVESFSDYPPLGRFAVRDMRQTVAVGVIKAVDKKAAGAGKVTKSAQKAQKAK*   mouse
401 IVDMVPGKPMCVESFSDYPPLGRFAVRDMRQTVAVGVIKAVDKKAAGAGKVTKSAQKAQKAK*   human
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Fig. 4. Comparison of the amino acid sequences as deduced from published eucaryotic EF-1α gene sequences [9–17].

degree and position of sequence conservation in EF-1α. Since our previous comparison [2] a complete mouse and *Xenopus laevis* sequence have been published [16,17]. With the *Apis mellifera* sequence now determined by us we can compare sequences derived from a wide spectrum of the evolutionary tree that ranges from yeasts to mammals. This new comparison further reveals an extreme sequence conservation. Even by including 12 elongation factor sequences of 8 different species in our comparisons, the *Apis mellifera* sequence differences are, with two exceptions in amino acid 186 and 315, restricted to positions that had already found to be different between the as yet examined species.

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