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Elongation factor 1α genes of the red alga *Porphyra purpurea* include a novel, developmentally specialized variant

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Abstract

The life cycle of the red alga *Porphyra purpurea* alternates between two morphologically distinct phases: a shell-boring, filamentous sporophyte and a free-living, foliose gametophyte. From a subtracted cDNA library enriched for sporophyte-specific sequences, we isolated a cDNA encoding an unusual elongation factor 1α (EF-1α) that is expressed only in the sporophyte. A second EF-1α gene that is expressed equally in the sporophyte and the gametophyte was isolated from a genomic library. These are the only EF-1α genes detectable in *P. purpurea*. The constitutively expressed gene encodes an EF-1α very similar to those of most eukaryotes. However, the sporophyte-specific EF-1α is one of the most divergent yet described, with nine insertions or deletions ranging in size from 1 to 26 amino acids. This is the first report of a developmental stage-specific EF-1α outside of the animal kingdom and suggests a fundamental role for EF-1α in the developmental process.

Introduction

Elongation factor 1α (EF-1α) is a well-studied housekeeping protein found in all eukaryotic cells, where it plays a central role in protein synthesis. It is part of the elongation factor 1 complex, which also includes EF-1β and EF-1γ. EF-1α facilitates the GTP-dependent binding of aminoacyl-tRNA to the A site of the ribosome during the elongation phase of translation, while EF-1β/γ promotes the exchange of GDP for GTP on EF-1α [25]. In this process, EF-1α must bind to GTP/GDP, aminoacyl-tRNAs, the 80S ribosome and the EF-1β/γ complex. In addition, EF-1α appears to be a major component and regulator of the cytoskeleton [4]. Due to these multiple interactions, it

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U08841 (EF-1α*tef-s*) and U08844 (EF-1α*tef-c*).

is not surprising that most of the EF-1αs characterized to date are highly conserved in their amino acid sequences.

EF-1α is essential for the production of all cellular proteins and at least one active copy of the EF-1α-encoding gene (*tef*) is necessary for cell viability [5]. During certain developmental stages, EF-1α may be extremely abundant; it comprises 5% of the total protein in wheat germ extracts [3]. Many organisms contain multiple copies of *tef* and even organisms with markedly reduced genomes such as *Arabidopsis thaliana* [18] or *Saccharomyces cerevisae* [5] contain four and two active *tef* genes, respectively. In almost all cases, these gene families encode nearly identical proteins. Little is known about the developmental pattern of expression of these genes.

Diverse EF-1αs have so far only been found in *Drosophila* and *Xenopus*. In both cases, the encoding genes exhibit tissue or developmental stage-specific patterns of expression. *Drosophila* has two EF-1αs with very different patterns of expression; F1 is expressed throughout the life cycle, while F2 is mainly expressed only in the pupal stage [10, 32]. Three EF-1αs have been characterized from *Xenopus laevis*. The somatic elongation factor, EF-1αS, is expressed at high levels in somatic tissues while the closely related EF-1αO is expressed only in the oocyte. The third EF-1α, 42Sp50, is highly divergent in sequence, forms part of the RNA storage particle and is also expressed only in the oocyte [7]. A major function of the 42Sp50 protein appears to be the storage of tRNAs for later use in oogenesis and early embryogenesis. However, purified 42Sp50 can function as an EF-1α, but is unique in that it exchanges GDP for GTP without the assistance of EF-1β/γ and binds to uncharged tRNA more tightly than charged tRNA [31]. This suggests that 42Sp50 is unlikely to promote efficient protein synthesis. Rats and humans also encode a developmentally specific, but strongly conserved EF-1α, statin, that is expressed only in terminally differentiated tissues such as heart, brain and muscle [14, 17].

The red alga *Porphyra purpurea* has a life cycle that alternates between filamentous, diploid sporophytes and foliose, haploid gametophytes. As an initial approach to understanding developmental regulation of gene expression in *P. purpurea*, we constructed two subtracted cDNA libraries and isolated several cDNAs representing genes that are expressed in a phase-specific manner [19]. Among the cDNAs for genes that are only expressed in the sporophyte was one that encodes an EF-1α. Following this identification, a constitutively expressed *tef* gene was also isolated. Here, we describe the isolation and characterization of these two *tef* genes from *P. purpurea*. We present evidence that these are the only members of the *P. purpurea tef* gene family and that one is expressed in both phases while the other is specific to the sporophyte. We also report the phylogenetic relationship of these two EF-1αs to those from other eukaryotes.

Materials and methods

Genomic DNA manipulation and analysis

P. purpurea sporophytic cultures grown in modified D-11 medium [21] were used for DNA isolation. Nuclear DNA was extracted and purified according to Rice and Bird [24], except that the proteinase K treatment was omitted. For Southern hybridization, 12 μg of nuclear DNA was digested with 120 units of restriction enzyme (*Bam*HI, *Kpn*I, *Pst*I or *Sst*I) at 37 °C overnight. The restricted DNA samples were electrophoresed on an 0.6% agarose gel and transferred onto Zeta Probe GT nylon membranes (BioRad). Blotted membranes were hybridized to probe DNAs and washed as previously described [19], except that the final wash was in 2 × SSC, 0.1% SDS, at 68 °C.

Bacteriophage clones harboring *tef* genomic sequences were obtained by screening a *P. purpurea* genomic library kindly provided by Dr R. MacKay. Plaque lifts were prepared according to standard procedures [27]. Membranes were hybridized and washed under the same conditions as the genomic Southern hybridizations. Recombinant bacteriophage DNA was prepared

from positive clones by the method of Sambrook *et al.* [27]. The insert DNA was mapped by Southern hybridization using the ECL kit (Amersham). The DNA fragment containing *tef* was subcloned into the plasmid vector pUC18 and both strands were sequenced from double-stranded plasmid template using an ABI 373 sequencer.

cDNA characterization

Poly(A)$^+$ RNA extraction, subtracted cDNA library construction and screening, northern hybridization and DNA sequencing were performed as described previously [19]. The missing 5′ end of the cDNA was obtained by PCR from single-stranded cDNA extracted from the unsubtracted sporophyte cDNA library [19], which presumably contains longer cDNA inserts. The 5′-end PCR primer is complementary to the polylinker sequence adjacent to the *Eco*RI site on the universal sequencing primer side of pBluescript II SK$^-$ and the 3′-end primer is a gene-specific sequence 120 bp from the 5′ end of the originally isolated cDNA. The PCR product of expected size was purified from an agarose gel by Geneclean (BIO 101), cloned into a TA cloning vector (Invitrogen) and sequenced as described previously [19]. Protein secondary structure prediction was carried out with the PHD program [26].

Phylogenetic analysis

Sequences were aligned on a SUN workstation using the GCG program Pileup with default gap penalties [6]. Minor modifications were made by eye to minimize insertion/deletion events. For phylogenetic analyses, regions not alignable with confidence among all taxa were deleted. Deleted regions include the amino-terminus, two small internal regions, the carboxyl-terminus, and all insertions unique to the *Porphyra* sequences. These correspond to positions 1–4, 117–129, 157–169, 188–218, 238–241, 307–309, 409–435 and 479–524 of the *Porphyra* alignment (Fig. 1). All parsimony analyses were performed on amino acid sequences using the program PAUP 3.1.1 [28]. Shortest tree searches utilized 50 replicates of random addition with branch-swapping by tree-bisection-reconnection. Bootstrap analyses consisted of 500 replicates of random addition. Distance analyses were done with the PHYLIP 3.5c program PROTDIST [8] using the Dayhoff amino acid substitution matrix, and trees were constructed by neighor joining.

Results

Isolation and characterization of a sporophyte-specific EF-1α cDNA

In an effort to isolate developmental phase-specific genes from *P. purpurea*, we constructed two phase-specific subtracted cDNA libraries [19]. Differential screening of a portion of each library using labelled first-strand cDNAs revealed many positive colonies, a small number of which were randomly selected for characterization. Among these cDNAs was one from the sporophyte-specific library whose deduced amino acid sequence showed strong similarity to EF-1α. As this 1.2 kb cDNA sequence contained only the C-terminal half of an EF-1α, we used PCR to isolate the 5′ portion of the gene from the unsubtracted sporophyte cDNA library. The combined sequence from these two clones is 1882 bp in length and contains a single open reading frame (ORF) encoding an entire EF-1α of 515 amino acids (Fig. 1). This ORF is preceded by 53 bp of 5′ leader sequence and followed by 281 bp of 3′-untranslated region without a poly(A) tail at its extreme 3′ end.

When northern blots containing poly(A)$^+$ RNA from both the sporophyte and the gametophyte were hybridized with a 3′ region-specific probe from this cDNA, a single 2.1 kb transcript was detected only in the sporophyte RNA (Fig. 2A). This sporophyte-specific EF-1α has been designated *Porphyra*-sporophyte (P-s), while its gene is referred to as *tef-s*.

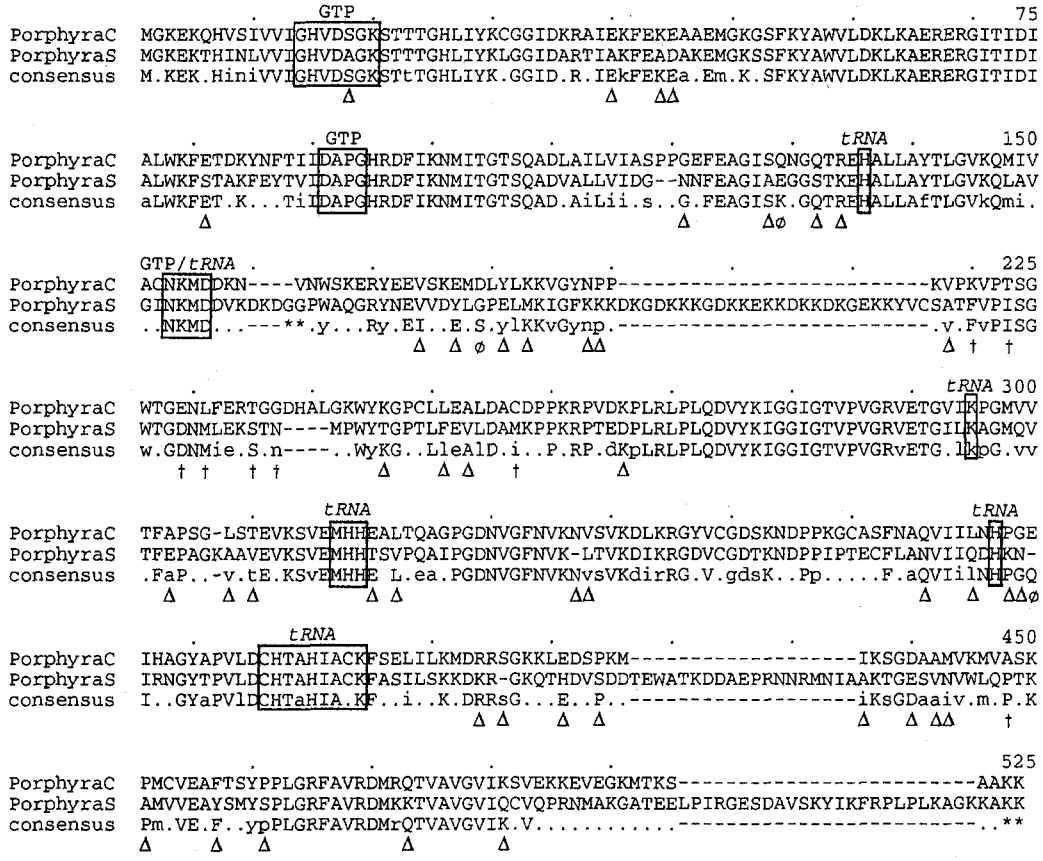

Fig. 1. Alignment of *Porphyra* EF-1αs with a eukaryotic EF-1α consensus sequence. The complete P-s and P-c deduced amino acid sequences are shown, aligned with a consensus sequence based on all known eukaryotic EF-1αs. Amino acids involved in GTP and tRNA binding are boxed. Dashes indicate gaps and pairs of asterisks indicate variable length regions. In the consensus sequence, upper case letters indicate strongly conserved amino acids while conservative positions are indicated in lower case letters, corresponding to the most common amino acid found at that position. Positions where either P-s (Δ), P-c (†) or both *Porphyra* EF-1αs (ϕ) differ from the consensus are indicated below the alignment.

Isolation and characterization of a second constitutively expressed EF-1α

Since *tef-s* is apparently only expressed in the sporophyte and since EF-1α is essential for translation, it was expected that there must be at least one more *tef* that is expressed in either the gametophyte or in both phases. Using the *tef-s* cDNA as a probe to screen an unamplified *P. purpurea* genomic library, four positive clones were obtained from ca. 200000 plaques. The cloned fragments were mapped, subcloned and sequenced. All four genomic clones contained a single *tef* gene whose sequence differed from *tef-s*. This second *tef* sequence encodes a single, uninterrupted ORF of 449 amino acids (Fig. 1). No genomic clones carrying *tef-s* were isolated during this screen.

To test the expression of this new *tef*, northern hybridization was performed under high stringency using the 3′-coding region of this sequence as a gene-specific probe. The 1.5 kb transcript of this new *tef* is present at equal levels in both the sporophyte and the gametophyte (Fig. 2B) indicating that it is constitutively expressed. We designate this gene as *tef-c* and its encoded EF-1α as *Porphyra*-constitutive (P-c). The hybridization signals to sporophyte RNA are of similar inten-

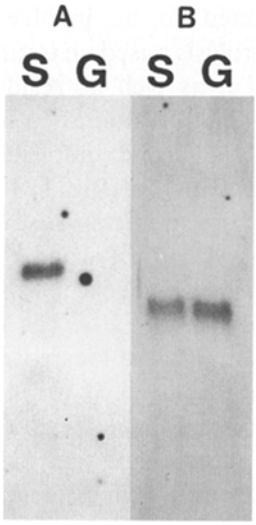

Fig. 2. Both *P. purpurea tef* genes are expressed at similar levels. One μg poly(A)$^+$ RNA from the sporophyte (S lanes) and the gametophyte (G lanes) were electrophoresed on a formaldehyde agarose gel, transferred onto a nylon membrane and hybridized to a ^{32}P-labelled DNA fragment. After washing, both membranes were exposed overnight. *A*. Hybridization with the 3′-specific probe of *tef-s* (from the *Xho*I site to the end of the cDNA including the 3′-untranslated region, Fig. 3A). *B*. Hybridization with the 3′-specific probe of *tef-c* (from the *Kpn*I site to the *Sst*I site, Fig. 3A).

sity for both *tef-c* and *tef-s* suggesting that these genes are expressed at similar levels in the sporophyte.

Estimation of gene copy number of tef *in* P. purpurea

The number of copies of *tef* in the *P. purpurea* genome was determined by separate Southern hybridizations with the 5′ and 3′ coding regions of the *tef-c* gene (Fig. 3). The 5′ half of *tef-c* produced two hybridization signals in the *Bam*HI, *Pst*I and *Sst*I digests (Fig. 3B). Based on similar hybridizations with 5′ and 3′ fragments of *tef-s*, sequencing data and the restriction mapping results from the genomic clones (data not shown), one fragment in each digestion can be assigned to *tef-c* (15 kb *Bam*HI, 6 kb *Pst*I and 8 kb *Sst*I fragments), while the other signal in each lane is produced by *tef-s* (1.9 kb *Bam*HI, 1.5 kb *Pst*I and 3.9 kb *Sst*I fragments). The 3′ *tef-c* probe (Fig. 3A), which overlaps the 5′ *tef-c* probe, hy-

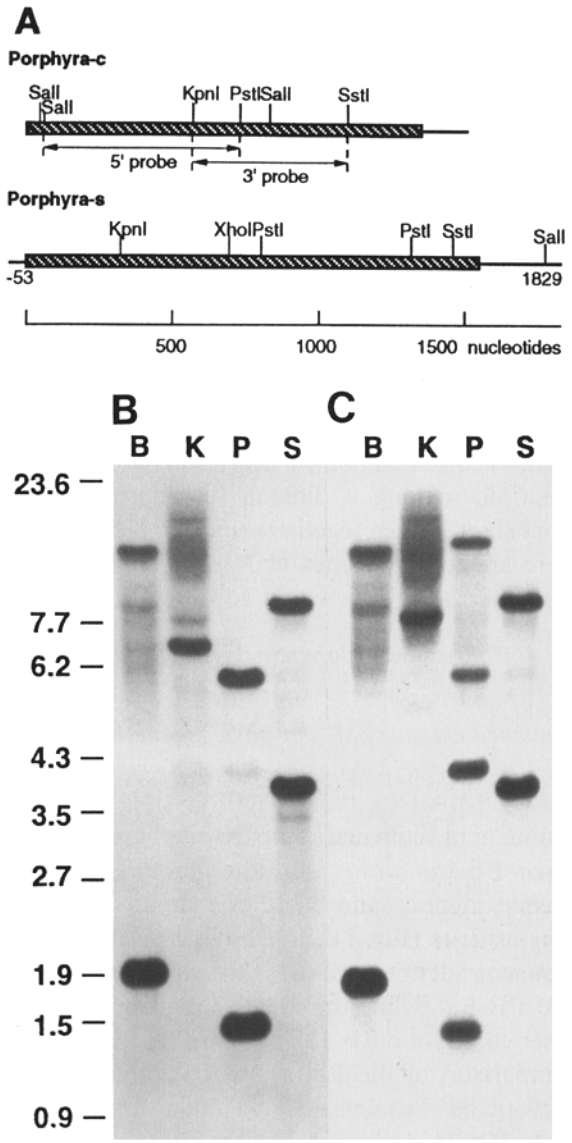

Fig. 3. P. purpurea encodes only two *tef* genes. *A*. Physical maps of *tef-c* genomic DNA and *tef-s* cDNA. Positions of relevant restriction enzyme digestion sites are those predicted from the nucleotide sequencing data. Hatched boxes represent coding regions. DNA fragments used as probes for Southern hybridization are indicated by arrows. *B*, *C*. Hybridizations with 5′ (*B*) and 3′ (*C*) coding regions of *tef-c* respectively. B, *Bam*HI; K, *Kpn*I; P, *Pst*I; S, *Sst*I. Size markers are in kilobases.

bridizes to the same *Bam*HI, *Pst*I and *Sst*I fragments as the 5′ probe and to two additional *Pst*I fragments (Fig. 3C). The two additional *Pst*I fragments result from a *Pst*I site in the 3′ region of both genes. According to the sequence data, there is a second *Pst*I site in the 3′ end of *tef-s*. However, a 513 bp *Pst*I fragment was not detected in the 3′ probe hybridization, suggesting that one of the two *tef-s Pst*I sites might be protected by DNA methylation. The hybridization signals obtained with *Kpn*I digests also appear to the consistent with those or the other enzymes except that some of the hybridizing fragments are large and either partially digested, or possibly degraded during isolation, making it difficult to determine their exact sizes. Taken together, our data indicate that there are two *tef* genes in *P. purpurea* and each gene is present as a single copy per haploid genome.

Sequence comparison and phylogenetic analysis of EF-1αs

Amino acid sequence comparison of the two *Porphyra* EF-1αs with each other reveals 63% sequence identity and 17% conservative residue substitutions (Fig. 1). P-s shows a relatively low sequence identity (60–67%) with other eukaryotic EF-1αs, while the identity between P-c and other eukaryotic EF-1αs is 70–78%. In addition, comparison of the P-s sequence with other eukaryotic EF-1αs revealed five unique insertions of 1, 4, 19, 25 and 26 amino acids (aa) (Fig. 1). Relative to other EF-1αs, P-s also has four one or two residue deletions. Among other eukaryotic EF-1αs single residue insertions/deletions are very rare and only one insertion longer than three aa has been reported [1]. The amino acid composition of the 26 aa insertion is also striking in that it contains 50% lysine residues. P-c also contains a unique insertion of 4 aa, which does not correspond to any of the P-s insertions.

Secondary structure predictions of the amino acid sequences of both *Porphyra* EF-1αs reveal high conservation of α-helix and β-sheet elements relative to the bacterial homolog, EF-Tu [2, 13]. Elements predicted to be involved in GTP-binding and hydrolysis based on tertiary structure studies of EF-Tu, as well as residues demonstrated by cross-linking studies to interact with the aminoacyl tRNA, are all completely conserved in both *Porphyra* EF-1αs (Fig. 1) [12, 20, 33]. Secondary structure predictions also show that all of the large insertions in P-s and P-c fall within pre-existing loops, with the exception of the large carboxy terminal insertion P-s. The 4 aa and the 26 aa insertions in P-s are located in the GTP-binding domain while the 18 aa insertion falls within a large loop in domain III. Both the 26 and 18 aa insertions apparently extend loops that protrude from the surface of the protein and may lie in close proximity to each other. The fourth P-s insertion, the 25 aa insertion near the carboxy-terminal end of EF-1α, is predicted to form an additional α-helix. The single insertion in P-c is also located within a loop of the GTP binding domain.

The relationship between the two *Porphyra* EF-1αs and other known EF-1α and EF-1α like proteins was investigated by phylogenetic analysis. All trees derived by either parsimony (Fig. 4) or distance methods (data not shown) show that both *Porphyra* proteins are of eukaryotic origin, although only very distantly related to each other (Fig. 4). All methods used place P-c in a similar position, close to plants, animals, fungi and the slime mold, *Dictyostelium*, although the exact placement of P-c among these taxa is ambiguous. Parsimony analysis places P-c as a separate branch immediately above green plants, while distance analyses place P-c directly on the same branch with green plants (data not shown).

In contrast, P-s falls much more deeply in the tree, although its position is again not clearly resolved. Although parsimony analysis puts P-s together with the EF-1α from the apicomplexan *Plasmodium* (Fig. 4), distance analysis places P-s as the second deepest branch among eukaryotes after *Giardia* EF-1α (data not shown). The association of P-s with *Plasmodium* is probably artefactual as the inclusion of additional archaebacterial sequences in parsimony analyses can move P-s to the same position as in distance trees (data

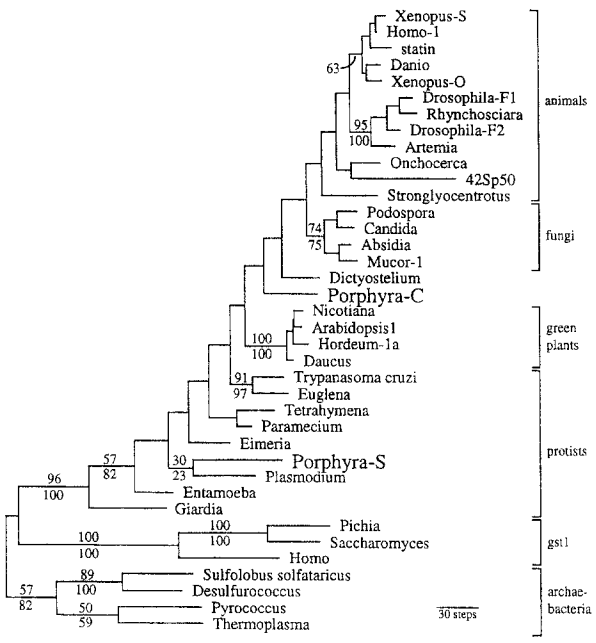

Fig. 4. The relationship among *Porphyra* and other eukaryotic EF-1α as predicted by phylogenetic analysis. The phylogenetic tree shown is one of four equal-length trees derived by parsimony analysis of amino acid sequences. Branches are drawn proportional to the number of inferred amino acid substitutions as indicated by the scale bar. Bootstrap values above 50% are indicated for major groups only. Numbers above the lines are from parsimony analysis, below the lines for distance analysis. This tree has a length of 1088 steps, a consistency index, excluding uninformative characters, of 0.540, and a retention index of 0.566. The other trees found at this length differ from the tree shown in placing *Stronglyocentrotus* alone as the single deepest branch among the animals and in placing *Tetrahymena* as separate, deeper branch to *Paramecium*.

not shown). This effect, together with the low overall similarity of P-s to other EF-1αs, suggests that P-s may not necessarily be an ancient protein, but rather a rapidly evolving sequence, i.e., a long branch that is artefactually attracted to other long branches in the tree such as *Plasmodium*, *Giardia* and the archaebacteria. Such 'long-branch effects' are particularly problematic for phylogenetic analysis [29]. It should be noted that P-s shows no affinity for any of the specialized EF-1αs currently known, such as 42Sp50 or the EF-1α-like, *gst1*-encoded protein required to G1-to-S phase transition [11].

Discussion

In this paper, we describe the isolation and characterization of two *P. purpurea tef* genes that are strikingly different from each other with respect to sequence and expression pattern. The *tef-c* gene encodes a typical eukaryotic EF-1α that is expressed in both the sporophyte and the gametophyte, while *tef-s* encodes an EF-1α that has an unusual primary structure and is expressed only in the sporophyte. Although developmental stage-specific *tef* genes have been demonstrated in *Drosophila*, *Xenopus* and mammals [7, 10, 32], this is the first example of a stage-specific *tef* outside of the animal kingdom. Our results show that developmental specialization of EF-1α is more widespread than previously thought, and that it has arisen independently at least twice during the evolution of multicellular eukaryotes.

Both *Porphyra tef* genes potentially encode functional EF-1αs. P-c is highly conserved in both primary sequence and predicted secondary structure. This, together with its high level of phase-independent expression, indicate that *tef-c* encodes a typical, eukaryotic, 'housekeeping' EF-1α responsible for the bulk of protein synthesis. Although P-s is very divergent in terms of primary structure, including an unprecedented number of insertions and deleteions, none of the amino acid substitutions are predicted to interfere with function. In addition, all four large insertions fall within pre-existing loops or at the carboxy-terminus, and secondary structure predictions suggest that P-s can fold properly. Therefore, *tef-s* also potentially encodes a functional EF-1α.

Nonetheless, the unusual sequence and structure of P-s, together with its restricted pattern of expression, suggest that *tef-s* may not encode an entirely typical EF-1α. In this respect it is important to note that a large body of evidence has now accumulated for multifunctionality of EF-1α. Besides its major role in the elongation step of protein synthesis, EF-1α has been shown to be a major component of the cytoskeleton as well as a possible regulator of its assembly in diverse eukaryotes ranging from ciliates [15] to man [4]. EF-1α also appears to be a major component of

the centrosome [16, 22], and is involved in ubiquitin-dependent degradation of proteins in animals [9].

One possible explanation for the high level of divergence found in P-s is that it is only performing a subset of the normal EF-1α functions. For instance, P-s may be specialized for high levels of protein synthesis. This would be consistent with the fact that P-s has retained all amino acid residues known to be required for this function. Since EF-1α appears to have other major cellular roles, an EF-1α involved only in making protein might no longer be constrained at amino acid positions required for other, non-protein-synthetic functions. Conversely, P-s could be specialized for one of the other EF-1α roles that might be required uniquely in the sporophyte. Some, if not all, of these roles also require GTP-hydrolysis, and some may also require tRNA binding, as is the case with 42Sp50. Thus, the necessary amino acid positions might still be conserved as is the case in P-s (Fig. 1). The large insertions in P-s may also be related to a specialized function. For instance, the highly positively charged 26 aa insertion that is predicted to protrude from the surface of the protein may allow novel interactions with negatively charged cellular components.

Although our phylogenetic analyses show an ancient origin for P-s, the low sequence similarity of the protein to all other known EF-1αs suggests that P-s has undergone accelerated evolution, a situation known to interfere with accurate phylogenetic analysis [29]. This is especially problematic when there is a lack of sequence information from closely related taxa. One striking example of this phenomenon is the 42Sp50 protein of *Xenopus*, a highly divergent, developmentally restricted EF-1α-like protein [31]. Early analyses placed 42Sp50 very deeply in the eukaryotic tree, similar to the position of P-s in our tree (Fig. 4). This lead to the suggestion that 42Sp50 might be an ancient protein. However, with the broader taxonomic representation now available, 42Sp50 groups together with other vertebrates, with a very long terminal branch indicating accelerated evolution (Fig. 4). Thus, we predict that, as more protistan sequences become available, P-s will show a longer terminal branch and a closer relationship with P-c, perhaps even supporting an origin from within the red algae.

If P-s is indeed derived from within the red algae, this would mean a relatively recent origin for the *tef-s* gene, followed by a striking acceleration in its evolutionary rate. This is very similar to the situation with 42Sp50 [31]. The fact that a similar phenomenon of highly specialized, developmental-stage-specific EF1-αs has been documented in *Xenopus* and now *P. purpurea* suggests a broad utility for EF1-α in developmental specialization, possibly involving a subset of the full range of EF-1α functions. As residues involved in these functions become identified, it may be possible to determine which roles the specialized EF-1α proteins have been selected for.

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