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Overexpression and Purification of Recombinant eRF1 Proteins of Rabbit and *Tetrahymena thermophila*

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Abstract—The polypeptide release factor (eRF1) gene was cloned from rabbit and its overexpression and purification system was established in parallel with that of the eRF1 gene of *Tetrahymena thermophila* that has been cloned recently in this laboratory. The rabbit eRF1 (Ra-eRF1) is composed of 437 amino acids and is completely identical to human eRF1 though 3% distinct in the nucleotide sequence. This is in sharp contrast to *Tetrahymena* eRF1 (Tt-eRF1) that is only 57% identical to human eRF1. The recombinant Ra-eRF1 was marked with a histidine tag, overexpressed, and purified to homogeneity by two-step chromatography using Ni-NTA-agarose and Mono Q columns. In contrast to Ra-eRF1, Tt-eRF1 formed aggregates upon overexpression in *Escherichia coli*, hence it was purified under denaturing conditions, and used to raise rabbit antibody. The resulting anti-Tt-eRF1 antibody proved useful for examining conditions for soluble Tt-eRF1 in test cells. Finally, a soluble Tt-eRF1 fraction was purified from *Saccharomyces cerevisiae* transformed with the Tt-eRF1 expression plasmid by three steps of affinity and anion exchange chromatography. The cloned Ra-eRF1 gene complemented a temperature-sensitive allele in the eRF1 gene, *sup45* (ts), of *S. cerevisiae*, though the complementation activity was significantly impaired by the histidine tag, whereas Tt-eRF1 failed to complement the *sup45* (ts) allele.

Key words: translation termination, polypeptide release factors, rabbit eRF1, *Tetrahymena* eRF1

Termination of translation during protein biosynthesis takes place on the ribosome in response to a stop, rather than a sense, codon in the decoding site. This process requires two classes of polypeptide release factors (RFs): a class I factor, codon-specific RF (RF1 and RF2 in prokaryotes; eRF1 in eukaryotes), and a class II factor, nonspecific RF (RF3 in prokaryotes; eRF3 in eukaryotes) that binds guanine nucleotides and stimulates class I RF activity [1, 2]. Prokaryotic RF1 and RF2 recognize UAG/UAA and UGA/UAA, respectively [3], and known eukaryotic eRF1s recognize all three stop codons [4].

Although the termination process was first discovered in the late 1960s, much of the mechanism has remained obscure. One of the reasons for relatively slow progresses in the study of translation termination mechanisms was the lack of a complete *in vitro* system that catalyzes initiation, elongation, and termination of protein synthesis equivalent to the *in vivo* process. The classical, but still useful, *in vitro* system of translation termination was to monitor the rate of N-formyl-methionine (fMet) release from the ribosome–fMet–tRNA complex at the stop codon [5, 6]. Obviously, this is not sufficient to investigate the whole mechanism of protein termination or to clarify the interplay between translation termination and the elongation or initiation step of protein synthesis. It was only recently in eukaryotes that a competition against suppressor tRNAs of exogenous eRF1 was investigated in the rabbit reticulocyte lysate system by monitoring terminated and readthrough products in polyacrylamide gel electrophoresis [7]. In prokaryotes the *in vitro* system is more advanced, but it was only recently that the complete and recycling translation system was reconstituted with initiation, elongation, and termination factors and used to analyze termination and recycling steps of protein synthesis *in vitro* [8, 9].

In this study, we cloned the rabbit eRF1 gene to facilitate the *in vitro* translation system composed of rabbit reticulocyte lysate under homologous conditions. One of the assay systems that we are engaged in the construction of is to measure competition between translation termination and frameshifting using the mammalian antizyme frameshift construct of mRNA (unpublished). Cloning and purification of rabbit eRF1, designated Ra-eRF1, is along this line.

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Moreover, once an efficient and sensitive termination assay is available in the complete *in vitro* translation system, it should provide us with a powerful tool to investigate the mechanism of the stop codon reassignment discovered in *Tetrahymena*, where UAA/UAG codons are decoded as glutamine [10-13]. It is not fully understood how UAA/UAG termination is suppressed efficiently by two glutaminyl-tRNAs cognate to UAA and/or UAG in the presence of eRF1 that is generally omnipotent to three stop codons in eukaryotes. Toward this aim, we recently cloned the eRF1 cDNA from *Tetrahymena thermophila* [14]. Here, we developed a comparative study of overexpression and purification of recombinant eRF1 proteins of both rabbit and *Tetrahymena*.

MATERIALS AND METHODS

Strains, plasmids, and media. Strains and plasmids used are listed in Table 1. Yeast cells were grown in YPD or synthetic complete (SC) media [18], and *Escherichia coli* cells were grown in LB medium supplemented with relevant antibiotics for selection (50 μg/ml ampicillin, 15 μg/ml tetracycline, or 50 μg/ml kanamycin) [19].

Cloning of rabbit eRF1 cDNA. The primers used for reverse transcription and polymerase chain reaction (PCR) are listed in Table 2. The hybridization screening of the lambda Zap^R II rabbit cDNA library (Stratagene, USA) for Ra-eRF1 cDNA was carried out using the human eRF1 cDNA probe (a gift from Dr. S. Hoshino) according to standard procedures [19]. The ECL kit

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant description	Source or reference
E. coli:		
BL21(DE3)	F$^-$ *ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen, USA
SOLR™	*e14$^-$ (mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::TN5(kan^r), uvrC, lac, gyrA96, relA1, thi-1, endA1, λ^R [F' proAB, lacI^{q}ZΔM15]Su$^-$* (nonsuppressing)	Stratagene, USA
S. cerevisiae:		
YPH499	a *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*	[15]
MT557/4d	*MATα sal4-2 ura3-1 ade2-1 leu2-1, 112*	[16]
Plasmids:		
pUC118	*E. coli* cloning vector, Ap^r	[17]
pET-30a(+) and pET-30b(+)	*E. coli* expression vectors, Km^r, T7lac promoter	Novagen, USA
pYES2	*S. cerevisiae* expression vector, Ap^r, URA3, promoter P_{GAL1}	Invitrogen, USA
pR-eRF1–25/1	Ra-eRF1 with N-terminal His_6 tag in pET-30-a(+)	this work
pTT-eRF1–38/1	Km^r, Tt-eRF1 with N-terminal His_6 tag under T7lac promoter in pET-30-b(+) vector	this work
pTT-eRF1–39/1	same with pTT-eRF1-38/1 but Tt-eRF1 without His_6 tag	this work
pTT-eRF1–4/12	Ap^r, URA3, Tt-eRF1 with N-terminal His_6 tag under P_{GAL1} promoter in pYES2	this work
pTT-eRF1–1/3	same with pTT-eRF1-4/12 but Tt-eRF1 without His_6 tag	this work
pR-eRF1–25/2	Ap^r, URA3, Ra-eRF1 without His_6 tag under P_{GAL1} promoter in pYES2	this work
pR-eRF1–25/3	Ap^r, URA3, Ra-eRF1 with N-terminal His_6 tag and 43 additional amino acid residues between His tag and N-terminal under P_{GAL1} promoter in pYES2	this work

Table 2. List of oligonucleotides

Oligonucleotide*	5′ to 3′ sequence	Source, purpose, target
5'-RACE Abridged Anchor Primer	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	GibcoBRL, 5′-RACE, poly-dG-dI primer
Primer A23 (+)	AGATCAACGTCAAAGAAATATTGAAC	Tt-eRF1, site-directed mutagenesis, TAA (8**) → CAA
Primer A24 (+)	CCTCCTAAGAAGCAAATTAACGATTCC	Tt-eRF1, site-directed mutagenesis, TAA (43**) → CAA
Primer A25 (+)	GAGTCAACCGTCAATCTGTACAAG	Tt-eRF1, site-directed mutagenesis, TAA (68**) → CAA
Primer A26 (+)	AGTCGCCACTCAAACTTTCATTTCC	Tt-eRF1, site-directed mutagenesis, TAA (210**) → CAA
Primer A27 (+)	GATAAAATTAATGTTCAAGGTTTAGTC	Tt-eRF1, site-directed mutagenesis, TAA (221**) → CAA
Primer A28 (+)	GAGTACTACACAGATGTTCGAC	Tt-eRF1, site-directed mutagenesis, TAG (239**) → CAG
Primer A29 (+)	TTGAACTCGCTCAAGAATCTTTAAC	Tt-eRF1, site-directed mutagenesis, TAA (271**) → CAA
Primer A30 (+)	CAAGTTCGTTCAAGAAAAGAACG	Tt-eRF1, site-directed mutagenesis, TAA (281**) → CAA
Primer A31 (+)	CAAGATACTATGCAACTCTTATTAG	Tt-eRF1, site-directed mutagenesis, TAA (309**) → CAA
Primer A32 (+)	AAGTCACAGAACAAATCACTCATAT	Tt-eRF1, site-directed mutagenesis, TAA (339**) → CAA
Primer A33 (+)	TTGGATCCATATGCACCATCATCATCATCATGAAGA-GAAAGATCAACGTCAAAG	5′-end PCR primer for expression plasmid of His_6-tagged Tt-eRF1, *Bam*HI and *Nde*I sites
Primer A34 (–)	TATAAGCTTATCAAATGAAGCCTTCTTCTTCTTCGTA	3′-end PCR primer for expression plasmid of Tt-eRF1, *Hind*III site
Primer A35 (+)	TATGGATCCATATGGAAGAGAAAGATCAACGTCA	5′-end PCR primer for expression plasmid of tag-free Tt-eRF1, *Bam*HI and *Nde*I sites
Primer A51 (–)	GAGGATCCGGGTACCATTTTTTTTTTTTTTTTT	rabbit 3′-RACE, poly-dT primer, *Bam*HI and *Kpn*I sites
Primer Z1(+)	TTGAATTCATGGCGGACGACCCCAGT	5′-end PCR primer for full-length coding region of Ra-eRF1 cDNA, *Eco*RI site
Primer Z17(+)	ATGAATTCGATTCAACCAAGCTATTGAGTT	rabbit 3′-RACE, *Eco*RI site
Primer Z23(–)	AACTCAATAGCTTGGTTGAA	rabbit 5′-RACE
Primer Z25(–)	GAGCGGCCGCCTAGTAGTCATCAAGGTCAAA	3′-end PCR primer for full-length coding region of Ra-eRF1 cDNA, *Not*I site

* Symbols (+) and (–) mean sense and antisense sequences, respectively.
** TAA and TAG codons in the reading frame of Tt-eRF1; the number refers to the codon position from the initiation codon.

(Amersham, UK) was used for detection of positive clones according to the manufacturer's instructions. For positive clones, *in vivo* excision of the pBluescript phagemid from the lambda Zap^R II vector was done using ExAssist/SOLR system (Stratagene, USA). Hybridization screening was combined with the 5′and 3′ RACE method essentially as described [20] using the 5′-RACE System for Rapid Amplification of cDNA Ends (GibcoBRL, USA). Reverse-transcribed total cDNA was prepared from rabbit muscle mRNA. For 3′RACE, primers Z17 and A51 were used. Two 3′RACE products (1.4 and 1 kb) that differ in 3′-UTR length were cloned into *Eco*RI/*Sma*I sites of pUC118. 5′ RACE products were amplified using 5′-RACE Abridged Anchor Primer and primer Z23. The complete Ra-eRF1 cDNA was amplified by PCR from total cDNA using primers Z1 and Z25. PCR product (1.3 kb) was subcloned into *Eco*RI/*Not*I sites of the pET-30a(+) expression vector, resulting in pR-eRF1-25/1. The Ra-eRF1 cDNA sequence was confirmed by DNA sequencing on both strands using ABI PRISM^{TM} dye terminator cycle sequencing ready reaction kit (Perkin Elmer, USA).

Overexpression of recombinant eRF1s. For Ra-eRF1 expression, *E. coli* BL21 (DE3) strain was transformed with plasmid pR-eRF1-25/1 containing eRF1 cDNA with a histidine (His_6) tag at the N-terminus under T7 promoter. Transformants were grown to OD_{600} = 0.5 and induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h at 37°C. For *Tetrahymena* eRF1 (designated Tt-eRF1) expression in *E. coli* BL21 (DE3), the coding part of Tt-eRF1 cDNA was recloned into pET-30b(+) vector under the T7 promoter, giving rise to two plasmid constructs (pTT-eRF1-38/1 and pTT-eRF1-39/1) with or without the His_6 tag at the N-terminus, respectively. Cells were grown to OD_{600} = 0.5 and induced with 0.1 mM IPTG for 10 h at 25°C. For Tt-eRF1 expression in the budding yeast *Saccharomyces cerevisiae* the coding part of Tt-eRF1 cDNA marked with the N-terminal His_6 tag was recloned into pYES2 vector under the P_{GAL1} promoter, resulting in pTT-eRF1-4/12. *S. cerevisiae* YPH499 cells transformed with pTT-eRF1-4/12 were grown at 25°C in SC medium lacking uracil and supplemented with galactose (for induction of P_{GAL1} promoter).

Purification of recombinant rabbit eRF1. Recombinant Ra-eRF1 was isolated from the soluble fraction of bacterial lysate using Ni-NTA-agarose (Qiagen, Germany) according to the manufacturer's instruction and purified on Mono Q column (Pharmacia, Sweden) by linear gradient 0.15-1.00 M KCl in buffer containing 50 mM Tris-HCl (pH 7.5) and 2 mM DTT. Ra-eRF1 fractions were combined, dialyzed against 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM DTT, and 5% glycerol, and stored at –80°C.

Purification of the recombinant *Tetrahymena* eRF1 under denaturing conditions. Overexpressed Tt-eRF1 formed inclusion bodies in *E. coli* and was isolated under denaturing conditions from *E. coli* BL21(DE3) cells transformed with plasmid pTT-eRF1-39/1. The inclusion bodies were collected by low speed centrifugation after cell lysis, washed with solution containing 2% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl (pH 8.0), and solubilized in 10 mM Tris-HCl (pH 8.0) solution containing 8 M urea. eRF1 was further purified by FPLC anion exchange chromatography using Mono Q column with linear gradient of 0-0.75 M NaCl in buffer containing 10 mM Tris-HCl (pH 8.0) and 8 M urea.

Purification of the recombinant *Tetrahymena* eRF1 under non-denaturing conditions. The native recombinant Tt-eRF1 protein was purified from *S. cerevisiae* YPH499 cells transformed with plasmid pTT-eRF1-4/12. Cells were disrupted by French Press in buffer containing 50 mM NaH_2PO_4 (pH 7.7), 300 mM NaCl, 10 mM $MgCl_2$, 20 mM imidazole, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by low speed centrifugation, and ribosomes were collected by centrifugation at 130,000*g*, and the resulting post-ribosomal supernatant was applied to chromatography in Ni-NTA-agarose (Qiagen) according to the manufacturer's instruction. Fractions containing Tt-eRF1 were subjected to chromatography in AF-Heparin Toyopearl 650 M (Tosoh Co.) with a linear gradient of 0.05-1.0 M NaCl in buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM β-mercaptoethanol. Tt-eRF1 was finally purified by anion exchange chromatography on a Mono Q column by linear gradient of 0-0.8 M NaCl in buffer containing 20 mM Tris-HCl (pH 8.0) and 1 mM DTT. Purified protein was dialyzed against 20 mM Tris-HCl (pH 7.6), 25 mM KCl, and 1 mM DTT.

Other DNA techniques. Site-directed mutagenesis was performed using Altered Sites II *in vitro* Mutagenesis System (Promega, USA) and primers A23 through A32 (see Table 2). The DNA fragments of the coding region of Tt-eRF1 cDNA for expression constructs were amplified by PCR using sets of primers A33 and A34 or A34 and A35 (Table 2). The preparation of rabbit RNA was conducted using QuickPrep (Pharmacia) according to the manufacturer's instruction. Yeast transformation with plasmid DNA was done using the lithium acetate method [21]. Other DNA manipulations were conducted according to standard methods [19].

RESULTS AND DISCUSSION

Cloning of rabbit eRF1. A rabbit cDNA library was screened using a human eRF1 cDNA as probe, and one positive clone was isolated. It contained a 327-bp insert, and the deduced coding sequence was completely identical to human eRF1 sequence [4]. Based on this sequence

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R     1 CCGCCGGGGAGGAGCAGCCGCTGCCGCCCAGGACTGGGCCCTTAGGGAGGAGGAGGCGAGAAGATGGCGGAC   72
H    73 ************************************************************************  144
R    73 GACCCCAGTGCTGCCGACAGGAACGTGGAAATCTGGAAGATCAAGAAGCTCATTAAGAGCTTGGAGGCGGCC  144
H   145 *****************************G******************************************  216
R   145 CGCGGCAATGGCACCAGCATGATATCATTGATCATTCCTCCCAAAGACCAGATTTCCCGAGTGGCAAAAATG  216
H   217 ********************************************************A***************  288
R   217 TTAGCAGATGAATTTGGAACTGCATCCAACATTAAGTCACGAGTAAACCGCCTTTCAGTCCTGGGAGCCATT  288
H   289 *****G*****G**************T*********************************************  360
R   289 ACATCTGTACAACAAAGACTCAAACTTTATAACAAAGTACCTCCAAATGGTCTGGTTGTTTACTGTGGAACA  360
H   361 ***********************************************************A************  432
R   361 ATTGTAACAGAAGAAGGAAAGGAAAAGAAAGTCAACATTGACTTTGAACCTTTCAAACCAATTAATACGTCA  432
H   433 ************************************************************************  504
R   433 TTGTATTTGTGTGACAACAAATTCCATACAGAGGCTCTTACAGCACTACTTTCAGATGATAGCAAGTTTGGC  504
H   505 ***********************************************************************A  576
R   505 TTCATTGTAATAGATGGTAGTGGTGCACTTTTTGGCACACTGCAGGGAAATACAAGAGAAGTCCTGCACAAA  576
H   577 *****************************************C**A*****C*********************  648
R   577 TTCACTGTGGATCTCCCAAAGAAACACGGTAGAGGAGGTCAGTCAGCCTTGCGTTTTGCCCGTTTAAGAATG  648
H   649 ************************************************************************  720
R   649 GAAAAGCGACACAACTATGTTCGGAAAGTAGCAGAGACTGCTGTACAGCTGTTTATTTCTGGGGACAAAGTG  720
H   721 ***********T********************************G***************************  792
R   721 AATGTGGCTGGTCTCGTTTTAGCTGGATCAGCTGACTTTAAAACTGAACTAAGTCAATCTGATATGTTTGAC  792
H   793 **************A**************C*****************************************T  864
R   793 CAGAGGTTGCAATCAAAAGTTTTAAAATTAGTTGATATATCCTATGGCGGTGAAAATGGATTCAACCAAGCT  864
H   865 ********A**************************************T************************  936
R   865 ATTGAGTTATCTACTGAGGTCCTCTCCAACGTGAAATTCATTCAAGAGAAGAAATTAATAGGACGATACTTT  936
H   937 *****************A******************************************************  1008
R   937 GATGAAATCAGTCAAGACACGGGCAAGTACTGTTTTGGAGTTGAAGATACGCTAAAAGCTTTGGAAATGGGA  1008
H  1009 ***********C**G***********************C***********A*****G***************  1080
R  1009 GCCGTAGAAATTCTAATAGTCTATGAAAATTTGGATATAATGAGATACGTTCTTCATTGCCAAGGCACAGAA  1080
H  1081 **T***************************C****************T************************  1152
R  1081 GAGGAGAAAATTCTTTACCTAACTCCAGAACAAGAGAAGGATAAATCTCATTTCACAGACAAAGAGACAGGA  1152
H  1153 **************C**T***********G*****A********************************C***  1224
R  1153 CAGGAACATGAGCTGATTGAGAGCATGCCCCTGTTGGAATGGTTTGCTAACAACTATAAAAAATTTGGAGCT  1224
H  1225 **************T**C******************************************************  1296
R  1225 ACATTGGAAATTGTCACAGATAAGTCACAAGAAGGATCCCAGTTTGTGAAAGGATTTGGTGGAATTGGAGGT  1296
H  1297 **G********************A***********G**T*********************************  1368
R  1297 ATCTTGCGGTACCGAGTAGATTTCCAGGGAATGGAATATCAAGGAGGAGACGATGAATTTTTTGACCTTGAT  1368
H  1369 **************************************C*********************************  1440
R  1369 GACTACTAGGTAGTCGACATGGGTCCGGCAAAACGTGCCTCGCCCTCCAGCATCCAACCCAAGGAGCATACC  1440
H  1441 *****************************************A******************************  1512
R  1441 CGTGGTGGAATCCAAACAGATCCCTGCCTTACAATTGGAACATTTCCAGAACTTAATCCATGAGCATTGGAT  1512
H  1513 *A**********************************************************************  1584
R  1513 ATTGAAAAGAAAACCGAAACAAAACCAGGCCCAACCCTACACTTTGGTTTGTCATGGTGTCAGCGCAGCAGC  1584
H  1585 ****************************A****G**************************************  1656
R  1585 CTACAACTAAGTTCCTAAATGCCACTTTGGACTAATTTAAAAAAGAATCCCAATTTTTACTTTTACTCGATG  1656
H  1657 *******************C********************************G**************G****  1728
R  1657 GTGAAATTGGTTGCTCTTGTATTTTATGAAAAAA--TGATTTTTTTAACCTTCATACATAGAAGCAAAAATA  1726
H  1729 **********************************AA************************************  1800
R  1727 CTTTAACTGCTGTAAACCTTCAAAAGTTAATAGAAGTGAGATCATACTGGTTTGTTTCTTATTTTGATTGGA  1798
H  1801 ************************************************************************  1872
R  1799 GAAAAATTAAATTGCTGCATTTCGCAGTGACCCATTTACATGGCATTCTCAGCTTAGACTGCATAAGAAGAA  1870
H  1873 **************************************************************G*********  1944
R  1871 ATATATGTGGTGAAATGTTGGAACCATTTCTCTCTTGGTCTCTGTTTAATGTTGAAAGGGTGAGCTAATAGG  1942
H  1945 ************************************************************************  2016
R  1943 AGGCAATGTCATCTTCACTCCCTCACACTCTCCCTTCCCCTAACAGACTGTCAATTTCAAGGATGCAAACTG  2014
H  2017 *****C*T***A**************G**AC***G*****CTC*******G**G***************T**  2088
R  2015 CATTGCAAGGTCAAACTGACTCAAGAAGCATCTGGGCCAGTGCACTGTTTACTTCCATGTGTTTGGCAGCCA  2086
H  2089 ********AA*************T*******T**************************C******C-**A**  2159
R  2087 CGTTTGGGCACAGCATTTGGGAGCCCTTTGTATCAGTTG-CTTTGACAAAGGTCCCCTAATCCT-AGCCTAT  2156
H  2160 *A****T**C*G**G********************A*GTT*--******G******TAT*A***T*A****C  2229
R  2157 TAGAAACCAGTTGGAGATGGATATGATGGGGCTTCTGTGCTGTTGCTGGGATTGGGAGAAATAAAACATGCA  2228
H  2230 *C******G***T*G**************************A******************************  2301
R  2229 ATTTCAGTGGAAAAAAAAAAAAAAAAA                                                2255
H  2302 ****A*****                                                                 2373
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Fig. 1. Sequence alignment of rabbit and human eRF1 cDNAs. Human eRF1 cDNA sequence (GenBank accession No. X81625), referred to as "H", was aligned with Ra-eRF1 cDNA (this work), referred to as "R", using the Genetyx Mac 10.1 program. The initiating codon (ATG) and termination codon (TGA) are boxed. Asterisks denote nucleotides identical to the Ra-eRF1 cDNA. The position of the end of the smaller 3′UTR is marked by an arrow. The location of the two putative polyadenylation signals in the 3′UTR is underlined.

information, 5′ and 3′ RACE were conducted, giving rise to one 5′ RACE product (0.9 kb) and two 3′ RACE products with sizes of 1.4 and 1.0 kb. Sequences of both 3′ RACE products were identical except for the 3′ UTR length; i.e., 861 and 514 bp sequences preceding poly(A), suggesting alternative polyadenylation at distinct sites (see Fig. 1). The 5′ and 3′ UTR sequences of Ra-eRF1 mRNA were highly homologous to those of human eRF1 mRNA (Fig. 1). A cDNA encoding full-length Ra-eRF1 was synthesized by PCR from rabbit muscle mRNA. The cloned Ra-eRF1 cDNA encoded a 1,314-bp open reading frame (ORF) flanked by the AUG initiator codon and the UAG terminator codon (Fig. 1). The deduced protein is composed of 437 amino acids (49 kD), and its protein sequence was completely identical to human eRF1 regardless of being 3% distinct in the

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   1  TTTGAAAATAATTGTATAAACTTTAAATTAACAAAAAAAAGTTAAATAAAAAAGAGCAAAAATAGATTAAATTTA   75
  76  AACCTAAAATAGTTAGCGTAAATGGAAGAGAAAGATCAACGTTAAAGAAATATTGAACATTTTAAGATCAAGAAA  150
                           METGluGluLysAspGlnArg***ArgAsnIleGluHisPheLysIleLysLys

 151  TTGATGACAAGACTTAGAAACACTAGAGGTTCTGGTACTTCTATGGTTTCTTTGATTATTCCTCCTAAGAAGTAA  225
      LeuMETThrArgLeuArgAsnThrArgGlySerGlyThrSerMETValSerLeuIleIleProProLysLys***

 226  ATTAACGATTCCACAAAGTTAATCAGTGATGAATTCAGTAAGGCCACTAATATTAAAGACAGAGTCAACCGTTAA  300
      IleAsnAspSerThrLysLeuIleSerAspGluPheSerLysAlaThrAsnIleLysAspArgValAsnArg***

 301  TCTGTACAAGATGCAATGGTTTCCGCCTTACAAAGATTAAAATTATATCAAAGAACTCCCAATAATGGTTTGATT  375
      SerValGlnAspAlaMETValSerAlaLeuGlnArgLeuLysLeuTyrGlnArgThrProAsnAsnGlyLeuIle

 376  CTTTATTGCGGTAAGGTTCTTAACGAAGAAGGAAAAGAAATTAAGCTTTTGATCGATTTCGAACCCTACAAGCCC  450
      LeuTyrCysGlyLysValLeuAsnGluGluGlyLysGluIleLysLeuLeuIleAspPheGluProTyrLysPro

 451  ATCAACACCTCTCTTTATTTCTGTGACAGTAAGTTCCACGTTGATGAATTGGGTTCACTTCTTGAAACCGACCCT  525
      IleAsnThrSerLeuTyrPheCysAspSerLysPheHisValAspGluLeuGlySerLeuLeuGluThrAspPro

 526  CCTTTTGGTTTCATCGTTATGGATGGTCAAGGTGCTCTCTATGCCAATCTCCAAGGAAATACAAAGACAGTTTTA  600
      ProPheGlyPheIleValMETAspGlyGlnGlyAlaLeuTyrAlaAsnLeuGlnGlyAsnThrLysThrValLeu

 601  AATAAATTCTCAGTCGAATTGCCCAAGAAGCACGGTAGAGGTGGTCAATCATCAGTTCGTTTTGCACGTCTTAGA  675
      AsnLysPheSerValGluLeuProLysLysHisGlyArgGlyGlyGlnSerSerValArgPheAlaArgLeuArg

 676  GTTGAAAAGAGACACAACTATCTTAGAAAGGTTTGTGAAGTCGCCACTTAAACTTTCATTTCCCAAGATAAAATT  750
      ValGluLysArgHisAsnTyrLeuArgLysValCysGluValAlaThr***ThrPheIleSerGlnAspLysIle

 751  AATGTTTAAGGTTTAGTCTTAGCTGGTTCTGGTGATTTCAAGAATGAATTGAGTACTACATAGATGTTCGACCCT  825
      AsnVal***GlyLeuValLeuAlaGlySerGlyAspPheLysAsnGluLeuSerThrThr***METPheAspPro

 826  CGTCTTGCTTGCAAGATTATCAAGATTGTTGATGTTTCTTATGGTGGTGAAAATGGTCTTAACCAAGCTATTGAA  900
      ArgLeuAlaCysLysIleIleLysIleValAspValSerTyrGlyGlyGluAsnGlyLeuAsnGlnAlaIleGlu

 901  CTCGCTTAAGAATCTTTAACTAACGTCAAGTTCGTTTAAGAAAAGAACGTTATCTCCAAATTCTTCGATTGCATC  975
      LeuAla***GluSerLeuThrAsnValLysPheVal***GluLysAsnValIleSerLysPhePheAspCysIle

 976  GCTATTGACTCCGGTACCGTTGTCTATGGTGTTCAAGATACTATGTAACTCTTATTAGATGGTGTTATTGAAAAC 1050
      AlaIleAspSerGlyThrValValTyrGlyValGlnAspThrMET***LeuLeuLeuAspGlyValIleGluAsn

1051  ATTCTTTGTTTCGAAGAACTCACTACCTTGAGAGTCACTCGTAAAAATAAAGTCACAGAATAAATCACTCATATA 1125
      IleLeuCysPheGluGluLeuThrThrLeuArgValThrArgLysAsnLysValThrGlu***IleThrHisIle

1126  TTCATTCCTCCTAATGAATTAAATAATCCTAAGCATTTCAAGGATGGTGAACATGAACTTGAAAAGATTGAAGTT 1200
      PheIleProProAsnGluLeuAsnAsnProLysHisPheLysAspGlyGluHisGluLeuGluLysIleGluVal

1201  GAAAACTTAACTGAATGGTTAGCTGAACACTACAGTGAATTCGGTGCTGAACTTTACTTTATTACTGATAAATCT 1275
      GluAsnLeuThrGluTrpLeuAlaGluHisTyrSerGluPheGlyAlaGluLeuTyrPheIleThrAspLysSer

1276  GCTGAAGGTTGCCAATTTGTCAAAGGTTTCTCTGGTATTGGTGGTTTCTTAAGATATAAGGTCGATTTAGAACAT 1350
      AlaGluGlyCysGlnPheValLysGlyPheSerGlyIleGlyGlyPheLeuArgTyrLysValAspLeuGluHis

1351  ATTGTTAACCCTAATGACGAATACAACTACGAAGAAGAAGAAGGCTTCATATGATCAATATTTTTATTTCTAAAT 1425
      IleValAsnProAsnAspGluTyrAsnTyrGluGluGluGluGlyPheIle

1426  TAAATTTTACATCTAAAAGATGTTTTAAAACATTCTGCTTAAGCACTCTAAAATATTAAGTTTATATTAAAGATT 1500
1501  TAATATTTTCTGTAATTCATTTCATTATGTACTATAGTATTATTACTCTTTGTATTTTGAGAGCTTTAAGCTTCC 1575
1576  TTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1609
```

Fig. 2. Nucleotide sequence of Tt-eRF1 cDNA. Initiation (ATG) and termination (TGA) codons are boxed. The underlined TAA and TAG codons are reassigned to glutamine (shown by asterisks). Deduced amino acid sequence is also shown.

nucleotide sequence, showing the high conservative structural and functional feature of the mammalian eRF1 family. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB029089.

Primary structure of *Tetrahymena thermophila* eRF1 cDNA. Tt-eRF1 cDNA has been cloned recently from *Tetrahymena thermophila* using the 5′- and 3′-RACE method [14]. The deduced Tt-eRF1 ORF is composed of a 1,308-bp sequence starting at the AUG initiator codon and ending at the UGA termination codon (Fig. 2). This ORF was interrupted by nine UAA and one UAG codons that are known to be reassigned to the glutamine codon in *Tetrahymena* [10-13] (Fig. 2). The predicted Tt-eRF1 protein contains 435 amino acids (molecular mass 49.5 kD) and shares 57% identity and 66% similarity with rabbit, human, and *Xenopus* eRF1s. Southern hybridization using the cloned Tt-eRF1 probe detected a unique signal in *Tetrahymena* DNA while not in DNAs from *E. coli*, *S. cerevisiae*, and *Schizosaccharomyces pombe* (Fig. 3), showing that the cloned Tt-eRF1 sequence is derived from *T. thermophila* and its sequence is not highly conservative compared with other eRF1s.

Overproduction and purification of rabbit eRF1. Ra-eRF1 cDNA was cloned into pET-30a(+) expression vector. This expression plasmid yielded a large amount of soluble His_6-tagged Ra-eRF1 protein in *E. coli* BL21 strain. Presence of the N-terminal His_6 tag enabled quick isolation of eRF1 using Ni-NTA resin. Recombinant Ra-eRF1 was purified from the soluble fraction of bacterial lysate using Ni-NTA-agarose and Mono Q column chromatographies to homogeneity (Fig. 4).

Overproduction and isolation of *Tetrahymena* eRF1 from *E. coli* under denaturing conditions. To establish an overexpression system of Tt-eRF1 in *E. coli* or in standard eukaryotes, ten internal UAA/UAG codons of Tt-eRF1 cDNA were changed to glutamine codons (CAG and CAA) by site-directed mutagenesis. Overexpression of both His_6-tagged and tag-free Tt-eRF1s was achieved by the T7 promoter-driven expression system in *E. coli*. Although both constructs gave very high level of protein expression in *E. coli* BL21 strain, the overproduced eRF1, in contrast to Ra-eRF1, was fully insoluble in both cases (data not shown), which greatly impeded the purification of active Tt-eRF1. Hence, we decided to isolate first denatured Tt-eRF1 to prepare antibody against the recombinant Tt-eRF1 for Western immunoblot detection. Tt-eRF1-containing inclusion bodies were isolated by centrifugation and Tt-eRF1 was purified by anion-exchange chromatography on Mono Q to homogeneity under denaturing conditions (Fig. 5). About 5 mg of solubilized Tt-eRF1 was purified and used for rabbit immunization, and high-titer anti-Tt-eRF1 antibody was prepared.

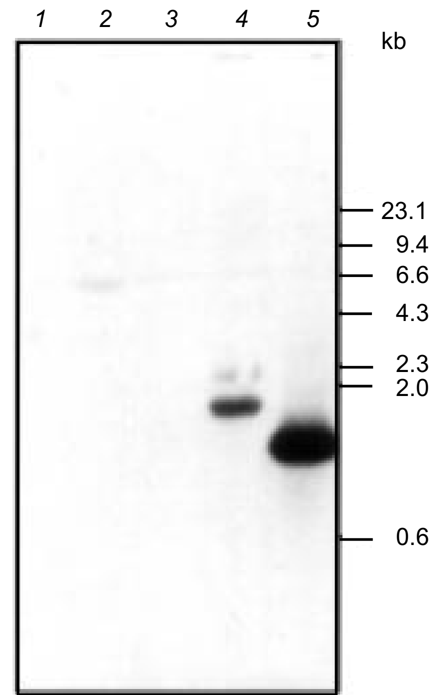

Fig. 3. Southern hybridization analyses using PCR fragment of Tt-eRF1 cDNA as a probe. Total genomic DNA (7-10 μg) of *E. coli* (lane *1*), *S. cerevisiae* (lane *2*), *S. pombe* (lane *3*), and *T. thermophila* (lane *4*) were digested with *Hind*III, separated by electrophoresis in 0.9% agarose gel, transferred to nylon filter, and hybridized with the ECL-labeled PCR fragment of Tt-eRF1 according to the manufacturer's instruction. The PCR fragment of Tt-eRF1 cDNA (lane *5*) was used as a positive control of hybridization. DNA size markers of *Hind*III digests of lambda DNA are shown.

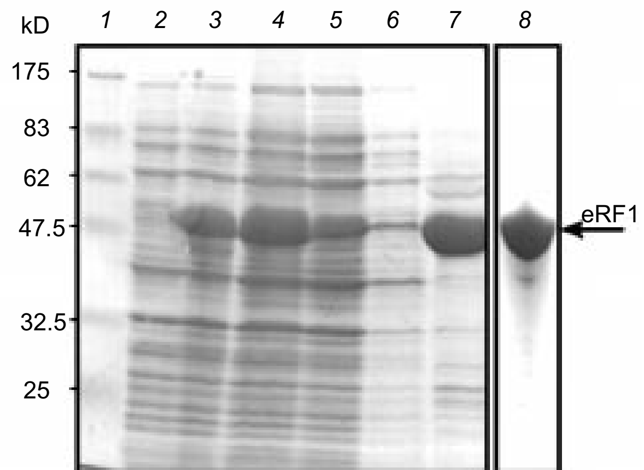

Fig. 4. Purification of recombinant Ra-eRF1 under native conditions. Lanes: *1*) protein markers; *2*, *3*) whole cell proteins before and after induction, respectively; *4*) cleared cell lysate; *5*) flow-through fraction of Ni-NTA column; *6*) wash; *7*) Ra-eRF1 eluted from Ni-NTA-agarose; *8*) Ra-eRF1 purified on Mono Q column.

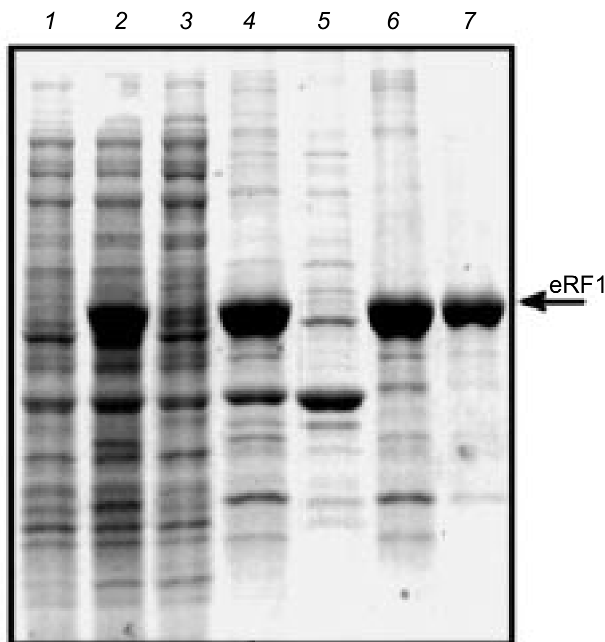

Fig. 5. Purification of His_6-tag free Tt-eRF1 under denaturing conditions. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining at different purification steps. Lanes: *1*, *2*) whole cell proteins before and after induction, respectively; *3*) cleared lysate; *4*) pellet of cell debris with Tt-eRF1 inclusion bodies; *5*) supernatant obtained after treatment of the pellet with 2% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl (pH 8.0); *6*) Tt-eRF1 protein solubilized in 8 M urea prior to Mono Q purification; *7*) Tt-eRF1 after Mono Q column chromatography.

Purification of the soluble *Tetrahymena* eRF1. Several attempts were made to increase the soluble fraction of Tt-eRF1 or to prevent the aggregate formation *in vivo* as well as *in vitro*, though without any improvement: 1) denaturation and renaturation treatment with several solubilization chemicals or gel-filtration; 2) co-expression of *E. coli* chaperons; 3) fusion with thioredoxin protein; and 4) test of several growth media or growth conditions (data not shown). Finally, we found that Tt-eRF1 remains in a soluble form once expressed in *S. cerevisiae* although the expression level was very low. Since the first affinity purification of His_6-tagged Tt-eRF1 with Ni-NTA-agarose was incomplete in the purity, the fraction was further subjected to chromatography on a Heparin column and on Mono Q column. A single protein was purified to near homogeneity (Fig. 6). The purified protein was reactive to anti-Tt-eRF1 antibody described above (not shown).

Functional test of recombinant eRF1s in yeast. Strain MT557/4d has a temperature-sensitive mutation in the eRF1 gene of *S. cerevisiae* (*sup45* ts allele; *sal4-2* [16]) and was used for heterologous complementation test of recombinant eRF1s. Both His_6-tagged and tag-free constructs of Tt-eRF1 and Ra-eRF1 cDNAs were cloned under the P_{GAL1} promoter in the pYES2 vector (plasmids pR-eRF1-25/2, pR-eRF1-25/3, pTT-eRF1-4/12, and pTT-eRF1-1/3; see Table 1), and transformed into MT557/4d strain. Growth of these transformants were monitored on uracil-free SC medium plates supplemented with galactose for induction of P_{GAL1} promoter at the permissive and non-permissive temperatures (Fig. 7). The data indicated that Ra-eRF1 complemented efficiently the *sup45* ts allele of *S. cerevisiae*, showing that eRF1 genes of rabbit and yeast are functionally exchangeable. This observation is consistent with the previous results that the eRF1 genes from *S. pombe* [22], *Xenopus* [23, 24], human, and hamster [24] are able to complement the lethal *sup45* mutation of *S. cerevisiae*. The recombinant Ra-eRF1, however, became less active for complementation upon tagging with His_6 (see Fig. 7), suggesting that the exogenous polypeptide including His_6 tag at the N terminus is slightly but significantly harmful to the function of Ra-eRF1.

In contrast to Ra-eRF1 and most other eRF1s so far tested, Tt-eRF1 (with or without His_6-tag) failed to complement the *sup45* ts mutation (Fig. 7). The disabil-

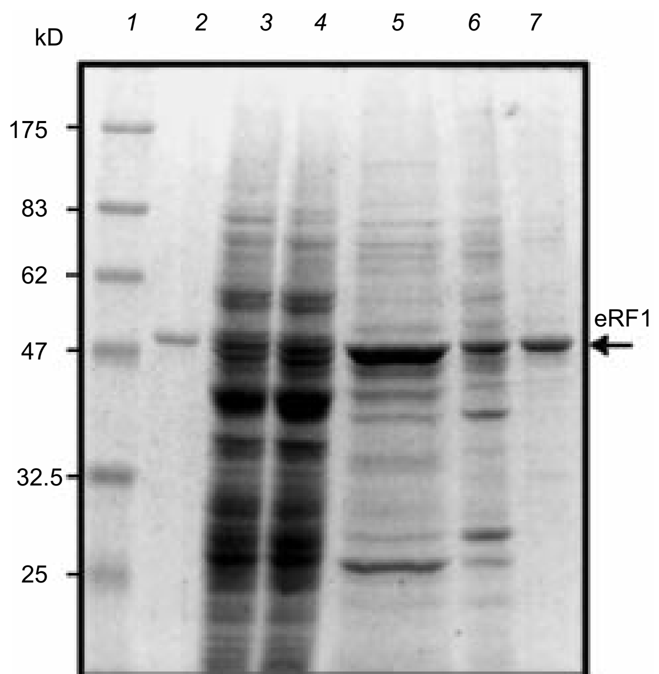

Fig. 6. Purification of native Tt-eRF1 protein synthesized in *S. cerevisiae*. Samples were analyzed by SDS-PAGE and Coomassie Blue staining at different purification steps. Lanes: *1*) protein markers; *2*) denatured Tt-eRF1 protein isolated from inclusion body in *E. coli* (control); *3*) total lysate; *4*) lysate after removal of ribosomes; *5*) Tt-eRF1 fraction after chromatography in Ni-NTA-agarose; *6*) Tt-eRF1 fraction after chromatography in Heparin Toyopearl; *7*) Tt-eRF1 purified by FPLC chromatography on a Mono Q column.

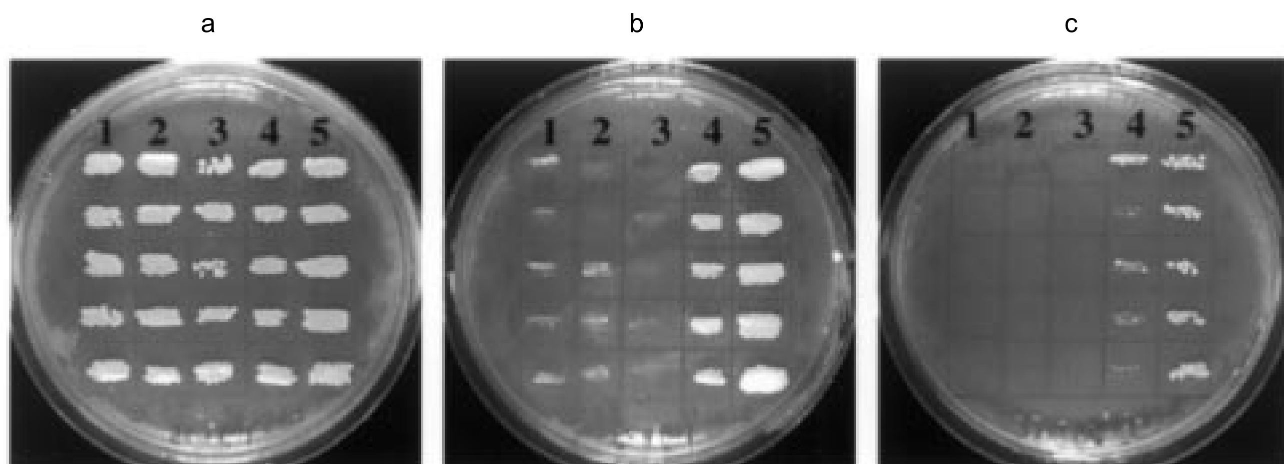

Fig. 7. Complementation test of eRF1 clones in *sup45* ts mutant of *S. cerevisiae*. *S. cerevisiae* strain MT 557/4d transformants (in quintet) were grown on the solid SC media lacking uracil and supplemented with galactose at 25 (a), 35 (b), or 37°C (c). eRF1 genes transformed: *1*) Tt-eRF1 (with His_6 tag); *2*) Tt-eRF1 (without His_6 tag); *3*) pYES2 vector (control); *4*) Ra-eRF1 (with His_6 tag); *5*) Ra-eRF1 (without His_6 tag).

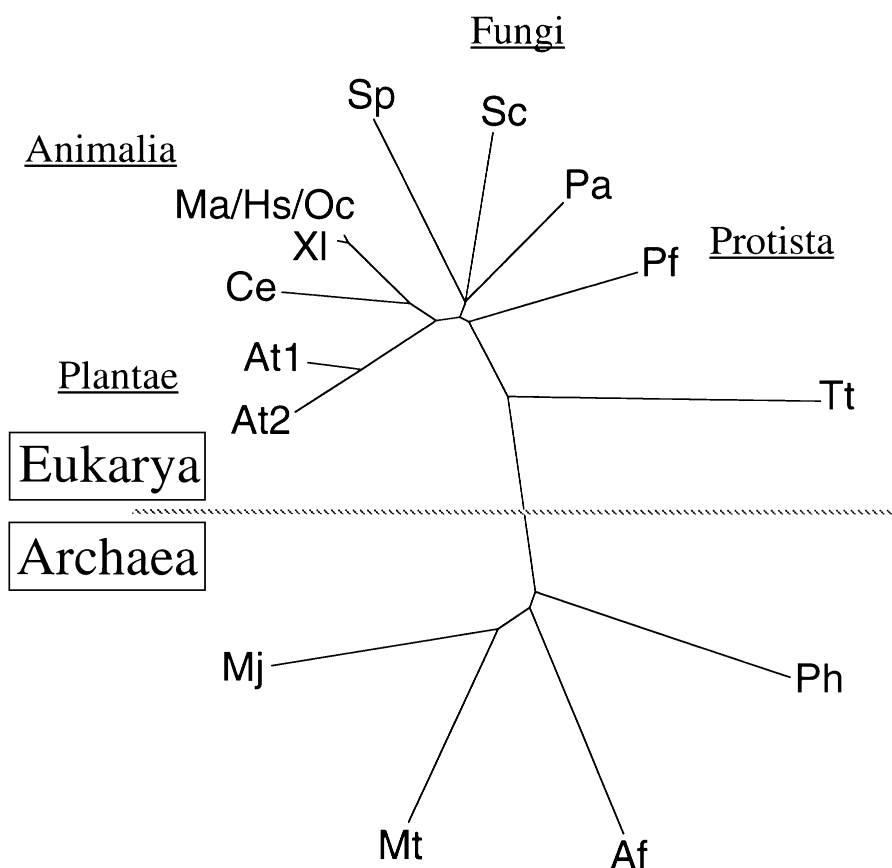

Fig. 8. Phylogenetic tree of the eukaryotic and archaebacterial release factors. Ph, *Pyrococcus horikoshii* (GenBank accession No. AP000006); Af, *Archaeoglobus fulgidus* (AE001020); Mt, *Methanobacterium thermoautotrophicus* (AE000864); Mj, *Methanococcus jannaschii* (U67526); Sp, *Schizosaccharomyces pombe* (D63883); Pa, *Podospora anserina* (AF053983); Sc, *S. cerevisiae* (X04082); At, *Arabidopsis thaliana* (AT81KBGEN, AB016886); Ce, *Caenorhabditis elegans* (CELT05H4); Xl, *Xenopus laevis* (Z14253); Ma, *Mesocricetus auratus* (MAC114); Hs, *Homo sapiens* (X81625); Oc, *Oryctolagus cuniculus* (rabbit, this work); Pf, *Plasmodium falciparum* (AE001402); Tt, *Tetrahymena thermophila* (AB026195).

ity of Tt-eRF1 for the complementation could be explained by assuming that Tt-eRF1 recognizes only UGA but not the other two codons, unlike yeast eRF1, or by assuming that Tt-eRF1 cannot interact properly with the heterologous ribosome derived from *S. cerevisiae*. The latter possibility should not be the sole reason for the disability but could well be supported by the large phylogenetic distance of Tt-eRF1 from yeast eRF1 (Fig. 8). It is quite remarkable that Tt-eRF1 is very isolated from most other eRF1s in the phylogenetic tree, showing only 57% identity to mammalian and yeast eRF1. Therefore, one could argue that *Tetrahymena* eRF1 might have evolved rapidly compared with other eRF1s. It remains to be examined whether eRF1 genes from other ciliates are also distantly related with the mammalian release factor genes.

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