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Overexpression of Human Release Factor 1 Alone Has an Antisuppressor Effect in Human Cells

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Two eukaryotic proteins involved in translation termination have recently been characterized in in vitro experiments. Eukaryotic release factor 1 (eRF1) catalyzes the release of the polypeptide chain without any stop codon specificity. The GTP-binding protein eRF3 confers GTP dependence to the termination process and stimulates eRF1 activity. We used tRNA-mediated nonsense suppression at different stop codons in a *cat* reporter gene to analyze the polypeptide chain release factor activities of the human eRF1 and eRF3 proteins overexpressed in human cells. In a chloramphenicol acetyltransferase assay, we measured the competition between the suppressor tRNA and the human release factors when a stop codon was present in the ribosomal A site. Whatever the stop codon (UAA, UAG, or UGA) present in the *cat* open reading frame, the overexpression of human eRF1 alone markedly decreased translational readthrough by suppressor tRNA. Thus, like the procaryotic release factors RF1 and RF2 in *Escherichia coli*, eRF1 seems to have an intrinsic antisuppressor activity in human cells. Levels of antisuppression of overexpression of both eRF3 and eRF1 were almost the same as those of overexpression of eRF1 alone, suggesting that eRF1-eRF3 complex-mediated termination may be controlled by the expression level of eRF1. Surprisingly, when overexpressed alone, eRF3 had an inhibitory effect on *cat* gene expression. The results of *cat* mRNA stability studies suggest that eRF3 inhibits gene expression at the transcriptional level. This indicates that in vivo, eRF3 may perform other functions, including the stimulation of eRF1 activity.

Translation is usually completed when a stop codon enters the A site of the ribosome. Recognition of the stop codon by the release factor liberates the nascent polypeptide (4, 7). The first eukaryotic release factor, named eukaryotic release factor 1 (eRF1), was recently identified, and the in vitro release factor activity of human and *Xenopus laevis* eRF1 was characterized (15). eRF1 recognizes all three nonsense codons (UAA, UGA, and UAG) and catalyzes peptidyl-tRNA hydrolysis in a GTP-independent reaction. Because translation termination was shown to be a GTP-dependent process (28), it was suggested that another release factor which confers the GTP requirement to translation termination exists in eukaryotes (15, 44). This second factor, named eRF3, which was first isolated from *X. laevis* (52), exhibits GTP binding motifs and stimulates eRF1 release factor activity in vitro but only in the presence of GTP. Although eRF3 does not recognize stop codons by itself, it binds to eRF1 to form an active termination complex. It was proposed that a quaternary complex composed of eRF1, eRF3, GTP, and the ribosome can govern eukaryotic translation termination at a stop codon of the mRNA (52). eRF3 is an eRF1- and ribosome-dependent GTPase (16) and is most likely the functional homolog of the recently sequenced prokaryotic release factor 3 (RF-3), a GTP-binding protein which stimulates RF-2 activity upon translation termination (19, 20, 34).

In the yeast *Saccharomyces cerevisiae*, it has been postulated that the products of two distinct genes, *SUP45* and *SUP35*, which have similar mutant phenotypes, were involved in translation termination (45). The *SUP45* and *SUP35* gene products belong to the highly conserved eRF1 and eRF3 protein families, respectively (15, 52). Both *sup45* and *sup35* mutants exhibit, among pleiotropic effects, either an omnipotent suppressor phenotype, i.e., suppression of all three nonsense codons (22, 25), or an allosuppressor phenotype, i.e., an ability to enhance nonsense suppression by a weak suppressor tRNA (8, 9). The effect of overexpression of the *SUP45* and *SUP35* genes of *S. cerevisiae* in a nonsense suppressor tRNA background has been studied recently. When overexpressed separately, the yeast Sup35 and Sup45 proteins have no effect on nonsense suppression, but the overproduction of both genes decreased stop codon suppression by the suppressor tRNA (43).

Here, we used the properties of nonsense suppression recently applied to mammalian cells in culture (38) to investigate the roles of eRF1 and eRF3 in human cells. After transient expression, the results of a competition reaction between nonsense tRNA suppression and termination were measured by a chloramphenicol acetyltransferase (CAT) assay. It is shown here that, for all three stop codons, overexpression of eRF1 alone favors translation termination efficiency over tRNA suppression. Thus, eRF1 has an antisuppressor effect by itself, in contrast to the situation in yeast. When eRF1 is overexpressed in combination with eRF3, antisuppression is not significantly enhanced, suggesting that eRF1 is the limiting factor for termination efficiency in human cells. Overexpression of eRF3 alone has an inhibitory effect on *cat* gene expression. The results of Northern blot hybridization and stability studies of *cat* mRNA suggest that eRF3 inhibits gene expression at the transcriptional level. Therefore, eRF3 may perform other functions in vivo, including the stimulation of eRF1 activity.

MATERIALS AND METHODS

Plasmid constructs. The parent plasmids pSV-tS Su^+ (Och), pSV-tS Su^+ (Am), and pSV-tS Su^+ (Opal), which contain the ochre, amber, and opal suppressor derivatives of the wild-type human serine tRNA gene, respectively, have been described previously (3). Plasmids ptRNAoc, ptRNAam, and ptRNAop (ptRNA series) were constructed by cloning the 900-bp *Sau*3A fragment containing the suppressor tRNA gene of the pSV-tS Su^+ series into the *Bam*HI site

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of pUC18 (51). The plasmid pMLPCAT (renamed pCATwt in this study) contains the *cat* gene from transposon Tn*9* under the control of the adenovirus major late promoter (13). The *Hin*dIII-*Nco*I fragments of plasmids pRSV-*cat*(oc27), pRSV*cat*(am27), and pRSV*cat*(op27) containing the ochre, amber, or opal nonsense codon at position 27 of the *cat* gene (2), respectively, were recloned into *Hin*dIII-*Nco*I-digested pCATwt to yield the expression plasmids pCAToc27, pCATam27, and pCATop27 (pCAT series).

The expression vector pBK-CMV was obtained from Stratagene. Plasmid pBSKDHG11 (a generous gift of M. Kress, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France) contains the human release factor 1 gene inserted into the multicloning site of pBluescriptII SK+ (Stratagene). The *Spe*I-*Sal*I fragment of pBSKDHG11 comprising 120 bp of the 5′ untranslated region and the entire open reading frame of the human eRF1 gene was cloned into *Nhe*I-*Sal*I-digested pBK-CMV to yield the human eRF1-expressing plasmid pCMVhRF1. Plasmid pCMVhRF3, which expresses human eRF3, was obtained by cloning the *Pst*I–Klenow fragment-filled-*Bam*HI fragment of plasmid pUC19GST1 (24) into pBK-CMV digested with the *Pst*I–Klenow fragment-filled-*Nhe*I fragment.

DNA transfection of human cells and CAT assays. Human 293 cells are an adenovirus-transformed embryonic kidney cell line (17). 293 cells were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% calf serum. Transfection of cells at 50% confluency was carried out with a maximum of 35 μg of DNA per 100-mm-diameter plate by the calcium phosphate coprecipitation method (18). Unless otherwise indicated, in suppression experiments, cells were routinely transfected with 5 μg of plasmid DNA of the pCAT series, 5 μg of DNA of the ptRNA series except for that of ptRNAoc (10 μg was used because of the weak suppression efficiency of the ochre suppressor tRNA [tRNAsu$^+$ ochre]), and 10 μg of plasmid DNAs of pCMVhRF1 and/or pCMVhRF3, which contain the human release factor genes. For all transfections, the amount of DNA in the calcium phosphate coprecipitate was equalized with plasmid pBK-CMV DNA in order to have similar amounts of the cytomegalovirus promoter in the transfected cells. After overnight incubation, the medium and precipitates were removed and replaced with 10 ml of fresh medium. Following a further 24-h incubation, the medium was removed and the cell monolayer was washed with phosphate-buffered saline (PBS; 10 mM phosphate buffer [pH 7.4], 140 mM NaCl). Cells were collected by scraping them in 10 ml of PBS, pelleting them, and resuspending them in 1 ml of PBS; 300 μl was used for CAT assays, 300 μl was used for Northern blot analysis, and the remaining 400 μl was stored as a dry pellet at −20°C or used for Western blot analysis.

For CAT assays, the cells were centrifuged and the pellet was resuspended in 300 μl of 250 mM Tris-HCl, pH 8. The cells were lysed by four cycles of freezing and thawing, and after 10 min of centrifugation, the supernatant was heated for 5 min at 60°C to inactivate cellular acetylases and centrifuged at 12,000 × *g* for another 10 min. Five, 10, or 15 μl of the resulting supernatant was used for CAT assays as previously described (40). Samples were assayed for total proteins with a Bio-Rad assay reagent. For each sample, CAT assays were performed in duplicate after determination of the linear range of the reaction. The volume of extract and the time of incubation were chosen so as to obtain 3,000 to 10,000 cpm. The mean value of blank and mock-transfected cell assays was 600 to 800 cpm. CAT activity was expressed as counts per minute per microgram of total proteins.

RNA isolation and analysis. Samples of the same cell cultures used for CAT assays were centrifuged for 2 min at 5,000 × *g* and mixed with 400 μl of the lysis solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 100 mM 2-mercaptoethanol [pH 7]), and total RNA was extracted as previously described (5). For the detection of *cat* mRNA and 18S rRNA, 15-μg samples of RNA were electrophoresed on 1.2% agarose–formaldehyde gels, blotted, and UV cross-linked onto a nylon membrane (Amersham). The membranes were probed in BLOTTO with randomly labelled denatured double-strand DNA as described previously (39). The ^{32}P-labelled *cat* probe was the *Hin*dIII-*Eco*RI 251-bp fragment of the *cat* gene. The membranes were washed under stringent conditions (55°C) and exposed to X-ray films. The hybridization signals were quantified with a model BAS1000 Fuji image plate scanner. The membranes were then stripped, reprobed with the 18S rRNA probe (the *Rsa*I-*Bam*HI 517-bp fragment of the 18S rRNA gene) and treated as described above for the *cat* probe.

For detection of suppressor tRNAs and 5.8S rRNA, 15-μg samples of RNA were electrophoresed in a 7 M urea–10% polyacrylamide gel. The gel was electroblotted and probed as described previously (12). The two following synthetic oligonucleotides labelled at their 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) were used: 5′CGAAGTGTCGATGA TCAAT3′, which was complementary to the 5.8S rRNA from nucleotides 86 to 104, and 5′TTTRRAGTCCATCGCC3′ (in which an R represents an A or G base), which was complementary to the anticodon loop of the human serine tRNA except for the anticodon sequence. The presence of a degenerate sequence complementary to the anticodon allowed us to visualize all three suppressor tRNAs (ochre, amber, or opal suppressor) with the same probe. Under the moderately stringent conditions of washing used (45°C), this degenerate oligonucleotide did not recognize the parental serine tRNA. After being probed with the suppressor tRNA probe, the membrane was washed at 65°C, reprobed with the 5.8S rRNA probe, and washed at stringent temperature (55°C). For each probe, the membrane was exposed to X-ray film and to the Fuji image plate and then the hybridization signals were quantified with a model BAS1000 Fuji image plate scanner.

Western blot analysis. For human eRF1 and eRF3 detection, transfected cells were collected as described above. The dry cell pellets were lysed in 500 μl of Laemmli loading buffer and sonicated, and 30 μl was loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel (29). For CAT protein detection, samples from the freeze-thaw supernatant were assayed for total proteins with the Bio-Rad assay reagent and 15 μg of proteins was loaded onto an SDS-12% polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane (Hybond C; Amersham). The membrane was incubated overnight in BLOTTO-Tween 20 buffer (5% nonfat milk and 0.5% Tween 20 in PBS) at 4°C and then washed in PBS-Tween 20 buffer (0.5% Tween 20 in PBS). The membrane was incubated for 2 h at room temperature in BLOTTO-Tween 20 buffer containing the diluted rabbit polyclonal antibodies. The anti-CAT rabbit antibodies were from 5 Prime-3 Prime. The anti-human eRF1 antibodies were produced by inoculating two rabbits according to usual protocols (21) with purified his-tagged TB3-1 (human eRF1) recombinant protein expressed from the plasmid pTB3-YuS-eRF1, which was constructed and kindly provided by Y. Stasiv and L. Frolova (Engelhard Institute of Molecular Biology, Moscow, Russia). The purification of the antibodies was performed either with an activated CNBr-Sepharose (Pharmacia) column saturated with the purified his-tagged TB3-1 recombinant protein or with the purified his-tagged XSup35C recombinant protein fixed to nitrocellulose membranes. After the primary antibody incubation, the membrane was washed three times in PBS-Tween 20 buffer and incubated for 1 h at room temperature in BLOTTO-Tween 20 buffer containing a 7,500-fold dilution of antirabbit polyclonal antibodies coupled with horseradish peroxidase (Promega). After extensive washes, the membrane was revealed with enhanced chemiluminescence reagents (New England Nuclear) according to the manufacturer's instructions.

RESULTS

Experimental system. Our goal was to study the effect of the human release factors eRF1 and eRF3 on translation termination in human cells. We designed a system to measure the variations in readthrough of a stop codon obtained by expression of a suppressor tRNA (tRNAsu$^+$). The decrease in the readthrough activity of the suppressor tRNA corresponds to antisuppression.

(i) Suppression efficiency measurement. The experimental system was based on the transient expression in human cultured cells of a *cat* reporter gene containing a nonsense codon in its open reading frame and on the concomitant expression of a suppressor tRNA and of human eRF1 and eRF3. The *cat* gene under the control of the adenovirus major late promoter contained one of the stop codons instead of the serine codon at position 27 of the open reading frame (plasmids pCATam27, pCAToc27, and pCATop27 have an amber [UAG], ochre [UAA], and opal [UGA] stop codon, respectively). Readthrough of these stop codons was accomplished by the expression of suppressor tRNAs, which were derivatives of a human serine tRNA carrying a proper mutation in the anticodon (2, 3). In order to obtain measurable levels of antisuppression, we limited the level of suppressor tRNAs. It has been shown (2) that the level of nonsense suppression is low when nonreplicating plasmids are used to express the suppressor tRNA genes. Therefore, the suppressor tRNA genes, under the control of their own promoter, were recloned into plasmid pUC18, yielding plasmids ptRNAam, ptRNAoc, and ptRNAop. With these plasmids, the CAT activity produced by suppression of a nonsense codon at position 27 of the *cat* gene was about 10% of the activity produced by expression of the wild-type *cat* gene (data not shown). In this case, the amount of suppressor tRNA was the limiting factor for suppression efficiency.

To optimize the suppressor tRNA gene expression and to ensure that the CAT activity produced by nonsense suppression was correlated with the amount of transfecting suppressor tRNA genes, CAT activity was measured in extracts of 293 cells cotransfected with the plasmid pCATop27, which carries a *cat* gene interrupted by a UGA stop codon at position 27, together with increasing amounts of plasmid ptRNAop DNA expressing tRNAsu$^+$ opal (Fig. 1). The level of synthesis of

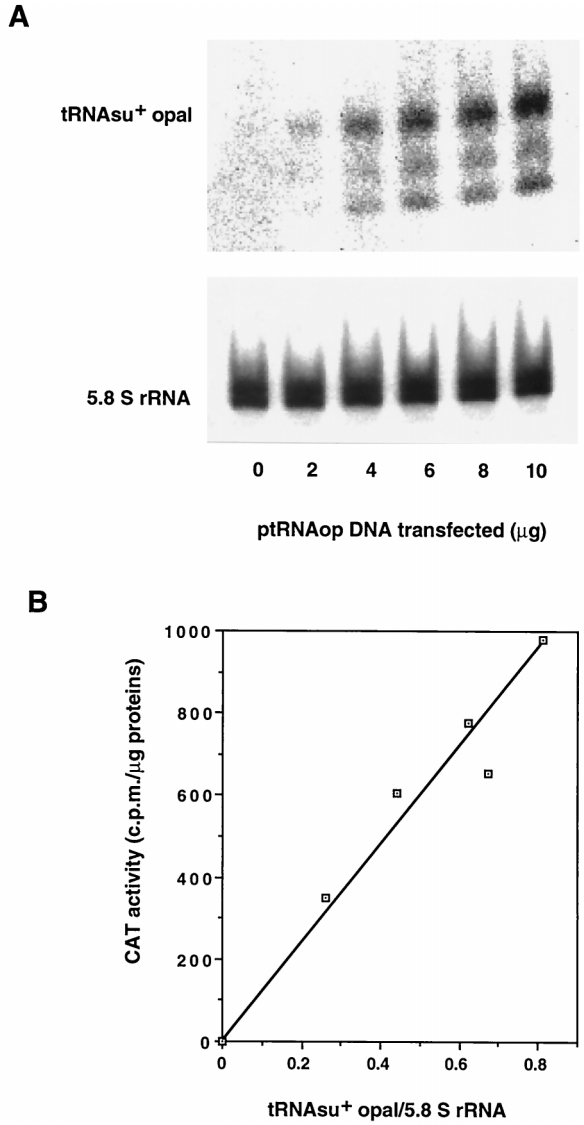

FIG. 1. Suppression of the opal stop codon in the *cat* reporter gene by $tRNAsu^+$ opal in human 293 cells. Parallel cultures of 293 cells were cotransfected with 5 μg of plasmid pCATop27 and increasing amounts of ptRNAop DNA. (A) Northern blot analysis of $tRNAsu^+$ opal and 5.8S rRNA in transfected cells. The amount of ptRNAop DNA transfected is indicated below each lane. Total cellular RNA (15 μg) was electrophoresed on a 10% denaturing acrylamide gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labelled $tRNAsu^+$ probe. The membrane was then stripped and reprobed with the ^{32}P-labelled 5.8S rRNA probe. For each probe, the signals were quantitated with a model BAS1000 Fuji image plate scanner and printed on photographic paper. The two minor bands below the main $tRNAsu^+$ opal band have a difference of size of only 1 nucleotide and likely correspond to intermediates in the addition of the CCA sequence at the 3′ terminus of the tRNA. The size of the human $tRNA^{Ser}$ is 85 nucleotides, while that of human 5.8S rRNA is 159 nucleotides. (B) CAT activity expressed in transfected cells results from the readthrough of the opal stop codon at position 27 of the *cat* gene by $tRNAsu^+$ opal. The CAT and total protein assays were performed in duplicate with 5 and 10 μg of cell extracts. The curve represents the mean value of CAT activity as a function of the relative amount of intracellular $tRNAsu^+$ opal quantitated on the Northern blot.

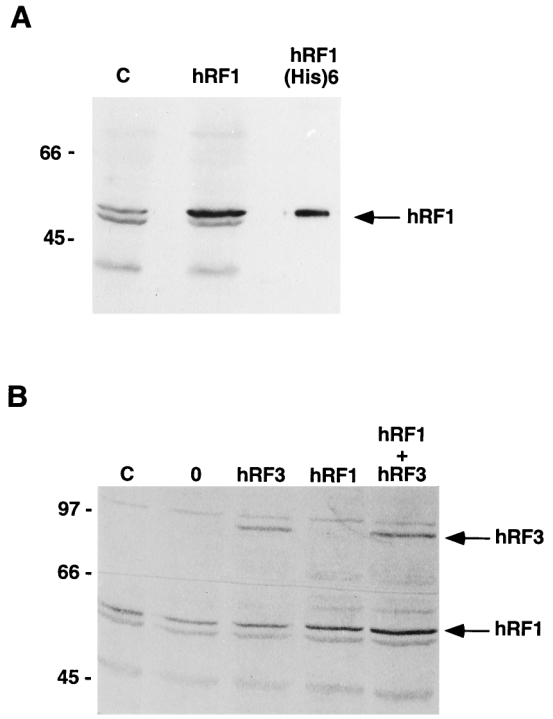

FIG. 2. Western blot analysis of the overexpression of the human release factors eRF1 and eRF3 in 293 cells. (A) Immunodetection of human eRF1. Cells were transfected with 10 μg of pCMVhRF1 DNA (lane hRF1) or untransfected control cells (lane C). Sonicated cell extracts were loaded onto an SDS–12% polyacrylamide gel. A sample of affinity-purified his-tagged human eRF1 recombinant protein was loaded on the same gel [lane hRF1-(His)6]). After transfer to nitrocellulose membranes, human eRF1 proteins were detected with purified polyclonal anti-human eRF1 rabbit antibodies (see Materials and Methods). The human eRF1 polypeptide is indicated by an arrow on the right, and molecular mass markers in kilodaltons are indicated on the left. (B) Cell samples from one of the antisuppression experiments presented in Fig. 5 and 6 were used for Western blot analysis. Shown are untransfected control cells (lane C) and cells cotransfected with the plasmids pCATam27, ptRNAam, and either pBK-CMV (lane 0), pCMVhRF3 (lane hRF3), pCMVhRF1 (lane hRF1), or pCMVhRF3 and pCMVhRF1 (lane hRF1+hRF3). After electrophoresis and transfer onto a nitrocellulose membrane, the membrane was cut and each part was revealed separately. Immunodetection of human eRF3 was performed with purified polyclonal rabbit antibodies directed against the *Xenopus* eRF3 protein (upper part). Immunodetection of human eRF1 was as described above (lower part). Overexpressed human eRF1 and eRF3 proteins are specified on the right (hRF1 and hRF3, respectively). Molecular mass markers in kilodaltons are indicated on the left.

$tRNAsu^+$ opal in the transfected cells was measured by Northern blotting and normalized to the level of 5.8S rRNA. As shown in Fig. 1A, the amount of $tRNAsu^+$ opal in the cells increased proportionally with the amount of ptRNAop DNA transfected. Moreover, the endogenous serine tRNA was not detected by the oligonucleotide probe under the stringent conditions used. The two signals of less intensity detected below the main tRNA band were likely to correspond to intermediates in the addition of the CCA sequence at the 3′ terminus by tRNA nucleotidyltransferase (10). The suppression efficiency measured by the CAT activity (Fig. 1B) was proportional to the amount of ptRNAop plasmid DNA transfected and to the amount of $tRNAsu^+$ opal in the cells. Thus, quantification of the suppressor tRNA was made for each experiment and was used as an internal standard for transfection efficiency measurement.

(ii) human eRF1 and eRF3 overexpression. Overexpression of human eRF1 in 293 cells was estimated by Western blot experiments. Cells were transfected with 10 μg of pCMVhRF1 plasmid DNA, and cell extracts were subjected to Western blot analysis. As shown in Fig. 2A, the rabbit anti-human eRF1 antiserum revealed the overexpression of a protein of 49,000 Da in pCMVhRF1-transfected cells corresponding in size to

the histidine-tagged human eRF1 protein purified from overexpressing *Escherichia coli* extracts and used for rabbit immunization. This polypeptide of 49,000 Da was also detected in control cells (lane C). The minor bands also visible below human eRF1 likely correspond to cross-reacting protein species.

We have previously demonstrated that translation was initiated at each of the first three AUG codons of the human eRF3 open reading frame (27). However, with different constructs containing the long and the medium-sized versions of the human eRF3 gene, which begin at the first or at the second AUG codon, translation was also initiated at the third AUG codon, probably by leaky scanning of ribosomes. In 293 cells, the major form of the human eRF3 protein was a polypeptide of 82,000 Da corresponding to translation initiation at the first AUG codon of the open reading frame (Fig. 2B). The short polypeptide of 65,000 Da translated from the third AUG codon was only faintly visible. In the antisuppression experiments presented here, we used the medium-sized version of the human eRF3 open reading frame (plasmid pCMVhRF3), which begins at the second AUG codon and expresses a protein of 80,000 Da as a major species and also the 65,000-Da polypeptide. We did not observe any difference when the antisuppression experiments were performed with a plasmid containing the short version of the gene and expressing only the protein of 65,000 Da (data not shown). It has been shown in in vitro experiments that the carboxy-terminal domain of eRF3 carries all the information necessary for the formation of the termination complex and to produce GTPase activity (16).

In antisuppression experiments, 293 cells were cotransfected with 5 μg of one of the plasmids containing a *cat* gene harboring a stop codon at position 27, 5 or 10 μg of a plasmid from the ptRNAsup series containing the cognate suppressor tRNA gene, 10 μg of one or both of the plasmids expressing the human release factor genes, and enough pBK-CMV plasmid DNA to equalize the total amount of transfecting DNA. As shown in Fig. 2B, the amount of human eRF1 or eRF3 proteins produced in transfected cells was not modified when the two factors were overexpressed together.

Overexpression of human eRF3 interferes with the transcription efficiency of the *cat* gene. As stated above, in our system, suppression was measured by the appearance of CAT activity, which was proportional to the quantity of suppressor tRNA. Thus, allosuppression increases CAT activity whereas, conversely, antisuppression reduces it. However, such variations may also be obtained by a modification of the expression of the reporter gene itself without being related to suppression. Thus, the effect of overexpression of eRF1, eRF3, or both on the expression of the wild-type *cat* gene was tested. This was accomplished by performing cotransfections with plasmid pCATwt, which expresses a wild-type *cat* gene, and the plasmids containing the human eRF1 and eRF3 genes. Figure 3A shows the means of three independent experiments by assigning a value of 100% to the CAT activity observed in control cells, which were cotransfected with pCATwt plasmid and with the original vector, pBK-CMV, to equalize the amount of DNA in the calcium phosphate coprecipitates. Examination of Fig. 3A shows that in cells expressing eRF1 alone, CAT activity reached 115% compared to that of control cells. Surprisingly, expression of eRF3 alone reduced CAT activity to about 60% of the level of the control, whereas a moderate decrease to roughly 90% of the level of the control was observed when both eRF1 and eRF3 were overexpressed.

Comparison of the cellular levels of either *cat* gene mRNA (Fig. 3B) or CAT protein (Fig. 3C) clearly showed that the decrease in CAT activity of cells which overexpressed eRF3 alone was a consequence of the partial inhibition of expression of the *cat* gene. The decrease in the steady-state level of *cat* gene mRNA (30 to 40% of that of the control cells) was reproducibly obtained in 293 cells overexpressing eRF3 (see also Fig. 4A). Coexpression of eRF1 and eRF3 had a far less significant negative effect on CAT activity, which was not detectable at the level of *cat* mRNA or CAT protein. This result may be explained by the formation of the eRF1-eRF3 termination complex. Such a complex formation between *Xenopus* eRF1 and eRF3 has been described in reports of in vitro experiments (52) and was also observed to occur between Sup45 and Sup35 proteins in yeast by Stansfield et al. (43). Thus, termination complex formation most likely prevented the inhibitory effect of human eRF3 on *cat* gene expression. When human eRF1 alone was overexpressed, the modest stimulatory effect on CAT activity detectable at the protein level (Fig. 3C) but not observed at the mRNA level (Fig. 3B) was consistent with a general increase in termination efficiency.

We cannot conclude from these results whether eRF3 had a direct effect on transcription efficiency. One possibility was that the effect of eRF3 on the steady-state level of *cat* mRNA was due to an alteration of mRNA stability. Parallel cultures of 293 cells were cotransfected with 5 μg of pCATam27, 10 μg of pCMVhRF3, or 10 μg of pBK-CMV. Actinomycin D at 5 μg/ml was added 48 h after transfections for various times as shown in Fig. 4, and total cellular RNAs were prepared. RNA levels were assayed by Northern blot analysis first with a *cat* gene probe, second with a probe prepared from a mouse actin gene, and third with a probe prepared from the *HSC73* gene (a generous gift of O. Bensaude). For each hybridization, the signals were quantitated with a Fuji image plate scanner and the membrane was subjected to autoradiography (Fig. 4A). The level of each mRNA at time zero was set to 100%, and the half-lives were measured on their decay curves (Fig. 4B). Like the actin mRNA, the *cat* mRNA was quite stable, demonstrating a half-life longer than 8 h, regardless of the concomitant expression of human eRF3. We used as a control the *HSC73* mRNA (whose half-life was about 5 h in our experiments), which has been described to be moderately stable in mammalian cells, with a half-life of 2 to 5 h, depending on the cell line (30). These results indicated that the mechanism by which eRF3 reduced the abundance of mRNA did not involve mRNA stability. It is also important to notice that (i) the presence of a nonsense codon in the *cat* gene did not markedly alter *cat* mRNA stability and (ii) when human eRF3 was overexpressed, the abundance of the mutated *cat* mRNA in Fig. 4A was reduced in the same proportion as the wild-type *cat* mRNA in Fig. 3B.

Overexpression of human eRF1 alone has an antisuppressor effect. To establish the functions of eRF1 and eRF3 in human cells, we performed experiments to determine competition between the suppressor activity of a modified serine tRNA and the chain termination activities of the human release factors eRF1 and eRF3 measured as antisuppression activities. Parallel cultures of 293 cells were transfected with one of the plasmids containing the mutated *cat* gene (pCAT series), together with plasmids containing the cognate suppressor tRNA (ptRNA series), and either pCMVhRF1, pCMVhRF3, or pCMVhRF1 plus pCMVhRF3. Transfection efficiency was estimated by Northern blot analysis with a specific suppressor tRNA oligonucleotide probe and a 5.8S rRNA oligonucleotide probe as described in Materials and Methods. An example of the Northern blot analysis of one transfection experiment is shown in Fig. 5. The suppressor tRNA oligonucleotide probe was designed to recognize all three suppressor tRNAs but not the endogenous parental serine tRNA, under

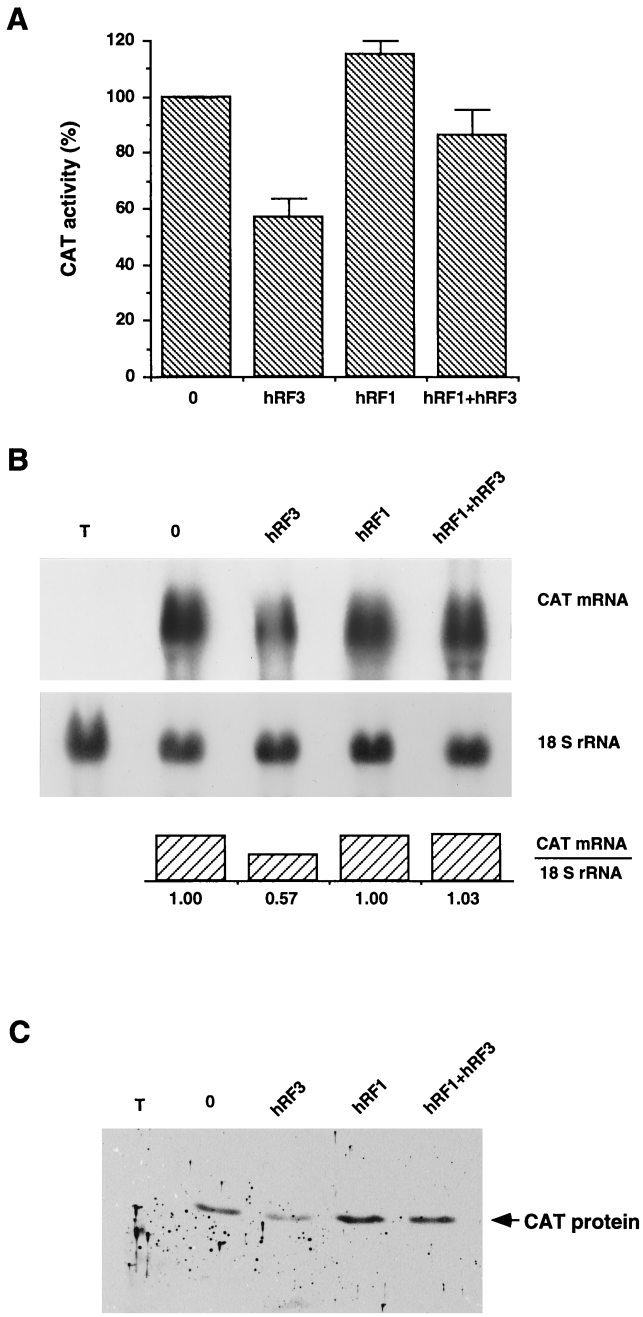

FIG. 3. Effect of overexpression of human eRF1 and eRF3 on wild-type *cat* gene expression. Parallel cultures of 293 cells were cotransfected with 5 μg of pCATwt plasmid DNA and either 10 μg of pCMVhRF1 DNA (lanes and columns hRF1), 10 μg of pCMVhRF3 DNA (lanes and columns hRF3), 10 μg each of both plasmids (lanes and columns hRF1+hRF3), or 20 μg of the vector pBK-CMV alone (lanes and columns 0). The amounts of DNA transfected were equalized in each plate with pBK-CMV DNA. After transfection and transient expression, cell extracts were prepared for CAT assays, Northern blot analysis, and Western blot analysis. (A) CAT activity of transfected cells. The data are means and standard errors for three independent transfection experiments. The level of CAT activity for cells transfected with the wild-type *cat* plasmid (pCATwt) and no eRF1- or eRF3-expressing plasmids (column 0) was assigned a value of 100%, and the relative level of CAT activity for eRF1-, eRF3-, or eRF1- plus eRF3-expressing cells was calculated. (B) Northern blot analysis of *cat* gene mRNA and 18S rRNA. Lane T contains untransfected 293 cells. Total cellular RNA (15 μg), separated on a 1% denaturing agarose gel, was blotted and probed with the *cat* probe or with the 18S rRNA probe. After autoradiography and quantitation of hybridization signals with a Fuji image plate scanner, the membrane was reprobed with the 18S rRNA probe and subjected to autoradiography and surface analysis as described above. The histogram below the autoradiograms represents the ratios of *cat* mRNA to 18S rRNA; the relative level of *cat* mRNA in control cells (column 0) was assigned a value of 1, and the relative levels of *cat* mRNA, indicated below each column, were calculated. (C) Western blot analysis of the CAT protein of one of the transfection experiments described above. Fifteen micrograms of total protein from the cell extract used for the CAT assays was loaded on an SDS–12% polyacrylamide gel. After transfer onto a nitrocellulose membrane, the CAT protein was revealed as described in Materials and Methods with commercial anti-CAT rabbit antibodies. Lane T contains untransfected 293 cells. The arrow on the right indicates the CAT polypeptide.

the moderately stringent washing conditions used (Fig. 5, lane T). However, because the probe was complementary to the anticodon loop of the tRNA, which bore a degenerate sequence at the anticodon position, the melting temperature for $tRNAsu^+$ ochre hybridization was lower than for the other $tRNAsu^+$ species. This explains the low signal observed for $tRNAsu^+$ ochre and the disappearance of the two signals of less intensity detected below the main tRNA bands for $tRNAsu^+$ amber and $tRNAsu^+$ opal. The hybridization signals were quantified, and the ratio of suppressor tRNA to 5.8S rRNA was measured for each sample.

The CAT activity of transfected cell extracts was assayed, and the values were corrected with the suppressor tRNA/5.8S rRNA ratio. CAT activity was then expressed as suppression efficiency (Fig. 6). The value for the CAT activity of the control cells (transfected with the pCAT and ptRNA series of plasmids only) was designated 100% suppression efficiency. As stated above, the overexpression of human eRF3 alone affected *cat* gene expression, and consequently, the CAT activity resulting from readthrough during protein synthesis must also have been affected in the same way. Therefore, the inhibition of CAT activity by 40% of the level of control cells by eRF3 overexpression (Fig. 3A) was added to the CAT activity measured in the suppression experiments. Even though the effects of overexpression of human eRF1 alone or of human eRF1 plus eRF3 on *cat* gene expression were less significant, we have corrected the CAT activity values measured as follows: CAT activity was reduced by 15% when eRF1 alone was overexpressed and was increased by 10% when both eRF1 and eRF3 were overexpressed. The results reported in Fig. 6 correspond to the means of four independent experiments. Whichever stop codon was present at position 27, human eRF3 overexpression had no effect on suppression efficiency. Conversely, overexpression of human eRF1 had a significant antisuppression effect on all three stop codons, i.e., the CAT activity was decreased to 40 to 60% of the level of the control. Surprisingly, when human eRF1 and eRF3 were overexpressed together, the level of antisuppression was only marginally higher than when eRF1 was overexpressed alone. Of course, we cannot exclude from these results the possibility that the eRF3 protein overexpressed was only partially active.

The same pattern of suppression and antisuppression by human eRF1 and eRF3 was obtained when stop codons at different locations were used—the CAG glutamine codons at positions 26 and 38 of the *cat* gene open reading frame were replaced by the amber UAG stop codon (described in reference 32)—in conjunction with the plasmid encoding $tRNAsu^+$ amber (data not shown). Comparing the antisuppression effect of eRF1 overexpression on the three amber stop codons only (at positions 26, 27, and 38), we did not observe any significant variation that could be interpreted as an influence of the sequence surrounding the stop codon. However, in our system, two different processes were involved in the antisuppression effect, i.e., nonsense suppression by a suppressor tRNA and translation termination by the release factors. Both of these

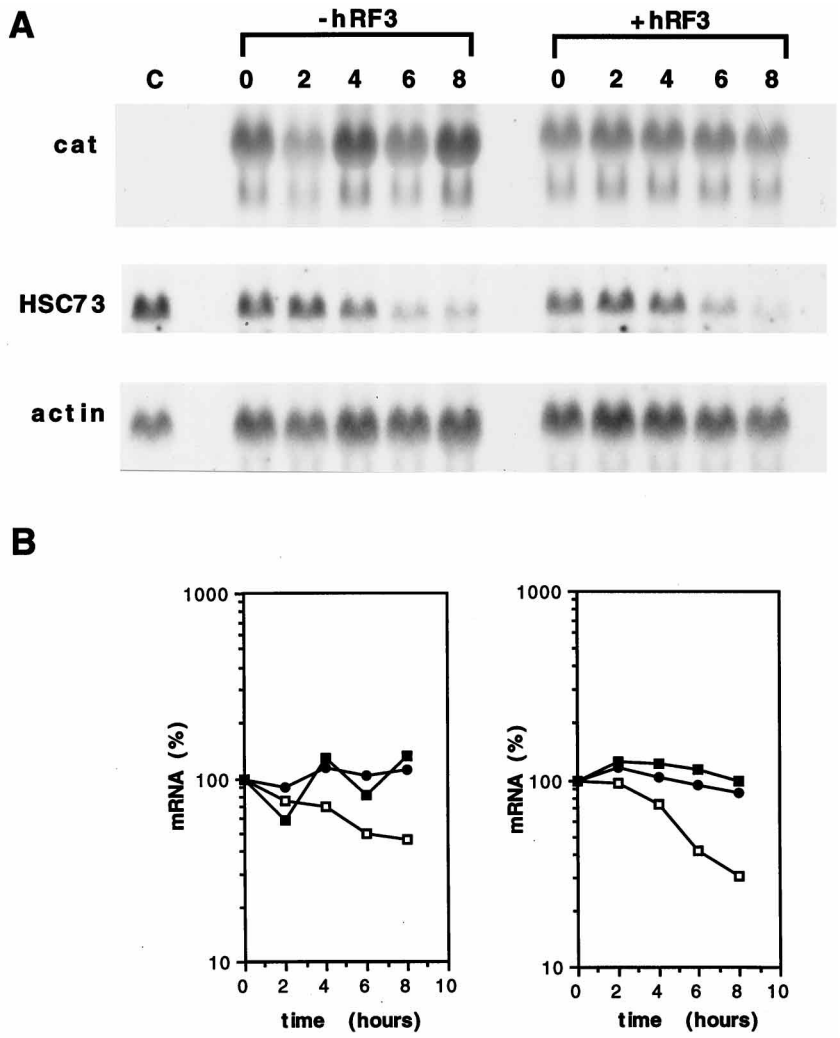

FIG. 4. Northern blot analysis of *cat* mRNA stability in cells overexpressing human eRF3. (A) Parallel 293 cell cultures were cotransfected with the plasmid pCATam27 and either plasmid pBK-CMV (−hRF3) or plasmid pCMVhRF3 (+hRF3). Lane C contains untransfected control cells. Forty-eight hours after transfection, the transfected cells were treated with actinomycin D (5 μg/ml) for the times indicated. Northern blot analysis was performed with 15 μg of total cellular RNA. The nitrocellulose membrane was probed first with a *cat* probe; then the membrane was washed and reprobed with the actin probe, and after being rewashed, the membrane was finally reprobed with the *HSC73* probe. The probes are specified on the left of the autoradiograms. For each hybridization, the signals were quantitated with a Fuji image plate scanner and the membrane was subjected to autoradiography. (B) Kinetics of mRNA decay in 293 cells cotransfected with pBK-CMV on the left or pCMVhRF3 on the right. The results of the image plate scanning were plotted as a function of the time of drug treatment. The level of each mRNA at time zero (i.e., at the time of drug addition) was set to 100% and used to normalize decay curves for each mRNA species. Filled squares, *cat* mRNA; filled circles, actin mRNA; open squares, *HSC73* mRNA.

processes were based on stop codon recognition, and both of them have been described to be influenced in different ways by the 3′ context of the stop codon (31, 33). Thus, the 3′-context influence on termination efficiency may be masked in our system by the parallel effects on suppression.

DISCUSSION

Recently, in vitro experiments have identified two release factors, eRF1 and eRF3, in higher eukaryotes (15, 52). Although eRF1 possesses intrinsic polypeptide chain release factor activity, eRF1-mediated hydrolysis of the ester bond between the polypeptide chain and the tRNA is markedly increased by the addition of eRF3 and GTP to the reaction mixture. eRF3 binds to GTP on its own and has a GTPase activity which requires the presence of eRF1 and ribosomes (16). In *S. cerevisiae*, both the eRF1 and the eRF3 gene (previously named the *SUP45* and *SUP35* genes, respectively) are essential, and mutants of these genes have omnipotent nonsense suppressor phenotypes (26), suggesting that both gene products are required for translation termination. However, overexpression of eRF1 and eRF3 genes in nonsense suppressor tRNA-containing strains of *S. cerevisiae* gave rise to conflicting results. Stansfield et al. (43) claimed that the antisuppressor phenotype was obtained only when both genes were simultaneously overexpressed. Ter-Avanesyan et al. (47) have demonstrated that overexpression of the complete open reading frame of the eRF3 gene induced a suppressor effect and that overexpression of deleted variants lacking the first ATG codon have an antisuppressor effect in various strains. It is well documented now that the N-terminal region of the eRF3 protein is nonessential except for the propagation and maintenance of the allosuppressor [*psi*$^+$] phenotype (6). The [*psi*$^+$] phenomenon was explained recently by the prion model (50),

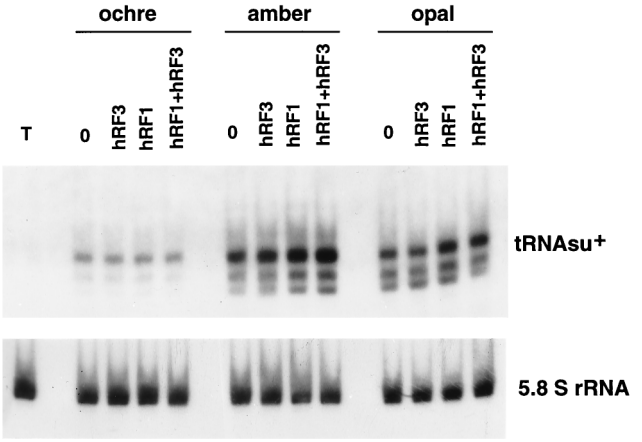

FIG. 5. Example of a Northern blot for determination of suppressor tRNA expression used for quantitation of transfection efficiency in the suppression experiment. Parallel cell cultures were cotransfected with the pCAT series of plasmids containing one of the stop codons (ochre, amber, or opal) at position 27 and the cognate plasmid from the ptRNA series as indicated at the top. In addition, the coprecipitate contains either pCMVhRF1 (lanes hRF1), pCMVhRF3 (lanes hRF3), pCMVhRF1 and pCMVhRF3 (lanes hRF1+hRF3), or pBK-CMV (lanes 0). Lane T contains untransfected control cells. Fifteen micrograms of total cellular RNA was subjected to electrophoresis, transfered to a nylon membrane, and hybridized with the ^{32}P-labelled tRNAsu^{+} probe and the ^{32}P-labelled 5.8S rRNA probe as described in the legend to Fig. 1A. For each probe specified on the right, the membrane was autoradiographed and the hybridization signals were quantitated with a Fuji image plate scanner.

the N-terminal region being considered the prion-forming domain. Aggregation of eRF3 protein has been observed in [*psi*$^{+}$] strains (35, 36), and it was suggested that the aggregated form of eRF3 cannot function in translation termination and was responsible for an enhanced nonsense suppression phenotype (48). In addition, Stansfield et al. (41) have shown recently that the allosuppressor phenotype observed in some yeast strains is likely due to a reduction in the level of eRF1 molecules and suggested that the abundance of eRF1 plays an important role in translation termination efficiency.

In this study, we have undertaken the analysis of the functions of the human release factors when they are overexpressed in human cell cultures. For this purpose, we have applied the techniques and properties of nonsense suppression that have recently been investigated in detail in mammalian cells (32, 38). Our approach was based on the effect of transient overexpression of the release factors on nonsense suppression at various stop codons in a *cat* reporter gene.

Our predominant finding is that human eRF1 has an antisuppressor activity when it is overexpressed in cells. This activity was observed for all three stop codons and in different codon contexts. This result is consistent with the release factor activity attributed to eRF1 in in vitro experiments. Therefore, we suggest that, in vivo, eRF1 is involved in translation termination at the three stop codons and has a stop codon-recognizing activity. Thus, eRF1 can compete with the dominant effect of a suppressor tRNA. Moreover, the increase in the wild-type CAT protein synthesis observed in pCMVhRF1-transfected cells (Fig. 3) indicates that eRF1 overproduction has a stimulatory effect on overall translation and suggests that in human 293 cells, the eRF1 level is a factor limiting the efficiency of translation termination, probably by limiting the formation of the termination complex on translating ribosomes.

The antisuppressor activity of eRF1 in human cells is consistent with the antisuppressor activity demonstrated for the *E. coli* and mitochondrial release factors RF1, RF2, and mRF1 (37, 49). An antisuppressor activity has also been demonstrated for *X. laevis* and human eRF1 in reticulocyte lysates (11). One could question the role of eRF3 in vivo. This role was established in the in vitro system, in which the release factor activity of eRF1 alone was observed only at the saturation level of the stop codons. The release activity at nonsaturation levels of stop codons was dependent upon the presence of eRF3 and GTP (15). Since eRF3 binds GTP, we hypothesize that the proportion of active binary complexes is sensitive to

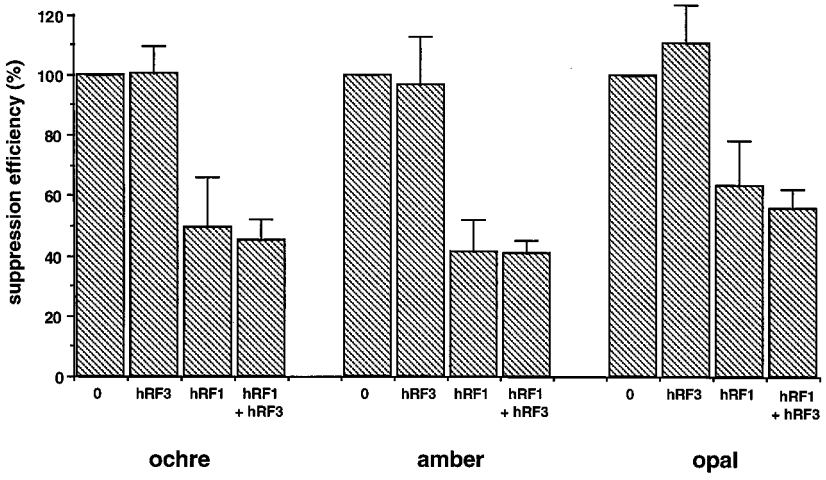

FIG. 6. Influence of overexpression of the human release factors eRF1 and eRF3 on stop codon suppression. Parallel cell cultures of 293 cells were cotransfected as indicated in the legend to Fig. 5. Cell samples were used for a CAT assay and Northern blot analysis as shown in Fig. 5. For each stop codon, the value of CAT activity, expressed in counts per minute per microgram of protein, was corrected with the relative amount of the suppressor tRNA (tRNAsu^{+}/5.8S rRNA ratio) quantitated on the Northern blot membrane. The level of CAT activity for cells that did not overexpress the release factors (columns 0) was assigned a value of 100%, and the relative levels of CAT activity for human eRF1-, eRF3-, and eRF1- plus eRF3-expressing cells were calculated. As indicated in the text, these values were also corrected by addition of the value for inhibitory effects (43% for the hRF3 columns and 13.5% for the hRF1+hRF3 columns) or subtraction of the value for stimulatory effects (15.4% for the hRF1 columns) of the release factors on wild-type *cat* gene expression (see the text and Fig. 3A). The data represent means and standard errors from four independent transfection experiments.

the energy charge of the cell and also to the recycling of the eRF3-GDP complex. Reminiscent of the situation with eukaryotic initiation factor 2 (eIF-2) and eEF-1α (23), two other GTP binding factors involved in translation initiation and elongation, the eRF3-GDP complex must exchange GDP for GTP, a reaction that requires catalysis. Thus, the removal of eRF3-GTP products from the equilibrium reaction mixture by termination complex formation, i.e., ribosomes-eRF1-eRF3-GTP, contributes to a reaction flux toward the eRF3-GTP complex. In our experiments, the presence of an excess of eRF3 poorly influences the efficiency of translation termination even if the reaction flux is modified in favor of eRF3-GTP formation, probably because the amount of eRF1 is the limiting factor for translation termination complex formation.

A consequence of this hypothesis is that the efficiency of translation termination is dependent on the variation in the eRF1 level in the cells. This is also suggested by the results obtained with eRF1 mutants in yeast (41). We have observed in Western blot experiments that there are some important variations in the intracellular level of the eRF3/eRF1 ratio in various tissues of *X. laevis* and mouse (data not shown). It will be of interest to compare the efficiencies of translation termination in the different tissues of higher eukaryotes.

Alternatively, it is possible that eRF3 is not directly involved in the release of the nascent polypeptide from the ribosome but that it induces the dissociation of the termination complex and the recycling of eRF1 molecules. In the in vitro assay, such an activity of eRF3 allows the recycling of the tetranucleotide carrying the stop codon and explains the enhancement of the release activity of eRF1 only at the nonsaturation levels of stop codons. In vivo, the recycling activity of eRF3 is essential only at a low level of eRF1, when all eRF1 molecules are engaged in translation termination complexes. It seems that this is the case with *S. cerevisiae*, in which it has been found that the cellular level of eRF1 is low and that eRF1 is always associated with ribosomes, with about one eRF1 molecule per 20 ribosomes (42). Recycling of eRF1 molecules is absolutely necessary in this situation. This hypothesis also explains why the overexpression of eRF3 alone may have an antisuppression effect (47). The allosuppressor phenotype in *sup35* mutants (mutations in the eRF3 gene) and in [*psi*$^+$] strains (inactivation of eRF3 protein by aggregation) of *S. cerevisiae* can be explained by the absence of eRF1 recycling (36). In mammalian cells, it is possible that the antisuppressor effect of eRF3 can be observed only at a level of eRF1 lower than that of 293 cells.

A puzzling observation is that in *E. coli*, the *prfC* gene, which encodes the polypeptide chain release factor RF3, is not essential (19, 34). Moreover, the homolog of the *prfC* gene was not found in the sequence of the complete genomes of the bacterium *Mycoplasma genitalium* and of the archaeon *Methanococcus jannaschii* when the homolog of the RF1 gene was present (1, 14). Thus, we can speculate that, in this species, either RF3 is not necessary or another GTP-binding protein can perform the function attributed to RF3. To date, an absolute requirement for eRF3 activity in translation termination seems to be specific to *S. cerevisiae.*

One surprising finding during this work was the inhibitory effect on gene expression of human eRF3 when it was overexpressed alone in cells. Although we cannot conclude at which step of gene expression this inhibition occurs, it is suggested by Northern blot analysis and mRNA stability experiments that transcription may be affected (Fig. 3 and 4). Recent results from Ter-Avanesyan et al. (46) emphasize that the eRF3 protein is involved in the regulation of transcription in yeast. When both human eRF1 and eRF3 are overexpressed, the inhibitory effect on transcription is partially prevented, as demonstrated in Fig. 3. Taken together, our data suggest that the inhibitory effect of human eRF3 on transcription is abolished when the efficiency of translation termination is high. Further studies on this inhibitory effect will help us to understand the possible involvement of eRF3 in the transcription process.

The experimental system we describe here in combination with in vitro biochemical studies may be used to establish the precise function of factors involved in the control of the termination of translation and to study other recoding events, like frameshifting.

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