Characterization of the Translation-dependent Step during Iron-regulated Decay of Transferrin Receptor mRNA*

(Received for publication, August 10, 1998, and in revised form, April 2, 1999)

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Iron regulates the stability of the mRNA encoding the transferrin receptor (TfR). When iron is scarce, iron regulatory proteins (IRPs) stabilize TfR mRNA by binding to the 3′-untranslated region. High levels of iron induce degradation of TfR mRNA; the translation inhibitor cycloheximide prevents this. To distinguish between cotranslational mRNA decay and a trans effect of translation inhibitors, we designed a reporter system exploiting the properties of the selectable marker gene thymidine kinase (TK). The 3′-untranslated region of human transferrin receptor, which contains all elements necessary for iron-dependent regulation of mRNA stability, was fused to the TK cDNA. In stably transfected mouse fibroblasts, the expression of the reporter gene was perfectly regulated by iron. Introduction of stop codons in the TK coding sequence or insertion of stable stem-loop structures in the leader sequence did not affect on the iron-dependent regulation of the reporter mRNA. This implies that global translation inhibitors stabilize TfR mRNA in trans. Cycloheximide prevented the destabilization of TfR mRNA only in the presence of active IRPs. Inhibition of IRP inactivation by cycloheximide or by the specific proteasome inhibitor MG132 correlated with the stabilization of TfR mRNA. These observations suggest that inhibition of translation by cycloheximide interferes with the rate-limiting step of iron-induced TfR mRNA decay in a trans-acting mechanism by blocking IRP inactivation.

Regulated degradation of mRNA constitutes an important mechanism for differential gene expression (reviewed in Refs. 1 and 2). Changes in the stability of specific transcripts allow the cell to react quickly to alterations of physiological conditions by manipulating the pool of translatable transcripts. Iron-dependent regulation of transferrin receptor (TfR) mRNA expression represents one of the most intensively studied eukaryotic model systems for regulated mRNA turnover (reviewed in Refs. 3–5). Under low iron conditions, mammalian cells induce iron uptake by increasing the number of TfRs on the cell surface (6–9). This rise in receptor expression is mediated by a dramatic increase in TfR mRNA stability and is dependent on the presence of the 3′-untranslated region of the TfR transcript (10). Five RNA motifs termed iron-responsive elements (IREs) were identified within this region (11, 12). In iron-deprived cells, specific RNA-binding factors, the iron regulatory proteins (IRPs), bind to these regions and prevent the degradation of TfR mRNA, presumably by masking a rapid turnover determinant (13, 14). IRPs also recognize IREs located in the 5′-UTR of ferritin, erythroid δ-aminolevulinic acid synthase, and succinate dehydrogenase b mRNAs; in these cases, binding causes translational repression (15–20).

Two distinct IRPs have been identified in cells of different mammalian species (14, 15, 21–25). Both proteins recognize naturally occurring IREs (23, 24), although with different affinity (26). IRP-1 binds equally well to IREs from ferritin, TfR, m-acotase, and erythroid δ-aminolevulinic acid synthase, whereas IRP-2 has a higher affinity for the ferritin IRE. In addition the RNA binding activities of IRP-1 and IRP-2 are regulated by distinct molecular mechanisms (27–30). In iron-replete cells, IRP-1 contains an iron-sulfur cluster and has low affinity to IREs; in iron-deprived cells, in contrast, the protein is converted into its apo-protein form with high RNA binding affinity (28, 29, 34). A similar type of IRP regulation was previously observed in rabbit cells in response to modulation of cell growth (35, 36).

Finally, the IRE binding activities of IRP-1 and IRP-2 were shown to be differentially regulated in response to non-iron environmental conditions, such as nitric oxide signaling (37–39), oxidation (40, 41), and hypoxia (42–44).

Under high iron conditions, in the absence of active IRPs, TfR mRNA is rapidly degraded. A potential endonucleolytic cleavage site has been identified within the 3′-UTR of the TfR transcript (45). The cutting site was mapped within a previously characterized destabilizing domain close to the IREs (46). The corresponding endonuclease, however, still awaits identification.

As inhibition of transcription by high concentrations of α-amanitin interferes with TfR mRNA decay, it was suggested that RNA polymerase III transcripts may play a role in the degradation process (47). In addition, inhibition of translation by cycloheximide and puromycin was reported to block TfR mRNA decay (11, 47, 48). This effect could either be due to the involvement of highly unstable factors in the degradation of the transcript (trans effect) or to the fact that translation per se is necessary for the decay of TfR mRNA (cis effect). Manipulating
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the translatability by insertion of a ferritin IRE within the 5'-UTR of TIR transcripts, Koeller et al. (48) demonstrated that inhibition of translation in cis had no effect on the rapid turnover of constitutively unstable TIR mutant constructs and TIR/c-fos hybrids. These results would point toward a trans effect of protein synthesis in the case of transferrin receptor mRNA and also of the AU-rich elements (AREs) containing c-fos transcript. However, the constructs used in their study did not allow one to distinguish whether inhibition of translation affects the degradation step or the regulatory system.

We designed a novel reporter system that reflects all aspects of the iron-dependent regulation of TIR transcripts. To this end, the 3'-UTR of human transferrin receptor gene containing all five IREs and the rapid turnover determinant (11, 46) was linked to the coding part of mouse thymidine kinase (TK) cDNA. Efficient inhibition of TK protein synthesis could be easily assayed by enzyme activity assays, immunoblots, and, on a cellular level, by selection for HAT-sensitive transfecteds. Using this reporter system, we set out to characterize the translation-dependent step during the iron-dependent decay of TIR mRNA.

MATERIALS AND METHODS

Plasmid Constructions—All expression vectors are based on the mammalian expression vector pSVL (Amersham Pharmacia Biotech). The 2.3-kb BamHI/BglII fragment of the plasmid pD-TR1 (49), representing the 3'-UTR of the human transferrin receptor cDNA with the complete regulatory region, was ligated in the correct orientation into the BamHI site of the construct pSVLTKΔ30 described previously in Ref. 50. This parental reporter plasmid was termed pTK-hTIR.

The frameshift constructs pSTOP1 and pSTOP2 were obtained by linearization with ApaI and ClaI, respectively, of the plasmid pTK-hTIR, followed by a Klenow fill-in reaction and religation. The changes in the translation reading frame were confirmed by sequencing and expression of the encoded polypeptides by in vitro transcription/translation in reticulocyte lysates (Promega).

To create the construct pSL, the original NcoI site at position 333 of pSVL was converted to a StuI site by standard PCR reaction. 1130 base pairs of the pSVL vector containing the SV40 intron were deleted by ligation to the StuI site at position 1463. The resulting plasmid pSL lacks the SV40 intron and a stretch of untranslated nucleotides of pSVL; it encodes a transcript with a 5' leader sequence shortened from 265 to 50 bases.

For the plasmids pSTEM1 and pSTEM2, encoding for mRNAs with stem-loop structures in their 5'-UTR, synthetic oligonucleotides were phosphorylated with T4 polynucleotide kinase (Promega), annealed, and ligated into the XhoI site of pTK-hTIR. Oligonucleotides 1 (5'-CTCG ACC GCG GCC GCC GCG ACC AAC AAA AGG CCG CCG GCC GCC GCG ACC GCG GCC GCC GCG ACC GCC GCG GCG-3') and 1a (5'-CTCG ACC GCG GCC GCC GCG ACC AAC AAA AGG CCG CCG GCC GCC GCG ACC GCG GCC GCC GCG ACC GCC GCG GCC GCG-3') were annealed for stem-loop STEM1, which corresponds to STEM2 in Ref. 51. Oligonucleotide 2 (5'-ACT AGC AGC GGA GCA CCA AAT TCG TGC GGC GCC GCG GCC GCG GCC-3') of stem-loop STEM2 is similar to STEMN in Ref. 51. The presence of inserted oligonucleotides was confirmed by sequencing.

Cell Culture and DNA Transfection—Mouse LTK- fibroblasts (ATCC CCL 1.3) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (30 mg/liter penicillin, 50 μg/ml streptomycin-sulfate). Transfection and selection for TK expressing transfectants was done as described (50). Cell lines expressing TK frameshift mutants and stem-loop constructs were established by co-transfection with 20 ng of a plasmid conferring hygromycin resistance. Stable transfectants were selected in medium containing 250 μg/ml hygromycin B (Roche Molecular Biochemicals). Pools of 50–100 clones and single colonies were propagated for analysis in further experiments. The regulation of reporter gene expression was essentially the same in mixed cell populations and single clones. Iron chelation was performed by the addition of 50 μM desferrioxamine (Desferal, gift from Ciba-Geigy), where indicated, was added as sodium citrate at 25 μM. To inhibit protein synthesis, cycloheximide was added to the medium at 10 μg/ml. For proteasome inhibition studies, cells were treated with 50 μM MG132 (Peptides International, Louisville, KY).

RNA Isolation and Northern Blot Analysis—Isolation and analysis of cytoplasmic mRNA was performed as described previously (52). The 1.2-kb fragment of the murine TK cDNA (53), the 2.25-kb cDNA of the murine transferrin receptor cDNA clone TR2 (54), and the 1.4-kb fragment of glyceraldehyde-3-phosphate dehydrogenase (55) were used as probes. DNA fragments were labeled with [α-32P]dCTP (Rotem Industries, Israel) using the random-primed DNA labeling kit (Roche Molecular Biochemicals).

Polyosome Profile Analysis—Iron-deprived cells were harvested by trypsination and washed with 1X phosphate-buffered saline. To prepare cytoplasmic lysates, cells were thoroughly resuspended in 1 ml of ice-cold lysis buffer (1 mM potassium acetate, 2 mM magnesium acetate, 250 mM sucrose, 1.5 mM MgCl2, 10 mM dithiothreitol, 100 μg/ml cycloheximide) by pipetting 10 times. Cells were kept on ice for 10 min; then the nuclei were pelleted by centrifugation at 1,500 × g for 10 min at 4 °C. The supernatant was layered on a continuous sucrose gradient (15–40% sucrose in 10 mM Tris acetate, pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, 10 mM diethiothreitol, 100 μg/ml cycloheximide) and separated by centrifugation at 38,000 rpm for 2 h at 4 °C in a SW41Ti rotor (Beckman). Fractions (500 μl) were collected, and each fraction was vigorously mixed with 200 μl of a buffer containing 7 mM urea, 1% SDS, 0.35 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 400 μl of phenol-chloroform. After centrifugation at 14,000 × g for 30 min at 4 °C, 600 μl of the aqueous phase were precipitated with 1 ml of ethanol containing 20 μg of yeast tRNA carrier. The RNA pellet was dissolved in 20 μl of water and loaded on 12% denaturing gels. RNA of all fractions was visualized by electrophoresis, transferred to GeneScreen nylon membranes (NEN Life Science Products), and submitted to Northern blot analysis. A similar protocol was used for the analysis of transcripts after EDTA-induced ribosome release. Cycloheximide was substituted by 10 mM EDTA in both the lysis buffer and the 15–40% sucrose gradients in these experiments. Similarly, high salt gradients contained 500 mM NaCl and 10 mM EDTA.

Protein Extraction and Immunological Methods—Cyttoplasmic proteins were prepared according to the procedure described by Sherr and Kelly (56). Cells were trypsined, pelleted at 200 × g for 4 min, washed, and lysed in digitonin buffer (0.8 mg/ml digitonin, 250 mM sucrose, 1.5 mM MgCl2, 160 mM KCl, 3 mM 2-mercaptoethanol, 50 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μg/ml cycloheximide) for 15 min on ice. Cell fragments were removed by centrifugation at 1,500 × g for 10 min at 4 °C, and the supernatant was used for further analysis. Protein concentrations were determined by the Coomassie Brilliant Blue protein assay (Bio-Rad). Thymidine kinase enzyme assays, immunoprecipitation, and Western blot analysis of the epitope-tagged mouse thymidine kinase protein have been described (50).

Preparation of RNA Transcripts—[32P]-Labeled RNA was transcribed from the linearized plasmid pSPT-fer containing the IRE from the 5'-UTR of human ferritin H chain mRNA as described by Mullner et al. (14). Transcription was carried out in the presence of 1.5 mM ATP, GTP, and UTP, 60 μCi of [α-32P]CTP (800 Ci/mmol, NEN Life Science Products), and T7 RNA polymerase (Promega).

RNA-Protein Band Shift Assays—The IRE-IRE interaction was analyzed as described previously (12, 14). Briefly, protein extract (1–2 μg) was incubated for 10 min at room temperature with an excess (0.2 ng) of [32P]-labeled IRE in vitro transcript in a total volume of 20 μl. After addition of heparin for another 10 min, RNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel and processed for autoradiography.

RESULTS

TK-hTIR Reporter mRNA Stability Is Regulated by Iron—To analyze the effect of translation on the iron-dependent decay of RNA molecules containing TIR control elements, we established a selectable reporter system. The coding region of the mouse thymidine kinase cDNA was fused to the entire 3'-UTR of human TIR cDNA to give the construct TK-hTIR (Fig. 1A). This mRNA, which is expressed from the constitutive SV40 promoter, contains all elements that have been demonstrated to be essential for iron-dependent regulation of mRNA stability (12). The open reading frame encodes a highly stable COOH-terminally-truncated mouse thymidine kinase protein TK-hTIR which contains a c-myc epitope at its amino terminus (50). The expression of this protein has been shown to be independent of growth conditions (50). Cellular levels of the epitope-tagged TK polypeptide can be easily monitored by immunological techniques.
Mouse LTK cells were stably transfected with the construct TK-hTfR to give the corresponding cell line LTK-hTfR. To test whether levels of TK-hTfR mRNA were regulated by iron availability, logarithmically growing cells were depleted of iron by the addition of 50 μM desferrioxamine to the medium. After different periods of time, cytoplasmic RNA was prepared and analyzed by Northern hybridization. LTK cells stably transfected with the parental construct pSVLTK30 giving cell line LTK served as a control to rule out an effect of the iron status on the coding region of the reporter transcript. TK-hTfR mRNA was about 12-fold induced upon iron deprivation (Fig. 1A), whereas the expression of TK transcripts in the control cells was not increased by desferrioxamine (Fig. 1B). As shown in the lower panel, rehybridization with a probe specific for the coding part of murine TfR mRNA revealed a similar induction pattern for the endogenous mTfR transcript. TK-hTfR mRNA expression is therefore clearly dependent on intracellular iron levels. To confirm that the iron-dependent regulation of TK-hTfR reporter mRNA is due to a change in the mRNA stability, we determined the mRNA half-life both under conditions of iron deprivation and abundance. TK-hTfR cells were iron-starved by addition of the iron chelator desferrioxamine for 18 h or iron-loaded by addition of ferric ammonium citrate to the medium for 12 h. Transcription was inhibited by the addition of DRB, and TK-hTfR mRNA levels were determined by Northern blot analysis after different periods of time. In iron-depleted cells, we observed a half-life of TK-hTfR reporter mRNA of about 6 h (Fig. 1C, left panel). The presence of ferric ammonium citrate in the culture medium dramatically reduced the half-life to about 50 min (Fig. 1C, right panel). Similar data have previously been shown for murine TfR mRNA (47) and were confirmed by rehybridization with a probe specific for the endogenous murine TfR transcripts (data not shown). These results clearly indicate that the reporter mRNA and the endogenous TfR mRNA both are regulated by the same mechanism.

Iron-dependent Degradation of TK-hTfR Reporter mRNA Is Uncoupled from Ongoing Translation—A number of rapidly degraded transcripts are markedly stabilized upon the addition of global translation inhibitors. A similar effect of cycloheximide on iron-mediated decay of TfR mRNA has been reported previously (11). Similarly, we observed inhibition of TK-hTfR mRNA degradation by cycloheximide and puromycin in the course of iron repletion in our reporter system (data not shown). Two mechanisms have been proposed to account for the ability of inhibitors of protein synthesis to stabilize labile mRNAs; either highly unstable proteins are involved in the degradation of these mRNAs (trans effect), or translation of the labile mRNAs itself is required for decay (cis effect).

In order to distinguish between these alternatives, two approaches to influence protein synthesis from our reporter mRNA were chosen. First, we investigated whether complete translation of the coding region is a prerequisite for TK-hTfR mRNA degradation by introducing stop codons into the open reading frame of pTK-hTfR. Plasmids pSTOP1 and pSTOP2 encode truncated TK proteins of 51 and 128 amino acids, respectively. In vitro expression in a reticulocyte lysate transcription/translation system yielded polypeptides of the calculated sizes of 5.6 and 14 kDa, respectively (data not shown). The corresponding cell lines LSTOP1 and LSTOP2 expressed no full-length TK protein, as judged by Western blot analysis (Fig. 2A) and enzyme activity assays (data not shown), and were not viable in HAT medium, which selects for transfectants expressing functional TK protein. Although truncated TK protein could be immunoprecipitated from in vitro translation reactions, we were unable to do so in cellular extracts of LSTOP1 and LSTOP2 cells, indicating that the polypeptides might be unstable in vivo. Nevertheless, although the reporter mRNAs of LSTOP1 and LSTOP2 cells were not fully translated, their expression remained strictly iron-regulated (Fig. 2B). We conclude that translation of the complete coding region is not required for the enhanced turnover of TK-hTfR mRNA in response to iron.

In order to investigate whether the polysome distribution of the TK-hTfR reporter RNAs was affected by premature termination of translation, we analyzed the association of these transcripts with ribosomes in linear 15–40% sucrose gradients. The resulting RNA distribution, analyzed on ethidium bromide-stained gels, clearly revealed fractions containing 40, 60, and 80 S ribosomal particles as well as polysomes (Fig. 2C). The localization of the STOP1 mRNA was essentially identical to that of the fully translated TK-hTfR transcript, with both RNAs being predominantly detectable in the polysome fractions 11–15. The same distribution also was found for the STOP2 transcripts (data not shown). Thus, premature termination of translation was without significant consequence for the polysome distribution on the mutant mRNAs.

Interestingly, TK-hTfR reporter mRNA sedimented faster than parental TK mRNA, which was primarily found in fractions 10–13 (Fig. 2C). The relative shift in apparent density was independent of the presence of divalent cations, as removal of ribosomes and other divalent cation-dependent binding proteins by EDTA failed to abrogate the difference in sedimentation. As shown in Fig. 2D, the TK transcript sedimented slowly in an EDTA-containing sucrose gradient (fractions 5–8), whereas TK-hTfR transcripts and endogenous mouse TfR mRNA showed higher sedimentation rates (fractions 7–10). Thus, transcripts containing the 3′-UTR of the TfR mRNA seem to form larger RNA-protein complexes. It is unlikely that this is due to the binding of IRPs to IREs, because a similar polysome profile was found for the low abundant TfR mRNA (and the TK-hTfR transcripts) under high iron conditions, at which IRPs are dissociated from IREs (14 and data not shown). Even in a gradient with more stringent conditions (500 mM NaCl, 10 mM EDTA) TK mRNA sedimented about two fractions slower than TK-hTfR and mTfR transcripts (Fig. 2E). Presumably, other EDTA-insensitive and high salt-resistant
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**Fig. 2. Expression of reporter constructs with premature stop codons.** A, TK protein expression in the different reporter cell lines. Cytosolic proteins were isolated from LTK-hTfR, LSTOP1, and LSTOP2 cells after treatment with Desferal for 18 h. TK protein was immunoprecipitated from 60 µg of total protein from LTK-hTfR cells or 1200 µg of total protein from the other cell lines and analyzed on a Western blot. B, expression of reporter transcripts with nonsense codons under low and high iron levels. Logarithmically growing LSTOP1 and LSTOP2 cells were iron-starved for 18 h (DES) or iron was added for 4 h (FE) and mRNA levels determined by Northern blot analysis. The blots were sequentially hybridized with radiolabeled probes for TK and GAPDH as loading control. A schematic drawing of the reporter mRNAs STOP1 and STOP2 with a translation stop at codons 51 and 128, respectively, is shown on the left. C, polySome profiles of LTK, LTK-hTfR, and LSTOP1 cells. Extracts from iron-deprived cells were separated on 15–40% sucrose gradients as described under “Materials and Methods.” RNA was prepared from all 22 fractions and separated on denaturing 1.2% agarose gels. Distribution of ribosomal complexes is represented by the ethidium bromide stain of one typical gel. mRNA distribution was visualized by Northern hybridization with a radiolabeled TK probe. D, sucrose gradients of LTK and LTK-hTfR cells after EDTA-induced mRNA release from ribosomes. Cellular lysates of iron-starved cells were separated on 15–40% gradients containing 10 mM EDTA. Top panel shows representative distribution of ribosomal RNA from a typical experiment. Northern analysis in the lower three panels was performed with radiolabeled fragments of TK cDNA and mouse TfR cDNA. PolySome profiles of LTK and LTK-hTfR cells under high salt conditions. Cellular lysates from iron-deprived cells were separated on 15–40% gradients containing 500 mM NaCl and 10 mM EDTA. Representative distribution of ribosomal RNA is shown on top panel. Distribution of TK, TK-hTfR, and mouse TfR RNA was visualized by Northern analysis (lower three panels); Northern blots were hybridized with radiolabeled TK cDNA and mouse TfR cDNA probes. Each experiment was performed twice with the same results.

Factors are bound to the TfR-untranslated region, increasing sedimentation of the respective mRNAs.

Next, we addressed the question whether targeted inhibition of translation initiation might interfere with the iron-induced destabilization of TK-hTfR reporter mRNA. To prevent ribosome association on TK-hTfR mRNA, two hairpin structures (STEMB and STEMN in Ref. 51), which had been previously shown to efficiently block initiation of protein synthesis in eukaryotic cells, were introduced into the 5'UTR of the reporter. As such secondary structures are only effective if they are located close to the transcription start site, we first drastically shortened the 265-nucleotide leader sequence of TK-hTfR mRNA. The resulting SL mRNA differs from TK-hTfR mRNA only in possessing a shorter 5'UTR. As expected, expression of the fully translated SL mRNA was regulated by the iron level (Fig. 3A, upper panel). To block translation initiation of SL mRNA, two different hairpin cDNAs were introduced into the unique XhoI cloning site 50 base pairs downstream of the transcription initiation site resulting in plasmids pSTEM1 and pSTEM2. By stably transfecting LTK cells with these constructs, we established the cell lines LSTEM1 and LSTEM2.
Both cell lines were HAT-sensitive, indicating that TK protein synthesis was efficiently inhibited. TK protein was undetectable in cytosolic extracts of these cells, as judged by immuno-precipitations and enzyme activity assays (Fig. 3B and data not shown). Translational inhibition of the stem-loop structures seems to be very efficient, because owing to a half-life of more than 18 h (50), TK protein would accumulate even at low translation rates. To directly demonstrate that the hairpin insertion affected translation initiation of STEM1 mRNA, we evaluated the association with ribosomes in polysome gradients (Fig. 3C). Although the control SL mRNA was predominantly found in polysome-bound fractions (fractions 12–17), insertion of the hairpin (as shown for STEM1) resulted in a shift of the transcripts toward polysome-free fractions (fractions 8–12). The fact that STEM1 transcripts do not shift beyond the 40 S complexes is most probably due to the formation of high molecular weight RNA-protein complexes. This idea is further corroborated by only a minor shift in the polysome profile of STEM1 mRNA after EDTA induced ribosome release (Fig. 3D, compare also with Fig. 2D). Similar results were obtained with cell line LSTEM2 (data not shown). Despite this efficient block of TK translation by insertion of stem-loops, both STEM1 and STEM2 transcripts are still regulated by changes in iron levels as shown by Northern blot analysis (Fig. 3A). Therefore, we concluded that introduction of stable secondary structures into the 5′-UTR of TK-hTIR transcripts results in a considerable change in the polysome profiles of the mRNAs but has no effect on their destabilization by iron. These data are in good agreement with the results of Koeller et al. (48) and indicate a trans effect of translation inhibitors.

Cycloheximide Fails to Stabilize TfR mRNA when Iron Is Abundant—Subsequently, we focused our attention on how global translation inhibitors stabilize TfR mRNA by a trans-acting mechanism. Koeller et al. had suggested that a short-lived component of the degradation apparatus might be affected by global translation inhibitors (48). In their system, translation inhibitors markedly increased levels of a TfR construct, which was intrinsically and constitutively unstable owing to mutation within its regulatory region. To test the effect of cycloheximide on the levels of destabilized mouse TfR mRNA, the translation inhibitor and iron salt were added to iron-depleted L fibroblasts at different time points (Fig. 4A). mTfR mRNA was stabilized when iron-starved cells were treated with cycloheximide and eventually incubated in in new medium containing iron salt and cycloheximide (Fig. 4A, lane b). Similarly mTfR mRNA could be stabilized when iron salt and the translation inhibitor were applied simultaneously (lane c). In contrast, cycloheximide did not affect the low expression of TfR mRNA in iron-loaded cells when added after 4 h of iron treatment (Fig. 4A, lane d). The fact that the translation inhibitor is not sufficient to stabilize TfR transcripts in iron-loaded cells strongly argues against the idea that a labile factor of the degradation machinery is a target for cycloheximide.

In order to exclude the possibility that the translation inhibitor influences transcription of mouse TfR mRNA, we uncoupled RNA synthesis from the degradation process with the transcription inhibitor DRB. The half-lives of both endogenous TfR and TK-hTIR reporter mRNAs were measured in iron-loaded cells as in Fig. 1C, except they were treated with cycloheximide for 2 h prior to DRB addition. As depicted in Fig. 4B, cycloheximide was not sufficient to impede the rapid decay of the transcripts destabilized by iron treatment. In fact the half-life of TK-hTIR mRNA in iron-replete cells was, as analyzed by densitometric scanning of autoradiograms, very similar (about 50 min) in the presence or absence of cycloheximide (compare Fig. 4B with Fig. 1C, right panel). Therefore, the degradation of TfR mRNA under high iron conditions is equally efficient in the absence of de novo synthesis of proteins; consequently, cycloheximide failed to induce TfR mRNA levels when added to iron-loaded L fibroblasts. Previous studies (11, 47) have shown that cycloheximide prevents TfR mRNA degradation when added simultaneously with iron salts; in contrast, we observed that the translation inhibitor was not sufficient to interfere with TfR transcript decay in iron-replete cells (Fig. 4, A and B). This implies that the stabilizing effect of translation inhibitors is dependent on the iron level at the time point of cycloheximide addition.

Inhibition of IRP Inactivation Mediates Stabilization of TfR mRNA during Iron Repletion—Our data suggest that, in mouse L fibroblasts, the mechanism by which inhibitors of protein synthesis stabilize TfR mRNA involves rather the system that regulates TfR mRNA stability (i.e. the IRPs) than factors degrading TfR mRNA. Therefore, we set out to investigate whether the presence of active IRPs in iron-loaded cells is sufficient for TfR mRNA stabilization.

Recently, it was demonstrated that cycloheximide prevented the iron-induced proteolysis of IRP-2 (27, 29). Thus, it was suggested that a labile, yet unidentified factor had to be synthesized in order to inactivate this iron regulatory protein. The same component might also be the key protein whose function is necessary for the iron-dependent degradation of TfR mRNA. To address this question, we first verified the effect of cycloheximide on IRP inactivation in our model system. Desferal-treated LTK-hTIR cells were incubated with iron salts in the absence (Fig. 5A, lanes 1 and 2) or presence (Fig. 5A, lanes 3 and 4) of the translation inhibitor. It can be seen that cycloheximide had only a slight effect on the deactivation of IRP-1, but completely inhibited the disappearance of IRP-2 during...
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FIG. 5. Effect of cycloheximide and MG132 on IRP activity and TfR mRNA expression. A, gel retardation assays of cellular extracts with a radiolabeled transcript of a human ferritin IRE. Extracts were prepared from LTK-hTfR cells iron-deprived for 18 h (DES, lanes 1 and 5). Where indicated, 10 μg/ml cycloheximide was added during the last hour of iron chelation (DES-CHX, lanes 3 and 7). Desferal-treated cells were eventually iron-repleted for 3 h in new medium containing 20 μg/ml ferric ammonium citrate (FE, lanes 2 and 6) or both iron salt and the translation inhibitor (FE-CHX, lanes 4 and 8); cells in lane FE-CHX received cycloheximide treatment throughout the last 4 h before harvesting. In vitro reduction with 2% 2-mercaptoethanol to restore IRP activity is indicated by 2-ME (lanes 5–8). RNA-protein complexes containing IRP-1 or IRP-2 are indicated by arrows. B, Northern blot analysis of cytoplasmic RNA from cells treated with Desferal (lanes 1, 3, and 5) or iron-repleted with ferric ammonium citrate (lanes 2, 4, and 6) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of cycloheximide as described under A. Where indicated (lane 5), iron-starved cells were incubated with 50 μM proteasome inhibitor MG132 for 1 h. In lane FE-MG132 (lane 6), iron-starved cells were treated with MG132 for 1 h; subsequently they were incubated in new medium containing iron salt and the inhibitor for another 3 h. Northern blots were probed with radiolabeled cDNA probes for murine TfR and GAPDH. C, RNA band shift assay with MG132-treated cells. Cells were iron-starved or repleted with iron salt as described in A. Where indicated, cells were incubated with 50 μM MG132 during the last hour of iron starvation. Cells in lane FE-MG132 received MG132 treatment throughout the last 4 h before harvesting. Experiments were performed three times with similar results; the variation, as determined by densitometric scanning, was less than 7%.

In this report, we have analyzed the translation-dependent step during the iron-mediated degradation of TfR mRNA. Interrelationships between protein synthesis and mRNA degradation have been observed for a number of different eukaryotic transcripts in specific cellular systems (recently reviewed in Ref. 59). For example, inhibition of translation in cis was observed to have completely different effects on the stability of different transcripts. On one hand, insertion of a stable secondary structure in the otherwise stable yeast phosphoglycerate kinase 1 mRNA resulted in about 7-fold reduction of its half-life (60). On the other hand, stem-loop insertion into the 5’-UTR stabilized transcripts containing the AU-rich element of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA (61), but was without consequence for the stability of chloramphenicol acetyltransferase transcripts in yeast (62).

The best understood mammalian example for a direct link between translation and mRNA decay is the autoregulation of β-tubulin expression (63, 64). Polysome-associated tubulin transcripts have been shown to be targeted as a substrate for degradation through recognition of the amino-terminal tetrapeptide sequence of the nascent β-tubulin protein after its emergence from the ribosome. Cotranslational mRNA degradation was also reported for the cell cycle-dependent regulation of histone mRNA expression (65). In both cases the presence of a polysome-associated nuclease was suggested (66, 67).

Another group of highly unstable mRNAs encoding proteoncogenes, cytokines, and transcription factors contains AU-rich elements (AREs) within their 3’-UTRs (reviewed in Ref. 68). AU-rich sequences of the GM-CSF and c-fos 3’-UTRs have been shown to destabilize the otherwise stable β-globin reporter transcripts (69, 70). Numerous studies have addressed the mechanism of the decay of ARE-containing transcripts (70–73). The common degradation pathway involves two steps: shortening of the poly(A) tail and subsequent degradation of the mRNA body (71, 74–77). In the case of c-myc mRNA, the deadenylation step was identified as the translation-dependent step during the decay (78). In contrast, deadenylation does not seem to be a prerequisite for TfR mRNA degradation as it was shown that the average length of poly(A) tails of both degradation intermediates and the full-length TfR transcripts are very similar (45).

Studies concerning a cis effect of translation on the decay of AU-rich transcripts yielded divergent results. Although the GM-CSF ARE-directed decay was shown to be translation-dependent (61, 79), degradation of transcripts containing the c-fos ARE was found to be uncoupled from protein synthesis in cis (48). Direct comparison of the two destabilizing elements by different methods in different systems again gave contradictory results (57, 80).

We have analyzed the effect of translation on the decay of TfR mRNA by using a TK-hTfR reporter that contains all five stem-loops and the rapid turnover determinant of the human wild-type TfR mRNA. In their studies, Koeller et al. (57, 58) had used a synthetic minimal construct (TRS-1) containing three stem-loops and the rapid turnover determinant. TRS-1 was recently shown to contain a mutation in stem-loop C at a key residue for IRP binding (81). Although iron-dependent regulation of both TK-hTfR and TRS-1 transcripts is similar to the one of endogenous TfR mRNA, the two reporter constructs showed clear differences in the response to cycloheximide. The steady state

equally potent in inhibiting iron-induced TfR mRNA decay as cycloheximide (Fig. 5B, lanes 4 and 6). These data strongly support the idea that cycloheximide stabilizes TfR mRNA in mouse fibroblasts by impairing the function of a labile protein involved in IRP inactivation.

DISCUSSION

In this report, we have analyzed the translation-dependent step during the iron-mediated degradation of TfR mRNA. Interrelationships between protein synthesis and mRNA degradation have been observed for a number of different eukaryotic transcripts in specific cellular systems (recently reviewed in Ref. 59). For example, inhibition of translation in cis was observed to have completely different effects on the stability of different transcripts. On one hand, insertion of a stable secondary structure in the otherwise stable yeast phosphoglycerate kinase 1 mRNA resulted in about 7-fold reduction of its half-life (60). On the other hand, stem-loop insertion into the 5’-UTR stabilized transcripts containing the AU-rich element of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA (61), but was without consequence for the stability of chloramphenicol acetyltransferase transcripts in yeast (62).

The best understood mammalian example for a direct link between translation and mRNA decay is the autoregulation of β-tubulin expression (63, 64). Polysome-associated tubulin transcripts have been shown to be targeted as a substrate for degradation through recognition of the amino-terminal tetrapeptide sequence of the nascent β-tubulin protein after its emergence from the ribosome. Cotranslational mRNA degradation was also reported for the cell cycle-dependent regulation of histone mRNA expression (65). In both cases the presence of a polysome-associated nuclease was suggested (66, 67).

Another group of highly unstable mRNAs encoding proteoncogenes, cytokines, and transcription factors contains AU-rich elements (AREs) within their 3’-UTRs (reviewed in Ref. 68). AU-rich sequences of the GM-CSF and c-fos 3’-UTRs have been shown to destabilize the otherwise stable β-globin reporter transcripts (69, 70). Numerous studies have addressed the mechanism of the decay of ARE-containing transcripts (70–73). The common degradation pathway involves two steps: shortening of the poly(A) tail and subsequent degradation of the mRNA body (71, 74–77). In the case of c-myc mRNA, the deadenylation step was identified as the translation-dependent step during the decay (78). In contrast, deadenylation does not seem to be a prerequisite for TfR mRNA degradation as it was shown that the average length of poly(A) tails of both degradation intermediates and the full-length TfR transcripts are very similar (45).

Studies concerning a cis effect of translation on the decay of AU-rich transcripts yielded divergent results. Although the GM-CSF ARE-directed decay was shown to be translation-dependent (61, 79), degradation of transcripts containing the c-fos ARE was found to be uncoupled from protein synthesis in cis (48). Direct comparison of the two destabilizing elements by different methods in different systems again gave contradictory results (57, 80).

We have analyzed the effect of translation on the decay of TfR mRNA by using a TK-hTfR reporter that contains all five stem-loops and the rapid turnover determinant of the human wild-type TfR mRNA. In their studies, Koeller et al. (57, 58) had used a synthetic minimal construct (TRS-1) containing three stem-loops and the rapid turnover determinant. TRS-1 was recently shown to contain a mutation in stem-loop C at a key residue for IRP binding (81). Although iron-dependent regulation of both TK-hTfR and TRS-1 transcripts is similar to the one of endogenous TfR mRNA, the two reporter constructs showed clear differences in the response to cycloheximide. The steady state

iron repletion as shown previously (27, 29). Concurrently, mTIR mRNA was stabilized by the translation inhibitor (Fig. 5B, compare lanes 2 and 4). These results fit well into a model according to which a block in IRP-2 degradation by cycloheximide leads to stabilization of TfR mRNA. One prediction would be that stabilization of active IRPs would lead to stable TfR mRNA in iron-replete cells.

To test this hypothesis, we utilized the specific proteasome inhibitor MG132 that had been previously shown to protect IRP-2 against iron-mediated proteolysis (57, 58). RNA-protein band shift analysis confirmed this (Fig. 5C, lanes 1 and 5). In MG132-treated cells, the effect of MG132 on the mRNA binding activity of IRP-2 was similar to that of cycloheximide. The drug efficiently blocked the inactivation of IRP-2 (Fig. 5C). In addition, a considerable fraction of IRP1 (about 25%) remained active in the presence of the proteasome inhibitor (Fig. 5C). This result was reproducible in three independent experiments with less than 7% variation. Similar results were obtained with another proteasome inhibitor, MG115 (data not shown). Importantly, MG132 was
levels of TRS-1 and a constitutively unstable derivative were induced by the translation inhibitor in iron-loaded cells (48), suggesting that cycloheximide affects the function of a short-lived participant in mRNA turnover. In contrast, we find that TK-tIR transcripts and endogenous mouse TfR mRNA both are rapidly degraded in iron-loaded L fibroblasts in the presence of cycloheximide.

However, cycloheximide interferes with iron-induced decay of TfR mRNA when added simultaneously with iron salts to L cells (11, 47). These results strongly argue against the idea that translation inhibitors act via a labile protein directly mediating the iron-dependent endonucleolytic cleavage of TfR transcripts. The simplest hypothesis to explain the iron-dependent effect of cycloheximide is that the regulatory system of TfR stability is a target for the translation inhibitor.

The regulatory proteins that determine the fate of TfR mRNA display unique features. IRP-1 is mutually active as cytosolic aconitase or mRNA-binding protein (41, 57, 58, 82, 83). The enzymatically active form contains a cubane 4Fe-4S cluster and has no RNA binding activity. Disassembly of the Fe-S structure in iron-depleted cells results in a gain of RNA binding activity but a loss of enzymatic activity. IRP-2, on the other hand, has no aconitase activity, but contains a unique domain of 73 amino acids that is required for its iron-dependent decay (24, 28, 29).

Stabilization of IRP-2 by cycloheximide and MG132 prevents not only proteolysis but also the inactivation of this regulatory protein (27–29). Here, we demonstrate that this block in IRP inactivation correlates with the stabilization of TfR transcripts. Surprisingly, MG132 also affected the iron-dependent decrease in IRP-1 binding activity. This result is divergent from data obtained with hamster FTO2B cells (29), but was also found in independent studies with mouse L cells (84), indicating cell type-specific differences in the regulation of IRP activity. A possible explanation for the effect of MG132 on IRP-1 inactivation might be the involvement of another labile factor that is a target of the proteasome. Due to the effect of MG132 on both IRPs, it is difficult to distinguish which of the two proteins mediates the effect of the proteasome inhibitor on TfR mRNA decay. Most likely both RNA-binding proteins, IRP-1 and IRP-2, stabilize TfR mRNA by binding to its IREs. This idea is supported by the findings that both proteins bind TfR and ferritin IREs supported by the findings that both proteins bind TfR and IRP-2, stabilize TfR mRNA by binding to its IREs. This idea is

**Acknowledgments**—We thank F. Yeong, L. Kuhn, E. Müllner, A. von Gabain, and B. Henderson for critically reviewing this manuscript.

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