Effect of Transcription Inhibitors on the Iron-dependent Degradation of Transferrin Receptor mRNA*

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Transferrin receptor (TfR) mRNA expression is tightly linked to intracellular iron levels. Upon iron deprivation, the iron regulatory protein (IRP) stabilizes TfR mRNA by binding to stem-loop structures in its 3′-untranslated region, whereas increased iron levels result in inactivation of the mRNA-binding protein and rapid degradation of TfR mRNA. Although IRP and the regulation of its RNA binding activity have been studied extensively, little is known about the mechanism of TfR mRNA degradation. In order to get more information about factors involved in this process we investigated the in vivo IRP-RNA interaction and the effect of transcription inhibitors on the iron-dependent decay of TfR mRNA. Here we demonstrate that part of the active IRP co-localizes with TfR mRNA to the rough endoplasmic reticulum. High intracellular iron levels led to a drastic reduction of this active RNA-bound IRP in vivo, indicating that IRP dissociates prior to TfR mRNA decay. Furthermore, the transcription inhibitor actinomycin D and translation inhibitor cycloheximide suppressed TfR mRNA degradation but did not interfere with the IRP dissociation step. Other inhibitors of RNA polymerase II had no effect on iron-dependent degradation of TfR mRNA. However, high concentrations of a-aminonon known to block transcription by RNA polymerase III interfered with mRNA decay suggesting the involvement of polymerase III transcripts in the degradation pathway.

Cellular iron acquisition is a prerequisite for cell proliferation. In most vertebrate cells, iron uptake is mediated by transferrin receptor (TfR), whose mRNA expression is modulated inversely proportional to iron availability. This regulation occurs mainly at the post-transcriptional level through the control of mRNA stability. A change in mRNA turnover is mediated by sequences within the 3′-untranslated region (UTR) of the TfR transcript (1). This 3′-UTR contains both rapid turn-over determinant(s) and five hairpin elements referred to as iron responsive elements (IREs) (2, 3). Deletion of the entire 3′-UTR or the rapid turnover determinant yields a constitutively high expression of TfR (3), whereas mutagenesis of all IREs results in rapid unregulated degradation of TfR mRNA (4). IREs are recognized by a cytosolic RNA-binding protein named iron regulatory protein (IRP), previously also referred to as IRE-binding protein (5, 6), iron regulatory factor (7), ferritin repressor protein (8) or p90 (9). The RNA binding activity of IRP is reversibly regulated by intracellular iron levels and gets induced under conditions of iron deprivation. IRP binds also to single copy IREs in other mRNAs, notably in the 5′-UTR of mRNAs coding for H- and L-ferritins (2, 10) as well as the erythroid form of 5-aminolevulinate synthase (11, 12). Here, IRP binding initiates translation. In the case of TfR mRNA at least four of the five IREs are simultaneously bound by IRPs (7, 13), whereas only three of these elements are required for the iron-dependent control of RNA degradation (3, 4). Recently, a potential endonucleolytic cleavage site has been mapped near to one of these indispensable IREs (14). These results, together with the fact that the IRP activity in vivo parallels TfR mRNA levels, suggest that binding of IRP to IREs masks the recognition site for a putative nuclease. However, the pathway of TfR decay and the components involved in this process remain to be characterized. One of the factors essential for the degradation seems to be a short-lived polypeptide because inhibition of protein synthesis by cycloheximide blocks the iron-dependent decay of TfR mRNA (3). This hypothesis is supported by results of Koeller et al. (15), who found that translation inhibition through an IRE in the 5′-UTR had no influence on the decay of a constitutively unstable TfR mRNA. Furthermore, inhibition of transcription by actinomycin D has also been reported to interfere with the iron-dependent degradation of TfR mRNA (3).

In this report we investigated whether the previously observed effects of transcription and translation inhibitors can be attributed to impaired IRP function and/or whether transcription by a particular RNA polymerase is required for the TfR mRNA degradation pathway. We show by cell fractionation that part of the IRP co-localizes with TfR mRNA in the rough endoplasmic reticulum and gets iron-dependently released. However, dissociation of IRP from this fraction was neither impaired by actinomycin D nor cycloheximide, although TfR mRNA degradation was blocked. Specific inhibition of RNA polymerase II-dependent mRNA synthesis failed to interfere with the decay process. However, treatment of mouse L cells with high concentrations of a-aminon significantly inhibited TfR mRNA degradation. These results suggest that polymerase III transcripts may play a crucial role in the pathway of TfR mRNA decay.

**MATERIALS AND METHODS**

Cdl Culture–Mouse Ltk− fibroblasts were grown in minimal essential medium supplemented with 10% fetal calf serum. Iron chelation...
was performed by the addition of 50 µM desferrioxamine (Desferal; gift from Ciba-Geigy) for 20 h, whereas iron was added as ferric ammonium citrate at 20 µM. Where indicated, cells were treated with growth medium containing the following inhibitors of transcription: actinomycin D (Boehringer Mannheim) at 6 µg/ml, a-amanitin (Boehringer Mannheim) at 10 µg/ml or 200 µg/ml, 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (Sigma) at 30 µg/ml, and cordycepin (Boehringer Mannheim) at 25 µg/ml. To inhibit protein synthesis, cycloheximide (Boehringer Mannheim) was added to the medium at 10 µg/ml.

Fractionation of Cellular RNA and Protein—The fractionation scheme is shown in Fig. 1. 2 × 10^7 Ltk− cells were gently lysed in 2 ml of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100) supplemented with 0.5 ml of buffer P-40. After centrifugation (1500 × g for 8 min) at 4°C, the supernatant was supplemented with 1 ml of buffer A, loaded in two equal parts on top of a 30% sucrose cushion (1.5 ml in buffer A, 0.5% Nonidet P-40), and spun for 75 min at 100,000 × g (Beckman SW50.1). The high speed supernatant was harvested in six 0.5-ml fractions, whereas the corresponding pellets were resuspended in 0.1 ml of buffer A. One set of high speed fractions was used for RNA preparation, and the other was analyzed for IRP activity and the rough endoplasmic reticulum (ER) marker protein ribophorin I. In order to recover IRP from ribosome-associated RNA, the resuspended polysomal pellet was adjusted to 0.4 M KCl and extracted by shaking for 30 min at 4°C. After extraction, the polysomes were pelleted, and the supernatant was diluted to 80 mM KCl and centrifuged at 100,000 × g to sediment IRP-bound polysomes. The pellet from the low speed centrifugation contained the nuclei as well as a substantial fraction of ER membranes. After a wash with 0.5 ml lysis buffer, the membranes were extracted from the pellet by a wash with 0.5 ml of lysis buffer supplemented with 0.2% deoxycholate (DOC). The remaining nuclei were pelleted by another low speed centrifugation (1500 × g for 8 min) at 4°C and resuspended in 0.5 ml of lysis buffer. RNA was prepared from 0.4 ml of the wash fraction, the DOC extract, and the nuclear fraction. 50 µl of the resuspended nuclear pellet were adjusted to 0.4 M KCl, and IRP was extracted by shaking for 30 min at 4°C. For Western blot analysis, 50 µl of the nuclear fraction were extracted with lysis buffer containing 1% DOC.

Simplified Extraction of Cytosolic and Membrane-associated Proteins—Cytosolic extracts were prepared from Ltk− fibroblasts by gentle lysis in buffer C containing 10 mM Hepes, pH 7.5, 3 mM MgCl2, 40 mM KCl, 5% glycerol, and 0.2% Nonidet P-40. Nuclei and membranes were pelleted by centrifugation for 4 min at 14,000 × g, and the cytosolic supernatant was frozen in aliquots at −70°C. The pellet containing the nuclei as well as most of the ER membrane association was washed once with buffer C and divided into two equal parts. RNA-bound IRP was extracted from one half by shaking with buffer C with 0.4 M KCl (final concentration) for 30 min at 4°C. Nuclei were removed by centrifugation, and the supernatant was diluted with buffer C to 40 mM KCl and frozen in aliquots at −70°C. The other half of the resuspended pellet was extracted with 0.4 M KCl supplemented with 0.5% DOC. Nuclei were removed by centrifugation and the DOC extract was extracted with lysis buffer containing 1% DOC.

RNA Isolation and Northern Blot Analysis—Cells were lysed in buffer A with 0.2% Nonidet P-40 and 0.5% DOC, and the lysate was spun for 4 min at 14,000 × g. The supernatant was transferred to a microcentrifuge tube containing an equal volume buffer B (10 mM Tris-HCl, pH 7.5, 7.5 mM uracil, 1% SDS, 0.35 mM NaCl, 10 mM EDTA) and 2 volumes phenol/chloroform. After vortexing and centrifugation (14,000 × g for 15 min), the aqueous phase was transferred to a new tube, and RNA was precipitated with 2.5 volumes of ethanol. The integrity and concentration of the RNA was checked on denaturing 1% agarose gels containing formaldehyde. Equal amounts of RNA (20 µg) or the total RNA from each of the cell fractions were then separated on 1% agarose gels containing formaldehyde and transferred to GeneScreen nylon membranes (DuPont NEN). RNA was cross-linked to the membranes by ultraviolet light and hybridized by a novel “sandwich” method. After prehybridization for 2 h, membranes were incubated overnight at 42°C without any agitation between sheets of Whatman filter paper. Filters were probed with 5 × SSPE (0.3 M NaCl, 27 mM NaH2PO4, pH 7.4, 2 mM EDTA), 100 mM sodium phosphate, pH 6.8, 50% formamide, 5 × Denhardt’s solution, 10 mM EDTA, 1% lauroyl sarcosine, 100 µg/ml yeast tRNA, and radiolabeled DNA probe (106 cpm/ml). Hybridization probes were labeled with [α-32P]dCTP (800 Ci/mmol, Amersham Corp.) using a random primed DNA labeling kit (Boehringer Mannheim). The following CDNA were used as probes: the 2.2-kilobase fragment of the murine transferrin receptor cDNA clone pTR-2 (16), the 3.2-kilobase pair DNA encoding the mouse plasmidogen activator inhibitor-1 (PAI-1) (17), or the 1.4-kilobase pair fragment of the rat glyceraldehyde-3-phosphate dehydrogenase CDNA (18).

Western Blot Analysis—50 µl of the different fractions obtained by the fractionation method were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters using a Hoefer TransPhor transfer cell as described by the manufacturer. Proteins were visualized on the filter by reversible staining with Ponseau S and destained in water. Filters were soaked in 1 × phosphate-buffered saline, 1% polyvinylpyrrolidone, 0.1% Tween 20, and 1% nonfat dry milk and incubated overnight with polyclonal rabbit anti-rabbit ribophorin I (serum 19) followed by a 1-h incubation with horseradsh peroxidase-coupled anti-rabbit IgG. The antigen was detected by an ECL kit as described by the supplier (Amer sham Corp.).

Preparation of RNA Transcripts—[32P]-labeled RNA was transcribed from the linearized plasmid pSP73-fer containing the IRE from the 5′-UTR of human ferritin H chain mRNA as described by Müller et al. (7). Transcription was carried out in the presence of 1.5 mM ATP, GTP, and UTP, 60 µCi of [α-32P]CTP (800 Ci/mmol, Amersham Corp.), and T7 RNA polymerase (Promega).

RNA-Protein Band Shift Assays—The IRP-IRE interaction was analyzed as described previously (7). Briefly, protein extract (1–2 µg) was incubated for 10 min at room temperature with an excess (0.2 ng) of [32P]-labeled RNA in a final volume of 20 µl. Unprotected probe was degraded by incubation with 1 unit of RNase T1, for 10 min, and harspin (5 mg/ml) was added for another 10 min to prevent nonspecific binding. RNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel and processed for autoradiography.

RESULTS

Transferrin Receptor mRNA and IRP Co-localize to the Rough Endoplasmic Reticulum—IRP is a key post-transcriptional regulator of proteins involved in mammalian cell iron metabolism. Whereas ferritins and erythroid 5-aminolevulinate synthase are regulated at the level of translation in the cytoplasm, TfR is modulated by changes in mRNA stability and translated on the rough ER. In order to determine the distribution of cellular IRP and TfR mRNA between the cytoplasm and ER membranes and to evaluate the effect of transcription inhibitors on IRP, we first developed a cell fractionation scheme (Fig. 1). To validate this scheme, each of the fractions was analyzed for IRE binding activity, mRNA content, and the presence of ER.

In a typical experiment we tested whether TfR mRNA and IRP changed their cellular localization in response to iron availability (Fig. 2). Cells were treated overnight with the iron chelator desferrioxamine to induce the RNA binding activity of IRP and TfR mRNA or incubated for additional 3 h with ferric ammonium citrate to repress this induction. Cells were then lysed, and fractions were isolated (Fig. 1). Each fraction was analyzed on Western blots with a polyclonal serum raised against the rough ER marker protein ribophorin I (19). Ribophorin was detected mainly in the low speed pellet and DOC extract, as well as to some extent in the pellet of the high speed centrifugation (Fig. 2B). The distribution of TfR mRNA in iron chelator-treated cells, as measured by Northern blot analysis, correlated perfectly with that of ribophorin (Fig. 2A). More than 95% of the total TfR mRNA was found associated with ER membrane fractions. Some TfR mRNA that remained associated with nuclei after extraction with 0.2% DOC could be removed entirely with 1% DOC (not shown). As expected, administration of iron salt to chelator-treated cells strongly diminished the total amount of TfR mRNA. But there was no evidence for TfR mRNA dissociation from the ER containing fractions.

In parallel, IRP was determined both in cytosolic and membrane fractions. Soluble IRP was directly tested for IRE binding in the six sucrose fractions, whereas membrane- and polyclone-associated IRP was first dissociated by high salt extraction with 0.4 M KCl and separated from remaining insoluble material by centrifugation. As shown in Fig. 2C (upper part), most IRP was present in the sucrose fractions and activated due to iron chelation. This soluble IRP was clearly inac-
were further analyzed. The high speed supernatant and corresponding poly-

some pellet was washed, and membrane-associated proteins, including ribo-

some, were extracted with 0.2% deoxycholate (DOC). Remaining nuclei

were repelleted. The high speed supernatant and corresponding poly-

glycerol cushion were used to pellet.

Some of the ER membranes remained associated with nuclei and could be pelleted by low speed centrifugation at 1500 × g. Some membranes, however, formed smaller ER vesicles, which were separated from cytosol by high speed centrifugation through a 30% sucrose cushion. The low speed pellet was washed, and membrane-associated proteins, including ribo-
somes, were extracted with 0.2% deoxycholate (DOC). Remaining nuclei

were repelleted. The high speed supernatant and corresponding poly-
some pellet (P), as well as the low speed wash (W), DOC-extract (D), and

nuclei (N) were harvested and further analyzed.

Activation of IRP has not yet received a rational explanation. In order to
test whether the inhibitors stabilize the IRE-IRP interaction and thereby maybe prevent RNA degradation, we compared the in vivo kinetics of IRP inactivation after iron salt treat-
ment, its disappearance from ER membranes, and the amount of TfR mRNA in these fractions. For such time course experi-
ments, we simplified the cell fractionation procedure by omit-
ing the sucrose cushion. After gentle lysis of cells in hypotonic buffer with 0.2% Nonidet P-40, nuclei and attached ER mem-

branes were immediately pelleted by a short centrifugation. Membrane-associated RNA was then directly isolated from the nuclear pellet by extraction with 0.5% DOC, while RNA-bound IRP was recovered from duplicate pellets by high salt extrac-
tion with 0.4M KCl.

A first set of experiments was performed without the inhibi-
tors. TfR mRNA was more than 20-fold induced after desfer-
rrixamine treatment (Fig. 3A), whereas subsequent addition of ferric ammonium citrate resulted in a rapid disappearance of TfR mRNA within 2 h. In control hybridizations, murine plas-
mogen activator inhibitor 1 (PAI-1) mRNA, unlike TfR mRNA, was markedly induced by iron (Fig. 3A). A similar effect was also observed after the addition of new medium with desfer-
rrixamine and is presumably due to fresh serum.2 Analysis of free cytosolic IRP revealed that IRE binding activity was

2 C. Seiser, unpublished observation.
modulated as a function of iron levels, in parallel to changes in TfR mRNA (Fig. 3B). Inactivated IRP in cytosolic extracts could also be reactivated by in vitro incubation with 2% β-me to yield a constant total IRE binding activity. Membrane-associated IRP was also perfectly regulated by modulations of intracellular iron (Fig. 3C). However, unlike inactivated IRP of cytosol, it responded only slightly to 2% β-me. The experiment was performed twice with the same result.

Thus, inhibition of neither transcription nor translation interfered with the iron-induced dissociation of IRP from ER membranes. It seems therefore that the degradation of TfR mRNA, rather than IRP inactivation, depends on RNA and protein synthesis. Consequently, the simplest explanation for the action of these inhibitors would be to postulate a labile trans-acting factor encoded by a very unstable mRNA. Similar conclusions were reached by Koeller et al. (15), who found that the degradation rate of a constitutively unstable TfR transcript was unaffected by a cis-acting translation block through an IRE in the 5′ UTR. Both results suggest the presence of a labile protein essential for TfR mRNA degradation.

Transcription by RNA polymerase III but not polymerase II is required for TfR mRNA degradation—in order to test for this rise. Inactivation of cytosolic IRP, however, was not influenced by the inhibitor (Fig. 4B).

FIG. 3. Iron-dependent regulation of membrane-associated TfR mRNA and IRP. Exponentially growing Ltk− cells (EXP) were treated with desferrioxamine for 20 h (DES) and then transferred into medium containing 20 μg/ml ferric ammonium citrate (FE). After different periods of time, cells were harvested and lysed, and membrane fractions were isolated by a 0.5% DOC extraction of a rough nuclear pellet as described under “Materials and Methods.” A, Northern blot analysis of membrane-associated RNA (20 μg/lane) was performed by sequential hybridization with labeled cDNA fragments for mouse TfR and PAI-1. B, IRE binding activity of IRP in the cytosolic fractions was determined by a gel retardation assay using a human ferritin H chain IRE probe as described under “Materials and Methods.” Where indicated, samples were preincubated in 2% β-me. C, IRP bound to membrane-associated RNA was recovered by high salt extraction (0.4 M KCl) of the rough nuclear pellet. The extract was diluted to 80 mM KCl, and the IRE binding activity determined as above. Where indicated, samples were preincubated in 2% β-me. The experiment was performed twice with the same result.

FIG. 4. Actinomycin D inhibits the iron-dependent decay of TfR mRNA but not the dissociation of the IRP-RNA complex. Exponentially growing Ltk− cells (EXP) were treated for 20 h with desferrioxamine (DES), and the transcription inhibitor actinomycin D (6 μg/ml) was then added for 1 h to the medium (D/A). Thereafter, fresh medium containing the inhibitor and 20 μg/ml ferric ammonium citrate was added for different periods of time, and cells were processed by the simplified cell fractionation (see “Materials and Methods”). A, membrane-associated RNA was analyzed on a Northern blot by hybridization with labeled cDNA fragments for mouse TfR and PAI-1. B, cytosolic proteins were analyzed for IRE binding activity in the presence or absence of 2% β-me. C, RNA band shift assays were performed using equal amounts of high salt-extracted protein (1 μg) from the low speed pellet as described under “Materials and Methods.” Where indicated, samples were preincubated in 2% β-me. The figure shows typical results from two experiments.

The transcription of TfR mRNA was unaffected by actinomycin D, a compound known to inhibit transcription by intercalation into DNA, did not affect the dissociation of IRP from membrane-bound transcripts.

Similar results were obtained with the translation inhibitor cycloheximide. It inhibited completely the iron-dependent decay of TfR mRNA (Fig. 5A). In contrast, inactivation of cytosolic IRP as well as the dissociation of membrane-associated IRP were unaffected (Fig. 5, A and B). Treatment of cells with cycloheximide for 1 h still in the presence of desferrioxamine led to a slight reduction of the total cytosolic IRP activity. The induction of PAI-1 mRNA was not influenced by cycloheximide.

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Transcription by RNA polymerase III but not polymerase II is required for TfR mRNA Degradation—In order to test for this rise. Inactivation of cytosolic IRP, however, was not influenced by the inhibitor (Fig. 4B). More importantly, membrane-associated IRP declined with the same kinetics as in the absence of actinomycin D (compare Fig. 4C with Fig. 3C). Thus, actinomycin D, a compound known to inhibit transcription by intercalation into DNA, did not affect the dissociation of IRP from membrane-bound transcripts.

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regulation of transferrin receptor mRNA stability

whether the hypothetical trans-acting factor is encoded by an unstable transcript, we investigated how inhibitors of mRNA synthesis other than actinomycin D affect the iron-dependent decay of TfR mRNA; DRB and α-amanitin at a low concentration (10 μg/ml) specifically inhibit RNA polymerase II, whereas cordycepin interferes with the poly(A)-tail formation of mRNAs (26, 27).

TfR mRNA in Ltk− fibroblasts was first induced by desferrioxamine. Prior to iron levels, transcription inhibitors were added for 1 h and then maintained for 3 h further after the addition of iron salt. In contrast to actinomycin D, cordycepin at low concentrations of α-amanitin nor DRB had an effect on the iron-induced decay (Fig. 5B). However, the RNA polymerase II-specific inhibitors abolished transcription of PAI-1 mRNA. This excludes the possibility of insufficient cellular uptake of these drugs. Expression of glyceraldehyde-3-phosphate dehydrogenase mRNA, a highly stable transcript, was not affected by the inhibitors. Similarly, poly(A)-tail shortening, a prerequisite for the degradation of c-myc mRNA (28) did not seem to affect the TfR mRNA half-life; cordycepin had no effect on iron-dependent decay or steady state levels of TfR mRNA, even in a more extended kinetic study, whereas c-myc transcript levels were strongly reduced (data not shown). These results are consistent with the recently published observation that shortening of the poly(A)-tail length is not necessary to create a 3′ degradation intermediate of TfR mRNA (14). By using DRB rather than actinomycin D, we have also been able to measure the rate of TfR mRNA decay with accuracy (data not shown). The half-life of TfR mRNA was about 3.5 h in exponentially growing Ltk− cells in normal medium. This value increased to more than 6 h in cultures treated with 50 μM desferrioxamine. In contrast, high intracellular iron levels reduced the half-life of TfR mRNA to less than 60 min.

Taken together, the data indicate that mRNA synthesis by RNA polymerase II is not essential for the iron-dependent regulation of TfR mRNA expression. Because in contrast to the other inhibitors, actinomycin D affects all three RNA polymerases, we tested the possibility that RNA polymerase III might be necessary for TfR mRNA degradation. This enzyme is responsible for the transcription of tRNAs, 5S ribosomal RNA, and some other small RNA molecules. High concentrations of α-amanitin are known to block polymerase III by direct interaction with the protein (29, 30). We therefore tested the effect of a high dose of α-amanitin (200 μg/ml) on the regulation of TfR mRNA stability. Pretreatment with the inhibitor for 1 h resulted in nearly complete inhibition of iron-induced degradation during the first 2 h (Fig. 7). After 4 h, 20% of the TfR mRNA were still left, whereas TfR transcript levels in cells treated with iron alone were undetectable. Simultaneous addition of 200 μg/ml α-amanitin and iron salt, however, had no inhibitory effect on the TfR mRNA decay. As expected, induction of the control PAI-1 mRNA was completely abolished by α-amanitin. These results suggest that the presence of labile polymerase III transcripts is essential for the degradation of TfR mRNA. The stabilization of TfR mRNA in cells treated with α-amanitin was not complete, but the decay is clearly delayed by the drug. The slight difference in the effect of actinomycin D and α-amanitin is possibly due to a slower uptake of the latter by the cell. This could also explain why α-amanitin has no effect when added simultaneously with iron salt.

DISCUSSION

The iron-dependent regulation of TfR mRNA is one of the best studied examples for a post-transcriptional control of mRNA stability. According to the prevalent model, iron-deprived IRP, the [Fe-S]-free apoprotein form of cytoplasmic acinatase (21, 31–33) acts as a RNA-binding protein with high affinity for IREs and thereby protects the TfR mRNA 3′ UTR from endonucleolytic attack (4, 7). This hypothesis is based on functional assays with numerous 3′-UTR mutants (3, 4) and has recently gained support from the identification of a putative endonuclease cleavage site next to the IREs (14). The model implies that iron favors dissociation of IRP from TfR mRNA, which then becomes accessible to a nuclease. However, neither the in vivo IRE-IRP dissociation nor the enzymes in-

FIG. 6. Effect of different RNA synthesis inhibitors on TfR mRNA decay. Murine Ltk− fibroblasts were treated with desferrioxamine for 20 h (DES) and then for 3 h with ferric ammonium citrate (FE). Where indicated, actinomycin D (6 μg/ml), cordycepin (25 μg/ml), α-amanitin (10 μg/ml), or DRB (30 μg/ml) was added together with the iron salt (FE+) after a 1-h preincubation with the inhibitor in the presence of desferrioxamine. The Northern blot was hybridized with probes for murine TfR, PAI-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are representative for two independent experiments.

Effect of cycloheximide on the iron-dependent degradation of TfR mRNA and the IRP-IRE interaction. Exponentially growing Ltk− cells (EXP) were treated with desferrioxamine for 20 h (DES). 1 h after the addition of 10 μg/ml cycloheximide to the medium (D/C), new medium with 20 μg/ml ferric ammonium citrate and cycloheximide (CHX + FE) was added for different periods of time. Membrane-associated RNA and IRP bound to this RNA were prepared as described under “Materials and Methods.” A, membrane-associated RNA was analyzed on a Northern blot using radiolabeled cDNA fragments for mouse TfR and PAI-1 as probes. B, a gel retardation assay with cytosolic extracts was performed. Where indicated, samples were preincubated in 2% β-me. The decay of TfR mRNA; DRB and α-amanitin interferes with the poly(A)-tail formation of mRNAs (10 mM DES) and then for 3 h with ferric ammonium citrate (FE). Where indicated, actinomycin D (6 μg/ml), cordycepin (25 μg/ml), α-amanitin (10 μg/ml), or DRB (30 μg/ml) was added together with the iron salt (FE+) after a 1-h preincubation with the inhibitor in the presence of desferrioxamine. The Northern blot was hybridized with probes for murine TfR, PAI-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are representative for two independent experiments.

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Effect of cycloheximide on the iron-dependent degradation of TfR mRNA and the IRP-IRE interaction. Exponentially growing Ltk− cells (EXP) were treated with desferrioxamine for 20 h (DES). 1 h after the addition of 10 μg/ml cycloheximide to the medium (D/C), new medium with 20 μg/ml ferric ammonium citrate and cycloheximide (CHX + FE) was added for different periods of time. Membrane-associated RNA and IRP bound to this RNA were prepared as described under “Materials and Methods.” A, membrane-associated RNA was analyzed on a Northern blot using radiolabeled cDNA fragments for mouse TfR and PAI-1 as probes. B, a gel retardation assay with cytosolic extracts was performed. Where indicated, samples were preincubated in 2% β-me. The decay of TfR mRNA; DRB and α-amanitin interferes with the poly(A)-tail formation of mRNAs (10 mM DES) and then for 3 h with ferric ammonium citrate (FE). Where indicated, actinomycin D (6 μg/ml), cordycepin (25 μg/ml), α-amanitin (10 μg/ml), or DRB (30 μg/ml) was added together with the iron salt (FE+) after a 1-h preincubation with the inhibitor in the presence of desferrioxamine. The Northern blot was hybridized with probes for murine TfR, PAI-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are representative for two independent experiments.
volved in RNA degradation have been characterized. Most intriguingly, we have observed that cycloheximide and actinomycin D are inhibitory to rapid degradation of TfR mRNA (3). As the decay of several other short-lived mRNAs was reported to be blocked by translation and/or transcription inhibitors too, it seemed of interest to elucidate whether the inhibitors stabilize TfR mRNA by effects on IRP or at a subsequent step. To approach this question we developed a cell fractionation procedure that permits the simultaneous analysis of rough ER-associated IRP and TfR mRNA. The method takes advantage of a mild detergent lysis that leaves the rough ER mostly intact and attached to nuclei, such that it is readily separated from the cytosol by low speed centrifugation. mRNA was then extracted with DOC, and IRP was dissociated in high salt. Using this procedure we find an iron-dependent association of IRP with the ER, which comprises about 14% of the active cellular IRP under iron-chelating conditions, and virtually none under conditions of high iron supply. This relative amount as compared with total IRP may be slightly overestimated, because we did not measure all IRP in the cytosol where some is also bound to mRNAs (34). As IRP interacts strongly with IREs in a 1:1 stoichiometry but was never reported to bind to other cellular components, we conclude that rough ER-associated IRP consisted mainly of active IRP that was bound in vivo to transcripts with an IRE. This conclusion is supported by the properties of the extracted IRP, as well as some IRP-2, that showed a high IRE binding affinity and no further in vitro activation by 2% β-mer.

Very importantly, IRP in ER fractions coincided with the presence of TfR mRNA, suggesting strongly its interaction with this mRNA, but possibly also other, hitherto unidentified, IRE-containing transcripts. Whereas high iron, either in the absence or presence of cycloheximide and actinomycin D, dislodged IRP from the rough ER, TfR mRNA decayed only in the absence of inhibitors but remained ER-bound and stable in their presence. This demonstrates for the first time iron-dependent in vivo dissociation of IRP from IRE-containing mRNAs without an obligatory degradation of these mRNAs or their release from the ER. The results are in agreement with the prevalent model, according to which the initially active RNA-protecting IRP gets inactivated by iron and dissociates from TfR mRNA, thereby unmasking a ribonuclease cleavage site. Thus, actinomycin D and cycloheximide interfere with this latter step by stabilizing the unprotected TfR transcript but have no effect on IRP dissociation.

A link between translation and RNA degradation has been shown in numerous instances, notably through the inhibition of mRNA decay by cycloheximide (35–37). In the case of early immediate transcripts with AU-rich elements, like c-myc and c-fos mRNA, degradation is preceded by poly(A)-tail shortening (38). For c-myc mRNA this step was reported to be blocked by translation inhibition (28). In the yeast Saccharomyces cerevisiae, a similar deadenylation step is presumably followed by decapping and 5′ to 3′ exoribonuclease degradation (reviewed in Ref. 39), and cycloheximide seems to inhibit the decapping reaction rather than translation elongation (40). For vertebrate mRNAs the role of decapping is not yet clarified, and certain data speak in favor of a trans- rather than a dis-acting effect of cycloheximide on c-fos and TfR mRNA degradation (15). When translation rates of chimeric mRNAs with 3′ instability elements of c-fos or TfR mRNA were modulated 20-fold through a 5′ IRE, no change in RNA half-life was observed (15). Moreover, in contrast to most other degradation pathways, decay of TfR mRNA is not preceded by the shortening of its poly(A)-tail (14). The trans-acting factor that is sensitive to translation inhibition remains to be characterized. Yet, our study eliminates IRP as a possible target of cycloheximide.

The conclusion concerning actinomycin D is similar to that for cycloheximide: actinomycin D does not block RNA turnover by interference with IRP inactivation. Thus, its effect on the iron-dependent degradation of TfR mRNA must be at a subsequent step. This block was strongest when the inhibitor was added to iron-deprived cells simultaneously with or shortly before the iron salt (this report and Ref. 3) and was present during the time period when most of the TfR mRNA gets degraded. However, in a quite different physiological situation, a similar effect has been observed; in the mouse T cell line B6.1, a decrease in TfR mRNA expression after IL-2 withdrawal was also blocked by actinomycin D (41). These findings seem somewhat different from a study where chimeric TfR mRNA constructs were analyzed in murine B6 fibroblasts and where actinomycin D was successfully used as a transcriptional inhibitor to determine mRNA stability in different iron conditions (15). In these experiments, however, the iron source hemin was added many hours before the transcription inhibitor. It seems possible, therefore, that actinomycin D affects an iron-dependent step in mRNA decay other than IRP inactivation and fails to block TfR degradation when added long after iron. Alternatively, different cell lines might respond to actinomycin D in different ways. Independent of the possible cause for these differences, it was rewarding to find that DRB, a ribonucleotide analog that interacts with the RNA polymerase II transcription complex (42, 43), apparently did not interfere with RNA decay and provided an excellent tool to measure the stability of TfR mRNA. Consequently, it became possible for the first time to measure accurately the half-life of wild-type TfR mRNA in conditions of high intracellular iron levels. A half-life of less than 1 h was found, which is in good agreement with the results obtained with TfR mRNA constructs containing the minimal regulatory region (15). As expected, the stability of TfR mRNA increased dramatically to a half-life of more than 6 h after treatment of cells with desferrioxamine.

Actinomycin D-induced stabilization was observed for several other mRNAs besides TfR mRNA. The transcript for the RIIβ subunit of protein kinase A is transiently induced by cAMP and thought to be stabilized in the nucleus by polymerase II transcription inhibitors (44). Likewise, there is a transcription-dependent degradation of neurofilament mRNA in axotomized sensory neurons (45). During differentiation of rat myoblast cells in culture, creatine phosphokinase is strongly inducible by insulin but disappears rapidly after hormone removal. This apparently cytoplasmic degradation of mRNA is inhibited by actinomycin D (46). A dexamethasone-induced modulation of the mRNA turnover has been observed for sev-
eral AU-rich mRNAs and is, at least in the case of the uroki-
nase type plasminogen activator mRNA, inhibited by actino-
mycin D (47). In some cases DRB was shown to exhibit an effect
similar to that of actinomycin D (44, 45). These results were
usually interpreted by a model that predicts the involvement in
mRNA turnover of a labile protein encoded by an unstable
transcript. In the case of TFR mRNA decay, however, the effect
of actinomycin D seems to be distinct because specific inhibi-
tors of mRNA synthesis like DRB or α-amanitin (at 10 μg/ml)
fail to interfere with the iron-dependent decay of TFR mRNA
(Fig. 6).

Thus, it remains an important question how actinomycin D
acts. Besides its effect on transcription by intercalating into
DNA, actinomycin D might interfere with mRNA degradation
by intercalating into double-stranded RNA regions. However,
this would not provide a satisfactory explanation for why the
high concentrations of α-amanitin, a transcription inhibitor
which directly interacts with RNA polymerases, can also affect
TFR mRNA degradation. In fact it is known that actinomycin D
and high concentrations of α-amanitin are able to inhibit RNA
polymerase III, an enzyme responsible for the synthesis of
small transcripts like 5 S ribosomal RNA and transfer RNA
(30). Because we demonstrate that both inhibitors are able to
block TFR mRNA decay, whereas other conditions of transcrip-
tional inhibition do not, we propose that a RNA polymerase III
product might be important as a component of the TFR mRNA
decay machinery. Small RNAs have been shown to play a role in
RNA splicing (48) and processing of histone pre-mRNAs
(49–51) and are suspected as potential components for mRNA
turnover and translation. RNase P and RNase MRP are related
ribonucleoproteins involved in cleaving of tRNA precursors and
processing of mitochondrial primer RNAs (52, 53). Both func-
tions require the presence of RNA components. A small RNA
polymerase III transcript was also reported to be responsible
for the regulation of translation of the myosin heavy chain
mRNA (54, 55). Furthermore, a potential role of polymerase III
transcripts from B2 repeats was discussed in the regulation of
mRNAs containing AU-rich elements (56), and Brewer and
Ross (57) purified a labile destabilizer that accelerates the
gartrator of RNA synthesis in an in vitro system. The factor
contains an essential nucleic acid component. It will be of
particular importance now to biochemically characterize the trans-
acting factors that are involved in the iron-dependent
degradation of TFR mRNA, in order to establish firmly the role
of polymerase III transcripts in the endonucleolytic cleavage of
this mRNA.

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