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Iron regulates ferritin mRNA translation through a segment of its 5′ untranslated region

(translational control/regulatory sequence/secondary structure)

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ABSTRACT **In previous studies, we showed that acute administration of iron to intact rats or to rat hepatoma cells in culture induces synthesis of the iron-storage protein ferritin by activating translation of inactive cytoplasmic ferritin mRNAs for both the heavy (H) and the light (L) subunits. In the course of activation, these ferritin mRNAs are recruited onto polysomes. To elucidate the structural features of these mRNAs involved in the translational response to iron, a chimera was constructed from the 5′ and 3′ untranslated regions (UTRs) of ferritin L subunit mRNA fused to the reading frame of the mRNA of bacterial chloramphenicol acetyltransferase (CAT). This chimera and deletion constructs derived from it were introduced into a rat hepatoma cell line by retrovirus-mediated gene transfer. The complete chimera showed increased CAT activity in response to iron enrichment of the medium, whereas deletion of the first 67 nucleotides of the 5′ UTR, which contain a highly conserved sequence, caused loss of regulation by iron. Whereas cis-acting sequences located in the 5′ flanking regions of many genes have been repeatedly implicated in modulating their transcriptional expression, we report here a specific regulatory translational sequence found within the 5′ UTR of a eukaryotic mRNA.**

The iron-storage protein ferritin ($M_r \approx 450,000$) consists in mammals of two types of subunits, a heavier H ($M_r \approx 22,000$) and a slightly lighter L ($M_r \approx 20,000$) type. Iron administered to rats (1) or added to rat hepatoma cells in culture (2) rapidly increases synthesis of both subunits by mobilizing inactive ferritin L and H mRNAs onto polysomes. This report explores features of the rat ferritin L mRNA involved in this activation process. The 5′ untranslated regions (UTRs) of both ferritin mRNAs are unusually long (210 and 168 nucleotides, respectively, for the L and H subunits of the rat), thus differing from most eukaryotic messages with 5′ UTRs commonly of 30–60 nucleotides (3). As determined by computer-aided analysis, the first 75 nucleotides of the 5′ UTR of L and H subunits in human, rat, and frog yield putative stem-loop structures with calculated free energies of formation (ΔG values) of −46.1 kcal/mol for rat L mRNA (Fig. 1) and −40.3 kcal/mol for rat H mRNA (1 kcal = 4.18 kJ). Within this stem-loop structure there is a highly conserved stretch of 28 nucleotides showing almost perfect homology in both H and L mRNAs so far isolated from human (4, 5), rat (6, 7), chicken (8), and frog (9) compared with overall homology of the complete L and H subunit mRNAs of only about 50% in rat and human. To examine the molecular basis for the translational regulation of ferritin mRNA, notably involvement of the untranslated regions, a chimeric gene containing the 5′ and 3′ UTRs of the ferritin L subunit mRNA fused to the reading frame and 3′ UTR of the bacterial chloramphenicol acetyltransferase (CAT) gene has been constructed. This chimeric gene and two deletion constructs were introduced into rat hepatoma cell line FTO-2B by means of retroviral infection.

FIG. 1. Secondary structure within the 5′ UTR of ferritin mRNA. Secondary stem-loop structure within the first 76 nucleotides of the 5′ UTR of rat ferritin L mRNA, showing the position of the conserved sequence (boxed in). The numbers at the bottom denote the first and last nucleotides of the 5′ UTR represented in this figure.

MATERIALS AND METHODS

Cell Culture, DNA Transfection, and Viral Infection. The FTO-2B cell line was grown in Dulbecco's modified Eagle's medium (DMEM) and F-12 medium (1:1) containing 10% fetal calf serum. Transfections were done by the calcium phosphate technique (10). Eighteen hours after glycerol shock, the culture medium was collected and used to infect FTO-2B cells (11). Forty-eight hours later cells were selected in complete medium containing G418 (GIBCO) at 500 μg/ml. After the appearance of resistant colonies, the cells either were pooled or were isolated by using cloning cylinders.

DNA Manipulation for Construction of Plasmids. Restriction enzymes (New England Biolabs or Boehringer Mann-

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Abbreviations: CAT, chloramphenicol acetyltransferase; H, heavy; L, light; UTR, untranslated region; LTR, long terminal repeat.

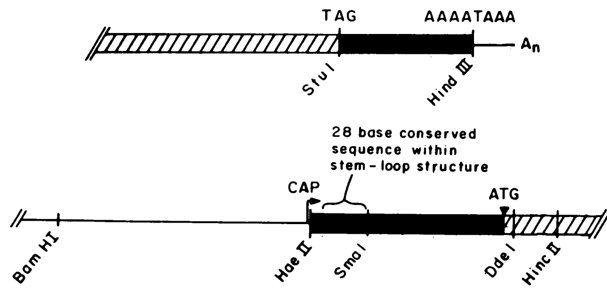

FIG. 2. Restriction sites in the 3′ UTR of the L cDNA (*Upper*) and the 5′ UTR of the L gene (*Lower*), used in construction of the fusion gene. Here and in Figs. 3 and 4 the solid bars represent the UTRs and the hatched bars represent the coding regions. The thin line in *Upper* represents nucleotides immediately preceding the poly(A) tail (A_n) and that in *Lower* denotes the 5′ flanking region of the ferritin gene.

heim) were used according to the suppliers' specifications. Linker ligations were performed (12). DNA fragments were purified by excising the band from a gel, then electroeluting the DNA in a dialysis bag. Double-stranded sequencing of the fusion gene was carried out by using a pUC vector (13). Plasmid DNA was prepared on a small scale (14) for restriction analysis or on a large scale (15) for preparative purposes.

Iron Treatment of Cells and CAT Assays. Cells transfected with pZip-5CAT3 were stimulated with iron by adding ferric ammonium citrate (green) to give 60 μM iron in the medium for 4 hr. With other vectors, cells were treated with iron from hemin (100 μM) for 12–16 hr, additional hemin being added to make the concentration in the medium 200 μM for another 4 hr before harvesting. Cells were lysed by repeated freezing and thawing, and CAT activity was assayed (16).

Plasmid Construction. The murine retroviral vectors pWE (17), pLJ(DOL-) (18), and pZipneoSV(X) (11), which contain the gene for neomycin (or G418) resistance, were used. In pWE this selectable marker is driven by the viral long terminal repeat (LTR) promoter, while the test gene is driven by an internal promoter sequence of the β-actin gene. In pLJ the test gene is driven by the viral LTR promoter and the selectable neomycin resistance marker is driven by an internal simian virus 40 promoter. In pZipneoSV(X) both the test and the selectable marker gene are transcribed on a full-length genomic RNA by the viral LTR promoter. To insert the CAT reading frame we used pMC, a pUC plasmid containing the CAT gene derived from pSV2cat. The 5′ UTR of the CAT gene was removed and the initiator codon ATG was destroyed by BAL-31 resection up to the adenylate nucleotide and cloned in the *Bam*HI site of pUC-13 with *Bam*HI linkers ligated at both ends of the gene.

Fusion of the Complete 3′ UTR of Ferritin L cDNA to the 3′ End of CAT. The 3′ UTR of ferritin L cDNA lies within a 144-base-pair (bp) *Stu* I–*Hin*dIII fragment (Fig. 2 *Upper*). This fragment was excised from the L cDNA insert of pRLFL3 (19) and purified by gel electrophoresis. The 3′ end of the CAT gene in pMC was released by a partial *Bam*HI digestion. The ends of the *Bam*HI site were made flush by T4 DNA polymerase and then the DNA was cut at the *Hin*dIII site located downstream from the CAT gene within the multiple cloning site of the pUC vector. The 3′ UTR *Stu* I–*Hin*dIII fragment was cloned in the filled-in *Bam*HI and

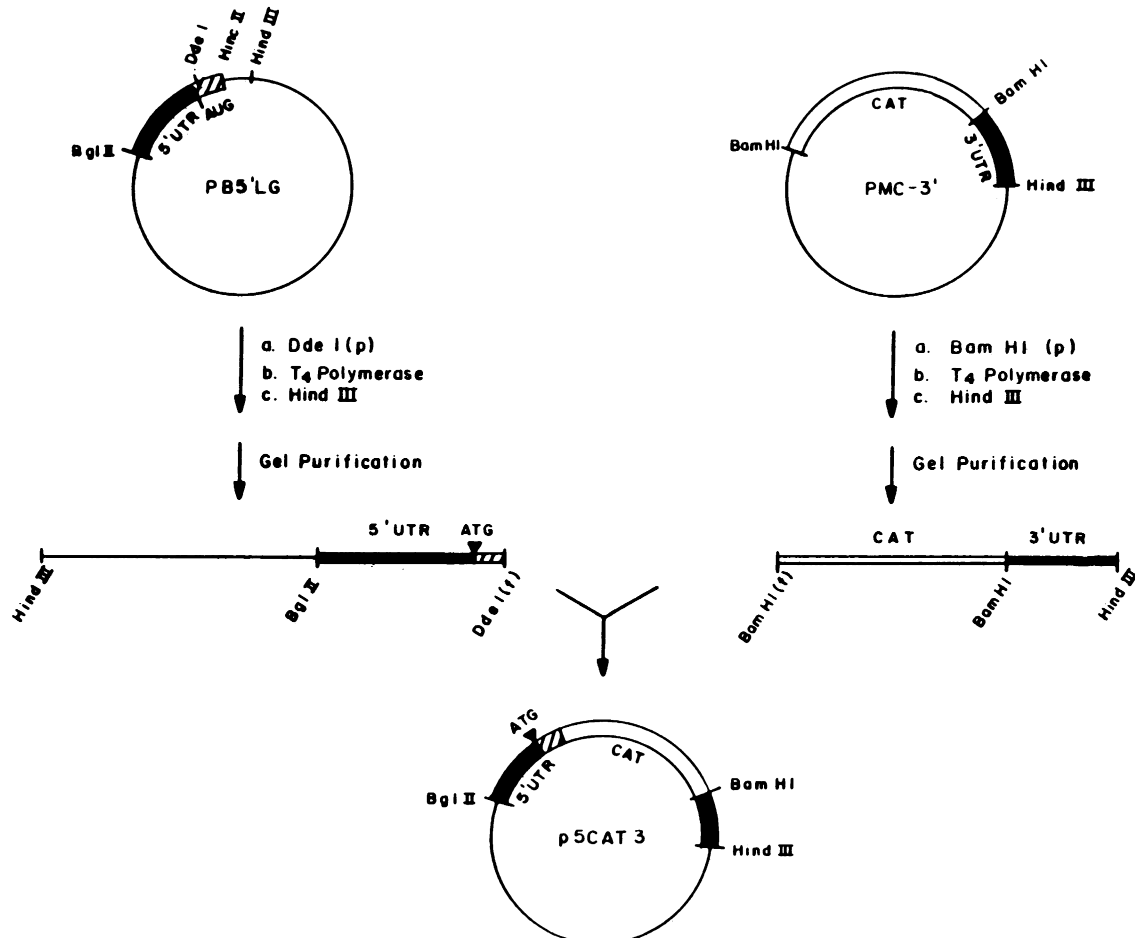

FIG. 3. Experimental scheme for constructing p5CAT3. (See text for details.)

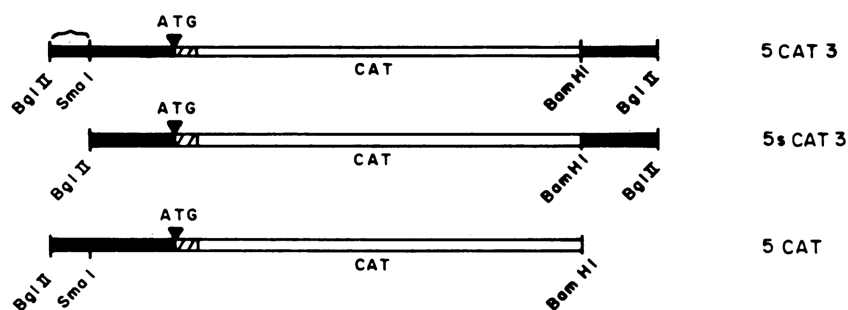

FIG. 4. Structure of the complete chimera 5CAT3 and its deletion mutants. (See text for details.)

*Hin*dIII site of pMC. The blunt-ended ligation of *Stu* I and filled-in *Bam*HI ends regenerated a *Bam*HI site. This resulting plasmid, pMC-3′ (Fig. 3), has the 3′ UTR of ferritin ligated in the correct orientation downstream to the 3′ UTR of the CAT gene, which is 90 bp long.

Fusing the Complete 5′ UTR of the Ferritin Gene to the 5′ End of the CAT Reading Frame. Ferritin L gene sequences are numerically indicated with reference to the cap site, +1. A 5′ end piece of the ferritin L gene (6) within a *Bam*HI–*Hin*cII fragment (Fig. 2 *Lower*) was subcloned in pUC13, forming p5′LG for further manipulation. The insert of p5′LG was isolated and cut with *Hae* II (+4). The *Hae* II end was made flush with T4 DNA polymerase, ligated to *Bgl* II linkers, cut with *Hin*cII (+260), and subcloned in a pUC13 vector to form pB5′LG (Fig. 3).

The complete chimera 5CAT3 was constructed by joining parts of pMC-3′ and pB5′LG as depicted in Fig. 3. pB5′LG was partially digested with *Dde* I (there are six other *Dde* I sites in the vector) to release it at the coding region of ferritin (+209), 8 bp after the ATG. The *Dde* I site was filled in with T4 DNA polymerase and the rest of the coding region of ferritin was removed by digesting with *Hin*dIII at the multiple cloning site. The vector DNA, containing the complete 5′ UTR and 11 nucleotides of the coding region of ferritin, was purified by gel electrophoresis. pMC-3′ was then cut partially with *Bam*HI to release the 5′ end of the CAT gene, filled in with T4 DNA polymerase, and cut with *Hin*dIII to release the CAT-3′UTR insert. This fragment was purified by gel electrophoresis and ligated to the purified vector band forming p5CAT3. The resulting plasmid, p5CAT3, thus contains the full chimeric ferritin-CAT construct, in which the reading frame of CAT is maintained by the initiator codon of ferritin. The 5′ and 3′ junctions of this ferritin-CAT chimera were confirmed by DNA sequencing and multiple restriction analyses.

Construction of Deletion Mutants of the Chimera. Two deletion mutants of the full-length chimera 5CAT3 were prepared (Fig. 4). 5sCAT3 was constructed by deleting the first 67 nucleotides of the 5′ UTR of 5CAT3. A unique *Sma* I site at position +67 was utilized to remove the region that contained the highly conserved 28-bp sequence (+33 to +61) that forms a part of the stem-loop structure (Fig. 1). *Bgl* II linkers were attached to the *Sma* I end of the 5′ UTR. 5CAT was constructed by deleting the entire 3′ UTR of ferritin from 5CAT3 by cutting at the *Bam*HI site at the junction of CAT and ferritin 3′ UTRs. The constructs were then transferred from the pUC vector into the *Bam*HI site of the retroviral vectors.

The orientations of chimeras 5CAT3, 5sCAT3, and 5CAT in the retroviral vectors pZipneoSV(X), PLJ, and PWE were investigated by an internal and unique *Eco*RI site within the CAT gene's coding region. These chimeras lie in a 5′-to-3′ position downstream from the left LTR.

RESULTS AND DISCUSSION

The ferritin-CAT constructs used in this study were assigned the names 5CAT3, 5sCAT3, and 5CAT (Fig. 4). 5CAT3 contained the entire 5′ UTR (210 nucleotides) of rat ferritin L mRNA, its initiation codon, and three additional codons of ferritin fused to the CAT gene whose 5′ UTR had been removed and its initiator codon AUG destroyed by BAL-31 resection up to the adenylate nucleotide position. The first 144 nucleotides of the 3′ UTR of ferritin cDNA from the stop codon to the polyadenylylation signal were attached to the 3′ UTR of CAT. In construct 5sCAT3, the 5′ UTR was shortened by removing the first 67 nucleotides. In construct 5CAT, the complete 3′ UTR of ferritin was removed. Permanently infected FTO-2B cell clones containing these constructs were tested for CAT expression in the presence or absence of iron, added to the medium in experiments with pZipneo as ferric ammonium citrate and for other vectors as hemin, which releases iron intracellularly through the action of heme oxygenase (2). Since the inserted 5′ UTR of the ferritin mRNA does not carry the 5′ flanking region, a transcriptional response to iron can be ruled out.

Transfections Using pZipneoSV(X) Vector. Initially, the complete ferritin–CAT chimera 5CAT3 was cloned in pZipneoSV(X) and introduced into the rat hepatoma cell line FTO-2B by calcium phosphate/DNA-mediated gene transfer. CAT activity was greater in iron-treated cells than in control cells (Fig. 5). However, the overall expression of CAT was very low due to poor transfectibility of this rat cell line, and no stable clone could be obtained in FTO-2B. To overcome this, retroviral infection was used to introduce the chimeric gene into FTO-2B to obtain stable cell clones. However, when permanently infected FTO-Zip5CAT3 cells were assayed for CAT expression, no activity was detected. To survive G418 selection, these cells must have maintained selectable gene expression while losing CAT gene activity, probably due to a splicing event of the viral genomic RNA deleting the ferritin–CAT chimera. In pZipneoSV(X) vector, viral splice donor and acceptor sites are present on either side of the *Bam*HI site where the gene of interest has been inserted. Others have observed similar deletions of the inserted test gene when using this vector (18). Accordingly, we turned to modified retroviral vectors.

Transfections Using pWE and pLJ (DOL-). Recently several vectors have been constructed in which the viral splice sites have been removed (17, 18). The test gene and the selectable marker genes are driven by independent promoters. Two such vectors, pWE and pLJ (DOL-), were used to eliminate the problem of deletion that occurs when pZipneoSV(X) is used. The ferritin CAT chimera 5CAT3 and the two deletion constructs 5sCAT3 and 5CAT were inserted into the *Bam*HI sites of pWE and pLJ. These vectors were introduced into FTO-2B cells via the packaging cell line ψ-2 (11). Permanently infected FTO-2B cells were grown and tested for CAT inducibility in the presence or absence of iron. Fig.

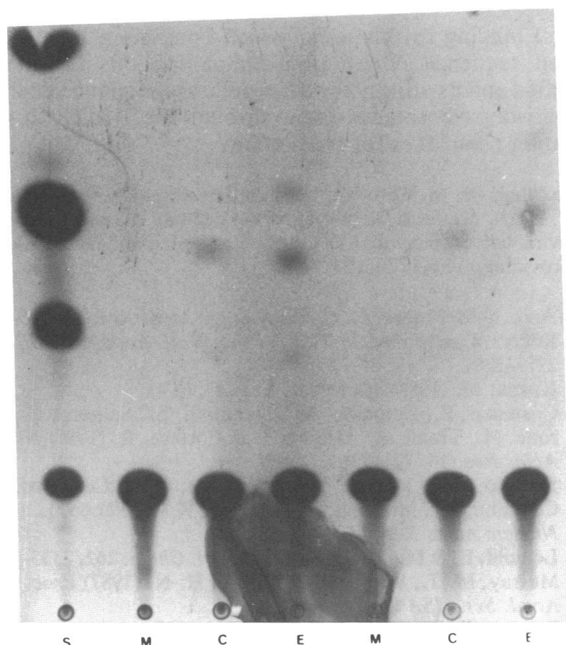

FIG. 5. Induction of CAT activity in iron-stimulated FTO-2B cells transfected with pZip5CAT3. Replica plates of FTO-2B cells were transfected with pZip5CAT3 by the calcium phosphate technique. Mock-transfected cells (M) did not receive any DNA in the calcium phosphate precipitate. Forty-eight hours after transfection, experimental cells (E) were treated with ferric ammonium citrate at 10 μg/ml for 4 hr or left untreated (C). The cells were harvested by scraping and CAT activity assays were performed as described by Gorman *et al.* (16). The CAT activity of 1 unit of commercial CAT was used as a standard (S) to show the mono-, di-, and triacetylated forms of [^{14}C]chloramphenicol. CAT activity was quantitated by densitometric scanning of several autoradiograms. The differences between the various treatment groups are statistically significant by the Student *t* test ($P < 0.005$ for five replications).

6 (lanes 1 and 2) shows that, when FTO-2B cells carrying pWE-5CAT3 were stimulated with hemin, there was a 5-fold increase in CAT activity ($P < 0.005$ for six replications). When cells were treated with desferal, an intracellular iron chelator (lane 3), CAT activity fell to half that of the control (lane 2). As in Fig. 5, these results indicate that the untranslated regions of ferritin L mRNA must contain an iron-responsive element(s) since, after fusion of this iron-regulatory sequence to the CAT gene, iron inducibility of ferritin is conferred on the foreign protein CAT.

Regulation of eukaryotic *transcription* is mediated by the interaction of negatively and/or positively acting cellular factors (proteins) with specific consensus sequences in the 5′ flanking regions of genes. Similarly, our evidence shows that ferritin L message contains a regulatory sequence that increases *translation* of ferritin mRNA in response to iron, activation resulting from removal of a factor repressing translation rather than stimulation of translational activity for the following reasons. With few exceptions, eukaryotic messages are almost all polysome-associated, whereas, in the absence of iron stimulation, ferritin mRNAs are largely found as inactive mRNA–protein complexes (1, 20). This suggests that repression of ferritin translation is due to binding of a negative trans-acting molecule(s) to a regulatory element in the ferritin untranslated sequences, which prevents the message from interacting with the translational machinery of the cell. Indeed, a protein interacting with the conserved sequence of the 5′ UTR of rat L and H ferritin mRNAs has been identified (E. Leibold and H.N.M.).

Relevant to this conclusion is the response obtained in cells carrying 5CAT3 in the vector pLJ. In contrast to cells

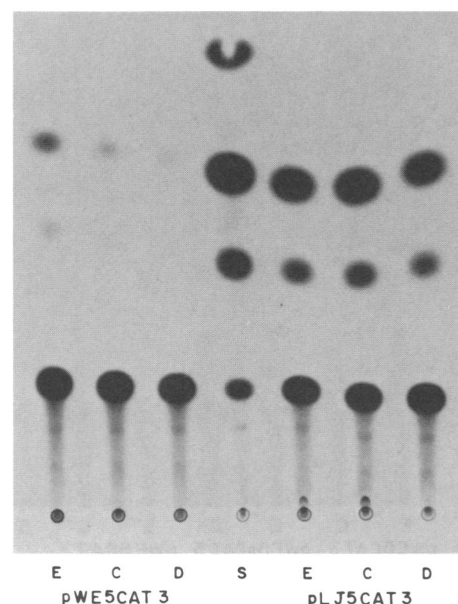

FIG. 6. CAT inducibility by iron of cells permanently infected with pWE-5CAT3 and pLJ-5CAT3. Equal number of cells (5×10^6) permanently infected with pWE-5CAT3 and pLJ-5CAT3 was plated 24 hr before treatment. Experimental (E) cells received 100 μM hemin for 14 hr. Additional hemin was added to make the final concentration 200 μM for 4 hr before harvesting the cells. Control cells (C) were untreated. A third batch of cells was treated with desferal at 10 μg/ml for 18 hr (D). CAT activity was assayed with a standard (S) as described for Fig. 5.

containing pWE-5CAT3, these cells do not demonstrate iron inducibility of CAT (Fig. 6, lanes 5, 6, and 7). However, the overall CAT activity is much greater in control and iron-induced or desferal-treated pLJ-5CAT3 cells than in the case of pWE-5CAT3, because of the strong promoter activity of the vector pLJ transcribed from the murine retroviral LTR, whereas in pWE the ferritin–CAT gene is driven by the weaker β-actin gene promoter. This presumably occurs because the repressor protein is present in limited amounts in the cell and is readily titrated out by overproduction of the chimeric mRNA of pLJ-5CAT3 containing the repressor binding site. In consequence, repression of translation is obliterated in control cells carrying this vector. Such titration of repressor molecules by excess production of regulatory sequences has been demonstrated in other systems (21).

To delineate the location of the iron-responsive element to either the 3′ or the 5′ UTR of ferritin mRNA, cells permanently infected with the deletion constructs were tested for CAT inducibility by iron. In Fig. 7 (lanes 1 and 2) the complete chimera in the pWE vector confirms the induction by iron seen with the same vector in Fig. 6 (lanes 1 and 2). In cells expressing the deletion construct pWE-5CAT lacking the 3′ UTR (Fig. 7, lanes 5 and 6), CAT inducibility is maintained, showing that the 3′ UTR is not essential for the action of iron and implying that the iron-regulatory element resides only in the 5′ terminus. When the 5′ UTR was shortened by removing the first 67 nucleotides, which contain the conserved 28-nucleotide sequence and the secondary stem-loop structure (pWE-5sCAT3), the iron inducibility of the chimeric gene was lost (lanes 3 and 4). Thus deletion of this region leads to loss of translational repression in control cells, presumably because the specific repressor molecule(s) can no longer bind to the message.

In addition to causing insensitivity to iron, removal of the first 67 nucleotides of the 5′ UTR resulted in a considerable increase in the translation of CAT by control cells containing pWE-5sCAT3 (Fig. 7, lane 4) compared with those having the

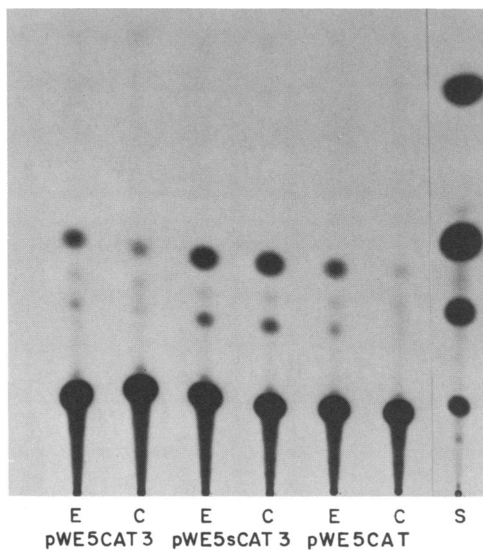

FIG. 7. CAT inducibility of the ferritin–CAT chimera and its deletion constructs carried on the vector pWE. Equal numbers of FTO-2B cells, permanently infected with pWE-5CAT3, pWE-5sCAT3, or pWE-5CAT, were monitored for CAT inducibility in the presence of iron. Experimental cells (E) and control cells (C) were treated as described for Fig. 6. CAT activity was assayed with a standard (S) as described for Fig. 5. Data represent six replications.

complete 5′ UTR (lane 2). Secondary structure in the 5′ UTR is known to reduce the efficiency with which messages are translated (22–24). Thus certain viral messages, for example that of alfalfa mosaic virus, lack secondary structure at the 5′ terminus and are translated with a higher efficiency than those with secondary structures, presumably due to the diminished requirement for the unwinding activity of cap binding protein complexes (25). Thus when the first 67 nucleotides of the 5′ UTR are deleted, not only is the specific repression (controlled by intracellular iron levels) of ferritin mRNA lost but also the general requirement for translational factors (cap binding proteins) is reduced (22). A similar example of modulation of translational efficiency by secondary structure has been reported (26) in which a stem-loop structure near the 5′ terminus of the protooncogene c-*myc* is thought to hinder its translation, since loss of this structure in some lymphoid neoplasms results in a surge of increased c-*myc* translational efficiency.

The 5′ leader sequences of the mRNAs for adenoviral messages (27), GCN4 mRNA in yeast (28), and heat shock mRNAs in *Drosophila* (29) are also thought to be involved in their preferential translation over other messages in response to a change in physiological status—e.g., starvation and heat shock. In our case, translation of ferritin is regulated in response to a specific inducer, iron. Our results indicate that ferritin expression is regulated by an iron regulatory element within the first 67 nucleotides of the 5′ UTR of the mRNA. We propose that, when intracellular iron is low, ferritin synthesis is repressed due to negative trans-acting molecule(s) binding to this area, which contains a highly conserved sequence. Upon iron stimulation this factor(s) is modified and its affinity for the regulatory sequence decreases, allowing ribosomes to scan through the 5′ UTR to form initiation complexes for translation.

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