Acad. Sci. U.S.A. **80**, 1454 (1983); S. B. Rodan *et al.*, *J. Clin. Invest.* **72**, 1511 (1983).
4. E. L. Simpson *et al.*, *N. Engl. J. Med.* **309**, 325 (1983).
5. J. M. Moseley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5048 (1987).
6. G. J. Strewler *et al.*, *J. Clin. Invest.*, in press; A. F. Stewart, T. Wu, D. Goumas, W. J. Burtis, *Biochem. Biophys. Res. Commun.* **146**, 672 (1987); M. Mangin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
7. L. J. Suva *et al.*, *Science* **237**, 893 (1987).
8. G. W. Tregear *et al.*, *Endocrinology* **93**, 1349 (1973).
9. M. Rosenblatt, *N. Engl. J. Med.* **315**, 1004 (1986).
10. The peptide hHCF-(1–34)NH_2 was synthesized by modifications of the Merrifield solid-phase technique (*11*) with an Applied Biosystems 430A Synthesizer and purified by gel filtration followed by preparative high-pressure liquid chromatography (HPLC). The peptide was chemically analyzed and found to be authentic and of high purity (>99%) by HPLC, amino acid analysis, Edman sequence analysis, fast atom bombardment mass spectrometry and proton nuclear magnetic resonance spectroscopy. PTH peptides were purchased from Bachem (Torrance, CA) (*11*).
11. R. B. Merrifield, *Adv. Enzymol. Relat. Subj. Biochem.* **32**, 221 (1969).
12. N. Horiuchi *et al.*, *Am. J. Physiol.* **244**, E589 (1983).
13. Across several assays, bovine PTH is as potent as or more potent than the human homolog. Formal determination of potency cannot be made with this assay; potencies are compared and estimated on the basis of the concentrations of peptide required to produce similar calcemic responses. In each of the assay systems utilized, the form of PTH used most frequently in previous studies was selected as the reference peptide.
14. Renal handling of calcium could not be assessed in this animal model because thyroparathyroidectomized animals need to be sustained by a calcium-containing infusion.
15. A. F. Stewart *et al.*, *N. Engl. J. Med.* **303**, 1377 (1980); D. A. Bushinski, G. S. Riera, M. J. Favus, F. L. Coe, *J. Clin. Invest.* **76**, 1599 (1985); H. N. Hulter, B. P. Halloran, R. D. Toto, T. C. Peterson, *ibid.*, p. 695.
16. G. J. Strewler *et al.*, *Endocrinology* **119**, 303 (1986).
17. M. E. Goldman, M. Chorev, J. E. Reagan, L. H. Caporale, M. Rosenblatt, *J. Bone Mineral Res.* **2**, (Abstr. 284) (1987).
18. S. B. Rodan *et al.*, *J. Clin. Invest.* **72**, 1511 (1983).
19. R. A. Nissenson, G. J. Strewler, R. D. Williams, S. C. Leung, *Cancer Res.* **45**, 5358 (1985).
20. N. Horiuchi, M. F. Holick, J. T. Potts, Jr., M. Rosenblatt, *Science* **220**, 1053 (1983).
21. Y. Cheng and W. H. Prisoff, *Biochem. Pharmacol.* **22**, 3099 (1973).
22. Animal care was provided in accordance with NIH and institutional guidelines. Surgery was performed under anesthesia induced by a single injection of chloral hydrate (100 mg/250 g of body weight) or by injection of ketamine HCl (100 mg/ml) at 1.0 ml/kg intraperitoneally. For nephrectomy and subsequent infusion, rats were given one injection of dial-urethane [(w/v) 10% 5,5-diallylbarbituric acid, 40% ethyl urea, 40% urethane, 0.5% EDTA] 1 ml/kg intraperitoneally.
23. Statistically significant differences were observed among groups by one-way analysis of variance ($P < 0.01$) in all parameters measured. Values that are significantly different from control are indicated by * ($P < 0.5$) or *($P < 0.01$) [C. W. Dunnett, *J. Am. Stat. Assoc.* **50**, 1096 (1955)].
24. We are grateful to M. Chorev for critical reading of the manuscript, M. Buddle for technical assistance, S. Camburn for secretarial assistance, and the Audio-Visual Department of Helen Hayes Hospital. This work was supported, in part, by NIH grants AR 36446 and AR 39191 (T.L.C.) and Merck Sharp & Dohme Research Laboratories.

28 August 1987; accepted 22 October 1987

Parathyroid Hormone-Related Protein of Malignancy: Active Synthetic Fragments

BRUCE E. KEMP, JANE M. MOSELEY, CHRISTINE P. RODDA, PETER R. EBELING, RICHARD E. H. WETTENHALL, DAVID STAPLETON, HANNELORE DIEFENBACH-JAGGER, FIONA URE, VALDO P. MICHELANGELI, HOLLIS A. SIMMONS, LAWRENCE G. RAISZ, T. JOHN MARTIN

Peptides corresponding to the amino-terminal region of the parathyroid hormone–related protein (PTHrP) of humoral hypercalcemia of malignancy were synthesized. A 34–amino acid peptide, PTHrP(1–34), was two to four times more potent than bovine or human PTH(1–34) in bioassays promoting the formation of adenosine 3′,5′-monophosphate (cAMP) and plasminogen activator activity in osteogenic sarcoma cells and adenylate cyclase activity in chick kidney membranes. Like parathyroid hormone itself, in which the activity resides in the first 34 residues, PTHrP peptides of less than 30 residues from the amino terminus showed substantially reduced activity. PTHrP(1–34) had only 6% of the potency of bovine PTH(1–34) in promoting bone resorption in vitro. PTHrP(1–34) strongly promoted the excretion of cAMP and phosphorus and reduced the excretion of calcium in the isolated, perfused rat kidney consistent with the symptoms seen in malignant hypercalcemia.

STUDIES OF HUMORAL HYPERCALCEMIA of malignancy (HHM) have provided evidence that tumors produce a protein that acts through the parathyroid hormone (PTH) receptor but is immunologically distinct from PTH (*1*). A PTH-related protein (PTHrP) was isolated from a lung cancer cell line, and 8 of its 16 amino-terminal residues were identical with human PTH (*2*). The amino acid sequence to residue 50 was subsequently determined and a full-length complementary DNA encoding a 141–amino acid protein was isolated (*3*). The homology with PTH is restricted to the amino-terminal region. Since the biological activity of the 84–amino acid residue PTH molecule is contained within the first 34 residues (*4*), we investigated the biological actions of synthetic peptide analogs of the amino-terminal sequence of PTHrP (*3*). We found that PTHrP(1–34) has substantial biological activity in five PTH response systems, and that PTHrP peptides of chain length less than 30 residues have greatly attenuated activities.

The activity of the synthesized and purified PTHrP(1–34) (*5*) was studied in the PTH-responsive osteogenic sarcoma cell line UMR 106-01 (*6*). PTHrP(1–34) stimulated adenosine 3′,5′-monophosphate (cAMP) formation in a dose-dependent manner with a potency four times greater than that of either human or bovine PTH(1–34); the parent protein is six times more potent than bovine PTH(1–34) in the same assay system (*2*). PTHrP(1–34) was approximately equal in potency to rat PTH(1–34) (Fig. 1A) which is several times more potent than either the human or bovine PTH(1–34) (*7*). Similar relative potencies of PTHrP(1–34) and PTH(1–34) were evident in bioassays based on measurements of the activities of membrane adenylate cyclase from chicken kidney.

We also synthesized a series of shorter PTHrP peptides. These were appreciably less potent in the osteogenic sarcoma cell cAMP response (Fig. 1B). Synthetic PTHrP(1–29) had about 10% of the activity of PTHrP(1–25) and less than 0.01% of the activity of PThrP(1–34). There was no measurable activity in the shorter peptides, PTHrP(1–20) and PTHrP(1–14). Virtually identical relative activities were obtained in the chicken kidney adenylate cyclase response. Thus the region of PTHrP responsible for its known biological activities is contained within the amino-terminal 34 residues, a situation analogous to that with PTH (*4*).

PTHrP(1–34) was also more potent than PTH(1–34) in stimulating tissue plasminogen activator (tPA) activity produced by osteogenic sarcoma cells (*8*). In contrast, the relative potencies in promoting bone resorption in vitro were reversed. The effects of PTHrP(1–34) and bovine PTH(1–34) on bone resorption were assayed as the release of previously incorporated ^{45}Ca from cul-

B. E. Kemp, J. M. Moseley, C. P. Rodda, P. R. Ebeling, D. Stapleton, H. Diefenbach-Jagger, F. Ure, V. P. Michelangeli, T. J. Martin, University of Melbourne, Department of Medicine, Repatriation General Hospital, Heidelberg, Victoria, Australia 3081.
R. E. H. Wettenhall, La Trobe University, Department of Biochemistry, Bundoora, Victoria, Australia 3083.
H. A. Simmons and L. G. Raisz, Division of Endocrinology, University of Connecticut Health Sciences Center, Farmington, CT.

tured long bone shafts of 19-day rat fetuses as described (*9*). Synthetic PTHrP(1–34) stimulated bone resorption appreciably at concentration of 25 and 100 ng/ml (Fig. 2A), and was thus approximately 5% as potent as PTH(1–34). The maximum response and the slope of the dose-response curve for PTHrP(1–34) were similar to that for bovine PTH(1–34) in this culture system, with 94% of ^{45}Ca released from the bone at the highest concentration after 5 days of culture. The relative potencies of PTHrP and PTH analogs were not altered by inclusion of albumin and ACTH in the long-term organ cultures to reduce possible proteolytic degradation (*10*). This was of particular concern because of the Arg-Arg-Arg sequence (residues 19–21) present in PTHrP (*3*). Adequate amounts of the full-length recombinant PTHrP are not yet available and, consequently, it is not known whether the parent protein is more potent at promoting bone resorption than PTHrP(1–34). From the data available, it seems reasonable to suggest that if sufficiently high levels of circulating PTHrP were reached in patients, excessive bone resorption might occur.

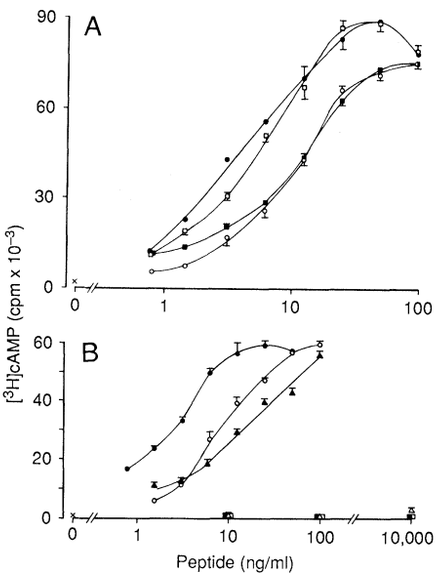

Fig. 1. (**A**) Cyclic AMP formation in osteogenic sarcoma cells. The [^{3}H]cAMP was assayed in intact UMR 106-01 sarcoma cells labeled with [^{3}H]adenine (*6, 7*). Bovine PTH(1–34) amide, rat PTH(1–34) amide, and human PTH(1–34) amide were obtained from Beckman (California), Bachem (California), and Amour Pharmaceutical (Illinois), respectively. Points are means ± SEM of triplicates for: ●, PTHrP(1–34); □, rPTH-(1–34); ○, bPTH(1–34) and ■, hPTH(1–34). (**B**) Effect of peptide chain length on bioactivity of PTHrP. Synthetic peptides were assayed for stimulation of [^{3}H]cAMP formation in intact UMR 106-01 cells labeled with [^{3}H]adenine (*6, 7*). Points are means ± SEM of triplicates for: ●, PTHrP(1–34); ○, bPTH(1–34); ▲, PTHrP(1–29); △, PTHrP(1–25); ■, PTHrP(1–20); and □, PTHrP(1–14).

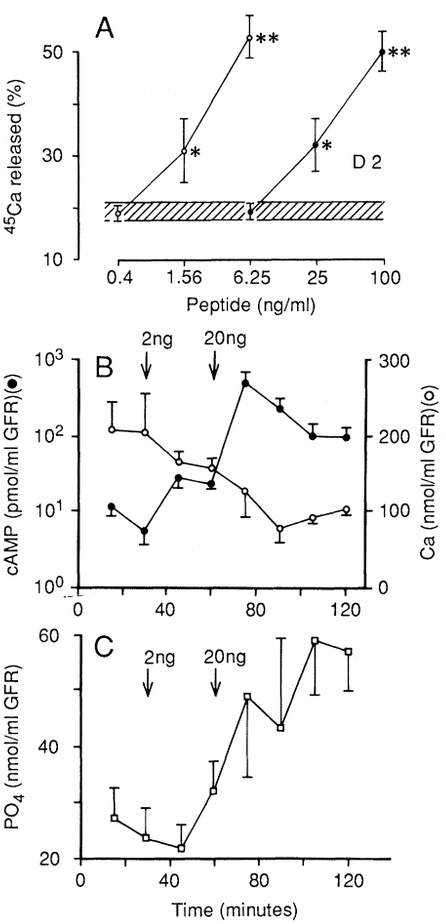

Fig. 2. (**A**) Effect of PTHrP(1–34) on bone resorption. Comparison of the stimulation of bone resorption by: ○, bPTH(1–34) and ●, PTHrP(1–34). Points are means ± SEM (n = 8) for $^{45}Ca^{2+}$ release from fetal rat long bones cultured for 2 days. The value for control unstimulated culture ± SEM is indicated by the cross-hatched horizontal lines (*9, 10*). The probability values P for 0.05 and 0.01 significance are indicated by one and two asterisks, respectively. (**B**) Effect of PTHrP(1–34) on cAMP excretion and calcium retention in kidney. Rat kidneys cannulated via the ureter and right renal artery were perfused (at 140 mmHg) with Krebs-Henseleit high bicarbonate buffer containing 6.7% bovine serum albumin supplemented with D-glucose (5 mM), cysteine (0.5 mM), glycine (2.5 mM), and glutamic acid (0.5 mM). The preparation was stable for 2 to 3 hours. Both urine and perfusate were sampled at 15-minutes intervals. PTHrP(1–34) was added at 31 minutes to give a final concentration of 2 ng/ml in the perfusate and an additional dose was given at 61 minutes to give 20 ng/ml. The concentrations of calcium and phosphate in the perfusate were 2.25 mM and 1.15 mM, respectively. Glomerular filtration rates (GFR) determined by [^{3}H]inulin clearance were above 0.29 ml/min. cAMP concentrations were determined as described previously (*6*). Values shown are means ± SEM for four perfusions. There was no apparent change in the cAMP concentrations in the absence of added hormone. (**C**) Effect of PTHrP(1–34) on phosphorus excretion in kidney. Phosphate concentrations were determined as described by Chen *et al.* (*13*). Values shown are means ± SEM for four perfusions. Phosphate concentration rose by less than 5% of the basal level over the duration of the perfusion in the absence of added hormone.

The bone-resorbing effects of PTH and prostaglandins are thought to be mediated by cells of the osteoblast lineage, and to be related to initial actions upon cAMP formation (*11*). Thus, it would be predicted on the basis of the present adenylate cyclase data with synthetic PTHrP(1–34) and our previous results with native PTHrP (*2*) that PTHrP and PTHrP(1–34) would be more potent bone resorbers than PTH(1–34). The apparent dissociation between the bone resorption and cAMP responses raises a number of questions regarding the regulatory pathways operating in osteoblast-like cells or osteoclast precursors as well as the possible function of the carboxy-terminal structure of PTHrP in bone resorption associated with HHM.

Increased nephrogenous cAMP, increased phosphorus excretion, and reduced calcium excretion occur in HHM as they do in primary hyperparathyroidism (*1*). We have used an isolated rat kidney preparation, perfused in closed circuit (*12*), to demonstrate that PTHrP(1–34) promotes the excretion of cAMP and phosphorus and the retention of calcium. PTHrP(1–34) increased cAMP excretion by the perfused kidney at a perfusate concentration of 2 ng/ml; this excretion increased dramatically at 20 ng/ml (Fig. 2B). There was a corresponding decrease in calcium and increase in phosphorus excretion (Fig. 2, B and C), although these last changes were more variable and only significant at the higher dose. These actions of synthetic PTHrP(1–34) on the kidney are entirely consistent with the clinical manifestations of the HHM syndrome and point to the kidney as one of the major targets.

REFERENCES AND NOTES

1. A. F. Stewart *et al.*, *N. Engl. J. Med.* **203**, 176 (1980); G. R. Mundy and T. J. Martin, *Metabolism* **31**, 1247 (1982); S. B. Rodan *et al.*, *J. Clin. Invest.* **72**, 1511 (1983); G. J. Strewler *et al.*, *ibid.* **71**, 769 (1983); W. J. Burtis *et al.*, *J. Biol. Chem.* **262**, 7151 (1987).
2. J. M. Moseley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5048 (1987).
3. L. J. Suva *et al.*, *Science* **237**, 893 (1987).
4. G. W. Tregear *et al.*, *Endocrinology* **93**, 1349 (1973); J. T. Potts, Jr., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 63 (1971); J. T. Potts, Jr., *et al.*, *Adv. Prot. Chem.* **35**, 323 (1982); M. Rosenblatt and J. T. Potts, Jr., *Endocrine Res. Commun.* **4**, 115 (1977).

5. Peptides were synthesized as the carboxyl-terminal amide form by the solid-phase synthesis procedure [R. S. Hodges and R. B. Merrifield, *Anal. Biochem.* **65**, 241 (1975)]. Amino acid derivatives protected with the *t*-butyloxycarbonyl group in the α-amino position were obtained from the Protein Research Foundation (Osaka, Japan). The peptides were assembled by means of an Applied Biosystems Peptide Synthesizer Model 430, with ninhydrin testing at each step [V. Sarin *et al.*, *Anal. Biochem.* **117**, 147 (1981)]. The completed peptides were simultaneously deprotected and cleaved from the resin in anhydrous HF containing 10% (v/v) anisol [J. M. Stewart and J. D. Young, in *Solid Phase Peptide Synthesis* (Freeman, San Francisco, 1966), pp. 44 and 66]. Poor yields (<2%), of synthetic product were obtained when *t*Boc-*N*-tosyl-im-histidine was used, whereas yields of approximately 17% were obtained with the use of *t*-butyloxycarbonyl-*N*-dinitrophenol-im-histidine. Dinitrophenol deprotection was done prior to HF cleavage by treating the resin with 2-mercaptoethanol 20% (v/v) and diisopropyl ethylamine 2% (v/v) in dimethylformamide [S. Kent and I. Clark-Lewis, in *Synthetic Peptides in Biology and Medicine*, K. Alitalo, P. Partarmen, A. Vaheri, Eds. (Elsevier, Amsterdam, 1985), pp. 29–57]. Peptides were extracted from the resin with 60% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid solvent, subjected to rotary evaporation, and lyophylized from water. The peptides were purified by sequential ion-exchange chromatography (either SP-Sephadex or CM-Sephadex) and preparative low-pressure reversed-phase chromatography (25 by 400 mm column, C18, 250 Å, 35 to 70 μm Amicon resin) with an acetonitrile gradient in the presence of 0.1% (v/v) trifluoroacetic acid. The synthetic PTHrP(1–34) migrated as a major peak coincident with PTH-like biological activity. Biologically active synthetic PTHrP(1–34) was obtained from three independent syntheses. Amino acid analyses of purified peptides were in good agreement with theoretical values. For example, the average of two analyses of 24-hour hydrolysates of PTHrP(1–34) yielded the following number of residues: Asx (2.03), Thr (0.98), Ser (2.15), Glu (3.46), Gly (1.52), Ala (3.29), Val (1.10), Ile (2.86), Leu (4.96), Phe (2.06), His (4.63), Lys (2.14), and Arg (2.64).
6. S. M. Forrest *et al.*, *Calcif. Tissue Int.* **37**, 51 (1985); N. C. Partridge *et al.*, *Cancer Res.* **43**, 4308 (1983); T. J. Martin *et al.*, *Methods Enzymol.* **145**, 324 (1987). PTH-like activity was assayed by using the cAMP response in intact, clonal osteogenic sarcoma cells (UMR 106-01) (*2, 6*) that were labeled by incubation with [^{3}H]adenine and then treated with 1 m*M* isobutylmethylxanthine. The [^{3}H]cAMP generated was then measured. Cells were counted in a Coulter Model DN Counter. Adenylate cyclase activity was assayed in partially purified chick kidney plasma membranes [N. H. Hunt *et al.*, *J. Endocrinol.* **69**, 401 (1976)].
7. H. T. Keutmann *et al.*, *Endocrinology* **117**, 1230 (1985).
8. J. A. Hamilton *et al.*, *Biochem. Biophys. Res. Commun.* **122**, 230 (1984); J. A. Hamilton *et al.*, *Endocrinology* **116**, 2186 (1985); E. H. Allan *et al.*, *Biochim. Biophys. Acta* **888**, 199 (1986).
9. L. G. Raisz and I. Niemann, *Endocrinology* **85**, 446 (1969).
10. J. H. Dominguez and L. G. Raisz, *Calcif. Tissue Int.* **30**, 205 (1980); P. J. Bergman, *et al.*, *Endocrinology* **116**, 1729 (1985).
11. G. A. Rodan and T. J. Martin, *Calcif. Tissue Int.* **33**, 349 (1981); T. J. Martin, *Pharmacol. Ther.* **21**, 209 (1983).
12. B. D. Ross *et al.*, *Am. J. Physiol.* **225**, 1165 (1973).
13. P. S. Chen *et al.*, *Anal. Chem.* **28**, 1756 (1956).
14. Supported by grants from the National Health and Medical Research Council of Australia, the Anti-Cancer Council of Victoria, and the Australian Government Department of Veteran's Affairs. Equipment support was provided by the Ian Potter Foundation and the Buckland Foundation. C.P.R. is a Wenkart Foundation Scholar. We thank W. Adam for assistance in setting up the kidney perfusion assay and K. Kasperowicz for manuscript typing.

18 September 1987; accepted 3 November 1987

Identification of the Iron-Responsive Element for the Translational Regulation of Human Ferritin mRNA

MATTHIAS W. HENTZE, S. WRIGHT CAUGHMAN, TRACEY A. ROUAULT, JAVIER G. BARRIOCANAL, ANDREW DANCIS, JOE B. HARFORD, RICHARD D. KLAUSNER

Regulated translation of messenger RNA offers an important mechanism for the control of gene expression. The biosynthesis of the intracellular iron storage protein ferritin is translationally regulated by iron. A *cis*-acting element that is both necessary and sufficient for this translational regulation is present within the 5′ nontranslated leader region of the human ferritin H-chain messenger RNA. In this report the iron-responsive element (IRE) was identified by deletional analysis. Moreover, a synthetic oligodeoxynucleotide was shown to be able to transfer iron regulation to a construct that would otherwise not be able to respond to iron. The IRE has been highly conserved and predates the evolutionary segregation between amphibians, birds, and man. The IRE may prove to be useful for the design of translationally regulated expression systems.

IN THE PAST FEW YEARS, INTENSIVE scientific efforts have led to fascinating insights into transcriptional regulatory mechanisms (*1*). In contrast, there is only a limited list of eukaryotic genes for which translational regulation of messenger RNA (mRNA) utilization has been reported (*2*). As one of the earliest examples, Munro and his colleagues demonstrated that the biosynthesis of the intracellular iron storage protein ferritin is regulated by iron in the absence of alterations in mRNA levels and proposed that this regulation took place at the translational level (*3*). It was shown that cytoplasmic ferritin mRNA underwent a redistribution from an inactive ribonucleoprotein pool to translationally active polyribosomes after iron induction (*4*). Ferritin is a particularly useful model to study the translational regulation of a eukaryotic gene. The protein is highly conserved in all eukaryotic cells (*5*), and the regulation of its biosynthesis by iron is rapid, reversible, and covers more than a 100-fold range (*6, 7*). We have recently shown that the expression of the human ferritin H chain is translationally regulated by iron in transiently transfected and stably transformed murine fibroblasts (*7, 8*). The presence of an iron-responsive cis-acting element in the translated 5′ leader region of the ferritin mRNA was necessary to achieve regulation. Furthermore, the presence of the complete 5′ leader sequence proved to be sufficient to regulate the expression of a chloramphenicol acetyl transferase (CAT) construct that was expressed from the ferritin promoter and encoded a ferritin-CAT fusion protein. Quantitative S1 nuclease protection assays demonstrated that the resulting iron regulation of CAT occurred in the absence of any changes in the level of specific mRNA (*8*). The purpose of the present study was to identify the iron-responsive element (IRE) and to begin to characterize its structure and function.

Our initial strategy was to localize the IRE by progressive deletional analysis. Figure 1A shows the nucleotide sequence of the 5′ leader region of the human ferritin H-chain complementary DNA (cDNA). Fragments containing the ferritin promoter and portions of this 5′ leader region were generated by Bal 31 digestion from a parent construct, pFPL CAT (*8*). The 3′ end points of these fragments as well as the nature of an internal deletion were determined by DNA sequencing (Fig. 1A) (*9*). Expression constructs containing the minimally deleted L1 (Δ−1/−5) and maximally deleted L5 (Δ−1/−206) ferritin promoter plus 5′ leader fragments were generated with either CAT (*10*) or human growth hormone (hGH) (*11*) protein coding regions. Iron responsiveness of these constructs was determined by treating transient transfectants with hemin (H) as an iron source or desferrioxamine (D) as an iron chelator and determining the level of CAT activity or the hGH level relative to an identical untreated control (C) transfectant. The H/D ratio for each deletion construct indicates the range of iron regulation that was observed for that construct under the experimental conditions. This ratio and thus the iron responsiveness observed with construct L1-CAT (Fig. 1B, inset) was identical to that seen with the parent construct, pFPL CAT (*8*). In the construction of L1-CAT, the ferritin ATG present in pFPL CAT was removed, leaving the CAT ATG as the translation start codon. The observation of identical iron

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.