Translational enhancement of H-ferritin mRNA by interleukin-1 β acts through 5' leader sequences distinct from the iron responsive element

J.T.Rogers*, J.L.Andriotakis, L.Lacroix, G.P.Durmowicz, K.D.Kasschau and K.R.Bridges

Division of Hematology–Oncology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

Received February 14, 1994; Revised and Accepted May 24, 1994

ABSTRACT

Interleukin-1 β (Il-1 β), a key cytokine in the acute phase response, elevates hepatic expression of both the heavy (H) and light (L) ferritin subunits without influencing the steady-state levels of either ferritin transcript. Transfection experiments with human hepatoma cells reveal that sequences within the 5' untranslated region (5'UTR) of H-ferritin mRNA confer translational regulation to chimaeric chloramphenicol acetyl transferase (CAT) mRNAs in response to Il-1 β in the absence of marked changes in CAT mRNA levels. Il-1 β dependent translational enhancement is mediated by a distinct G+C rich RNA sequence within 70 nucleotides (nt) of the start codon. The upstream Iron Responsive Element RNA stemloop does not confer increased expression to CAT mRNA in Il-1 β stimulated hepatoma transfectants. A 38 nucleotide consensus sequence within the 5'UTRs of the mRNAs encoding the hepatic acute phase proteins α_1 -antitrypsin (α_1 AT), α_1 -acid glycoprotein (AGP) and haptoglobin (Dente *et al.*, 1985) is similar to sequences in the G+C rich H-ferritin mRNA translational regulatory element. Deletion of three nucleotides from this region of the 61 nt G+C rich element in the H-ferritin mRNA 5' leader eliminates Il-1 β translational enhancement of the CAT reporter transcripts.

INTRODUCTION

The inflammatory cytokines Il-1 β and Il-6 alter the transcription of a number of liver genes encoding serum proteins (1–3). The concentration of these proteins, termed acute phase reactants (APRs), rises sharply during inflammation as a part of an adaptive response to infection and physiological stress (4). Il-1 β (MW, 17.4 kDa) is the first cytokine to be released from macrophages during inflammation (5). It transduces signals through ubiquitous Il-1 receptors which not only stimulate increased transcription of hepatic acute phase genes but also alter the translational efficiency of hepatic mRNAs (6–8). There is an average four-fold increase in the translation of H- and L-ferritin mRNAs in

human hepatoblastoma (HepG2) cells in response to Il-1 β , although the levels of both transcripts remains unchanged (8). Translational mechanisms are also responsible for elevated expression of the APR gene encoding C-reactive protein (CRP) in Il-1 β stimulated hepatocellular Hep3B cells (9). This two-fold Il-1 β response augments a one thousand fold induction of CRP transcription by Il-6 (9). The hepatic acute phase genes, including α_1 AGP and α_1 AT, respond to the inflammatory cytokines Il-1 β and Il-6 with a three to five fold increase in gene transcription (2, 3, 10).

Ferritin consists of a mixture of 24 heavy (H, 21 kD) and light (L, 19 kD) subunits which co-assemble to form a heteropolymeric shell (11). Cellular ferritin stores iron in an oxidized state as ferric oxyhydroxide [Fe(III)O.OH] (12). The increase in translational efficiency of H- and L-ferritin mRNAs by rat liver cells in response to inflammation defines ferritin as an acute phase reactant (13–15). Both transcriptional and translational mechanisms contribute to the elevation of ferritin levels during inflammation, an event that results in sequestration of iron into tissues to deprive microorganisms of serum iron and diminish oxidant damage to cell structures (16–21).

Both H- and L-ferritin mRNAs are translationally regulated by iron in eukaryotic cells by the relief of translational repression at conserved RNA stem-loops, the Iron Responsive Elements (IREs), in 5' untranslated regions (5'UTRs) of each message (22, 23). The highly conserved IREs regulate translation of human, rodent, and amphibian ferritin transcripts from sites adjacent to the 5' cap sites (24–27). In conditions of intracellular iron depletion by the iron chelating agent desferrioxamine, the IRE binds to an iron-inducible repressor, the IRE binding protein (IRE-BP) or iron regulatory factor (IRF) (28, 29). This inhibits the initiation of ferritin mRNA translation by the small 43S ribosome subunit (30). The IRE-BP is a cytosolic enzyme which exhibits aconitase activity in the presence of iron and binds to the IRE when iron is chelated from the cell (31–32). Under the same conditions five IREs within the 3'UTR of the transferrin receptor (TfR) mRNA bind to IRE-BP (33, 34). Detachment of the IRE-BP from the IRE during iron influx increases translation of the H- and L-ferritin mRNAs to elevate the iron storage

*To whom correspondence should be addressed

capacity of the cell (30, 35). This uncoupling event also permits ribonuclease access to TfR transcripts thereby destabilizing the receptor transcript and decreasing TfR-mediated iron uptake through the cell membrane (33, 34).

This report shows that $\text{Il-1}\beta$ increases the translational efficiency of both the H- and L-ferritin mRNAs. We describe a G+C rich element within the 5' UTR of H-ferritin mRNA which confers $\text{Il-1}\beta$ -dependent translation to a CAT reporter mRNA. This *cis*-acting H-ferritin mRNA element is distinct from the IRE with its 5' terminus 77 nt downstream from the IRE. The sequence is similar to a motif previously reported by Dente *et al.*, (1985) to be upstream of the start codons of other hepatic acute phase response mRNAs (36).

MATERIALS AND METHODS

Preparation of constructs

H-ferritin 5' untranslated region. The H-ferritin genomic subclone, pUC-HFER, contains a 458 bp SstI fragment from the H-ferritin gene cloned into pUC12 (Gift of J.Drysdale). The insert comprises 164 bp of H-ferritin gene sequence upstream of the 5' cap site and 294 bp of the first exon including the 5' untranslated region. Figure 1 shows the distribution of restriction enzyme sites within the H-ferritin 5' UTR (37). A HinfI sequence is 18 bp from the transcription start site of the H-ferritin gene. The StyI and DdeI sites are respectively 120 bp and 181 bp downstream from the HinfI site. The DdeI recognition site is 9 bp from the H-ferritin initiation codon. The box represents the 20 nucleotides (nt) acute box consensus region which is 22 nt upstream from the H-ferritin initiation codon and is described in the text.

Expression vectors. The vector, pSV2CAT, contains a unique StuI site 45 bp downstream of the SV40 early T-antigen promoter which was used as the cloning site for preparing all the constructs except H-IRECAT (38, 39). The 458 SstI bp insert of pUC-HFER was digested with combinations of HinfI, StyI, and DdeI. Appropriate fragments were gel purified; the ends were filled in with DNA polymerase (Klenow fragment; Boehringer, Indianapolis, IN) and then subcloned into StuI restricted pSV2CAT by blunt end ligation. The construct, pSV2(5'UTR)-CAT, was prepared with the 181 bp HinfI–DdeI fragment inserted into the StuI site of pSV2CAT. The pSV2(Ac)CAT and pSV2(rev)CAT constructs were prepared by inserting the 61 bp StyI–DdeI fragment into the unique StuI site in the forward and reverse orientations. H-IRECAT was prepared by gel purifying the 302 bp SstI–StyI fragment from pUC-HFER, and 'blunt ending' the 5' end of the StyI site with DNA polymerase (Klenow fragment). This modification was followed by ligation into the unique SstI and HincII sites in the polylinker of pUC₁₂CAT. The pUC12CAT vector contains the CAT gene inserted into the HindIII site of pUC12. The identity of all constructs were confirmed by double-stranded DNA sequencing to preclude the presence of artificial AUG sites upstream of the CAT initiation codon.

The pSV2(Δ 3)CAT construct. pSV2(Δ 3)CAT was prepared by inserting a 58 bp DNA cassette encoding a 3 bp deletion of the H-ferritin gene 61 bp StyI–DdeI into a site adjacent to the unique StuI site in the 5'UTR of the CAT gene in pSV2CAT. A 96 nt oligonucleotide encoding this deletion was a substrate for 36 rounds of a PCR thermocycling reaction using two small 19mers

Figure 1. Eukaryotic expression constructs containing different segments of the H-ferritin 5' noncoding region preceding the CAT reporter gene. RNA sequences which are transcribed from pSV2CAT sequences are represented by a thin line. H-ferritin gene derived RNA sequences are depicted by a thick line. All the constructs contain the pSV2CAT early SV40 promoter except for H-IRECAT which transcribes RNA from the ferritin heavy-subunit gene promoter. The pSV2(5'UTR)CAT construct contains a 181 HinfI–DdeI fragment encompassing the complete 5'UTR of H-ferritin mRNA less 18 nt downstream from the 5' cap site and 9 nt immediately upstream from the initiation codon. The pSV2(Ac)CAT construct was prepared by insertion of a 61 bp StyI–DdeI fragment in front of the H-gene start codon into the 5' leader of pSV2CAT. The 61 bp StyI–DdeI fragment was inserted in reverse orientation into the StuI site of pSV2CAT in order to construct pSV2(rev)CAT. Both pSV2(Ac)CAT and pSV2(rev)CAT transcribe hybrid CAT mRNAs from the same promoter and each has a 159 nt chimeric 5' leader. The 182 bp H-ferritin promoter region is present in H-IRECAT and transcribes the first 142 nt of H-ferritin mRNA as part of a hybrid H-ferritin/CAT transcript.

annealed at each end. The resulting double-stranded DNA was digested with StuI and HindIII to generate a convenient cloning cassette for insertion in between the compatible restriction sites in pSV2CAT.

pBluescript constructs: A 254 bp HindIII–EcoRI fragment from pSV2CAT contains 36 bp of the CAT gene 5' UTR and 218 bp of the 5' end of the coding sequence of CAT gene (Fig. 3A). This fragment was subcloned from pSV2CAT into compatible restriction sites in pBluescript (pBS). Exons 2–4 of the L-ferritin gene are encoded by a 0.63 kb PstI fragment isolated from the L-ferritin cDNA clone, pBRLFER, which was subcloned into the single PstI site of pBS [(40) (Fig. 3B)].

Cell culture

HepG2 hepatoma cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, essential amino acids, penicillin, and streptomycin (GIBCO).

Transfections and CAT assays

Twenty micrograms of DNA from each construct was transfected into HepG2 cells (2×10^7 cells) by the calcium phosphate

technique (41). Cells were trypsinized after 6 hr, reseeded at equal density into an appropriate number of separate 10 cm² culture plates before exposure to iron (2.5 μ M Fe₂Tf), 1 ng/ml Il-1 β or to intracellular iron chelation [200 μ M desferrioxamine (Df)] for 12–16 hr. Lysates were prepared by harvesting cells into a buffer containing 125 mM Tris, pH 7.6, 0.5% Triton. Each lysate was assayed for protein content to confirm that an equivalent number of cells had been reseeded prior to stimulation. The level of CAT activity in each lysate was assayed by two methods: 1) CAT activity was determined by incubating equal amounts of the lysates with 2×10^5 cpm [¹⁴C]-chloramphenicol (CAP) and 0.67 mg acetylcoenzyme-A for 1–12 hours at 37°C. The reactions were extracted with ethyl acetate and the products were separated by thin-layer chromatography (TLC) and autoradiographed. CAT conversion was quantified by determining the ratio of acetylated forms of [¹⁴C]-CAP to non-acetylated substrate using a Betagen 603 Blot Analyzer (Betagen Corp, Waltham, MA). 2) CAT activity was also determined by directly counting the amount of the CAT reaction product diffusing into liquid scintillation fluid (42). Each lysate was incubated at 37°C with [³H]-acetylcoenzyme-A (0.1 μ Ci) and 2 mM chloramphenicol (CAP) in 200 μ l of 100 mM Tris.Cl (pH 7.8) which had been overlaid by 5 mls of liquid scintillant (Econofluor, NEN). The CAT catalyzed acetylation of CAP was monitored by counting the accumulation of tritiated acetyl-CAP product in the organic scintillation fluid over a time period from 2 hr to 48 hr. The slope of these time courses accurately reflects CAT protein levels in each lysate.

RNA isolation and polyribosome gradients

HepG2 cells (2×10^7 cells) were grown for 12 hours in the presence of Il-1 β (60 ng/ml), or for three hours with 114 μ M ferric ammonium citrate (pH 7.0) as an iron source or left unstimulated. Total RNA was isolated by guanidinium thiocyanate lysis and ethanol precipitation (43). Polyribosomes were fractionated by harvesting 2×10^7 cells into 1 ml of polysome lysis buffer [0.15 M NaCl, 10 mM Tris.HCl (pH 7.2), 0.5% Nonidet P-40, 10mM MgCl₂, 50 μ g of cycloheximide/ml, 20 mM dithiothreitol, 100 units/ml RNasin] followed by sedimentation through a 10–50% sucrose gradient as previously described (39). Polyribosome, 80S, 40S, and ribosome-free fractions were identified using an ISCO fractionator (Lincoln, NE) and by ethidium staining after separation on a 1.2% agarose gel. The fractions associated with the polyribosomes were pooled, phenol extracted, and ethanol precipitated. Total and polyribosomal RNA was quantitated by Northern blotting with nitrocellulose membranes and hybridization of the filter-bound RNA with randomly primed H-ferritin cDNA (8). In most experiments filters were rehybridized with either a L-ferritin cDNA probe or an α_1 AT cDNA probe (10, 40).

RNase protection

Ferritin and CAT mRNAs from HepG2 transfectants were characterized and quantitated by RNase protection (44). A 480 nt ³²P-labeled cRNA, antisense to exons 2–4 of the L-ferritin mRNA, was transcribed with T3 polymerase from PvuII digested DNA template isolated from the L-ferritin subclone (pBS-LF_{er}) (8). T7 polymerase transcribed a 261 nt ³²P-labeled cRNA from HindIII digested DNA template isolated from the CAT gene subclone (pBSCAT-1). Labeled CAT and L-Ferritin cRNAs (1×10^6 cpm) were each hybridized to 20 μ g of hepatoma RNA in a buffer containing 80% formamide, 40 mM Pipes (pH 6.7),

0.4 M NaCl, 1 mM EDTA for 16 hr at 45°C. Digestion with RNase A (40 μ g/ml; Boehringer Mannheim) and RNase T1 (2 μ g/ml; Boehringer Mannheim) removed unhybridized cRNAs. Protected cRNAs were separated by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. HaeIII-digested T_x174 DNA fragments were kinase labeled and used as molecular weight markers to confirm the lengths of protected fragments.

Slot blotting

Endogenous ferritin mRNA and CAT mRNA levels were quantitated by slot blotting. Transfected and untransfected hepatoma RNA (20 μ g) was denatured for 15 mins at 65°C in a buffer containing 6.15 M formaldehyde, 0.15 M sodium citrate, 1.5 M NaCl. The RNA was applied onto slots aligned on nitrocellulose membranes. For the purpose of quantitation, 1 ng–0.01 pg pSV2CAT was denatured in 0.3 M NaOH at 60°C for 30 mins, neutralized in 1M NH₄Acetate (pH 7.4) and applied to slots aligned on nitrocellulose membranes. A 0.63 kb PstI fragment of DNA from the L-ferritin cDNA clone (40) and a 1.635 kb HindIII–BamHI fragment coding for the complete CAT gene from pSV2CAT were gel purified. The DNA fragments were labeled by randomly priming (specific activity = 2×10^8 cpm/ μ g) and used as hybridization probes. Overnight hybridization of the filters was carried out in 20 ml of a buffer consisting of 50% formamide, 50 μ g/ml denatured salmon sperm DNA, 5 $\times$ SSC, 0.1% sodium dodecyl sulfate and 5 $\times$ Denhardt's solution with the addition of 50 ng randomly primed ³²P-labeled CAT gene and L-ferritin cDNA probes. The filters were washed three times in 0.5 $\times$ SSC, 0.1% sodium dodecyl sulfate at 55°C so that levels of radioactive CAT gene probe which specifically hybridized to each RNA slot could be quantitated accurately using a Betagen 603 Blot Analyzer (Betagen Corp, Waltham, MA).

RESULTS

We previously showed that Il-1 β induces an average two to four-fold, and a maximal twenty-fold, increase in the synthesis of both H- and L-ferritin subunits (8). L-ferritin was shown to be recruited to polyribosomes without affecting L-ferritin mRNA levels. Iron increases ferritin synthesis by forty-fold (35). Figure 2A demonstrates by Northern blotting that H-ferritin mRNA levels are unchanged after 12 hr Il-1 β stimulus or iron exposure. Sucrose gradients were also loaded with lysates prepared from control, Il-1 β and iron treated HepG2 cells in order to separate ribosome associated mRNAs from free mRNAs. Northern blotting and autoradiographic densitometry showed a three-fold increased accumulation of H-ferritin mRNA in polyribosomes after 12 hr Il-1 β stimulation of the cells (Fig. 2A). HepG2 cells exposed to iron as ferric ammonium citrate for three hours also exhibited a three fold recruitment of H-ferritin mRNA into the polyribosomes compared to unstimulated counterparts (Fig. 2A). Five separate experiments confirmed that Il- β stimulates an average two-fold increased accumulation of H- and L-ferritin mRNAs within HepG2 polyribosomes without changing the steady state levels of ferritin mRNA (Fold induction: Mean = 2.07, Standard error = 0.21, N = 5).

The effect of Il-1 β exposure on the polyribosomal distribution of both L-ferritin and α_1 AT mRNAs in HepG2 cells has also been examined by sucrose gradient analysis. The histogram in Figure 2B shows an experiment where the proportion of L-ferritin and α_1 AT transcripts were quantitated in pooled fractions across 10–50% sucrose gradients after sedimentation of lysates from

either unstimulated or IL-1 β stimulated HepG2 cells. Six hours of IL-1 β stimulus resulted in a recruitment of 22.6% of L-ferritin mRNA to the polyribosome fractions whereas 13.4% of L-ferritin mRNA was present within the polyribosome fractions of control HepG2 lysates (panels A and B). The filters were rehybridized with a α_1 AT cDNA probe in order to detect the polyribosome distribution of the efficiently translated α_1 AT mRNA. More than 70% of α_1 AT mRNA was determined to be associated with HepG2 monosomes and polyribosomes regardless of IL-1 β stimulus (panels C and D).

Constructs were prepared using the eukaryotic expression vector, pSV2CAT, to determine whether 5' UTR sequences from the H-ferritin transcript could confer IL-1 β responsive translational activation to CAT mRNA when inserted in front of the CAT start codon after transfection into HepG2 cells (Table I). Four of the five informative constructs, pSV2(5'UTR)CAT, pSV2(Ac)CAT, and pSV2(rev)CAT, and pSV2(Δ 3)CAT were made from pSV2CAT (Fig. 1). These constructs use the same SV40 early T-antigen promoter to transcribe CAT mRNAs with hybrid H-ferritin/CAT 5' leaders (39). The H-IRECAT construct is transcribed from the H-ferritin gene promoter.

The pSV2(5'UTR)CAT construct consists of 181 bp of the complete 208 bp 5' noncoding region from H-ferritin cDNA inserted into the 98 bp CAT gene 5' noncoding region (Fig. 1). Table 1 displays the data accumulated from 7 separate pSV2(5'UTR)CAT transfections of HepG2 cells showing that H-ferritin mRNA 5' noncoding sequences confer an average 1.84-fold increase in CAT activity in response to IL-1 β and a 1.4-fold increase in response to iron. In contrast, pSV2CAT transfectants exhibit a 1.23-fold increase in CAT activity in

Figure 2. A. IL-1 β does not change HepG2 H-ferritin mRNA levels but increases the proportion H-ferritin mRNA associated with the polyribosomes. RNA from human hepatoma (HepG2) cells was treated with: 1) no supplement 2) 60 ng/ml IL-1 β for 12 hr 3) 114 μ M ferric ammonium citrate for 3 hr. Samples (20 μ g RNA) were Northern blotted prior to probing the nitrocellulose filters with randomly primed labelled H-ferritin cDNA (35). For preparation of RNA associated with polyribosomes, post mitochondrial homogenates from control, IL-1 β stimulated, and iron treated cells were resolved on sucrose gradients prior to fractionation. The polyribosome fractions were identified by the UV absorbance profile and by ethidium bromide staining after RNA separation on a 1.2% agarose gel. The polyribosomes fractions were pooled, phenol extracted and ethanol precipitated. The H-ferritin mRNA content associated with the polyribosomes was estimated by RNA blotting and probing with a 32 P-labelled cDNA probe (35). B. The effect of IL-1 β on the polyribosomal distributions of L-ferritin mRNA and α_1 AT mRNAs. Two separate flasks of HepG2 (1×10^7 cells) were stimulated with 1ng/ml IL-1 β for 6 hr or grown without supplement. Lysates of each culture were fractionated by centrifugation through 10–50% sucrose gradients and the polyribosomal, 80S, 43S, and free fractions [ribonucleoproteins (RNP)] were identified by UV absorption using an Isco Fractionator (top panels). The fractions were confirmed by RNA gel electrophoresis and ethidium bromide staining since the RNP fractions contain no ribosomal (rRNA), the 43S fraction contains only 18S rRNA, the 80S fraction exhibits heavily stained 18S and 28S rRNA and the polyribosome fractions contain lighter staining 18S and 28S rRNA. Pooled fractions were slot blotted in duplicate and the filters were hybridized separately with labeled L-ferritin and α_1 AT cDNAs. The autoradiographs were scanned and quantitated by densitometry so that the distributions of each hepatic transcript throughout the gradients could be calculated. The Y axis shows the percent of L-ferritin mRNA and α_1 AT mRNA which sediments in each fraction as a percent of the total mRNA loaded onto each sucrose gradient. Histograms on the left panels are the polysome distributions of each mRNA in control cells and those on the right panels represent IL-1 β stimulated cells. Panel A is L-ferritin mRNA, control; panel B is L-ferritin mRNA, IL-1 β ; panel C is α_1 AT-mRNA, control; panel D is α_1 AT-mRNA, IL-1 β .

response to $\text{Il-1}\beta$ ($N=17$) and exhibit no iron response ($N = 11$). $\text{Il-1}\beta$ inductions of CAT levels exhibited by pSV2(5'UTR)CAT transfectants were consistently larger than those exhibited by pSV2CAT controls. The difference in $\text{Il-1}\beta$ inducibility between pSV2(5'UTR)CAT and pSV2CAT was small but significant ($P = 0.009$ between pSV2CAT and pSV2(5'UTR)CAT transfectants). The smaller than expected induction of CAT activity by iron in pSV2(5'UTR)CAT transfectants was consistent with a repositioning of the IRE to a site further downstream from the H-ferritin mRNA 5' cap site (Discussion).

In order to identify which portion of the H-ferritin mRNA 5'UTR contains the putative $\text{Il-1}\beta$ translational regulatory element, the 5' and 3' segments of the H-ferritin gene 5'UTR were independently subcloned in front of the CAT reporter gene. The constructs pSV2(Ac)CAT and pSV2(rev)CAT contain a 61 bp G+C rich 3' segment in forward and reverse orientation respectively (Fig. 1). The construct, pSV2(Ac)CAT, tested whether the 61 bp G+C rich region between the StyI–DdeI sites of the H-ferritin gene 5'UTR could confer $\text{Il-1}\beta$ responsive translation to the reporter transcript. This region is within 70 nt upstream of the H-ferritin start codon. Table 1 shows that $\text{Il-1}\beta$ stimulated an average 2.2-fold induction of CAT levels in HepG2 cells transfected with pSV2(Ac)CAT over 23 separate transfections. The difference in $\text{Il-1}\beta$ responsiveness demonstrated by pSV2CAT and pSV2(Ac)CAT transfectants is statistically significant. The data in Table 1 was used to calculate a significance probability value P inductions of CAT levels exhibited by pSV2CAT and pSV2(Ac)CAT transfectants in response to $\text{Il-1}\beta$. Stimulation of pSV2(Ac)CAT transfectants by iron did not change CAT expression.

Transcripts from the pSV2(rev)CAT construct are well matched controls for $\text{Il-1}\beta$ mediated translational enhancement by pSV2(Ac)CAT mRNAs since the 5'UTR and complete mRNA lengths of both sets of transfected CAT mRNAs are identical after transcription from the SV-40 promoter (39). This construct was prepared by subcloning the 61 bp StyI–DdeI fragment from the H-subunit gene into the single StuI site of pSV2CAT in the reverse orientation to pSV2(Ac)CAT. Transfectants of pSV2(rev)CAT transcribe an antisense version of the 61 nucleotide G+C rich H-ferritin mRNA sequence within the 5'UTR of the hybrid CAT transcript. The average data from nine pSV2(rev)CAT transfections show that $\text{Il-1}\beta$ induces a

background 1.23-fold increase in CAT activity, identical to the induction in pSV2CAT transfectants (Table 1). We concluded that there are structural features of the sense RNA strand from the G+C rich region of the H-ferritin mRNA transcript which mediate the $\text{Il-1}\beta$ action.

The H-IRECAT construct uses the H-ferritin gene promoter to transcribe a hybrid RNA with a correctly positioned IRE, attached to the CAT coding region (37). The transcript lacks the G+C rich (82% G+C rich nucleotides) sequence between the StyI site and the H-ferritin initiation codon (Fig. 1). H-IRECAT transfected cells exhibited an average 11.8-fold increase in CAT expression in response to iron and desferrioxamine over five separate transfections (Table 1). The H-IRECAT transfectants were not $\text{Il-1}\beta$ responsive indicating that a functional IRE is not sufficient to confer $\text{Il-1}\beta$ -dependent translation to a CAT mRNA (Table 1).

RNAse protection was used to quantitate CAT mRNAs generated from the transfected constructs as well as endogenous L-ferritin mRNA before and after $\text{Il-1}\beta$ induction (44). RNAse protection confirmed that the levels of protected CAT mRNA in pSV2(Ac)CAT transfected HepG2 cells do not change in response to $\text{Il-1}\beta$ (Fig. 3A, lanes 3 and 4). Similarly, $\text{Il-1}\beta$ does not stimulate an increase in CAT mRNA levels in pSV2(rev)CAT (Fig. 3A, lanes 5 and 6) and pSV2CAT (Fig. 3A, lanes 1 and 2) transfected HepG2 cells. Endogenous L-ferritin mRNAs are transcribed at equal levels in control and $\text{Il-1}\beta$ stimulated pSV2(Ac)CAT transfectants (Fig. 3B, lanes 1 and 2). Hybridization with a 530 nt cRNA antisense to exons 2–4 of the L-ferritin gene generated a 450 nt protected cRNA fragment [(Fig. 3B) (40)]. The protected L-ferritin fragment is broader and smaller than the input cRNA since the L-ferritin mRNA population displays a range of poly A sizes at the 3' terminus and the cRNA probe is complementary with the 3' end of the L-ferritin mRNA (Rogers *et al.*, unpublished observations). These data confirm that both the levels of CAT mRNAs and L-ferritin mRNA are unchanged in pSV(Ac)CAT transfectants after stimulation with $\text{Il-1}\beta$ (Fig. 3A, lanes 3 and 4 for CAT mRNA; Fig. 3B, lanes 1 and 2 for L-ferritin mRNA). Two features of these experiments confirmed the specificity of the RNAse protection technique. First, untransfected HepG2 RNA protects the 450 nt band from RNAse digestion after hybridization with the 530 nt antisense L-ferritin cRNA (Fig. 3B, lane 3) whereas the same untransfected HepG2 RNA does not generate the 254 nt protected CAT mRNA (Fig. 3A, lanes 7 and 8). Second, neither CAT nor L-ferritin antisense cRNAs are protected from RNAse T1 digestion by 20 μg tRNA (Fig. 3A, lane 9; Fig. 4B, lane 4).

CAT mRNA levels were also measured in RNA preparations from control or $\text{Il-1}\beta$ -stimulated pSV2CAT and pSV2(Ac)CAT HepG2 transfectants by counting slot blots in order to confirm the RNAse protection results. The results of eight separate pSV2(Ac)CAT transfections confirmed that $\text{Il-1}\beta$ induces an average 1.23-fold increase in the levels of CAT mRNA (Table 2). The observed 1.23-fold induction of CAT mRNA levels is not sufficient to account for the 2.2-fold elevation of CAT activity in pSV2(Ac)CAT transfectants (Table 1). pSV2CAT transfectants did not exhibit an $\text{Il-1}\beta$ -dependent increase in CAT mRNA levels (Tables 1 and 2).

The effect of $\text{Il-1}\beta$ on the translational efficiencies of CAT mRNAs transcribed from the pSV2CAT and pSV2(Ac)CAT constructs was calculated by standardizing the CAT activity by the amount of CAT mRNA transcribed after transfection (Fig.

Table 1. Fold increase in CAT activity

Construct	$\text{Il-1}\beta$	N	Iron	N
pSV2(Ac)CAT	2.20 ± 0.13	23	1.00 ± 0.08	10
pSV2(rev)CAT	1.23 ± 0.10	9	1.14 ± 0.08	5
pSV2(5'UTR)CAT	1.84 ± 0.16	7	1.40 ± 0.09	11
pSV2CAT	1.23 ± 0.11	17	1.08 ± 0.08	11
H-IRECAT	1.80 ± 0.12	6	11.80 ± 4.00	5
pSV2(Δ 3)CAT	1.37 ± 0.23	6	ND	ND

The average induction of CAT activity by $\text{Il-1}\beta$ or iron in pSV2(Ac)CAT, pSV2(rev)CAT, pSV2(5'UTR)CAT, pSV2CAT and H-IRECAT transfectants. Maps of the constructs are shown in Figure 1 and the analysis described in this table are the average increase in CAT activity from several experiments after the induction of each transfectant by either $\text{Il-1}\beta$ and iron. The value N is the number of separate transfections for each construct. The constructs were matched against each other in several transfections. This analysis takes independent values from different experiments and indicates the standard error as $\pm$ SEM (standard error of the mean).

Figure 3. The effect of Il-1β on the levels of CAT mRNA and endogenous L-ferritin mRNA in pSV2(Ac)CAT, pSV2(rev)CAT and pSV2CAT transfectants. Antisense RNAs were used to detect the expression of (A) either the transfected CAT transcript or (B) the endogenous L-ferritin mRNA in HepG2 cells by RNase protection. The arrow indicates the size of the input antisense cRNAs (261 nt for CAT cRNA and 530 nt for the L-ferritin cRNA). The RNaseT1 protected cRNAs are smaller than the input cRNAs and migrated slightly faster during electrophoresis (254 nt for CAT and 450 nt for the L-ferritin cRNA). The lanes represent RNase protections using the following transfected or untransfected RNA sources: Gel A CAT mRNA detection: lane M, ϕ X174 DNA digested with HaeIII; lane 1, pSV2CAT; lane 2, pSV2CAT + Il-1β; lane 3, pSV2(Ac)CAT; lane 4, pSV2(Ac)CAT + Il-1β; lane 5, pSV2(rev)CAT; lane 6, pSV2(rev)CAT + Il-1β; lane 7, HepG2; lane 8, HepG2 + Il-1β; lane 9, tRNA; lane 10 Input CAT cRNA. Gel B L-Ferritin mRNA detection: lane M, ϕ X174 DNA digested with HaeIII; lane 1, pSV2(Ac)CAT; lane 2, pSV2(Ac)CAT + Il-1β; lane 3, HepG2; lane 4, tRNA; lane 5, Input L-ferritin cRNA.

4A and B). This ratio is an *in vivo* index of the translational efficiency of CAT mRNAs. HepG2 cells were transfected, trypsinized, and reseeded in equal cultures (10^7 cells) prior to overnight exposure to Il-1β for 16 hours. Figure 4A is a representative experiment comparing the rate of CAT mRNA translation in lysates prepared from control and Il-1β stimulated pSV2CAT transfectants. Each time point is the average of a duplicate assay and the slopes of the data generate the reaction rates. Lysates prepared from pSV2CAT transfectants which had

been exposed to Il-1β for 12 hours exhibited no increase in the rate of CAT synthesis per picogram of CAT mRNA. In contrast the experiment in Figure 4B shows that the rate of CAT synthesis per picogram of CAT mRNA in pSV2(Ac)CAT transfectants was 2.7 fold elevated after 12 hours stimulus with Il-1β. These data confirm that the G+C rich RNA sequences within 70 nt upstream of the H-ferritin start codon confer Il-1β-dependent translation to CAT mRNAs in HepG2 cells. In support of this conclusion we have observed that pSV2(Ac)CAT transfectants of another

Table 2. Fold increase in CAT mRNA

Construct	IL-1 β	N
pSV2(Ac)CAT	1.23 $\pm$ 0.20	8
pSV2CAT	0.98 $\pm$ 0.13	7

The average induction of CAT mRNA levels by IL-1 β in pSV2(Ac)CAT and pSV2CAT transfectants. RNA was prepared from control or IL-1 β -stimulated transfectants. The relative levels of CAT mRNA was quantitated by slot blotting twenty micrograms of each RNA population and counting the specific hybridization signal of a labelled CAT gene probe at each slot using a Betagen 606 Analyzer (Materials and Methods). The means and standard errors were calculated from N separate transfections.

human hepatoma cell line, Hep3B, exhibit a four fold increase in the rate of CAT synthesis per picogram CAT mRNA in response to IL-1 β [Rogers *et al.*, (1994) manuscript in preparation].

A deletion in the H-ferritin mRNA *cis*-element abolishes IL-1 β -dependent translation

The construct pSV2(Δ 3)CAT contains a three nucleotide deletion (5' CAC3') from the 61 bp G+C rich segment of the H-ferritin gene 5'UTR inserted in front of a CAT reporter gene (Fig. 5A). Figure 5B is a typical experiment comparing IL-1 β dependent increases of CAT activity in lysates prepared either from wildtype pSV2(Ac)CAT or pSV2(Δ 3)CAT HepG2 transfectants. In this experiment IL-1 β stimulated a 1.8-fold increase of CAT activity in pSV2(Ac)CAT transfectants whereas CAT gene expression is not elevated in pSV2(Δ 3)CAT deletion mutant transfectants (Fig. 5B). Six separate transfections of HepG2 cells showed that IL-1 β confers an average 1.37-fold increase in CAT levels in pSV2(Δ 3)CAT transfectants compared with a 2.2-fold induction of CAT activity in wild type pSV2(Ac)CAT transfectants (Table 1). Both pSV2(Δ 3)CAT and pSV2(Ac)CAT transcribe hybrid H-ferritin /CAT reporter transcripts from the same SV40 early T antigen promoter (39).

Sequences within the H-ferritin mRNA *cis*-acting element align with a consensus reported for three acute phase mRNAs

Dente *et al.*, (1985) described a 38 nucleotide consensus within the 5'UTRs of the mRNAs encoding the hepatic acute phase reactants α_1 AT, AGP, and haptoglobin (36). Our computer searches of the complete H-ferritin gene and 5' leader identified that the consensus is similar to a part of the G+C rich *cis*-acting element of the H-ferritin mRNA [(Fig. 5A) (45)]. This consensus maps between the StyI and DdeI sites within 70 nt upstream of the H-ferritin gene start codon and is 105 nt downstream from the IRE stemloop. We have used the term 'the acute box' to describe the consensus since the motif is present within the 5' leaders of the acute phase genes coding for haptoglobin, α_1 AT and α_1 AGP, and H-ferritin although the sequence is matched to a lesser extent for haptoglobin mRNA 5'UTR sequences (2, 10, 37). Actin mRNA 5'UTR sequences are not similar to the consensus (46).

DISCUSSION

We have mapped the site of IL-1 β regulation of ferritin heavy subunit translation to a *cis*-acting StyI–DdeI fragment within 70 nt upstream of the H-gene start codon. This fragment encodes a 61 nt RNA sequence containing 82% G+C nucleotides (Fig.

Figure 4. The effect of IL-1 β on the comparative translational efficiencies of CAT mRNAs in pSV2(Ac)CAT and pSV2CAT transfectants: A and B: The effect of IL-1 β on CAT activity per picogram of CAT mRNA in HepG2 cells transfected with pSV2(Ac)CAT and pSV2CAT. HepG2 cells (2×10^7 cells) were transfected with either 20 μ g of pSV2CAT or pSV2(Ac)CAT DNA for 8 hrs and then split equally. One of the reseeded cell cultures was stimulated with 1ng/ml IL-1 β for 16 hrs and both were subsequently harvested into 1 ml CAT lysis buffer each (Materials and Methods). RNA was isolated from 0.7 vol of the lysates and the remainder was assayed for CAT activity in duplicate. Transfected RNA (20 μ g) was slot-blotted and CAT mRNA levels were quantitated by counting the amount of specific hybridization of a labeled CAT gene probe to each slot using a Betagen 603 Blot Analyzer. The number of picograms (pg) of CAT mRNA within each 20 μ g HepG2 RNA population was calculated by comparison with the CAT probe that hybridized to 10 ng, 1ng, 100 pg, 10 pg, 1 pg 0.1 pg and 0.01 pg of denatured pSV2CAT DNA which had been applied to a series of slots. Each graph represents a time course for the acetylation of chloramphenicol with [3 H]acetyl-CoenzymeA and reflects the levels of CAT enzyme in each lysate corrected for the pg CAT mRNA in the sample.

1, Fig. 5A and Table I). The element is downstream from the IRE and independently confers 2.2-fold enhanced translation to chimaeric CAT mRNAs in HepG2 hepatoma cells stimulated with IL-1 β (Table I and Fig. 4). Hep3B cells, transfected with pSV2(Ac)CAT, exhibit a four fold increase in CAT mRNA translation (Rogers *et al.*, in preparation). These data indicate that the IRE is not involved in IL-1 β mediated translational regulation of H-ferritin mRNA but is iron responsive. We confirm the results of Goosen *et al.*, (1990) showing that iron regulation is blunted in pSV2(5'UTR)CAT transfectants because the construct transcribes hybrid CAT mRNAs where the 35 nt IRE stem-loop is 53 nt from the 5' cap site (47). The IRE is 26 nt from the natural H-ferritin mRNA 5' cap site and 147 nt upstream

A CCAAGGCCCGCCGCGCTCCAGGCGCGCAGCCACCGCGCGCGCCGCTCTCCTTAGTCGCCCATG H ferritin-mRNA.
 5'CTGCAGCNTGCAGCCACCAGCACTGNCCTGGCNTCCAG3' (Dente et al., 1985).

Figure 5. A. The H-ferritin mRNA *cis*-acting sequence. The StyI and DdeI restriction enzyme sites delimit the H-ferritin gene 5' UTR sequences inserted into the pSV2(Ac)CAT and pSV2(rev)CAT constructs. Bold lettering depicts these sites and also the three nucleotides deleted from this region of the H-ferritin gene 5'UTR in pSV2(Δ3)CAT. Computer analysis demonstrated a similarity between the H-ferritin 5'UTR sequence and a consensus reported for the 5'UTR sequences of three mRNAs coding for the acute phase proteins, haptoglobin, α_1 -acid glycoprotein, and α_1 -antitrypsin (Dente *et al.*, 1985). **B.** The effect of Il-1 β on CAT activity in pSV2(Δ3)CAT transfectants. HepG2 cells were transfected with 20 μ g of either pSV2(Δ3)CAT or pSV2(Ac)CAT for 8 hours and were then split equally. One of each of the duplicate cultures was stimulated with Il-1 β (1ng/ml) for 26 hours. Each of the cultures were harvested into CAT lysis buffer and the levels of CAT enzyme present in each lysate was quantitated by the liquid scintillation method (Materials and Methods). The CAT enzyme activity in each lysate is represented by the slopes of curves indicated and is most accurately estimated from the linear part of each reaction time course.

from the H-ferritin start codon. Similar position effects might diminish the action of the G+C rich H-ferritin mRNA regulatory element in response to Il-1 β in pSV2(5'UTR)CAT transfectants. A three nucleotide deletion mutant version of this *cis*-acting H-ferritin 5'UTR element no longer confers Il-1 β dependent translational enhancement to a CAT reporter mRNA (Fig. 5, bold lettering). The $\Delta 3$ mutant version of the H-ferritin mRNA 5'UTR disrupts the predicted wild type secondary structure which may account for the lack of responsiveness of the mutant to Il-1 β . H-ferritin mRNA sequences within the *cis*-acting enhancer are computer predicted to form a stemloop 77 nucleotides downstream from the IRE and 17 nt from the start codon although there is no experimental proof of this structure [$\Delta G = -16.7$ kcal/mol for the G+C rich enhancer compared to -40.3 kcal/mol for the rat L-ferritin IRE, (24, 48)].

Direct estimates of the level of CAT mRNA transcribed from pSV2(Ac)CAT transfectants confirm that the H-ferritin mRNA G+C-rich sequence elevates CAT expression in response to Il-1 β primarily at the translational level (Figs 3 and 4). The small elevation in CAT mRNA levels accounts for the Il-1 β -dependent increase in CAT enzyme activity in pSV2CAT and pSV2(rev)CAT transfectants (Table 2). Indirect evidence also suggests that the G+C rich region in the H-ferritin gene 5'UTR is important for translational control and is not a transcriptional regulatory element. First, Il-1 β induces a modest increase in CAT mRNA levels via the same T-antigen promoter in all the pSV2CAT derived constructs (39). Second transcriptional enhancers are defined as regulatory sequences which augment gene expression from a variety of promoters in a distance and orientation independent manner (49). The pSV2(rev)CAT construct has the H-ferritin gene sequences inserted into the

5'UTR in the reverse orientation to pSV2(Ac)CAT and does not exhibit elevated CAT levels in response to Il-1 β , suggesting that the 61 nt G+C rich element is not a typical transcriptional regulatory element.

The precise mechanism by which the G+C rich region of H-ferritin mRNA 5'UTR regulates Il-1 β induced translation in HepG2 cells (50) is being investigated by two lines of evidence. Firstly we identified the sequence as a potential Il-1 β dependent enhancer because of sequence similarity to a consensus reported between the 5'UTRs in AGP, α_1 AT and haptoglobin mRNAs [(Fig. 5A) (36)]. Computer searches showed that 21 nt of the H-ferritin mRNA 5' leader within 70 nt of the start codon are similar to a 38 nt consensus sequence in the 5'UTRs of these liver acute phase genes (36, 45). This report does not attribute a role to the acute box consensus as a general *cis*-acting translational enhancer of acute phase mRNAs and only refers to the translation of H-ferritin mRNAs. In this regard the sequence of the G+C rich region in H-ferritin mRNA aligns most stringently with the AGP and α_1 AT gene 5'UTRs and exhibits less sequence identity with the haptoglobin gene 5' leader. The possible role of the consensus in other acute phase responsive mRNAs remains unclear. Secondly, the first step in translation occurs when the 43S small ribosome subunit attaches to mRNAs by first binding to the m⁷G(5')ppp(5') structure uniquely present at the 5' termini of eukaryotic mRNAs (51, 52). This attachment is limited by iron-dependent binding of the IRE-BP to the IRE in ferritin transcripts (30, 31). The second step in translation occurs when the 60S ribosome subunit attaches to the 43S ribosomal scanning complex at the AUG in an optimal surrounding sequence (The Kozak consensus, CCPuCCATGG) (51, 52). Secondary structure formed from sequences around the

H-ferritin mRNA acute box may represent a target for 60S ribosomal recognition of the H-ferritin mRNA scanning complex prior to peptide synthesis in response to $\text{Il-1}\beta$. This hypothesis is consistent with the fact that $\text{Il-1}\beta$ does not enhance H- or L-ferritin mRNA translation when iron chelation results in a tight interaction between the IRE-BP and its cognate IRE thereby blocking formation of the 43S scanning complex (8). This model is also consistent with a report showing that a G+C rich variable portion within 28S rRNA (bases 820–890) in the 60S ribosomal subunit extensively complements the H-ferritin mRNA 5'UTR in a region of over 50 nt around the acute box ferritin start codon (53).

ACKNOWLEDGEMENTS

We are grateful to Dr Joel Belasco, Dr Deborah Galson and Professor H.F. Bunn for helpful comments in preparation of the manuscript. We thank Dr J. Drysdale for the H-ferritin clone. Dr D. Darling and Dr W. Chin supplied the vector for the H-IRECAT construct. This work was supported by a grant from the National Institutes of Health (NIH R01 HL45794 to K.B. and R29 AI32717-01 to J.R.).

REFERENCES

- Akira, S., Hirano, T., Taga, T., and Kishimoto, T. (1990) *FASEB J.* **4**, 2860–2867.
- Dente, L., Pizza, M.G., Metcalpe, A., and Cortese, R. (1987) *EMBO J.* **6**, 2289–2296.
- Morrone, G., Ciliberto, G., Oliviero, S., Arcone, R., Dente, L., Content, J., and Cortese, R. (1988) *J. Biol. Chem.* **263**, 12554–12558.
- Kushner, I. (1982) *Ann. NY. Acad. Sci.* **389**, 39–48.
- Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7907–7911.
- Heguy, A., Baldari, C.T., Macchia, G., Telford, J. and Melli, M. (1992) *J. Biol. Chem.* **267**, 2605–2609.
- Wilson, D.R., Juan, T.S.C., Wilde, M.D., Fey, G.H., and Darlington, G.J. (1990) *Mol. Cell. Biol.* **10**, N° 12, 6181–6191.
- Rogers, J.T., Bridges, K.B., Durmowicz, G., Glass, J., Auron, P.E., and Munro, H.N. (1990) *J. Biol. Chem.* **265**, N° 24, 14572–14578.
- Ganter, U., Arcone, R., Toniatti, C., Morrone, G., and Ciliberto, G. (1989) *EMBO J.* **8**, 3773–3779.
- Ciliberto, G., Dente, L., and Cortese, R. (1985) *Cell* **41**, 531–540.
- Arosio, P., Adelman, T. G., and Drysdale, J.W. (1978) *J. Biol. Chem.* **253**, 4451–4458.
- Theil, E.C. (1987) *Ann. Rev. Biochem.* **56**, 289–315.
- Campbell, C.H., Solgonick, R.M., and Linder, M. (1989) *Biochem. Biophys. Res. Commun.* **160**, 453–459.
- Konijn, A.M., and Herskho, C. (1977) *Br. J. Haematol.* **37**, 7–16.
- Konijn, A.M., Carmel, N., Levy, R., and Herskho, C. (1981) *Br. J. Haematol.* **49**, 361–370.
- Weinberg, E.D. (1978) *Microb. Rev.* **42**, 45–56.
- Lalonde, R.G. and Holbein, B.E. (1984) *J. Clin. Invest.* **73**, 470–475.
- Starke, P.E., and Farber, J.L. (1985) *J. Biol. Chem.* **260**, 10099–10104.
- Halliwell, B., and Gutteridge, J.M.C. (1984) *Biochem. J.* **219**, 1–14.
- Torti, S.V., Kwak, E.L., Miller, S.C., Miller, L.L., Ringold, G.M., Myambo, K.B., Young, A.P., and Torti, F.M. (1988) *J. Biol. Chem.* **263**, 12638–12644.
- Miller, L.L., Miller, S.C., Torti, S.V., Tsuji, Y. and Torti, F.M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4946–4950.
- Klausner, R.D., Rouault, T.A. and Harford, J.B. (1993) *Cell* **72**, 19–28.
- Eisenstein, R.S., and Munro, H.N. (1990) *Enzyme* **44**, 42–56.
- Aziz, N., and Munro, H.N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8478–8482.
- Hentze, M.W., Caughman, S.W., Rouault, T.A., Barriocanal, J.G., Dancis, A., Harford, J.B., and Klausner, R.D. (1987) *Science* **238**, 1570–1573.
- Murray, M.T., White, K., and Munro, H.N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7438–7442.
- Harrel, C.M., McKenzie, A.R., Patino, M.M., Walden, W.E., and Theil, E.C. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4166–4170.
- Leibold, E.A., and Munro, H.N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2171–2175.
- Rouault, T.A., Hentze, M.W., Caughman, S.W., Harford, J.B., and Klausner, R.D. (1988) *Science* **241**, 1207–1210.
- Walden, W.E., Daniels-McQueen, S., Brown, P.H., Gaffield, L., Russell, D.A., Bielser, D., Bailey, L.C., and Thach, R.E. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9503–9507.
- Haile, D.J., Rouault, T.A., Tang, C.K., Chin, J., Harford, J.B., Klausner, R.D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7536–7540.
- Emery-Goodman, A., Hirling, H., Scarpellino, L., Henderson, B., and Kuhn, L.C. (1993) *Nucl. Acids. Res.* **21**, 1457–1461.
- Mullner, E.W., Neupert, B., and Kuhn, L.C. (1989) *Cell* **58**, 373–382.
- Koeller, D.M., Horowitz, J.A., Casey, J.L., Klausner, R.D., and Harford, J.B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7778–7782.
- Rogers, J.T., and Munro, H.N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2277–2281.
- Dente, L., Ciliberto, G., and Cortese, R. (1985) *Nucl. Acids. Res.* **13**, 3941–3952.
- Constanzo, F., Colombo, M., Staempfli, S., Santoro, C., Marone, M., Rainer, F., Delius, H., and Cortese, R. (1986) *Nucl. Acids. Res.* **14**, 721–436.
- Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
- Jones, N.C., and Rigby, P.W.J. & Ziff, E.B. (1988) *Genes Dev.* **2**, 267–281.
- Dorner, M.H., Salfeld, J., Will, H., Leibold, R.A., Vass, J.K., and Munro, H.N. (1985) *Proc. Natl. Acad. Sci. USA* **82** 3139–3143.
- Graham, F.L., and Van der Eb, A.J. (1983) *Virology* **52**, 456–467.
- Sleigh, M.T. (1986) *Anal. Biochem.* **156**, 251–262.
- Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) *Biochemistry* **18**, 5294–5299.
- Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, and Green, M.R. (1984) *Nucl. Acids. Res.* **12** N° 18 7035–7056.
- Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucl. Acids. Res.* **12**, 387–395.
- Nakajima-Iijima, S., Hamada, H., Reddy, P., and Kakunaga, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6133–6137.
- Goossen, B., Caughman, S.W., Harford, J.B., Klausner, R.D., and Hentze, M.W. (1990) *EMBO J.* **9**, 4127–4133.
- Zuker, M. (1989), *Methods Enzymol.* **180**, 262–288.
- Gillies, S.D., Morrison, S.L., Oi, V.T., and Tonegawa, S. (1983) *Cell* **33**, 717–728.
- Knowles, B.B., Howe, C.C., and Aden, D.P. (1980) *Science* **209**, 497–499.
- Kozak, M. (1989) *Mol. Cell. Biol.* **9**, N° 11, 5134–5142.
- Kozak, M. (1986) *Cell* **44**, 283–292.
- Jain, S.K., Crampton, J., Gonzalez, I.L., Schmickel, R.D., and Drysdale, J.W. (1985) *Biochem. Biophys. Res. Commun.* **131**, 863–867.