In order to investigate positive and negative regulatory elements of the erythropoietin (Epo) gene, synthetic oligonucleotides were designed to control Epo transcription by means of an antigene strategy. We devised a new method for detecting regulatory elements of genes that have a weak promoter. Synthetic oligonucleotides were incubated with Hep3B cells in the presence or absence of CoCl₂ or hypoxia. To exclude the effect of translational regulation, Epo mRNA concentration was determined by competitive polymerase chain reaction.

The addition of antisense oligonucleotide for CACCC elements decreased the production of Epo mRNA in a dose-dependent fashion when cells were stimulated by CoCl₂ or hypoxia. In contrast, the addition of antisense oligonucleotide for the GATA element caused a dose-dependent stimulation of Epo mRNA production either in the presence or absence of CoCl₂ or hypoxia. Triple helix formation was revealed by electrophoresis. CACCC elements of the Epo gene, whereas the GATA element was a negative regulatory element. Furthermore, by gel mobility shift assays, we demonstrated evidence for the presence of factors in Hep3B cell nuclear extract that specifically bind to CACCC or GATA elements. Based on these observations, we presented the possibility that triple helix formation could serve as a novel means for transcriptional regulation of the gene.

Erythropoietin (Epo) is produced in the kidney and in fetal liver in response to hypoxia (1) as well as to CoCl₂ (2). However, little is understood about the mechanism by which hypoxia leads to increased expression of the Epo gene. The Epo gene promoter lacks canonical CAAT and TATA elements (3). Furthermore, there is no conserved lymphokine element that has been detected in human and mouse interleukin-3, granulocyte-macrophage colony-stimulating factor, and granulocyte-colony-stimulating factor gene promoter regions (4). Goldberg et al. (5) have demonstrated that, in the human hepatoma cell line Hep3B, Epo production can be induced in response to hypoxia or cobalt chloride treatment. Furthermore, the level of Epo mRNA was markedly increased with such stimuli (5, 6).

Placing a series of truncation fragments upstream of a reporter gene is a commonly employed method for detecting regulatory elements of a gene. However, this approach may not be effective if the gene under investigation has weak promoter activity. We previously showed, using growth hormone reporter gene constructs and Hep3B cells, the presence of promoter and enhancer elements within the 5'-flanking region, the first intron, and the 3'-flanking region of the human Epo gene that are responsive to both hypoxia and cobalt treatment (7). The effects of these regions, however, were weaker than those expected, suggesting that these regulatory regions include both positive and negative regulatory elements. Analysis of deletion mutants of the reporter gene construct did not give us a clearcut result, probably due to the weak activity of the Epo promoter.

In this regard, it is interesting to note that enhancer activity was identified in the 3'-flanking sequence of the Epo gene (8, 9). Blanchard et al. (8) have demonstrated that oxygen-responsive elements include a steroid receptor response element. However, the function of the promoter and upstream promoter regions remains to be clarified.

Recent studies reveal that DNA can be targeted by oligonucleotides that inhibit the first step of gene expression, namely transcription (10, 11). This approach is called "antigene strategy" (12, 13). Oligonucleotide-directed triple helix formation inhibits the association of sequence-specific DNA-binding proteins such as transcription factors (13-15). It is thus expected that triple helix formation will interfere with biological processes including transcription. Triple helix formation is based on Hoogsteen or reversed Hoogsteen H-bonding between a duplex base pair and an oligonucleotide base (16, 17). Further, a triple helix-forming oligonucleotide blocks transcription of the c-myc gene by inhibiting binding of a transcription factor to one of the regulatory sequences upstream from the transcription start site (14).

In order to investigate positive and negative regulatory elements of the Epo gene in more detail, synthetic oligonucleotides were designed to control Epo transcription by means of an antigene strategy. To exclude the confounding effect of alterations in efficiency of translation, Epo mRNA concentration was determined by competitive PCR (18), because the advantages of this technique are that quantitation is independent of the many variables that affect amplification and it is more sensitive than Northern blotting or ribonuclease protection assays. In this report we demonstrated that CACCC elements at -60 bp from the CAP site are positive regulatory elements of the Epo gene, whereas the GATA element at -30 bp is a negative regulatory element. Furthermore, factors in the nuclear extracts from Hep3B cells were found to specifically bind to CACCC elements or the GATA element. These data imply that both positive and negative regulation is important in the expression of the Epo gene.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and RNA Preparation**

The Hep3B cell line was obtained from the American Type Culture Collection. Hep3B cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum.
bovine serum (Hazleton) in 25-cm² tissue culture flasks. Cells were maintained in a humidified 5% CO₂, 95% air incubator at 37 °C. One day prior to experimentation, the cells were harvested and plated at a density of 3 x 10⁶ cells/25-cm² flask. Cells were grown in the presence or absence of 50 μM CoCl₂ or hypoxia (1% oxygen), as described previously (5). After 24 h of incubation with 50 μM CoCl₂ or 1% oxygen, extracts from stimulated or unstimulated cells were prepared. Total cellular RNA was harvested by conventional methods (19).

Competitive PCR

Epo Map with Position of Oligonucleotide Primers—Epo-A and Epo-B flanking primers were 30 bp, had 67 and 50% G + C contents, respectively, and lacked 3' complementarity between primer pairs (see Fig. 1A).

Reverse Transcription of RNA into cDNA—Epo mRNA was reverse transcribed from total RNA into Epo cDNA and was co-amplified with a competitive template by competitive PCR (18). After initial denaturation of the RNA at 65 °C to eliminate possible secondary structure, 5 μg of RNA from Hep3B cells was reverse transcribed in a reaction mixture containing 20 nmol of each dNTP (U. S. Biochemical Corp.), antisense (Epo-B) primer (40 pmol), 1 unit of RNasin (Boehringer Mannheim), and 16 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) in a 40-μl total volume of 1x PCR buffer (final concentration in each), 0.2 μM each primer, and 0.01% (w/v) gelatin. Each reaction mixture contained dNTPs (200 μM final concentration in each), 0.2 μM each primer, and 5 units/ml of Taq polymerase (Cetus) in 1 x PCR buffer in a final volume of 100 μl. In all experiments, the presence of possible contaminants was checked by control reactions in which amplification was carried out on samples in 1) the absence of reverse transcriptase and 2) lysis buffer alone. Samples were amplified by 60 cycles at 94 °C for 1 min, 62 °C for 2 min, and 72 °C for 3 min, containing various amounts of Epo genomic DNA competed against a fixed volume (10 μl) of Epo cDNA. An aliquot of each reaction mixture was subjected to electrophoresis on 1% agarose, 2% NuSieve gels. The gel was stained with ethidium bromide, photographed, and analyzed by densitometry (Immunomedica Co. Ltd.).

Oligonucleotides for Antigen

Oligonucleotides were synthesized as A₁ (which included CACC elements –60 bp from the CAP site) and its complement A₂, as well as B₁ (which included the GATA element –30 bp from the CAP site) and its complement B₂ (see Fig. 2A). Also synthesized were C₁ (which lacked CACC elements from A₁) and its complement C₂, as well as D₁ (which lacked AGATAA from B₁) and its complement D₂ (see Fig. 2A). These were synthesized by the β-cyanoethylphosphoramidite method on an Applied Biosystems 392 automated synthesizer and purified on NAP™-10 columns (Sephadex G-25) (Pharmacia LKB Biotechnology Inc.) equilibrated with water.

Electrophoresis

Triple helix formation was measured by an electrophoresis assay in Mg²⁺-containing buffer, as described previously (14). To assay for triple helix formation, 3²P-labeled sense strand oligonucleotide was titrated with unlabeled antisense strand oligonucleotide (in 20 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 10% sucrose) and then loaded on a 10% polyacrylamide gel containing 30% acrylamide, 0.5% bisacrylamide gel (containing 90 mM MOPS, 100 mM KCl, and 0.01% of poly[d(A-T)]) electrophoresis was performed at 10 V/cm for 2 h at 4 °C. Gels were vacuum-dried and autoradiographed with intensifying screens at –80 °C for 2–24 h.

DNA Binding Assays

Nuclear extracts were prepared by published methods (20, 21). Protein concentration was determined by a Bio-Rad assay with bovine serum albumin standards. Sense strand oligonucleotides were end-labeled with 3²P polynucleotide kinase (Toyobo) and annealed to a 4-fold excess of the corresponding unlabeled antisense oligonucleotide. A probe (2.0 ng) was used in each binding reaction. The binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol, and 75 mM KCl. An equilibrium mixture of poly(dI-C) and poly[d(A-T)] (Sigma) was used as nonspecific competitor at a concentration of 1.5 μg in the reaction mixtures. Binding reactions (25 μl) were incubated for 15 min at 4 °C and electrophoresed on 5% nondenaturing polyacrylamide gels in 0.25 x TBE buffer (22 μM Tris borate, 22 mM

Fig. 1. Competitive PCR. A. A map with position of oligonucleotide primers. The flanking primers were as follows: Epo-A, 5'-GTCCGGCAGCGCGCCAGTTAAGTCTCGGCAG-3'; Epo-B, 5'-AGATGTCATTGCTGGCGATTGTTCCAT-3'. B, a titration curve of Epo genomic DNA/Epo cDNA. Lanes (left to right) contain various amounts of Epo genomic DNA (1000, 800, 600, 400, 200, 100, 80, 60, 40, 20, and 10 pg) and 0 pg) and 0 pg competed against a fixed volume (10 μl) of Epo cDNA. The reactions were amplified for 60 cycles and subjected to electrophoresis. The gel was stained with ethidium bromide, photographed, and analyzed by densitometry. C, plot of the ratio of Epo genomic DNA/Epo cDNA. Data are plotted as log ratio of Epo genomic DNA/Epo cDNA versus log input Epo genomic DNA (pg).

RESULTS

Quantitation of Epo cDNA by Competitive PCR—To exclude the effect of translational regulation, the alteration of transcription should be measured in relation to mRNA levels. For this purpose, the quantitation of Epo cDNA in the reverse transcription reaction was carried out using an Epo genomic template as shown in Fig. 1. A C. Primers were chosen from exons 4 and 5 that gave a 485-bp fragment when Epo cDNA was

boric acid, 0.5 mM EDTA) at room temperature at 150 V for 1.5 h as described previously (20). Gels were vacuum-dried and autoradiographed with intensifying screens at –80 °C for 2–24 h.

Fragmenting of Epo cDNA by Competitive PCR.
amplified and a 619-bp fragment when Epo genomic DNA was amplified (Fig. 1A). A single concentration of Epo genomic DNA was co-amplified with a dilution series of known concentrations of Epo genomic DNA (Fig. 1B). After electrophoresis in a 2% NuSieve, 1% agarose gel, the gel was stained with ethidium bromide, photographed, and analyzed by densitometry. Data were plotted as log ratio of Epo genomic DNA/Epo cDNA versus log input Epo genomic DNA (Fig. 1C). The amount of Epo genomic DNA was multiplied by the ratio of Epo cDNA bp/Epo genomic DNA bp to correct for increased label/ethidium staining/mol by the larger fragment. Epo genomic DNA/Epo cDNA can then be plotted as a function of the amount of known competitive Epo genomic DNA (Fig. 1C). When Epo genomic DNA and Epo cDNA product are in equivalence (i.e. ratio = 1.0), the starting concentration of Epo cDNA prior to PCR is equal to the known starting concentration of competing Epo genomic DNA. The concentration of Epo cDNA calculated from this plot by competitive PCR was 38.0 pg. We determined that 80 fg of Epo mRNA/pg of RNA was at the limit of detectability in our assay. The measured change in the amount of Epo mRNA with CoCl2 treatment was a 20-fold increase over control and a 100-fold increase upon stimulation by hypoxia.

**Addition of Oligonucleotides Resulted in Changes of the Steady State Level of Epo mRNA**—The effects of the addition of synthetic oligonucleotides on the steady state level of Epo mRNA in Hep3B cells were examined. The oligonucleotides were added to Hep3B cell cultures, and the level of Epo mRNA was quantitated to examine the transcriptional activity of the Epo gene in Hep3B cells treated with hypoxia or CoCl2. In the presence of 50 µM CoCl2, the addition of oligonucleotide A2 resulted in a decrease of the Epo mRNA level, whereas cells treated with A1 oligonucleotide showed the same level of Epo mRNA as control (Fig. 2B). The addition of A2 significantly decreased the level of Epo mRNA dose dependently in Hep3B cells stimulated with CoCl2 or hypoxia (Fig. 2C). In contrast, the addition of B2 caused stimulation of Epo mRNA production in the absence of CoCl2 or hypoxia (Fig. 3A). Furthermore, B2 caused a dose-dependent stimulation of Epo mRNA production in the presence of CoCl2 or hypoxia (Fig. 3B). On the other hand, neither A1 nor B1 affected the production of Epo mRNA (Figs. 2C and 3B). Furthermore, C2, which lacked the CACCC elements from A2, and D2, which lacked the AGATAA element from B2, did not induce any significant change in the production of Epo mRNA (Figs. 2D and 3C). Double-stranded oligonucleotide A (A1 + B2) or B (B1 + B2) did not affect the production of Epo mRNA (Figs. 2E and 3D). In the oligonucleotide treatments, control oligonucleotides did not indicate nonspecific effects (data not shown). Neither A2 nor B2 had a stimulatory effect on total RNA synthesis (data not shown), indicating that the observed effects of A2 and B2 on Epo mRNA production are specific.

**Triple Helix Formation Was Demonstrated by an Electrophoresis Assay**—To further delineate the nature of this phenomenon, triple helix formation was determined by an electrophoresis assay as described by Cooney et al. (14). Oligonucleotide A1 was 32P-end-labeled with polynucleotide kinase and then titrated with unlabeled A2. The titration produced two distinct species. In the presence of Mg2+ at low concentrations of A2 the duplex formed, whereas at higher concentrations a complex formed by the binding of a second A2 molecule that migrated more slowly as a triple helix band (Fig. 4A). Densitometry indicated that the intensity of the triple helix band of 24 µM was stronger than that of 12 µM (data not shown). Triple helix formation could not be detected in the absence of Mg2+ (Fig. 4B). This is consistent with the fact that the colinear triple helix is specifically stabilized by multivalent ions. Oligonucleotide A2 was 32P-end-labeled and then titrated with unlabeled A1. Titration produced the duplex, but the binding of a second A1 equivalent to form a triple helix could not be detected at any tested oligonucleotide concentration, up to 24 µM (Fig. 4C). Without the component of CACCC elements from oligonucleotide A, the titration of C2 did not produce a triple helix with 32P-end-labeled C1 (data not shown).
Fig. 3. Effect of synthetic oligonucleotides on the production of Epo mRNA. Conditions are the same as in Fig. 2. A, Hep3B cells (3 x 10^5/flask) were incubated with or without 5 μM B2. Lanes (left to right) contain various amounts of Epo genomic DNA (1000, 500, 100, 50, 10, and 1 pg) competed against a fixed volume (10 μl) of Epo cDNA. B, various concentrations of B1 or B2 were added to Hep3B cells in the presence of 50 μM CoCl2 or 1% oxygen. C, D1 or D2 was added to Hep3B cells in the presence of 1% oxygen. The amounts of Epo mRNA present at 100% were 8.1 pg (B, CoCl2), 67.0 pg (B, hypoxia), 76.2 pg (C), and 59.7 pg (D)/total μg of RNA.

Factors in Nuclear Extract of Hep3B Cells Specifically Bound to CACC or GATA Elements—Nuclear extracts were prepared from Hep3B cells grown under control conditions or in the presence of 50 μM CoCl2 or 1% O2. The presence of DNA-binding proteins in the nuclear extracts was assayed by gel mobility shift assays. Three DNA-protein interactions were identified as retarded complexes by mobility shift gel electrophoresis using A (CACC elements) as a probe (Fig. 5A). The addition of double-stranded nonradiolabeled CACC element oligonucleotides (A1+2) demonstrated two specific DNA-protein complexes (circle, triangle) and a single nonspecific complex (square) (Fig. 5A). The patterns of the upper specific complex (circle) were similar in extracts prepared from normoxia-, cobalt-, and hypoxia-treated Hep3B cells. However, the intensities of the bands of the lower specific complex (triangle) and the nonspecific complex (square) from hypoxia-treated cell extracts were stronger than those from control and cobalt-treated Hep3B cells (Fig. 5A). These results indicated that these factors were inducible by hypoxia treatment. In the case of the GATA element, the addition of double-stranded nonradiolabeled GATA element (B1+2) also demonstrated two specific DNA-protein complexes (circle, triangle) and a single nonspecific complex (square) (Fig. 5B). However, the band of the upper specific complex (circle) was not seen consistently (Figs. 5D and 6B). The intensities of the bands of the specific complexes and the nonspecific complex from control cell extracts were stronger than those from cobalt- and hypoxia-treated cells (Fig. 5B), suggesting that these factors were depressible by CoCl2 or hypoxia treatment. Furthermore, in the gel mobility shift assay, the addition of antisense single-stranded nonradiolabeled CACC element (A2) and also antisense single-stranded nonradiolabeled GATA element (B2) oligonucleotides showed the same pattern of competition as the addition of double-stranded (A1+2 or B1+2) oligonucleotides (Fig. 5, C and D). These results demonstrated the presence of factors in nuclear extracts of Hep3B cells that specifically bind to CACC elements or the GATA element and also strongly indicated the formation of a triple helix. The large signal in Fig. 5C that migrates between the free probe and nonspecific band that is present in lanes 2–4 was not seen in Fig. 5A. Therefore, the large signal had no reproducibility. Because nuclear extracts were crude, unknown small molecular weight substances might appear. To further investigate the factors that bind to CACC or GATA elements, similar experiments were carried out with mutated CACC element (CACC to TTTC) or mutated GATA element (AGTAA to AGCGAA or to CGCGAT) oligonucleotides. Mutated CACC element oligonucleotides did not reveal retarded bands on a gel mobility shift assay (Fig. 6A), suggesting that interaction with the trans-acting factor that recognizes CACC elements is important. Similarly, we found that mutation of the GATA element oligonucleotides no longer produced retarded bands (Fig. 6B). This result strongly suggested that interaction with one of the GATA factors is important for this GATA element.

**DISCUSSION**

A 50-bp oxygen-responsive enhancer element located 100 bp downstream from the polyadenylation site of the Epo gene has been shown (8, 9); however, cis-acting elements upstream from the CAP site remain to be clarified. We show that CACC elements at –60 bp from the CAP site are positive regulatory elements, whereas the GATA element at –30 bp is a negative regulatory element.

Beru et al. (22) describes nuclear factors from the kidney that bind to the Epo gene at a position –61 to –45 relative to the start site of transcription (Ep17); this region partly overlaps oligonucleotide A from our experiments. One of these factors is a 47-kDa protein, whereas the other is a ribonucleoprotein. The amounts that bind to Ep17 are greatly reduced in nuclear extracts from the kidneys of animals stimulated with CoCl2. They find that the amounts of two high molecular mass RNA species (I and II) are greatly reduced in extracts obtained from the cobalt-injected animals. They suggest that the ribonucleoprotein that binds to Ep17 might work as a repressor. The 47-kDa protein, which is not affected by the stimulation with CoCl2 and thus appears to be constitutive, might be a positive trans-acting factor whose action in the uninduced state is blocked by the ribonucleoprotein that binds to the same element.

We demonstrate that the addition of oligonucleotide A2 in
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**Fig. 4. Triple helix formation.** A, A₂ binding to A duplex was measured in Mg²⁺-containing buffer, as described in Ref. 14. The³²P-labeled A₁ concentration was held constant throughout at 5 nM. Unlabeled A₂ concentrations were as follows: 0, 0.012, 0.12, 1.2, 12, and 24 μM. S, single strands; D, duplex; T, triple helix. B, to determine if triple helix formation is dependent on divalent ion binding, binding was analyzed at 4 °C in an electrophoresis buffer containing EDTA (90 mM Tris-borate (pH 8.0), 5 mM EDTA). C, A₁ binding to A duplex was measured in Mg²⁺-containing buffer, under the same conditions as in A. The³²P-labeled A₂ concentration was held constant throughout at 5 nM. Unlabeled A₁ concentrations were as follows: 0, 0.012, 0.12, 1.2, 12, and 24 μM. *Upper panel,* short time exposure (2 h); *lower panel,* long time exposure (24 h).

**Fig. 5. Gel mobility shift assay.** A, gel mobility shift assay for CACCC elements. End-labeled probes were incubated with 7.0 μg of nuclear extract from Hep3B cells. The circle and triangle indicate complexes that migrate as a discrete band and are specifically inhibited by the CACCC elements. The triangle and square indicate complexes that are inducible by hypoxia. B, gel mobility shift assay for GATA element. Conditions are the same as in A. The circle and triangle indicate complexes that migrate as a discrete band and are specifically inhibited by the GATA element. The circle, triangle, and square indicate complexes that are inducible by hypoxia. C, competition by single-stranded and double-stranded oligonucleotide on gel mobility shift assay for CACCC elements. 5.7 μg of nuclear extract from Hep3B cells was used. S, sense oligonucleotide (A₁); A, antisense oligonucleotide (A₂); D, double-stranded oligonucleotide (A₁+A₂). The circle and triangle on the left identify the specific complexes formed on CACCC elements. The square indicates the nonspecific complex. D, competition by single-stranded and double-stranded oligonucleotides on gel mobility shift assay for the GATA element. Conditions are the same as in C. S, sense oligonucleotide (B₁); A, antisense oligonucleotide (B₂); D, double-stranded oligonucleotide (B₁+B₂). The triangle on the left identifies the specific complex formed on the GATA element. The square indicates the nonspecific complex. 300 ng (6 μl) of competitor DNA was added to each reaction mixture from A to D.
cobalt- or hypoxia-induced cell extracts decreases the expression of Epo mRNA in a dose-dependent fashion. This result supports the findings of Beru et al. (22) as the binding of oligonucleotide A₂ would block binding of the 47-kDa protein to CACCC elements. We find that upon the addition of A₂ in the absence of stimulation, the expression of Epo mRNA is not detected (data not shown), since A₂ binds to CACCC elements tightly by triple helix formation. Therefore, in the uninduced state, neither the ribonucleoprotein nor the 47-kDa protein can bind to the CACCC elements. Taken together, our results and that of Beru et al. (22) suggest a model for regulation of Epo gene expression (Fig. 7). In the uninduced state, the 47-kDa protein is synthesized, as Beru et al. (22) have proved.

Another trans-acting factor (which has been called G) is believed to be synthesized and to act as a repressor. The 47-kDa protein is thought to bind to CACCC elements, and G is believed to bind to the GATA element (Fig. 7A). In the induced state (Fig. 7B), the inducer is presumably synthesized via an oxygen-sensing mechanism as previously described by Goldberg et al. (6). This inducer decreases the activity of the repressor by binding to it as Beru et al. (22) have shown. Therefore the 47-kDa protein can bind to the CACCC elements, allowing gene transcription. Since the addition of A₂ blocks the CACCC elements, A₂ decreases the production of Epo mRNA in the presence or absence of CoCl₂ or hypoxia induction. Furthermore, the addition of oligonucleotide B₂ increases the production of Epo mRNA, as B₂ binds to the GATA element and interferes with the binding of the repressor. In the induced state, the addition of B₂ increases the production of Epo mRNA higher than hypoxia alone, in a dose-dependent fashion. The inducer may completely inhibit the activity of the remaining repressor allowing the 47-kDa protein to bind to CACCC elements, thus stimulating the production of Epo mRNA. The finding of inducible factors identified at the CACCC elements and repressible factors at the GATA element after stimulation by hypoxia also supports the idea that CACCC elements are positive regulatory elements, whereas the GATA element is a negative regulatory element.

The DNA-binding protein GATA-1 has been found to be a positive-acting transcription factor known to regulate most or all erythroid cell-specific genes (23, 24). However, the mouse albumin gene enhancer has been revealed to contain a GATA element that acts as a negative regulatory element (25). In the case of CACCC elements, Cooney et al. (14) have already demonstrated triple helix formation. We demonstrated triple helix formation by an electrophoresis assay for CACCC elements. However, we did not succeed in detecting triple helix formation for the GATA element by this method, perhaps because the synthetic oligonucleotide B contained only 6 bp (AGATAA) of the GATA element and was too short to reveal a triple helix band by molecular weight differences (14). However, the result of competition using the single-stranded GATA element (B₂) in a gel mobility shift assay strongly suggested evidence for triple helix formation.

Confidence in the interpretation of this in vitro study would be enhanced by demonstrating evidence for in vivo triple helix formation at the CACCC and GATA elements of action of the oligonucleotides by in vivo footprinting. However, no one has yet been able to demonstrate the regulatory regions upstream of the CAP site of the human Epo gene by footprinting, perhaps because Epo's 5'-flanking region is very GC-rich and therefore...
The alteration of transcription should be assayed by examination at the uninduced state. To exclude the effect of translation, mRNA levels can be measured by competitive PCR. Our approach, combining the use of antigene and competitive PCR, is a useful tool for defining the regulatory sequences of relatively weak promoters.

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