Holthuis, J., Owen, T., van Wijnen, A., Wright, K., Ramsey-Ewing, A., Kennedy, M., Carter, R., Cosenza, S., Soprano, K., Lian, J., Stein, J., & Stein, G. (1990) *Science 247*, 1454-1457.

Kroeger, P., Stewart, C., Schaap, T., van Wijnen, A., Hirshman, J., Helms, S., Stein, G., & Stein, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3982-3986.

Labarca, C., & Paigen, K. (1980) *Anal. Biochem. 102*, 344-352.

Owen, T. A., Holthuis, J., Markose, E., van Wijnen, A. J., Wolfe, S. A., Grimes, S., Lian, J. B., & Stein, G. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5129-5133.

Pardee, A. B., Dubrow, R., Hamlin, J. L., & Kletzien, R. F. (1978) *Annu. Rev. Biochem. 47*, 715-750.

Pauli, U., Chrysogelos, S., Stein, G., Stein, J., & Nick, H. (1987) *Science 236*, 1308-1311.

Pauli, U., Chrysogelos, S., Stein, J., & Stein, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 16-20.

Plumb, M., Stein, J., & Stein, G. (1983) *Nucleic Acids Res. 11*, 2391-2410.

Prescott, D. M. (1987) *Int. Rev. Cytol. 100*, 93-128.

Ramsey-Ewing, A., van Wijnen, A., Stein, G. S., & Stein, J. L. (1992) (submitted for publication).

Sierra, F., Lichtler, A., Marashi, F., Rickles, R., Van Dyke, T., Clark, S., Wells, J., Stein, G., & Stein, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1795-1799.

Stein, G., Lian, J., Stein, J., Briggs, R., Shalhoub, V., Wright, K., Pauli, U., & van Wijnen, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1865-1869.

Tucker, R. W., & Boone, C. W. (1981) in *Progress in Clinical and Biological Research* (Cunningham, D., Goldwasser, E., Watson, J., & Fox, C. F., Eds.) Vol. 66A, pp 589-592, Alan R. Liss, New York.

van Wijnen, A. J., Wright, K. L., Lian, J. B., Stein, J. L., & Stein, G. S. (1989) *J. Biol. Chem. 264*, 15034-15042.

van Wijnen, A. J., Ramsey-Ewing, A. L., Bortell, R., Owen, T. A., Lian, J. B., Stein, J. L., & Stein, G. S. (1991) *J. Cell. Biochem. 46*, 174-189.

Wright, K. L., Ramsey-Ewing, A., Aronin, N., van Wijnen, A., Stein, G. S. & Stein, J. (1992) (submitted for publication).

Zambetti, G., Dell'Orco, R., Stein, G., & Stein, J. (1987) *Exp. Cell Res. 172*, 397-403.

Zetterburg, A., & Larson, O. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5365-5369.

The Iron Regulatory Region of Ferritin mRNA Is Also a Positive Control Element for Iron-Independent Translation†

David J. Dix, Peng-Nian Lin, Yoko Kimata, and Elizabeth C. Theil*

Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-7622

Received October 18, 1991; Revised Manuscript Received December 10, 1991

ABSTRACT: The iron regulatory element (IRE) in the 5′-untranslated region of ferritin mRNA interacts with a specific regulator protein (P-90, IRE-BP, or FRP) to block translation. High cellular iron changes the IRE/P-90 interaction to relax the translational block and allow polyribosome formation. We now show that the IRE and base-paired flanking regions also enhance translation in the absence of P-90, explaining the high translational efficiency of deregulated ferritin mRNA observed previously. The effect of the IRE on translational efficiency was examined by comparing four sets of mRNAs: (1) ±IRE in animal (frog) ferritin, regulated translationally by iron in vivo; (2) ±animal IRE fused with plant (soybean) ferritin, regulated transcriptionally by iron in vivo; (3) repositioned IRE in animal ferritin; (4) mutated IRE in animal ferritin with G16A substitution, which decreases P-90 binding (negative control). The IRE region increased translational efficiency of both the animal ferritin and the heterologous IRE/soybean ferritin fusion mRNAs; the effect was observed in cell-free translation systems from either plants (wheat germ) or animals (rabbit reticulocyte). Repositioning the IRE further from the 5′ cap eliminated positive control of translation. The single base mutation had no effect, indicating that positive and negative translational control involves different sections of the IRE region. Thus, the IRE region in ferritin mRNA encodes both positive translational control and, when combined with the regulator protein P-90, negative translational control.

A highly conserved, 28-nt, complex stem–loop structure (IRE/iron regulatory element) is present in the 5′-untranslated region (UTR) of all known vertebrate ferritin mRNAs (Theil, 1990) and in the 3′ UTR of transferrin receptor (TfR) mRNAs (Klausner & Harford, 1989). The IRE is the first example of an RNA element which mediates coordinate regulation of two mRNAs encoding metabolically related proteins (ferritin for iron storage, TfR for iron uptake) by the same signal and may be a model for coordinate regulation of mRNAs encoding other metabolically related proteins.

In ferritin mRNAs, the IRE is structurally integrated with base-paired flanking regions close to the 5′ cap. The flanking sequences are conformationally altered (increased base-stacking) when the mRNA regulator protein P-90 binds the IRE (Harrell et al., 1991); P-90 specifically blocks translation of ferritin mRNA at low levels of intracellular iron (Walden et al., 1988, 1989; Brown et al., 1989). Increased intracellular iron leads to increased ferritin synthesis, apparently through changes in the IRE/P-90 interaction which relax structure in the flanking regions (Harrell et al., 1991); under such conditions, ferritin mRNA forms polyribosomes either in vivo (Zahringer et al., 1976) or in vitro in cell-free extracts lacking

† Partial support was provided by National Institutes of Health Grants DK20251 (E.C.T.) and DK08615 (D.J.D.) and by the North Carolina Agricultural Research Service (E.C.T.).

0006-2960/92/0431-2818$03.00/0

P-90 (Dickey et al., 1988). In contrast to the base-paired sequences which flank the single copy of IRE sequence in the 5′ UTR of ferritin mRNA, five copies of the IRE are present in the 3′ UTR of TfR mRNA without base-paired flanking regions; in this different context, the IRE controls iron-dependent mRNA degradation rather than mRNA storage and translation (Casey et al., 1988, 1989).

Another feature of ferritin mRNA translation, in addition to the iron-dependent regulation of polyribosome formation, is the competitive efficiency of deregulated ferritin mRNA. Competitive efficiency of deregulated ferritin mRNA has been observed both in vivo and in vitro (Schaefer & Theil, 1981; Shull & Theil, 1982, 1983). We now show that the IRE region increases in vitro translation of deregulated ferritin mRNA either in the native (animal) ferritin mRNA context or in a heterologous fusion between the animal IRE and a plant (soybean) ferritin mRNA, which is regulated transcriptionally by iron in vivo (Lescure et al., 1991). IREs have not been identified in plant ferritin mRNAs (Lescure et al., 1991). This positive translational effect of the IRE region occurs in both the animal and plant cell-free translation systems (rabbit reticulocyte and wheat germ). Thus, the structure of the IRE region of ferritin mRNA must encode information for a second, distinct translational effect which is recognized by both plant and animal protein synthesis apparati and functions independently of P-90 regulator protein and iron.

Experimental Procedures[1]

Full-length (wild-type) animal ferritin mRNA (892 nt, with 141-nt 5′ UTR) was transcribed from pBFH-1, a cDNA encoding the bullfrog ferritin H-chain subunit mRNA which was produced by inserting 60 nucleotides containing the IRE and flanking regions, corresponding to the 5′-untranslated region lacking in pTZ5F12 (Didsbury et al., 1986). ΔIRE animal ferritin mRNA was transcribed from pTZ5F12. Plant ferritin mRNA (1120 nt with 89-nt 5′ UTR) was transcribed from pSOF, which is the soybean cDNA IC1 (Lescure et al., 1991) in the *Eco*RI site of pTZ18U (U.S. Biochemicals, Cleveland, OH). The fusion construct was transcribed from pISF, which has 101 nt of the bullfrog IRE region at the 5′ end of pSOF. The IRE-position mutant was produced by moving 60 nt of the IRE and flanking region 73 nt from the 5′ end at a *Hha*I site of pTZ5F12. A G/A mutation in the IRE loop (CAGUG→CAGUA) was prepared from pBFH-1 by site-directed mutagenesis. The same sequence, which occurs in a bullfrog pseudogene (Dickey et al., 1987) and a cloned transferrin receptor IRE (Koeller et al., 1989), leads to a large decrease in P-90 binding (Koeller et al., 1989; Harrell, 1991). Capped and uncapped transcripts were transcribed according to the method of Fletcher et al. (199) except that [^{3}H]UTP or [^{35}S]CTP were incorporated as labels. RNA concentrations were determined by cutting bands from 1 M urea/agarose gels, melting them in 1 M HCl, and determining DPM by scintillation counting. Globin mRNA was an equimolar mixture of α and β isoforms purified from rabbit reticulocytes (Life Technologies, Inc., Gaithersburg, MD).

In vitro translation in wheat germ extract (WGE) (Promega Corp., Madison, WI) followed previously published procedures (Shull & Theil, 1982, 1983). Rabbit reticulocyte lysate (RRL) was prepared and used as previously described (Dickey et al., 1988). [^{35}S]Methionine was used to label synthesized protein in both systems. Incubation, at 25 °C for WGE and 30 °C for RRL, was for 60 min. Protein synthesis directed by the various mRNAs was determined by TCA precipitation. Subunit synthesis was analyzed by gel electrophoresis and autoradiography (Dickey et al., 1987). RNA stability in experiments without [^{35}S]methionine was analyzed by TCA precipitation of ^{3}H-labeled mRNAs or by electrophoresis in 7 M urea/acrylamide gels and autoradiography of ^{35}S-labeled mRNAs.

[1] Experiments were approved by the University Animal Care Committee.

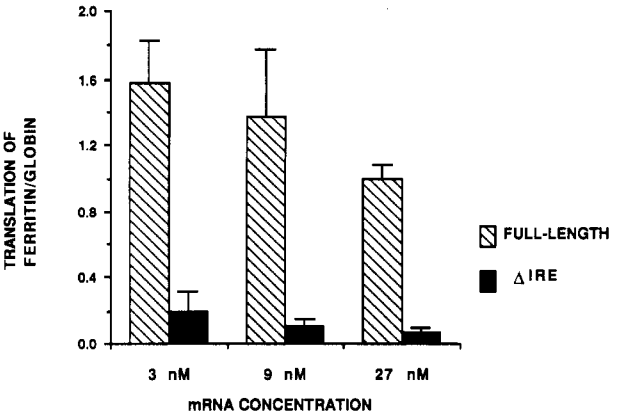

FIGURE 1: Translation of ferritin mRNAs, with and without the IRE region, in wheat germ extracts (WGE). CPM of TCA-insoluble [^{35}S]methionine of translation products from full-length frog ferritin mRNA and ΔIRE frog ferritin mRNA expressed relative to translation of rabbit globin mRNA. The results are presented as the average of four experiments, with the error represented as the standard deviation. The IRE in the 5′ UTR of ferritin mRNA enhances translational efficiency 10–19-fold at all concentrations of mRNA in WGE, which does not have endogenous IRE binding protein. Translation of deregulated full-length ferritin mRNA is slightly more efficient than translation of globin mRNA at all mRNA concentrations [(CPM × 10^{-3}/nM mRNA of 9.99 ± 1.03 and 7.99 ± 0.318, respectively].

Results and Discussion

The effect of the IRE on translational efficiency was analyzed by measuring protein synthesis under the direction of six varieties of capped and uncapped in vitro transcripts of both animal and plant ferritin cDNAs: animal ferritin mRNA plus and minus the IRE region; plant ferritin mRNA, which has no detectable IRE or translational control (Lescure et al., 1991), plus and minus the animal IRE region; animal ferritin mRNA with a mutated IRE, or with a repositioned IRE in the 5′-untranslated region. To determine types of cell-free extracts which could recognize the regulatory features of the IRE, both wheat germ extracts (WGE) and rabbit reticulocyte lysates (RRL) were used. WGE contains no endogenous P-90, and the concentrations of ferritin mRNA used with RRL were high enough to saturate the endogenous P-90.

The translational efficiency of the animal ferritin mRNA minus the IRE region (ΔIRE) was 10–16-fold less than full-length animal ferritin mRNA (Figure 1) at all concentrations of mRNA tested in wheat germ extracts. To test whether the enhancer effect of the IRE region could be transmuted to another mRNA, we fused the animal IRE region to a plant ferritin mRNA and compared translational efficiencies. Soybean ferritin mRNA was chosen because iron regulation of ferritin synthesis in soybeans appears to be only transcriptional, and no IRE has been detected in the plant ferritin mRNA (Lescure et al., 1991). The animal IRE also enhanced translation of the soybean ferritin mRNA (Table I). Context-dependent features of IRE structure (Harrell et al., 1991) may account for the quantitative difference in enhancement of translation in vitro between the natural animal ferritin mRNA and the animal IRE/soybean ferritin fusion

Table I: Effects of Deletion, Fusion, Repositioning, and Mutation of the IRE Region on Translational Efficiency of Ferritin mRNAs

mRNAs	ratio of translational efficiency[a]
wild-type frog ferritin vs ΔIRE frog ferritin	19.4 ± 3.23
fusion (frog IRE + soybean ferritin)[b] vs wild-type soybean ferritin	2.24 ± 0.676[c]
wild-type frog ferritin vs IRE repositioned 73 nt from the 5′ cap	5.26 ± 0.138[d]
wild-type frog ferritin vs IRE-loop mutation G16A	1.07 ± 0.0804

[a] Protein synthesis directed by 27 nM mRNA as determined by [^{35}S]methionine incorporation into TCA-insoluble translation product in wheat germ extracts. Results presented as the average of four, nine, three, and four experiments, respectively, with the error represented as the standard deviation. [b] Regulation of soybean ferritin by iron is only transcriptional; no natural IRE has been detected (Lescure et al., 1991). [c] The difference in the nine experiments between ±IRE is significant at the 99.9% confidence level. The quantitative difference in the effect of the IRE on translational efficiency in the wild-type (full-length) and fusion constructs is likely due to other sequences in the wild-type ferritin mRNA which have previously been shown to influence structure in the IRE and flanking regions (Harrell et al., 1991; Dickey et al., 1988). [d] Analyzed in rabbit reticulocyte lysate, where the ratio of translational efficiency for wild-type frog ferritin mRNA to ΔIRE + FL frog ferritin mRNA is 9.30 ± 3.11 (see Figure 2).

construct. The importance of context suggested by results with the fusion transcript is emphasized by the effect of moving the IRE within the animal ferritin sequence. Moving the IRE 73 nucleotides from the 5′ cap had almost the same effect on translational efficiency as deleting the IRE (Table I). In contrast, when the context was unchanged but A for G was substituted at position 16 in the IRE loop, translational efficiency was unchanged (Table I), even though this mutation greatly decreases regulator protein binding (Koeller et al., 1989; Harrell, 1991). Such results also show that translational enhancement can be separated from translational repression by a single mutation in the IRE loop and that the information for the positive translational control may reside in the stem of the hairpin loop. The competitive efficiency of deregulated ferritin mRNA previously observed in red cell poly(A)$^+$ RNA in whole cells (Schaefer & Theil, 1981) and cell-free extracts (Shull & Theil, 1982, 1983) was reproduced with the in vitro transcripts (Figure 1).

In order to test the generality of recognition of the IRE for translational efficiency, frog ferritin mRNA (±IRE) was also translated in RRL. At ferritin mRNA concentrations (27 nM) far exceeding the binding capacity of endogenous P-90 regulator, full-length ferritin mRNA was translated 9.3-fold more efficiently than ΔIRE ferritin mRNA (Figure 2). At low concentrations of ferritin mRNA (3 nM), the effect of P-90 could be observed as a disproportionate decrease in ferritin synthesis which reproduces the inhibition previously observed with poly(A)$^+$ RNA (Dickey et al., 1988). In contrast to ferritin mRNA with the IRE, translation of the ΔIRE ferritin mRNA was not regulated by P-90 in RRL; even at low mRNA concentrations, translation changed proportionately with mRNA concentration under all conditions tested.

There were no differences in the stability of the various mRNAs during translation. For example, the ratio of recovered [^{3}H]UTP (TCA-insoluble) after translation for ΔIRE/full-length ferritin mRNA was 0.822 ± 0.102 in RRL and 0.896 ± 0.082 in WGE (n = 5 and 4, respectively); similar results were obtained with in vitro transcripts of the animal/plant ferritin fusion, the IRE-position mutation, and the G/A mutation. Analysis of ferritin transcripts in denaturing acrylamide gels before and after translation confirmed, qualitatively, the quantitative results from TCA precipitation

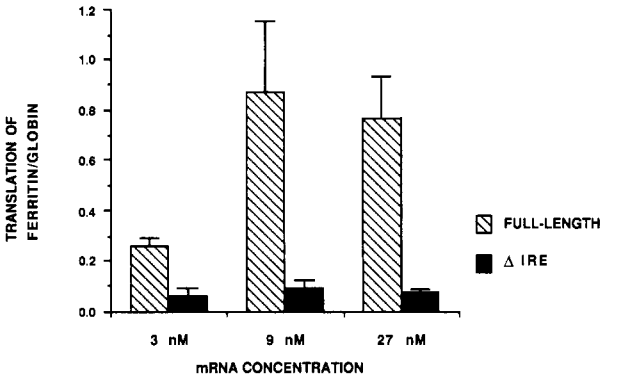

FIGURE 2: Translation of ferritin mRNAs, with and without the IRE region, in rabbit reticulocyte lysate (RRL). CPM of TCA-insoluble [^{35}S]methionine of translation products from full-length frog ferritin mRNA and ΔIRE frog ferritin mRNA expressed relative to translation of rabbit globin mRNA. The results are presented as the average of six experiments, with the error represented as the standard deviation. The IRE region's effect on ferritin mRNA translational efficiency is independent of repression in RRL and occurs at all mRNA concentrations. The largest increase (9.3-fold) in translational efficiency is evident at 27 nM mRNA, where P-90 binding and negative translational control of wild-type ferritin mRNA are absent. Translation of full-length ferritin mRNA at 3 nM is disproportionately low, indicating the effect of endogenous P-90 regulator protein at the lower concentrations of ferritin mRNA.

Table II: Translational Efficiency of Uncapped Ferritin mRNAs in Rabbit Reticulocyte Lysates

mRNAs[a]	protein synthesis (CPM × 10^{-6})[b]
full-length frog ferritin mRNA	0.753 ± 0.224
ΔIRE frog ferritin mRNA	≤0.03[c]

[a] High mRNA concentrations (27 nM) were selected to saturate endogenous P-90 regulator (see Figure 2). [b] [^{35}S]Methionine incorporation into TCA-insoluble translation product. Results presented as the average of five experiments, with the error represented as the standard deviation. [c] No significant translation was detected of uncapped ΔIRE ferritin mRNA, which is equivalent to a 6-fold reduction in efficiency relative to the capped ΔIRE mRNA. The greater dependence on the 5′ cap of ΔIRE ferritin mRNA suggests that the IRE region is recognized by translation initiation factors and could provide a structural analogue to the cap (Carberry & Goss, 1991).

of the mRNAs. The structural integrity of the various animal and plant ferritin transcripts was also confirmed by the synthesis of identical 20- and 32-kDa ferritin subunits, respectively, as observed by SDS–PAGE and autoradiographic analysis of translation products. The plant ferritin product was a precursor of mature subunits containing the transit peptide (Ragland et al., 1990).

The presence of a 5′ cap enhances the effect of the IRE on translational efficiency but is not required for positive control (Table II). Uncapped full-length ferritin mRNA was translated 2.5-fold less efficiently than the capped mRNA. However, uncapped ΔIRE ferritin mRNA was ≥6.0-fold less efficiently translated in RRL than its capped analogue, indicating a greater dependence on the presence of a 5′-cap structure in mRNAs without an IRE. The greater cap dependence for translation of ΔIRE ferritin mRNA suggests that the IRE itself might contribute to the formation of the initiation complex. The effect of moving the IRE further from the 5′ cap on both translational efficiency in vitro (Table I) and iron-dependent regulation in transfected cells (Goossen et al., 1990) supports such an idea.

What structural features within the IRE regulatory region function as the translational enhancer is not yet known. Since mutation of the IRE loop residue 16 from G to A alters P-90 binding to the in vitro transcript of an H′ ferritin pseudogene

(Dickey et al., 1987; Harrell, 1991), but had no effect on the translational efficiency conferred by the IRE (Table I), structures outside the loop are likely to be important for positive translational control. The influence of context on IRE-enhanced translation further suggests that interactions with other parts of ferritin mRNA are involved. Examples of structural effects of context in natural ferritin mRNA include the sequestering of sequences in the IRE-flanking region from nuclease probes; in an IRE-containing oligoribonucleotide (n = 55), these same sequestered sequences become nuclease-sensitive (Harrell et al., 1991). Structural features for the ferritin IRE region are based upon computer-predicted stabilities and a phylogenetically conserved hairpin loop. However, evidence for tertiary interactions either within the ferritin mRNA IRE region or with sequences outside the region has also recently been obtained (Harrell et al., 1991; Wang et al., 1990, 1991). Regulatory structures with defined tertiary features have been observed in other translationally regulated mRNAs such as the pseudoknot exemplified by gene 32 mRNA of phage T4 (McPheeter et al., 1988; Shamoo et al., 1991) and S4-α mRNA of *Escherichia coli* (Tang & Draper, 1989, 1990).

Initiation is a likely step at which the IRE enhancer function is exerted, based on earlier studies of deregulated translation of ferritin mRNA with cycloheximide in whole cells (Schaefer & Theil, 1981). The importance of the IRE in initiation is supported by in vitro results with uncapped mRNAs (Table II) and the 80% reduction in translation rate when the IRE was moved downstream from the cap (Table I). Cap binding proteins (CBPs) critical to translation initiation appear to recognize more than just the 5′ cap of mRNA (Fletcher et al., 1990; Anthony & Merrick, 1991), and capped oligoribonucleotides with hairpin–loop structures near their 5′ ends have recently been shown to have increased affinity for CBPs eIF-4F and isoeIF-4F (Carberry & Goss, 1991). Thus, the translational enhancer activity of the IRE, which is within 17 nt of the 5′ cap of all ferritin mRNAs (Wang et al., 1991), could reflect a favorable conformation for cap/CBP interactions.

The IRE region is the first example, to our knowledge, of a structural element in the 5′ UTR of an mRNA which functions both as a translational repressor when bound by its protein regulator and as a translational enhancer in the absence of regulator. Since the results in both the animal and plant translation systems are similar, a generalized mechanism for positive control by the IRE is indicated. The IRE enhancer activity in ferritin mRNA may be a model for other translationally regulated mRNAs in which enhancer activity has yet to be investigated. Other mRNAs with specific regulatory sequences in the 5′ UTR may be potential candidates for positive translational control. In prokaryotes, such mRNAs include *Escherichia coli* S4-α mRNA (Tang & Draper, 1989, 1990; Decker & Draper, 1987), phage R17 replicase mRNA (Romaniuk et al., 1987), phage T_4 gene 32 mRNA, and gene 38 mRNA (McPheeter et al., 1988; Shamoo et al., 1991). In eukaryotes, ornithine decarboxylase mRNA (Manzella & Blackshear, 1990; Manzella et al., 1991) and HIV TAR-containing mRNA (Gunnery et al., 1990; Sengupta et al., 1990) have regulatory structures in their 5′ UTRs which might encode positive translational control.

Determining precisely how two types of translational regulatory information are encoded in the IRE region will depend on understanding the complete, three-dimensional structure in wild-type and mutant ferritin mRNAs. Unusual features already known include the regularly spaced hypersensitivity to cleavage by Fe-EDTA (Harrell et al., 1991) that suggests a hairpin loop either folded upon itself or lying on a surface formed by interactions with other parts of the mRNA. An additional structural distinction of the IRE region in wild-type ferritin mRNA is sequestering of sequences from large, structural probes such as protein nuclease in the absence of the specific regulator protein P-90, and conformational changes in the sequestered regions when P-90 binds (Harrell et al., 1991). Determining the relationship between the structure of the ferritin mRNA regulatory region, its context in the 5′ UTR, and its translational enhancer and repressor function will show how mRNA influences the dramatic changes in rates of protein synthesis, exemplified by ferritin synthesis in vivo (Drysdale et al., 1968; Theil, 1978).

Registry No. Iron, 7439-89-6.

References

Anthony, D. D., & Merrick, W. C. (1991) *J. Biol. Chem. 266*, 10218.

Brown, P. H., Daniels-McQueen, S., Walden, W. E., Patino, M. M., Gaffield, L., Bielser, D., & Thach, R. E. (1989) *J. Biol. Chem. 264*, 13383.

Carberry, S. E., & Goss, D. J. (1991) *Biochemistry 30*, 4542.

Casey, J. L., De Jeso, B., Krishnamurthy, R., Klausner, R. D., & Harford, J. B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1787.

Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D., & Harford, J. B. (1989) *EMBO J. 8*, 3693.

Decker, I. C., & Draper, D. E. (1987) *J. Mol. Biol. 196*, 323.

Dickey, L. F., Sreedharan, S., Theil, E. C., Didsbury, J. R., Wang, Y. H., & Kaufman, R. E. (1987) *J. Biol. Chem. 262*, 7901.

Dickey, L. F., Wang, Y. H., Shull, G. E., Wortman, I. A., & Theil, E. C. (1988) *J. Biol. Chem. 263*, 3071.

Didsbury, J. R., Theil, E. C., Kaufman, R. E., & Dickey, L. F. (1986) *J. Biol. Chem. 261*, 949–955.

Drysdale, J. W., Olafsdottir, E., & Munro, H. N. (1968) *J. Biol. Chem. 243*, 552.

Fletcher, L., Corbin, S. D., Browning, K. S., & Ravel, J. M. (1990) *J. Biol. Chem. 265*, 19582.

Goossen, B., Caughman, S. W., Harford, J. B., Klausner, R. D., & Hentze, M. W. (1990) *EMBO J. 9*, 4127.

Gunnery, S., Rice, A. P., Robertson, H. D., & Mathews, M. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8687.

Harrell, C. M. (1991) M.S. Thesis, North Carolina State University.

Harrell, C. M., McKenzie, A. R., Patino, M. M., Walden, W. E., & Theil, E. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4166.

Klausner, R. D., & Harfard, J. B. (1989) *Science 246*, 870–872.

Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L.-N. L., Klausner, R. D., & Harford, J. B. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3574.

Lescure, A. M., Proudhon, D., Pesey, H., Ragland, M., Theil, E. C., & Briat, J. F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8222.

Manzella, J. M., & Blackshear, P. J. (1990) *J. Biol. Chem. 265*, 11817.

Manzella, J. M., Rychlik, W., Rhoads, R. E., Hershey, J. W. B., & Blackshear, P. J. (1991) *J. Biol. Chem. 266*, 2383.

McPheeter, D. S., Stormo, G. D., & Gold, L. (1988) *J. Mol. Biol. 201*, 517.

Ragland, M., Briat, J.-F., Gagnon, J., Laulhere, J.-P., Massenet, O., & Theil, E. C. (1990) *J. Biol. Chem. 263*, 18339–18344.

Romaniuk, P. J., Lowary, P., Wu, H. N., Stormo, G., & Uhlenbeck, O. C. (1987) *Biochemistry 26*, 1563.
Schaefer, F. V., & Theil, E. C. (1981) *J. Biol. Chem. 256*, 1711.
Sengupta, D. N., Berkhout, B., Gatignol, A., Zhou, A., & Silverman, R. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7492.
Shamoo, Y., Webster, K. R., Williams, K. R., & Konigsberg, W. H. (1991) *J. Biol. Chem. 266*, 7967.
Shull, G. E., & Theil, E. C. (1982) *J. Biol. Chem. 257*, 14187.
Shull, G. E., & Theil, E. C. (1983) *J. Biol. Chem. 258*, 7921.
Tang, C. K., & Draper, D. E. (1989) *Cell 57*, 531.
Tang, C. K., & Draper, D. E. (1990) *Biochemistry 29*, 4434.
Theil, E. C. (1978) *J. Biol. Chem. 253*, 2902.
Theil, E. C. (1990) *J. Biol. Chem. 265*, 4771.
Walden, W. E., Daniels-McQueen, S., Brown, P. H., Gaffield, L., Russell, D. A., Bielser, D., Bailey, L. C., & Thach, R. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9503.
Walden, W. E., Patino, M. M., & Gaffield, L. (1989) *J. Biol. Chem. 264*, 13765.
Wang, Y. H., Sczekan, S. R., & Theil, E. C. (1990) *Nucleic Acids Res. 18*, 4463.
Wang, Y. H., Lin, P. N., Sczekan, S. R., McKenzie, A. R., & Theil, E. C. (1991) *Biol. Met. 4*, 56.
Zahringer, J., Baliga, B. S., & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 857.

Effect of the (+)-CC-1065–(*N*3-Adenine)DNA Adduct on in Vitro DNA Synthesis Mediated by *Escherichia coli* DNA Polymerase†

Daekyu Sun and Laurence H. Hurley*
The Drug Dynamics Institute, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712
Received September 16, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: (+)-CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis*. Previous studies have shown that the potent cytotoxic and antitumor activities of (+)-CC-1065 are due to the ability of this compound to covalently modify DNA. (+)-CC-1065 reacts with duplex DNA to form an *N*3-adenine DNA adduct which lies in the minor groove of the DNA helix overlapping with a 5-base-pair region. As a consequence of covalent modification with (+)-CC-1065, the DNA helix bends into the minor groove and also undergoes winding and stiffening [Lee, C.-S., Sun, D., Kizu, R., & Hurley, L. H. (1991) *Chem. Res. Toxicol. 4*, 203–213]. In the studies described here, in which we have constructed site-directed DNA adducts on single-stranded DNA templates, we have shown that (+)-CC-1065 and select synthetic analogues, which have different levels of cytotoxicity, all show strong blocks against progression of Klenow fragment, *E. coli* DNA polymerase, and T4 DNA polymerase. The inhibition of bypass of drug lesions by polymerase could be partially alleviated by increasing the concentration of dNTPs and, to a small extent, by increasing polymerase levels. Klenow fragment binds equally well to a DNA template adjacent to a drug modification site and to unmodified DNA. These results taken together lead us to suspect that it is primarily inhibition of base pairing around the drug modification site and not prevention of polymerase binding that leads to blockage of DNA synthesis. Unexpectedly, the exact termination site of the in vitro DNA synthesis by Klenow fragment is not dependent on the species of covalently bound drug molecule but on the sequence to the 5′ side of the drug-modified adenine. Misincorporation of dA for dG by Klenow fragment occurred at the secondary pausing site specifically for (+)-CC-1065 contained within the covalently modified sequence 5′-GATTA-3′. Although (+)-CC-1065 and its analogues evaluated in this study did not produce dramatically different effects on DNA polymerases when the drugs were bound to a single-stranded template, polymerization from a primer site containing a drug lesion in the duplex region did show a selective inhibitory effect with (+)-CC-1065 and (+)-AB′C′. When this observation is considered alongside results of experiments showing selective inhibition by these same compounds of T4 ligase and helicase II, the winding phenomena uniquely found with these compounds may be associated with the potent biological effect known as delayed lethality.

(+)-CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis*, which is active against several experimental tumors in vivo and is at least 50–1000 times more cytotoxic than other clinically used anticancer drugs (Hanka et al., 1978; Neil et al., 1981; Bhuyan et al., 1982; Li et al., 1982). There is overwhelming evidence that DNA is the principal biological target of (+)-CC-1065 and that the alkylating properties of this drug molecule are largely responsible for the potent cytotoxic effects produced via the formation of the (+)-CC-1065–DNA adduct (Li et al., 1982; Reynolds et al., 1986). Previous studies have revealed that (+)-CC-1065 forms a covalent adduct through *N*3 of adenine in a highly sequence-specific manner, in which the drug molecule lies within the minor groove covering 4- and 1-base-pair regions to the 5′ and 3′ sides, respectively, of the covalently modified adenine (Reynolds et al., 1985; Scahill et al., 1990; Hurley et al., 1990; Lin & Hurley, 1991).

Recently we have demonstrated that (+)-CC-1065 induces local bending (17–19° in toward the minor groove), winding, and helix-stiffening of DNA molecules as a consequence of *N*3-adenine covalent adduct formation (Lee et al., 1991; Sun & Hurley, 1992a). Previous work has shown that, in L1210 cells, (+)-CC-1065 inhibited DNA synthesis much more than it inhibited RNA or protein synthesis (Bhuyan et al., 1982).

† This research was supported by the Public Health Service (CA-49751), The Welch Foundation, and the Burroughs Wellcome Fund.
* Address correspondence to this author.

0006-2960/92/0431-2822$03.00/0