particles most probably sedimented continuously from suspension at a slow rate and were not derived from an episodic source. The present-day rate of oceanic pelagic sedimentation is <1 cm per 1000 years. The reduced C content of the black slates is comparable to that of modern oceanic pelagic muds, in which the reduced C concentrations are closely correlated with planktonic productivity (*23*).

Local small-scale redox perturbations may cause a dispersion of the C isotopic composition of graphite that forms during metamorphism (*5, 6, 24*). The effect of these perturbations is observed as an increase in the range of isotopic compositions as sample volumes decrease. Earlier studies of C isotopic compositions of Isua graphite have shown a general increase in the range of $\delta^{13}C$ values with a decrease in sample volume (*5*). Ion microprobe analysis has shown that the variation in one sample can be up to ~40 per mil (within the analytical errors) (*6*). These results suggest that C isotope compositions are heterogeneous on a grain scale. I thus studied sample volumes that were greater than 5 to 10 cm^3 and analyzed 2-g aliquots of well-mixed sample.

The sedimentary rocks have low $\delta^{13}C$ values (–18.77 to –19.11 per mil), and the $\delta^{13}C$ values increase in rocks that are recrystallized and that contain coarse graphite. The ^{13}C values in the least modified samples vary over a narrow range (–19.07 to –19.11 per mil) and show no correlation with the abundance of organic C in the rock. These data suggest that the $\delta^{13}C$ values in these samples were not greatly disturbed by secondary reaction with transient carbonic fluids, although systematic isotopic modification during the transition from some other reduced C compound to graphite cannot be precluded. The variation in C isotope composition for all Isua reduced C (Fig. 3) could be the result of isotopic dispersion by postdepositional geochemical processes (*24*), affecting C deposits with primary $\delta^{13}C$ values close to –19 per mil.

The most voluminous C reservoir in the Isua supracrustal belt is metasomatic carbonate rocks, which have an average $\delta^{13}C$ of -2.5 ± 1.7 per mil (*2*). The isotopic composition of these rocks therefore likely reflects the composition of regional carbonic metamorphic fluids; metasomatic veins and strongly deformed rocks also have high $\delta^{13}C$ values of –11.40 to –14.10 per mil. Thus, the sedimentary reduced C is significantly depleted in ^{13}C, in relation to the hydrothermal and strongly metamorphosed graphite. The increase in ^{13}C in recrystallized samples could thus be caused by a progressive reaction with hydrothermal fluids with a C isotopic composition that is biased by the regional carbonate C composition.

The presumed primary $\delta^{13}C$ values of about –19 per mil of the sedimentary graphites are within the range of biologically reduced C and within the range of reduced C compositions of most modern marine sediments (*25*). The sedimentological and geochemical evidence thus indicates a biogenic origin of the C forming the graphite globules. In analogy to modern oceanic pelagic shales, the precursor organic detritus of the graphite globules could have been derived more or less continuously from planktonic organisms that sedimented from the surface waters. Thus, these organisms could have been photoautotrophic.

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Coordinated Regulation of Iron-Controlling Genes, H-Ferritin and *IRP2*, by c-MYC

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The protein encoded by the c-*MYC* proto-oncogene is a transcription factor that can both activate and repress the expression of target genes, but few of its transcriptional targets have been identified. Here, c-MYC is shown to repress the expression of the heavy subunit of the protein ferritin (H-ferritin), which sequesters intracellular iron, and to stimulate the expression of the iron regulatory protein–2 (IRP2), which increases the intracellular iron pool. Down-regulation of the expression of H-ferritin gene was required for cell transformation by c-MYC. These results indicate that c-MYC coordinately regulates genes controlling intracellular iron concentrations and that this function is essential for the control of cell proliferation and transformation by c-MYC.

The c-*MYC* proto-oncogene is involved in the control of cell proliferation, differentiation, and apoptosis (*1*). Structural alterations of the c-*MYC* locus deregulate c-MYC expression and contribute to tumorigenesis by various mechanisms (*1*). The c-MYC protein is a transcription factor that functions by means of heterodimerization with MAX, a related protein that, like c-MYC, contains basic (b), helix-loop-helix (HLH), and leucine zipper (LZ) domains but lacks the transactivation domain present in the NH_2-terminus of c-MYC (*1*). MYC-MAX complexes stimulate transcription of target genes containing MYC-MAX binding sites (*1*). In addition, MYC can act as a transcriptional repressor of genes containing the initiator ele-

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ment (Inr) in their promoter region (*2*).

The precise function of the c-MYC protein, and in particular the mechanism by which it promotes cell proliferation in normal and neoplastic cells, is not known. Few target genes of c-MYC transcriptional activation have been identified, and those that have are involved in heterogeneous functions including DNA synthesis, cell cycle control, glucose metabolism, and protein translation (*3*). Even fewer genes have been identified as repression targets, and their contribution to c-MYC function is still unclear (*1*, *3*).

To identify novel c-MYC target genes, we used human Epstein-Barr virus (EBV)–immortalized B cells (CB33) engineered by transfection to express large amounts of either c-MYC–MAX (CBMyc.Max) or MAX-MAX (CBMax) complexes, representing the two extreme regulatory situations to amplify the response of MYC-activated or MYC-repressed genes (*4*). CBMyc.Max cells had a short doubling time, were clonogenic in vitro, and were tumorigenic in vivo, whereas CBMax cells proliferated slowly and lacked any transformation-related phenotype (*4*). In these cell lines, an RNA subtraction hybridization protocol (*5*) selected for isolating c-MYC–repressed genes (that is, overexpressed in CBMax cells) led to the recurrent isolation of cDNAs coding for the heavy subunit of ferritin (H-ferritin). Ferritin is a protein that regulates intracellular iron concentrations by sequestering it within multimeric complexes made by subunits of H-ferritin and L-ferritin, the light subunit (*6*).

H-ferritin RNA levels were decreased by 70% in CBMyc.Max cells and inversely correlated with c-*MYC* RNA levels (Fig. 1A). H-ferritin RNA levels significantly increased after 12-*O*-tetradecanoylphorbol 13-acetate (TPA)–induced terminal monocytic differentiation of U937 cells, a process that requires down-regulation of c-*MYC* (*7*). In contrast, expression of the RNAs encoding L-ferritin and transferrin receptor, a protein involved in iron uptake by the cell (*6*), was not affected in the same cells. Immunoprecipitation and immunoblot analysis showed that the changes in H-ferritin RNA levels were associated with proportionate changes in H-ferritin protein levels (Fig. 1B). Finally, H-ferritin RNA levels were increased by more than twofold in c-*myc*–deficient Rat1 fibroblasts (*8*) (Fig. 1C). These results indicate that there is an inverse relation between the expression of the c-*MYC* and H-ferritin genes, and our results are consistent with previous findings of up-regulation of H-ferritin gene expression in resting and terminally differentiated cells that lack c-*MYC* expression (*7*, *9*).

To establish a direct link between c-MYC and H-ferritin expression, we examined expression of the H-ferritin gene in cells where c-*MYC* expression can be induced in the absence of cell proliferation. We engineered a B cell line (EREB.TCMyc) in which proliferation and c-*MYC* expression could be independently controlled (*10*). In EREB.TCMyc cells, immortalization is conditional upon estrogen (E2) induction because the EBV gene encoding EBNA-2 is expressed as a chimeric fusion with the hormone-binding domain of the estrogen receptor (*10*); conversely, exogenous c-*MYC* expression can be induced by tetracycline (TC) withdrawal

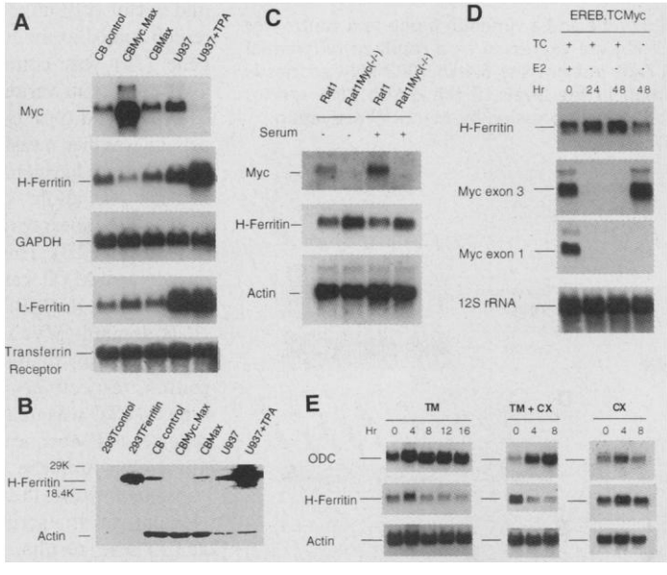

Fig. 1. Inverse relation between c-*MYC* and H-ferritin gene expression. (**A**) Northern blot analysis of total cellular RNA from CB33 cells transfected with plasmid vectors (*4*) expressing c-*MYC* and *MAX* (CBMyc.Max), *MAX* (CBMax), or a control vector (CB control); from naïve U937 cells; and from U937 cells treated with TPA (U937+TPA). The same blot was sequentially hybridized with the indicated probes (*26*) (GAPDH, glyceraldehyde phosphate dehydrogenase). (**B**) Immunoprecipitation and immunoblot analysis of the cell lysates from the same cells with an antibody to H-ferritin and an antibody to actin as a control for protein loading (*27*). Lysates from 293T cells transiently transfected with an H-ferritin expression vector (*22*) or with a control vector are also shown for positive identification of the H-ferritin band. Note that no β-actin protein is detectable in 293T extracts because only 5 μg of extracts was used for immunoprecipitation to avoid overloading of the H-ferritin–transfected lane. Molecular sizes are indicated in kilodaltons. (**C**) Northern blot analysis of Rat1 fibroblasts containing a bi-allelic targeted deletion of the *myc* locus [Rat1Myc(−/−); (*8*)]. (**D**) Repression of H-ferritin upon induction of c-*MYC* in the absence of cell proliferation. Northern blot analysis was performed of RNA from EREB.TCMyc cells upon estrogen (E2) withdrawal and c-*MYC* activation by TC withdrawal with the indicated probes. The exon 3 genomic c-*MYC* probe detects both endogenous and exogenous (pTCMyc plasmid) c-*MYC* RNA species; the exon 1 c-*MYC* probe detects only the endogenous c-*MYC* RNA because exon 1 sequences are not present in the transfected pTCMyc plasmid. Twenty-four hours after E2 withdrawal, cells were examined for proliferation by flow-cytometric analysis of DNA content and found arrested at G_0/G_1 as previously described (*10*, *12*); no change in cell cycle activity was detectable after induction of c-*MYC* by TC withdrawal. (**E**) Down-regulation of H-ferritin gene expression by c-MYC is independent of new protein synthesis. Northern blot analysis was performed of serum-starved Rat1MycER^{TM} (MycER^{TM}) cells upon activation of MycER^{TM} by TM (500 nM), by TM in the presence of CX (10 μg/ml), or with CX alone. The same blots were sequentially hybridized to an ornithine decarboxylase (ODC) probe as a control for a c-MYC–induced gene (*3*), to an H-ferritin probe, and to an actin probe as a control for RNA loading.

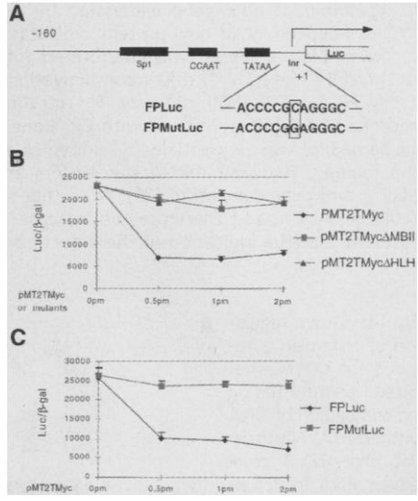

Fig. 2. Transcriptional repression of the H-ferritin promoter by c-MYC. (**A**) Representation of the reporter gene construct (FPLuc) containing the H-ferritin proximal promoter region (*15*) linked to the Luc coding domain. A single base pair substitution was introduced in the Inr element of the H-ferritin promoter within FPLuc to create FPMutLuc. (**B**) Results of transient transfection assays in which the indicated amounts (pm, picomoles) of a c-*MYC* wild-type expression vector (Myc) or mutants of the same vector (MycΔHLH and MycΔMBII) (*16*) were cotransfected into CV1 cells with 0.5 μg of the FPLuc plasmid by the $CaPO_4$ precipitation method. The total amount of transfected DNA and pMT2T sequences were kept constant in each experiment by adding pMT2T DNA. At 48 hours after transfection, cells were harvested and transcriptional activity was assayed as a function of Luc activity. The values are expressed as Luc activity after normalization with β-galactosidase activity expressed from a cotransfected plasmid expressing the bacterial β-galactosidase gene (0.5 μg; pCMVβgal). Each transfection was performed in triplicate and standard deviation bars are shown. (**C**) CV1 cells were cotransfected as in (B) with the pMT2TMyc vector and 0.5 μg of the FPLuc or FPMutLuc plasmid.

because these cells have been transfected with a TC-repressed c-MYC vector (*10*). In these cells, estrogen removal leads to growth arrest (*10*), documented by the complete down-regulation of c-*MYC* expression (Fig. 1D, $TC^+/E2^-$ lanes), and to an increase in H-ferritin RNA levels. In these quiescent cells, induction of exogenous (but not endogenous; see Fig. 1B legend) c-*MYC* RNA causes a decrease in H-ferritin RNA levels (Fig. 1D, $TC^-/E2^-$ lanes). Because c-*MYC* induction is not sufficient to induce cell cycle entry in EREB.TCMyc cells (*11, 12*), these results indicate that c-MYC induces the down-regulation of H-ferritin RNA by a mechanism independent of c-MYC–induced changes in cell cycle activity.

To investigate whether c-MYC–mediated down-regulation of the H-ferritin gene was direct, we studied H-ferritin RNA levels in a rat fibroblast cell line engineered to express an inactive c-MYC–estrogen receptor fusion protein ($MycER^{TM}$). By treating the cells with 4-hydroxytamoxifen (TM), which specifically activates $MycER^{TM}$ (*13*), and by simultaneous treatment with the protein synthesis inhibitor cycloheximide (CX), we examined whether expression of the H-ferritin gene could be down-regulated upon activation of preexisting $MycER^{TM}$ in the absence of new protein synthesis. Treatment with TM (with or without CX treatment, but not CX alone) led to a ~60% reduction in H-ferritin RNA expression within 12 hours (Fig. 1E). Because the half-life of H-ferritin RNA is 10 to 12 hours (*14*), the kinetics of c-MYC–induced down-regulation and the independence of new protein synthesis are consistent with a direct effect of c-MYC on H-ferritin gene expression.

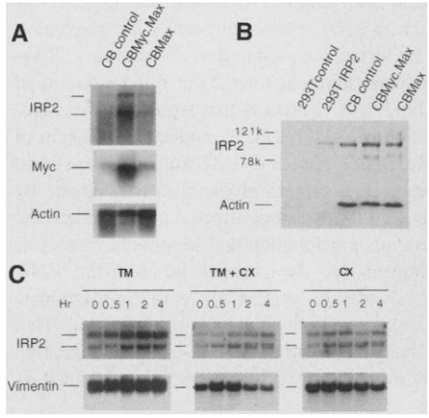

Fig. 3. Transcriptional activation of *IRP2* gene expression by c-MYC. (**A**) Northern blot analysis of total cellular RNA from CBMyc.Max, CB-Max, or CB cells with the indicated probes (*28*). (**B**) Immunoblot analysis of cell lysates (50 μg) from the same cells with an antibody to IRP2 (*29*). Lysates from 293T cells transiently transfected with an *IRP2* expression vector or with a control vector are also shown for positive identification of the IRP2 band. Note that no β-actin protein is detectable in 293T extracts because only 5 μg of extracts was loaded to avoid overloading of the *IRP2*-transfected lane. (**C**) Up-regulation of *IRP2* gene expression by c-MYC is independent of new protein synthesis. Northern blot analysis was performed of Rat1MycER™ ($MycER^{TM}$) cells upon activation of MycER by TM (500 nM), by TM in the presence of CX (10 μg/ml), or with CX alone. The same blot was sequentially hybridized to an *IRP2* probe and a vimentin probe as a control for RNA loading. Two *IRP2* mRNA species (6.4 and 3.7 kb) are expressed as a result of differential splicing and polyadenylation (*29*). Note that the 3.7-kb, but not the 6.4-kb, *IRP2* RNA species is stabilized by CX, and therefore the increase (fivefold) in the levels of the 6.4-kb RNA species represent the true indication of the induction of *IRP2* gene expression by MycER™ activation.

To ascertain whether c-MYC down-regulates H-ferritin gene expression at the transcriptional level, we studied whether c-MYC could repress the transcription of a reporter gene driven by the H-ferritin promoter (*15*). The H-ferritin promoter contains an Inr element, the domain involved in transcriptional repression by c-MYC (Fig. 2A) (*2*). A plasmid vector (FPLuc) containing this promoter region linked to the luciferase (Luc) reporter gene (*15*) was cotransfected into monkey CV1 cells with various amounts of a vector carrying wild-type c-*MYC* (*16*). Luciferase activity was then measured 48 hours after transfection as a function of H-ferritin promoter activity. The results showed that c-MYC repressed FPLuc expression in a dose-dependent fashion (Fig. 2B). This repression was not induced by c-MYC carrying mutations in the c-*MYC* Box II (MBII) (MycΔMBII) or the HLH domain (MycΔHLH), which are necessary for transrepression and heterodimerization, respectively (*2, 16*). Finally, wild-type c-MYC was unable to repress a mutant reporter (FPMutLuc) (*15*) carrying a point mutation within the H-ferritin Inr element, consistent with the involvement of this element in transcriptional repression (*2, 16*). These results, together with run-on transcription assays showing a decrease in H-ferritin gene transcription in CBMyc.Max cells (*11, 17*), are consistent with a role for c-MYC in controlling H-ferritin gene transcription.

We next investigated whether c-MYC controlled the expression of other genes involved in iron homeostasis. c-MYC did not influence the expression of the genes coding for the transferrin receptor, which controls iron import into the cell, or L-ferritin, which together with H-ferritin is involved in intracellular iron transport and sequestration (*6*) (Fig. 1A). Conversely, c-*MYC* overexpression in Rat1 fibroblasts (*17*) and CB33 B cells (Fig. 3, A and B) was associated with increased levels of expression of iron regulatory protein–2 (IRP2 gene). IRP2 is

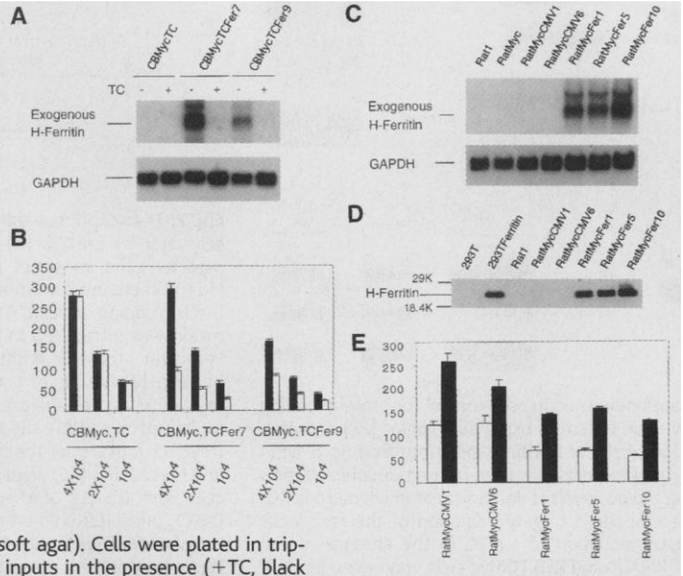

Fig. 4. Down-regulation of H-ferritin is required for c-MYC–mediated transformation and stimulation of DNA synthesis. (**A**) Northern blot analysis of total cellular RNA from CB-Myc cells transfected with a pTCFer expression vector (two clones, CBMycTCFer7 and -9), or with a control (pTC) vector (CBMycTC). The same blot was sequentially hybridized with an SV40 poly(A) probe to detect exogenous H-ferritin expression and a GAPDH probe as a control for RNA loading. (**B**) Clonogenicity assay of the same cells in semi-solid medium (soft agar). Cells were plated in triplicate at three different inputs in the presence (+TC, black bars) or absence (−TC, white bars) of tetracycline (*30*). (**C**) Northern blot analysis of total cellular RNA from Rat1 cells transfected with a c-*MYC* (RatMyc) or control (Rat1) expression vector, and RatMyc cells transfected with a vector (pHeBoCMVFerritin) expressing H-ferritin (three clones, RatMycFer1, -5, and -10) or with a control (pHeBoCMV) vector (two clones, RatMycCMV1 and -6). The same blots were sequentially hybridized with an SV40 poly(A)$^+$ probe and a GAPDH probe as a control for RNA loading. (**D**) Immunoprecipitation and immunoblot analysis of the cell lysates from the same cells with an antibody to H-ferritin (*27*). Transiently transfected 293T cells are also shown for positive identification of the H-ferritin band. (**E**) Clonogenicity assay of the same cells in semi-solid medium (soft agar) in the presence or absence of hemin (100 μM). Cells were plated in triplicate; values of plating efficiency showed a linear distribution at three different inputs (10^4, 5×10^3, and 2.5×10^3); only values relative to inputs of 1×10^4 cells are shown.

an RNA-binding protein that, upon iron deprivation, binds to iron-responsive elements (IREs) in the transcripts of various iron-controlling genes and regulates their stability or rate of translation (*18*). Increased *IRP2* RNA and protein levels were associated with increased IRE-specific RNA-binding activity by IRP2 in the same cells; this effect was not seen for the related protein IRP1 (*17*). Run-on transcription analysis showed that induction of *IRP2* by c-MYC occurred at the transcriptional level (*17*). Finally, the induction (fivefold) (*17*) of *IRP2* by c-MYC was rapid and independent of new protein synthesis in MycER^{TM} cells (Fig. 3C), suggesting direct transcriptional activation of *IRP2* by c-MYC.

We next examined whether down-regulation of H-ferritin or up-regulation of *IRP2* was required for c-MYC to promote proliferation and cell transformation. We could not obtain cell clones expressing exogenous IRP2, most likely because IRP2 concentrations are regulated at the level of protein stability, and therefore, excess IRP2 was rapidly degraded in transfected cells (*19*). We then tested whether reexpression of high levels of H-ferritin could revert c-MYC–induced transformation. Toward this end, a vector expressing H-ferritin upon TC withdrawal (pTCFer) (*20*) was transfected into c-MYC–transformed CB33 cells (Fig. 4A) and in a Burkitt lymphoma cell line (BL59) (*17*) carrying a c-*MYC* gene constitutively activated by chromosomal translocation (*21*); in addition, a vector expressing H-ferritin (pHeBoCMV-Ferritin) (*22*) was stably transfected into MYC-transformed Rat1 cells (Fig. 4C). In all three cell types, H-ferritin expression led to a significant decrease in in vitro clonogenicity (Fig. 4, B and E), a crucial trait of the transformed phenotype. This effect could be rescued by treatment with hemin, a chemical carrying iron (Fig. 4E), but not by protoporphyrin IX (iron-free hemin), consistent with an effect of H-ferritin on the intracellular iron pool (*23*). Finally, c-MYC induced an increase in DNA synthesis that was abolished by H-ferritin reexpression and restored by hemin treatment in Rat1 cells (*17*), consistent with a role of iron in regulating the activity of ribonucleotide reductase, the rate-limiting enzyme for DNA synthesis (*24, 25*). Thus, down-regulation of H-ferritin gene expression is necessary for c-MYC to induce cell proliferation and transformation.

Our observations indicate that c-MYC coordinately regulates a gene expression program controlling iron homeostasis (*6*, *18*). H-ferritin sequesters iron, and therefore its down-regulation can increase the availability of intracellular iron (*6*, *18*). This effect can be amplified by IRP2 up-regulation because IRP2 binds to an IRE in the H-ferritin RNA and inhibits its translation (*18*), thus further decreasing H-ferritin levels. Furthermore, IRP2 binds to IREs in the transferrin receptor RNA and inhibits its degradation (*18*), thus enhancing iron import. Therefore, c-MYC–mediated regulation of H-ferritin and *IRP2* can lead to complementary effects that would increase the intracellular iron pool.

The finding that c-MYC controls iron metabolism is consistent with the observations that iron chelation leads to growth arrest (*23, 25*) and decreased synthesis of the cell cycle regulators p34^{cdc2} and cyclin A (*25*), whereas increased iron availability up-regulates the activity of ribonucleotide reductase (*24*). However, other iron-dependent cellular functions, including oxygen transport, mitochondrial energy metabolism, and electron transport, may also be influenced by c-MYC.

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27. For immunoprecipitation, we incubated 1 mg of the cell lysates (lysis buffer: 1% Triton-X 100, 50 mM tris, pH 7.5, 10% glycerol, 150 mM NaCl, 30 mM $MgSO_4$, 8 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride) with a polyclonal antiserum to ferritins (Boehringer Mannheim) for 2 hours using protein A Sepharose. Immunoprecipitates were electrophoresed on a 12% SDS-PAGE minigel, transferred to nitrocellulose, and then incubated with a monoclonal antibody to human H-ferritin [P. Santambrogio *et al.*, *J. Biol. Chem.* **268**, 12744 (1993)]. Signal detection was carried out with sheep antibody to mouse immunoglobulins and an ECL kit (Amersham).
28. The human and rat *IRP2* probes were generated by RT-PCR amplification of mRNAs from human lymphoblastoid cells and Rat1 cells, respectively. The primers used for the generation of the *IRP2* probe were 5′-CCATAGCAGGCACAGTGAAT and 3′-GGAA-TCTGCATTTTCTCCTG (both identical for human and rat).
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