c-Myc Represses and Miz-1 Activates the Murine Natural Resistance-associated Protein 1 Promoter

Iron is essential for growth, and impaired iron homeostasis through a non-conserved mutation within murine Nramp1, also termed Slc11a1, contributes to susceptibility to infection. Nramp1 depletes the macrophage cytosol of iron, with effects on iron-regulated gene expression and iron-dependent processes. Wu and colleagues (Wu, K.-J., Polack, A., and Dalla-Favera, R. (1999) Science 283, 676–679) showed converse control of iron regulatory protein expression (IRP2) and H-ferritin by c-Myc, suggesting a role for c-Myc in enhancing cytoplasmic iron levels for growth. We investigated if c-Myc also regulates Nramp1 expression. We show an inverse correlation with cell growth, and in co-transfection experiments c-Myc represses the Nramp1 promoter. Within the Nramp1 promoter we identified six non-canonical E boxes, which are not important for c-Myc repression. By deletion analysis the repressor site maps to one or more initiator elements flanking the transcriptional initiation site. Co-transfections with the c-Myc interacting zinc finger protein (Miz-1) show that Miz-1 can overcome c-Myc repression of Nramp1, and, from a deletion construct lacking E box sites, Miz-1 activates the Nramp1 promoter. These studies reinforce the link between c-Myc and iron regulation and provide further evidence that c-Myc negatively regulates genes that decrease the iron content of the cytosol. The results provide further support for a divalent cation antiporter function for Nramp1.

A biallelic phenotype in mouse, termed Ity/Lsh/Bcg, was identified in response to infection by obligate intracellular macrophage pathogens (1–3). Nramp1* (also termed Slc11a1) was isolated as the positional gene candidate for Ity/Lsh/Bcg (4). In subsequent studies the full-length sequence of the encoded polypeptide was identified (5). Mouse strains resistant to infection encode Gly at codon 169 within Nramp1 whereas susceptible mice encode Asp (6). The G169D polymorphism is sufficient to explain the outcomes of model infections within inbred mouse strains at pre-T cell stages of infection, as confirmed by gene targeting and transgenesis experiments (7, 8). Allele D169 is phenotypically null (7). When Nramp1 was cloned, the biochemical basis for its control over the proliferation of intracellular pathogens was not obvious, but the sequence suggested a transporter function (4). Subsequent studies showed the encoded polytopic integral membrane Nramp1 protein was expressed in a perinuclear location, on intracellular late endosomal/lysosomal membranes (9–11). Nramp1 underwent rapid recruitment to the periphery of a pathogen-containing vesicle (10, 11) and displayed a more peripheral location in response to treatment with interferon-γ (11). These observations led to the suggestion that growth control of microbial pathogens could be achieved by the transport of some toxin into the lumen of the phagosome or by the sequestration of some essential nutrient. The identity of a candidate transport substrate was revealed from studies on a highly sequence-related gene, Nramp2 (DMT1/DCT1/SLC11A2). Nramp2 was initially isolated as an orphan gene (12) but was re-isolated by functional cloning through a divalent cation/iron uptake assay (13). In addition, identical mutations, G185R, within Nramp2 in the mkm mouse and the Belgrade rat are associated with impaired intestinal and erythroid cell iron uptake (14). Based on the striking sequence similarity between the two polypeptides (15), Nramp1 was also predicted to transport divalent cations or iron within murine macrophages, and infection susceptibility occurs through impaired divalent cation transport.

The many pleiotropic effects attributed to Nramp1 (16) should be explained by differential cation transport. Because Nramp1 protein is expressed within internal membranes, the differential partitioning of divalent cations between the cytosol and the lumen of the internal vesicle should contribute to the pleiotropic effects described (16). Inducible nitric-oxide synthase (iNos) is expressed at quantitatively higher levels in functional Nramp1 macrophages (17–20). Ferrous iron provides the link between iNos expression and Nramp1, because Dlaska and Weiss (21) revealed that iron loading inhibits iNos expression via the NF-IL6 transcription factor. Enhanced iNos expression in Nramp1 functional strains could be explained by depletion of iron availability within the cell cytosol that is...
sensed by transcription factors such as NF-IL6. However, there is controversy surrounding the direction of Nramp1-mediated cation transport. Some experimental evidence supports cation/ferrous ion influx, into the vesicle lumen, as measured by direct transport of cations (22), and analysis of Nramp1 transport activity in *Xenopus* oocytes (23). Other data support transport out of the intracellular vesicle lumen (24, 25). Divalent cations were proposed to accumulate or transit the cytosol, but these were not assayed directly (24). Based on the two transport hypotheses; Nramp1-mediated antimicrobial mechanisms have been suggested as reduct-mediated killing (22, 23) or iron starvation (24, 25). Additionally, Nramp1 not only depletes iron from the cytoplasm but also is proposed to operate in a pathway ultimately leading to iron sequestration from the macrophage (26, 27). New approaches to delineate Nramp1 function are therefore required to resolve the controversy of transport and antimicrobial mechanisms.

We reported that transfectants of the *Nramp1* 
RAW264.7 cell, expressing the functional *Nramp1* allele, constitutively exhibit lower redox-active cytoplasmic iron levels relative to control transfectants or parental cells (28). In these cells iron-regulated processes are affected, including IRP2 activity and the iron-regulated protein kinase C isoform, protein kinase Cβ1 (28). The changes are supportive with Nramp1 limiting cytoplasmic iron availability and transport into the vesicle lumen. Cytoplasmic iron depletion may be important for maintaining macrophage function during inflammation when high levels of reactive oxygen and nitrogen intermediates are generated and to minimize the effects of oxidative stress on cell viability (29). Cytosolic iron depletion could explain the elevated expression of *iNos* in Nramp1 functional cells (17–20).

Links between the c-Myc family of basic helix-loop-helix transcription factors and iron homeostasis have recently been reported (30). A role for c-Myc was described in elevating cytoplasmic iron for cell proliferation, with stimulatory and inhibitory effects on the expression of genes that either increase or decrease, respectively, the extent of the LIP. Further data in support of a link between c-Myc and iron have been provided by the expression cloning, in a ferrous transport-defective yeast strain, of a novel complementing maize myc transcription factor (31). Kyriakou and colleagues (32) have described iron-dependent growth of peripheral blood mononuclear cells mediated via c-Myc. We therefore sought to investigate a role for c-Myc in the control of Nramp1. Our results indicate that c-Myc represses *Nramp1* expression by acting at the Inr that spans the transcriptional initiation site (33). Furthermore, Miz-1 (34) positively regulates *Nramp1* expression, and in co-transfection experiments Miz-1 overcomes the inhibitory effects of c-Myc. The data provide an independent method that lends further support to the hypothesis that Nramp1 reduces the iron pool within the cytoplasm and therefore functions as a proton divalent cation antiporter (23).

**EXPERIMENTAL PROCEDURES**

**Preparation of Nramp1-expressing RAW264.7 Clones—**Nramp1-transfectant RAW264.7 cell clones have been prepared to study *Nramp1* function, as described previously (28), with the full-length *Nramp1* cDNA in the sense, lines R32 and R37, or antisense, line R21, orientations driven from the human β-actin promoter.

**Anti-Nramp1 Antibodies—**Antibodies immunoreactive to Nramp1 were prepared from recombinant GST-Nramp1 fusion proteins to the N-terminal domain as described previously (26). Western blotting was performed as before (28).

**Analysis of Cell-proliferative Responses—**Cell proliferation was determined on lines R21, R32, R37, parental cells, or primary bone marrow cells, as indicated, by staining 96-micro-well plates at time points with 0.5% crystal violet in 20% methanol and by determining the extent of either [3H]thymidine or BrdUrd incorporation (Amersham Biosciences). Each sample was performed in replicates of at least three. Post crystal violet staining, bound dye was resolubilized in 100% methanol, and absorbances at 540 nm were determined. Samples were diluted to 1 × 10^4 cells/ml, a necessary 1–2 × 10^5 necessary, and plated at day 0 for growth experiments. Statistical analysis was performed using Student’s t test.

**CBA Bone Marrow-derived Macrophages and N11 Microglial Cells—**Bone marrow-derived macrophages were flushed out of the femurs of young adult males and matured in the presence of 20% L929 media as a source of macrophage colony stimulating factor. Cells were harvested and resuspended in DMEM/F12 for Nramp1 expression determination and for cell growth quantitation. N11 microglial cells that express mature 90- to 100-kDa Nramp1 protein were used for analysis of Nramp1 regulation following serum removal and replacement and were maintained as described previously (9).

**Genomic Cloning and DNA Sequence Analysis—**Genomic phage clones were isolated from a C57BL/6J/BRAfJ FIII library (Stratagene) by screening with a PCR-derived probe spanning the 5′ end of the published murine *Nramp1* gene sequence (33). The probe extended from −265 bp of the major transcriptional initiation site to a BamHI site 43–48 bp 3′ of the last base of exon 2. From the screen two clones were isolated, A1.4 and A2.3, that were similar by restriction analysis. A SalI restriction fragment of −9 kb was subcloned into pBluescript and sequenced (Oswel, Southampton). The sequence of the genomic segment used in these studies has been deposited in the EMBL data base (accession number AJ458183). The sequence is also available from the mouse genome data base (www.ensembl.org/ Mus_musculus/). Nramp1 5′-flanking sequence was analyzed for transcription factor binding sites (35). A restriction fragment used for promoter studies was from an Xbal site at −1555 bp, relative to the major transcriptional initiation site (33), to a synthetic BamHI site introduced immediately downstream of exon 1. This modification also converts the natural *Nramp1* ATG translational initiation codon to TTG. The 1655-bp fragment was cloned via XhoI and BamHI into the CAT reporter plasmid pBLCAT3, called pH84, and used in transactivation experiments (see below). Other constructs in pBLCAT3 were prepared by cloning more 3′ HindIII, −868 bp (pHB6), or Spal −71 bp (pHB8) restriction sites, numbered relative to the transcriptional initiation site. The HindIII 5′ truncation removes four of the six identified E-box sites, and the Spal truncation removes all the E-box sites. Other constructs were prepared from synthetic oligonucleotides (Oswel, Southampton); phb20, pHb21, and pHb22 have similar 3′ termini at +34 bp and 5′ termini at −71 bp, −34 bp, and +7 bp, respectively. An expression construct based on pCAT-enhancer (Promega) was also constructed for studies in Raw264.7 cells, based on pHb20/1, and termed pHb20E/21E. All promoter construct inserts were sequenced by Oswel.

**c-Myc and Miz-1 Transactivation Studies—**Transfection studies were performed in COS-1 cells and Raw 264.7 cells using LA (Invitrogen) or by electroporation. Briefly, 1–3 × 10^6 cells/ml (COS-1), 1 × 10^7 cells/ml Raw264.7) were transfected by electroporation (450 V, 500 microfarads (Raw264.7 cells)) at room temperature in 0.5 ml of complete media using 10 μg of *Nramp1* promoter CAT constructions, 1 μg for LA experiments, and the indicated amount of pEF-c-Myc (provided by Yongfeng Shang, Harvard Medical School) or pEF empty plasmid. LA transfections were performed according to the manufacturer’s instructions. pCMV MIZ-1 expression plasmid was provided by Frank Hanel, Hans Knoll-Institut for Naturstoff-Forschung, Heidelberg. Plasmid DNA used in transfections were prepared using Machery-Nagel Maxi Prep kits, and the total quantity of DNA in any transfection was normalized to 20, 2, or 3 μg for LA transfections, with non-recombinant expression plasmid, or as indicated. Electroporated cells were immediately placed back in culture and harvested 48 h later. Cells were washed and lysed by multiple cycles of freeze thaw. Protein concentrations of soluble fractions were determined (Pierce), and 50 μg of soluble proteins (20 μg of LA), or as indicated, used for chloramphenicol acetyltransferase (CAT) activity determination by standard methods. CAT activities were quantitated using a PhosphorImager (Amersham Biosciences). CAT activities are described as percentage conversion of substrate to product/substrate plus product for 50 or 20 μg of soluble protein except where indicated. The human β-actin promoter

---

2 C. H. Barton and S. T. Baker, unpublished.
CAT construct was used as a transfection control between experiments, and a range of protein amounts were assayed (not shown). Experiments on the repression of \( \text{Nramp1} \) by c-Myc have been repeated on more than 20 independent occasions with various vectors and plasmid DNA preparations. The transfection efficiency for Raw264.7 cells was considerably lower than COS-1 cells, and for analysis of reporter gene expression we used an enhancer vector (Promega) to increase reporter gene expression levels. Direct analysis of reporter gene expression in Raw264.7 cells has also been undertaken using a semi-quantitative approach of digital microscopy. pEGFP-N3 (CLONTECH) was modified to remove the endogenous CMV promoter by digestion with \( \text{Ase} \)I and \( \text{Bgl} \)II, 5′/H11032 of the promoter and within the polylinker, respectively. Ends were repaired, DNA molecules were purified/ligated, and clones were recovered (p\( \text{CMV-EGFP-N3} \)). \( \text{Nramp1} \) promoter fragments, as described above for pHB4, were cloned into this plasmid, called pHB15, and transfected, as above, and cells were plated onto coverslips in single wells of eight-well plates (Nunc). After 48 h, cells were analyzed for fluorescence. Ten randomly selected fields at 40 magnification (Zeiss digital microscope) were collected and analyzed for fluorescence above background using Metamorph version 4.6 imaging software (Universal Imaging Corp.). Results are presented as the mean fluorescence intensity over background for each field.

**RESULTS**

**Decreased Proliferation of \( \text{Nramp1} \)-expressing RAW264.7 Cell Clones**—\( \text{Nramp1}^{G169} \) transfectants of the BALB/c-derived RAW264.7 (\( \text{Nramp1}^{D169} \)) macrophages show decreased proliferation compared with control cells not expressing the functional \( \text{Nramp1} \) allele. A representative experiment is shown (Fig. 1a) conducted for \( \text{Nramp1} \)-expressing lines R32 and R37 and control, antisense line R21 and parental cells. The former express the mature 90- to 100-kDa \( \text{Nramp1} \) polypeptide, whereas the latter two clonal cell lines do not (Fig. 1d). The enhanced cell proliferation for \( \text{Nramp1}^{D169} \) cells is maintained over all cell densities analyzed. DNA synthesis data are normalized for cell number using a colorimetric assay (Fig. 1b). Greatest differences, ~3-fold, are detected when using 10,000–20,000 cells per well. Addition of low molecular weight iron to cultures abolished the significant differences obtained between lines expressing and not experiencing \( \text{Nramp1} \) protein (Fig. 1c), whereas differences without iron were significant (\( p < 0.05 \)), suggesting \( \text{Nramp1} \) expression limits iron availability for cell proliferation.

**Proliferative Response of CBA Bone Marrow Cells Precedes \( \text{Nramp1} \) Induction**—On successive days in culture, samples were taken from bone marrow cells, CBA strain (\( \text{Nramp1}^{G169} \)), for \( \text{Nramp1} \) Western blotting (Fig. 2, a and b). Cell numbers were assessed in parallel cultures by a colorimetric assay, and DNA synthesis was quantitated by BrdUrd incorporation (Fig. 2c). Lanes R21 and R37 (Fig. 2a) are positive control extracts from Raw264.7 control and \( \text{Nramp1}^{G169} \)-expressing transfec-
tants (see Fig. 1c). Lanes 1–5 are from CBA bone marrow cells cultured from 1 to 5 days in the presence of macrophage growth factor (Fig. 2a). Confirmation that protein is loaded onto all tracks was provided by Amido Black staining an Immobilon membrane after immunodetection (Fig. 2b). Immunoreactive Nramp1 protein was not detectable at days 1 and 2, although full-length c-Myc immunoreactivity was present (not shown). The mature Nramp1 90- to 100-kDa protein was induced from day 3 and persisted until day 5, and the 45-kDa aglycosyl species could be detected on day 5 (Fig. 2a). The experiment shown is a representative of four such experiments, all of which demonstrate Nramp1 induction at day 3. Over the 5-day period in culture, the number of cells, assayed using the colorimetric assay, increased 6-fold (Fig. 2c). DNA synthesis peaked at day 2, and a temporal separation was apparent between DNA synthesis and the onset of appearance of Nramp1 immunoreactivity (Fig. 2a). After the peak in DNA synthesis, BrdUrd incorporation levels remained higher than at day 1. Further evidence for a link between Nramp1 expression and cell growth was provided in microglial N11 cells, these cells endogenously expressed mature 90- to 100-kDa Nramp1. Following 48 h of serum starvation levels of the Nramp1 protein were low, and addition of serum (10%) to serum-starved cultures revealed a further decrease of the 90- to 100-kDa Nramp1 before an increase at 24 and 32 h (Fig. 3a). Amido Black staining of the membrane revealed equivalent protein loading (Fig. 3b). In contrast, Lamp1 expression is increased after serum addition at 8 h (not shown). To study the basis for this regulation the flanking region of the Nramp1 gene was isolated and analyzed to assess if there is a mechanistic link between cell proliferation and/or c-Myc and Nramp1 expression.

**Isolation and Analysis of Nramp1 5′-Flanking Sequence**—A restriction map of an isolated clone is shown together with locations of exons identified by comparison of cDNA (accession number X75355) and genomic sequences (Fig. 4a). Also aligned with the linear map of the genomic clone are the constructs...
FIG. 4. *Nramp1* genomic sequence of 5’ flanking region. *a*, restriction map of phage λ clone isolated from a C57BL/6CBA-F1J mouse library. Below, a map shows the location of exons, *black boxes* labeled I–IV, identified within the clone and determined by comparison of the murine genomic and cDNA sequences. The *arrow* indicates the major transcriptional start site determined previously (33). Fragments of the *Nramp1* promoter used in this study correspond to XbaI, HindIII, and SpI restriction endonuclease cleavage sites, corresponding to constructs pH4, pH6, and pH8 and constructs prepared from synthetic oligonucleotides, pH20, pH21, and pH22. The 3’-ends of constructs pH4/6/8 correspond to a synthetic BamHI site located in place of the ATG translational start codon at +99 bp and constructs pH20/21/22 to +34 bp. In expanded format is shown the location of candidate elements within the construct pH4 as indicated. *b*, sequence of murine 5’ flanking sequence, including exon one (*underlined*) to the 3’ splice site. Sequence extends up to the XbaI site of construct pH4. Restriction sites used in the preparation of the constructs are indicated in *boldface*, as are the six non-canonical E-boxes (*MycMax*), the two initiator elements (*Inr*), and the Sp1 site in pH20. A GT repeat element is shown in *italics* and *underlined*. The first two amino acids of Nramp1 are shown in *single-letter code*. The sequence of the promoter fragment used in this study has been deposited with the EMBL data base (accession number AJ458183).
prepared for this study. These are from 5’ restriction sites XbaI, HindIII, and Spel I to a synthetic 3’ BamHI site, which removes the translational initiation site, and are labeled pH4, pH6, and pH8, respectively, and three constructs were prepared from synthetic oligonucleotides, pH20, pH21, and pH22. A preliminary analysis of the murine 

The table below summarizes the consensus sequences within c-myc-repressed genes:

| Gene    | Inr element |
|---------|-------------|
| Ad-MLP  | TCACTCT     |
| C/EPP   | TCACTCT     |
| MT-1    | TCACTAC     |
| NCAM    | TACCTCA     |
| LFA-1   | TCACTT      |
| HLA-A2  | TACAGTT     |
| HLA-C   | TACAGTT     |
| Cav-1   | TACGTTC     |
| Nramp1 #1 | CCACCTC    |
| Nramp1 #2 | TACCTCG    |
| Consensus | YYAN(T/A)YY |

The consensus sequence is YYAN(T/A)YY.

The figure on the right illustrates the repression of Nramp1 promoter activity by co-transfected c-Myc. a, shown is a representative experiment of acetylated chloramphenicol product volume quantitation expressed as a percentage of substrate + product (CAT conversion, %). Plasmids used were Nramp1 CAT constructions pH4, pH6, and pH8/20/21/22 incorporating 6, 2, and 0 of the putative E-box elements, respectively (solid bars). Nramp1 promoter plasmids were co-transfected with 2 μg of pEF-c-Myc (open bars) provided by Yongfeng Shang (Harvard Medical School), and the total amount of plasmid in all transfections was normalized to 3 μg with non-recombinant expression plasmid. Triangles indicate the degree of inhibition of promoter activity by co-transfected c-Myc. b, titration of pH4 with 0–2 μg of c-Myc in COS-1 cells using LA. Results are presented as CAT activity relative to untreated cultures. A significant reduction in reporter gene activity was observed with respect to no added c-Myc (*, p = 0.023; **, p = 0.012; ***, p = 0.0015; ****, p = 0.00021).
DNA in transfections was normalized to 20 &H9262 LA with pHB20E (1 &H9262 modulation of c amount of pEGFP-N3 plasmid. Raw264.7 cells were transfected with indicated amount of pEGFP-N3, and FI was determined. c, dose-dependent modulation of Nramp1 promoter by co-transfected c-Myc. FI recorded is plotted against the amount of c-Myc used in the transfection 0–10 &g, all DNA in transfections was normalized to 20 &g of DNA. Student’s t test showed significance compared with pHB15 (0 &g of c-Myc) p < 0.008 for 2, 5, and 10 &g of c-Myc. d, dose-dependent reduction in CAT reporter gene activity with increasing c-Myc. Raw264.7 cells were transfected using LA with pHB20E (1 &g) and the indicated amount of c-Myc, total DNA in all transfections was 5 &g. Student’s t test compared with pHB20E alone; *, p = 0.035; **, p = 0.029.

COS-1 cells is shown (Fig. 5b), and the inhibition by c-Myc was statistically significant (p = 0.001, comparing 0 and 2 &g of added c-Myc). In some experiments we observed a biphasic response to c-Myc, which happened on a regular basis, and higher c-Myc levels were again inhibitory. The basis for this biphasic response is not known but has been described previously on c-Myc titration experiments with the AdML and cyclin D promoters (34). In our hands it does not appear to occur at a reproducible level of co-transfected c-Myc, and in this experiment occurred at 0.2 &g of added c-Myc, whereas 0.5 &g of c-Myc was again inhibitory.

Analysis of Nramp1 Promoter Activity in Raw264.7 Cells—Nramp1 is a macrophage-specific gene in mouse and therefore attempts were made to analyze the promoter activity in cell lines of this lineage. However, as a consequence of low transfection efficiency it was not feasible to undertake this study using either CAT or luciferase reporters in our hands with pHB4. We therefore evaluated the use of quantitative digital microscopy for eGFP reporter gene expression in single cells.

To evaluate the stability of the eGFP fluorophore for the study proposed here, Raw264.7 cells were transiently transfected with eGFP plasmid and analyzed 48 h later. A representative field was identified, captured, and then constantly exposed to the UV light for a period of up to 10 min. Images were captured at time intervals during this period of persistent UV exposure. All images were analyzed for fluorescence intensity using the Metamorph software package. Results indicate (Fig. 6a) little change in the fluorescence intensity over a period of 5 min. However, from 5 to 10 min a decline of 1.5 log units of fluorescence intensity was determined. This experiment indicates that, if images are collected within the 5-min window, it is possible to use this method for quantitative analysis. In another experiment Raw264.7 cells were transfected from 1 to 20 &g of eGFP-N3 wild-type plasmid (Fig. 6b). Ten fields for each plasmid dose were evaluated, and data show a dose-dependent increase in fluorescence intensity with increasing plasmid dose. This method was employed to evaluate Nramp1 promoter activity in Raw264.7 cells following transient transfection (Fig. 6c). As in COS-1 cells, increasing c-Myc plasmid in the transfection caused a dose-dependent inhibition of Nramp1 promoter activity (compare data with that of Fig. 5b).

Using a construct containing an SV40 enhancer element, it was possible to measure Nramp1-driven reporter gene activity in transiently transfected Raw264.7 cells using the CAT reporter gene. In this experiment a significant dose-dependent inhibition of the reporter gene activity from pHB20E was observed that reproduced the inhibition curve obtained by digital microscopy in Raw264.7 cells and of c-Myc inhibition in COS-1 cells (Fig. 6d, compare with Figs. 6c and 5b). These data indicate that the effects we describe in COS-1 cells for c-Myc-mediated inhibition of Nramp1 are also relevant to macrophage lineage cells. The differences between 0 &g of co-transfected c-Myc and 2 &g are significant (p < 0.0001). The differences in the dose-response curves between Figs. 6, c and d, were due to the transfection method employed (Fig. 6c, electroporation, Fig. 6d, LA) and the amount of Nramp1 promoter construct used. In both cases the ratio of promoter to co-transfected c-Myc is the same, 5 &g in Fig. 6c corresponds to 0.5 &g in Fig. 6d. Using LA in Raw264.7 cells similar effects to those described in COS-1 cells were observed on deleting –868 bp to –71 bp (data not shown) as described (Fig. 5a).

Modulation of Nramp1 Promoter Activity by Sequence Variation within the GT Repeat—Previous work has identified func-
tional sequence polymorphisms within the human NRAMP1 promoter that correlate with infectious and autoimmune diseases susceptibility and resistance (39). The polymorphism is associated with a simple tandem, microsatellite-like repeat and is suggested to function as an enhancer element (39). As we show sequence variation within this region in mouse in G169 and D169 allelic mouse strains, we evaluated if promoter activity is influenced by this sequence variation. Surprisingly, we found that the promoter within a G169 allele strain, CBA, displayed less functional activity (2- to 3-fold, p < 0.000075) than that found in a D169 strain, C57Bl/6. However, both promoters were still susceptible to an equivalent (2-fold) inhibition by c-Myc co-transfection (not shown). Activities of both promoters were not significantly different with 2 µg co-transfected c-Myc.

**Miz-1 Relieves the Inhibition of c-Myc and Transactivates the Nramp1 Promoter**—One or more Inrs are located within the smallest construct tested that reveals c-Myc inhibition. Recent studies have shown that Miz-1 can bind to Inrs and function as a positive regulator of gene expression (34). c-Myc antagonizes the effects of Miz-1. Using a co-transfection approach in COS-1 cells (Fig. 7a) 1 µg of c-Myc produced a 50% reduction in activity (**, p = 0.0015), as before (Fig. 5b). Co-transfection of 1 µg of Miz-1 suppressed the repression of c-Myc on Nramp1 promoter activity for construct pH4, such that the promoter activity was not significantly different from the control (promoter alone) but significantly greater than c-Myc treated (**, p = 0.036). Surprisingly, Miz-1 could not significantly enhance the activity of Nramp1 (pHB4) on c-Myc untreated cells in this experiment. We performed a dose-response analysis of Miz-1 with promoter constructs pH4/20/22 (Fig. 7b). As before in untreated cultures (Fig. 5a) pH20 was more active than pH4. Miz-1 co-transfection caused a 5- and 6-fold induction of pH4 and pH20, respectively, that achieved significance for pH4 only at the highest doses of Miz-1, and for all except the lowest dose of Miz-1 for pH20 over untreated cultures. Therefore, pH20 appears to be more sensitive to transactivation to low doses of Miz-1. In contrast no increase in pH22 was observed.

**DISCUSSION**

In this report we present functional data on the murine Nramp1 promoter and show inhibition of Nramp1 expression during cell growth. We show that ectopic Nramp1 expression can reduce cell proliferation. Together these data are supportive for a link between Nramp1-dependent iron homeostasis and transcription factors associated with cell growth. A recent paper by Wu and colleagues (30) showed c-Myc regulates the expression of genes that modulate the extent of iron within the labile iron pool (30), specifically that c-Myc enhances the LIP for cell proliferation. These data led us to investigate the murine Nramp1 promoter for regulation by c-Myc. In the extended murine Nramp1 promoter sequence presented, we identified six c-Myc-Max binding, E-box sites (35). However, although we observed DNA protein complexes binding to site 5 (data not shown), these do not appear important for the inhibitory effects.
of c-Myc on Nramp1 expression, and a c-Myc site 5 mutant did not show any altered transcriptional responses (data not shown). However, deletion of c-Myc-Max site 6, and its flanking region, does correlate with an increase in promoter activity and increased responsiveness to Miz-1 transactivation, particularly at low Miz-1 doses. Peukert and colleagues (34) showed induction of the AdML and cyclin D promoters by Miz-1, and the cyclin D construct containing a number of E box sites was induced to a lesser extent, suggesting that the presence of E box sites within the promoter can interfere with the ability of Miz-1 to transactivate. This hypothesis is consistent with the increase in Nramp1 promoter activity we observed on deleting from −868 to −71 bp, including E box site #6, but we cannot rule out roles for other factor binding sites. However, the smallest construct we tested was still sensitive to c-Myc inhibition, indicating that E box site #6 is not of primary importance for c-Myc repression. This deletion construct contained two Inrs, a target for c-Myc repression. The Inr has been described as a core promoter element that can replace the TATA box in TATA-less promoters (43). Gene repression for generating the transformed phenotype has been shown to be of equal importance as gene-inductive events (44), and some, but not all, c-Myc gene repression is mediated through the Inr.

We have not undertaken a more detailed analysis of the −868 and −71 bp region and the associated negative regulatory element, but it does harbor the polymorphic microsatellite element described here, and by others in the human NRAMP1 promoter (39). In studies on the human promoter, the polymorphic repeat was suggested to function as an enhancer element and alleles driving high expression protect against infectious but, conversely, promote susceptibility to autoimmune diseases such as rheumatoid arthritis (39, 40). Recent studies have also indicated that promoter polymorphism contributes to susceptibility to Crohn’s disease, including the identification of a novel allele (41). Although the results of this study do not contradict the work in human, we favor the model of a negative-acting element being located within the region of the promoter where the GT element is located. This is based on the enhancement of promoter activity we see when this region is deleted. We propose that the repressor, between −868 and −71 bp in the C57Bl/6 strain is attenuated. The proposed attenuation contributes to stronger promoter activity. We have no evidence for or against the repressor being attributed directly to the microsatellite itself, but suggest that the altered spatial (2.04 nm) and angular separation (216°) of factor binding sites on either side of the repeat element may be sufficient to change potential protein-protein interactions and consequently the activity of the putative repressor. Our current experiments are directed at identifying the nature of the potential repressor element by further deletion mutagenesis. Both promoter alleles are subject to the same level of repression by c-Myc, consistent with c-Myc operating downstream of the polymorphic site at the initiator, and promoter activities of the two allelic variants of c-Myc-repressed Nramp1 are not significantly different.

In human, NRAMP1 alleles with low promoter activity associate with protection against autoimmune disease, including rheumatoid arthritis. However, a major driving force for keeping the high activity promoter in the gene pool is to provide protection against infectious disease (16). The major genetic determinant that contributes to infectious disease susceptibility in mouse is the G169D polymorphism (6), and this polymorphism has not been described in human. We do not propose that the mouse promoter polymorphism identified will dominate the G169D mutation, because the D169 allele is null. However, potential promoter sequence variation within G169 strains could lead to differential iron homeostasis and contrib-
itself has a major effect on the distribution of divalent cations within a macrophage and support our and others collective hypothesis of an iron secretory role for Nramp1 (26, 27). An iron secretory role for Nramp1 could rationalize data on Nramp1 function, both influx into the vesicular lumen and deprivation from pathogens of essential iron for intracellular growth.

Our model (Fig. 8) is supportive of an interaction between c-Myc (cellular growth) and Nramp1 and iron. Expression of Nramp1 from a heterologous expression plasmid disrupts this normal regulatory control allowing effects of ectopic Nramp1 expression on growth to be established. We do not believe this arises from Nramp1 overexpression, because levels of protein in the cell lines and primary macrophages are comparable and dilute in parallel (not shown). These current data and those of deprivation from pathogens of essential iron for intracellular growth within a macrophage and support our and others collective expression on growth to be established. We do not believe this normal regulatory control allowing effects of ectopic vitamin D, c-Myc suppression, and TGF-β signaling pathways and co-activator proteins have been identified (45, 46). This is of interest given that recent studies by Roig and colleagues (47) showed vitamin D is a potent agonist for human NrAMP1 induction and the well established links between vitamin D, c-Myc suppression, and TGF-β (48, 49). In current experiments we are actively pursuing the role of Miz-1 function for NrAMP1 regulation based on the model of p15ink4b (45, 46).

Previous studies in vivo have shown restricted expression of Nramp1, to specific macrophage populations undergoing erythropagocytosis, and macrophages within an experimental brain lesion (26), but so far no mechanistic basis for this differential control within macrophage sub-populations has been put forward. There are data suggesting Miz-1 translocation to the nucleus involves microtubule depolymerization (50), and oxidant stress has been reported to promote microtubule disorganization (51). A mechanism of microtubule disruption, leading to Miz-1 nuclear translocation, could lead to Nramp1 induction. That Nramp1 may be induced by microtubule dynamics could have a physiological basis, because a role for microtubules in phagosome movement has also been described (51), and some data suggest an interaction between Nramp1 and microtubules (36). Thus, the ingestion of particulates by macrophages could be one signal that leads to the induction of Nramp1 in specific macrophage populations. Future studies will be aimed at examining the effect Miz-1 on Nramp1 regulation by a range of stimuli.

Acknowledgment—We are particularly grateful to Frank Hanel for providing the Miz-1 expression plasmid.

REFERENCES

1. Plant, J., and Glynn, A. A. (1974) Nature 248, 345–347
2. Bradley, D. J. (1974) Nature 250, 353–354
3. Gros, P., Skamene, E., and Forget, A. I. (1981) J. Immunol. 127, 2417–2421
4. Vidal, S., Malo, D., Vogan, K., Skamene, E., and Gros, P. (1993) Cell 73, 469–485
5. Barton, C. H., White, J. K., Roach, T. I. A., and Blackwell, J. M. (1994) J. Exp. Med. 179, 1683–1687
6. Malo, D., Vogan, K., Vidal, S., Hu, J., Cellier, M., Schurr, E., Fuku, A., Bumstead, N., Morgan, K., and Gros, P. (1994) Genomics 23, 51–61
7. Vidal, S., Tremblay, M. L., Cellier, M., Gauthier, K., Skamene, G., Malo, D., Skamene, E., Olivier, M., Joshy, S., and Gros, P. (1995) J. Exp. Med. 182, 655–666
8. Govoni, G., Vidal, S., Gauthier, S., Skamene, E., Malo, D., and Gros, P. (1994) Infect. Immun. 62, 2920–2929
9. Atkinson, P. G., Blackwell, J. M., and Barton, C. H. (1997) Biochem. J. 325, 779–786
10. Gougenheid, S., Finner, E., Desjardins, M., and Gros, P. (1997) J. Exp. Med. 185, 717–730
11. Searle, S., Bright, N. A., Roach, T. I. A., Atkinson, P. G., Barton, C. H., Meleno, R. H., and Blackwell, J. M. (1998) J. Cell. Sci. 111, 2853–2866
12. Gougenheid, S., Cellier, M., Vidal, S., and Gros, P. (1996) Genomics 25, 514–525
13. Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Bron, W. F., Nussberger, S., Gollan, J. L., and Hediger, M. A. (1997) Nature 388, 422–428
14. Fleming, M. D., Tenero, C. C., Su, M. A., Foerman, D., Beier, D. R., Dietrich, W. F., and Andrews, N. C. (1997) Nat. Genet. 16, 383–386
15. Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W., and Gros, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10989–10993
16. Blackwell, J. M., Searle, S., Goswami, T., and Miller, E. N. (2000) Microbes. Infect. 2, 317–321
17. Barrera, L. F., Kramnik, I., Skamene, E., and Radzioch, D. (1994) Immunology 82, 457–464
18. Barton, C. H., Whitehead, S. H., and Blackwell, J. M. (1995) Mol. Med. 1, 267–279
19. Forunicola, S., Roach, T. I., and Blackwell, J. M. (1994) Immunology 82, 45–50
20. Ables, G. P., Takamatu, D., Noma, H., El-Shalay, S., Jin, J. K., Taniguchi, T., Sekikawa, K., and Watanabe, T. (2001) J. Interferon Cytokine Res. 21, 53–62
21. Dinckka, M., and Weiss, G. (1999) J. Immunol. 162, 6171–6177
22. Kuhn, D. E., Baker, B. D., Lafaure, W. P., and Zwillings, B. S. (1999) J. Leukocyte Biol. 66, 113–119
23. Govoni, G., Skamene, E., Babalj, A., Patic, S., Opolka, G., and Ganten, D. (1998) J. Exp. Med. 182, 483–493
24. Malo, D., Vogan, K., Vidal, S., Hu, J., Cellier, M., Schurr, E., Fuks, A., and Blackwell, J. M. (1996) FEBS Lett. 383, 189–194
25. Kojima, Y., Kinouchi, Y., Takahashi, S., Negoro, K., Hiwatashi, N., and Shimosegawa, T. (2001) Am. J. Physiol. Gastrointest. Liver Physiol. 281, G833–G847
26. Atkinson, P. G., Blackwell, J. M., and Brock, J. (2002) Biochem. J. 363, 89–94
27. Baker, S. T., Barton, C. H., and Biggs, T. E. (2000) J. Leukocyte Biol. 67, 561–567