microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family

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The microphthalmia (mi) gene appears essential for pigment cell development and/or survival, based on its mutation in mi mice. It has also been linked compellingly to the human disorder Waardenburg Syndrome. The mi gene was recently cloned and predicts a basic/helix-loop-helix/leucine zipper (b–HLH–ZIP) factor with tissue-restricted expression. Here, we show that Mi protein binds DNA as a homo- or heterodimer with TFEB, TFE3, or TFEC, together constituting a new MiT family. Mi can also activate transcription through recognition of the M box, a highly conserved pigmentation gene promoter element, and may thereby determine tissue-specific expression of pigmentation enzymes. Six mi mutations shown recently to cluster in the b–HLH–ZIP region produce surprising and instructive effects on DNA recognition and oligomerization. An alternatively spliced exon located outside of the b–HLH–ZIP region is shown to significantly modulate DNA recognition by the basic domain. These findings suggest that Mi’s critical roles in melanocyte survival and pigmentation are mediated by MiT family interactions and transcriptional activities.

[Key Words: microphthalmia; dimerization; DNA binding; M box; b–HLH–ZIP]

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A striking inheritable disorder of development in the mouse is microphthalmia (mi), a syndrome first recognized >50 years ago as a coat color mutation (Hertwig 1942). The human mi gene has also been linked compellingly to the human pigment cell disorder Waardenburg Syndrome (Hughes et al. 1994). Mutations at the mouse mi locus result in pigment cell defects in the skin (producing white spotting), eyes (producing small eyes), and inner ears (resulting in deafness). Mast cell defects have also been recognized for certain mi alleles, a pattern resembling the melanocyte/mast cell pattern of Sl/kit-defective mice and suggesting a connection between these factors in signaling (Dubreuil et al. 1991; Ebi et al. 1992). Bone resorption and other neural crest or neuroepithelial defects have also been observed for certain mi alleles (for review, see Green 1989), suggesting that Mi protein may function in part through oligomeric interactions with other factors.

The devastating consequences of mi mutations on melanocyte development suggest that Mi is a key regulator of melanocyte growth or survival. The mi gene was cloned recently and shown to predict a basic/helix-loop–helix/leucine zipper (b–HLH–ZIP) factor [Hodgkinson et al. 1993; Hughes et al. 1993; Tachibana et al. 1994]. Mammalian b–HLH–ZIP factors and the related b–HLH group contain several important regulators of cell proliferation and development such as Myc/Max [Blackwood and Eisenman 1991; Prendergast et al. 1991] and MyoD-related factors (for review, see Olson 1990, and references therein; Weintraub 1994). Given the effects of mi mutations on melanocyte biology, mi may regulate comparable pathways in melanocytes. Although a significant number of b–HLH–ZIP factors are known, biological activities are clear for only a few, primarily Myc and its related partners (see Prendergast and Ziff 1992; Ayer and Eisenman 1993; Zervos 1993). Only very few candidate target genes have been identified so far that are regulated by these factors. The striking biological consequences of mi mutations suggest a major role in melanocyte development and even point to specific candidate target genes.

Melanocytes represent a neural crest-derived lineage whose pigmentation function is easily assessed because...
melanocytes are not critical to survival of the whole animal. A great deal has been learned about pigmentation enzymes and their regulation through study, for example, of albino (tyrosinase) mutants (for review, see Halaban and Moellmann 1993). Determination of regulatory elements critical for pigmentation have revealed an 11-bp sequence known as the M box containing the core motif CATGTG which is highly conserved in the promoters of the three major pigmentation enzyme genes tyrosinase, and tyrosinase-related proteins 1 and 2 (Shibahara et al. 1991; Lowings et al. 1992; Yavuzer and Goding 1994). The presence of tissue-specific transcription factors capable of interacting with and transcriptionally activating elements such as this may shed light on the regulation of pigmentation and perhaps other melanocyte-specific functions.

The opportunity to examine structure/function relationships for transcription factor mutations coordinately in vitro and in mice has rarely been possible. The multitude of mi mouse mutants provides a unique opportunity to examine biochemical consequences of biologically important b-HLH-ZIP mutations. The b-HLH-ZIP family contains a short ~20-amino-acid basic domain rich in basic amino acids that makes sequence-specific DNA contacts. Carboxy-terminal to it is the HLH-ZIP containing two amphipathic helices separated by a flexible loop and a carboxy-terminal leucine zipper. The HLH-ZIP mediates dimeric interactions necessary for DNA binding (Ferre-D'Amare et al. 1993). Restricted heterodimerization plays a major role in regulating developmental programs ranging from inhibition of myogenesis by the HLH protein Id (Benezra et al. 1990) to cooperation in cellular transformation by Myc/Max (Blackwood and Eisenman 1990; Prendergast et al. 1991; Kato et al. 1992; Amati et al. 1993). The ability to group these factors into families, based on dimerization specificities, provides a useful handle for analysis of their biological roles.

Most b-HLH-ZIP proteins recognize the hexamer core sequence CACGTG or the related sequence CATGTG, whereas AP-4 (Hu et al. 1990) and most b-HLH proteins recognize CAGNTG hexamers. A hexamer containing the CACGTG sequence is present in the mouse immunoglobulin heavy chain enhancer and was used to isolate and characterize the transcription factor TFE3 (Beckmann et al. 1990; Roman et al. 1992). Although most b-HLH-ZIP factors interact avidly with cognate targets, it has been difficult to elucidate tissue-specific activities, in part because most of these factors are expressed ubiquitously. In this regard, mi, which is tissue restricted, is an attractive candidate as an M-box activator and regulator of pigmentation gene expression.

The studies described here identify Mi's DNA-binding activity and its ability to form stable DNA-binding heterodimers with TFEB, TFE3, and TFEC, three other b-HLH-ZIP factors. Collectively, these four proteins comprise a distinct family that likely modulates the biological activity of Mi through hetero-oligomer formation. The biological importance of Mi's protein–DNA and protein–DNA interactions have been revealed through a characterization of the proteins encoded by seven mutant mi mouse alleles (Steingrimsson et al. 1994). These mutations cluster within or near the b-HLH-ZIP motif of mi and display striking effects on heterodimerization and DNA binding that largely explain the unique severity and inheritance patterns of the different mi mouse strains. Novel structural features of b-HLH-ZIP biochemistry have also been revealed, such as the surprising ability of an alternative exon outside of the basic domain to modulate b-HLH-ZIP-dependent DNA recognition. Finally, Mi was shown to transcriptionally activate a reporter driven by the pigmentation promoter M-box element, suggesting that this family of factors plays a central role in the tissue-specific development of melanocytes.

Results

DNA recognition by Mi

The Mi protein produces a gel shift complex (Fig. 1) with DNA containing the CACGTG hexanucleotide derived from adenovirus major late promoter (MLP). Several deletions were made to determine the protein domains required for DNA binding (Fig. 1A). It was possible to truncate from the amino terminus to the beginning of the basic domain and from the carboxyl terminus to the end of the leucine zipper domain without loss of DNA binding (Fig. 1B, lanes 2–4). Further deletion from the carboxyl terminus removed part of the leucine zipper and abolished DNA binding (Fig. 1B, lane 5). Therefore, the leucine zipper was essential for stable complex formation.

Sequence specificity of DNA recognition was verified by competition analysis using both CACGTG (Fig. 1C) and CATGTG (Fig. 1D) probes. In each case, a double point mutant (GAGGTG) failed to compete the specific complex at concentrations effectively competed by unlabeled CACGTG competitor.

Mi is a member of a discrete family of b–HLH–ZIP factors

Stoichiometry of protein to DNA in the bound complexes was examined by mixing full-length Mi protein with the isolated b–HLH–ZIP region (Fig. 2, lanes 2 and 3, respectively). A single new intermediate mobility gel shift complex was observed (Fig. 2, lane 4). Overexposure failed to reveal additional intermediate complexes, suggesting that the protein–DNA stoichiometry is 2:1. Experiments were also undertaken to determine whether Mi is capable of forming DNA-binding heterodimers with several other b–HLH–ZIP proteins. Only three proteins, TFEB, TFE3, and TFEC, were found to form intermediate mobility complexes with Mi (Fig. 2, lanes 5–13). In these mixing experiments TFE3 (but not Mi) preferentially heterodimerizes, probably reflecting different kinetics from Mi. In contrast, no heterodimers
were observed upon mixing Mi with E47S (Fig. 2, lanes 14–16), Max, Myc, upstream stimulatory factor (USF), or several non-HLH-containing transcription factors (data not shown). Therefore, of the known and tested candidate partners, Mi appears to be capable of forming stable DNA-binding heterodimers with only TFEB, TFEC, and TFE3. With the additional observation that TFEB and TFEC form stable heterodimers (Fig. 2), all combinations of these four proteins have now been shown to heterodimerize with one another but not with any other known b–HLH–ZIP proteins (Fig. 2; Fisher et al. 1991; Zhao et al. 1993), indicating that they constitute a discrete group of interactive proteins, which we refer to as the MiT family.

Mutant alleles affect MiT interactions

Recent molecular genetic studies of Steingrimsson et al. (1994) suggest that dominant-negative Mi mutations are dominantly inherited while regulatory mutations or mutations that prevent or reduce Mi protein dimerization are recessively inherited. To examine this possibility, mutant proteins corresponding to the seven mi mutations characterized by Steingrimsson et al. (1994) were produced and tested directly for their ability to bind DNA as homodimers or as heterodimers with TFE3. Identical results were obtained when heterodimerization was tested with wild-type Mi, TFEB, or TFEC (data not shown). The seven mutations and their properties are summarized in Table 1.

When tested for homodimeric DNA binding, all three semidominant and two recessive mutant proteins failed to bind DNA (Fig. 3A, lanes 3–8). Only the helix 1 mutant D222N (mi<sup>+</sup>) mutant protein, which is inherited recessively, appeared to bind DNA normally. Quantitative affinity measurements revealed mi<sup>+</sup> to bind with a $K_d$ only 6% greater than that of wild-type Mi (using forms containing the 6-residue alternative insert), a difference within the 10% standard error of our measurements (data not shown). When mixed with TFE3, mi<sup>+</sup> was the only mutant protein able to produce a heterodimeric complex with TFE3 (Fig. 3A, lanes 10–16).

Examination of heterodimer mixing experiments using the mutant Mi proteins (Fig. 3A) revealed a striking loss of TFE3 homodimer activity in many reactions. Addition of mi, Mi<sup>+</sup>, Mi<sup>−</sup>, or mi<sup>−</sup> proteins essentially ablated TFE3 homodimeric DNA-binding activity (Fig. 3A, lanes 12,13,15,16). In contrast, the recessive allele
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mi<sup>ce</sup> contains a stop codon that removes the leucine zipper and did not affect TFE3-binding activity (Fig. 3A, lane 11). All three semidominant alleles contain basic domain mutations, failed to bind as homodimers, and additionally suppressed DNA binding by TFE3 in an apparently dominant-negative fashion.

Surprisingly, of the three recessive mutant proteins, mi<sup>wt</sup> bound DNA indistinguishably from wild-type protein despite its helix 1 mutation (and the striking phenotype of mi<sup>wt</sup> mice) (Fig. 3A, lanes 6,14), suggesting that this mutation might disrupt a function other than DNA binding. The recessive allele, mi<sup>ei</sup>, contains a stop codon at the beginning of the leucine zipper, failed to bind DNA, and was also incapable of suppressing the DNA-binding activity of TFE3 (Fig. 3A, lanes 3,11) behaving "recessively" in vitro. A third recessive mutation, mi<sup>ew</sup>, contains a 25-amino-acid deletion that removes the amino-terminal half of the basic region but does not involve the HLH-ZIP. This mutant failed to bind DNA as a homodimer and also suppressed the DNA-binding activity of TFE3 (Fig. 3A, lanes 8,16). As a basic domain deletion, this in vitro behavior was expected for mi<sup>ei</sup>. Its recessive inheritance is surprising, however. Importantly, this discrepancy between the biochemical behavior of the mi<sup>ei</sup> protein and the genetic behavior of the mi<sup>ew</sup> allele suggests that these deleted 25 amino acids carry out a second function (aside from DNA binding).

To verify that the TFE3 suppression seen by proteins encoded by the semidominant alleles occurred through protein–protein interactions, coimmunoprecipitations were performed using <sup>35</sup>S-labeled mutant Mi proteins, unlabeled recombinant TFE3, and a TFE3-specific antibody. Antibody specificity was verified by supershift of a TFE3/DNA complex but failure to supershift Mi or other b-HLH-ZIP proteins (data not shown). Specificity was indicated further by the dependence for TFE3 in the coimmunoprecipitations (Fig. 3B, lanes 1,2) as well as the dependence of antibody (data not shown). The labeled Mi protein migrates as a doublet of ~15 Kd. TFE3-specific antibodies coimmunoprecipitated wild-type Mi, the three semidominant proteins mi<sup>ei</sup>, Mi<sup>ew</sup>, and Mi<sup>wt</sup>, as well as the recessive protein mi<sup>ew</sup> (Fig. 3B, lanes 2–6), consistent with a dominant-negative inhibition of DNA binding by the products of the semidominant alleles. A similar coimmunoprecipitation pattern was also observed for mi<sup>ew</sup> (data not shown). The zipperless recessive protein mice did not efficiently coprecipitate (Fig. 3B, lane 7), although a weak signal was observed, possibly reflecting a propensity to form HLH-mediated tetramers in the absence of DNA (Fisher et al. 1991; Anthony-Cahill et al. 1992; Farmer et al. 1992; Fairman et al. 1993).

**Alternative splice affects basic domain function**

The mi message exists in splice forms either encoding or lacking 6 amino acids just amino-terminal to the basic domain (Hodgkinson et al. 1993). The mi<sup>sp</sup> mutation affects the polypyrimidine tract of the splice acceptor and precludes formation of Mi protein containing the 6-amino-acid insert (Steingrimsson et al. 1994). These mice produce normal pigment but exhibit a measurable decrease in the pigmentation enzyme tyrosinase within skin (Wolfe and Coleman 1964). Despite the subtlety of its homozygous phenotype, the mi<sup>sp</sup> allele enhances the effective phenotype of semidominant mi alleles in a compound heterozygote (Wolfe and Coleman 1964). To examine biochemical relevance of this alternative splice, wild-type Mi proteins with and without the 6-amino-acid insert were examined (Fig. 4A, lanes 2,3). Although the two proteins bound DNA similarly, quantitative measurements revealed that the splice form containing the insert bound with 20% higher affinity than the form lacking the insert (K<sub>d</sub> = 290 and 349 μM, respectively, in presence of poly[dI-C]]. No large effect was observed for the alternative 6-amino-acid insert on heterodimeric binding of wild-type Mi with TFE3 (Fig. 4A, lanes 4–6).

Surprisingly, however, the presence of the 6-amino-acid insert had a profound effect on DNA binding of the basic domain mutant I212N [Mi<sup>wt</sup>], the allele that displays interallelic complementation. As shown in Figure 4B, presence of the insert restored heterodimeric DNA binding by Mi<sup>wt</sup> with a wild-type partner (Fig. 4B, lanes 1–4,9,11). In contrast, presence of the upstream insert did not restore heterodimeric DNA binding for a different basic region mutant [mi], indicating the specificity of this effect for Mi<sup>wt</sup> (Fig. 4B, lanes 5–7,10). Thus, presence of the upstream insert restored DNA binding to the Mi<sup>wt</sup> protein if the heterodimer partner was wild type.
suggesting that this 6-amino-acid insert acts to stabilize the basic domain/DNA complex. Interestingly, the I212N mutation in the Mi\textsuperscript{vh} protein is the only basic region mutant predicted to face away from DNA in the basic domain α-helix, on the solvent-exposed face (Ferré-D’Amaré et al. 1993, Fisher et al. 1993, Steingrimsson et al. 1994). The restoration of DNA binding for Mi\textsuperscript{vh} may account for the interallelic complementation characteristic of this allele.

**Mi overexpression transcriptionally activates an M box-driven reporter in fibroblasts**

We have tested the ability of mi to activate transcription of a reporter driven by the M-box pigmentation gene promoter element (Shibahara et al. 1991; Lowings et al. 1992, Yavuzer and Coding 1994) because of our demonstration that Mi is capable of binding its CATGTG core sequence in vitro (Fig. 1D). Cotransfection of mi and the M-box reporter into NIH-3T3 cells resulted in Mi-dependent activation of the luciferase gene to levels >13-fold above controls (Fig. 5). Stimulation of the luciferase activity was dependent on both the presence of the M-box element in the reporter construct and on the cotransfection of mi. Although identical to the immunoglobulin enhancer element μE3 element at its core [CATGTG], the M box differs in flanking positions, which are conserved from mouse to human in the three pigmentation enzyme genes tyrosinase, and tyrosinase-related proteins 1 and 2. Recognition of M-box elements by Mi may constitute a critical component in the elaboration of melanocyte-specific gene expression.

**Discussion**

The experiments presented here demonstrate that the Mi protein is a transcription factor that forms homo- and heterodimeric DNA-binding complexes within a small family of proteins and whose complexity of allelic interactions may be largely explained by these features. Biochemical analysis of Mi demonstrated its capacity to specifically recognize the DNA core sequences CACCTC and CATGTG (Fig. 1). This DNA binding appeared to be dimeric based on mixing experiments that result in the formation of a single intermediate mobility complex (Fig. 2). Although this observation does not formally prove 2:1 stoichiometry of protein to DNA, the DNA cocrystallographic analyses of Max and USF showed dimeric protein interaction with the cognate DNA template (Ferré-D’Amaré et al. 1993, 1994). Additionally, the importance of Mi’s leucine zipper was demonstrated by the loss of DNA binding upon its deletion. A substantial body of data indicate that the leucine zipper is necessary for dimerization and DNA binding by b-HLH-ZIP proteins (Dang et al. 1989; Gregor et al. 1990; Beckmann and Kadesch 1991; Blackwood and Eisenman 1991; Fisher et al. 1991; Prendergast et al. 1991, Blanar and Rutter 1992; Roman et al. 1992).

**Mi belongs to a discrete MiT family**

Based on the phenotypic complexity of heterozygous combinations of mi alleles (for review, see Green 1989), it is likely that mi function depends on heterodimer formation during development. Heterodimeric DNA binding was seen for Mi protein in combination with TFE3, TFE3, or TFEC (Fig. 2). With the observation that TFEB and TFEC were also capable of heterodimerization and DNA binding, all dimeric combinations of these factors have now been demonstrated (Fig. 2; Fisher et al. 1991; Zhao et al. 1993). Heteromeric DNA-binding interactions are otherwise quite restricted for these proteins, as none of them have been shown to heterodimerize with other HLH or HLH-ZIP factors. Whereas TFEB and TFE3 are ubiquitous factors (Beckmann et al. 1990, Carr and Sharp 1990) and TFEC is tissue restricted (Zhao et al. 1992).
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important in the human pigmentation disorder Waardenburg Syndrome, which is dominantly inherited and was recently linked to the human mi locus (Hughes et al. 1994). The behavior of these mutants, particularly those with unanticipated protein function, may aid in the

Figure 3. DNA-binding properties of mi mutants. [A] Wild-type Mi (Mi-WT) and six different mutant Mi proteins were synthesized in vitro as amino-terminal deletions beginning with amino acid 109 (to visualize intermediate mobility complexes). Proteins were tested in DNA-binding assays either alone [lanes 2–8] or in post-translational mixes with TFE3 [lanes 10–16]. The semidominant alleles mi, Mi°', Mi^w, and the recessive alleles mi°', mi''', and mi^w were tested. The positions of two background reticulocyte bands are indicated (*), the lower one being remote from the strong signals and demonstrating evenness of sample loading. [B] Immunoprecipitation of wild-type [WT] and mutant Mi proteins with unlabeled recombinant TFE3, using a TFE3 specific antibody. Specificity is seen in lane 1, where lack of TFE3 results in no coprecipitation. Wild-type Mi, the three dominant-negative proteins (mi, Mi°', and Mi^w), and mi^w coprecipitate efficiently with TFE3 [lanes 2–6]; mi°', a zipperless protein, is very weakly coprecipitated, perhaps through a propensity to form HLH-dependent tetramers [lane 7].

1993], it will be important to determine the developmental expression of these factors within cell lineages affected by mi mutations. Thus, these four proteins represent a distinct MiT family that likely participates in pivotal developmental pathways, although other family members might exist as well.

Biochemical lesions and biological consequences

We show here that dominant-negative protein behavior appears to explain semidominant inheritance of mi alleles. This is relevant for mouse mi and is likely to be

Figure 4. Alternative splice restores heteromeric DNA binding by Mi°'. [A] DNA binding by two splice forms of Mi. Wild-type Mi protein [AΔ109;CA308] either lacking [WT −] or containing [WT +] the 6-amino-acid alternative exon was tested for DNA binding using the CACGTG probe, either alone or in the presence of TFE3. No obvious differences in DNA binding or heterodimerization were apparent. Several background bands (*) represent reticulocyte proteins capable of DNA binding. [B] Six-amino-acid insert restores heteromeric DNA binding by Mi°'. The basic domain mutant Mi°' [AΔ109;CA308] was synthesized either with [Wh +] or without [Wh −] the 6-amino-acid insert and tested for DNA binding (CACGTG) in the presence of TFE3 [lanes 1–8] or alone [lane 9]. A truncated form of Mi°' contains only the b–HLH–ZIP [Wh-b]. Another basic domain mutant, mi, was also synthesized from amino acid 109 [AΔ109;CA308] in the presence [mi +] or absence [see Fig. 3] of the 6-amino-acid insert and tested for DNA binding with TFE3 [lane 6] or alone [lane 10]. Presence of the 6 amino acids restored heterodimeric DNA binding to the Mi°' mutant (→) without affecting the mi protein. Several background reticulocyte lysate bands are observed [see lane 12, unprogrammed lysate].
Mi proteins producing recessive inheritance are also instructive regarding b-HLH-ZIP function and highlight functionally relevant regions unlikely to produce the dominant inheritance of Waardenburg Syndrome. Importantly, two (mi\textsuperscript{vte} and mi\textsuperscript{wsw}) of the three display biochemical behavior that is not expected. The third, mi\textsuperscript{wsw}, introduces a stop codon at the carboxyl terminus of the HLH domain (Steingrimsson et al. 1994), thereby truncating the leucine zipper. The transcription factor USF, however, appears to be capable of binding DNA without its leucine zipper (Gregor et al. 1990; Ferré-D’Amaré et al. 1994). By failing to dimerize, the mi\textsuperscript{wsw} protein should exert no dominant-negative effect at the level of DNA binding, as was observed in mixing experiments (Fig. 3). The weak coimmunoprecipitation of mi\textsuperscript{wsw} by TFEB (Fig. 4) suggests that the HLH domain alone can measurably oligomerize, perhaps as a tetramer, in the absence of DNA (Anthony-Cahill et al. 1992; Farmer et al. 1992; Fairman et al. 1993; Fisher et al. 1993).

The mi\textsuperscript{wsw} allele predicts a 25-amino-acid deletion (Steingrimsson et al. 1994) that begins amino-terminal to (and deletes much of) the basic domain. This protein failed to bind DNA as either a homodimer or heterodimer. Like the semidominant alleles, it repressed DNA binding by wild-type protein because the HLH–ZIP domains were intact. Interestingly, the mi\textsuperscript{wsw} allele is inherited recessively suggesting that dominant-negative function is not fully realized in vivo. Potential explanations include the loss of a nuclear localization signal or decrease in protein stability.

The D222N mutations [mi\textsuperscript{wsw}] produces a helix 1 mutation (Steingrimsson et al. 1994) with virtually no measurable effect on DNA binding (Fig. 3) but produces progressive, aging-dependent melanocyte death (Lerner 1986; Lerner et al. 1986). It is possible that the small [6%] difference in \(K_{d}\) produced by this mutation is sufficient to produce the aging-dependent vitiligo in these mice. Alternatively, this helix 1 mutation may affect tetramerization, a property of many HLH proteins. TFEB has been shown previously to exist in a tetrameric state in solution that dissociates into DNA-binding dimers upon addition of DNA (Fisher et al. 1991). Similar tetramers have been observed for several other HLH-containing proteins including Myc (Dang et al. 1989), MyoD (Anthony-Cahill et al. 1992), and myogenin (Farmer et al. 1992). The Id protein’s inhibition of MyoD DNA binding appears to be mediated by tetrameric complexes (Fairman et al. 1993) consistent with the observation that tetrameric forms cannot bind DNA (Fisher et al. 1991). The aspartate 222 mutated in mi\textsuperscript{wsw} (Steingrimsson et al. 1994) is located within the four-helix bundle predicted from the Max/DNA cocrystal structure (Ferré-D’Amaré et al. 1993) and could participate in interhelical salt bridges, although its disruption does not appreciably affect dimerization.

**Alternative splice modulates DNA binding**

Although b–HLH–ZIP DNA binding is generally thought to occur independently of major influences outside this
domain, we observed here a noteworthy effect on DNA binding by the presence or absence of the 6-amino-acid alternative insert (Hodgkinson et al. 1993) upstream of the basic region. Wild-type protein shows only a modestly (20%) enhanced DNA affinity in the presence of this insert, but a basic domain mutation (I212N, the M\(^{\text{Iw}}\) mutation, Steingrimsson et al. 1994) could be strikingly rescued for heterodimeric DNA binding by the insert (Fig. 4). This observation suggests that the b\(-\)HLH–ZIP, and more specifically the I212 site in the basic region, are subject to functionally important intramolecular interactions, an observation that may extend to other b\(-\)HLH[ZIP] factors. The location of the 6-amino-acid insert, amino-terminal to the basic domain, corresponds to the site of a 9-amino-acid alternatively spliced insert in Max (Blackwood and Eisenman 1991). Kinetic data suggest that Max has a slower off rate and altered affinity in the presence of its 9-amino-acid insert (Bousset et al. 1993; Kretzner et al. 1993). Virtually all b\(-\)HLH–ZIP proteins contain consensus casein kinase II sites at this same location (see Fisher et al. 1993, and references therein). Phosphorylation appears to alter Max DNA binding in the direction of lower affinity (Berberich and Cole 1992, Bousset et al. 1993), resulting in preferential heterodimeric DNA binding with Myc. The presence and configuration of negatively charged moieties near the basic domain may influence protein–DNA stability through repulsive forces with the DNA backbone. Similar influences of acidic residues upstream of the basic domain of E12 significantly suppress homodimeric DNA binding in this b\(-\)HLH factor (Sun and Baltimore 1991), suggesting that comparable mechanisms operate in other basic domain-containing transcription factors. The b\(-\)HLH–ZIP protein USF contains a direct repeat peptide sequence that resembles an immunoglobulin hinge motif [Gregor et al. 1990]. The presence of proline near the amino terminus of all b\(-\)HLH–ZIP basic domains suggests that the peptide backbone is kinked in such a fashion that the upstream amino acids may reach back in the vicinity of the basic domain. It is also interesting that the I212 mutation [Steingrimsson et al. 1994] occurs on the solvent exposed surface of the basic domain. Although this position is not likely to contact DNA [Fisher et al. 1993; Ferré-D’Amari 1993; Steingrimsson et al. 1994], it is strikingly conserved as a hydrophobic residue in all CAGCTG-binding b\(-\)HLH–ZIP proteins and is usually an arginine in CAGCTG binding ones (Dang et al. 1992). Because the b\(-\)HLH–ZIP basic domain is an intrinsically unstable \(\alpha\)-helix [Fisher et al. 1993; Ferré-D’Amari et al. 1994], interactions on this other face may affect DNA binding by influencing \(\alpha\)-helical folding. Although the mechanism by which the upstream region influences DNA binding remains unclear, it is likely to be functionally important because of its biological consequences in mice carrying the \(m^{Ipp}\) or \(M^{Iw}\) mutations. The mild “enhancing” phenotype of \(m^{Ipp}\) lacking the insert [Wolfe and Coleman 1964] and the interallelic complementation of \(M^{Iw}\) [Grüneberg 1952, Hollander 1968; Konyukhov and Osipov 1968, Steingrimsson et al. 1994] might both be explained \(m^{Ipp}\) more straightforwardly) by this unique biochemistry, representing novel mechanisms for influencing genetic behavior.

Mi activates the pigmentation gene M-box element

One example of the biological activity of Mi was demonstrated by its ability to trans-activate a reporter element driven by the M box [Fig. 5]. This element contains 11 bp that are perfectly conserved in the promoters of the three major pigmentation enzyme genes in both mouse and human and consists of 11 bp with a hexamer core of CATGTG [Shibahara et al. 1991, Lowings et al. 1992, Yavuzer and Goding 1994]. The immunoglobulin enhancer \(\mu\)E3 site contains the same core CATGTG and can be transcriptually activated by Mi (data not shown). It is attractive to speculate that through M-box recognition, Mi provides a melanocyte-specific signal that activates the pigmentation program, potentially qualifying it as a master gene for melanocyte development. Although the M box can be bound by different b\(-\)HLH–ZIP proteins such as USF [Yavuzer and Goding 1994], Mi’s transactivation motif[s] might provide melanocyte-specific signals. This idea is consistent with the observation that the M box is a melanocyte-specific enhancer element only when it is linked to the TATA box of a pigmentation gene promoter [Lowings et al. 1992]. Therefore, even if bound at an M-box site, different activator domains might not function like that of Mi. Importantly, whereas Mi is expressed in a few tissues other than pigment cells, the alternative splice form in melanocytes appears to be unique [Hodgkinson et al. 1993] and may represent a truly melanocyte-specific b\(-\)HLH–ZIP factor. It will be important to examine MiT family expression in cells affected by \(mi\) mutations. Two of Mi’s dimerization partners have been shown to encode transcriptional inhibitory activity. TFEC represses TFE3-dependent transcription [Zhao et al. 1993] and an alternative splice form of TFE3 has also been shown to repress the longer transcriptionally active form of TFE3 [Beckmann et al. 1990, Roman et al. 1991]. Thus, regulated MiT protein dimers might direct the tissue-specific expression of pigmentation program genes.

Mi also functions in melanocytes as a lineage-restricted survival factor. During melanocyte development, cells harboring \(mi\) mutations appear to die, rather than (e.g.) survive without producing pigment. The prospect that pigmentation enzymes and melanocyte survival genes are downstream effectors of Mi represents one of very few known transcription factor targets for the b\(-\)HLH–ZIP family. An understanding of the role of Mi in melanocyte development may provide insight into pathways of cellular proliferation and death in which other b\(-\)HLH–ZIP proteins, like Myc/Max, are known to play roles.

Materials and methods

DNA clones

The wild-type \(mi\) cDNA derived from melan-c cells was ex-
pressed in vitro from the clone pBS-Mi, which contains the cDNA inserted into the EcorI site of pBluescript SK+. This cDNA lacks the 6-amino-acid alternative exon. Mutants corresponding to the alleles mi [del 77S–77T], Mi\(^{77T}\), Mi\(^{77A}\), Mi\(^{77G}\), Mi\(^{77C}\), and the recessive alleles m\(^{i1}\) [C916T], and m\(^{i2}\) (G793A) [Steinbring et al. 1994] were generated by site-directed mutagenesis of pBS-Mi using the method of Eckstein according to the recommendations of the manufacturer (Amerham). Templates for m\(^{i1}\) and m\(^{i2}\), and constructs containing the 6-amino-acid alternative exon were expressed from PCR-derived fragments made from wild-type as well as mi and Mi\(^{77}\) mutant tissues. Expression vectors were verified by DNA sequencing. TFEB expression vectors were obtained by cloning an oligonucleotide containing four tandem repeats of the M box (AGTCATGTGCT) into the BamHI-KpnI site of pBluescript SK+. This allowed fragments made from wild-type as well as Mi\(^{77}\) mutants to be used. TFEB expression vectors were provided by Dr. T. Kadesch (Beckman et al. 1990). TFEB expression vectors were provided by Dr. B. de Crombrugghe (Zhao et al. 1993). E47S was expressed from the plasmid pE47S [Mürr et al. 1989]. His fusion Mi was expressed from a plasmid containing the BamHI-BamHI insert fragment from pBS-Mi inserted into the BamHI site of pET15b [Novagen]. For mammalian expression of Mi, the cDNA was cloned into the HindIII and XhoI sites of pRC-CMV (Invitrogen). The luciferase reporter plasmid was made by cloning an oligonucleotide containing four tandem repeats of the M box (AGTCATGTGCT) into the KpnI-XhoI sites of the luciferase reporter plasmid pGL2 promoter (Promega).

**Protein expression**

In vitro-translated proteins were made in rabbit reticuloocyte lysate (Promega) using RNA from in vitro transcription using T7 RNA polymerase according to the manufacturer’s recommendations (Pharmacia) for pBS-Mi and the corresponding mi, Mi\(^{77}\), Mi\(^{77A}\), Mi\(^{77G}\), and Mi\(^{77C}\) mutants as well as TF3. Full-length Mi proteins were obtained by linearizing with Smal, and carboxy-terminal deletions at amino acids 319 and 261 were obtained by linearizing with XmnI and Avall, respectively. TFEB and E47S were transcribed using T3 RNA polymerase [Fisher et al. 1991] (Pharmacia). Amino-terminal deletions and the DNA-binding domain of TFEC were made by amplifying discrete fragments using 5’ primers that begin at the described residue and append an initiation ATG, Kozak sequence, and T3 RNA polymerase promoter [derived from the plasmid pBS-ATG, Baldwin et al. 1990] followed by transcription and translation in vitro. In vitro-translated proteins were quantitated by TCA precipitation and SDS-PAGE and equivalent quantities were added to gel shift assays. Recombinant TFEB was synthesized as described [Fisher et al. 1993]. Recombinant His fusion Mi protein was synthesized in the bacterial strain BL-21, purified using nickel chelate chromatography (Qiagen), and eluted with 100 mM imidazole.

**Electrophoretic mobility shift assay, affinity measurements, and immunoprecipitation**

DNA-binding assays were performed as described [Fisher et al. 1993] in 20-μl reactions containing 5% glycerol, 100 mM KCl, 10 mM Tris (pH 7.4), 1 mM DTT, and ~5×10\(^5\) cpm of \(^{32}\)P-end-labeled probe DNA. In mixing experiments, separately translated proteins were incubated at 37°C for 30 min prior to the addition of probe DNA. CACGTG, CATGTG, and double point mutant probes were used as described [Fisher et al. 1991]. Polyacrylamide gels (6% Tris-glycine–EDTA) were run and subjected to autoradiography after drying. Competitors were prepared as described previously [Fisher et al. 1991]. Reactions probed with the CACGTG probe contained 1 μg of poly[d(C–T)] per 20-μl reaction, whereas those containing CATGTG probe contained 0.5 μg. \(K_d\) was determined by calculating half saturation from the initial [linear] slope of protein titrations under conditions of probe excess. Proteins were derived from in vitro translation reactions and were quantitated by determining probe saturation in gel shift using probe of known specific activity. Mi will aggregate with DNA in the absence of poly[d(C–T)], therefore this nonspecific competitor was added to all reactions [as above]. \(K_d\) measurements therefore reflect its presence. Equilibrium conditions were established by incubation at 30°C for 25 min. Quantitation was carried out using a PhosphorImager [Molecular Dynamics]. Immunoprecipitations were performed by mixing the various proteins under gel shift conditions [excluding poly [d(C–T)] and DNA probe] at 37°C for 1 hr, followed by addition of 3 μl of rabbit anti-TFEB antisera and freshly washed protein A-Sepharose (Pharmacia), incubation at 4°C for 2 hr, and three washes with PBS containing 0.1% NP-40 prior to elution in loading buffer and SDS-PAGE.

**Transient transfections and luciferase assay**

NIH-3T3 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 5% calf serum/5% fetal calf serum, 4 mM l-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO BRL). Cells were split 24–36 hr prior to transfection such that cells were ~60% confluent at the time of DNA addition, and were refed with fresh medium 8 hr prior to transfection. Transfections were carried out by calcium phosphate/DNA coprecipitation according to Kingston (1993) and harvested after 24 hr. Three 6-cm plates were each transfected with 0.25 μg of luciferase reporter plasmid, 1 μg of β-galactosidase control plasmid pRSV-β-Gal [Edlund et al. 1985], 4.7 μg of cytomegalovirus (CMV)-driven expression vector pRC-CMV [Invitrogen], and 4.05 μg of carrier DNA pBS-SK [Stratagene].

At harvest, plates were washed once with phosphate-buffered saline, lysed, and analyzed using a Monolight 2010 Luminometer according to the recommendations of the manufacturer [Analytical Luminescence Laboratory, San Diego, CA]. β-Galactosidase activity in cell lysates as a measure of relative transfection efficiency was used to adjust luciferase data and was assayed as described [Sambrook et al. 1989].

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