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Contrasting roles for Myc and Mad proteins in cellular growth and differentiation

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ABSTRACT **The positive effects of Myc on cellular growth and gene expression are antagonized by activities of another member of the Myc superfamily, Mad. Characterization of the mouse homolog of human *mad* on the structural level revealed that domains shown previously to be required in the human protein for anti-Myc repression, sequence-specific DNA-binding activity, and dimerization with its partner Max are highly conserved. Conservation is also evident on the biological level in that both human and mouse *mad* can antagonize the ability of c-*myc* to cooperate with *ras* in the malignant transformation of cultured cells. An analysis of c-*myc* and *mad* gene expression in the developing mouse showed contrasting patterns with respect to tissue distribution and developmental stage. Regional differences in expression were more striking on the cellular level, particularly in the mouse and human gastrointestinal system, wherein c-Myc protein was readily detected in immature proliferating cells at the base of the colonic crypts, while Mad protein distribution was restricted to the postmitotic differentiated cells in the apex of the crypts. An increasing gradient of Mad was also evident in the more differentiated subcorneal layers of the stratified squamous epithelium of the skin. Together, these observations support the view that both downregulation of Myc and accumulation of Mad may be necessary for progression of precursor cells to a growth-arrested, terminally differentiated state.**

Cessation of proliferation and onset of terminal differentiation represent pivotal events in the maturation program of all committed cell types. The genetic elements and molecular mechanisms governing these processes are poorly understood. Multiple studies have pointed toward the c-Myc oncoprotein as a potential regulator of these processes, since the loss of c-*myc* gene expression appears to be a required genetic event for progression of maturing cells through this critical developmental transition (for review see ref. 1). Additional support for the regulatory role of Myc derives from both its biochemical profile and its biological activities. In particular, Myc functions in part as a sequence-specific transcription factor and can stimulate transcription of reporter genes bearing a Myc consensus binding site (2, 3). Candidate genes that appear to be regulated by Myc are involved in DNA synthesis (4) and cell cycle regulation (5), further supporting the view that c-Myc promotes cellular growth. The forced expression of c-*myc* in cultured cells and transgenic mice can lead to deregulated growth and malignancy and can impede terminal differentiation in diverse cell types (for review see ref. 1).

Disruption of c-Myc function by gene targeting is associated with severe growth retardation and congenital defects in many developing organs and embryonic structures during early ontogeny and ultimately leads to a lethal condition before or during the onset of organogenesis *circa* embryonic day 10 (ref. 6; H.-W.L. and R.A.D., unpublished observations). The pleiotropic effects associated with c-Myc deficiency most likely relate to the generalized pattern of c-*myc* expression in both the embryo proper and extraembryonic tissues during this stage of normal development (7, 8). By embryonic day 18.5, c-*myc* transcripts are no longer observable in organs that have completed proliferation and instead are restricted to those which maintain proliferating precursor populations (e.g., intestinal crypts, basal skin layer, and thymus; ref. 9).

Efforts to elucidate how the actions of Myc are regulated have pointed to mechanisms that limit the amount of c-*myc* mRNA and protein (for review see ref. 10) and to those that block Myc activities such as induction of cellular growth (11, 12) and transcriptional activation (13). The latter mechanism involves interactions among an extended family of related basic–helix–loop–helix/leucine zipper (bHLH/LZ) proteins that includes Myc, Max, Mad, and Mxi1 (13–16). Myc transactivation function appears to be highly dependent upon its heterodimerization with Max (refs. 14, 15, and 17 and references therein) and the binding of the heterodimer to the Myc consensus sequence (18, 19). Heterodimers formed between Max and Mad or Mxi1 lack transactivation activity and may inhibit the expression of Myc-regulated targets through their capacity to competitively occupy the CACGTG consensus (13, 16) and to actively repress those targets (20, 21). The concept that Mad and Mxi1 may function as repressors of Myc activity is supported by the biological observations that human *mad* or *mxi1* overexpression can suppress transformation activity of c-*myc* in cultured cells (11) and block progression across the G_1/S transition of the cell cycle (12).

From a developmental standpoint, a rise in *mad* mRNA levels (22) and a switch in Max-associated complexes from Myc/Max to Mad/Max (23) accompany *in vitro* differentiation of human myeloid cell lines. As findings in these and other cell culture-based differentiation systems suggest opposing patterns of Myc and Mad expression with progression toward the terminally differentiated state, we sought to understand the relationship of Myc and Mad to this process in normal mouse and human tissues. The crypts of the gastrointestinal system and stratified squamous epithelium of the skin were chosen for detailed studies since they exhibit abundant levels of steady-state *mad* mRNA and are regionally compartmentalized with respect to growth and differentiation. Notably, cessation of proliferation and acquisition of a more differentiated phenotype along the vertical axis of colonic crypts and stratified squamous epithelium are associated with the loss of c-Myc expression and activation of Mad expression. The finding of overlapping patterns of Myc and Mad expression in the skin is in accord with the view that Myc and Mad exert opposite effects on genetic pathways central to the transition from an immature proliferating cell type to a fully differentiated one.

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Abbreviations: REF, rat embryo fibroblast; bHLH/LZ, basic–helix–loop–helix/leucine zipper.

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The spatially distinct localization of Myc and Mad in the intestinal epithelium argues for a specific *in vivo* role for Mad in processes of terminal differentiation that is removed from its role as a putative regulator of Myc in these cells.

MATERIALS AND METHODS

Isolation of cDNA Clones, Analysis of DNA/RNA, and Construction of Plasmids. For the isolation of mouse *mad* cDNA, a human *mad* fragment (ref. 13; a gift from Bob Eisenman) containing the open reading frame was used to screen at low stringency an amplified oligo(dT)- and random oligonucleotide-primed λZAPII cDNA library generated from RNA derived from newborn mouse brain (Stratagene). Purification of recombinant clones, subcloning, probe preparation and radiolabeling, Northern blotting and hybridizations, and reverse transcription–polymerase chain reaction (RT–PCR) were performed as described (24). Nucleotide sequence of the mouse *mad* cDNA¶ was determined and analyzed as described (25). Total RNA derived from a panel of newborn and adult mouse tissues was isolated by the LiCl/urea method (26) and subjected to RNase protection assays (24).

Expression Constructs and Rat Embryo Fibroblast (REF) Cooperation Assays. Expression constructs for mouse *mad* and human *mad* were generated by placing their respective cDNAs in both orientations between two tandemly repeated Moloney murine leukemia virus long terminal repeats in pVNic (24). The mouse c-*myc* and Ha-*ras*$^{Val\text{-}12}$ expression constructs have been described (27). Early-passage cultures of REFs were prepared and cotransfected by the calcium phosphate precipitation method (27). The malignant phenotype of transformed foci was assessed by morphological criteria under a light microscope and in the soft-agar assay (27).

Immunohistochemistry. Staged embryonic and postnatal mouse tissues were embedded in OCT cryopreservative solution (Miles), fresh-frozen in liquid N_2, and then stored at −70°C. Immunohistochemical stains were performed on cryostat-cut sections (5 μm) by the avidin–biotin immunoperoxidase method (28). Adjacent sections were either stained with hematoxylin and eosin or incubated with an anti-Mad antibody (Santa Cruz Laboratories) at a dilution of 1:500, an anti-Myc antibody (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:1000, or an anti-laminin antibody (Sigma) at a dilution of 1:1000. Sections were then incubated with secondary biotinylated goat anti-rabbit immunoglobulins followed by avidin–biotin-peroxidase complexes (Vector Laboratories). To verify the specificity of anti-Myc and anti-Mad immunohistochemical staining, antibodies were preincubated with an excess of synthetic peptides to which the antibodies were raised (concentrations of 0.1–0.2 μg/μl). Diaminobenzidine was used as the chromogen and nuclei were counterstained with hematoxylin or ethyl green and photographed on a Nikon Optiphot microscope.

RESULTS and DISCUSSION

Key Structural Features of Mouse Mad. Human *mad* (13) probes were employed under low-stringency conditions to screen a mouse newborn brain cDNA library. Nucleotide sequence analysis of a recombinant phage clone identified an ATG-initiated open reading frame capable of encoding a putative protein of 227 aa with a predicted molecular mass of 25,500 Da. That this protein indeed represents the mouse homolog of human Mad (13) is supported by their shared amino acid identity of 89% compared with only 61% when aligned with the related human Mxi (16). The majority of the cross-species amino acid differences between mouse and human Mad proteins are highly conservative substitutions, with the exception of an extended repeat of Asp-Val pairs positioned near a highly charged acidic region in the C-terminal portion of the mouse Mad protein. A possible function for this 2-aa repeat is not apparent from a database search; RT–PCR studies do not support the existence of mouse *mad* transcripts capable of encoding an Asp-Val repeat equivalent in size to that of the human protein (data not shown).

Homology between Mad and Max (14) bHLH/LZ regions (51% similarity) suggests that the DNA-binding/dimerization motifs of Mad will assume a structure similar to that determined crystallographically for Max—namely, one that comprises two continuous α-helices (basic region plus helix I and helix II plus leucine zipper) separated by a loop (19). With respect to the basic region, residues that have been shown crystallographically for Max (19) and by site-directed mutagenesis for E-box-binding proteins (ref. 38 and references therein) to confer sequence-specific DNA recognition and to participate in anchoring the protein to the major groove are also found in the basic region of mouse Mad. Aside from maintenance of key residues implicated in DNA-binding and dimerization function, the mouse Mad protein also contains specific residues that have been hypothesized (25) to govern the selection or dismissal of dimerization partners through favorable or unfavorable charge interactions on the electrostatic surface.

N-terminal to the bHLH/LZ region of mouse Mad is a region (aa 1–32) markedly conserved throughout vertebrate evolution; it exhibits an overall identity of 88% with human Mad (13) and is highly homologous (75% similar) to similarly positioned regions in both human (16) and mouse (20) Mxi. The secondary structure of this region, as assessed by the PREDICTPROTEIN algorithm (European Molecular Biology Laboratory), is predicted to be strongly α-helical in nature (data not shown). For mouse Mxi, this region has been shown to be essential for potent repression of Myc transforming activity (20) and for binding to the mammalian homolog of the yeast transcriptional repressor Sin3 (20, 21). The conservation of the primary and secondary structure of this domain suggests that its activities and interactions may be highly similar if not identical in the Mad and Mxi proteins.

C-terminal to the bHLH/LZ region of mouse Mad is a highly charged region (aa 136–219) that is rich in serine and acidic residues, previously termed "similarity region 2" on the basis of homology between human Mad and Mxi proteins (13, 16). Within similarity region 2 of mouse Mad is a subregion (aa 147–178) of extreme negative charge. This subregion could conceivably facilitate interactions with positively charged histone proteins which could then lead to nucleosome unfolding and allow access of Mxi1/Max or Mad/Max complexes to DNA; similar protein/histone interactions have been postulated for excision-repair proteins (29). Alternatively, this acidic region may allow for sliding of the complexes on chromatin in search of specific DNA-recognition sequences, an activity that has been suggested for a negatively charged subregion in the Myc protein (30).

Cross-Species Conservation of Suppression by Mad of Myc Transforming Activity. We demonstrated recently that human *mad* is a powerful inhibitor of *myc/ras* cotransformation activity in the REF cooperation assay (11). The highly quantitative nature of this biological assay (31) was used in the present study to determine whether the mouse homolog of *mad* possessed antioncogenic activities comparable to those of the human gene. Inhibition was assessed by measuring the number of transformed foci generated in cotransfections of mouse c-*myc* and activated Ha-*ras*$^{Val\text{-}12}$ in the presence or absence of an equimolar amount of human or mouse *mad* expression construct. In multiple independent cotransfections, a significant reduction in the number of foci was observed

¶The mouse *mad* sequence has been deposited in the GenBank database (accession no. L38926).

when either human *mad* or mouse *mad* was added to the c-*myc*/*ras* cotransfections (Fig. 1). The decrease in the number of foci was found, on average, to be greater with the human gene than with the similarly designed mouse expression construct. Since virtually all of the other cross-species amino acid differences between the human and mouse *mad* open reading frames are highly conservative substitutions, this diminished activity may assign significance to the extended Asp-Val repeat in the mouse protein (see above).

The suppressive potential of mouse *mad* was readily evident with other parameters used to assess the degree of malignant transformation. Cell lines established from the few foci arising in *myc*/*ras*/mouse *mad* cotransfections exhibited a less transformed morphology than cell lines generated from *myc*/*ras*-induced foci. The addition of *mad* to the cotransfections was also associated with a poorer potential for anchorage-independent growth in that only 20% of *myc*/*ras*/mouse *mad*-derived cell lines were capable of growth in soft agar, compared with 80% for c-*myc*/*ras* (data not shown). Interestingly, the two *myc*/*ras*/mouse *mad* lines that exhibited anchorage-independent growth had undetectable levels of *mad* transcripts (data not shown). This observation suggests that strong selective pressure against *mad* overexpression is operative during the progression toward a highly malignant phenotype, and only those foci that eliminate or diminish *mad* expression can sustain growth in soft agar.

Contrasting Patterns of Myc and Mad in Developing Tissues. As Mad clearly antagonizes the ability of Myc to neoplastically transform cells in culture, an analysis of *mad* and *myc* mRNA and protein expression was performed to understand the functional relationships among these members of the Myc superfamily in the growth and development of normal cells. As an initial assessment, RNase protection assays were used to compare the steady-state levels of *mad* and *myc* mRNAs in a panel of developing newborn tissues and terminally differentiated adult tissues (Fig. 2). Progressive development and growth arrest in most organ systems were accompanied by a dramatic decrease in c-*myc* mRNA, as described previously (32), and by a maintenance or substantial increase

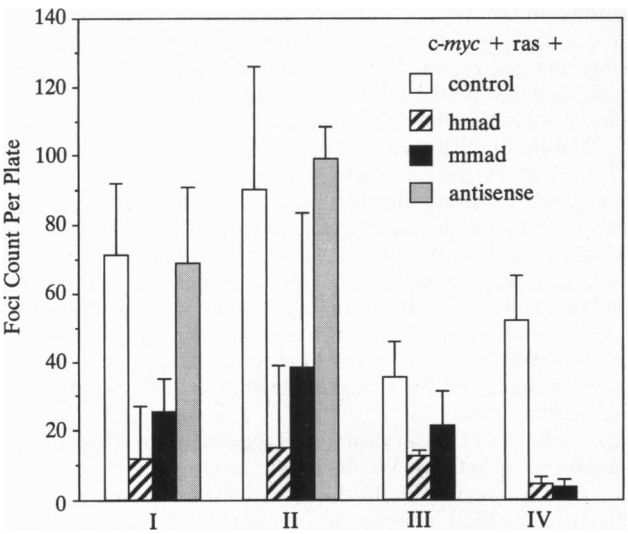

FIG. 1. Anti-*myc* activity of *mad* in the REF cooperation assay. In each experiment, primary plates were transfected with 2 μg of the mouse c-*myc* expression construct, 2 μg of Ha-*ras*$^{Val-12}$, 2 μg of the appropriate *mad* expression construct [human (h), mouse (m), or mouse antisense] or empty vector (control), and 30 μg of genomic carrier DNA. The average number of foci per plate from six plates (derived from two transfected plates that were split 1:3) was determined ≈11 days after transfection in four separate experiments (I–IV).

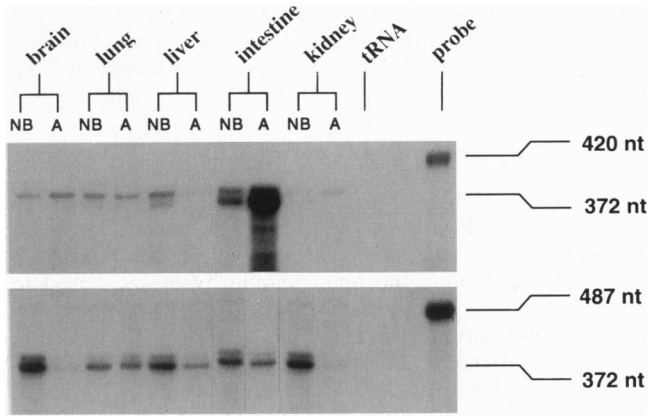

FIG. 2. Analysis of the distribution of mouse *mad* (*Upper*) and c-*myc* (*Lower*) transcripts in newborn (NB) and adult (A) tissues by RNase protection assay. For mouse *mad*, the probe was a 420-bp (insert plus linker sequences) *Eco*RI–*Kpn* I fragment from the cDNA clone containing 5′ untranslated and 5′ coding sequences. For mouse c-*myc*, the probe was a 487-bp (insert plus linker sequences) *Pvu* II–*Xho* I fragment from the pmc-Myc54 cDNA clone (kind gift of Ken Marcu, State University of New York at Stony Brook) containing exon 3 sequences. Each probe was transcribed *in vitro* with [α-^{32}P]CTP, hybridized to 40 μg total RNA from the various tissues listed or to yeast tRNA, and digested with RNases A and T1. The sizes of the two probes and their respective protected bands (372 nt for mouse *mad* and 372 nt for mouse c-*myc*) are indicated. The doublet observed in both experiments most likely represents terminal digestion resulting from partial denaturation at the ends of the RNA duplex under the high-stringency conditions employed.

in *mad* gene expression (Fig. 2, compare *mad* and c-*myc* signals in newborn versus adult intestine and brain). Notably, tissues that did not exhibit this contrasting relationship (e.g., liver) showed persistently high or rising levels of mouse *mxi1* mRNA in adult tissue (L.C. and R.A.D., unpublished data), a finding consistent with the notion that the structurally related Mad and Mxi1 may serve redundant roles in terminally differentiated tissues. In mature organ systems which undergo perpetual renewal (such as the gastrointestinal tract and skin) or extensive postnatal development (such as the lung), high levels of mRNA for both c-*myc* and *mad* continued to be present in the adult.

Although these expression patterns suggest differential activities for *myc* and *mad* with progression through development, the cell type heterogeneity of whole organs precludes a detailed view of how *myc* and *mad* expression relates to processes of growth and differentiation of the individual cell lineages within each organ. Consequently, an immunohistochemical analysis of Myc and Mad protein in the gastrointestinal tract and skin was performed to determine their patterns of expression at the microanatomical level (Fig. 3). Selection of the gut and epidermis was dictated by the high levels of *mad* mRNA in these tissues and by the well-characterized morphological and biological changes that accompany epithelial cell growth and differentiation therein.

In the colon, the base of each crypt contains multipotent stem cells which undergo several rounds of cell division before committing along several possible lineages to become goblet cells, enterocytes, and enteroendocrine cells (33). These postmitotic committed lineages continue to differentiate as they migrate vertically toward the gut lumen and, upon reaching the apex, undergo apoptosis and are extruded into the lumen (33). In multiple sections of the gastrointestinal tract of the adult mouse, the lower portion of the colonic crypts, comprising slowly proliferating progenitor cells, showed a strong nuclear-associated immunoreactivity to a c-Myc-specific antibody (Fig. 3*A*). Similarly, the midportion of the crypts, populated by maturing and rapidly proliferating cells, displayed positive

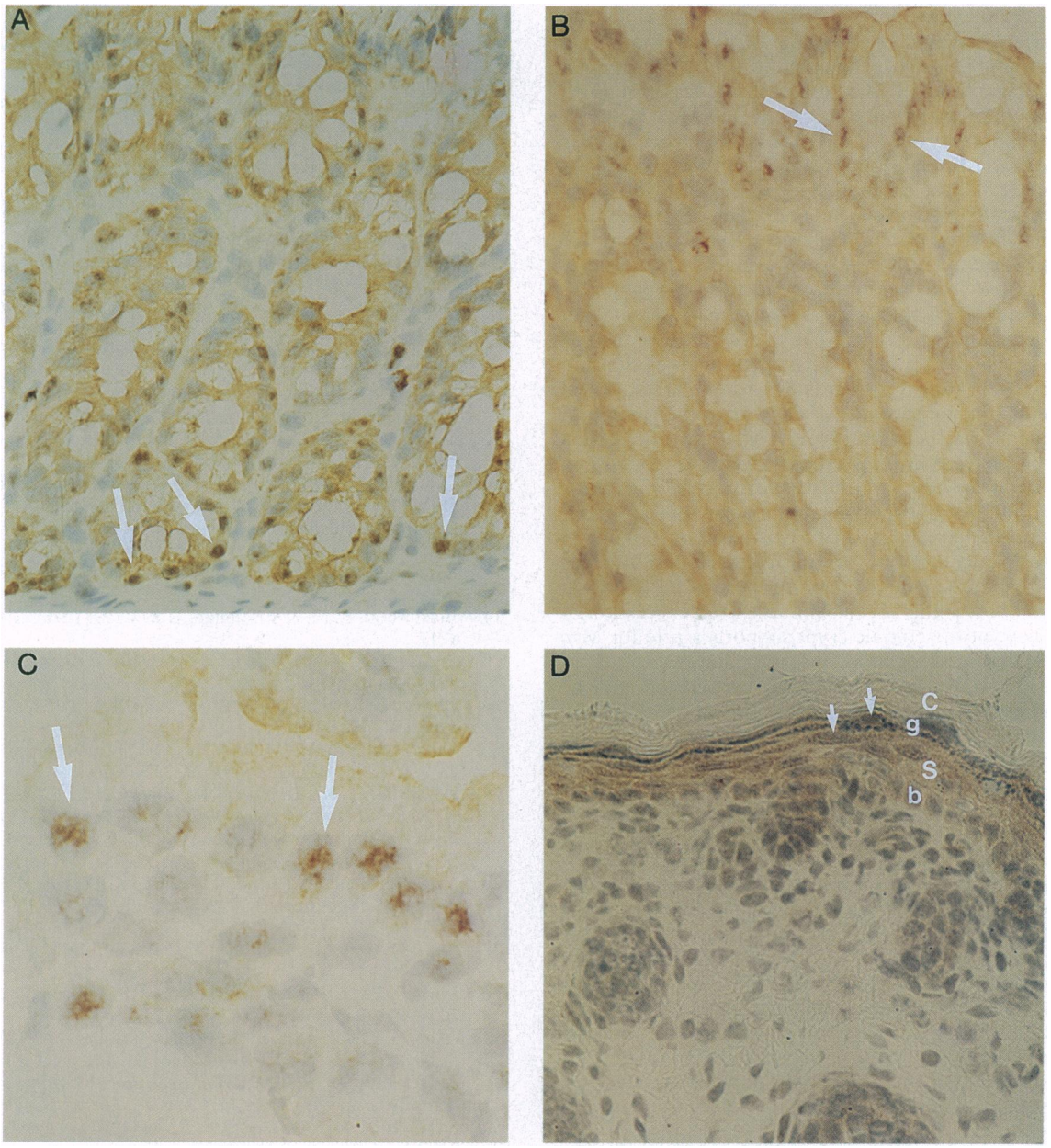

FIG. 3. Localization of Mad and c-Myc proteins in the mouse intestine and skin by immunohistochemistry. In *A–C*, photomicrographs of sections through colonic crypts in adult and newborn mice are oriented so that the base of the crypts is at the bottom. (*A*) Intense nuclear staining of c-Myc (arrows) in proliferating and nonproliferating but still differentiating epithelial cells of the lower and middle sections of adult mouse colonic crypts. Occasional peroxidase-positive cells are present in the cellular lamina propria between the crypts in this horizontal section. (×130.) (*B*) Strong nuclear immunoreactivity of Mad (arrows) in epithelial cells located in the luminal portion of the adult glands. (×130.) (*C*) Strong nuclear-associated staining of Mad in luminal cells of the newborn intestine. (×650.) (*D*) An increasing gradient of Mad expression is observed in more superficial suprabasal layers of the skin (arrows denote strongly immunoreactive nuclei). (×260.) c, Corneum; g, granular layer; s, spinous layer; b, basal layer.

c-Myc staining. However, c-Myc immunoreactivity was undetectable in sections through the luminal portion of the crypts wherein differentiated nondividing cells reside. This regional pattern of Myc protein expression is identical to that observed at the mRNA level (9). In sharp contrast to c-Myc, intense nuclear immunostaining for Mad was observed in cells of the apical or luminal portion of the adult colonic crypts but not in the cells of the middle and lower portions of colonic crypts (Fig. 3*B*). While a similar pattern of expression was observed in the colonic tissues of newborn mice, the distribution of c-Myc was more diffuse and cytoplasmic, whereas Mad proteins were clearly nuclear-associated in the apical cells of the colonic crypts (Fig. 3*C*; only Mad is shown). Analogous results were obtained when normal adult human colonic tissue samples were analyzed (data not shown).

The discrete cell layers of the stratified squamous epithelium of the epidermis include the basal layer, in which some precursor cells are proliferatively active; the spinous and granular layers, consisting of actively differentiating cells; and the stratum corneum, comprising terminally differentiated cells (34). Definitive localization of the basal layer was made possible by anti-laminin staining of the basement membrane upon which the basal layer sits (data not shown). Adjacent sections stained with the anti-Mad antibody showed low or undetectable cytoplasmic and nuclear staining in the proliferating and less mature cells of the basal layer and an increasing gradient of strong cytoplasmic and nuclear immunoreactivity in cells of the maturing suprabasal layers (Fig. 3*D*). Ultimately, Mad expression was lost as terminally differentiated keratinocytes reached the superficial stratum corneum and underwent apoptosis and denucleation (Fig. 3*D*).

Biochemical and cell culture-based studies on the physical and functional relationships between Myc and Mad have fueled the notion that Mad antagonizes the transcriptional activation by Myc/Max of Myc-responsive genes during cell cycle exit and differentiation. Specifically, this view has been supported by the ability of Myc and Mad to associate with Max and bind common target sequences (13) and the capacity of Mad to suppress the transactivation by Myc in reporter assays (13, 21). Intracellularly, this competition model appears to hold true under circumstances wherein Myc and Mad are coordinately expressed, as Mad expression can effectively interfere with the growth-promoting function of Myc in the REF assay (ref. 11; this study) and arrest human astrocytoma cells with aberrantly expressed Myc in the G_1 phase of the cell cycle (12). A requisite feature of such a yin–yang relationship in cell lineages progressing towards the growth-arrested and fully differentiated state would be some degree of temporal and/or spatial overlap between Myc and Mad expression. This appears to be the case in the stratified squamous epithelium where some, albeit weak and variable, expression of Mad is detectable in the c-Myc-expressing basal layer. In sharp contrast, the compartmentalization of Myc and Mad proteins in anatomically distinct zones of the gut suggests separable roles for these two proteins in the regulation of cellular proliferation and differentiation in this organ. The restricted expression of Myc to the base of the colonic crypt supports a role for Myc in active cellular proliferation and/or maintenance of an undifferentiated state. The absence of Myc and the appearance of Mad expression in highly differentiated gut epithelial cells well after their withdrawal from the cell cycle suggest that Mad performs functions beyond competing with Myc for commonly regulated gene targets in some cell lineages. Rather, in tissues wherein Mad is expressed but Myc is not, Mad may play a role in regulation of genes required for completion (rather than initiation) of the terminal differentiation program. Lastly, in tissues where *mad* gene expression is extremely low or is downregulated during development, this differentiation function may be served by the highly related Myc antagonist, Mxi1. Indeed, in the developing kidney, very abundant levels of *mxi1* mRNA are present and these levels increase substantially with advancing age (data not shown).

Our findings suggest that a given cell may need both the down regulation of Myc and the up regulation of Mad (or Mxi) to achieve its developmental end point. Support for this view comes from other studies in which molecular strategies designed to neutralize the actions of Myc (e.g., antisense synthetic oligonucleotides or RNA) have proven only partially effective in triggering terminal differentiation and, in most instances, have served only to accelerate differentiation upon exposure to differentiation-inducing agents (35, 36). A study assessing the phenotypic impact of Myc neutralization (e.g., dominant negative forms of Myc) coupled with direct induction of Mad expression may provide insight into how members of the Myc superfamily participate and cooperate mechanistically in important developmental transitions.

Note. After submission of this manuscript, Västrik *et al.* (37) reported similar activities for mouse Mad in the REF assay as well as similar patterns of expression by RNA *in situ* hybridization to developing mouse tissues.

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