Coordinated regulation of c-Myc and Max in rat liver development

Jennifer A. Sanders and Philip A. Gruppuso

Department of Pediatrics, Rhode Island Hospital and Brown University, Providence, Rhode Island

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Sanders, Jennifer A., and Philip A. Gruppuso. Coordinated regulation of c-Myc and Max in rat liver development. Am J Physiol Gastrointest Liver Physiol 290: G145–G155, 2006.—The processes of liver development and regeneration involve regulation of a key network of transcription factors, the c-myc/max/mad network. This network regulates the expression of genes involved in hepatocyte proliferation, growth, metabolism, and differentiation. In previous studies on the expression and localization of c-Myc in the fetal and adult liver, we made the unexpected observation that c-Myc content was similar in the two. However, c-Myc was localized predominantly to the nucleolus in the adult liver. On the basis of this finding, we went on to characterize the expression patterns of the other members of the network, max and mad, comparing their regulation during late fetal development with the proliferation of mature hepatocytes that is seen in liver regeneration. We found that Max content, rather than being constitutive, as predicted by other studies, was elevated in the fetal liver compared with the adult liver. Its content correlated with hepatocyte proliferation during the perinatal transition. In contrast, mad4 expression was decreased in the fetal liver compared with the adult liver. Nucleolar localization of c-Myc coincided with changes in Max content. To explore this relationship, we overexpressed Max in cultured adult hepatocytes. High levels of Max resulted in a shift in c-Myc localization from nucleolar to diffuse nuclear. In contrast, liver regeneration was associated with an increase in c-Myc content but no change in Max content. We conclude that the regulation of Max content during liver development and its potential role in determining c-Myc localization are means by which Max may control the biological activity of the c-Myc/Max/Mad network during liver development.

DURING the last 3 days of gestation in the rat, hepatocytes proliferate rapidly, resulting in a tripling of liver mass. This burst of proliferation is followed by a transient growth arrest at term and a short period of hepatocyte proliferation during the first postnatal week as hepatocytes gradually transition to the quiescent adult hepatocyte phenotype (15). However, adult hepatocytes retain their ability to reenter the cell cycle in response to a growth stimulus, such as two-thirds partial hepatectomy. During the regenerative process, ~90% of hepatocytes enter the cell cycle within the first 24 h (32). Although our laboratory has identified several marked differences in comparing fetal and adult hepatocyte proliferation in vivo (1, 5, 16), the mechanisms involved in these developmental changes in hepatocyte proliferation and growth have not been fully elucidated.

A key regulatory network for the diverse cellular processes that are required for hepatocyte growth and proliferation involves the myc family of transcription factors. c-myc has been assigned roles in hepatocyte proliferation during liver development and regeneration, control of hepatic metabolism, and the dysregulated growth that occurs during heptocarcinogenesis (8, 19, 28, 33). c-Myc is considered to be an extremely short-lived protein whose expression is tightly regulated in many cell types. This protein is normally expressed at high levels in proliferating cells and at diminishing levels as cells undergo growth arrest and differentiation (14).

All of the biological activities of c-Myc require its binding partner, Max. c-Myc-Max heterodimers bind DNA at CACGTG elements (E boxes) to either activate or repress transcription of target genes. In contrast to c-Myc, Max is considered to have ubiquitous expression and to be highly stable, resulting in Max levels that under usual conditions exceed those of c-Myc (35). Max interactions are promiscuous, as Max also dimerizes to another short-lived family of proteins, Mad (2, 23). The mad family of transcription factors oppose the biological activities of c-Myc by competing for binding to Max and repressing the transcription of a subset of c-Myc-activated genes. This repression is mediated through binding to the same E boxes as c-Myc and recruitment of the Sin3 repressor complex through the Sin3 interaction domain of Mad (2). In contrast to c-Myc, Mad members (Mad1 and Mad4) are expressed during growth arrest and in differentiated cells, whereas Mad3 is expressed in proliferating cells, where it plays a role in S phase progression (11, 12, 25).

In the current model for the c-myc/max/mad network, Max is constitutively expressed while the transcriptional activity of the network relies on the differential expression patterns of c-Myc and Mad (14, 21). In previous studies, our laboratory showed that c-Myc protein content was similar in proliferating the fetal and quiescent adult liver (29). However, c-Myc was localized to the nucleolus in the adult liver, whereas the fetal liver displayed a diffuse nuclear pattern. This observation suggested an alternative model for control of the network. We have therefore analyzed the expression pattern of other members of the c-myc/max/mad network to determine if their regulation might be critical to the control of hepatocyte proliferation during liver development.

MATERIALS AND METHODS

Animals. Male and timed-pregnant female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Two-thirds partial hepatectomy was performed on adult male rats (125–150 g) under isoflurane anesthesia. Sham-operated animals underwent liver exteriorization without excision. Cesarean sections were performed on timed-pregnant rats under pentobarbital anesthesia (50 mg/kg body wt, administered by an intraperitoneal injection) on embryonic days 19 (E19) or 21 (E21, term). Fetal livers were harvested from the first four to six pups in each litter so as to avoid profound effects of stress. Livers from postnatal animals up to 2 wk of age were harvested without anesthesia. All animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Rhode Island Hospital.

Address for reprint requests and other correspondence: P. A. Gruppuso, Div. of Pediatric Endocrinology and Metabolism, Rhode Island Hospital, 593 Eddy St., Providence, RI 02903 (e-mail: Philip_Gruppuso@brown.edu).

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approved by the Rhode Island Hospital Animal Care and Use Committee and were in accordance with criteria outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, Revised 1985).

Hepatocyte isolation and transient transfection. Hepatocytes from adult male rats were isolated as previously described (9, 30). The isolation procedure results in cultures that are 90% hepatocytes with the remainder a mixture of nonparenchymal cells. Transfection of adult hepatocyte cultures was performed as previously described (29). The conditions used were chosen to maximize cell viability. As a result, transfection efficiencies were ~10%. The expression vector for hemagglutinin (HA)-tagged Max (HA-Max) was kindly provided by Dr. Michael Cole (Dartmouth Medical School; Lebanon, NH). The plasmid for HA-3Cl-Myc was generously provided by Dr. William Tansey (Cold Spring Harbor Laboratory; Cold Spring Harbor, NY). Hepatocytes were incubated for 5 h at 37°C. The transfection was terminated by the addition of 5% fetal calf serum. Hepatocytes were fixed and processed for immunofluorescence microscopy 24 h posttransfection.

Cotransfection and luciferase assay. Cultured adult hepatocytes were cotransfected with 0.8 μg HA-Max or empty vector (EV) and 0.7 μg of either pGL3C luciferase reporter, pGL3C containing a proximal E box binding site, or pGL3C containing a proximal mutated E box binding site. Transient transfections were performed as described in Hepatocyte isolation and transient transfection. All luciferase reporter constructs (34) were kindly provided by Dr. James Padbury (Women and Infants Hospital of Rhode Island; Providence, RI). Luciferase assays were performed according to the manufacturer’s instructions 24 h posttransfection (BD Pharmingen; San Diego, CA). Luciferase activity was measured from triplicate wells per construct in an automated luminometer (Analytical Luminescence Laboratory).

RNase protection assays and RT-PCR. RNA was isolated from triplicate frozen livers obtained from E19 fetuses, control adult rats, and rats killed 2, 6, or 24 h after sham surgery or partial hepatectomy. The isolation procedure used homogenization in guanidinium thiocyanate followed by cesium chloride density centrifugation (6). RNase protection assays were performed according to the manufacturer’s instructions using the miMyc multiprobe template with mouse RNA and yeast tRNA serving as positive and negative controls, respectively (BD Biosciences; San Diego, CA). Preliminary studies comparing mouse and rat RNA showed no differences in the protected fragments obtained using the mouse Myc multiprobe template. GAPDH was used as an internal control to normalize expression data. For RT-PCR, cDNA was synthesized according to the manufacturer’s instructions using Superscript first-strand synthesis for the RT-PCR kit (Invitrogen Life Technologies; Carlsbad, CA). Primer sequences used for amplification of c-Myc, the primary antibody (Upstate) was incubated overnight at 4°C with a 1:10 dilution of each antibody. The resultant supernatant was diluted fivefold in ChIP dilution buffer, and samples were incubated at 4°C, and immunoprecipitated with either 2 μg Max antibody or rabbit IgG (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitates were collected by the addition of 60 μl salmon sperm DNA-protein A agarose for 1 h at 4°C. The supernatant from the IgG precipitation was retained as the input. Immunoprecipitates were washed with 1 ml of the following buffers for 5 min at 4°C with rotation: low-salt buffer, high-salt buffer, LiCl buffer, and twice with 0.1% Tris-EDTA buffer (TE) buffer. Protein-DNA complexes were eluted from the antibody with 1% SDS and 0.1 M NaHCO3 and brought to a final concentration of 0.2 M NaCl. Cross-links were

Immunochemical precipitation. Liver cryosections (6 μm) and primary adult hepatocyte cultures were fixed in methanol for 10 min and incubated with Max or influenza HA primary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. For immunodetection of c-Myc, the primary antibody (Upstate) was incubated overnight at 4°C. Detection employed Alexa Fluor 594- or Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes; Eugene, OR). Omission of the primary antibody was used as a negative control for all antibodies employed. Indirect immunofluorescent microscopy was used to acquire images of three ×40 fields per tissue cryosection, which were then subjected to image analysis. The total cell number was determined using manual counting of 4’,6-diamidino-2-phenylindole (DAPI)-counterstained images (Vector Laboratories; Burlingame, CA). Confocal images were acquired with a Nikon PCM 2000 (Nikon; Melville, NY). Serial optical sections were performed with Simple 32, C-imaging computer software (Compix; Cranberry Township, PA). Z-series sections were collected at 0.5 μm with a ×60 PlanApo lens and a scan zoom of ×2. Images were processed and reconstructed with NIH Image shareware.

Chromatin immunoprecipitation. Protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 34.4 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) were added to all reagents before use for chromatin immunoprecipitation. The frozen liver (0.3–0.4 g) was thawed in 5 ml ice-cold buffer A1 [containing (in mM) 15 HEPES (pH 7.5), 300 sucrose, 60 KCl, 15 NaCl, 2 EDTA, 0.5 EGTA, and 14.2-mercaptoethanol], placed in a douce homogenizer, and homogenized for five strokes with the loose pestle and two strokes with the tight pestle. The homogenate was transferred to a 15-ml conical tube and allowed to settle on ice for 5 min. The top 4 ml were then transferred to a new 15-ml conical tube and centrifuged at 1,000 g for 5 min at 4°C. The nuclear pellet was resuspended in 5 ml ice-cold buffer A2 (buffer A1 plus 0.5% Nonidet P-40) by trituration with a Pasteur pipette. The resuspended nuclei were layered onto 10 ml ice-cold buffer B [containing 15 mM HEPES (pH 7.5), 30% sucrose, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, and 14 mM 2-mercaptoethanol] in a 15-ml conical tube and centrifuged at 1,600 g for 5 min at 4°C. The resulting nuclear pellet was resuspended in 0.5 ml ice-cold buffer C (20 mM Tris (pH 7.9), 50% glycerol, 75 mM NaCl, 0.5 M EDTA, and 0.85 mM DTT). Approximately 2 × 106 nuclei were added to 5 ml ice-cold PBS containing 10% glycerol. Protein and DNA were crosslinked by adding formaldehyde (1% final) to the nuclei and incubating for 10 min at room temperature with rotation. Cross-linking was terminated by adding glycerine to a final concentration of 0.125 M and incubating for 5 min at room temperature with rotation. Nuclei were pelleted by centrifugation at 1,000 rpm for 5 min at 4°C and washed twice with ice-cold PBS containing 10% glycerol. Chromatin immunoprecipitations were performed using a ChIP assay kit from Upstate Biotechnology (cat no. 17-295). Nuclei were lysed in 200 μl SDS lysis buffer, and DNA was sheared to lengths between 200 and 1,000 bp by sonication with a Cole Parmer Ultrasonic homogenizer 4710 series, setting 2, 30% duty cycle, 3 × 10-s pulse. Samples were centrifuged at 13,000 rpm for 10 min at 4°C. The resultant supernatant was diluted fivefold in ChIP dilution buffer, precleared with 75 μl salmon sperm DNA-protein A agarose for 30 min at 4°C, and immunoprecipitated with either 2 μg Max antibody or rabbit IgG (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitates were collected by the addition of 60 μl salmon sperm DNA-protein A agarose for 1 h at 4°C. The supernatant from the IgG precipitation was retained as the input. Immunoprecipitates were washed with 1 ml of the following buffers for 5 min at 4°C with rotation: low-salt buffer, high-salt buffer, LiCl buffer, and twice with 0.1% Tris-EDTA buffer (TE) buffer. Protein-DNA complexes were eluted from the antibody with 1% SDS and 0.1 M NaHCO3, and brought to a final concentration of 0.2 M NaCl. Cross-links were
reversed by heating at 65°C for 4 h, and eluates were treated with 10 mg/ml proteinase K at 45°C for 1 h. DNA was recovered by phenol chloroform extraction and ethanol precipitation. DNA samples were analyzed by PCR using the following primers to amplify rat genomic sequences: nucleolin, 5'-CGC GTC GCA GGC AGT G-3' and 5'-TCC ATC TAC CGT CAC GGT CAG-3'; and carbamoyl-phosphate synthase (glutamine-hydrolyzing/aspartate carbamoyltransferase/di-hydroorotase (CAD), 5'-GCC GTC GCA GTC GTG CT-3' and 5'-ACC GAC CCG TCC TCC AA-3'. Optimal PCR cycle numbers for exponential amplification were defined in preliminary range-finding experiments. Gels were stained with ethidium bromide and photographed under UV illumination.

Densitometry and statistical analyses. Quantification of bands from RNase protection assays and Western immunoblots was performed by digital image analysis using a Hewlett-Packard Scanjet 5470c scanner and Gel-Pro Analyzer software (Media Cybernetics; Silver Spring, MD). Results were analyzed using a Student's t-test for E19 versus adult comparisons and ANOVA with a post hoc Tukey test for all other comparisons (GraphPad Prism; San Diego, CA).

RESULTS

c-myc/max/mad network expression during liver development and regeneration. RNA was isolated from triplicate E19 fetal and adult rat livers, and the expression of various genes involved in the c-myc/max/mad network were analyzed by RNase protection assay (Fig. 1A). The members of the network associated with growth and proliferation, c-myc and mad3, were elevated in E19 liver compared with adult liver, whereas those associated with growth arrest, mad4, and the transcriptional repressor sin3, which is recruited by mad family members, were decreased in E19 liver compared with adult liver (Fig. 1B). An unexpected finding was that max expression was 10-fold higher in fetal liver than in the adult liver. We also examined the expression of these genes during liver regeneration as a control for our fetal versus adult comparisons. Adult rats were subjected to two-thirds partial hepatectomy or sham surgery, and RNA was isolated at 2, 6, and 24 h postsurgery. No significant changes in c-myc, max, or mad4 were observed after sham surgery (data not shown). In agreement with previously published data (24), c-myc expression and max expression were induced during early liver regeneration, with peaks at 2 and 6 h posthepatectomy, respectively. As expected, mad4 was decreased during the first 24 h of liver regeneration (Fig. 1B). mad1 and mxi were expressed at very low levels, and no significant changes were observed during either perinatal liver development or liver regeneration (data not shown).
Max content is regulated in the developing liver. On the basis of the observation that max steady-state mRNA levels were regulated during liver development and regeneration, we evaluated Max protein content in the fetal and adult liver. Nuclear extracts were prepared from six E19 and adult rat livers and analyzed by Western immunoblot analysis (Fig. 2). Max was identified as a doublet with apparent molecular masses of 22 and 21 kDa. In accordance with the results from the RNase protection assays, Max was found to be 10-fold higher in fetal liver nuclei compared with adult liver nuclei. Max protein was not detectable in Western immunoblot analysis conducted on whole homogenates from fetal and adult liver. To further investigate this result, fetal and adult liver cryosections were immunostained for Max, and image analysis was performed (Fig. 3). Staining of triplicate cryosections revealed a diffuse nuclear pattern that appeared to exclude the nucleolus in ~75% of fetal nuclei. Approximately 25% of nuclei in the adult liver showed positive Max staining. However, the staining pattern differed in the fetal and adult liver. The adult liver displayed diffuse cytoplasmic staining along with punctate staining of the nucleus.

To characterize the kinetics of this decrease in Max content during the fetal to adult hepatocyte transition, we prepared nuclear extracts from livers at various developmental ages and analyzed them by Western immunoblot analysis for Max (Fig. 4). Max content was high in the E19 fetal liver and declined sharply at term (E21). This was followed by a modest increase during the first postnatal week. When these changes in in vivo Max content were compared with the results of previous studies conducted in our lab describing in vitro DNA synthesis by freshly isolated hepatocytes of various developmental ages (9), a parallel between Max content and hepatocyte proliferation was readily apparent.

Max content during liver regeneration. Given the correlation between Max content and hepatocyte proliferation during perinatal development, we hypothesized a similar relationship during liver regeneration. Nuclei were isolated from triplicate control adult rats and from livers obtained 2, 6, 24, or 48 h after sham surgery or two-thirds partial hepatectomy and analyzed by Western immunoblot analysis for Max (Fig. 5). Results using multiple samples prepared during three separate experiments (n = 7 per group) revealed considerable animal-to-animal variation in Max content in both the sham and partial hepatectomized animals. This variation could not be attributed to differences in protein loading as total protein content analyzed by Coomassie stain was similar in all samples. We considered it possible that some of the variability was associated with surgical stress and the performance of replicate experiments on separate days. To compare the immunoblots for Max across multiple experiments, densitometric data for each experiment were expressed as a ratio of partial hepatectomy to sham and combined. This analysis revealed no change in Max content at 2 h. We observed a trend toward increased Max content in the partial hepatectomy animals at all other times analyzed (6, 24, and 48 h), but these changes did not reach statistical significance. Because we were unable to detect Max in Western immunoblots of whole liver homogenates, we used immunofluorescence to detect changes in total liver Max content during liver regeneration (data not shown). Results revealed no apparent differences between partial hepatectomized and sham-operated animals at times up to 24 h after surgery.

In the face of the absence of Max regulation during liver regeneration, we suspected a primary role for control of c-Myc content. Nuclear extracts were isolated from triplicate control adult rats and livers obtained 2, 6, 24, or 48 h after sham surgery or partial hepatectomy. Immunoblot analysis (Fig. 6) identified three isoforms of c-Myc with apparent molecular masses of 64, 62, and 55 kDa. These are within the previously identified molecular weight range for the c-Myc protein in the
rodent (31). In accordance with RNA data, c-Myc protein content was significantly higher 6 and 24 h after partial hepatectomy than in the corresponding sham-operated animals.

Expression of Max and c-Myc localization. In combination with our previous studies (29), the above results indicated that c-Myc and Max content are regulated differently during liver development and regeneration. Our prior study (29) also demonstrated that c-Myc localizes to the nucleolus in adult liver and in quiescent adult hepatocyte cultures. To test the hypothesis that the nucleolar localization of c-Myc might be a function of Max expression, we performed c-Myc immunostaining on liver cryosections cut from the same developmental ages.

Fig. 3. Max localization in E19 fetal and adult livers. To determine Max localization, E19 and adult cryosections were fixed in methanol and stained for Max. A: representative confocal images show immunofluorescent detection of Max (left) and propidium iodide (PI) counterstaining (middle); merged images are shown on the right. B: triplicate E19 and adult cryosections were fixed, processed for immunofluorescent detection of Max, and counterstained with DAPI. Representative images were captured at ×40. The percentage of nuclei positive for Max are shown as means + 1SD. *P < 0.0005.

Fig. 4. Association between hepatic Max content and hepatocyte proliferation during perinatal liver development. A: triplicate livers were obtained from fetal rats (E19 and E21) and newborn rats [postnatal days (P) 1, 3, and 7]. Nuclear extracts were prepared and analyzed by Western immunoblot analysis for Max (top) and by Coomassie stain for total protein content (inset). Numbers to the left of the autoradiogram represent the apparent molecular mass (in kDa). B: the graph shows the results of the immunoblot analyzed by densitometry (filled bars) and [3H]thymidine incorporation over the first 24 h in culture by hepatocytes isolated from fetal, neonatal, and adult rats (open bars). Densitometric data are expressed as means + 1SD. *P < 0.05, E19 vs. all other developmental ages.
used to assess Max content (Fig. 7A). As observed previously, c-Myc displayed a diffuse nuclear pattern in the E19 fetal liver. At term, c-Myc was still diffusely localized, but discrete concentrations of c-Myc within the nucleus were observed. On the basis of dual immunodetection of c-Myc and nucleolin, these discrete speckles were previously identified as nucleoli (29). During the first postnatal week, c-Myc displayed progressive localization to the nucleolus, and the percentage of nuclei that were diffusely stained decreased significantly. By postnatal day 14, c-Myc was exclusively localized to nucleoli.

A recent report (10) has established a role for ARF in regulating the localization and function of c-Myc. To investigate whether ARF played a role in the nucleolar localization of c-Myc observed in the adult rat liver, total RNA was isolated from duplicate E19 fetal and adult rat livers, and RT-PCR was performed to analyze the relative expression level of ARF mRNA (Fig. 7B). ARF expression was barely detectable in both fetal and adult liver, although a strong signal was observed in the positive control (rat placenta). These results suggest that ARF is not a likely candidate for...
the regulation of c-Myc nucleolar localization in the rat liver.

A comparison of the ontogeny of c-Myc localization with changes in Max content (Fig. 4) was consistent with the possibility that Max could be involved in c-Myc localization. As noted above, we (29) have previously found that c-Myc was localized to the nucleolus in cultured adult hepatocytes. We therefore considered these primary cultures to be an appropriate model system to study the effect of Max overexpression on c-Myc localization. Cultured adult rat hepatocytes were transiently transfected with HA-Max or EV. Cultures were fixed 24 h posttransfection and processed for fluorescent immuno-detection of HA-Max and endogenous c-Myc. Results (Fig. 8A) showed that HA-Max-positive adult hepatocytes had a diffuse nuclear pattern for c-Myc staining, whereas the surrounding untransfected hepatocytes displayed nucleolar c-Myc localization. Transfection with EV had no effect on c-Myc localization (data not shown).

To further examine the role of Max in c-Myc localization, we transfected adult hepatocyte cultures with a HA-tagged c-Myc deletion mutant (ΔC1-Myc) lacking residues 367–439, which encode the helix-loop-helix and leucine zipper domains of c-Myc protein. Given the region of deletion, this mutant should be unable to bind Max protein. ΔC1-Myc displayed a similar localization pattern to that observed with full-length HA-Max. The majority of adult hepatocytes displayed a diffuse nuclear staining that excluded the nucleolus. However, diffuse nuclear staining the included the nucleolus was also observed (Fig. 8B). These results suggest that the means by which c-Myc localizes to the nucleolus may involve mechanisms other than a direct protein-protein interaction with Max.

Effect of Max overexpression on c-Myc-mediated transcription of an E box reporter. To examine the effect of overexpression of Max and the subsequent shift in c-Myc localization from the nucleolus to the nucleus on c-Myc transcriptional activity, we performed transient transfection experiments in cultured adult hepatocytes utilizing the HA-Max expression vector and a luciferase reporter construct containing a proximal E box binding site. Hepatocytes were harvested 24 h after transfection, and luciferase assays were performed (Fig. 9). Although transfection efficiencies were low using the conditions employed, cotransfection of HA-Max and an E box-containing reporter resulted in a significant increase in luciferase activity compared with controls (cotransfection of EV plus an E box-containing reporter and transfection of HA-Max with the reporter alone). Mutation of the E box binding site abolished the observed increase in luciferase activity.

c-Myc/Max binding to regulatory regions of target genes is regulated in the fetal and adult liver. To further assess the functional consequences of decreased Max content and nucleolar localization of c-Myc, we performed chromatin immunoprecipitation assays to investigate c-Myc/Max transcriptional activity in E19 fetal and adult rat livers using the known c-Myc target genes nucleolin and CAD. In addition to being a well-documented c-Myc target gene, we (16) have previously identified nucleolin as overexpressed in fetal liver compared with adult liver using suppressive subtraction hybridization. To assess the expression of CAD, RNA was isolated from duplicate E19 and adult livers, and RT-PCR was performed. CAD expression was found to be approximately threefold higher in E19 liver compared to adult liver (data not shown). As a first step in performing the chromatin immunoprecipitation assays,
we tested a panel of 10 antibodies (5 for c-Myc and 5 for Max) for their ability to coimmunoprecipitate c-Myc and Max from rat liver nuclear extracts. One of the five c-Myc antibodies tested was able to specifically immunoprecipitate the c-Myc-Max complex from rat liver. However, the efficiency of the immunoprecipitation was extremely low, and the antibody did not yield results in chromatin immunoprecipitation experiments (data not shown). We found that one of the Max antibodies tested was able to efficiently immunoprecipitate the c-Myc-Max complex from rat liver. Therefore, this antibody was chosen for use in the chromatin immunoprecipitation assays. Intact nuclei were isolated from four E19 fetal and adult livers, Chromatin immunoprecipitation assays were performed, and Max binding to the 5′ regulatory region of nucleolin and CAD was analyzed by PCR (Fig. 10).

Although we observed some variation across different E19 and adult samples and across separate experiments, fetal samples showed generally enhanced Max binding to both nucleolin and CAD promoters compared with adult samples. In contrast, the signal intensity for both nucleolin and CAD was similar in fetal and adult input samples, indicating that comparable amounts of genomic DNA were present in the starting samples. As expected, no signal was detected for nucleolin or CAD in the IgG control and mock immunoprecipitation reactions. Notwithstanding the relatively qualitative nature of the chromatin immunoprecipitation analysis, and given that nucleolin and CAD are expressed at a significantly higher levels in the fetal liver compared with the adult liver, we interpreted these data as indicating that c-Myc-dependent transcriptional activation of nucleolin and CAD is enhanced in the fetal liver compared with the adult liver.

**DISCUSSION**

In many in vitro systems, the regulation of the c-myc/max/mad network has been shown to rely on the opposing expression patterns of c-myc and mad family members, whereas max expression is constitutive (22, 25). We (29) have reported previously that c-Myc protein content is not changed even though c-myc RNA expression is decreased as proliferating hepatocytes growth arrest during liver development, but c-Myc localization switches from a diffuse nuclear to a nucleolar pattern (29). In the present study, we found that Max expression at both the RNA and protein level coincided with the pattern of hepatocyte proliferation during perinatal liver development. We found Max to be 10-fold elevated in the proliferating fetal liver compared with the quiescent adult liver. We also demonstrated a difference in Max localization in the fetal and adult liver. In the fetus, the majority of hepatocytes displayed a diffuse nuclear pattern for Max. In the adult, cytoplasmic staining for Max was present and fewer hepatocyte nuclei were positive. The kinetics of the decrease in Max content was rapid and correlated with hepatocyte proliferation during the perinatal period. We also examined the expression of mad4 and mad3 in the fetal and adult liver. In agreement with previous studies in other systems, we found mad4 to be
Induced in the quiescent adult, whereas mad3 was elevated in the fetus (12, 25).

These results show that, in contrast to previous in vitro studies suggesting that max expression is constitutive (3, 14, 17), changes in max expression and Max protein content is a component of the temporal regulation of the c-myc/max/mad network in the developing liver. The decrease in Max content as hepatocytes mature is similar to the changes in Max expression that occur during differentiation of chondrocytes (36). In this in vivo model, Max was present in the nucleus of proliferating chondrocytes and decreased as the chondrocytes matured. However, in the rat epiphyseal plate, unlike the liver, c-Myc content followed chondrocyte proliferation and growth with levels decreasing dramatically as the chondrocytes differentiate. The developing murine prostate gland represents yet another pattern of c-myc/max/mad network regulation (17, 27).

In this in vivo model, mad1 and mad4 were continually expressed during both proliferative and differentiating phases of prostate development (20).

To examine the regulation of Max in mature hepatocytes induced to proliferate, we used the model of liver regeneration after partial hepatectomy. We found no change in Max protein content during the earliest stage of liver regeneration examined (2h). However, a trend toward increasing Max content was observed beginning at 6 h through 48 h posthepatectomy. In contrast, c-Myc protein content increased rapidly (2 h), peaked at 6 h, and remained elevated during the first 24 h of liver regeneration. The increase in c-Myc content occurred before the increase in Max and was more pronounced, suggesting that the regulation of c-Myc plays a key role in regulating the hepatic c-Myc/Max/Mad network during liver regeneration. In contrast to Mauleon et al. (24), we did not observe a significant increase in c-myc expression in sham-operated animals at any time point after surgery. This discrepancy may be result of the use of different anesthesia. Again, we found expression of mad4 and mad3 at the RNA level to follow the expected pattern. These results suggested that, in contrast to the developing liver, the regulation of the c-Myc/Max/Mad network during liver regeneration depended on alterations in c-Myc and mad4, whereas the Max content was stable.

Our laboratory has found that the regulation of mitogenic signaling pathways and cell cycle control differ in the fetal liver compared with the adult and regenerating liver (4, 5). In the fetal liver, several signaling pathways, including those that involve ERK1, ERK2, phosphatidylinositol 3-kinase, Akt, and ribosomal protein S6 kinases 1 and 2, are inactive and can only be minimally stimulated in response to growth factors. In the adult liver, these pathways are active and responsive to mitogens. We (4) have also demonstrated that fetal hepatocyte proliferation in vivo is resistant to rapamycin, whereas hepatocyte DNA synthesis during liver regeneration is rapamycin sensitive. Rapamycin resistance is a characteristic usually associated with tumor-derived cell lines (13, 18), leading us to speculate that the fetal versus adult difference in the Myc/Max/Mad control may be relevant to hepatic carcinogenesis. This is supported by recent preliminary studies showing that several signaling genes that are overexpressed in the fetal liver relative to the adult liver (16) are also overexpressed in hepatocellular carcinoma cell lines (N. Brim, R. Jimenez, and P. Gruppuso, unpublished observations).

During the course of the present study, we observed progressive nucleolar localization of hepatic c-Myc during the first postnatal week. Recent reports suggest a feedback loop exists between ARF and c-Myc where c-Myc increases ARF expression, resulting in ARF binding to c-Myc and inhibition of c-Myc’s ability to activate transcription (7). Datta et al. (10) found that ARF can physically interact with c-Myc and that this interaction results in relocalization of c-Myc to the nucleolus and inhibition of c-Myc transcriptional activation. A study by Qi et al. (26) also found that ARF and Myc interact but that this interaction occurs in the nucleoplasm and results in the inhibition of c-Myc transcriptional activity but not repression of target genes. We found that ARF expression was barely detectable in both the E19 fetal and adult liver, indicating that ARF is not involved in the nucleolar localization of hepatic c-Myc. However, a temporal correlation between Max content...
and c-Myc localization was observed. In general, the expression of Max was inversely associated with the nucleolar localization of c-Myc. Further experimentation revealed that overexpression of HA-Max in cultured adult hepatocytes resulted in a shift in c-Myc localization from nucleolar to diffuse nuclear, suggesting Max may contribute to the regulation of c-Myc localization. We did not elucidate the mechanism by which Max affects c-Myc’s localization. The regulation appears to involve more than a direct interaction between the two proteins. A transiently transfected deletion mutant of c-Myc lacking the Max dimerization domain displayed diffuse nuclear localization. However, we did observe an increase in c-Myc/Max activity in transient transcription assays upon overexpression of HA-Max in cultured adult hepatocytes. This increase may be a result of the shift in c-Myc localization from the nucleolus to the nucleus. However, it is also possible that Max content is limiting in cultured adult hepatocytes like the adult liver and that overexpression of Max results in the formation of c-Myc/Max complexes in the nucleus. We also observed a decrease in c-Myc/Max binding at nucleolin and CAD promoters in the adult liver compared with the fetal liver, suggesting that transcriptional activation of these genes by c-Myc is decreased in the adult. A previous study described in fibroblasts using green fluorescent protein (GFP) fusions of c-Myc, Max, and Mad family members showed that proteins of the Myc network could affect the localization of other members. This effect was codominant and dynamic, with the less abundant member of the heterodimer assuming the pattern of its more abundant partner (37). These results using overexpression studies of GFP fusions are similar to our in vivo data, where endogenous c-Myc displayed diffuse nuclear staining in tissue samples where Max expression was found to be elevated, and in our vitro studies in which the overexpression of Max was associated with diffuse nuclear localization of c-Myc.

In summary, our findings indicate that Max expression at the protein level is regulated during liver development and that this regulation may contribute to the nucleolar localization of c-Myc. In contrast, nuclear Max content is only slightly, if at all, increased during liver regeneration, whereas the increase in c-Myc content is profound. These findings may indicate two distinct modes of regulation of the hepatic c-myc/max/mad network, one that incorporates the constitutive expression of Max and is operative when mature hepatocytes are induced to proliferate and a second that is operative during liver development and may involve a primary regulatory role for Max.

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