MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1037–1048 Vol. 17, No. 3
0270-7306/97/$04.00+0
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A Minimal Regulatory Region Maintains Constitutive Expression of the *max* Gene

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Received 27 August 1996/Returned for modification 23 October 1996/Accepted 27 November 1996

Max is a basic helix-loop-helix/leucine zipper protein that forms heterodimers with the Myc family of proteins to promote cell growth and with the Mad/Mxi1 family of proteins to inhibit cell growth. The role of Max as the obligate binding partner for these two protein families necessitates the observed constitutive expression and relatively long half-life of the *max* mRNA under a variety of growth conditions. In this study, we have used the chicken *max* gene to map DNA elements maintaining *max* gene expression in vertebrate cells. We have identified a minimal regulatory region (MRR) that resides within 115 bp of the *max* translation initiation site and that possesses an overall structure typical of TATA-less promoters. Within the MRR are two consensus binding sites for Sp1, a ubiquitously expressed transcription factor that plays a role in the expression of many constitutive genes. Interestingly, we show that direct binding by Sp1 to these sites is not required for MRR-mediated transcription. Instead, the integrity of a 20-bp DNA element in the MRR is required for transcriptional activity, as is the interaction of this DNA element with a 90-kDa cellular protein. Our data suggest that it is the persistence of this 90-kDa protein in vertebrate cells which drives *max* gene expression, insulates the *max* promoter from the dramatic changes in transcription that accompany cell growth and development, and ensures that adequate levels of Max will be available to facilitate the function of the Myc, Mad, and Mxi1 families of proteins.

Max is a nuclear phosphoprotein of the basic helix-loop-helix/leucine zipper (bHLH/LZ) class of transcription factors (13, 42, 55, 62). The Max protein associates with the Myc family of oncoproteins in vitro and in vivo (1–3, 12, 13, 42, 51, 54, 62, 63) and forms heterodimers which bind to a core consensus DNA sequence, CACGTG, referred to as the Myc E box (3, 12, 13, 42, 63). Myc-Max heterodimer formation has been shown to be essential for all of the known biological functions of Myc, including the induction of cell growth (1), the triggering of apoptosis under specific growth conditions (1), cellular transformation (2, 54, 62), and the regulation of target gene transcription (3, 27, 59). Several groups have observed a dual effect of Max on Myc function, with an increase in Myc-mediated cellular transformation noted when Max levels are elevated modestly and a decrease in transformation when Max levels are extremely high (2, 57, 61). Since Max does not possess a transcription activation domain (42, 55) and forms homodimers that bind to the same target DNA sites as Myc-Max heterodimers (10–12, 42, 51, 62), it has been proposed that a small increase in Max provides the protein necessary to maximize Myc-Max activity, while high Max levels promote the formation of inactive Max-Max homodimers (4, 32, 57, 63). With the recent discovery of the Mad (7, 36) and Mxi1 (77) proteins—additional bHLH/LZ proteins that lack transcription activation domains and preferentially dimerize with Max (not Myc) to bind to Myc E-box DNA (6, 77)—understanding the regulation of Myc function at the molecular level has become even more complex. For the Mad proteins and, to a lesser extent, for Mxi1, maximal protein expression is observed following the terminal differentiation of certain cell types (6, 20, 36, 46, 77), while Myc expression is associated with proliferating, undifferentiated cells (15, 25, 56). On the other hand, Max levels remain relatively constant under all growth conditions (11, 12, 44, 46, 66), further supporting the notion that cells must maintain appropriate levels of Max in order to ensure that the proliferation-specific and differentiation-specific bHLH/LZ factors (for which Max is the sole dimerization partner characterized to date) function efficiently. The importance of *max* gene expression to the coordinated development of an intact organism has been demonstrated by the recent observation that *max* null animals die earlier in embryogenesis than c-*myc* or N-*myc* null animals (19, 48, 69). For these reasons, we have focused our efforts on investigating the mechanism of transcriptional regulation of the *max* gene in an effort to understand how Max levels are maintained during cellular growth and differentiation.

The *max* gene is conserved in evolution, and *max* cDNAs have been cloned and characterized from a number of organisms (13, 26, 37, 44, 62, 66). However, outside of partial sequence information on the genomic organization of the human *max* gene (13, 53, 71), the complete structure of a *max* genomic DNA has been described only for chicken (68). In this study, we have examined the 5′ flanking region of the chicken *max* gene and have identified a minimal regulatory region (MRR) that resides 115 bp immediately upstream of the methionine initiator codon and is absolutely essential for maintaining transcriptional activity in chicken, human, and mouse cells. The MRR contains several transcription initiation sites, two Sp1 consensus binding sites, and no consensus TATA or CAAT elements, all of which are considered typical structural features of the promoters of cellular housekeeping genes (8, 22). Although Sp1 has been implicated as an important factor in the transcriptional regulation of many TATA-less promoters (16, 28, 39, 52), our data indicate that direct binding by Sp1 to the Sp1 consensus DNA sites located in the chicken *max* MRR is

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not necessary for MRR-directed reporter gene expression. Instead, we have identified a core sequence from positions −91 to −110 in the MRR which binds several cellular proteins, including a 90-kDa protein whose association with the core sequence is correlated with MRR transcriptional activity. The identification of this 90-kDa protein as a cellular factor that is responsible, in large part, for maintaining *max* gene expression in cells suggests that the activity of this protein will have an indirect impact on the function of bHLH/LZ factors, such as Myc and Mad, and thus play an important role in regulating the proliferation and differentiation of cells.

MATERIALS AND METHODS

Plasmids. pMaxCAT was constructed in two steps by first inserting an *Xba*I/*Sma*I fragment containing 2,089 bp of chicken *max* 5′ flanking DNA plus first exon and intron DNA (68) into the *Xba*I/*Sma*I sites in E1BTATACAT (50) and replacing the E1B TATA box with chicken *max* promoter sequence and then by cleaving this DNA with *Sma*I/*Nae*I and religating to create a chloramphenicol acetyltransferase (CAT) reporter controlled by DNA spanning positions −6 to −2089 of chicken *max*, where position +1 is the first A of the ATG initiator codon. The 5′ and 3′ deletions of pMaxCAT were made by using an Erase-a-Base Kit (Promega). 5′ deletions into the chicken *max* promoter were made from the *Xba*I site and protected by using the 5′ *Pst*I site in the CAT vector polylinker. 3′ deletions into the promoter were made from the *Xma*I site and protected by using a 3′ *Sac*I site in the polylinker. 5′[−77] was made by restricting 5′[−234] with *Xho*I and *Eag*I, blunt ending the linear molecule with Klenow DNA polymerase and deoxynucleoside triphosphates, and religating. 3′[−47] was made similarly using internal *Eco*RI and *Bss*HII cleavage sites. The structures of all pMaxCAT derivatives used in this study were verified by DNA sequencing (TAQuence; U.S. Biochemical). pBScm12 5′[−234] was constructed by inserting a *Hin*dIII (blunt-ended)/*Nar*I fragment excised from 5′[−234] into an *Xba*I (blunt-ended)/*Nar*I-digested pBScm12BglII vector. This generated a chicken *max* genomic clone with a 5′ deletion up to 234 bp relative to the methionine initiator codon. pBScm 5′[−234] (which was used for site-directed mutagenesis) was prepared by using a *Hin*dIII/*Eco*RI fragment excised from 5′[−234] and inserted into the *Hin*dIII/*Eco*RI site of pBluescript II KS+. pADH-RI contains the *Drosophila* alcohol dehydrogenase promoter and actin 5C poly(A) addition site inserted into an *Eco*RI/*Bgl*II-modified version of pUC118 (17). pADH-Sp1 contains the full-length Sp1 cDNA (40) ligated to *Eco*RI-linearized pADH-RI. The pSV$_2$CAT reporter contains the CAT coding sequence under transcriptional control of the simian virus 40 promoter/enhancer (30).

Cell culture. Primary chicken embryo fibroblasts (CEFs) were obtained from 11-day-old chicken embryos removed from eggs, minced, and trypsinized for 20 min at 37°C as described previously (24). HeLa cells, CEFs, and C3H10T1/2 mouse fibroblasts were maintained in basal modified Eagle medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and, for CEFs only, 1% chicken embryo extract (GIBCO). *Drosophila* Schneider 2 (S2) cells were maintained at ambient room temperature in 1× Schneider's medium (Sigma) supplemented with 12.5% heat-inactivated FBS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Stable transfections. Stable transfection of C3H10T1/2 cells was performed by the calcium phosphate-DNA precipitation method described previously (18). Briefly, 24 h prior to transfection, cells were seeded at 5×10^5/100-mm-diameter plate in complete medium. Precipitates containing 30 μg of carrier DNA, 50 ng of pKO*neo* (70), and 150 ng of each test construct were added to each plate. Cultures were split 1:6 24 h following transfection and maintained in complete medium containing Geneticin (G418; 400 μg/ml [active concentration]; GIBCO) to select for neomycin-resistant cells. After 14 days, G418-resistant colonies were counted, and approximately 500 colonies from each experimental group were pooled and grown to semiconfluency in complete medium plus G418.

Transient transfections and CAT assays. Transient transfections were performed by the calcium phosphate-DNA precipitation method described previously (55). For HeLa cells, CEFs, and C3H10T1/2 cells, precipitates containing 5 μg of CAT reporter plasmid and 5 μg of the RSV*lacZ* expression vector (38) were added to cells seeded 24 h prior to transfection at 5×10^5/100-mm-diameter plate. Five hours following the addition of precipitates, the cells were shocked osmotically with 20% glycerol (in serum-free basal modified Eagle medium) for 2 min, refed complete medium, and maintained in complete medium for an additional 40 to 45 h. *Drosophila* S2 cells were seeded at 5×10^6/60-mm-diameter plate 24 h prior to transfection. Precipitates containing 7.5 μg of CAT reporter plasmid and 2.5 μg of pADH-RI vector DNA or pADH-Sp1 DNA were added to each plate. Cells were fed complete medium 15 to 18 h following the addition of precipitates and maintained in complete medium for an additional 40 to 45 h. Transfected HeLa cells, CEFs, and C3H10T1/2 cells were harvested by scraping into 1 ml of cold CMF saline (130 mM NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 2.7 mM KCl [pH 7.4]). The cells were pelleted, washed, and resuspended in 230 μl of 0.25 M Tris–5 mM EDTA (pH 8.0) at 4°C, and the cell membranes were disrupted by sonication for 20 s. The β-galactosidase activity measured in each cell extract was used to normalize the amount of extract used to assay CAT activity as described previously (76). S2 cell extracts were prepared as outlined above except that each extract was normalized by using protein content (Protein Assay; Bio-Rad) prior to the CAT assay. Percent conversion of ^{14}C-chloramphenicol (ICN) to the acetylated form by CAT was determined by scintillation counting. All assays were maintained within the linear range of CAT activity, and the activities reported represent the average of at least three independent determinations.

RPA. The template for pMaxCAT riboprobe synthesis was prepared by cleaving 5′[−234] with *Pvu*II, isolating a 419-bp fragment containing 150 bp of CAT, 234 bp of 5′ flanking *max* sequence, and 35 bp of polylinker, and ligating this fragment into the *Sma*I site of pBluescriptII KS+ to create RP(CAT 5′[−234]PvuII). The template for generating a riboprobe for the endogenous chicken *max* transcript was prepared by subcloning a 400-bp fragment containing the chicken *max* first exon, first intron, and 200 bp of 5′ *max* flanking sequence into the *Hin*dIII/*Sac*I site of the pBluescript II KS+ to create pMaxBS 5′.40. [α-^{32}P]UTP (Amersham) was used with the MAXIscript *in vitro* Translation Kit (Ambion) to generate riboprobes from *Eco*RV-linearized RP(CAT 5′[−234]PvuII) and *Hin*dIII-linearized pMaxBS 5′.40. Full-length, radiolabeled RNA was purified by electrophoresis through a 5% polyacrylamide gel followed by elution overnight at 37°C in 0.5 M ammonium acetate–1 mM EDTA–0.2% sodium dodecyl sulfate (SDS). The labeling efficiency was determined by scintillation counting, and approximately 10^5 cpm of each labeled riboprobe was used per reaction. The RNase protection assay (RPA) was performed with an RPA II kit (Amersham). Briefly, the labeled riboprobes were annealed to total RNA isolated from C3H10T1/2 cells stably transfected with pMaxCAT or from untransfected CEFs. Single-stranded RNA unprotected following annealing was digested with a mixture of RNase A (2.5 U/ml) plus RNase T_1 (100 U/ml), and the resistant double-stranded RNA fragments was sized on a 6 or 10% denaturing polyacrylamide gel. Two control reactions were performed in which each riboprobe was annealed to total RNA isolated from yeast and incubated with the RNase mixture or with the reaction buffer alone. A dideoxy DNA sequencing reaction of pMaxCAT was used as a standard to determine the size of the protected fragments and hence the transcription start points for pMaxCAT, and a DNA sequencing reaction of pBluescript II KS+ was used as a size marker to map the transcription start points of the endogenous chicken *max* gene.

Site-directed mutagenesis. Single-stranded pBScm5′[−234] template DNA was prepared from bacterial cultures as described by the supplier (Oligonucleotide-directed *in vitro* Mutagenesis System; Amersham). Synthetic oligonucleotides (obtained from the Purdue University Center for Computational Biochemistry) were designed such that one unique restriction site (*Bam*HI or *Nhe*I) was introduced into the MRR sequence at the Sp1^a site (CCGCCC→GATCCC) or the Sp1^b site (GGGCGG→GGCTA), respectively. Inadvertently, a G→C mutation was introduced at position −96 in Sp1^a and a single G was deleted in Sp1^b. The mutated oligonucleotides were phosphorylated with T4 polynucleotide kinase and ATP and annealed to the pBScm5′[−234] template DNA. Reagents supplied by the Oligonucleotide-directed *in vitro* Mutagenesis System (Amersham) were used to incorporate these sequence changes into double-stranded pBScm5′[−234] DNA, and the presence of the mutations was verified by restriction mapping and DNA sequencing (TAQuence; U.S. Biochemical). 5′[−115]^a and 5′[−115]^b were generated by cleaving pBScm5′[−234]^a and pBScm5′[−234]^b with *Hin*dIII and *Xma*I, ligating the mutant 5′[−234] fragments into *Hin*dIII/*Xma*I-cleaved E1BTATACAT, removing excess polylinker sequence with a *Sac*I digest, and then cleaving with *Hin*dIII and *Sac*II to truncate the 5′ region at position −115. pMaxCAT^a and pMaxCAT^b were made by replacing the *Sac*II/*Xma*I fragment in pMaxCAT with mutant *Sac*II/*Xma*I fragments isolated from pBScm5′[−234] derivatives and then restricting with *Sac*I to remove excess polylinker DNA. The constructs containing the Sp1$^{a/b}$ mutation were made by isolating a 155-bp *Hin*dIII/*Eag*I fragment containing Sp1^a from 5′[−234]^a and ligating it to *Hin*dIII/*Eag*I-cleaved 5′[−234]^b. This construct, designated 5′[−234]$^{a/b}$, was restricted with *Hin*dIII/*Sac*II to create 5′[−115]$^{a/b}$. pMaxCAT$^{a/b}$ was made by replacing the *Sac*II/*Xma*I fragment in pMaxCAT with a *Sac*II/*Xma*I fragment isolated from 5′[−234]$^{a/b}$. 5′[−115]mI, -II, and -III were made by using complementary synthetic oligonucleotides possessing three consecutive, 10-bp mutations (boldface) in the region between −77 and −115:

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mI  5′-agctGGACTATGACTTGTGCAATGCCCCGCGCTCAGGCAGGC
          CCTGATACTGAACACGTTACGGGGCGCGAGTCCGTCCGCCGG-5′
mII 5′-agctGGACTCGCCGCCCCAACCGATGGATCGCTCAGGCAGGC
          CCTGAGCGGCGGGGGTTGGCTACCTAGCGAGTCCGTCCGCCGG-5′
mIII 5′-agctGGACTCGCCGCCCCACAATGCCCCGATAGGCTCATGGC
          CCTGAGCGGCGGGGGTGTTACGGGGCTATCCGAGTACCGCCGG-5′
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The oligonucleotides were gel purified, annealed as described previously (64) to generate the double-stranded fragments diagrammed above, and subcloned into *Hin*dIII/*Eag*I-cleaved 5′[−115] to replace the wild-type MRR sequence between positions −77 to −115.

Northern blot hybridization. A hybridization probe specific for chicken *max* mRNA was obtained by restricting pBScm12BglII (68) with *Xho*I and *Bgl*II and isolating a 400-bp fragment corresponding to the 3′ untranslated region of the chicken *max* gene transcript. The human *max* probe was a 560-bp *Hin*dIII/*Eco*RI

digest from pBSmaxII (18), while the probe for c-*myc* contains 580 bp of coding sequence isolated following a *Bgl*II/*Sal*I digest of pBS*myc*, which has the v-*myc* coding region inserted into pBluescript KB+. Total RNA was isolated as described previously (18) from proliferating cells, from quiescent cells, or from quiescent cultures stimulated for 1, 2, or 6 h with 10% FBS and 10 μg of cycloheximide (Sigma) per ml. Twenty micrograms of RNA from each sample was electrophoresed through 1% agarose-formaldehyde gels, transferred to nylon membranes (Nytran; Schleicher & Schuell), and prehybridized, hybridized, washed, and exposed to X-ray film as described previously (18). The probes were radiolabeled with [α-^{32}P]dCTP (6,000 Ci/mmol; Amersham) by using an Oligolabeling kit (Pharmacia), and unincorporated nucleotides were removed from the probes by using G-50 spun columns (64).

Nuclear extract isolation. Nuclear extracts were isolated from C3H10T1/2 cells, CEFs, and S2 cells as described previously (47). Briefly, cells were grown to semiconfluency, rinsed with CMF saline at 4°C, and scraped into lysis buffer (2 ml/plate), at which time the number of nuclei/milliliter was determined prior to centrifugation and resuspension in nuclear extraction buffer at a concentration of 2.5×10^7 nuclei/ml. The protein concentration of each extract was determined by using the Bio-Rad Protein Assay. HeLa nuclear extracts were purchased from Sigma.

EMSA. Probes and competitors representing the −77 to −115 region of the MRR were prepared by cleaving 5′[−115] with *Xho*I and *Eag*I. The −56 to −115 and the −6 to −77 competitors were prepared by restricting 5′[−115] with *Xho*I plus *Bss*HII and *Eag*I plus *Eco*RI, respectively. The 87-bp nonspecific DNA competitor was isolated from the pSP72 vector by using an *Nde*I/*Xho*I digest. Probes were end labeled with Klenow DNA polymerase and [α-^{32}P]dCTP or a combination of [α-^{32}P]dCTP and [α-^{32}P]dATP (6,000 Ci/mmol; Amersham). The Sp1 consensus oligonucleotide (Promega) was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol; Du Pont). All probes were purified as described previously (64), and approximately 2×10^5 cpm of each probe was incubated with nuclear extracts at 4°C for 30 min or with purified Sp1 protein (Promega) at room temperature for 20 min. Competitors were added 5 min before the addition of radiolabeled probe DNA. The binding buffer for the nuclear extracts consisted of 25 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 5 mM dithiothreitol, 15 mM $MgCl_2$, 10% glycerol, and 2 μg of poly(dI-dC), and the electrophoretic mobility shift assay (EMSA) profiles were resolved by electrophoresis through 5% nondenaturing polyacrylamide gels in medium-high-ionic-strength buffer (12.5 mM Tris [pH 8.5], 98 mM glycine, 0.5 mM EDTA). The binding buffer and electrophoresis parameters used for the Sp1 binding reactions were recommended by Promega. Electrophoresis was conducted for 3 h at 100 V, after which time the gels were vacuum dried and exposed to X-ray film overnight with an intensifying screen at −80°C.

DNase I footprint analysis. pBScm12 5′[−234] was cleaved with *Apa*LI or *Eco*RI in the presence of calf intestinal alkaline phosphatase (Boehringer Mannheim) to create the coding and noncoding strand probes, respectively. Following inactivation of the enzymes and ethanol precipitation of the DNA, the complementary restriction enzyme digestions (*Eco*RI for noncoding and *Apa*LI for coding) were performed in the absence of calf intestinal alkaline phosphatase. The probe DNA was isolated following electrophoresis through a 5% nondenaturing polyacrylamide gel and elution in 0.5 mM ammonium acetate–10 mM magnesium acetate overnight at 37°C. The probes were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol; Du Pont). Binding reactions were carried out as described for the EMSA with 2×10^4 cpm (~1 ng) of each probe, 19 μg of HeLa nuclear extracts (Promega), and a 100-fold molar excess of competitor DNA where indicated. The final volume of each reaction was increased to 60 μl and the $MgCl_2$ concentration was adjusted to 7 mM before treatment with 0.075 μg (DNA only) or 2 μg (protein plus DNA) of DNase I (Worthington Biochemical) per ml at room temperature for 1 min. Reactions were stopped by adding 60 μl of buffer containing 1% SDS, 50 mM EDTA, 0.2 μg of tRNA per μl, and 200 mM NaCl. Then 4×10^3 cpm of each reaction mixture was electrophoresed through an 8% denaturing polyacrylamide gel. A Maxam-Gilbert G/A sequencing reaction for each probe was prepared by using the DuPont/NEN Maxam-Gilbert DNA sequencing system and electrophoresed in parallel to map the location of the footprinted region on both DNA strands.

Photoaffinity cross-linking. EMSA gels were irradiated with 312-nm UV light and cross-linked protein-DNA complexes isolated as outlined in reference 31, except that Millipore Ultrafree-15 centrifugal filters were used to concentrate the complexes following elution from the gels. The purified complexes then were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (10% gel); following staining of the gels with Coomassie brilliant blue to visualize the protein markers (High MW and Low MW; Bio-Rad), the gels were dried and exposed to X-ray film. EMSA using the AP-1 oligonucleotide (Promega) and bacterially synthesized glutathione *S*-transferase (GST)–c-Jun and GST–B-ATF fusion proteins were performed as described above except that the binding reaction mixture was electrophoresed through a 5% nondenaturing polyacrylamide gel, using medium-high-ionic-strength buffer. A function of the form $y = b_1 \exp[b_2 X] + b_3 X^2$ was derived from the migration of the protein markers, using a nonlinear least squares curve fit method to estimate the size of the protein-DNA complexes. The molecular weight of the protein in the complex was estimated by subtracting the molecular mass of the DNA component, which was 39 kDa for the −77 to −115 probe and 15 kDa for the AP-1 oligonucleotide probe.

RESULTS

Chicken *max* expression is unaltered by cell proliferation events. The human and rodent *max* genes are expressed constitutively in a wide range of tissues and cell lines, and mammalian *max* mRNA levels remain unchanged as cells are stimulated to proliferate or induced to differentiate (10, 12, 46, 72). This pattern of *max* gene expression is in contrast to the expression pattern of the c-*myc* gene, which is low in quiescent cells, rapidly induced following the treatment of cells with mitogens, and down-regulated when cells are triggered to terminally differentiate (15, 43). Although it is recognized that differences in transcriptional regulation underlie the contrasting pattern of *max* and c-*myc* mRNA accumulation in mammalian cells, the greater stability of the *max* mRNA compared to the c-*myc* mRNA (72) also plays a significant role.

To establish if the avian *max* and c-*myc* genes are expressed similarly to their mammalian counterparts, Northern blot analysis was performed on total RNA isolated from various chicken tissues. Not surprisingly, both *max* and c-*myc* mRNAs were expressed ubiquitously, and the relative level of each mRNA in individual tissues did not indicate that these genes were coregulated at the transcriptional level (data not shown). Northern blot analysis also was used to establish the levels of *max* and c-*myc* mRNAs in proliferating CEFs, quiescent CEFs, and CEFs stimulated with 10% FBS for 1, 2, 4, and 6 h. While *max* mRNA levels remained uniform under all growth conditions, the basal level of c-*myc* mRNA in quiescent cultures was increased approximately 10-fold following serum stimulation (data not shown). To investigate if avian *max* mRNA accumulates in cells under conditions where mRNA turnover is inhibited, quiescent CEFs were stimulated with 10% serum in the presence of the protein synthesis inhibitor cycloheximide at 10 μg/ml. Total RNA was isolated from the cultures after 1, 2, and 6 h of treatment and analyzed by Northern blot hybridization for c-*myc* and *max* mRNA (Fig. 1). Throughout the assay period, the levels of *max* mRNA remained constant while c-*myc* mRNA levels increased dramatically, underscoring the difference in the stability of the chicken *max* and c-*myc* mRNAs and the critical role that mRNA turnover plays in ultimately determining the intracellular levels of these two proteins.

Transfected C3H10T1/2 mouse fibroblasts express the chicken *max* gene. Current evidence supports the hypothesis that the function of the Myc, Mad, and Mxi1 proteins depends on cells maintaining adequate levels of Max (reviewed in reference 34). Therefore, to investigate how a consistent level of *max* gene expression is maintained in cells, C3H10T1/2 mouse fibroblasts were stably transfected with a 12-kb *Bgl*II chicken genomic DNA fragment (pBScm12BglII) containing the chicken *max* gene (68) and a derivative of this genomic clone (pBScm12BglII 5′[−234]) which is truncated 234 bp 5′ to the translation initiation site (designated +1). Total RNA isolated from CEFs, control C3H10T1/2 cells, and the transfected cultures was analyzed by Northern blot hybridization using a ^{32}P-labeled *max* probe designed to detect both the >2.0-kb mouse *max* mRNA (62) and the 1.5-kb chicken *max* message (68) (Fig. 2). The results show that both chicken *max* genomic clones are transcribed in C3H10T1/2 cells, indicating that the proteins necessary for directing chicken *max* gene expression are present in this mouse cell line and that these proteins mediate *max* gene transcription by interacting with sequences found within the pBScm12BglII 5′[−234] genomic clone.

The MRR maps to a 110-bp fragment within the 5′ flanking region of the chicken *max* gene. Sequence analysis of pBScm12BglII (68) revealed that the 5′ flanking region of

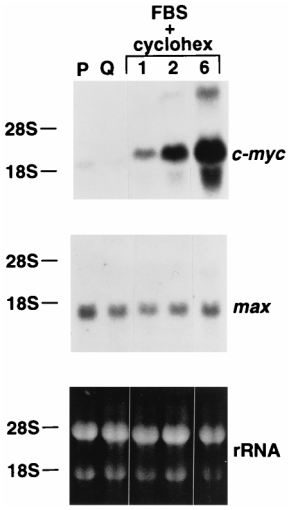

FIG. 1. Expression of chicken c-*myc* and *max* mRNAs in serum-stimulated, cycloheximide-treated CEFs. Total RNA was isolated from proliferating (P) CEFs, CEFs rendered quiescent (Q) by growth in 1% supplemented medium for 48 h, and CEFs stimulated with 10% FBS and 10 μg of cycloheximide per ml for the times indicated. Twenty micrograms of each RNA was analyzed by Northern blot hybridization as described in Materials and Methods for the expression of c-*myc* (upper panel). The c-*myc* probe was removed from the filter by boiling in 0.1% SDS, and the filter was rehybridized to detect expression of *max* (middle panel). Migration of the 18S and 28S rRNAs was determined by ethidium bromide staining of the gel prior to transfer (lower panel) and is marked to the left of each blot.

chicken *max* displays similarity to the promoter regions of cellular housekeeping genes in that it possesses a high GC content, lacks TATA and CAAT consensus sequences, and contains multiple Sp1 transcription factor binding sites (8, 22, 23). To identify the *cis*-acting DNA sequences that direct expression of the chicken *max* gene, we constructed a CAT reporter gene (pMaxCAT) containing the region from −6 bp to −2088 bp relative to the *max* initiator methionine codon (designated +1). pMaxCAT was subjected to exonuclease III and S1 nuclease treatments as described in Materials and Methods to generate a series of 5′ and 3′ deletions of the full-length promoter region (Fig. 3). Five micrograms of each gene construct was used to transfect C3H10T1/2 cells, and after 48 h, cell extracts were prepared, normalized to the β-galactosidase activity of a cotransfected RSVlacZ gene, and assayed for CAT gene expression as described previously (76). The CAT activity generated from each construct is presented in Fig. 3 as fold increase over the activity measured in a control group transfected with E1BTATACAT, a reporter gene controlled by a minimal promoter which exhibits a basal level of CAT expression in C3H10T1/2 cells (50, 55). The 27-fold increase in CAT activity produced by pMaxCAT demonstrates that DNA elements within the 5′ flanking region control transcription of the *max* gene. The results of the 5′ deletion series show that removal of sequences 5′ to position −115 in the *max* promoter (5′[−115]) does not significantly alter CAT expression compared to the full-length promoter region (27.7-fold versus 27.6-fold). However, deletion of an additional 38 bp (5′[−77]) reduces promoter activity essentially to basal levels (2.2-fold over the level for E1BTATACAT). pMaxCAT, 5′[−115], and 5′[−77] were tested for the ability to direct CAT gene expression in CEFs and in human HeLa cells, and an activity profile similar to that observed for C3H10T1/2 cells was obtained (data not shown). These deletion studies define the MRR for the chicken *max* gene as the DNA sequence from −6 to −115 relative to the methionine initiator codon. The basal level of CAT expression directed by the 5′[−77] and 3′[−96] deletions suggests that within the MRR, sequences mapping between −77 to −115 are particularly critical for maintaining normal levels of transcription. The observation that 3′[−52] displays 40% of the CAT activity of pMaxCAT suggests that sequences 3′ to −77 play an accessory role, if any, in the transcriptional regulation of the chicken *max* gene.

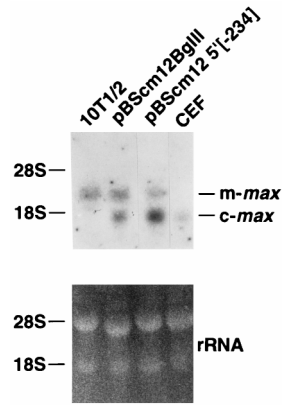

FIG. 2. Expression of chicken *max* genomic clones in C3H10T1/2 fibroblasts. Total RNA was isolated from control C3H10T1/2 cells (10T1/2), control CEFs (CEF), or C3H10T1/2 cells stably transfected with pBScm12BglII or pBScm12 5′[−234]. Twenty micrograms of each RNA was analyzed by Northern blot hybridization as described in Materials and Methods, using a mixture of human and chicken *max* probes and a final wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2 mM EDTA–0.2% SDS at 65°C (upper panel). The smaller size of the chicken *max* mRNA (68) allows for simultaneous detection of mouse (m-*max*) and chicken max (c-*max*) transcripts. Migration of the 18S and 28S rRNAs was determined by ethidium bromide staining of the gel prior to transfer (lower panel) and is marked to the left of the blot.

The endogenous chicken *max* gene and the pMaxCAT reporter construct initiate transcription at multiple start points. Having identified the MRR of the chicken *max* gene, we next sought to define the transcription start points of the gene by using RNase protection of mRNA transcribed from pMaxCAT in C3H10T1/2 cells and from the endogenous chicken *max* gene in CEFs. For pMaxCAT, a ^{32}P-labeled antisense riboprobe spanning 147 bp of the CAT gene and 234 bp of upstream *max* sequence was annealed to total RNA isolated from C3H10T1/2 cells stably cotransfected with a neomycin resistance gene and pMaxCAT. Following treatment with RNase A and RNase T_1, the fragments protected from RNase digestion were separated electrophoretically on a denaturing polyacrylamide gel. Three major transcription start points at −54, −60, and −76 bp were identified by comparison to the nucleotide sequence of pMaxCAT initiated at the base pair representing the 3′ end of the synthesized riboprobe (Fig. 4A). For mapping the transcription start points of the endogenous chicken *max* gene, a similar approach using a ^{32}P-labeled riboprobe generated from pBScm12BglII and total RNA isolated from CEFs was used (data not shown). As indicated in the MRR sequence (Fig. 4B), the three start points identified for pMaxCAT and the four identified for the chicken *max* gene map to the same region of the MRR. Interestingly, there may be a cell-specific preference for start points, since the major initiation at −76 bp of pMaxCAT in C3H10T1/2 cells is not used in CEFs, and the start point at −47 bp in CEFs is not observed in C3H10T1/2. Alternatively, since the start points for pMaxCAT transcription were not mapped in CEFs, this discrepancy also could reflect a shift in initiation due to the fusion of the *max* promoter to the CAT gene sequence. Regardless of the source of this minor

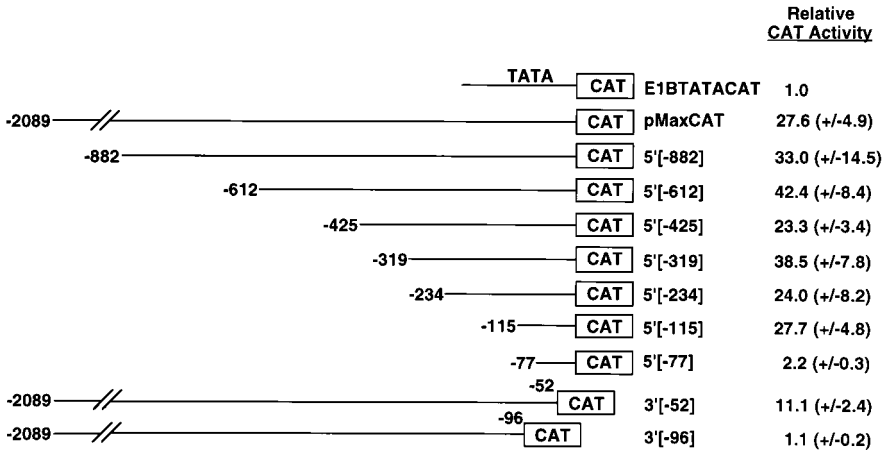

FIG. 3. Identification of the MRR of the chicken *max* gene. Nested deletions in pMaxCAT were performed as outlined in Materials and Methods. C3H10T1/2 cells were transiently transfected with each construct, and CAT activity in cell extracts was measured 45 to 50 h after transfection. Relative CAT activity is expressed as fold increase over that of E1BTATACAT, which is set to 1.0. Each value represents the average of at least three independent determinations, with the standard error of each mean presented in parentheses.

variation, we take the overall correspondence in start point selection to indicate that the transcription initiation of pMaxCAT in C3H10T1/2 cells mimics the behavior of the endogenous *max* gene in CEFs. The presence of multiple start points within the 5′ flanking sequence of the chicken *max* gene was expected and reflects a pattern of transcription initiation that is common to many constitutive, TATA-less promoters (14, 28, 33, 52).

Analysis of the proteins that bind to the MRR. To define the sites of protein interaction along the MRR, we used EMSA and DNase I footprinting. For the EMSA, a ^{32}P-labeled DNA probe containing the entire MRR (−6 to −115) was incubated with nuclear extracts prepared from C3H10T1/2 cells. Following resolution of the protein-DNA complexes on a native polyacrylamide gel, multiple complexes were detected and are designated 1 (a doublet), 2, 3, and 4 (Fig. 5). The MRR probe also was used for EMSA with nuclear extracts prepared from HeLa cells and from CEFs, and the same pattern of shifted bands was detected (data not shown). All four complexes are specific to the MRR, since their intensities are not reduced in binding reactions containing a 100-fold molar excess of an unlabeled, nonspecific competitor DNA (Fig. 5, lane 7) but are reduced when a 100-fold molar excess of unlabeled MRR DNA is added to the reaction (Fig. 5, lane 3). Interestingly, competition with the −77 to −115 subregion of the MRR competes complexes 2 and 4, while competition with the −6 to −77 subfragment reduces the appearance of bands 1 and 3, suggesting that there are at least two classes of protein-DNA complexes that interact with distinct regions of the MRR. Given that the region of the MRR implicated in the control of *max* gene expression maps to −77 to −115 (Fig. 3), these EMSA data strongly suggest that complexes 2 and 4 likely contain the proteins most critical for maintaining *max* gene transcription.

To map more precisely the regions of protein-DNA interaction within the MRR, DNase I footprinting was used. A DNA probe containing the MRR plus an additional 58 bp of polylinker DNA 5′ to the MRR was end labeled on either the coding or the noncoding strand, incubated with HeLa cell nuclear extract, and treated with DNase I as described in Materials and Methods. Following resolution of the DNase I digestion products on denaturing polyacrylamide gels, the regions of protein-DNA interaction across the MRR were determined by comparing the intensity of the banding pattern in the protected sample to that of the banding pattern from a sample treated with DNase I alone. The correspondence of protected regions to bases within the MRR was determined by using a Maxam-Gilbert G/A sequencing reaction of each end-labeled probe (Fig. 6). For both sets of reactions (coding strand and noncoding strand), a core region of the MRR from −84 to −109 was protected. With a 100-fold molar excess of the −56 to −115 subfragment of the MRR as an unlabeled competitor for the coding strand, the DNase I sensitivity of this region was restored (Fig. 6A, lane 4). Curiously, protection of additional regions of the MRR (in particular, reproducible footprints resulting from proteins binding downstream of position −77 in the MRR) was not detected in these assays. This may reflect the reduced stability of the protein-DNA complexes mapping to this region of the MRR. However, the single region footprinted in these experiments (−84 to −109) is contained within the region of the MRR that competes complexes 2 and 4 in EMSA (Fig. 5) and is the region of the MRR that is essential for full transcriptional activity of the chicken *max* promoter (Fig. 3).

***max* promoter activity does not rely exclusively on the binding or on the transcriptional activity of Sp1.** The ubiquitously expressed transcription factor Sp1 binds GC-rich DNA elements and has been found to play a major role in the positive regulation of many TATA-less promoters (16, 28, 39, 52). The MRR contains two Sp1 consensus binding sites designated Sp1^a and Sp1^b (Fig. 4B). The Sp1^a site resides in the region of the MRR protected from DNase I digestion by protein binding (Fig. 6), and the Sp1^b site maps near the conserved cluster of transcription start points determined both for pMaxCAT and for the endogenous chicken *max* gene (Fig. 4B). Thus, the binding of Sp1 to one, or perhaps to both, of these sites could be responsible for the maintenance of chicken *max* gene expression in cells.

The role of the Sp1 consensus binding sites in regulating the transcription of the chicken *max* gene was tested by using oligonucleotide site-directed mutagenesis of pBScm5′[−234] to alter the Sp1^a site, the Sp1^b site, or both sites (Sp1$^{a/b}$) (see Materials and Methods for details). Mutations were confirmed by DNA sequencing, and subcloning strategies were used to generate both pMaxCAT and 5′[−115] derivatives modified with each alteration. Control and mutant constructs were

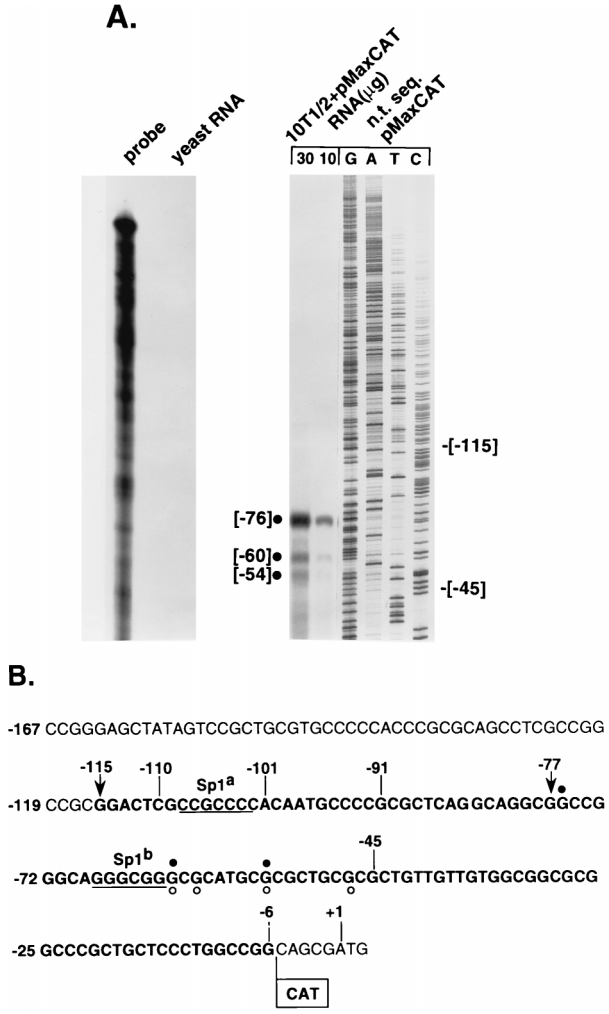

FIG. 4. (A) Transcriptional start points of pMaxCAT mapped by RPA as described in Materials and Methods. Ten and 30 μg of total RNA from C3H10T1/2 cells stably transfected with pMaxCAT were annealed to a ^{32}P-labeled riboprobe covering 5′ CAT sequences and 147 bp of the chicken *max* promoter region. Dideoxy nucleotide sequencing (n.t. seq.) reactions (lanes G, A, T, and C) of the same region of pMaxCAT covered by the riboprobe (base pair positions indicated to the right of the sequence) were used to identify the 5′ base of each protected fragment (filled circles). As a control, the riboprobe was annealed to 10 μg of yeast RNA and treated with RNase (yeast RNA) or with RNase buffer only (probe). (B) Nucleotide sequence of the 5′ flanking region of chicken *max* showing the MRR in boldface type. The 5′[−115] and 5′[−77] deletion sites are indicated by vertical arrows. The sequences of the two consensus binding sites for Sp1 (Sp1^a and Sp1^b) are underlined. Open circles under the sequence indicate the transcriptional start points mapped for the endogenous chicken *max* gene in CEFs (data not shown), and filled circles above the sequence indicate the transcriptional start points mapped in panel A for pMaxCAT. The location of the initiator methionine codon for the chicken *max* gene is indicated (+1), as is the *Nae*I restriction site (−6) used to link the *max* promoter region to the CAT gene.

tested for promoter activity by transient transfection of C3H10T1/2 cells, and the resultant CAT activities were expressed as fold increases over the basal activity of E1BTATA CAT. As shown in Fig. 7, mutation of the Sp1 consensus binding sites does not decrease reporter gene activity. This experiment was repeated with human HeLa cells and CEFs as recipients, and the same profile of activities was observed (data not shown). We conclude that ablation of both Sp1 consensus binding sites in the MRR does not adversely affect the ability of this DNA element to direct gene transcription.

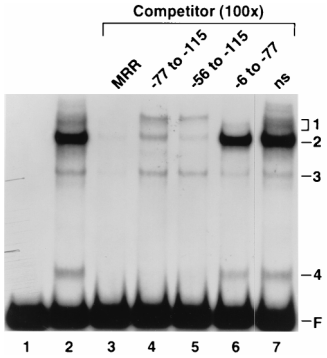

FIG. 5. Profile of protein-DNA complexes formed on the chicken *max* MRR. EMSAs were performed as described in Materials and Methods with a ^{32}P-labeled MRR probe (lane 1) and nuclear extract prepared from C3H10T1/2 cells (lanes 2 to 7). Protein-DNA complexes on the MRR (lane 2) were analyzed by competition with a 100-fold molar excess of unlabeled MRR (lane 3), −77 to −115 DNA (lane 4), −56 to −115 DNA (lane 5), −6 to −77 DNA (lane 6), and a nonspecific DNA fragment (ns; lane 7). The major protein complexes binding to the MRR are numbered 1 (a doublet) through 4 and are indicated to the right of the autoradiogram. The migration of the free, unbound MRR probe DNA is indicated as F.

The observation that the consensus Sp1 sites are not essential for the transcriptional activity of the MRR suggests that the observed protein complexes that bind to the MRR may not contain Sp1. To examine this, EMSAs were performed with C3H10T1/2 cell nuclear extract and ^{32}P-labeled DNA probes representing the wild-type MRR and the MRR containing the Sp1 site mutations (MRR^a, MRR^b, and MRR$^{a/b}$) (see Materials and Methods for details). To ensure that Sp1 binding activity was present in our nuclear extracts and that the Sp1 protein was able to bind to DNA under our experimental conditions, we included a control in which a ^{32}P-labeled Sp1 consensus oligonucleotide was incubated with C3H10T1/2 nuclear extract. Following electrophoresis through a native polyacrylamide gel, similar binding profiles were obtained with all MRR probes, with the exception that complex 1 in the MRR^b and MRR$^{a/b}$ probe lanes appears to consist of only a single band, whereas MRR and MRR^a probes produce a complex 1 doublet (Fig. 8A, lanes 2, 4, 6, and 8). Supershift assays using an Sp1-specific antibody and competition EMSA in which protein binding to the MRR was challenged with an excess of Sp1 DNA confirmed that the upper band of the MRR complex 1 behaves in a manner consistent with it containing Sp1 (data not shown). To further examine the possibility that additional complexes appearing in the MRR profile contain an Sp1 complex that is able to bind to mutated DNA sites, or that binds cooperatively to those mutated sites with another protein, competition assays were again used (Fig. 8B). In one set of reactions, the MRR$^{a/b}$ probe was reacted with nuclear extracts in the presence of excess unlabeled MRR$^{a/b}$ or Sp1 DNA; in a second set of reactions, the labeled Sp1 oligonucleotide was reacted with nuclear extracts in the presence of excess unlabeled Sp1 or MRR$^{a/b}$ DNA. For both probes, competition with the homologous DNA was effective at a 50-fold molar excess, whereas competition with heterologous DNA was ineffective, even at a 100-fold molar excess. This provides additional evidence that the Sp1 present in the C3H10T1/2 nuclear extract does not contribute significantly to the protein complexes which form on the MRR. As a final demonstration that direct binding by Sp1 is not correlated with MRR transcriptional activity, EMSAs were performed with purified Sp1 protein under conditions which are optimal for Sp1 binding activity (see Materials and Methods for details) (Fig. 8C). As expected,

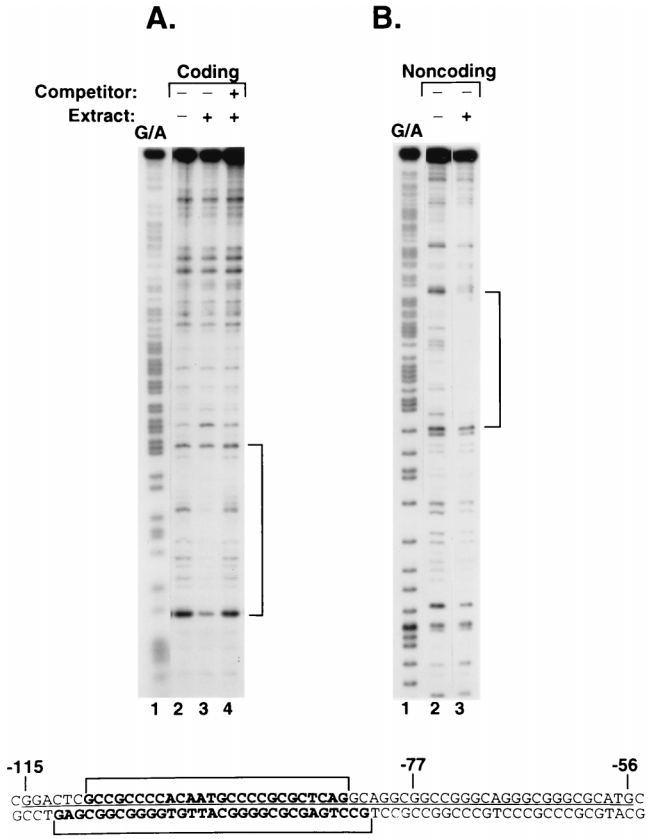

FIG. 6. DNase I footprinting of proteins binding to the chicken *max* MRR. A DNA probe containing the MRR and an additional 58 bp of DNA 5′ to the MRR region was ^{32}P labeled on the either the coding or noncoding strand and digested with DNase I following incubation with HeLa cell nuclear extracts as described in Materials and Methods. A Maxam-Gilbert G/A sequencing reaction of the probe DNA (lanes 1) was electrophoresed in parallel to locate the bases protected from DNase I digestion. (A) Footprint of the coding strand (lane 3) showing protection of bases −84 to −109 (bracket). A reaction in which a 100-fold molar excess of unlabeled −56 to −115 DNA was added as a competitor (lane 4) in a parallel binding reaction to lane 3 shows that the protection can be reversed. (B) Footprint of the noncoding strand showing protection (lane 3) of bases −113 to −82 (bracket). Below the footprinting gels is the DNA sequence of the −56 to −115 region showing the region of protein binding on each strand (brackets) and the −56 to −115 competitor DNA (underlined).

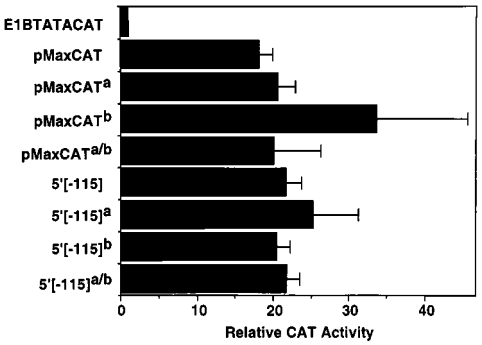

FIG. 7. The Sp1 consensus binding sites are not essential for MRR transcriptional activity. The Sp1 consensus sites within the MRR (Fig. 4B) were altered by site-directed mutagenesis as described in Materials and Methods, and the mutated MRRs were examined for activity following replacement into pMaxCAT (pMaxCAT[a], pMaxCAT[b], and pMaxCAT[a/b]) or the 5′[−115] reporter gene (5′[−115][a], 5′[−115][b], and 5′[−115][a/b]). C3H10T1/2 cells were transiently transfected with each construct, and cell extracts were assayed for CAT activity. Relative CAT activity is expressed as fold increase over that of E1BTATACAT, which is set at 1.0. Each value represents the average of at least three independent determinations, and the error bars indicate the standard error of each mean.

based on the presence of consensus Sp1 binding sites in the probes, the MRR, MRR[a], and MRR[b] DNA generate diffuse binding profiles containing a minor, high-molecular-weight component that comigrates similarly to the complex resulting from the binding of Sp1 to the Sp1 oligonucleotide (lanes 2, 4, 6, and 10), while the double mutant, MRR[a/b], does not (lane 8). Since reporter genes containing the Sp1[a/b] mutation are active transcriptionally (Fig. 7), we conclude that direct binding by Sp1 (or other members of the Sp1 family that share its recognition sequence) is not necessary for the activity of the chicken *max* promoter.

To confirm the data generated from EMSA by a different experimental approach, we next tested the activity of the pMaxCAT and 5′[−115] reporter genes in *Drosophila* S2 cells (65). These cells were selected based on the documented lack of Sp1 protein or Sp1 activity in this cell line (17) and on the observation that promoters regulated by Sp1 (e.g., the simian virus 40 promoter/enhancer) are activated transcriptionally in these cells following coexpression of Sp1 (17). S2 cells were cotransfected with 7.5 μg of pSV_2CAT, pMaxCAT, or 5′[−115] and 2.5 μg of a *Drosophila* expression vector containing the Sp1 cDNA. While the low, basal activity of pSV_2CAT was enhanced 7-fold following cotransfection of the Sp1 expression vector, the pMaxCAT and 5′[115] reporters were not and, in fact, produced lower levels of CAT activity than either pSV_2CAT or E1BTATACAT alone (Fig. 9 and data not shown). This result provides in vivo evidence that Sp1 alone cannot activate the chicken *max* promoter and suggests that if Sp1 plays a role in *max* gene transcription, it must do so by interacting indirectly with MRR binding proteins that also are absent in the S2 cell line. In support of this inference, EMSA with the full-length MRR probe and nuclear extract prepared from *Drosophila* cells reveal a binding profile dramatically different from that observed with mammalian or avian nuclear extracts (data not shown).

Transcription from the MRR is dependent on protein binding to positions −91 to −110. On the basis of transcription assays, EMSA, and DNase I footprinting, it is apparent that the DNA sequence between −77 and −115 binds proteins that are essential for the full activity of the *max* MRR. Based on our studies with the Sp1 sites that reside within (or very close to) that region, mutations that prevent the binding of Sp1 do not decrease the ability of the MRR to activate transcription. Therefore, to obtain additional information on the *cis*-acting DNA elements that are critical for the full activity of the MRR, we synthesized three double-stranded oligonucleotides containing mutations that affect contiguous, 10-bp segments of the DNA sequence between positions −77 and −115 (see Materials and Methods for details and sequences). These oligonucleotides, designated mI, mII, and mIII (with mutations spanning positions −110 to −101, −100 to −91, and −90 to −81, respectively), were used to replace the −77 to −115 region in the 5′[−115] reporter construct, and each gene construct was tested for transcriptional activity in C3H10T1/2 cells. As shown in Fig. 10A, 5′[−115]mI and 5′[−115]mII generate CAT activities that are not significantly different from the basal activity of E1BTATACAT, indicating that both of these 10-bp mutations completely abolish the function of the MRR in this assay. On the other hand, 5′[−115]mIII displays a level of CAT activity that is 10-fold above that of E1BTATACAT and approximately 40% of the activity of the wild-type MRR. These

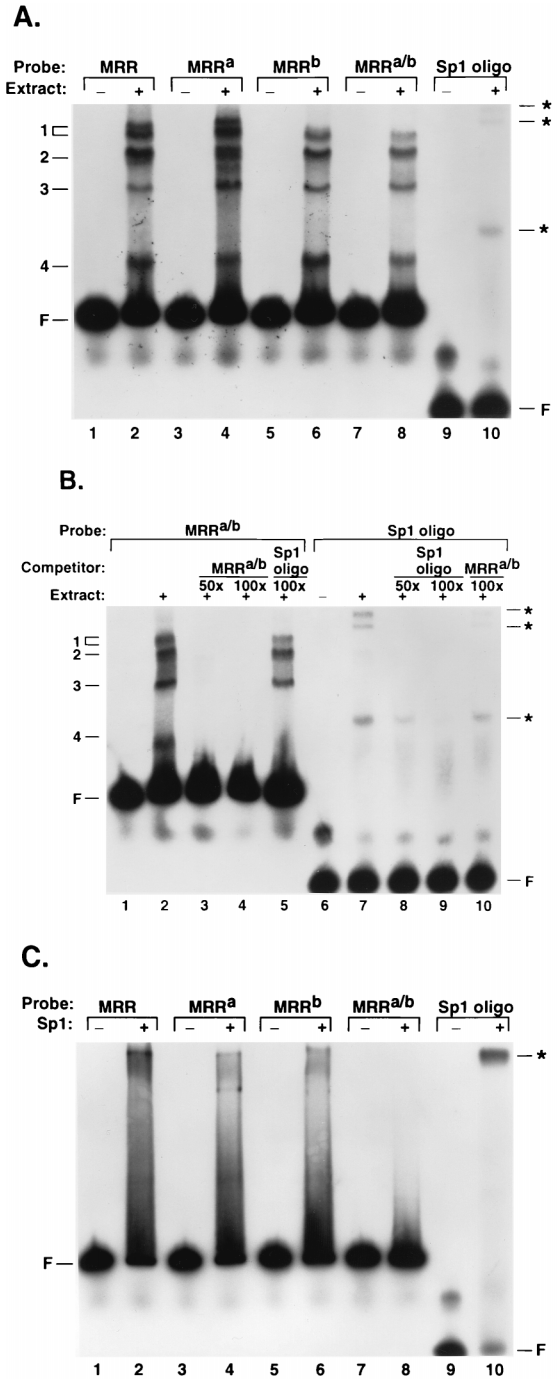

FIG. 8. Mutation of the Sp1 consensus sites does not alter the profile of the major protein-DNA complexes formed on the MRR. EMSAs were used to compare the profiles of protein binding to the wild-type MRR (−6 to −115) and to MRR probes containing the $Sp1^a$, $Sp1^b$, or $Sp1^{a/b}$ mutation. (A) The indicated radiolabeled probe DNA was incubated with C3H10T1/2 nuclear extract, and complexes were resolved by EMSA as described in Materials and Methods. A radiolabeled Sp1 oligonucleotide was used to verify that the C3H10T1/2 nuclear extract contains Sp1 protein. Lanes 1, 3, 5, 7, and 9 are probe alone; lanes 2, 4, 6, 8 and 10 are probe bound to extract. The numbers on the left indicate the migration of specific protein-MRR DNA complexes and are directly comparable to the complexes shown in Fig. 5. The asterisks on the right indicate the migration of protein-Sp1 DNA complexes. F marks the migration of the free probes. (B) Complexes formed by reacting C3H10T1/2 nuclear extract with the $MRR^{a/b}$ probe (lanes 1 and 2) were used in competitive EMSA with the indicated fold molar excess of homologous, unlabeled DNA (lanes 3 and 4) or Sp1 oligonucleotide (lane 5). The reverse experiment in which complexes bound to the Sp1 oligonucleotide (lanes 6 and 7) were challenged with the indicated fold molar excess of homologous competitor (lanes 8 and 9) or $MRR^{a/b}$ DNA (lane 10) was performed. Numbers to the left of the autoradiogram indicate the migration of specific protein-MRR DNA complexes. The asterisks to the right indicate the migration of protein-Sp1 DNA complexes. F indicates the migration of free, labeled probe DNA. (C) The experiment in panel A, repeated with purified Sp1 protein instead of C3H10T1/2 nuclear extract under binding conditions recommended by the supplier. Lanes 1, 3, 5, 7, and 9 are probe only; lanes 2, 4, 6, 8, and 10 are probe plus Sp1. The arrow on the right indicates the migration Sp1 protein bound to its consensus oligonucleotide. F indicates the migration of the free probes. While the Sp1 consensus sites in the wild-type MRR and the single-site mutants bind Sp1, the double mutant does not bind Sp1, despite retaining full transcriptional activity (Fig. 7).

observations agree with the results from DNase I footprints and strongly implicate the core sequence from −91 to −110 as being most critical to maintaining MRR function.

To investigate the protein binding profiles of these variants compared to the wild-type −77 to −115 region, EMSA were performed (Fig. 10B). A labeled probe, representing the wild-type −77 to −115 sequence, was allowed to bind C3H10T1/2 nuclear extract in the presence of unlabeled homologous competitor DNA (−77 to −115) or in the presence of mI, mII, or mIII DNA. A reaction in which a 100-bp fragment of pSP72 vector DNA was added as a nonspecific competitor served as a negative control (Fig. 10B, lane 10). A comparison of the EMSA profile of the −77 to −115 subregion of the MRR (Fig. 10B, lane 2) to that of the full-length MRR (Fig. 5, lane 2) shows the presence of the expected complexes 2 and 4 as well as a minor, higher-molecular-weight complex that we have designated 1′ since it is unique to the −77 to −115 probe and does not correspond to the doublet previously noted in Fig. 5 as complex 1. The mI oligonucleotide was not effective at competing any of the bands (lanes 4 and 5), and the mII oligonucleotide competed only complex 4 (lanes 6 and 7). However, the mIII probe, which, in the context of the MRR, maintains 40% of wild-type transcriptional activity, effectively competed complex 4 at a 100-fold excess and the majority of complex 2 at a 500-fold excess (lanes 8 and 9). This result suggests that the transcriptional activity of 5′[−115]mIII is due to its ability to bind complex 2, since mII apparently binds complex 4 yet does not display transcriptional activity. As for the novel complex 1′, this too was competed by mIII, but the significance of this observation is not clear, based on the fact that this complex is observed only in EMSA with the −77 to −115 probe. We conclude from these studies that the region designated most critical to MRR function (−91 to −110) interacts with a protein complex defined by EMSA as complex 2.

The protein binding to the −91 to −110 region of the *max* MRR is expressed in multiple cell lines and has an approximate molecular mass of 90 kDa. Efficient transcription of the pMaxCAT reporter gene in mouse, human, and chicken cells (Fig. 3 and data not shown), coupled with the absence of MRR transcriptional activity in insect cells (Fig. 9), suggests that the protein(s) that binds to the MRR (more specifically, complex 2 binding activity) is expressed ubiquitously in vertebrates. To examine this prediction, EMSAs were performed with nuclear extracts from C3H10T1/2 cells, CEFs, HeLa cells, and *Drosophila* S2 cells and the −77 to −115 subregion of the wild-type MRR as the probe. Figure 11A shows that strong complex 2 binding is observed with extracts prepared from cellular sources where the chicken *max* promoter is active (lanes 2 to 4), while no binding is observed in nuclear extract from *Drosophila* cells (lane 5).

To establish the number of proteins and the approximate molecular weights of the proteins that comprise complex 2 binding activity, we performed photoaffinity cross-linking. The

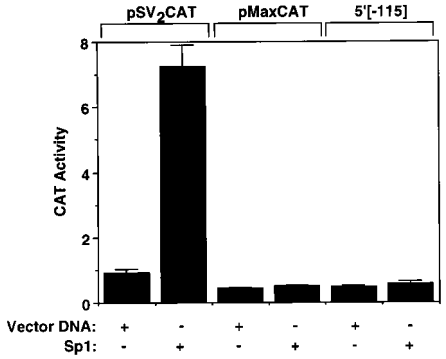

FIG. 9. The MRR is not transcriptionally active following ectopic expression of Sp1 in *Drosophila* S2 cells. S2 cells were transiently transfected as described in Materials and Methods with pMaxCAT, 5′[−115], or pSV_2CAT and, where indicated by +, the pADH vector (vector DNA) or pADH-Sp1 (Sp1). CAT activity was measured in cell extracts prepared from each group as described in Materials and Methods and is reported relative to the percent conversion of ^{14}C-chloramphenicol to the acetylated form by the pSV_2CAT reporter. Each value represents the average of five independent determinations; the error bars the indicate the standard error of each mean.

MRR −77 to −115 region was ^{32}P labeled and reacted with C3H10T1/2 nuclear extract, and the complexes were resolved by electrophoresis through a 5% native polyacrylamide gel. Following protein-DNA cross-linking under conditions optimal for generating one cross-linking event per protein-DNA complex (see Materials and Methods for details), complex 2 was eluted from the gel and concentrated by centrifugation, and the purified, cross-linked protein-DNA complex was subjected to SDS-PAGE (10% gel). As shown in Fig. 11B, a single radiolabeled band of approximately 130 kDa was observed. Subtracting the contribution of the radiolabeled −77 to −115 DNA (39 kDa) from the molecular weight of the complex, we estimate the size of the complex 2 protein to be approximately 90 kDa. As a positive control for the cross-linking procedure, a protein-DNA complex consisting of a ^{32}P-labeled AP-1 oligonucleotide and a protein heterodimer of bacterially expressed GST–c-Jun and GST–B-ATF fusion proteins was analyzed in parallel. As shown in Fig. 11B, lane 2, resolution of this complex revealed two proteins of 57 and 55 kDa, which, following subtraction of the molecular weight of the AP-1 oligonucleotide (15 kDa), correspond precisely to the sizes of the GST–c-Jun (42 kDa) and GST–B-ATF (40 kDa) fusion proteins (21). We conclude from this experiment that there is a single, 90-kDa protein species that contacts the MRR through the −91 to −110 core region associated with promoter activity, although the limitations of this technique prevent us from ruling out the possibility that additional proteins interact with this 90-kDa protein in vivo to mediate full MRR transcriptional activity.

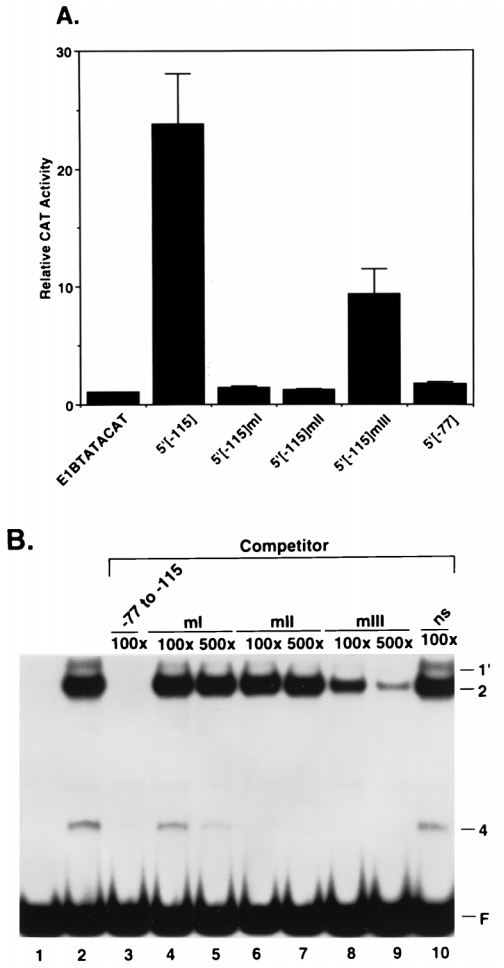

FIG. 10. Site-directed mutagenesis of the −77 to −115 region of the MRR identifies a functional element residing between −91 and −103 of the MRR. (A) 5′[−115] reporter genes engineered to contain nonoverlapping 10-bp changes in the sequence of the −77 to −115 region were tested for the ability to direct transcription in C3H10T1/2 cells as described in Materials and Methods. Relative CAT activity is expressed as fold increase over the activity of E1BTATCAT, which is set at 1.0. Each value represents the average of six independent determinations for 5′[−115] and mII and seven for E1BTATACAT, 5′[−77], mI, and mIII. Error bars indicate the standard error of each mean. (B) A radiolabeled probe covering −77 to −115 of the MRR (lane 1) was reacted with C3H10T1/2 nuclear extracts (lane 2) and used in competitive EMSA with the indicated fold molar excess of DNA representing a homologous sequence (−77 to −115) (lane 3) or the −77 to −115 region containing each of the 10-bp mutations: mI (lanes 4 and 5), mII (lanes 6 and 7), and mIII (lanes 8 and 9). Lane 10 shows the binding profile of the −77 to −115 probe competed with nonspecific DNA (ns). The numbers to the right indicate the migration of protein-DNA complexes bound to the −77 to −115 region of the MRR; with the exception of the 1′ complex which is unique to this probe, the remainder of the complexes are comparable to those formed on the full-length MRR (Fig. 7). F indicates the migration of the free probe DNA.

DISCUSSION

The emerging picture of Max as the sole heterodimerization partner for a growing family of bHLH/LZ factors that are expressed differentially during cell growth and terminal differentiation suggests that all components of the regulatory system maintaining intracellular Max levels are critical to the coordinated development of an organism. The *max* gene is single copy in human, mouse, and chicken cells (29, 58), and based on the early embryonic lethality of *max* null mice (48), the absence of Max cannot be compensated for by other cellular proteins. Cells are partially insulated against the deleterious effects of transient imbalances in the expression of the *max* gene since the *max* mRNA is very stable (72) and theoretically is available to be translated into protein long after the cessation of *max* gene transcription. However, posttranslation mechanisms of this sort are of short-term benefit, and ultimately it is the protein(s) that ensures constitutive transcription of the *max* gene under various growth conditions that is most critical to maintaining adequate intracellular levels of Max. Therefore, it is imperative that the molecular mechanism of *max* transcriptional control be defined, since it is likely that perturbation in the function of this regulatory network will mimic the indispensable role of the Max protein in vertebrate development.

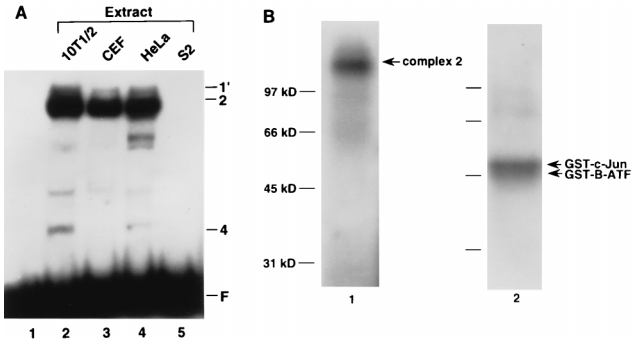

FIG. 11. (A) The protein binding profiles of the MRR −77 to −115 region were compared by EMSA with nuclear extracts prepared from cells in which the MRR is active (10T1/2 cells, CEFs, and HeLa cells) and nuclear extracts from *Drosophila* S2 cells, where the MRR is transcriptionally silent (Fig. 9). Complexes 1′, 2, and 4 are observed in 10T1/2 cells, CEFs, and HeLa cells (lanes 2, 3, and 4, respectively), while no protein binding is detected in S2 nuclear extract (lane 5). F indicates the migration of the free probe DNA. (B) Photoaffinity cross-linking was performed as described in Materials and Methods following binding of the −77 to −115 probe DNA to C3H10T1/2 nuclear extracts and resolution of the protein-DNA complexes by gel electrophoresis. MRR complex 2 was purified and analyzed by SDS-PAGE using low-molecular-weight (lane 1) and high-molecular-weight (not shown) markers. A control cross-linking reaction with a radiolabeled AP-1 DNA and two fusion proteins (GST–c-Jun and GST–B-ATF) which bind to AP-1 DNA as a heterodimer (21) was analyzed by SDS-PAGE (lane 2) and shows the resolution of two proteins of the appropriate molecular weights. Protein standards, the migration of which was established by Coomassie blue staining of the gel prior to autoradiography, are indicated to the left of each panel.

The complexity and large size of the human and mouse *max* genomic loci (67) have hampered efforts to analyze *max* gene regulation in these species. Fortunately, the chicken *max* gene is contained on a 6-kb *Bgl*II fragment (68), and in this study, we demonstrate that this fragment directs production of chicken *max* mRNA in chicken, mouse, and human cells. More importantly, the 5′ flanking sequence of the *max* gene contained within this fragment directs reporter gene expression in these same cell types, indicating that the *cis-trans* regulatory system responsible for *max* gene transcription is conserved across vertebrate species and is located within this 2-kb genomic sequence. Not surprisingly, inspection of the 5′ flanking DNA of the chicken *max* gene reveals many features common to constitutive, eukaryotic promoters, including the lack of TATA and CAAT consensus DNA sequences and a high GC content with potential binding sites for Sp1 (8, 22). The 5′ region also does not appear to have any initiator elements (reviewed in references 8 and 74), which may explain the presence of several transcription initiation sites (Fig. 4) (28, 33). Our studies show that in contrast to many TATA-less promoters (16, 28, 39, 52), the MRR of the *max* promoter does not require direct binding by Sp1 for activity, despite containing two Sp1 consensus sites which are capable of binding purified Sp1 protein in vitro (Fig. 8C). Instead, EMSA, DNase I footprinting, and site-directed mutagenesis implicate a 20-bp region of the MRR between positions −91 and −110 as being critical to the maintenance of promoter activity. The center of this element is positioned 25 bp from the 5′-most transcription initiation start point mapped for the pMaxCAT reporter gene in C3H10T1/2 cells (Fig. 4B) and thus is located in an appropriate site to bind proteins that facilitate transcription initiation.

As a first step toward identifying the key cellular proteins that bind to the −91 to −110 region of the MRR, we used competitive EMSA. Although we detected minor differences in protein binding profiles which were probe dependent, a major protein-DNA complex, designated complex 2, was correlated strictly with promoter function. Interestingly, complex 2 is detected in assays using nuclear extracts prepared from cells in which the *max* promoter is active but is not observed with nuclear extracts from insect cells where the *max* promoter is silent. While this evidence strongly supports the conclusion that the formation of complex 2 is necessary for *max* promoter activity, it does not address whether complex 2 is sufficient for promoter activity, and it remains formally possible that additional cellular factors interact with complex 2 in vivo to facilitate *max* gene expression. To establish the number and relative sizes of the proteins that comprise complex 2, photoaffinity cross-linking under conditions optimal for generating one cross-linking event per molecule were performed (5). Results with this technique show that complex 2 contains a single DNA binding protein with a molecular mass of approximately 90 kDa (Fig. 11B). Outside of the one Sp1 binding site ($Sp1^a$) located between positions −103 and −109, which we show is not necessary for transcription from the MRR (Fig. 7), a database search indicated that the MRR region from −77 to −115 contains loose consensus binding sites for AP-2 (52 kDa) (75), LSF (63 kDa) (35), BGP1 (66 kDa) (49), and GCF (97 kDa) (9). GCF may be within the right size range, but to date, GCF has been shown to function exclusively as a transcriptional inhibitor (41, 45). Therefore, we do not believe that the protein in complex 2 is any of these previously characterized transcription factors. To determine if the DNA element implicated in the binding to the 90-kDa protein is present in the sequences of other eukaryotic genes, we used the MRR sequence from −77 to −115 in an Entrez BLAST search. The human beta-kinesin gene (accession number X69658) and *lyl-1* gene (accession number M22638) contain sequences with 88 and 84% similarity to the minus strand of the MRR region from −88 to −112 and from −81 to −106, respectively. In the beta-kinesin gene, this element is located 35 bp upstream of the TATA box in a region containing several binding sites for Sp1. In the *lyl-1* gene, the MRR homology element is located within the first intron 1,126 bp upstream of the methionine initiation codon.

In attempts to develop an assay that can be exploited to isolate a cDNA encoding the 90-kDa MRR binding protein, tandem copies of the wild-type −77 to −115 region have been ligated upstream of E1BTATACAT, and in preliminary studies, this construct drives CAT gene expression in C3H10T1/2 cells (60). This wild-type DNA element, in conjunction with mutated elements (for example, the $Sp1^a$ element), will be used for expression library screening in *Escherichia coli*, or alternatively, library screening in a yeast one-hybrid system (73), to isolate cDNAs encoding the proteins that bind to this core DNA sequence. One possible outcome of the screen would be the identification of a novel transcription factor that binds specifically to the MRR core and that possesses a domain that functions to activate transcription. However, it also is possible that the 90-kDa protein is a histone-like protein which functions through the MRR to increase the accessibility of the *max* promoter to factors that do activate transcription directly. In this regard, during the cloning and sequencing of the chicken *max* gene, we encountered many technical difficulties that we attributed to the presence of extensive secondary structure in the naked DNA, a feature that could negatively impact transcription of the locus. Certainly it would be of interest in future studies to map the DNase I-hypersensitive sites in the *max* gene promoter region, as well as to perform in vivo footprinting to address this issue experimentally.

In performing the experiments in this study, we were aware of the inherent contradiction in attempting to characterize regulatory proteins that mediate constitutive gene expression.

In fact, using the chicken *max* gene, we tested a number of cell growth conditions and explored a variety of model systems of cellular differentiation to uncover a situation in which *max* gene expression was regulated in the traditional sense. Our lack of success in these efforts, while initially disappointing, also was intriguing since it implies that *max* transcription is unaffected by intracellular signaling pathways that are known to alter cellular gene expression, either by inducing or repressing genes encoding transcription factors or by posttranslationally modifying existing factors to alter their activities. Future experiments in which we characterize the 90-kDa protein described in our studies and investigate how this protein fits into a molecular network that has escaped regulation by a host of intracellular signaling pathways will lead to the identification of novel cellular proteins whose functions are critical to the growth and survival of eukaryotic organisms.

ACKNOWLEDGMENTS

We extend thanks to Steve Konieczny for supplying us with the pADH-R1 and pADH-Sp1 expression vectors, Rick Peters for assistance in interpreting the results of the cross-linking experiments, Ourania Andrisani for advice on preparing nuclear extracts, and Allan Bieber for supplying the *Drosophila* S2 cells.

This work was supported by grants awarded to E.J.T. from the National Cancer Institute and from the American Cancer Society. M.A.P. was supported by a Special Initiatives Fellowship from Purdue University and is currently a predoctoral trainee of NIH T32 GM08298. T-L.K. was a postdoctoral trainee of NIH T32 DK07532. E.J.T. is a Scholar of the Leukemia Society of America.

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