A Minimal Murine Msx-1 Gene Promoter

ORIENTATION OF ITS cis-REGULATORY MOTIFS AND THEIR ROLE IN TRANSCRIPTIONAL ACTIVATION IN CELLS IN CULTURE AND IN TRANSGENIC MICE*

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To dissect the cis-regulatory elements of the murine Msx-1 promoter, which lacks a conventional TATA element, a putative minimal Msx-1 promoter DNA fragment (from −1282 to +106 base pairs (bp)) or its congeners containing site-specific alterations were fused to luciferase reporter and introduced into NIH3T3 and C2C12 cells, and the expression of luciferase was assessed in transient expression assays. The functional consequences of the sequential 5′ deletions of the promoter revealed that multiple positive and negative regulatory elements participate in regulating transcription of the Msx-1 gene. Surprisingly, however, the optimal expression of Msx-1 promoter in either NIH3T3 or C2C12 cells required only 165 bp of the upstream sequence to warrant detailed examination of its structure. Therefore, the functional consequences of site-specific deletions and point mutations of the cis-acting elements of the minimal Msx-1 promoter were systematically examined. Concomitantly, potential transcriptional factor(s) interacting with the cis-acting elements of the minimal promoter were also studied by gel electrophoretic mobility shift assays and DNase I footprinting. Combined analyses of the minimal promoter by DNase I footprinting, electrophoretic mobility shift assays, and super shift assays with specific antibodies revealed that 5′-flanking regions from −161 to −154 and from −26 to −13 of the Msx-1 promoter contains an authentic E box (proximal E box), capable of binding a protein immunologically related to the upstream stimulating factor 1 (USF-1) and a GC-rich sequence motif which can bind to Sp1 (proximal Sp1), respectively. Additionally, we observed that the promoter activation was seriously hampered if the proximal E box was removed or mutated, and the promoter activity was eliminated completely if the proximal Sp1 site was similarly altered. Absolute dependence of the Msx-1 minimal promoter on Sp1 could be demonstrated by transient expression assays in the Sp1-deficient Drosophila cell line cotransfected with Msx-1-luciferase and an Sp1 expression vector pPacSp1. The transgenic mice embryos containing −165/106-bp Msx-1 promoter-LacZ DNA in their genomes abundantly expressed β-galactosidase in maxillae and mandibles and in the cellular primordia involved in the formation of the meninges and the bones of the skull. Thus, the truncated murine Msx-1 promoter can target expression of a heterologous gene in the craniofacial tissues of transgenic embryos known for high level of expression of the endogenous Msx-1 gene and found to be severely defective in the Msx-1 knock-out mice.

Homeobox (Hox) genes of vertebrates are closely related in sequence and genomic organization to the homeotic genes of Drosophila. Most vertebrate Hox genes are located in four unique clusters in the genome (e.g. HoxA, HoxB, HoxC, and HoxD complexes), each cluster consisting of about 10 genes; there is striking correlation between the linear order of Hox genes on the chromosome and their regional expression in the developing embryo (1, 2). In contrast, the members of the Msx class of hox genes, which also share remarkable homology to the msh gene of Drosophila, are found physically unlinked in the vertebrate genome (3, 4). Although Hox genes encode transcription factors, characterized by the presence of a highly conserved 60-amino acid-long helix-turn-helix DNA binding domain, the homeodomain, the downstream genetic targets of their regulation, and the underlying molecular mechanisms of their action are only beginning to be unraveled (5–7).

In the developing embryo, Hox genes play a central role in positional specification, pattern formation, and organogenesis; it is thought that inductive interactions among the various cell layers, mediated through the action of intercellular ligands with their receptors, and a cascade of signaling events regulate the temporal and spatial expression of Hox genes (4, 8–15). Inappropriate ectopic expression of Hox genes or their elimination by genetic “knock-out” leads to severe developmental anomalies (16–18).

Hox genes Msx-1 and Msx-2, the best studied members of the Msx family, have been shown to be expressed most conspicuously in the areas of epithelial-mesenchymal interactions (4). High levels of Msx-1 gene expression observed in the developing limb bud (19–26), regenerating limbs (27) or fins (28), developing eyes (29, 30), or molar teeth (31, 32) imply that Msx-1 plays a critical role during organogenesis. Defective expression of Msx-1 in the limb bud mesenchyme of chicken mutants limbless and talpid has been reported; apparently the embryos of limbless mutants failed to assemble an active apical ectodermal ridge, and the underlying mesoderm expressed little or no Msx-1 transcripts (33, 34). Implantation of apical ectodermal ridge from a wild type embryo above the limbless mesoderm restored Msx-1 gene expression (33). Therefore, it appears that the cells of apical ectodermal ridge, either through cell-cell contact or through diffusible factors, regulate Msx-1 gene transcription (23, 24, 29–36).

Concomitant alterations of Msx-1 gene expression and mirror image duplications of digits in response to 9-cis-retinoic acid were also studied in the transgenic mice harboring truncated Msx-1 promoters. These studies demonstrated that the expression of Msx-1 is significantly affected by the deletion of the upstream region containing the proximal E box, which is necessary for the optimal expression of the gene in vivo. In addition, we observed that the expression of Msx-1 is down-regulated by the insertion of an upstream stimulatory factor (USF-1) binding site, which is capable of regulating the expression of a heterologous gene in transgenic mice. These findings suggest that the cis-acting elements of the Msx-1 promoter play an important role in the regulation of gene expression in vivo and in vitro.
Acid (37) or fibroblast growth factor-2 or -4 (38–42) suggest that these phenomena may be causally related to each other, and therefore, the molecular mechanisms of Msx-1 gene regulation warrant further investigation. Earlier we described the structural organization of the coding and noncoding sequences of the Msx-1 gene and reported data that suggested that Msx-1 gene expression may be subject to autoregulation (43). We carried out a detailed functional analysis of ~5 kb of 5'-flanking genomic DNA of Msx-1 with an aim to elucidate the putative cis-acting elements which mediate Msx-1 gene transcription in NIH3T3 and C3C12 cells. We report that a 165/ +106-bp minimal Msx-1 promoter, containing sequence motifs capable of interacting with helix-loop-helix proteins (proximal E box) and a ubiquitous transcriptional modulator, Sp1 (proximal Sp1), is sufficiently active in driving the expression of luciferase in cells in culture. Furthermore, our analysis of the bacterial LacZ expression driven by the minimal Msx-1 promoter in transgenic mice suggests that the minimal Msx-1 promoter is exquisitely activated in the structures derived from epithelial and mesenchymal cell layers during craniofacial morphogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 cells (ATCC, CRL1658) and C3C12 cells (ATCC, CRL1772) were bought from the American Tissue Culture Collection, Bethesda, MD; cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. 1282 bp upstream and 106 bp downstream of the transcription start point was amplified by PCR from genomic DNA with an aim to elucidate the putative cis-acting elements which mediate Msx-1 gene transcription in NIH3T3 and C3C12 cells. We report that a 165/+106-bp minimal Msx-1 promoter, containing sequence motifs capable of interacting with helix-loop-helix proteins (proximal E box) and a ubiquitous transcriptional modulator, Sp1 (proximal Sp1), is sufficiently active in driving the expression of luciferase in cells in culture. Furthermore, our analysis of the bacterial LacZ expression driven by the minimal Msx-1 promoter in transgenic mice suggests that the minimal Msx-1 promoter is exquisitely activated in the structures derived from epithelial and mesenchymal cell layers during craniofacial morphogenesis.

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Activation of Murine Msx-1 Promoter

RESULTS

Selection of Cells to Study Msx-1 Promoter Function by Transient or Stable Expression Assays—Since Msx-1 shows a complex pattern of expression elicited at many sites of epithelial and mesenchymal cell interactions during embryogenesis (4), ideally, the Msx-1 promoter activation should be studied in vitro under conditions which mimic cell-cell interactions leading to organogenesis. While a number of in vitro organ cultures have been developed for such studies (e.g. cultured limb buds), it is not possible to efficiently transfect Msx-1 promoter-reporter constructs into all cells in such an organ culture system. Therefore, we surveyed a number of established cell lines for high levels of Msx-1 expression since such cells are likely to contain the necessary trans-acting factors to activate the Msx-1 promoter. As shown in Fig. 1, the steady state levels of Msx-1 transcripts in the NIH3T3 fibroblasts, C2C12 myoblasts, or a Msx-1 promoter. As shown in Fig. 1, the steady state levels of Msx-1 expression since such cells are likely to contain the necessary trans-acting factors to activate the Msx-1 promoter. As shown in Fig. 1, the steady state levels of Msx-1 expression since such cells are likely to contain the necessary trans-acting factors to activate the Msx-1 promoter.

moter-reporter constructs in C2C12 and NIH3T3 cells. Both NIH3T3 and C2C12 cells are readily transfectable by the LipofectAMINE™ method used in our studies. Although we tested a subset of the Msx-1 promoter-luc or Msx-1 promoter-LacZ constructs in stably transfected clones of C2C12 cells, we will restrict our discussion to transient expression assays, since no major discrepancy was noted in the promoter activation studied with the two types of assays.

Serial Truncations of the 5′-Flanking Sequences Reveal Positive and Negative cis-Acting Elements and Delimit a Minimal Msx-1 Promoter—Our previously published computer-based homology analysis of the Msx-1 promoter revealed several putative cis-acting elements (43). To experimentally dissect the functional Msx-1 promoter, serially truncated 5′-flanking Msx-1 genomic DNA fragments were ligated in front of the luciferase reporter in the pGL2-Basic Vector (Fig. 2); promoter activities were deduced from quantitation of luciferase assays in transiently transfected NIH3T3 and C2C12 cells. Both of these cell lines express moderate levels of Msx-1 and are therefore expected to contain the adequate levels of trans-acting factors to support the activation of Msx-1 promoter-luciferase constructs. Although we sequentially analyzed 5 kb of 5′-flanking Msx-1 DNA for promoter activity, the sequences between −5 kb and −1282 bp had no detectable enhancement over the −1280-bp promoter in our assays. Therefore, the expression of −1282/106-bp promoter (full-length promoter) was arbitrarily fixed as 100%, and the activities of all other constructs were compared with the full-length promoter. Compared with the full-length promoter, while some deletions in the 5′-flanking DNA of Msx-1 caused a modest to severe decline in luciferase activity (e.g. −1168/+106, −509/+106, and −127/ +106 bp constructs), the removal of some DNA sequences (e.g. −886/+106, −811/+106, −268/+106, and −165/+106 bp constructs) led to enhanced expression of the reporter gene (Fig. 3). It should be stressed however that although the ap-

2 C. Guron and R. Raghow, unpublished observations.

3 T. Takahashi and R. Raghow, unpublished data.
parent positive or negative modulatory consequences of particular deletions were rather modest, the overall quantitative patterns were strikingly similar and reproducible in both NIH3T3 and C2C12 cells (Fig. 3). Unlike NIH3T3 cells, C2C12 cells are capable of undergoing myogenic differentiation when they are cultivated in serum-free DMEM for many hours. Thus, it is conceivable that there are additional factors in C2C12 cells which may preferentially interact with some cis-regulatory sequences of the Msx-1 promoter. The activity of the 2165/1106-bp promoter compares favorably with the longer constructs, and further shortening the 2165/1106-bp promoter (e.g., 1105/1106- or 133/1106-bp promoter-luciferase constructs) abolished its activity completely in both cell lines (Fig. 3). Therefore, we have tentatively named the 165/1106-bp promoter as a “minimal” Msx-1 promoter.

The Proximal E Box and Sp1 Motifs Are Essential for Optimal Activity of the Minimal Msx-1 Promoter—Careful and detailed analysis of expression of sequentially deleted Msx-1 promoter-luciferase constructs in transient expression assays convinced us to examine the minimal promoter more rigorously to assess the contribution of the putative trans-acting factor(s) which may interact within the minimal promoter (165/1106 bp). The location in the truncated promoter of one of the three consensus E box elements (the proximal E box) and one of the three consensus GC boxes known to bind Sp1 (the proximal Sp1), predicted theoretically, prompted us to experimentally test the function of these two DNA elements individually. Msx-1 promoter-luciferase constructs, from which either proximal E box or Sp1, or both, had been deleted, were tested for promoter activities. We observed that deleting either one of these elements, regardless of the presence or absence of additional 5′-flanking DNA, caused nearly complete loss of the Msx-1 promoter activity (Fig. 4). Another, somewhat intriguing, observation came out of this analysis; we also noticed that while the terminal deletions in the Msx-1 upstream sequences had less severe effects on the promoter, the internal deletions almost completely abolished promoter function in both cell lines. We extended these data by introducing 4-bp block mutations in the proximal E box or Sp1 motifs individually and tested the effects of these perturbations in the context of the
As depicted in Fig. 5 mutations in either the proximal E box or the proximal GC box singly caused severe reduction in the expression of luciferase (1.2% and 4.1% activities remaining, respectively). Curiously, however, in contrast to what occurred with the block mutations at single sequence motifs, when both mutations were mobilized in the same construct a substantial level of luciferase activity was restored (Fig. 5). At present we can only speculate as to the cause of this phenomenon; conceivably, binding site(s) for an additional factor(s) were created as a result of combining both mutated E box and GC box sites on a contiguous fragment of DNA.

Nuclear Proteins from NIH3T3 and C2C12 Cells Bind to the Proximal E Box of the Msx-1 Promoter—Our transient expression data indicated that the proximal E box and Sp1 sites were critical for Msx-1 promoter activity. To explore directly whether putative transcription factors bound to sequences predicted by the deletion assays, we performed DNase I footprinting and EMSA experiments with radiolabeled DNA fragments encompassing the potential cis-regulatory sites incubated with nuclear extracts from NIH3T3 or C2C12 cells. When radiolabeled −91- to +106- and −268- to +106-bp DNA fragments were subjected to footprinting analyses, one clearly discernible area of protection from DNase I breakdown, Fp-1, could be consistently seen (Fig. 6); the identity of Fp-1, encompassing nucleotides numbered −26 to −13, was established to be an Sp1-like motif by more extensive analyses. The footprinted area marked Fp-2 was obtained less consistently; Fp-2 encompassed the sequence motif identified as the proximal E box by rigorous EMSA and site-specific mutagenesis experiments. Both putative motifs were recognized by a nuclear protein(s) from C2C12 cells (Fig. 6) and nuclear extracts prepared from NIH3T3 cells.3 We extended the DNase footprinting data by testing the specificity of binding of the putative factors with two types of EMSA experiments.
were used to competitively inhibit binding of the putative E box proteins to −165/−128 oligonucleotide while −165/−147 oligonucleotide inhibited binding very effectively (Fig. 7A, lane 3), the two truncated oligonucleotides, −156/−138 and −146/−128, did not displace the protein-bound DNA (Fig. 7A, lanes 4 and 5, respectively). The DNA binding region was narrowed to −165/−147 oligonucleotide by EMSA (Fig. 7B, lane 1) since neither −156/−138 (Fig. 7B, lane 2) nor −146/−128 (Fig. 7B, lane 3) oligonucleotides bound to trans-acting factor(s) from C2C12 extracts. To extend these observations, more specific mutations were created within the putative E box motif or immediately contiguous sequences, and the mutant double-stranded oligonucleotides were used as competitors to displace binding of the radioactively −165/−147 oligonucleotide. As shown in Fig. 7C, while M−165/−162 efficiently competed out binding of proteins to the proximal E box (lane 3), either M−161/−158 or M−157/−154 oligonucleotides failed to do so (Fig. 7C, lanes 4 and 5, respectively). Based on these data we conclude that the region of DNA encompassing nucleotides from −161 to −154 of Msx-1 promoter is essential for binding to the putative E box-recognizing factor(s). Identity of the proximal E box-binding factor was further investigated by competition with oligonucleotides with previously well-defined E box motifs; additionally, we carried out formation of DNA-protein complexes in the presence of antibodies to the basic helix-loop-helix proteins known to recognize the core E box motif in the context of additional contiguous sequences. As shown in Fig. 8, a consensus upstream stimulating factor-1 (USF-1) oligonucleotide competed with the Msx-1 oligonucleotide (Fig. 8, lane 4); a 50-fold excess of the consensus MEF-1 oligonucleotide was unable to dislodge this complex under identical conditions (Fig. 8A, lane 3). For unknown reasons, the apparent reduction of DNA-protein complex formation seen with MEF-1 oligonucleotide did not occur consistently.4 Furthermore, Msx-1 proximal

FIG. 5. Mutations in the proximal Sp1 or proximal E box elements of Msx-1 promoter eliminate its activity in C2C12 cells. Schematic representation of Msx-1 promoter and the locations of the mutated sites are indicated. The numbers indicate the 5′ and 3′ ends of each promoter insert, numbered in relation to the transcription start site. Luciferase expression is depicted as percent light intensity units, relative to the expression of the −886/+106-bp construct arbitrarily set at 100%. Paradoxically, while the individual mutations in Sp1 or E box motifs nearly completely inactivate the promoter, the activity of the promoter containing both sites mutated is substantially restored for unknown reasons.

FIG. 6. DNase I footprinting analysis of the Msx-1 promoter. Radioabeled DNA fragments were incubated with nuclear extracts from C2C12 cells, and the protected sequences were analyzed as described under “Experimental Procedures.” The data obtained with the labeled −91/+106-bp DNA are shown in the left autoradiograph (A) while the protection of the labeled −268/+106-bp DNA is illustrated on the right (B). A, lane 1, Maxam and Gilbert G + A sequencing ladder; lane 2, control DNase I reaction of probe DNA without nuclear extract; lane 3, 20 μg of nuclear extract; lane 4, 20 μg of nuclear extract in the presence of 50 ng of a Sp1 consensus oligonucleotide as competitor; lane 5, radiolabeled probe incubated with 5 footprint units of human Sp1 protein. B, lane 1, G + A sequence ladder; lane 2, control DNA without cell extract; lane 3, DNase I reactions with 20 μg of cell extract; lane 4, DNase I footprint in the presence of self-competitor; lane 5, radiolabeled probe incubated with 5 footprint units of human Sp1 protein. The boxes on the right side of each autoradiograph denote the boundaries of the footprinted regions marked Fp-1 (the proximal Sp1) and Fp-2 (the proximal E box); the precise sequence of Fp-1 is depicted on the right side of A.

E box-protein complexes could be readily supershifted with antibodies to USF-1 (Fig. 7B, lane 3); similar incubation of these complexes with antibodies to USF-2 resulted in a weakly supershifted band (Fig. 8B, lane 4).4 We should also mention here that polyclonal antibodies to a number of other E box-binding proteins which include MyoD, Myf5, Myf6, and myogenin failed to interact with the proximal E box DNA-protein complexes.5

As shown in an earlier experiment (Figs. 2 and 3), the deletion of sequences encompassing the distal E box element had a positive effect on the promoter activity in both NIH3T3 and C2C12 cells. When promoter constructs containing point mutations in the distal E box were quantitatively assessed for their positive effect on the promoter activity in transiently transfected C2C12 cells, the results corroborated the data obtained with terminal deletions; the presence of the Msx-1 promoter of a mutated distal E box element (disabled to form DNA-protein complexes) substantially

S. Shetty and R. Raghow, unpublished observations.

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boosted the Msx-1 promoter activity in a reproducible manner (Fig. 9). Thus, we were curious to compare the putative nuclear factor(s) binding to the distal E box (21164 to 21159) with the factor(s) that recognized the proximal E box (2159 to 2154). We discovered that the distal E box oligonucleotide, which formed three prominent protein-DNA complexes as revealed by EMSA (Fig. 10, lane 2), competed effectively with itself (lane 3), but failed to dislodge trans-acting factor(s) bound to the prox-

**FIG. 7.** C2C12 nuclear protein(s) binds to the proximal E box motif of the Msx-1 promoter. A, nuclear extracts from C2C12 cells were incubated with proximal E box probe (−165/−128) in the absence or presence of unlabeled double-stranded competitors and subjected to EMSA. Lane 1, probe alone; lanes 2–5, 8 μg of C2C12 nuclear extract; lanes 3–5, 50-fold molar excess of competitors, −165/−147 (lane 3), −156/−138 (lane 4), and −146/−128 (lane 5). Specific DNA-protein complexes are indicated with an arrow: B, the labeled oligonucleotides −165/−147 (lane 1), −156/−138 (lane 2), and −146/−128 (lane 3) were used as probes and incubated with C2C12 nuclear extract. C, the radiolabeled oligonucleotide (−165/−147) was incubated with C2C12 nuclear extracts in the absence or presence of unlabeled competitors as indicated. Lane 1, probe alone; lanes 2–5, 13 μg of C2C12 nuclear extract; lanes 3–5, 40-fold molar excess of competitors, M−165/−162 (lane 3), M−161/−158 (lane 4), and M−157/−154 (lane 5).

**FIG. 8.** DNA-protein complexes formed with the proximal E box of Msx-1 promoter can be competitively abolished with a consensus USF-1 oligonucleotide and supershifted with an antibody against USF-1. The DNA-protein complex formation (arrowhead) is inhibited by the presence of 50-fold molar excess of USF-1 oligonucleotide (A, lane 4) while a similar concentration of MEF-specific oligonucleotide is much less effective (lane 3). Antibodies to USF-1 efficiently supershift the complex as marked by SS (B, lane 3); under identical conditions of incubation antibodies to USF-2 are at least 20-fold less effective in producing the supershifted complex (B, lane 4).

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**FIG. 9.** Expression of luciferase driven by the full-length (−1282/+106), wild type distal E box-containing truncated (−1168/+106), or a mutated E box-containing truncated (−1168M) promoter in transiently transfected C2C12 cells. Sequence of the wild type distal E box and its mutated counterpart is shown in A. The percent luciferase activities, relative to the full-length promoter, indicate that mutation of the distal E box leads to enhancement of the truncated promoter as shown in B. The bars represent S.E. of means of five independent experiments and have been corrected for the experimental variations in the transfection efficiency.

imal E box motif (Fig. 10, lane 1). The proximal E box oligonucleotide bound to protein formed one shifted band (Fig. 10, lane 9) and was an excellent self-competitor (Fig. 10, lane 10) but failed to inhibit DNA-protein complex formation with the distal E box oligonucleotide (Fig. 10, lane 5). As expected, a mutated distal E box oligonucleotide did not dislodge DNA-protein complexes formed with either the distal (lane 4) or the proximal (Fig. 10, lane 12) E box oligonucleotides. The apparent lack of reciprocity displayed in the cross-competition experiments suggests that an apparently unique protein factor(s) bind to the distal and proximal E box motifs.

**The Minimal Msx-1 Promoter Has a Bona Fide Sp1 Binding Motif—** Transient expression assays demonstrated that the Msx-1 promoter containing only 32 bp upstream of the tran-
FIG. 10. Different nuclear protein(s) bind to the proximal and distal E boxes of Msx-1 promoter. Electrophoretic mobility shift assay with oligonucleotide containing distal E box and competition assay with distal and proximal E box. Wild type distal E box oligonucleotide, −1168/−1147 (lanes 1–5), mutated distal E box oligonucleotide, −1168/−1147Mut (lanes 6 and 7), or the proximal E box oligonucleotide, −165/−146 (lanes 8–12) were end-labeled and incubated with C2C12 nuclear extracts, in the absence or presence of unlabeled competitors. Lanes 2, 5, 7, and 9–12, 10 μg of C2C12 nuclear extract; lanes 3–5 and 10–12, 40-fold molar excess of competitors, −1168/−1148Wild (lanes 3 and 11), −1168/−1148Mut (lanes 4 and 12), and −165/−146 (lanes 5 and 10).

sorbed start site was sufficiently active to warrant a more thorough characterization of the “core promoter.” Therefore, the core promoter was analyzed in detail by EMSA for binding of the putative transcription factor(s). Analyses using EMSA and an antibody-mediated electrophoretic mobility supershift revealed that both C2C12 and NIH3T3 nuclear extracts contained readily detectable levels of Sp1 proteins which interacted with Msx-1 oligonucleotide encompassing −32/+2 (Fig. 11, lanes 2 and 4, respectively). Binding could be competed with cold, wild type oligonucleotide (Fig. 11, lanes 3 and 5, respectively) but failed to be competed with mutant oligonucleotides, M−29/+19, M−18/+15, and M−14/+11 (Table I; data not shown). We extended the competitive binding assays by incubating radiolabeled −32/+2 oligonucleotides with either C2C12 nuclear extracts or with purified Sp1 and carried out binding reactions in the presence of a polyclonal antibody specific for human Sp1 and known to cross-react with murine, rat, and human Sp-1. The radiolabeled bands, representing Sp1-bound DNA complexes, could be readily supershifted, regardless of whether the source of Sp1 was nuclear extracts from C2C12 cells (Fig. 11B, lanes 2 and 3), from NIH3T3 cells (Fig. 11B, lanes 4 and 5) or purified Sp1 (Fig. 11B, lanes 6 and 7). It appears that proximal Sp1 oligonucleotide formed multiple complexes when incubated with nuclear extracts; this is in contrast to what occurred with the purified Sp1. We believe that this result is not unexpected since Sp1 in the cells may be present in a variety of posttranslationally modified states or may be bound to other factors. Based on these data we conclude that the core promoter binds to a trans-acting factor(s) which is immunologically related to the authentic Sp1.

FIG. 11. EMSA shows that nuclear extracts from C2C12 and NIH3T3 bind to the proximal Sp1 motif of the Msx-1 promoter. A, the proximal Sp1 oligonucleotide (−32/+2) was end-labeled and incubated with C2C12 or NIH3T3 nuclear extracts in the absence or presence of unlabeled double-stranded competitor. Lane 1, probe alone; lanes 2 and 3, C2C12 nuclear extract; lanes 4 and 5, NIH3T3 nuclear extract; lanes 3 and 5, 40-fold molar excess of unlabeled −32/+2 oligo. The arrows indicate specific DNA-protein complexes, and SS denotes the supershifted band. B, electrophoretic mobility supershift experiments using labeled proximal Sp1 site. Labeled oligonucleotides containing the proximal Sp1 motif were incubated with C2C12 or NIH3T3 nuclear extracts in the presence of anti-Sp1 polyclonal antibody. Lane 1, radiolabeled −32/+2 oligonucleotide alone; lanes 2 and 3, radiolabeled oligonucleotide incubated with 11 μg of C2C12 nuclear extract; lanes 4 and 5, 11 μg of NIH3T3 nuclear extracts; lanes 6 and 7, 5 footprint units of purified human Sp1 protein; lanes 3, 5, and 7, 2 μg of anti-Sp1 antibody.

Activation of Murine Msx-1 Promoter—Site-specific deletions and transgene experiments indicated that Msx-1 promoter activity was critically dependent on the intact GC box in both cell lines (Figs. 4 and 5). This could be most directly demonstrated by our finding that Sp1-deficient Drosophila SL-2 cells transfected with the core Msx-1 promoter-luciferase failed to express detectable levels of luciferase (Fig. 12). However, the expression of the reporter was dramatically enhanced when −32/+106 bp Msx-1 promoter-luciferase construct was cotransfected with pPacSp1 (an Sp1 expression vector designed to be expressed in Drosophila cells; Ref. 48). Specificity of Sp1-mediated Msx-1 promoter activation was substantiated by two different means. First, cotransfection of −32 Msx-1 promoter-luciferase construct with pPacSp(−), which contains Sp1 in an antisense orientation, had little effect on transactivation. Second, the −32/+106 Msx-1 promoter-luciferase construct containing a mutated Sp1 motif (−32M) cotransfected with pPacSp1 showed greatly reduced transactivation (Fig. 12). Currently, we do not understand why significant residual transactivation of the minimal Msx-1 promoter containing a mutated Sp1 motif continued to occur in SL-2 cells cotransfected with pPacSp1(+) plasmid (Fig. 12). Finally, the specificity of Sp1-dependent transactivation of the −32 Msx-1 promoter was attested by the absence of a similar transactivation of the control reporter plasmid pGL2-Basic (Fig. 12).

Cells Cultured in Serum-deprived Media Contain Diminished Levels/Activities of trans-Acting Factors Binding to the Minimal Msx-1 Promoter—Ectopic expression of Msx-1 in myoblasts has been shown previously to abrogate their ability to differentiate into myotubes; it was shown later that Msx-1...
TABLE I

**Oligonucleotides used in electrophoretic mobility shift assays**

|M | Sequence |
|---|---|
|Proximal E box|E box|
|−165/−128|5’GATGCCACCTGAGCTTATGAGCGGAAAAAGCTCCCCA3’|
|−165/−147|5’GATGCCACCTGAGCTTATGAC3’|
|−156/−138|5’CTGACTTTCAGCTGAGC3’|
|−146/−128|5’TAGCGGAGAGAAGCTCCCCA3’|
|M−165/−162|5’GGTCGAGCCGGACCCACCTGACTAGC3’|
|M−161/−158|5’GGTCGAGCCGGACGGATGTACCGCTTTAGCAGTG3’|
|M−157/−154|5’CCGAGCCGATGCCCCATGACCTTTAGCGGAA3’|
|Distal E box|E box|
|−1168/−1147 Wild|5’CAATTCACCTGCTCCCCC3’ |
|−1167/−1147 Mut|5’CAATTCACCTGCTCCCCC3’ |
|Proximal Sp1|Sp1|
|−32/+2|5’TCTCCGCCCGCCCTGCGCTGTTAGTGGCC3’|
|M−22/−19|5’GGTTCTCTCGGACGATCGCCTGCTTTAGTGCC3’|
|M−18/−15|5’GGTTCTCTCGGACGCCGGCTTTAGTGCC3’|
|M−14/−11|5’GGACCCGCCCTTTATACCTTTAGTGGGCGC3’|

**FIG. 12.** Transcriptional activation of the minimal Msx-1 promoter by Sp1 in Drosophila Schneider cell line, SL-2. Freshly seeded SL-2 cells were co-transfected with 0.5 µg of one of the denoted reporter plasmids with or without 50 ng of pFastSp1, in either sense (S) or antisense (AS) orientation, as described under “Experimental Procedures.” Reporter constructs are pGL2-Basic (B) or antisense (AS) orientation, as described under “Experimental Procedures.” Luciferase activity, obtained with cotransfection of the reporter plasmid pGL2-Basic with the sense of antisense Sp1 expression vector is shown for comparison. Cotransfection of SL-2 cells with −32W with pFastSp1(S) activated the Msx-1 promoter more than 100-fold; mutation in the Sp1 motif reduced this transactivation substantially (greater than 90%). Cotransfection of reporter constructs with pSpSp1(AS) was inconsequential under all conditions.

**FIG. 13.** The steady-state levels of Msx-1 mRNA and the putative trans-acting factors which bind to the minimal Msx-1 promoter decline in cells grown in serum-deprived media. Twenty µg of RNA extracted from C5C12 cells grown in normal serum-containing (U) or serum-deprived medium (D) for 24 h were electrophoresed and probed with radiolabeled Msx-1 cDNA (A, upper panel) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (A, lower panel). The steady-state levels of Msx-1 declined substantially in cells grown in the serum-deprived conditions; there was no significant change in the level of the glyceraldehyde-3-phosphate dehydrogenase RNA. A 5’-end-labeled SstI-BglII fragment of Msx-1 DNA (−165/+106 bp) was incubated with nuclear protein extracts prepared from C5C12 or NIH3T3 cells grown in serum-containing (+) or serum-deprived (−) conditions. Radiolabeled probe (P) without nuclear extracts was electrophoresed. The putative DNA protein complexes are denoted with arrowheads.

repressed transcription of MyoD (an essential prerequisite for myogenesis) by binding directly to the MyoD enhancer (74). Because C5C12 cells differentiate into myotubes when grown in serum-free media, we were curious as to the status of Msx-1 gene expression in C5C12 cells undergoing myogenesis. We found that the steady state levels of Msx-1 transcripts declined substantially in C5C12 cells grown for 24 h in serum-deprived medium (Fig. 13A), and less than 5% transcripts remained in cells grown under serum-deprived conditions for 72 h. We also saw a concomitant increase in the levels of a smaller molecular weight species of RNA which was detected with Msx-1 cDNA probes; we think that this represents a breakdown product of Msx-1 transcripts in the cells undergoing myogenesis. Unlike C5C12 cells which undergo myogenesis in vitro, there was no noticeable change in the morphology of NIH3T3 cells grown in low serum. Nevertheless, Msx-1 transcripts were similarly reduced in both NIH3T3 and C5C12 cells; the transient expression assays also revealed that the −165/+106 bp Msx-1 promoter-luciferase activity was reduced by more than 10-fold in both cells grown under serum-deprived conditions after transfection.3 Therefore, we examined the levels/activities of the trans-acting factors capable of binding to the minimal Msx-1 promoter in the nuclear extracts prepared from NIH3T3 and
C_{2}C_{12} cells grown in serum-free media. The EMSA revealed a drastic reduction in the amount/activity of the trans-acting factors which associated with the minimal promoter (Fig. 13B). Thus, regardless of the morphological transformation of the two cell lines grown under low-serum conditions, the factor(s) which activate the minimal Msx-1 promoter decline in both cells, concomitant with a decline in the steady state levels of Msx-1 transcripts.

The Minimal −165/+106-bp Msx-1 Promoter Drives Heterologous β-Galactosidase Gene Expression in the Craniofacial Tissues of Transgenic Mice—Based on the transgenic analysis of 13 kb of DNA around the Msx-1 locus, MacKenzie et al. (73) surmised that the pattern of Msx-1 gene expression during embryogenesis was determined by a complex set of cis-acting elements, including the two tissue-specific enhancers located −2 kb apart from each other. Since none of the putative promoter DNAs could drive the LacZ gene expression in absolute concordance with the endogenous Msx-1, they concluded that disparate sequence motifs, which act both independently and in concert, determine the complex pattern of Msx-1 gene expression in the embryo (73). With a long term goal to elucidate the mechanisms regulating the Msx-1 gene activation in the developing embryo, we have begun a systematic analysis of a number of Msx-1 promoter-LacZ constructs in transgenic mice, concomitant with promoter dissection studies using transient expression in cells in culture. We have analyzed a number of transgenic mice lines, harboring incrementally truncated Msx-1 promoter ligated to LacZ DNA, in their genome. Our data revealed that the −5-kb Msx-1-LacZ embryos expressed β-galactosidase at many sites reminiscent of expression of the endogenous gene; the serially truncated variants of the full-length promoter exhibited wide variations in their patterns of developmental stage-specific activation.6

In light of our data showing that a truncated Msx-1 promoter was highly active in both C_{2}C_{12} and NIH3T3 cells, we tested the activity of the minimal promoter in transgenic embryos. A comparison of the endogenous Msx-1 expression as judged by wholemount in situ hybridization of 11–12-day-old mouse embryos with Msx-1-specific antisense probes and corresponding stage embryos depicting β-galactosidase gene expression driven by the −165/+106 bp promoter are illustrated in Fig. 14. As has been reported previously (31, 32, 73), the endogenous Msx-1 gene is highly expressed in the dorsal neural tube, chordoid plexi of the third and lateral ventricles, meninges, and skull bone precursors; significant expression is also seen in the developing nasal, mandibular, and maxillary prominences and in the limbs of the embryos (Fig. 13, A and B). Apparently, the −165/106-bp minimal Msx-1 promoter dictates LacZ expression in a highly restricted manner in the craniofacial structures. In particular, the cellular primordia which are destined to generate upper and lower jaws, teeth, nose, and bones of the skull are positive for LacZ gene expression driven by the truncated Msx-1 promoter (Fig. 14, C and D). The craniofacial pattern of expression of the transgene is remarkably similar to the craniofacial pattern displayed by the endogenous Msx-1 gene (Fig. 14, compare A and B with C and D). Out of the four lines of transgenic mice containing the −165/+106-bp Msx-1-LacZ construct, we have analyzed two in great detail; transgenic embryos from both of these lines show remarkable similarity of LacZ expression in the craniofacial primordia as exemplified in Fig. 14. Our data strongly suggest that neither the site(s) of integration nor the copy number of the transgene in the genome significantly alter the specificity of the minimal Msx-1 promoter activation in the craniofacial tissues of the transgenic mice. Finally, it is significant that the expression of the transgene driven by the minimal promoter was conspicuously absent from the limb primordia and the dorsal neural tube, the two locations well known for high endogenous Msx-1 gene expression. Therefore, we conclude that the minimal Msx-1 promoter, consisting of an E box and a GC-rich motif, can target expression of a heterologous gene into specific craniofacial tissues; interestingly, the Msx-1-ablated mice also showed consistent and severe abnormalities in the same craniofacial locations which are preferentially targeted by the minimal Msx-1 promoter (72).

DISCUSSION

With an objective to extend our previous analysis of the murine Msx-1 promoter (43), we carried out systematic deletion and mutagenesis studies on a 5-kb 5′-flanking DNA of Msx-1 gene. The longest and the truncated variants of the putative promoter DNA fragments were used to drive the expression of

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reporter genes, luciferase, and bacterial LacZ. Using transiently transfected NIH3T3 and C2C12 cells, both of which express Msx-1 constitutively, we tested functional consequences of targeted alterations in the potential cis-acting sequences of Msx-1 gene on the expression of the reporter genes. Our initial studies showed that the deletions between −5 kb and −1.4 kb did not significantly affect Msx-1 promoter activity in either cell line. Therefore, we analyzed the −1.4-kb promoter in much greater detail. Our studies revealed that although a number of positive and negative cis-regulatory regions could be readily demonstrated through site-specific modifications of the −1.4-kb promoter (considered the full-length Msx-1 promoter), most of these perturbations had modest effects on the activity of the Msx-1 promoter. In fact, a relatively short DNA fragment containing 165 bp upstream of the transcription start point (TSP) and 106 bp downstream of the TSP retained strong promoter function in both NIH3T3 and C2C12 cells. We tentatively termed this as the minimal Msx-1 promoter. Guided by these observations, we examined in detail the minimal promoter for the cis-regulating sequence motifs and the potential transcription factor(s) to which the cis-elements bind. Transient expression assays with wild type or mutated Msx-1 promoter-luciferase were combined with DNase I footprinting, EMSA, and supershift analysis with specific antibodies to unravel the potential interactions between cis-acting motifs and their trans-acting factor(s) which modulate the activity of the minimal promoter. These data reveal that cis-regulatory motifs, located at −159 to −154 (the proximal E box) and at −26 to −13 (the proximal Sp1 site), were critical for activation of the minimal Msx-1 promoter in the NIH3T3 and C2C12 cells.

E box motifs are known to bind to a diverse group of basic helix-loop-helix DNA-binding proteins; these include the myogenic transcription factors MyoD, Myf5, Myf6, and myogenin (56–59) and members of the Myc/Max gene family of transcription factors which includes USF, TEF3, TEFB, Mxi1, Ap4, and FIP (60–69). Since a wide variety of transcription factor(s) bind to E box motifs, we explored the attributes of the two additional E boxes located upstream of the proximal E box. We observed that in contrast to the proximal E box, which contributed positively to the Msx-1 promoter activity, the distal E box (−1164 to −1159) acted as a negative element. Conspicuously, the middle E box (−882 to −877) neither affected promoter activity nor appeared to bind to transcription factor(s) by EMSA (data not shown). Two other observations with regard to the proximal and distal E boxes are also pertinent. First, the trans-acting factor(s) binding to the two sequence motifs are apparently unique as judged by the number of DNA-protein complexes formed by the proximal (one) and distal (three) E boxes and the failure of oligonucleotides representing proximal and distal E box motifs to cross-compete each other in DNA-protein interactions unraveled by EMSA. Second, we have observed that the trans-acting factor(s) associating with the proximal E box are inducible with serum; this finding is significant since the Msx-1 gene transcription declined precipitously in C2C12 and NIH3T3 cells grown in serum-free media. Therefore, we speculate that a serum-modulatable proximal E box-binding trans-acting protein(s) may be involved in proliferation versus differentiation signaling and Msx-1 gene expression.

The Msx-1 core promoter, with only 32 bp of sequences upstream of the TSP contains an authentic Sp1 recognition element. The truncated Msx-1 promoter lacks a TATA element but sequences around its TSP are CGCGTGC which are 86% homologous to a recently discovered, modified initiator element (Inr) in the promoter of Ha-ras (70). The location of the proximal Sp1 site in the Msx-1 promoter, 16 bp upstream of the Inr, is reminiscent of the situation in the human muscle phosphofructokinase P1 promoter which also lacks a TATA box but contains a Sp1 site (between +12 and +21) immediately adjacent to TSP (70). Promoters containing Sp1 binding sites and Inr elements and lacking a TATA box are thought to be activated through a TBP-mediated mechanism (70). Of the three putative Sp1 recognition GC motifs located on the Msx-1 promoter (at nucleotides −671 to −663, −490 to −485, and −25 to −17), only the proximal GC box (located at −25 to −17) was found to be obligatory for transcriptional activation of the minimal promoter in either cell line. It has been proposed that Sp1 may interact with the basal transcriptional apparatus through coactivators and is involved in binding with TATA-binding protein TFIIID. In TATA-less promoters, Sp1 is thought to recruit the basal transcription factors through a novel tethering activity, distinct from coactivators. The tethering factor(s) physically associates with TBP and functions to anchor the initiation complex to the promoter through binding to Sp1 (70, 71). Based on the current data, we cannot be certain whether Msx-1 promoter activation involves an Inr-mediated or Sp1-mediated mechanism.

Expression of Msx-1 gene can be readily detected at many well-defined locations in embryos from 9.5 to 12.5 days of development; these include areas of active organogenesis mediated through interactions between the epithelial and mesenchymal cell layers (4). The Msx-1 knock-out mice have unraveled another important paradox between the sites of its expression during embryogenesis and the phenotype of the Msx-1 null mice. Thus, although Msx-1 gene expression occurs rather widely in the embryo, Msx-1-deficient mice exhibit very specific defects, primarily restricted to craniofacial structures (72). Currently, it is not known whether the characteristic craniofacial dysmorphology seen in the Msx-1 null mice reflects an apparent failure of the compensatory mechanisms (e.g. expression of Msx-2) which rescue other locations of organogenesis in the embryo. The cis-acting elements which regulate Msx-1 gene expression at various sites in the developing embryo are poorly understood. Recent studies of MacKenzie et al. (73) have revealed that multiple positive and negative tissue-specific elements, including two enhancer sequences located far apart from each other in the Msx-1 promoter, dictate the complex spatiotemporal expression of Msx-1 during embryogenesis. However, these authors failed to obtain absolute concordance between the endogenous Msx-1 expression and the transgene expression dictated by several variants of the Msx-1 promoter designed from 13 kb of the genomic DNA (73). Based on these elegant analyses, it was concluded that the Msx-1 promoter is made up of cis-acting elements that act both independently and in concert with each other to generate the complex pattern of expression of Msx-1 seen during embryogenesis (73). A systematic analysis of a number of Msx-1 promoter-LacZ constructs in transgenic mice in our laboratory suggests that the pattern of the endogenous Msx-1 gene expression during embryogenesis is only partially reproduced by most of the promoter constructs; therefore, our observations fully corroborate the conclusion of MacKenzie and co-workers.2

In light of the extensive observations regarding the widespread Msx-1 expression during development, it is extremely significant that the minimal Msx-1 promoter, encompassing −165/+106 bp is activated with remarkable precision in the craniofacial structures found to be defective in the Msx-1 knock-out mice (72). The minimal Msx-1 promoter appears to be extraordinarily simple; it consists of two commonly found sequence motifs of eukaryotic promoters, the proximal E box and the GC box. The proximal E box binds to a protein factor which is immunologically related to USF-1. The GC box binds to a transcription factor, which is immunologically related to...
Sp1. Conceivably, additional factor(s) may also recognize the minimal promoter and participate in Msx-1 gene activation in vivo; such interactions may not only be mediated by direct DNA-protein complex formation but also by protein-protein interactions. Msx-1 itself is a transcription factor which inhibits its MyoD expression in fibroblast × T cell hybrids (74). It is not known if some myogenic transcription factor(s) also re-

shown to bind to the consensus sequence 5'-C/G)TAATTG-3'.

Msx-1 promoter itself contains two Msx-1 consensus binding sites and the binding of Msx-1 homeodomain polypeptide to the predicted Msx-1 motif was previously demonstrated (43). The murine Msx-1 promoter may be subject to autoregulation by DNA-protein and protein-protein interactions and thus further com-

plicates regulatory feedback loops orchestrating early development.

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