Developmental Biology **237,** 107–115 (2001)
doi:10.1006/dbio.2001.0356, available online at http://www.idealibrary.com on IDEAL®

NF-κB Mediates FGF Signal Regulation of *msx*-1 Expression

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The nuclear factor-κB (NF-κB) family of transcription factors is involved in proliferation, differentiation, and apoptosis in a stage- and cell-dependent manner. Recent evidence has shown that NF-κB activity is necessary for both chicken and mouse limb development. We report here that the NF-κB family member c-*rel* and the homeodomain gene *msx*-1 have partially overlapping expression patterns in the developing chick limb. In addition, inhibition of NF-κB activity resulted in a decrease in *msx*-1 mRNA expression. Sequence analysis of the *msx*-1 promoter revealed three potential κB-binding sites similar to the interferon-γ (IFN-γ) κB-binding site. These sites bound to c-Rel, as shown by electrophoretic mobility shift assay (EMSA). Furthermore, inhibition of NF-κB activity significantly reduced transactivation of the *msx*-1 promoter in response to FGF-2/-4, known stimulators of *msx*-1 expression. These results suggest that NF-κB mediates the FGF-2/-4 signal regulation of *msx*-1 gene expression. © 2001 Academic Press

Key Words: **NF-κB; c-*rel*; *msx*-1; FGF; limb bud.**

INTRODUCTION

Development of a functional limb requires the precise orchestration of proliferation and differentiation to produce all the tissues of the limb, and apoptosis to remodel the structure into its final form. The ubiquitous transcription factor family, NF-κB, has been implicated in each of these cellular processes. Dimers of the NF-κB family members (c-Rel, RelA, RelB, p50, and p52) are characteristically located in the cytoplasm of a cell in an inactive state due to association with an inhibitory molecule, IκB. Stimulation with cytokines, growth factors, or pathological agents targets IκB for phosphorylation by the IκB kinases, IKK-1 and -2, and subsequent degradation by the 26S proteosome. Freed from its association with IκB, NF-κB translocates to the nucleus and directs gene transcription.

The role of NF-κB has expanded from an immune response factor to include roles as a critical factor in the development of multiple organ systems including the liver, lung, and limbs. Inhibition of NF-κB activity in chick embryos via viral delivery of a *trans*-dominant inhibitor results in a dysmorphic apical ectodermal ridge (AER) and limb truncation (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998). Additionally, disruption of the *ikk1* locus in mice severely impairs signal activation of NF-κB and results in truncation of both the fore- and hindlimbs (Hu *et al.*, 1999; Takeda *et al.*, 1999). Several downstream targets of NF-κB have been provisionally identified in the chick limb. Inhibition of NF-κB activity alters the expression patterns of *shh*, *twi*, *bmp*-4, *lhx*-2, *fgf*-8, and *msx*-1. While these data demonstrate the influence of NF-κB activity on the expression of these genes, they do not establish a direct interaction with or a role for individual NF-κB family members. In addition, the upstream inducers of NF-κB in the limb remain elusive since the cytokines and pathological agents known to stimulate NF-κB activity are not widely expressed in the developing limb (e.g., interleukin-1 and bacterial lipopolysaccharide). We have shown previously that expression of c-*rel* in the Progress Zone is regulated by AER-derived factors and that recombinant FGF-4 can maintain c-*rel* expression in the absence of an AER (Bushdid *et al.*, 1998).

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0012-1606/01 $35.00

These data suggest that FGF family members may stimulate NF-κB activity in limb bud mesenchyme similar to the function of FGF-1 in T-cells (Byrd *et al.*, 1999). Furthermore, FGF-2 and -4 stimulation induces the expression of *msx*-1, one of the potential downstream targets of NF-κB (Wang and Sassoon, 1995; Vogel *et al.*, 1995; Kanegae *et al.*, 1998).

Experiments using avian embryos point to a critical role for AER-derived FGF stimulation in limb bud elongation. Beads soaked in FGF-2, -4, or -8 can substitute for AER-derived signals and induce limb outgrowth and patterning when implanted into an AER denuded limb bud (Fallon *et al.*, 1994; Niswander *et al.*, 1993; Crossley *et al.*, 1996). Furthermore, implantation of beads soaked in FGF-1, -2, -4, -8, or -10 into nonlimb flank mesoderm induces the initiation and maturation of a morphologically normal limb (Cohn *et al.*, 1995; Crossley *et al.*, 1996; Ohuchi *et al.*, 1997). These data support a functional redundancy among the members of the FGF family. In support of this, genetic disruption of the *fgf*-1, -2, or -4 gene locus does not reveal an apparent limb defect. Conversely, targeted inactivation of *fgf*-8 or -10 results in limb truncation or agenesis, respectively (Meyers *et al.*, 1998; Min *et al.*, 1998).

Expression of the homeodomain gene, *msx*-1, is dependent on AER-derived signals such as FGF-4 (Wang and Sassoon, 1995). During early stages of limb bud outgrowth, *msx*-1 mRNA is observed in mesenchymal cells extending from the proximo-anterior border of the limb through the distal Progress Zone and continuing to the proximo-posterior border. Administration of recombinant FGF family members [FGF-2 (Watanabe and Ide, 1993; Vogel *et al.*, 1995) and FGF-4 (Wang and Sassoon, 1995; Vogel *et al.*, 1995)] or FGF-expressing retroviruses restores the expression of *msx*-1 mRNA in AER-deficient limbs and promotes limb outgrowth. Therefore, *msx*-1 expression can serve as an indicator of FGF stimulation. Several studies have proposed that Msx-1 maintains the Progress Zone in a highly proliferative and undifferentiated state (Wang and Sassoon, 1995; Vogel *et al.*, 1995). Furthermore, the upregulation of *msx*-1 expression has been correlated with the ability of chick, mouse, and amphibian limbs to initiate and program digit regeneration (Kostakopoulou *et al.*, 1996; Reginelli *et al.*, 1995; Simon *et al.*, 1995; Crews *et al.*, 1995). Conversely, *msx*-1 expression has been shown to define the anterior and posterior necrotic zones, those regions destined to undergo apoptosis in the limb bud (Coelho *et al.*, 1993). Expression of *msx*-1 is also evident in the interdigital mesenchyme in the developing limb, areas that demonstrate high apoptotic activity (Macias *et al.*, 1997). These data suggest multiple and complex roles for Msx-1 in limb development and regeneration.

Both *in vitro* and *in vivo* experiments demonstrate that agents such as FGF, retinoic acid, and BMPs can regulate *msx*-1 expression [FGF (Wang and Sassoon, 1995; Watanabe and Ide, 1993; Dealy and Kosher, 1996), retinoic acid (Chen *et al.*, 1995), BMP (Vainio *et al.*, 1993; Barlow and Francis-West, 1997)]. The mechanism of *msx*-1 regulation by any of these factors is unclear. An enhancer element responsible for the retinoic acid induction in cultured cells was identified in the human *msx*-1 5′-flanking region (Shen *et al.*, 1994). In addition, the murine *msx*-1 gene contains several enhancer elements including three potential NF-κB binding sites as well as an Msx-1 consensus binding site, suggesting a possible autoregulatory transcription cascade (Kuzuoka *et al.*, 1994; Gonzalez *et al.*, 1998; Takahashi *et al.*, 1997; Shetty *et al.*, 1999; Chaudhary *et al.*, 2001).

Both c-*rel* and *msx*-1 are associated with apoptosis and proliferation, both are expressed by limb bud mesenchyme, and the expression of both is dependent on AER-derived FGFs. Therefore, we hypothesize that NF-κB transduces the FGF signal to regulate *msx*-1 gene expression.

MATERIALS AND METHODS

In Situ Hybridization

Chick embryos were analyzed by whole-mount *in situ* hybridization as described by Riddle *et al.* (1993). Sense and antisense c-*rel* and *msx*-1 cRNA was synthesized as reported previously (Bushdid *et al.*, 1998; Robert *et al.*, 1991). Briefly, embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA), washed briefly with PBS, and stored in 100% methanol at −20°C until processed. The day of hybridization, embryos were rehydrated into PBS + 0.1% Tween 20 (PBT). The embryos were permeablized with 10 μg/ml Proteinase K and refixed with 4% PFA for 20 min at room temperature. After washing in PBT, the embryos were prehybridized in hybridization buffer (50% deionized Formamide, 4× SSC, pH 7.0, 50 μg/ml tRNA; 0.5 μg/ml Heparin; 1% SDS) for 2 h and then hybridized at 70°C overnight with 1 μg/ml cRNA labeled with Digoxigenin. The embryos were washed in Solution I (50% deionized Formamide, 5× SSC, pH 7.0, 1% SDS) two times for 30 min at 70°C, one time for 30 min at 65°C, and then three times in Solution II (50% deionized Formamide; 2× SSC; 0.5% SDS) for 30 min at 65°C. The embryos were washed three times in TBS + 0.1% Tween 20 (TBST) before blocking in TBST + 10% heat-inactivated sheep serum for 2.5 h at room temperature. The block solution was removed and anti-DIG $F(ab)_2$ fragments conjugated to alkaline phosphatase at a 1:2000 dilution in TBST + 1% serum was added and the embryos incubated at 4°C overnight. Following extensive washing in TBST containing 1 mM Levamisole, labeled cRNA was visualized with NBT/BCIP colorimetric reaction. Embryos were graded into 80% glycerol for photography.

Electrophoretic Mobility Shift Assay

EMSAs were performed as previously detailed (Muraoka *et al.*, 2000). Oligos for each of the κB binding sites within the murine *msx*-1 5′-flanking region were designed as follows: κB1 (−41/−-18) 5′-acagcccaatggttctctccggacccgccc-3′, κB2 (−910/−882) 5′-caggaactgcttatttttcctactggccca-3′, and κB3 (−1037/−1008) 5′-gttccccactccaactttccttctttttgt-3′ (Kuzuoka *et al.*, 1994). CEF nuclear extract (20 μg) was used per binding reaction. Super-shift analysis included the addition of 1 μg preimmune Rabbit IgG or anti-chick c-Rel antisera (kind gift of Nancy Rice) to the initial binding reaction.

Site-Directed Mutagenesis of msx-1

Oligonucleotide site-directed mutagenesis was performed on the murine *msx*-1 5′-flanking region in the pGL2-Basic vector (Promega) according to the manufacturer's protocols (BioRad T7 Mutagenesis kit). The oligonucleotides used to introduce nonfunctional mutations into the individual κB binding sites were as follows: Msx1ΔκB1 (−27) 5′-gggggcgggtcccccgagattgattgggctgtgctc-3′, Msx1ΔκB2 (−868) 5′-gccagtaccccaaataagcagttc-3′, or Msx1ΔκB3 (−942) 5′-caaaaagaaccccagttggagtgg-3′. Two independently mutagenized clones for each κB binding site were isolated, fully sequenced, and analyzed in transient transfection assays.

Cell Culture and Transfections

Drosophila Schneider S2 cells were cultured and transfected as described previously (Rosay *et al.*, 1995). Primary chick embryonic fibroblasts were established as described by Hunter (1979) and transfected by standard calcium phosphate precipitation followed by a glycerol shock (90 s with 15% glycerol in DMEM) 6 h postprecipitation. All transfections were monitored for equalized efficiency using a β-galactosidase expression vector (1 μg/sample) as a control.

Virus Production

Adenovirus harboring the *trans*-dominant inhibitor of NF-κB activity, IκB-αΔN was produced and purified as detailed in Bushdid *et al.* (1998). Retroviral stocks were generated according to Morgan and Fekete (1996). Concentrated stocks were titered by infection of CEFs with serial dilutions of the viral stock and detection of infected cells with the 3C2 monoclonal anti-p19(gag) antibody. RCAS-alkaline phosphatase and RCAS-αΔN viral stocks were titered at 10^9 infectious units per ml. The prelimb fields of stage-10 chick embryos were infected by using pulled microcapillary pipettes attached to a Picospritzer II (General Valve Corp.) set at 10 psi with 30-ms pulses which delivered approximately 4 nl of virus per pulse. Approximately 48 nl was injected into each prelimb field.

Quantitative Reverse Transcribed Real-Time PCR

The right prelimb fields of stage-10 chick embryos were injected with either RCAS-alkaline phosphatase or RCAS-αΔN. Forty-eight hours postinjection, total RNA from individual infected limbs was isolated by using TriReagent. Random hexamers and MMLV-RT were used to reverse transcribe this RNA, at which point each sample was divided in two. Real-time PCR was used to quantitate the amount of *msx*-1 mRNA present in each sample using the following oligonucleotides derived from the chick *msx*-1 cDNA: *msx*-1[186] 5′-gatgggcggcgaggaggagag-3′ and *msx*-1[704] 5′-agcagctgggccgtggtgaagg-3′ (Nohno *et al.*, 1992). A PCR standard curve was established for quantitation by using 10-fold dilutions (1 ng to 0.1 pg) of the *msx*-1 cDNA as the template DNA (kind gift of Brigid Hogan). The PCR of reverse transcribed sample cDNA was run concurrent with the standard curve in the presence of the fluorescent dye SYBR-Green 1 and quantitated using the Roche LightCycler (Morrison *et al.*, 1998). A similar strategy was used for quantitation of GAPD in the duplicate samples in order to normalize the amount of cDNA per sample. Oligonucleotides to the avian GAPD cDNA were designed as follows: GAPD[234] 5′-gggcacgccatcactatcttcc-3′ and GAPD[579] 5′-gaggggccatccaccgtctt-3′ (Dugaiczyk *et al.*, 1983).

RESULTS

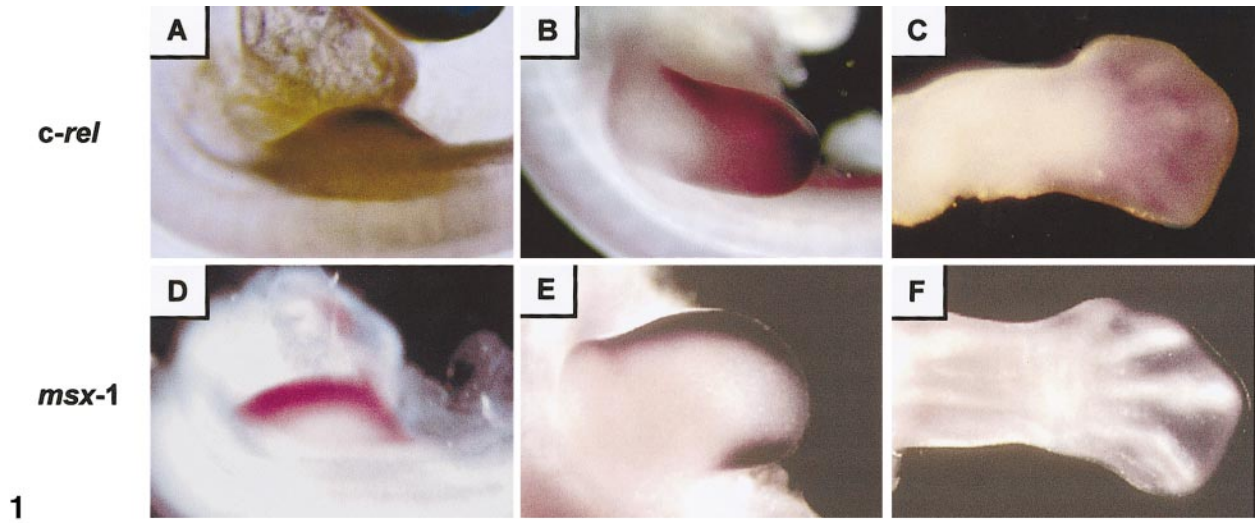
c-rel and msx-1 mRNA Expression Patterns Are Partially Overlapping

Whole-mount *in situ* hybridization was performed on embryos from stage 20 to stage 30 to define the temporal and spatial localization of c-*rel* in the developing embryo. c-*rel* mRNA was clearly detected in the mesenchyme of the limb bud at stage 20. The highest level of expression was detected along the distal aspect of the Progress Zone underlying the AER (Fig. 1A). By stage 26, c-*rel* was expressed in the Progress Zone but was not detected in the AER (Fig. 1B). The pattern of c-*rel* expression was limited to the lateral aspects of the elongating phalanges and the interdigital mesenchyme at stage 30 (Fig. 1C). By stage 35, c-*rel* expression was absent in the limb (data not shown). Whole-mount *in situ* hybridization for chick *msx*-1 detected expression in the Progress Zone of stage-20 and -26 chick limbs, the anterior and posterior aspects of stage-26 limbs, and the interdigital mesenchyme of stage-30 limbs (Figs. 1D–1F). This is consistent with the previously observed pattern of *msx*-1 mRNA expression (Ganan *et al.*, 1998; Coelho *et al.*, 1993; Kanegae *et al.*, 1998). These data demonstrate that *msx*-1 expression partially overlaps with c-*rel* expression in the Progress Zone and interdigital mesenchyme of the developing limb.

NF-κB Activity Is Necessary for Wild-Type msx-1 Expression

To determine whether inhibition of NF-κB activity alters *msx*-1 gene expression, we infected developing limbs with RCAS-αΔN, a replication competent retrovirus which expresses a mutant avian IκB-α that acts as a *trans*-dominant inhibitor of NF-κB activity (Bushdid *et al.*, 1998). Experiments using control retroviruses that express the human placental alkaline phosphatase gene demonstrated the optimal stage and location to inject the RCAS virus to obtain limited spread throughout the limb mesenchyme (Fig. 2). Infection of the limb with RCAS-αΔN has been previously shown to result in the inhibition of limb outgrowth and the loss of distal structures (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998). Injection of RCAS-αΔN into the right prelimb field resulted in an apparent decrease in *msx*-1 expression when compared to contralateral, uninfected limbs or to RCAS-alkaline phosphatase infected limbs (Figs. 2B and 2C). The reduction in *msx*-1 expression is unlikely to be the result of a generalized misregulation of transcriptional activity since *hoxd*10 expression is normal in RCAS-αΔN infected limbs (Fig. 2D).

Whole-mount *in situ* hybridization suggests a decrease in *msx*-1 expression after RCAS-αΔN infection. Real-time RT-PCR was used to quantitate the decrease in mRNA observed (Fig. 3). RNA from individual RCAS-alkaline phosphatase or RCAS-αΔN limbs was isolated and reverse transcribed. To standardize the assay, all RCAS-αΔN infected limbs collected for analysis demonstrated a 50%



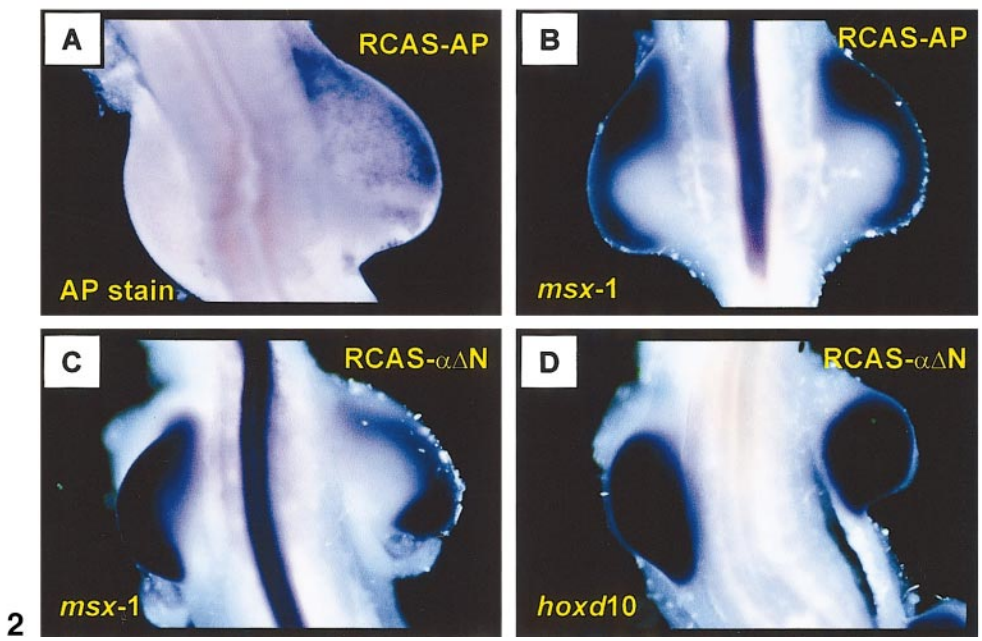


FIG. 1. c-*rel* and *msx*-1 have partially overlapping domains of expression. Whole-mount *in situ* hybridization for c-*rel* (A–C) and msx-1 (D–F). (A, D) *In situ* hybridization of stage-20 chick embryos reveals partially overlapping domains of expression for c-*rel* and *msx*-1 mRNA in forelimb mesenchyme. (B, E) By stage 25, c-*rel* is expressed in a broad domain in the distal portion of the limb, while the *msx*-1 expression domain is nested within the c-*rel* expression domain. (C, F) Both c-*rel* and *msx*-1 are expressed in the interdigital mesenchyme of stage-30 limbs.

FIG. 2. Retroviral delivery of the NF-κB inhibitor. (A) Infection of a stage-10 chick embryo prelimb field with RCAS-alkaline phosphatase results in normal limb outgrowth. Alkaline phosphatase activity was visualized with NBT/BCIP colorimetric detection ($n = 10$). (B) Infection with RCAS-alkaline phosphatase does not affect *msx*-1 expression ($n = 5$). (C) Infection with RCAS-αΔN results in a reduction in limb growth and in *msx*-1 expression when compared to control limbs ($n = 10$). (D) Inhibition of NF-κB activity does not affect expression of *hoxd*10 ($n = 5$).

reduction in size. This eliminates both minimally infected limbs as well as limbs too small to provide sufficient RNA. Standard curves were generated using *msx*-1 cDNA and GAPD for normalization of RNA between samples. A 50-fold reduction in *msx*-1 transcripts was detected after infection with RCAS-αΔN when compared to RCAS-alkaline phosphatase infected limbs (Fig. 3). Consistent with our *in situ* hybridization data, *msx*-1 mRNA is detected at low levels in the RCAS-αΔN infected limbs. These results demonstrate that *msx*-1 transcription is decreased in

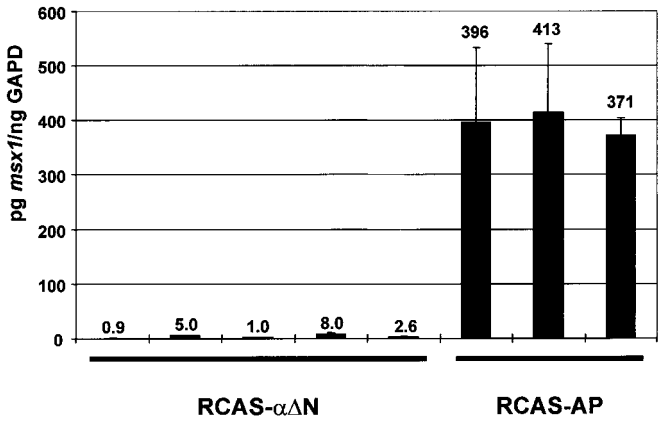


FIG. 3. Inhibition of NF-κB activity results in a 50-fold reduction in *msx*-1 transcripts. Reverse transcribed quantitative real-time PCR was used to measure the amount of *msx*-1 mRNA in RCAS-αΔN (n = 5) and RCAS-alkaline phosphatase (AP; n = 3) infected limbs. Total RNA from individual infected limbs was reverse transcribed, and the amount of *msx*-1 and GAPD mRNA quantitated using the Roche LightCycler. Each sample was measured in triplicate.

the absence of NF-κB activity *in ovo* and suggest that NF-κB factors regulate *msx*-1 gene expression.

The msx-1 5′-Flanking Region Binds NF-κB

The human *msx*-1 5′-flanking region contains at least one NF-κB binding site upstream of the start-site of transcription (Shen *et al.*, 1994). Sequence analysis of the human and murine *msx*-1 promoter sequences revealed three putative κB binding sites (Quandt *et al.*, 1995; Gonzalez *et al.*, 1998). We have termed these sites κB1, κB2, and κB3. All three sites are similar to the canonical κB binding site and are highly homologous to the IFN-γ high affinity binding site for the c-Rel oncoprotein (Fig. 4A). κB2 and κB3 bear the highest similarity to the IFN-γ c-Rel binding site. c-Rel binds the INF-γ binding site and activates gene transcription in the absence of association with other NF-κB family members (Sica *et al.*, 1992).

To determine whether NF-κB factors associate with the *msx*-1 κB binding sites *in vitro*, EMSA was performed. Equal amounts of purified, bacterially expressed c-Rel protein were used in binding assays with oligonucleotides for each putative *msx*-1 κB binding site (Fig. 4B). Recombinant c-Rel bound to all three *msx*-1 κB binding sites, but consistently demonstrated greater binding to κB3 (lane 2) than to either κB2 (lane 4) or κB1 (lane 6). The specificity of each binding reaction was demonstrated by the addition of unlabeled oligonucleotide (lanes 3, 5, and 7).

To determine the ability of native NF-κB complexes to bind the putative κB3 binding site, nuclear extracts were isolated from chick embryonic fibroblasts (CEFs) stimulated for 6 h with either 20 ng/ml TNF-α as a positive control or 20 ng/ml each FGF-2/-4 (Fig. 4C). TNF-α is a known inducer of NF-κB activity, while FGF-2/-4 are two of the AER-derived FGFs. The κB3 oligonucleotide bound a specific protein complex in CEF nuclear extracts stimulated with TNF-α or FGF-2/-4 (lanes 3 and 7), but exhibited minimal binding in unstimulated extracts (lane 1). This binding was eliminated by the addition of unlabeled κB3 oligonucleotides (lanes 2 and 6). These results suggest that FGF, a stimulus known to activate *msx*-1 gene expression, affects translocation of NF-κB complexes to the nucleus. In addition, incubation of the nuclear extracts with antisera to c-Rel (lanes 4 and 8) produces a specific supershifted band (arrow) that is not seen with preimmune antisera (lanes 5 and 9). These data confirm the presence of the NF-κB family member, c-Rel, in the nuclear protein complex that binds the κB3 oligonucleotide after stimulation with FGF-2/-4. Furthermore, these results suggest a direct interaction between NF-κB and the *msx*-1 promoter.

The κB Binding Sites in msx-1 Are Necessary for Activation by NF-κB

Transient transfections were performed by using 1.2 kb of the 5′-flanking region of *msx*-1 to drive expression of the *Photinus* luciferase cDNA reporter construct to determine if NF-κB factors are transcriptional activators of *msx*-1 gene expression (Fig. 5). Transfections were performed in *Drosophila* Schneider cells, a cell line that lacks endogenous NF-κB factors capable of binding mammalian κB binding sites (Inoue *et al.*, 1992). Only co-transfection of the reporter with one or a combination of NF-κB factors resulted in significant luciferase production. Consistent with EMSA analysis, co-transfection with c-Rel stimulated luciferase activity approximately 20-fold over control levels (Fig. 5). Co-transfection of c-Rel and p50 with the *msx*-1-luciferase construct produced an 18-fold increase in luciferase activity over controls while co-transfection with RelA resulted in an 11-fold stimulation of luciferase activity. These results suggest that NF-κB factors are able to transactivate the *msx*-1 promoter.

A series of deletion mutants was created to delineate more precisely the sequences in the *msx*-1 5′ flanking region required for transactivation by NF-κB. A significant reduction in luciferase activity in response to NF-κB was observed after deletion of sequences 5′ to nucleotide-884 [Msx(-884)] which removes two of the three κB binding sites (κB2 and κB3) from *msx*-1. Deletion of all sequences 5′ to nucleotide-164 [Msx(-164)] also abrogated reporter activity, suggesting that NF-κB acts at sequences between −1280 and −884 (Fig. 5).

To ascertain whether specific κB binding sites are required for NF-κB induced transactivation of *msx*-1, point mutations were introduced into the three κB binding sites either singly or in combination. These point mutations (ΔκB1, ΔκB2, ΔκB3, and ΔκBs) resulted in sequences that conform to no known transcription factor binding site. Co-transfection of Msx1(ΔκB3)-luciferase with the c-Rel expression vector resulted in a marked reduction in lucif-

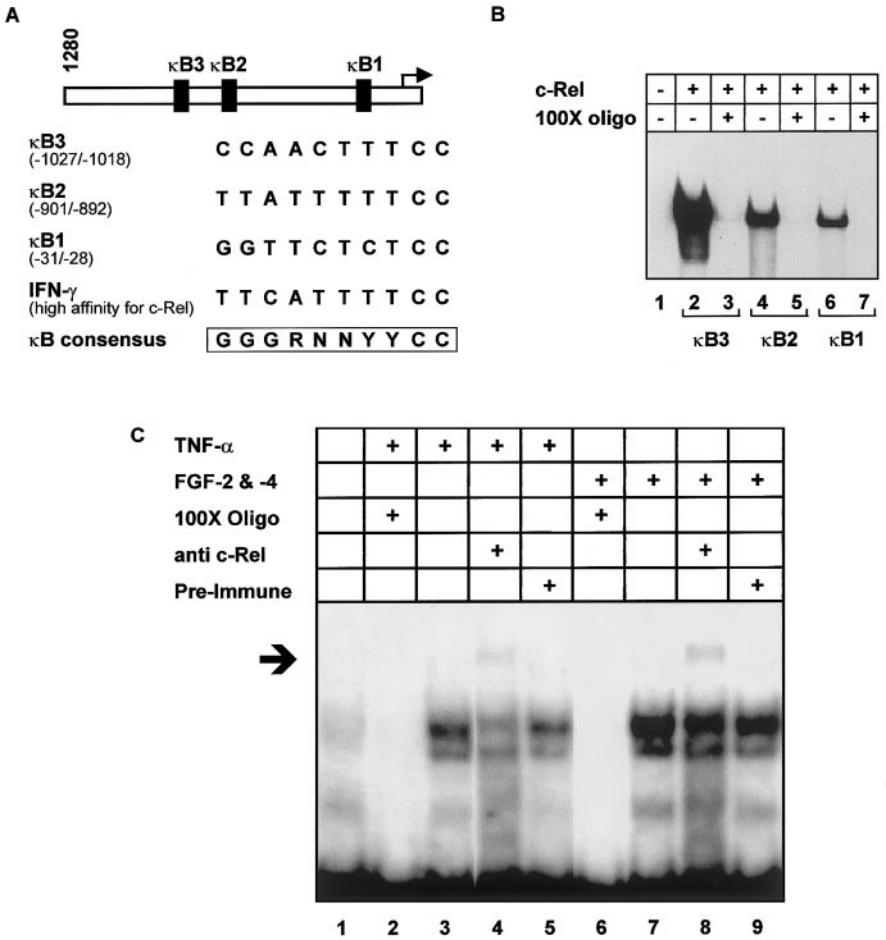


FIG. 4. *msx*-1 contains three κB binding sites. (A) Diagram of the *msx*-1 5′-flanking region. The potential κB binding sites are delineated and compared to the κB binding site of IFN-γ as well as the consensus κB binding site. (B) Each of the potential κB binding sites in *msx*-1 binds c-Rel as demonstrated by gel-shift analysis (lanes 2, 4, 6). In each case, binding is competed by 100× unlabeled oligonucleotide (lanes 3, 5, 7) while migration of the probe alone is demonstrated in lane 1. (C) c-Rel is a component of the FGF-stimulated DNA binding complex. Gel shift analysis of nuclear extracts derived from either TNF-α or FGF-2/-4 stimulated CEFs reveals a protein complex that binds to the κB3 binding site of *msx*-1 (lanes 3, 7). Excess unlabeled oligonucleotide competes with binding of the labeled probe (lanes 2, 6). Addition of anti c-Rel antisera to the binding reaction results in the appearance of a supershifted band (arrow) with both the positive control (TNF-α) and FGF-2/-4 stimulated extracts (lanes 4, 8). This supershifted band is not observed with the use of preimmune rabbit IgG (lanes 5, 9). Nuclear extracts from unstimulated cells exhibit minimal binding (lane 1).

erase activity when compared to the wild-type *msx*-1 luciferase construct. This suggests that κB3 is a critical site in *msx*-1 for NF-κB mediated transactivation. Mutation of either κB2 or κB1 slightly augmented c-Rel transactivation suggesting that κB3 mediates the majority of *msx*-1 transcriptional activation and that the other two κB-like binding sites might act to modulate the transcriptional response. Mutation of all three κB binding sites attenuated the NF-κB transcriptional regulation of *msx*-1 without significantly altering the basal transcriptional activity (Fig. 5). These data demonstrate that the *msx*-1 κB3 binding site is required for c-Rel and RelA mediated transcriptional activation.

NF-κB Mediates FGF-Induced msx-1 Transcriptional Activation

Inhibition of NF-κB activity in chick limb mesenchyme alters *msx*-1 gene expression suggesting that *msx*-1 is a downstream target of NF-κB mediated transactivation. To test this hypothesis, CEFs were transfected with either the wild-type *msx*-1 luciferase construct or the Msx1(ΔκBs) luciferase construct. CEFs were stimulated with 20 ng/ml each FGF-2/-4 for 6 h to examine the effects on NF-κB mediated transcription. FGF-2/-4 stimulated a sevenfold increase in luciferase activity using the wild-type *msx*-1 construct (Fig. 6A, lane 3). Mutation of the κB binding sites

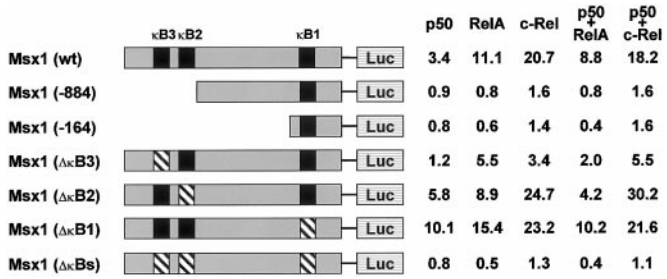


FIG. 5. Mutation analysis of the *msx*-1 5′-flanking region. Wild-type (solid boxes) and mutant (hashed boxes) *msx*-1 luciferase (Luc) constructs were co-transfected into *Drosophila* Schneider cells along with NF-κB members p50, RelA, and c-Rel. Fold stimulation over basal reporter activity is depicted. Each experiment was performed three times in triplicate.

abrogated FGF-2/-4 stimulation of reporter activity (Fig. 6A, lane 4). These data demonstrate the necessity of the κB binding sites for FGF-inducible *msx*-1 transcription.

To determine whether NF-κB activity is necessary for FGF regulation of *msx*-1 transcription, CEFs were transfected with the wild-type *msx*-1 construct and stimulated with 20 ng/ml each FGF-2/-4. These cells were either not infected, infected with a control virus (pAc), or infected with IκB-αΔN 24 h prior to stimulation. FGF-2/-4 stimulated a sevenfold increase in reporter activity alone or in the presence of control virus (Fig. 6B, lanes 2 and 4). Significantly reduced levels of luciferase activity were observed in cells infected with IκB-αΔN (Fig. 6B, lane 6). These results demonstrate that blockade of NF-κB activity inhibits FGF-2/-4 induced *msx*-1 transcriptional activation suggesting that NF-κB factors mediate FGF stimulation of *msx*-1 expression.

DISCUSSION

We demonstrate a role for NF-κB as a downstream effector for FGF regulation of *msx*-1 expression. The NF-κB family member, c-*rel*, and the homeodomain transcription factor, *msx*-1 are expressed in partially overlapping patterns in the chick limb. The expression pattern of *msx*-1 is nested within the larger region of c-*rel* expression. *In ovo* inhibition of NF-κB activity resulted in a 50-fold reduction in *msx*-1 expression. Furthermore, we identified three NF-κB binding sites in the *msx*-1 5′-flanking region that bind recombinant c-Rel. Inhibition of NF-κB activity, either through mutation of the κB binding sites or addition of IκB-αΔN, blocked FGF-2/-4-stimulated *msx*-1 transcriptional activation. These data provide the first direct evidence that NF-κB mediates FGF regulation of *msx*-1 expression.

FGF regulation of *msx*-1 expression has been well documented. Surgical ablation of the primary source of FGF in the limb, the AER, results in loss of *msx*-1 expression (Robert *et al.*, 1995). Moreover, the addition of FGF-4 to an AER-deficient limb rescues *msx*-1 expression (Wang and Sasson, 1995). Furthermore, exogenous FGF-4 is able to restore *msx*-1 expression to the remaining mesenchyme of an amputated limb (Kostakopoulou *et al.*, 1996). The components of the FGF/*msx*-1 signal pathway, however, have not been delineated. Here, we demonstrate that the transcription factor family NF-κB plays a role in this signal transduction pathway.

FGF signals are conveyed through a family of tyrosine kinase receptors, the FGF receptors (FGFR) -1, -2, -3, and -4. High affinity binding of FGF ligand in association with heparin sulfate induces the FGFRs to dimerize, transphosphorylate each other, and signal via the ras/mitogen activated protein kinase (MAPK) pathway. p38 MAPK and MEKK-1 are two components of the MAPK pathway known to be activated by FGF signaling (Faris *et al.*, 1996; Baron *et al.*, 2000). NF-κB can be stimulated through activation of IKK-1 and IKK-2 by p38 MAPK and MEKK-1 (Regnier *et al.*, 1997; Malinin *et al.*, 1997; Lee *et al.*, 1998; Liang and Gardner, 1999). The activated IKKs phosphorylate and inactivate the inhibitory molecule, IκB-α, thereby inducing NF-κB activity. This provides a plausible mechanism for NF-κB mediated FGF-stimulated regulation of *msx*-1.

In our studies, *msx*-1 expression served as a reporter for FGF

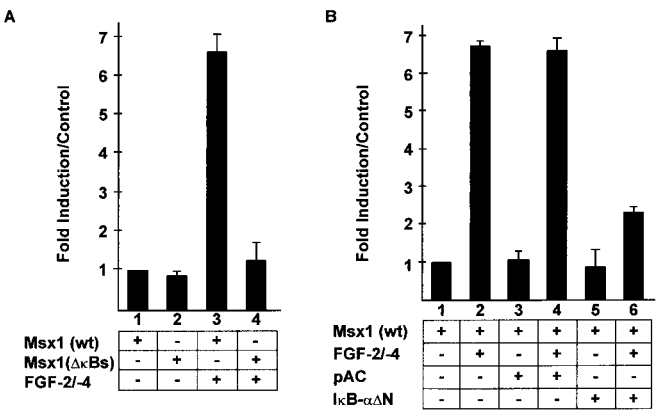


FIG. 6. The κB binding sites of *msx*-1 are necessary for FGF regulated transcription. (A) Wild-type or mutant *msx*-1 luciferase constructs were transfected into CEFs and stimulated with 20 ng/ml each FGF-2/-4. FGF-2/-4 stimulates a sevenfold increase in reporter activity using the wild-type *msx*-1 construct (lane 3). Mutation of the κB binding sites in *msx*-1 abrogates the ability of FGF-2/-4 to stimulate transcription through this sequence (lane 4). (B) NF-κB activity is necessary for FGF stimulation of transcription using *msx*-1. CEFs were transfected with the *msx*-1 luciferase construct followed by infection with either empty adenovirus (pAC) or adenovirus that expresses IκB-αΔN. Stimulation with FGF-2/-4 results in a sevenfold increase in reporter activity over basal levels (lane 2). Infection with control virus neither induces nor inhibits luciferase production (lanes 3, 4). Inhibition of NF-κB activity results in a significant reduction in luciferase activity upon stimulation with FGF-2/-4 (lane 6). Each experiment was performed three times in triplicate. Error bars indicate the standard error of the mean.

signal transduction. Our *in vitro* data demonstrate the requirement of NF-κB activity for FGF-2/-4 stimulation of *msx*-1 transcriptional activation. Furthermore, inhibition of NF-κB activity resulted in a reduction in *msx*-1 mRNA levels *in ovo*. The fact that *msx*-1 is reduced, but not absent, is not surprising given the complexity of the *msx*-1 promoter (Shen *et al.*, 1994; Gonzalez *et al.*, 1994; Kuzuoka *et al.*, 1994; Takahashi *et al.*, 1997). BMP-4, which is also expressed in the developing limb, can stimulate expression of *msx*-1 (Vainio *et al.*, 1993; Barlow and Francis-West, 1997). Previously, we have shown that inhibition of NF-κB results in an increase in *bmp*-4 expression (Bushdid *et al.*, 1998). We hypothesize that inhibition of NF-κB activity blocks FGF regulation of *msx*-1 while the concurrent increase in *bmp*-4 stimulates *msx*-1 expression. Thus, *msx*-1 expression is decreased, but not absent, in the infected limb bud.

Blocking NF-κB in the developing limb results in reduced limb size, lack of distal cartilage elements, and a dysmorphic AER (Bushdid *et al.*, 1998). This phenotype is similar to the truncated limb observed in *limb deformity* mice (Haramis *et al.*, 1995; Kuhlman and Niswander, 1997). These mice have a mutation in the *formin* gene locus and are unable to transmit Shh signals to stimulate AER formation and production of FGF. A primary aspect of FGF signaling in the limb involves cross communication between mesenchymal and epithelial cells, and several lines of evidence suggest that FGF-4 from the epithelium is necessary for the expression of *shh* in the Zone of Polarizing Activity (ZPA; Laufer *et al.*, 1994; Niswander *et al.*, 1994). These data support the hypothesis that AER-derived FGF-4 and Shh expressed in the ZPA maintain a positive feedback loop that is essential for proximo-distal and anteroposterior limb development. A breakdown in this reciprocal communication loop results in limb truncation, AER degradation, and loss of ZPA-regulated patterning (Haramis *et al.*, 1995; Kuhlman and Niswander, 1997).

The phenotype observed in IκB-αΔN-infected chick limbs suggests that NF-κB is necessary for maintenance of this reciprocal communication loop. Using *msx*-1 as a reporter of FGF signal transduction, we have shown that NF-κB is activated by FGF-2/-4 and binds the κB3 binding site in *msx*-1. Furthermore, inhibition of NF-κB activity blocks FGF-2/-4 signaling as measured by *msx*-1 transcriptional activation *in vitro* and *msx*-1 expression *in vivo*. Previously, we have shown that inhibition of NF-κB results in a loss of *shh* expression (Bushdid *et al.*, 1998). These data suggest that NF-κB in the limb transduces FGF signals derived from the AER and that inhibition of NF-κB activity blocks the Shh/FGF feedback loop. Our data suggest that the reciprocal communication by epithelial and mesenchymal cells in the limb is disrupted after the inhibition of NF-κB activity which results in the misregulation of multiple genes regulated by FGF, including *msx*-1.

In summary, we have demonstrated that NF-κB mediates FGF-2/-4 regulation of *msx*-1 expression. Whether NF-κB mediates FGF signal transduction in other cell types remains to be elucidated. Our data in the limb suggest the importance of NF-κB as a mediator of reciprocal communication between epithelium and mesenchyme. Such a role for NF-κB was previously suggested by the finding that inhibition of NF-κB activity results in limb truncation. Overall these data suggest that NF-κB is a likely component of the Shh/FGF feedback loop in the developing limb.

ACKNOWLEDGMENTS

We thank Brigid Hogan (Vanderbilt University), Nancy Rice (NCI–FCRDC), and Ron Emeson (Vanderbilt University) for reagents and advice. We also thank all the members of the Barnett laboratory for helpful discussions and critical reading of the manuscript. R.R. is a Senior Research Career Scientist of the Department of Veterans Affairs. J.V.B. is an Established Investigator of the American Heart Association. This work was supported by Grant RO1GM51249 from the National Institutes of Health.

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Received for publication March 7, 2001
Revised May 14, 2001
Accepted June 5, 2001
Published online July 30, 2001