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 kinase, IKK-1 and -2, and subsequent degradation by the 26S proteasome. 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, gff-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).
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 (Maicas 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 permeabilized 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 RNA; 0.5 μg/ml Heparin; 1% SDS) for 2 h and then hybridized at 70°C overnight with 1 μg/ml cRNA labeled with Diogoxigenin. 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, onetime 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), fragments conjugated to alkaline phosphatase at a 1:2000 dilution in TBST + 1% agarose 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**

EMSA were performed as previously detailed (Muraoka et al., 2000). Oligos for each of the β binding sites within the murine msx-1 5'-flanking region were designed as follows: xB1 (−41/−18) 5′-acagcccaatgtttctcgagcggc-3′, xB2 (−910/−882) 5′-caggactgtttttctactgcgca-3′, and xB3 (−1037/−1008) 5′-gtccccactcaacctcctggtttg-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 +B binding sites were as follows: Msx1a-B1 (-27) 5′-gagaggcgcctcagagttgggttcgctc-3′, Msx1a-B2 (-868) 5′-agcagacccaaattgaagtcg-3′, or Msx1a-B3 (-942) 5′-cagaaaaaacccaggccgag-3′. Two independently mutagenized clones for each +B binding site were isolated, fully sequenced, and analyzed in transient transfection assays.

Cell Culture and Transfections

Drosophila Schneider S2 cells were cultured and transected 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 monodonal anti-p19gag antibody. RCAS-alkaline phosphatase and RCAS-αΔN viral stocks were titered at 10^6 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 pre-limb 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 quantify 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′-gatgggctggcggagggaggg-3′ and msx-1[704] 5′-agcagcttcgggcgggaggg-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 Bridget 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′-ggggcgccattctccc-3′ and GAPD[579] 5′-gggggcatccacctgcttt-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, in 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 hoxd10 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 quantify 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%
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. 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 hoxd10 (n = 5).
We have termed these sites putative NF-κB binding sites and activates gene transcription in the absence of association with other 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 family members, c-Rel, in the nuclear protein complex that binds the κB 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. 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 luciferase activity, while GFP-2/-4 are two of the AER-derived FGFs. The κB 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 κB 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 κB oligonucleotide after stimulation with FGF-2/-4.

The Normal κB Binding Sites in 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 κB 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 κB 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 κB oligonucleotide after stimulation with FGF-2/-4. Furthermore, these results suggest a direct interaction between NF-κB and the msx-1 promoter.
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 FG F-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

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**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).
activity were observed in cells infected with IκB.B, lanes 2 and 4). Significantly reduced levels of luciferase reporter activity alone or in the presence of control virus (Fig. 6), to stimulation. FGF-2/-4 stimulated a sevenfold increase in

kappa-B binding sites or addition of IκB.B infected 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.

FGF Signal Regulation 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-κBΔN, 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.

Next, we analyzed the role of NF-κB activity in the regulation of msx-1 expression. In our studies, msx-1 expression serves as a reporter for FGF 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, trans-phosphorylate 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-κB, 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. 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.

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, trans-phosphorylate 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-κB, 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. These cells were either not infected, infected with a control virus (pAc), or infected with IκB-κBΔN 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-κBΔN (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.
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 fgrin 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; Lafer 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 Msx-1B-α3N-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 xB3 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.

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