Nuclear Factor I as a Potential Regulator during Postembryonic Organ Development*

Monika Puzianowska-Kuznicka and Yun-Bo Shi‡

From the Laboratory of Molecular Embryology, NICHD, National Institutes of Health, Bethesda, Maryland 20892-5430

Nuclear factor I (NFI) family members are transcription factors that are believed to also participate in DNA replication. We have cloned two Xenopus laevis NFI s that are up-regulated by thyroid hormone. They are 84–95% identical to their counterparts in birds and mammals. In contrast, the two Xenopus NFI s are much less homologous to each other, sharing only 58% homology, which largely resides in the DNA binding domain at the amino terminus. However, both NFIs can bind to a consensus NFI binding site and activate the transcription of a promoter bearing the site. Northern blot reveals that both NFI genes are regulated in tissue- and developmental stage-dependent manners. They are first activated, independently of thyroid hormone, to low levels at stages 23/24, around the onset of larval organogenesis. After stage 54, their mRNA levels are dramatically up-regulated by endogenous thyroid hormone, and high levels of their expression correlate with organ-specific metamorphosis. Furthermore, gel mobility shift assay indicates that the NFI proteins are present in different organs and that their levels are regulated similarly to the mRNA levels. These results strongly suggest that NFIs play important roles during postembryonic organ development, in contrast to the general belief that NFIs are ubiquitous factors.

The proteins of nuclear factor I (NFI) family are transcription factors encoded by multiple genes in birds and mammals (Gil et al., 1988; Santoro et al., 1988; Meisterernst et al., 1988; Paonessa et al., 1988; Inoue et al., 1990; Rupp et al., 1990). In addition, different forms of these factors can be generated by multiple alternative splicing of individual NFI genes (Santoro et al., 1988; Inoue et al., 1990; APT et al., 1994), although the functional difference among these various forms is still unclear. NFIs are sequence-specific DNA binding proteins that recognize a consensus NFI binding site made of TGGCA(N)3TGCCA (Nowock et al., 1985; Gronostajski, 1986; Nilsson et al., 1989). Upon binding to NFI binding sites, these NFIs can activate the transcription of the corresponding promoters (Jones et al., 1987; Cereghini et al., 1987; Santoro et al., 1988). While the mechanism of this transcriptional activation is still unknown, NFI binding sites have been found in a wide variety of genes (Raymondjean et al., 1988; Zorbas et al., 1992; Inoue et al., 1990), and the NFI genes are expressed in many different tissues (Cereghini et al., 1987; Paonessa et al., 1988; APT et al., 1994), suggesting that NFIs are crucial for cell function in many organs. In addition, NFIs have also been found to be required for the initiation of adenovirus replication both in vitro and in vivo (Nagata et al., 1982; Leegwater et al., 1985; Hay, 1985; Wang and Pearson, 1985; Bernstein et al., 1986; Gronostajski et al., 1988). This raises the possibility that NFIs may also participate in cellular DNA replication. However, it remains to be seen whether NFIs play specific roles during development.

We have identified two NFI genes that are up-regulated during the metamorphic transition in Xenopus laevis. Amphibian metamorphosis is an ideal model system to study postembryonic development (Tata, 1993). It systematically transforms every single organ/tissue of a tadpole, for example the total resorption of the tail, de novo development of the limb, and the remodeling of the simple tubular tadpole intestine into a complex, multiply folded adult organ (Dodd and Dodd, 1976; Gilbert and Frieden, 1986; Yoshizato, 1989). While different tissues undergo drastically different changes at distinct developmental stages, all are under the control of thyroid hormone (T₃) (Dodd and Dodd, 1976; Galton, 1983; Kikuyama et al., 1993). T₃ is believed to affect amphibian metamorphosis by regulating the transcription of specific target genes in different tissues through its nuclear receptors (Tata, 1993; Shi, 1994). The two NFI genes were isolated as two such T₃-activated genes during intestinal remodeling, a process that involves both apoptosis of the larval epithelial cells and proliferation and differentiation of the adult epithelial cells (McAvoy and Dixon, 1977; Ishizuya-Oka and Shimozawa, 1987, 1992).

We demonstrate here that the two Xenopus NFIs bind DNA specifically and activate transcription in an oocyte transcription system. More importantly, we show that the expression of the NFI mRNAs as well as the NFI or closely related proteins is up-regulated in the intestine during metamorphosis as the larval organ degenerates and adult intestine develops. Furthermore, the mRNA and protein levels are also high during both limb morphogenesis and tail resorption while very low in premetamorphic tadpoles or embryos. These results strongly implicate the participation of NFIs in frog organogenesis.

MATERIALS AND METHODS

Isolation and Sequencing of Full-length cDNAs for X. laevis NFI Genes—To clone the full-length cDNAs for T₃ (3,3',5'-triiodothyronine)-induced genes in the metamorphosing intestine, the PCR cDNA fragments isolated from a subtractive differentiation screen (Shi and Brown, 1993) were used to screen an intestinal cDNA library (Patterson et al., 1995). Sequence analysis showed that two of the genes encoded proteins homologous to NFIs previously cloned in other species. These two genes, IU16 and IU33 (Shi and Brown, 1993), were renamed as...
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Xenopus NFI-B1 and NFI-C1, respectively. The missing 3'-end of NFI-C1 gene and 5'-ends of NFI-B1 and NFI-C1 genes were cloned using the anchor PCR method of Frohman et al. (1988) and total RNA from stage 6 tadpoles.

Northern Blot Analysis—Tadpoles of indicated stages (Nieuwkoop and Faber, 1956) were treated with or without 5 nM T3, and RNA was isolated as described (Shi and Shi, 1990). Overproduction of Xenopus NFIs and Antibody Generation—The cDNA inserts from the original NFI cDNA clones and anchor PCR clones were used to construct overexpression vectors for the DNA binding domains and the full-length NFI-B1 and NFI-C1 in pET 15b and pET 28a vectors, respectively (Novagen). The clones were transformed into E. coli BL21 (DE3) (Promega). The bacteria were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 30°C. Under these conditions, the proteins produced were insoluble. To generate antibodies against the NFIs, the full-length NFI-B1 and DNA binding domain of NFI-C1 were isolated from the insoluble fraction and further purified on polyacrylamide gels with Chromatochrom stain (Promega). Gel slices containing NFI proteins were excised and used to immunize rabbits.

Cloning of the Full-length Open Reading Frames of NFI-B1 and NFI-C1 into the psP64pA vector and into Virotrans-Transfection—DNA encoding the entire NFI-B1 together with 52 bp of 3'-untranslated region was cloned into BamHI and SacI cloning sites of psP64pA vector (Promega), and the DNA coding region of NFI-C1 together with 384 bp of the 3'-untranslated region was cloned into the BamHI cloning site of the same vector. The resulting construct was linearized and transcribed with SP6 polymerase (Ambion). The purified mRNA was analyzed on 1.2% agarose/formaldehyde gel to determine the quantity.

Overproduction of NFI in X. laevis Oocytes and DNA Binding Assay—Stage 6 oocytes were injected with 25 pg of NFI mRNA/oocyte and incubated at 18°C overnight. The oocytes were homogenized in 70 mM KCl, 20 mM HEPES, pH 7.6, 1 mM dithiothreitol, 5% glycerol, 1 mM MgCl2, and 2 mM phenylmethylsulfonfluoride (10 μM! oocyte), and the protein extracts were prepared as described (Wong and Shi, 1995).

Specific DNA binding by NFI was analyzed by the gel mobility shift assay. 15 μL of the buffer containing 20 mM HEPES, pH 7.5, 5 mM MgCl2, 5 mM dithiothreitol, 10% glycerol, 0.5% Triton X-100, and proteinase inhibitors (5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, and 5 mM phenylmethylsulfonfluoride) was mixed with 2.5 μL of poly(dI-dC) (500 ng) and 32P-labeled double-stranded oligonucleotide (5 ng) containing the consensus palindromic binding site for NFI (ds-NFI, see below). The binding reaction was initiated by adding the above protein extracts to the mixture (2.5 μL of control or ds-NFI extract, or 0.5 μL of ds-NFI-1 extract supplemented with 2.0 μL of control extract; less NFI-C1 extract was used due to more efficient translation of NFI-C1 mRNA (see “Results!”). Samples were incubated for 20 min at room temperature and analyzed on a 6%, 0.5M acrylamide gel with Chromaphor stain (Promega). Gel slices containing the NFIs, the full-length NFI-B1 and DNA binding domain of NFI-C1 were isolated from the insoluble fraction and further purified on polyacrylamide gels with Chromatochrom stain (Promega). Gel slices containing NFI proteins were excised and used to immunize rabbits.

Cloning and Sequence Analysis of Xenopus NFI Genes—To study genes that are involved in the remodeling of the intestine from the larval to adult form, we previously isolated over 20 T3-up-regulated genes in the intestine of X. laevis by using a PCR-based subtractive differential screening method (Shi and Brown, 1993). The small PCR fragments of two such genes were used to screen a λ cDNA library made from intestinal mRNA of premetamorphic tadpoles treated with T3 for 18 h. Sequence analysis of the longest cDNA clones showed that both genes encoded members of the NFI gene family and were named NFI-B1 and NFI-C1 based on sequence homology to known NFIs (Fig. 1). However, the cDNA clones contained only part of the coding regions, as the NFI-B1 cDNA did not have an in-frame initiation codon and the NFI-C1 cDNA did not contain an in-frame stop codon at the 3'-end. In addition, no in-frame stop codon was present upstream of the first methionine codon of the NFI-C1 cDNA, suggesting the possible existence of an additional coding region upstream of this methionine codon.

To clone the missing coding regions, the anchor PCR method of Frohman et al. (1988) was used. The anchor PCR clones of NFI-B fell into three groups, NFI-B1, NFI-B2, and NFI-B3, respectively (Fig. 2). The NFI-B1 group of clones had completely identical DNA sequences in the region overlapping with the original cDNA clone, and their initiation codon lay 180 bp upstream of the 5'-end of the original cDNA. The NFI-B2 class of clones contained a deletion of 135 bp near the amino terminus and a few nucleotide sequence changes, resulting in 2 or 3 amino acid substitutions in the region overlapping the original clone. The last class, NFI-B3, had a deletion of 162 bp immediately after the initiation methionine of NFI-B1 and nucleotide sequence changes that produced two amino acid substitutions. Anchor PCR cloning of the 5'-end of NFI-C1 identified an in-frame stop codon upstream of the first methionine codon of the original cDNA, indicating that this cDNA clone contained the entire amino-terminal coding region. In addition, another clone (NFI-C2) was isolated that had a 27-bp insertion immediately after the methionine codon of the original NFI-C1 clone (Fig. 2). The anchor PCR cloning of the 3'-end of NFI-C1 resulted in four clones, one completely identical with the original cDNA clone and three other clones containing nucleotide sequence changes that resulted in only a single amino acid substitution in the region overlapping the original cDNA clone (Fig. 2B and data not shown). All anchor PCR clones were otherwise identical and encoded the carboxyl terminus of NFI-C1.

The anchor PCR cloning, therefore, revealed the existence of a family of NFI proteins in X. laevis, which can be divided into two subfamilies based on sequence homology (Fig. 1). The strong homology among subfamily members suggests that three members of the NFI-B subfamily are most likely derived from alternative splicing of a single gene, and the subtle differences in their sequences are probably due to polymorphism. Similarly, NFI-C1 and NFI-C2 are most likely encoded by a...
single gene that is alternatively spliced. Such a prediction is supported by the previous reports of different forms of avian and mammalian NFI proteins (Santoro et al., 1988; Rupp et al., 1990), where the sites of sequence divergence match exactly with what we have found for the *Xenopus* NFI proteins (Fig. 1).

Sequence comparison among *Xenopus*, chicken, and human NFI proteins showed a strong homology among the various NFIs (Fig. 1). In particular, the predicted *Xenopus* NFI-B1 protein is over 94% identical to the chicken NFI-B subfamily members with the DNA binding domain being essentially identical (Rupp et al., 1990). Similarly, the *Xenopus* NFI-C1 is most homologous to the chicken NFI-C subfamily members and human NFI/CTF (Santoro et al., 1988; Rupp et al., 1990). Overall, about 84% identity exists among the different NFI-C proteins, and again the DNA binding domain is the most conserved region.

In contrast to the extremely high degree of sequence conservation among the members of a given subfamily, members of different subfamilies are more divergent. Thus, *Xenopus* NFI-B1 shares only 58% identity with *Xenopus* NFI-C1 (Fig. 1, boldface letters). While the carboxyl terminus has only a low level of homology between NFI-B1 and NFI-C1 (42%), the DNA binding domains share over 86% identity.

Specific DNA Binding and Transcriptional Activation by *Xenopus* NFIs—The strong homology among the various NFIs (Fig. 1). In particular, the predicted *Xenopus* NFI-B1 protein is over 94% identical to the chicken NFI-B subfamily members with the DNA binding domain being essentially identical (Rupp et al., 1990). Similarly, the *Xenopus* NFI-C1 is most homologous to the chicken NFI-C subfamily members and human NFI/CTF (Santoro et al., 1988; Rupp et al., 1990). Overall, about 84% identity exists among the different NFI-C proteins, and again the DNA binding domain is the most conserved region.

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Specific DNA Binding and Transcriptional Activation by *Xenopus* NFIs—The strong homology among the various NFIs from different species suggest that they are likely to recognize at least some common binding sites. To study the function of the *Xenopus* NFIs, we chose a consensus binding site derived from (Santoro et al., 1988) and chicken NFI-C subfamily (Rupp et al., 1990). The DNA binding domains are bracketed. The sites of sequence divergence among different NFIs are putative alternative splicing sites and are indicated by arrows. Dots represent amino acid deletions, and dashes indicate identical amino acids. The boldface italic letters are amino acids that are conserved between *Xenopus* NFI-B1 and *Xenopus* NFI-C1 (58%), which concentrate in the DNA binding domain (86%).
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Fig. 3. Expression of NFI-B1 and NFI-C1 proteins in X. laevis oocytes. (A) [35S]methionine was coinjected with water (—) or NFI mRNAs into oocytes. Protein extracts were analyzed on a 10% gel. Dots indicate the positions of the overexpressed proteins. B and C, Western blot analysis of the same protein extracts electrophoresed on 7.5% gels with anti-NFI-B1 (B) or NFI-C1 (C) antibody. Note that both antibodies were specific to their antigens. The two bands of very similar sizes detected by the anti-NFI-C1 antibody that were also present in the water-injected oocytes (—) are probably non-NFI peptides. Dashes on the left indicate the positions of the size markers: 30, 46, 66, 97, and 220 kDa, respectively.

Fig. 4. Specific DNA binding by X. laevis NFIs. Extract from control (—), NFI-B1 (NFI-B1), or NFI-C1 (NFI-C1) mRNA-injected oocytes were used in the gel mobility shift assay with 5 ng of [32P]-labeled ds-NFI and the indicated amount of unlabeled ds-NFI or a nonspecific DNA (ds-NS) competitor.

Studies in birds and mammals (Nilsson et al., 1989) and used the Xenopus oocyte system to overproduce functional proteins. NFI-B1 and NFI-C1 mRNAs were prepared by in vitro transcription and microinjected into mature oocytes. As shown in Fig. 3A, when [35S]methionine was injected into oocytes, many proteins were labeled, demonstrating active translation of endogenous mRNA. When NFI-B1 or NFI-C1 mRNA was co-injected with [35S]methionine, a new labeled protein band appeared on the SDS-protein gel. Although the same amounts of mRNAs were injected, NFI-C1 mRNA was translated a few times more efficiently than NFI-B1 mRNA. The sizes of the new protein bands matched the expected sizes for NFI-B1 and NFI-C1. Furthermore, a polyclonal antibody raised against the full-length NFI-B1 recognized this same band present only in oocytes injected with the NFI-B1 mRNA but not in control or NFI-C1 mRNA-injected oocytes (Fig. 3B). Conversely, a polyclonal antibody against the DNA binding domain of NFI-C1 recognized the polypeptide produced by the injection of NFI-C1 mRNA. The anti-NFI-C1 polyclonal antibody also recognized two endogenous oocyte proteins of very similar sizes (Fig. 3B). These proteins are not likely to be members of the NFI family. This is because, as shown below, oocytes lack any detectable binding activity for the consensus NFI binding site that is recognized by both NFI-B1 and NFI-C1. In addition, the antibody was raised against the highly conserved DNA binding domain of NFI-C1 but failed to recognize NFI-B1. Thus, if these proteins are not members of the NFI family, they would be even more homologous to NFI-C1 than NFI-B1 and would be able to bind to the consensus site.

To study the DNA binding activity of the NFIs, a double-stranded oligonucleotide containing a consensus NFI binding site (ds-NFI) for avian and mammalian NFIs (Nilsson et al., 1989) was end-labeled and mixed with extracts isolated from uninjected or mRNA-injected oocytes. The resulting complex was analyzed by the gel mobility shift assay. While the uninjected oocyte extract gave no detectable complex (Fig. 4, lane 3), extracts from the oocytes preinjected with NFI-C1 or NFI-B1 mRNA formed a strong complex with ds-NFI (Fig. 4, lanes 5 and 12). The complex formed with NFI-C1 migrated faster than that with NFI-B1 (Fig. 4, compare lanes 5–11 with lanes 12–18), consistent with the smaller size of NFI-C1 (Fig. 1). The complexes could be efficiently competed out by the unlabeled ds-NFI itself. In contrast, even a 50-fold excess of a nonspecific double-stranded oligonucleotide (ds-NS) had no effect on the binding by either NFI-B1 or NFI-C1, demonstrating the specificity of the binding.

We next investigated whether the Xenopus NFIs were able to activate the transcription from a promoter bearing the NFI binding site. For this purpose, we inserted two copies of ds-NFI about 140 bp upstream of the major transcription start site of the SV40 early promoter in the CAT promoter vector. The original (CAT) or modified (CAT/NFI) vector was injected into Xenopus oocytes that had or had not been preinjected with the NFI mRNA. After overnight incubation, the transcribed RNA was analyzed by the primer extension assay. No signal was detected in the absence of injected promoter vector (Fig. 5, lanes 1, 4, and 7), demonstrating the specificity of the primer extension. Injection of both the CAT and CAT/NFI vectors gave low levels of transcription in oocytes un.injected with any NFI mRNA (Fig. 5, lanes 2 and 3). The levels of transcription from both vectors were comparable, and both vectors used the same expected major transcription start site.

When the CAT and CAT/NFI vectors were injected into oocytes that had been preinjected with either NFI-B1 (Fig. 5, lanes 5 and 6) or NFI-C1 (lanes 8 and 9) mRNA, they produced very different levels of transcription. The preinjection of NFI mRNAs did not alter the transcriptional activity of the CAT vector (compare lanes 5 and 8 with lane 2) but activated the transcription of the CAT/NFI vector by about 10-fold (compare lanes 6 and 9 to lane 3). As controls for the injection of the
mRNA, our DNA binding, \[^{35}S\]methionine-labeling, and Western blot analysis had consistently shown that both NFI-B1 and NFI-C1 were efficiently translated when their mRNAs were injected into the oocyte cytoplasm. However, we consistently observed more efficient translation of NFI-C1 mRNA, which might explain the slightly higher level of transcriptional activation by NFI-C1. In addition, when the injected promoter DNA was recovered after overnight incubation from the same oocytes used to assay the transcriptional activity and analyzed by hybridization (Fig. 5, lower panel), the results clearly demonstrated that equal amounts of promoter DNA were present in the nuclei of different samples. Thus, like their homologs in other vertebrates, both NFI-B1 and NFI-C1 can activate a promoter containing the consensus NFI binding site.

Organ-specific Developmental Regulation of Xenopus NFI Genes during Metamorphosis—The Xenopus NFI genes were initially isolated as genes that were activated by T₃ in the tadpole intestine and thus might participate in tissue remodeling during metamorphosis. To investigate this possibility further, the cDNAs derived from the original λ cDNA clones were used to probe Northern blots made of total RNA from different tissues during development. Under the hybridization conditions, no cross-hybridization was detected between NFI-B and NFI-C genes (Fig. 6), although the individual members of each subfamily could not be differentiated. The NFI-B and NFI-C probes detected the full-length mRNA of 10 and 8 kilobases, respectively, in different tissues. In the intestine, little NFI-B or NFI-C mRNAs were present in premetamorphic tadpoles (stages 54 and 56, Fig. 6). The mRNA levels for both genes were highly up-regulated during metamorphosis and remained high in the intestines of postmetamorphic frogs (stage 66). Similarly, in the tail, both NFI genes were highly expressed during tail resorption (stages 60–64), while they were repressed in premetamorphic tadpoles (stages 54 and 56). In contrast, high levels of the NFI mRNAs were present in the hind limb at stages 56–60, at the time of and immediately after limb morphogenesis. Subsequently, their expression was reduced to lower levels. These results strongly suggest that both NFI-B and NFI-C are involved in tissue remodeling during metamorphosis.

Thyroid Hormone Regulation of NFI Genes during Metamorphosis—Thyroid hormone is known to be the controlling agent of metamorphosis. It has been well established that a simple addition of T₃ to the rearing water can induce precocious metamorphosis in premetamorphic tadpoles. Thus if the NFI genes participated in metamorphosis, we would expect that they should be expressed during T₃-induced metamorphosis. Therefore, we treated premetamorphic tadpoles at stage 55 with 5 nM T₃, a concentration close to the peak plasma T₃ levels during natural Xenopus metamorphosis (Leloup and Buscaglia, 1977), and isolated RNA from the intestine and tail at various time points during treatment (limb was not used due to its small size). Northern blot analysis of the RNA showed that the mRNA levels for both NFI-B and NFI-C genes were up-regulated within 1 day of treatment and continued to increase, reaching the highest levels after 3–5 days in both the intestine and tail, similar to that observed during normal development (Fig. 7).

NFI Genes Are Activated during Larval Development—The above results suggest that both NFI-B and NFI-C are involved in the development of adult organs. As larval organogenesis occurs during embryogenesis, we asked whether the NFI genes were also expressed during this early developmental period. Thus, total RNA was isolated from the oocytes and whole embryos and tadpoles at different stages up to the end of metamorphosis (stage 66) and analyzed for the expression of NFI-B and NFI-C genes. The mRNAs for both genes were found to be absent in oocytes and early embryos. The genes were first activated around early tail bud stage (stage 23/24) (Fig. 8) and were expressed at relatively low levels around stages 33–45.
This period of NFI expression corresponds to the period of larval organogenesis (the tadpole hatches around stages 35/36 and begins to feed around stage 45; Nieuwkoop and Faber, 1956). Subsequently, these low levels of NFI expression persisted until after stage 54, when they were drastically up-regulated during metamorphosis, following the rise in the concentration of endogenous T₃ (Leloup and Buscaglia, 1977).

**NF1 Binding Activity Is Also Regulated in a Tissue-specific Manner during Metamorphosis—**To investigate the regulation of NFI proteins during development, we initially performed standard Western blot analysis using the specific antibodies described above. However, possibly due to the low abundance of these transcription factors, we failed to quantify the NFI proteins. Therefore, the gel mobility shift assay was employed together with the antibody supershift assay to determine the relative levels of NFI binding activity during development. To test the effect of the antibodies on the NFI DNA complexes, anti-NFI antibodies were added before or after the addition of the labeled ds-NFI to the extracts from oocytes that had been injected with NFI mRNAs (Fig. 9). Independent of the order of addition, the anti-NFI-B1 antibody efficiently supershifted the complex formed by NFI-B1 (Fig. 9, lanes 7 and 8, arrowhead) and to a much smaller extent the complex formed by NFI-C1 (lanes 12 and 13, star). On the other hand, the anti-NFI-C1 antibody had little effect on the complex formation by either NFI-B1 or NFI-C1 (lanes 9, 10, 14, and 15). Thus, the anti-NFI-B1 antibody was chosen for the studies on tissue extracts below.

Tissue extracts from the intestine, limb, and tail of tadpoles at different stages were prepared and subjected to DNA binding analysis. The binding activity for ds-NFI was found to be regulated identically as the NFI mRNA levels in all three organs during metamorphosis (Fig. 10 and data not shown). Thus in both the intestine (Fig. 10A) and tail (Fig. 10C), the NFI binding activity was low in tadpoles before stage 58 and was up-regulated during metamorphosis (stages 62 and 64). On the other hand, the ds-NFI binding activity was high in the limb at stage 56 when morphogenesis took place. Subsequently, the activity decreased as the hind limb underwent growth with little morphological changes (Nieuwkoop and Faber, 1956; Fig. 10B).

The specificity of the DNA binding by the extracts was confirmed by the ability of the unlabeled ds-NFI itself (Fig. 10, lanes 9–12) to compete efficiently for the complex formation and the inability of a nonspecific DNA (ds-NS, lanes 5–8) to do so. Furthermore, anti-NFI-B1 antibody could supershift most of the complexes formed (Fig. 10, lanes 13–16). Based on the mobilities of the supershifted complexes (bands labeled by arrowheads and stars; compare them with those in Fig. 9), it appeared that both NFI-B and NFI-C were present in these tissue extracts and regulated similarly. Thus, while the exact identities of the NFI proteins are unknown, these results strongly suggest that NFI-B and NFI-C or closely related pro-
proteins are the predominant proteins, if not the only ones, that are responsible for the binding to ds-NFI.

NFIs Are Present in Many Adult Organs—NFIs are known to be expressed in many tissues and cell types in mammals (Cereghini et al., 1987; Paonessa et al., 1988). Our Northern blot analysis above also showed that NFI mRNAs were present in frog intestine and limb (stage 66, Fig. 6). To determine whether the proteins are present in adult tissues, selected organs from postmetamorphic frogs were dissected to prepare whole cell protein extracts. Gel mobility shift assay clearly demonstrated the presence of NFI binding activity in all regions of the gastrointestinal tract as well as the limb and liver (Fig. 11). The binding activity was present at lower levels in the limb than in the intestine just like their respective NFI mRNA levels at stage 66 (Fig. 6), immediately after metamorphosis. It is interesting to note that as in the intestine and limb, the levels of the NFI binding activity in liver were very different in the frog compared with the tadpole (Fig. 11, compare lanes 13, 14–16, and 17). Premetamorphic tadpole liver had only low levels of NFI binding activity, while much higher levels were present in the samples. This is probably because proteinases were more abundant in the tail at these stages as the tail resorbs (Nieuwkoop and Faber, 1956). The protein degradation might be also responsible for the inefficient antibody supershifting.

Fig. 10. NFI binding activity is regulated similarly as the NFI mRNAs during development. Whole cell extracts were isolated from the intestine (A), hindlimb (B), and tail (C) of tadpoles at different developmental stages and analyzed for binding to labeled ds-NFI. Specific complexes were formed in the absence (lanes 1–4) or presence of a 20-fold excess of a nonspecific competitor (lanes 5–8) but not in the presence of a 20-fold excess of the unlabeled ds-NFI (lanes 9–12). The addition of the anti-NFI-B1 antibody could supershift most of the complexes formed (lanes 13–16). The arrowheads and asterisks indicate complexes of similar mobilities as the supershifted NFI-B1-DNA and NFI-C1-DNA complexes, respectively, shown in Fig. 9. Note that longer exposure was necessary for the tail samples (C) due to weaker binding activity and that more smear was present in stage 62 and 64 samples. This smear was likely due to protein degradation even though proteinase inhibitors were present in the samples. This is probably because proteinases were more abundant in the tail at these stages as the tail resorbs (Nieuwkoop and Faber, 1956). The protein degradation might be also responsible for the inefficient antibody supershifting.
The interesting regulation of the expression of these genes by T3 is a strong conservation of the sequence and function among the assays, and transcription activation experiments demonstrate amphibian metamorphosis. Sequence analysis, DNA binding factor family that are regulated by thyroid hormone during evolution (Kobel and Du Pasquier, 1986).

FIG. 11. NFI binding activity is present in adult organs. Whole cell extracts were made from different regions of the gastrointestinal tract, hind limb, and liver of young frogs and analyzed for ds-NFI binding activity. The binding activity was present in all tissues, and the binding could be competed out by a 20-fold excess of the unlabeled ds-NFI itself but not by a 20-fold excess of the nonspecific DNA (ds-NS). The adult liver complexes migrated faster, likely due to partial degradation of the NFI proteins. For comparison, stage 56 liver extract contained much less NFI binding activity than the frog liver but produced complexes of similar mobilities as those by the intestinal or limb extracts.

protection extract on an SDS gel did not reveal noticeable protein degradation. Alternatively, different NFI isoforms might be present in the tadpole and frog livers. In any case, the complex formation with all extracts was specific as judged from competition experiments and the ability of anti-NFI-B1 antibody to supershift most of the complexes formed (not shown). Thus, NFIs are present in a wide variety of frog tissues.

DISCUSSION

We have identified at least two genes of the NFI transcription factor family that are regulated by thyroid hormone during amphibian metamorphosis. Sequence analysis, DNA binding assays, and transcription activation experiments demonstrate a strong conservation of the sequence and function among the NFIs from Xenopus, chicken, and human. More importantly, the interesting regulation of the expression of these genes by T3 during metamorphosis provides strong evidence that these transcription factors are important for postembryonic organ development.

Xenopus NFIs Are Encoded by Multiple Genes That Are Alternatively Spliced—NFI was first identified as a component of the Hela cell nuclear extract that can enhance the initiation of adenovirus DNA replication (Nagata et al., 1982). Since then, the corresponding gene and several highly homologous genes have been cloned in birds and mammals (Santoro et al., 1988; Paonessa, 1988; Gil et al., 1988; Meisterernst et al., 1988; Inoue et al., 1990; Rupp et al., 1990). Sequence analysis of the two Xenopus NFI genes reported here demonstrates that they are the homologs of chicken NFI-B and NFI-C genes, respectively. Our anchor PCR cloning has revealed the existence of, at least at the amino end, multiple isoforms for both NFI-B and NFI-C subfamilies in Xenopus. These isoforms differ from each other by some sequence insertions or deletions. While it cannot be ruled out that they are encoded by different genes without cloning the full-length cDNAs, they are likely produced by alternative splicing. First, the sequences of different isoforms are essentially identical except for the insertions or deletions. Moreover, the points of sequence divergence are conserved across species and have been implicated or proven to be the sites of alternative splicing in other species (Santoro et al., 1988; Rupp et al., 1990). It should be pointed out that while this paper was submitted for review, Roulet et al. (1995) reported the cloning of Xenopus NFI-C1. Their NFI-C1 sequence differs slightly from ours. This is probably due to the fact the X. laevis is a pseudotetraploid organism with many of its genes duplicated during evolution (Kobel and Du Pasquier, 1986).

Both NFI-B and NFI-C Can Activate Transcription through a Consensus Binding Site—Both Xenopus NFI-B1 and NFI-C1 can bind to a consensus NFI binding site identified in birds and mammals (Nowock et al., 1985; Leegwater et al., 1985; Gronostajski, 1986; Jones et al., 1987; Nilsson et al., 1989). Although Xenopus NFI-B1 and NFI-C1 share only a low degree of homology with each other compared with their homologs in other species, their DNA binding domains are over 86% identical. Thus, it is not surprising that both can recognize the same NFI binding site.

Furthermore, when either NFI is introduced into Xenopus oocytes, it can activate the transcription from a promoter containing the NFI binding sites.

Currently, it is unclear how the transcription activation takes place. It is known that NFIs can bind DNA as homo- and heterodimers (Mermod et al., 1989; Gounari et al., 1990; Kruse and Sippel, 1994). Furthermore, it has been shown that the amino-terminal half, including the DNA binding domain, is sufficient for dimerization, site-specific DNA recognition, and adenovirus DNA replication (Mermod et al., 1989). In contrast, the carboxyl half of protein and the DNA binding domain are required to activate transcription (Mermod et al., 1989; Altmann et al., 1994; Xiao et al., 1994). It is interesting to note that given the sequence divergence between Xenopus NFI-B1 and NFI-C1 in the putative activation domain, which is only 42% conserved, both can activate transcription to a similar extent in the oocyte transcription system. It is known that the oocyte stores large quantities of different factors important for embryogenesis, especially during the period prior to the onset of zygotic transcription. Thus, it is very likely that Xenopus NFI-B1 and NFI-C1 interact with different factors in the transcriptional machinery to activate the promoter. It would be interesting to know the identities of such NFI-interacting factors.

Correlation of NFI Expression with Natural and T3-induced Metamorphosis—Both NFI-B and NFI-C genes are first activated relatively late, around tailbud stages, during embryonic development. This activation occurs before the development of the thyroid gland and is thus independent of thyroid hormone. Subsequently, the genes maintain low levels of expression throughout the rest of the embryonic period and early tadpole stages. After stage 54, their expression is drastically up-regulated in the tadpole by the rising levels of endogenous thyroid hormone. We have shown previously that this regulation by T3 occurs at the transcriptional level based on its resistance to protein synthesis inhibition (Shi and Brown, 1993). Furthermore, when premetamorphic tadpoles are treated with T3 for an extended period, which can induce precocious metamorphosis such as the intestinal length reduction and epithelial folding (Shi and Hayes, 1994), the expression of the NFI genes is induced similarly as during natural development.

During the premetamorphic stages (before stage 56), the NFI genes are expressed at very low levels in the intestine and tail. They are then drastically activated in the intestine from stage 58 to 66 when larval epithelium undergoes cell death and adult (secondary) epithelial cells as well as the connective tissue and muscle cells proliferate and differentiate (McAvo and Dixon,
NFIs as Regulators during Organ Development—NFIs cannot only regulate the expression of a wide variety of genes through their binding sites located in the promoter regulatory regions of these genes, but they also affect DNA replication and transcription. Thus, while it is unknown how the NFIs are regulated so differently in different organs, the close correlation of their expression with tissue remodeling during metamorphosis argues for a role of these transcription factors in organogenesis.

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Regulation of NFI Genes during Metamorphosis

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