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Characterization of Amphioxus IFN Regulatory Factor Family Reveals an Archaic Signaling Framework for Innate Immune Response

Shaochun Yuan,*1 Tingting Zheng,*1,2 Peiyi Li,* Rirong Yang,* Jie Ruan,* Shengfeng Huang,* Zhenxin Wu,* and Anlong Xu*†

The IFN regulatory factor (IRF) family encodes transcription factors that play important roles in immune defense, stress response, reproduction, development, and carcinogenesis. Although the origin of the IRF family has been dated back to multicellular organisms, invertebrate IRFs differ from vertebrate IRFs in genomic structure and gene synteny, and little is known about their functions. Through comparison of multiple amphioxus genomes, in this study we suggested that amphioxus contains nine IRF members, whose orthologs are supposed to be shared among three amphioxus species. As the orthologs to the vertebrate IRF1 and IRF4 subgroups, Branchiostoma belcheri tsingtauense (bbt)IRF1 and bbtIRF8 bind the IFN-stimulated response element (ISRE) and were upregulated when amphioxus intestinal cells were stimulated with poly(I:C). As amphioxus-specific IRFs, both bbtIRF3 and bbtIRF7 bind ISRE. When activated, they can be phosphorylated by bbtTBK1 and then translocate into nucleus for target gene transcription. As transcriptional repressors, bbtIRF2 and bbtIRF4 can inhibit the transcriptional activities of bbtIRF1, 3, 7, and 8 by competing for the binding of ISRE. Interestingly, amphioxus IRF2, IRF8, and Rel were identified as target genes of bbtIRF1, bbtIRF7, and bbtIRF3, respectively, suggesting a dynamic feedback regulation among amphioxus IRF and NF-κB. Collectively, to our knowledge we present for the first time an archaic IRF signaling framework in a basal chordate, shedding new insights into the origin and evolution of vertebrate IFN-based antiviral networks. The Journal of Immunology, 2015, 195: 000–000.

The IFN regulatory factors (IRFs) were initially identified as regulators of the type I IFNs system in the 1980s (1). To date, nine members, IRF1–9, have been determined in humans and mice, and an additional IRF-10 is specific to chicken and some species of teleosts, including Danio rerio and Paralichthys olivaceus (2–4). All vertebrate IRFs share a well-conserved DNA-binding domain (DBD) in the N-terminal region that recognizes DNA sequences similar to the IFN-stimulated response element (ISRE) (5). Except IRF1 and 2, vertebrate IRFs possess an ISRE-associated domain 1 (IAD1) in the C-terminal region that is responsible for homo- and heteromeric interactions with other family members or other transcription factors (5).

Studies during the past two decades have revealed five mammalian IRFs, IRF1, 3, 5, 7, and 8, which serve as positive regulators of type I IFN and IFN-stimulated genes (2, 6). IRF3 and 7, both residing in the cytosol in a latent form in unstimulated cells, are essential for the retinoic acid-inducible gene 1/melanoma differentiation-associated gene 5-mediated type I IFN gene induction (7). Upon viral infection, IRF3 is activated through phosphorylation by inhibitor of NF-κB kinase (IKK) ε and/or TANK-binding kinase 1 (TBK1), causing it to undergo nuclear translocation (8). The subsequent activation of IRF7 drives the induction of the IFN-α/β cascade in a positive feedback way. The formation of an IRF7 and IRF3 heterodimer, rather than an IRF3 homodimer, is presumed to be crucial for the production of IFN-α/β (9). In addition to the retinoic acid-inducible gene 1/melanoma differentiation-associated gene 5 signaling, mammalian IRFs play critical roles in the TLR-mediated IFN responses (7). The IFN-β induction by the TLR4–Toll/IL-1R domain–containing adapter inducing IFN-β pathway is mainly mediated by IRF3 rather than IRF7, whereas TLR9-MyD88–dependent type I IFN induction is mainly mediated by IRF7 in primary dendritic cells (10). The activation of IRF7 requires the formation of a complex consisting of MyD88, TNFR-associated factor (TRAF) 6, and IRF7 as well as TRAF6-dependent ubiquitination (10, 11). IRF5 and IRF1 have also been suggested to interact with MyD88 and to act as positive regulators of IFN gene induction (12). However, IRF4 can compete with IRF5, but not with IRF7, for MyD88 interaction, hence acting as a negative regulator of TLR signaling (13). In addition to the functions in pattern recognition, multiple IRFs (IRF1, 2, 4, and 8) have attracted attention, as they play central roles in the development of immune cells such as dendritic, myeloid, NK, B, and T cells...
(14). IRF1, 3, 5, and 8 can also modulate cellular responses involved in tumorigenesis (15).

In addition to a large number of studies in mammals, some IRFs in fish, such as IRF3, 5, 7, 8, 9, and 10 in Japanese flounder \( (Paralichthys olivaceus) \) (3, 16, 17); IRF1, 2, 3, 4, 7, and 8 in rainbow trout \( (Oncorhynchus mykiss) \) (18–20); IRF3, 7, and 9 in crucian carp \( (Carassius auratus) \) (21–23); and IRF1, 5, 7, and 10 in zebrafish \( (Danio rerio) \) (4, 24, 25) have also been found to regulate the induction of fish type I IFNs, indicating functional conservation of IRFs in fish and mammals. A search of genomic and expressed sequence tag databases also revealed the existence of IRF-like genes in invertebrate groups. For example, the IRF2 homolog identified in pearl oyster \( (Pinctada fucata) \) has been found to have similarity with vertebrate IRF1 and is involved in NF-kB activation (26). However, given that type I IFN genes have been found only in vertebrates, IFN-based antiviral responses are apparently not present in invertebrates (27, 28), suggesting that the IRF family has an ancient evolutionary origin and arose much earlier than the IFN system (5, 29). Thus, it is of interest to investigate the function of invertebrate IRFs in the absence of the IFN system and attempt to understand how the IFN-based antiviral response coevolved with the IRF family after the divergence of vertebrates from invertebrate-like ancestors. The amphioxus, of the most basal extant chordate lineage, is a key species model for investigating the subcellular localization, the full-length bbtIRFs were inserted into pEGFP-N1 (Clontech). To study the transcriptional activity, the full-length bbtIRFs were inserted into pCMV-BD (Promega). Vectors of bbtMyD88, bbtTRAF6, bbtRel, and bbtIκBα have been described in our previous studies (31–33).

Preparation of nuclear extract and EMSA

Nuclear extract from bbtIRF overexpressed cells was obtained using a NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Scientific). The protein concentration was measured by Pierce BCA protein assay kit (Thermo Scientific). Oligonucleotides for ISRE were synthesized and biotinylated by Invitrogen (ISRE sense, 5'-TGC AGG GAA ACT CAA ACT AAT-3'; ISRE antisense, 5'-ATT AGT TTC AGT TTC CTC GCA-3'; ISRE mutant sense, 5'-TGC AGG CAA ACT CAA ACT AAT-3'; ISRE mutant antisense, 5'-ATT AGT TTC AGT TTC CTC GCA-3'). Complementary oligonucleotide pairs were annealed at 95 °C for 10 min in 10 mM Tris and 1 mM EDTA and cooled slowly at room temperature to create double-stranded biotinylated and unbiotinylated probes. EMSA was performed using a chemiluminescent nucleic acid detection module (Thermo Scientific) according to the manufacturer’s protocol. For competitive coimmunoprecipitation (Co-IP) between the biotinylated ISRE motif and bbtIRFs, nuclear extracts were incubated with double-stranded biotinylated ISRE and Streptavidin Sepharose (GE Healthcare).

Culture of amphioxus intestinal cells and poly(I:C) transfection

Adult Chinese amphioxus \( B. belcheri \) were obtained from Zhangjiang, China, and reared in aerated sea water with algae. Three days before dissection, amphioxi were transferred to sea water without algae to evacuate the intestine. To reduce microbial abundance, on the day prior to dissection, amphioxi were transferred to sea water that was filtered with a 0.45-μm filter and contained 10 mg/ml penicillin. After amphioxi were anesthetized, the amphioxus intestines were extracted, dissected into pieces, and digested for 2 h at 37 °C with 1% collagenase type II (Life Technologies). Then the cells were suspended and cultured in medium (DMEM high glucose [HyClone], DMEM/F12 [HyClone], and Leiboviz’s L15 [HyClone]) supplemented with 10% FBS (Life Technologies) and antibiotics (penicillin and streptomycin; Life Technologies) at 23°C. To test the expression pattern of amphioxus IRFs, the primary amphioxus intestine cells were transfected with poly(I:C) (Sigma-Aldrich) at final concentration of 3 μg/ml by Lipofectamine 2000 and collected at 0, 4, 6, 8, 12, and 16 h after poly(I:C) transfection.

Sectional in situ hybridization was performed according to the protocol described previously (34). Luciferase reporter assay, Co-IP,


**Plasmid constructions**

For the expression of bbtIRFs in HEK 293T cells, PCR fragments encoding the full-length amino acids of bbtIRFs linked to 5'-Flag tag and 5'-hemagglutinin tag were inserted into the expression vector pcDNA 3.0 (Invitrogen) and designated bbtIRFs-Flag and bbtIRFs-hemagglutinin, respectively. The full-length bbtIRF8 was inserted into pCMV-Myc (Clontech) fused with 5'-Myc tag and designated bbtIRF8-Myc. To study the subcellular localization, the full-length bbtIRFs were inserted into pEGFP-N1 (Clontech). To study the transcriptional activity, the full-length bbtIRFs were inserted into pCMV-BD (Promega). Vectors of bbtMyD88, bbtTRAF6, bbtRel, and bbtIκBα have been described in our previous studies (31–33).

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Results

Identification and sequence analysis of nine amphioxus IRFs

Several studies have suggested that 10–13 IRF-like genes may exist in the Branchiostoma floridae genome (5, 29, 35). However, only nine (gene models 209310, 201596, 68560, 232921, 88707, 68559, 89979, 118813, and 178789) are confirmed to have corresponding orthologs in B. belcheri, whose genomic assembly has been recently completed (Fig. 1). Genomic comparison between B. floridae and B. belcheri showed that nine amphioxus IRFs have conserved genomic loci (Fig. 1). For further sequence comparison, the full lengths of nine IRFs were cloned from another Chinese lancelet (B. belcheri tsingtauense) cDNA library and designated bbtIRF1–bbtIRF9. All nine bbtIRFs contain the characteristic DBD domain of BbtIRF1–bbtIRF9. All nine bbtIRFs contain the characteristic DBD domain of vertebrate IRFs, the DBDs of bbtIRF1, 2, 4, 8, and 9 contain the well-conserved Trp repeats spaced by 11–24 residues, whereas the first Trp underwent mutation to Phe in bbtIRF3, 5, 6, 7, and the fourth Trp was mutated to leucine in bbtIRF6 (Fig. 1, Supplemental Fig. 1A). Genomic structure comparison further showed that the DBDs of nine bbeIRFs are well conserved in intron/exon organization, whereas the middle region and IAD1 show significant differences in the pattern of splice junction (Supplemental Fig. 1B). Moreover, the ratio of the rate of nonsynonymous to synonymous nucleotide mutations of amphioxus IRF DBD is <0.25, and that of amphioxus IRF IAD1 is <0.62 (Supplemental Table I). Thus, the DBD domain is likely under purifying selection pressure, suggesting that it might have developed a more specialized function than that found in IAD1.

Phylogenetic analysis based on deuterostome IRF DBDs suggested that predecessors of vertebrate IRF1 subgroup (SG) and IRF4SG might have already existed in the last ancestor of the deuterostome lineage, and it confirmed the orthology between amphioxus IRF1/IRF8 with vertebrate IRF1SG/IRF4SG (Fig. 2). The phylogenetic analysis also suggested that amphioxus has developed its exclusive IRF repertoire, such as IRF3, 5, 6, and 7 (Fig. 2). Many immune and stress gene repertoires have been shown to display rapid expansion and diversification both in the Florida and Chinese lancelet. A notable case is TLR, as 85% of lancelet TLRs became species specific (having no corresponding orthologs in the other lancelet species) within 130 million y ago (36). However, orthologs of nine amphioxus IRFs are supposed to be shared among three amphioxus species (Fig. 2), suggesting that nine amphioxus IRFs may have stabilized and specialized their functions within the last 130 million y.

Transcriptional activity of bbtIRFs

To reveal the functions of the amphioxus IRF family, full lengths of nine bbtIRFs were first inserted into pCMV-BD to make fusion proteins of Gal4 DBD and bbtIRFs. Then, luciferase reporter assays were performed with a reporter construct pL8G5, which contains both Gal4- and LexA-binding sites. LexA-Vp16 (a fusion protein of the LexA DBD and the Vp16 transactivation domain) dramatically stimulates the transcription of this reporter gene. When coexpressed with bbtIRF1, 3, 5, 7, and 8, transcriptional activity of LexA-Vp16 was significantly increased, suggesting that these five bbtIRFs are transcription activators. Alternatively, four bbtIRFs (bbtIRF2, 4, 6, and 9) function as transcription repressors (Fig. 3A). As transcription factors, vertebrate IRFs bind to ISRE (5′gNGAAANNGAAGACT), which is found in the promoters of type I IFN genes and many other genes that participate in immunity and oncogenesis. Further reporter assays showed that five transcription activators (bbtIRF1, 3, 5, 7, and 8) specifically activated ISRE reporter, but not NF-κB, AP-1, and PU box reporters in a dose-dependent manner (Fig. 3B, Supplemental Fig. 2A). Particularly, bbtIRF1 effectively recognizes the promoters of human IFN-α1, IFN-α2, IFN-α6, and IFN-β (Fig. 3C). EMSAs confirmed that bbtIRF1 and bbtIRF8 bind ISRE in a dose-dependent manner (Fig. 3D). Unlike mammalian IRFs, which can form heterodimers to exert their activity, no heterodimer of the transcription activators bbtIRF1, 3, 7, and 8 could be identified (Fig. 3E, Supplemental Fig. 2B, 2C). However, bbtIRF3, 5, 6, 7, and 8 with IAD1 did form homodimers, whereas bbtIRF1, 4, and 9 without IAD1 did not form homodimers.
not (Fig. 3F, 3G), suggesting that IAD1 might be essential for homodimerization.

As transcriptional repressors, bbtIRF2 and 4, but not bbtIRF6 and 9, could suppress the ISRE-dependent transcription activities of bbtIRF1, 3, 7, and 8 in a dose-dependent manner (Fig. 4A, Supplemental Fig. 2D). Further EMSAs confirmed that except bbtIRF6, bbtIRF2, 4, 5, and 9 can bind ISRE (Fig. 4B). Co-IP assays showed that neither bbtIRF2 nor bbtIRF4 can form heterodimers with any one of bbtIRF1, 3, 7, and 8 (Fig. 4C, Supplemental Fig. 2E). However, when coexpressed with bbtIRF1, 3, 7, and 8 in...
HEK 293T cells, bbtIRF4 could compete for the binding of ISRE (Fig. 4D). Thus, these results suggested that bbtIRF2 and 4 function as transcriptional repressors by competing with other IRFs for the binding of ISRE.

The presence of a TBK1–IRFs signal axis in amphioxus

Subcellular distribution of bbtIRFs was accessed using the GFP fusion proteins, and results showed that bbtIRF1, 2, and 4 were restricted to the cell nucleus, bbtIRF6, 8, and 9 were distributed widely throughout the cytoplasm and nucleus, and bbtIRF3, 5, and 7 were localized mainly in the cytoplasm (Fig. 5A). In vertebrates, cytoplasmic IRF3 can be activated through phosphorylation by IKK kinase and/or TBK1 (8, 37), resulting in the IRF3 dimerization and removal of an autoinhibitory structure to allow interaction with other coactivators and the translocation into nucleus. Because bbtIRF3, 5, and 7 are mostly distributed in the cytosol and have the nuclear localization sequences (Fig. 5B), to analyze whether a similar activation mechanism is present in amphioxus, we first performed Co-IP assays and showed that both bbtIRF3 and 7 interact with bbtTBK1, but not with bbtIKKα/β (Fig. 5C).

Then, reporter assays were performed and results showed that when coexpressed with bbtTBK1 in HEK 293T cells, the ISRE-dependent transcription activities of bbtIRF3 and 7 were increased in a dose-dependent manner (Fig. 5D). Moreover, in the presence of bbtTBK1, nuclear distribution of bbtIRF3 and 7 were increased and phosphoserines were detected in HEK 293T cells (Fig. 5E, 5F). Thus, we assumed the presence of a TBK1–IRFs signal axis in amphioxus.

Target genes of amphioxus IRF family

Because most amphioxus IRFs can bind to ISRE, we conducted genomic screening of IRF target transcripts by collecting the 2-kb sequences upstream of ATG of all amphioxus B. belcheri transcripts. Then, sequences with the ISRE mark sequence AA(G|C|A|G|N)(T|C|A|G|N)GAAA or its reverse strand sequence TTTC(G|C|T|A|N)(T|C|G|A|N)TT were identified and collected by a Perl script. Finally, a total of 214 candidate target genes of the amphioxus IRF family were identified. Gene functions assembled by KEGG showed that the putative target genes of amphioxus IRFs primarily participate in immune defense and cell growth and death, which are coincident with those in vertebrates (Fig. 6A). As shown in Fig 6B, some predicted genes contain pathogen-associated molecular pattern recognition domains, such as chitin-binding domain, Ig, leucine-rich repeats, and CLECT. Genes related to apoptosis, such as EDA-like (gene ID 154370), caspase–like (ID 112340), BecL1 (ID 197290), and genes involved in complement system, such as 042710 with fibrinogen-related domain and 060170 with C1Q domain were also identified. More interestingly, several transcriptional factors, including bbeRel (gene ID 274880), bbeIRF2 (ID 246490), and bbeIRF8 (ID 108280), were identified as putative IRF target genes in amphioxus.

Dynamic feedback regulation of amphioxus IRFs and NF-κB

To confirm the genomic screening results, the upstream sequences of 17 candidate target genes, including bbeIRF2 (gene ID 246490), bbeIRF8 (ID 108280), bbeRel (gene ID 274880), bbeEDA-like (ID 154370), and others were cloned from the B. belcheri genome and inserted into pGL3 luciferase vectors to test whether they could be recognized by bbtIRF1, bbtIRF3, bbtIRF7, or bbtIRF8 (Fig. 7A). Twelve of the 17 candidate genes were confirmed to be targets of bbtIRF1, 3, 7, or 8, indicating 80% accuracy. Promoter regions of bbeIRF2, bbeEDA-like, bbecaspase 8–like, BecBcL1, and 154440 with CLECT domain can be specifically recognized by bbtIRF1; promoters of bbeIRF8, bbeTrim2a-like (299800), and 060170 with C1Q domain are recognized by bbtIRF7; promoters of bbeRel, gene 142900 and 001450 are recognized by bbtIRF3; promoter of gene 170570 is recognized both by bbtIRF1 and bbtIRF8 (Fig. 7B, Supplemental Fig. 3A).

To further reveal the dynamic transcriptional regulation among IRFs and NF-κB in amphioxus, we cultured primary amphioxus

![FIGURE 4](http://www.jimmunol.org/) Transcriptional regulation among bbtIRFs. (A) The effects of bbtIRF2, 4, 5, 6, and 9 on the ISRE activation of bbtIRF1, 3, 7, and 8 were analyzed. Results were obtained from luciferase reporter assays by cotransfection of indicated bbtIRFs with the ISRE luciferase reporter into HEK 293T cells. The line chart corresponding to (A) is presented in Supplemental Fig. 2D. (B) EMSAs showed that bbtIRF2, 4, 5, and 9, but not bbtIRF6, can bind directly with the ISRE motif. (C) Co-IP results showed that no heterodimer can be formed between bbtIRF4 and any one of bbtIRF1, 3, 7, and 8. The homodimer of bbtIRF8 served as positive control. (D) Biotin pull-down assays showed that bbtIRF4 competitively binds ISRE motif with bbtIRF1, 3, 7, and 8. All Co-IP results are representative of at least two independent experiments.
intestine cells and transfected them with FITC-labeled poly(I:C) (Fig. 7C), as the digestive system is thought to comprise the major immune organs of amphioxus and contain many immune-related cells, including lymphocyte-like, monocyte-like, and macrophage-like cells (30). Then, RT-PCR assays were conducted and results showed that transcripts of bbeIRF1 and bbeIRF8 were upregulated in 2 h and sustained to 12 h, whereas transcripts of bbeIRF2 were upregulated in 16 h when amphioxus intestinal cells were transfected with poly(I:C) (Supplemental Fig. 3B). Using anti-bbeIRF8 mAb, which can recognize the Flag-tagged bbtIRF8 and the endogenous bbeIRF8 (Fig. 7D), we confirmed that the protein level of endogenous bbeIRF8 was significantly upregulated 8 h after poly(I:C) transfection (Fig. 7E). Because bbeRel was verified as the target gene of bbtIRF3, we further performed reporter assays and showed that bbtRel could mount the ISRE-dependent transcriptional activity of bbtIRF3 and 7, but not bbtIRF1 and 8 (Fig. 7F, Supplemental Fig. 3C). Moreover, when coexpressed with bbtMyD88, both bbtIRF3 and 7 can mount the NF-κB activation mediated by bbtMyD88 in a dose-dependent manner, suggesting that bbtIRF3 and 7 may be involved in amphioxus MyD88/NF-κB–dependent signaling (Supplemental Fig. 3D).

Discussion
Characterization of amphioxus IRFs presents a separate evolutionary event for nonvertebrate deuterostome IRFs

IRF-like genes have been found in sea sponges, tracing the origin of the IRF family to multicellular organisms (5, 29). However, invertebrate IRFs differ from vertebrate IRFs in genomic structure and syntenic gene arrangement (5, 29). For example, no IRF-like sequence was found in Drosophila and nematodes, but up to nine IRFs were found in cephalochordates. Several studies that tried to verify the relationship between amphioxus and vertebrate IRFs have linked bIRF1 (gene ID 209310, as BF4 in their study) to
vertebrate IRF1SG (including IRF1 and 2), and bIRF8 (ID 118813, as BF3 in their study) to vertebrate IRF4SG (including IRF4, 8, and 9) (5). Nehyba et al. (5) suggested that bIRF1 and bIRF8 locate in chordate linkage group 6 and chordate linkage group 3, which represent the origin loci of vertebrate IRF1SG and IRF4SG, respectively (5). Our phylogenetic analysis of nine amphioxus IRFs led to the similar observation and supported the orthology between nonvertebrate deuterostome IRF1 and vertebrate IRF1SG. Besides, we provided additional information to support the functional relationship between amphioxus IRF1/IRF8 and vertebrate IRF1SG/IRF4SG. We showed that among nine amphioxus IRFs, amphioxus IRF1 is the only one that could bind the promoters of human IFN-α1, IFN-α2, and IFN-α6. Moreover, both amphioxus IRF1 and 8 can bind ISRE and the promoter of human IFN-β and be upregulated when amphioxus intestinal cells were stimulated with poly(I:C), suggesting that amphioxus IRF1 and 8 have possessed characteristics key for the recruitment of the vertebrate IFN system.

In addition to genes with a clear relationship to vertebrate IRF1SG and IRF4SG, amphioxus has lineage-specific IRFs, such as IRF3, 5, and 7. In infected cells, mammalian IRF3 and 7 are activated by TBK1-mediated phosphorylation, leading to their dimerization.
nuclear translocation, and the transcription of type I IFN genes (6). In the present study, we showed that bbtIRF3 and 7 bind to the ISRE motif, reside in the cytoplasm, and are phosphorylated by bbtTBK1, suggesting the presence of a TBK1–IRFs axis in amphioxus. However, amphioxus IRF3 and 7 did not form heterodimers, showing differences between the vertebrate and amphioxus TBK1–IRFs axis. Besides serving as transcriptional activators, some amphioxus-specific IRFs, including IRF2, 4, 6, and 9, function as transcriptional repressors. Similar to mammalian IRF2, which can repress the IRF1 transactivation of certain promoters through competition for the same DNA-binding sites (1), bbtIRF2 and 4 mainly reside in the nucleus and function as transcriptional suppressors by competing with other IRFs for the binding of ISRE. An interesting observation is that amphioxus IRF4 and 8 emerged by lineage-specific duplication have contrary transcriptional activities, suggesting the functional specialization of amphioxus-specific IRFs. Collectively, the amphioxus IRF family not only has genes linked to the predecessors of vertebrate IRF1SG and IRF4SG, but it also has lineage-specific IRFs with specialized functions, suggesting a separate evolutionary event for nonvertebrate deuterostome IRFs.

Amphioxus IRFs shed light on the evolution of IFN-based antiviral responses

Although the IFN system has been found to be conserved in all tetrapods and fishes, gene models of IFNs have not been identified in the amphioxus genome (28, 38). By seeking the target genes of amphioxus IRFs, we found that the putative target genes of amphioxus IRFs are mainly involved in innate immunity, cell death, and growth. For example, bbtIRF1 activated the promoter of amphioxus EDA-like, caspase 8–like, and Bcl2L1, whose counterparts in vertebrates are involved in the development of the ectoderm and related to immune regulation (39). bbtIRF1 also recognized the promoters of genes with pathogen-associated molecular pattern recognition domains, such as CLECT, suggesting the dual roles of amphioxus IRF1 in both embryonic development and immune defense. An interesting finding is that amphioxus IRF2 was found as the target gene of bbtIRF1, whereas IRF8 was the target gene of bbtIRF7. The upregulation of bbeIRF2 and bbeIRF8 when amphioxus were stimulated with poly(I:C) suggests the feedback regulation among amphioxus IRFs.

The relationship between amphioxus IRFs and NF-κB is also of interest. In vertebrates, both transcription factors play an essential role in immune cell development and function, cooperatively regulating the expression of many cytokine genes. For example, IRF3 binds to the p65 subunit of NF-κB to transactivate a set of NF-κB–dependent genes without binding to an ISRE. IRF-1 interacts with NF-κB to induce the production of inducible NO synthase, which is relevant for the production of NO as a defense against bacterial infection and for elimination of tumor cells (40). We have suggested that, unlike IRFs, amphioxus NF-κB has not
experienced gene duplication. Only two NF-kB genes were present in both *B. floridae* and *B. belcheri* genomes (33). Thus, it is possible that amphioxus IRFs can interact with NF-kB to control the specificity and magnitude of their transcription events.

Our previous studies not only have identified gene models similar to vertebrate RIG-I-like receptors, but also indicated that amphioxus TLRs and Nod-like receptors expanded to 39 and 73 members, respectively (41). In the present study, we assumed the archaic IRF signaling network in amphioxus *B. belcheri* as follows: when amphioxus cells are stimulated with virus or other pathogens, bbeIRF3 and 7 can be phosphorylated by bbeTBK1 and then translocated into the nucleus for target genes transcription. The subsequent expression of bbeIRF8 and bbeRel leads to the magnification of IRF3/7-based responses to immune demands (Fig. 8). Because several STAT-binding sites were found in the promoter region of bbeIRF1, another path may be presented as follows: when amphioxus cells were stimulated by pathogens, the magnification of IRF3/7-based responses to immune demands.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


