Cloning and Functional Characterization of Hypoxia-Inducible Factor 1alpha Upstream Regions in Xenopus laevis

Conor W. Sipe

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CLONING AND FUNCTIONAL CHARACTERIZATION OF HYPOXIA-INDUCIBLE FACTOR 1α UPSTREAM REGIONS IN XENOPUS LAEVIS

A Thesis
Presented to
The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of

Master of Arts

by
Conor W. Sipe
2003
APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Arts

Conor W. Sipe

Approved, May 2003

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ABSTRACT

HIF-1, a basic helix-loop-helix transcription factor, is the critical mediator of oxygen-dependent gene expression in most organisms. Well-characterized in mammals, HIF-1 is a heterodimer composed of an alpha and beta subunit, although its biological activity is determined by the activity of the HIF-1α subunit. Following exposure to hypoxic conditions, HIF-1α protein is stabilized and translocates to the nucleus, where it activates the transcription of a number of hypoxia-inducible genes. While the post-translational control of HIF-1α has been well characterized, much less is known about its transcriptional regulation. In the present study, we set out to determine if transcriptional regulation of HIF-1α is a general feature of vertebrate development, and the degree to which HIF-1α promoter structure and regulation are conserved in a non-mammalian species. To this end, we have cloned the upstream regulatory regions of the HIF-1α gene in *Xenopus laevis* and analyzed the gene's embryonic expression pattern. Using transgenic techniques, we present the first in vivo characterization of a vertebrate HIF-1α promoter. These experiments, combined with in vitro transfection and the analysis of HIF-1α alleles, have allowed us to identify a short region of the promoter sufficient to drive normal HIF-1α expression during *Xenopus* development.
CLONING AND FUNCTIONAL CHARACTERIZATION OF THE UPSTREAM REGIONS OF HYPOXIA-INDUCIBLE FACTOR 1α IN XENOPUS LAEVIS
INTRODUCTION

As the final electron acceptor in the electron transport chain, oxygen is essential for the cellular production of ATP in aerobic environments. Consequently, few organisms in the animal kingdom can survive prolonged periods of anoxia. This need has led necessitated highly sensitive and regulated mechanisms to maintain oxygen homeostasis (reviewed in Hochachka and Lutz, 2001). A central mediator of oxygen-dependent gene expression is the HIF-1 complex. Well-characterized in mammals, HIF-1 is a heterodimer composed of an alpha and beta subunit, although its biological activity is determined by the activity of the HIF-1α subunit (Jiang et al., 1996; Jiang et al., 1997). Under normoxic conditions, HIF-1α protein is ubiquinated and rapidly degraded by the proteasome. However, following exposure to hypoxia, HIF-1α is stabilized and translocates to the nucleus, where it activates the transcription of a number of hypoxia-inducible genes by binding to a response element present in the upstream regulatory regions.

While the post-translational control of HIF-1α is well understood, particularly in mammals, much less is known regarding the relative importance of the transcriptional regulation of HIF-1α. Several studies have reported no significant changes in HIF-1α mRNA levels in response to hypoxia either in vitro in mammalian and fish cell lines (Wenger et al., 1997a; Wenger et al., 1997b;
Soitamo et al., 2001) or *in vivo* in mouse (Stroka et al., 2001). However, other reports demonstrated that while HIF-1α mRNA is constitutively expressed in cell culture, it is inducible to higher levels under low oxygen conditions (Wang et al., 1995). An increasing number of studies have consistently found that HIF-1α mRNA levels are upregulated in response to hypoxia; this has been demonstrated in the heart of chick embryo (Catron et al., 2001), ferret lung (Yu et al., 1998), and neonatal rat heart (Nguyen and Claycomb, 1999). Moreover, levels of human HIF-1α mRNA are increased in an early response to cardiac ischemia (Lee et al., 2000).

Although hypoxia is often associated with patho-physiologic conditions (reviewed in Semenza, 2000), it is also a natural aspect of development to which embryos must adapt, suggesting that HIF-1α expression is tightly regulated during development (Chen et al., 1999). Its critical role is illustrated by a homozygous null deletion of the HIF-1α gene in mouse; resulting embryos are developmentally arrested by embryonic day 8, exhibiting marked cardiovascular malformation and a failure of the neural tube to close (Iyer et al., 1998). The hypoxic conditions present during the latter part of gestation in developing rat brain have been demonstrated to induce a localized increase in HIF-1α transcripts (Royer et al., 2000). Furthermore, *in situ* hybridization data show spatial regulation of HIF-1α mRNA in the mouse embryo, with the highest levels present in the brain, heart, and kidney (Jain et al., 1998). Work in the chick embryo has shown increased HIF-1α expression in areas analogous to those identified in embryonic mouse (Takahashi et al. 2001). There is also evidence
for temporal regulation, as HIF-1α mRNA expression has been shown to be relatively high in early mouse and human fetal stages and decrease as gestation progresses (Madan et al., 2002).

The majority of work on the regulation of the HIF-1α system has been in mammalian systems, and although the mouse and human promoter regions have been characterized, the in vivo analysis of any vertebrate HIF-1α promoter has yet to be performed. Given the key role of hypoxia in embryogenesis and the wide scope of action that HIF-1α has in the cell (reviewed in Semenza, 2002), we wish to determine if control of HIF-1α at the transcriptional level is a general feature of its regulation during vertebrate development, and the degree to which HIF-1α promoter structure and regulation may be conserved in a non-mammalian species. In order to address these questions, we have cloned the upstream regulatory regions of HIF-1α in the amphibian *Xenopus laevis* and characterized the gene’s developmental expression pattern. The accessibility of large numbers of eggs, the ease of embryonic manipulation, and the availability of transgenesis in *Xenopus* makes the frog an excellent model for examining HIF-1α gene expression in vivo (Kroll and Amaya, 1996; Kroll and Kirschner, 1999). In the present study, we have generated transgenic *Xenopus* embryos in order to analyze the expression patterns of GFP reporter constructs driven by truncated portions of xHIF-1α 5’-flanking sequence. These experiments, coupled with in vitro transfection of these constructs and the sequence analysis of two xHIF-1α alleles, have allowed us to identify a short conserved region of the promoter sufficient for normal expression of xHIF-1α during *Xenopus* development.
EXPERIMENTAL PROCEDURES

Cloning of Xenopus HIF-1α cDNA

Clones containing the xHIF-1α cDNA were isolated by low stringency screening (Sambrook and Russell, 2001) of a Xenopus liver cDNA library using a radiolabeled probe consisting of the mouse EPAS1 cDNA sequence (GenBank accession NM_010137, a gift of S. McKnight). Phage DNA was isolated from positive clones using standard techniques (Sambrook and Russell, 2001). The xHIF-1α cDNA sequence has been deposited in GenBank under the accession number AY189821.

Isolation of HIF-1α Promoter Alleles and Sequencing

Genomic clones containing the xHIF-1α upstream region were isolated by low stringency screening (Sambrook and Russell, 2001) of a Xenopus genomic library (Clontech) with a 158 bp radiolabeled probe from exon 1 of the xHIF-1α cDNA (bases 128-285). Screening of approximately 2.5 x 10^5 phage plaques produced five positive clones whose DNA was subsequently isolated using standard techniques. Phage DNA from the initial clone was digested with Sacl to excise the entire insert from λ EMBL3 SP6/T7 and cloned into pBluescriptSK+ (Stratagene). This clone was digested with various restriction enzymes and Southern blot analysis was performed to identify and subsequently subclone xHIF-1α exon 1 and upstream regions. Hybridizing fragments were sequenced.
using the SequiTherm EXCEL II kit (Epicentre Technologies) on a LiCor 4200 sequencer. Sequence from additional clones was obtained using LiCor Dye800 terminator sequencing with one of the following primers: 5’-CTGGAGTTCTAGGAGAAGCTTTGC or 5’-GAACATGGTGCAAAAGCGTGCAACTTC.

Inserts from four remaining positive phage isolates were excised with Sacl and cloned into pBluescriptSK+. Both strands of each clone were sequenced using dye terminator sequencing with specific primers designed using the initial phage isolate HIF-1α 5’ sequence. The overlapping sequences were aligned using the Sequencher software package (GeneCodes) to obtain 3 kb of the 5’ flanking sequences for each genomic clone. Subsequent sequence analysis and manipulations were performed using the Vector NTI (Informax) and OMIGA (Oxford Molecular) software packages. The above sequences have been deposited in GenBank under the accession numbers AY262064-AY262068.

*In situ Hybridization*

Probes were transcribed from the pxHIF1 plasmid, containing the 1467 bp partial xHIF-1α cDNA in pBluescriptSK+, and labeled with digoxigenin-UTP. To generate the antisense probe, pxHIF1 was linearized with BamHI and transcribed with T3 RNA polymerase; the sense probe was generated by linearizing pxHIF1 with HindIII and transcribing with T7 RNA polymerase. Whole mount *in situ* hybridization was performed with staged embryos (Nieuwkoop and Faber, 1967) as described in Sive et al. (2000) with the following minor modifications: embryos
were fixed in MEMFA (100 mM MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, and 3.7% formaldehyde) for one hour at room temperature then successively washed for five minutes in 100% ethanol, 75% ethanol, 25% ethanol/75%PTw (10 mM phosphate buffer, pH 7.4, 150 mM NaCl and 0.1% Tween 20), and 100% PTw; following treatment with acetic anhydride, the embryos were rinsed twice for 5 minutes in PTw; to visualize antibody bound to probe RNA, embryos were incubated with 1 ml AP buffer (100mM Tris, pH 9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween 20, 2mM levamisol) containing 4.5 µl nitro blue tetrazolium (50 mg/ml) and 3.5 µl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml) at room temperature until staining became apparent. The embryos were then embedded in paraffin, cut into 10 µm sections, and mounted onto microscope slides for visualization by light microscopy.

Construction of GFP Reporter Plasmids

Oligonucleotide primers (Genelink) were used to create four different GFP constructs through amplification of specific xHIF-1α 5’ flanking regions from our original genomic clone. Except where noted, fresh PCR product was cloned into pCRII vector (Invitrogen) following the manufacturer’s instructions and then excised using EcoRI for subsequent cloning into pEGFP-1 vector (Clontech). Dye terminator sequencing as described above was used to confirm the identity and orientation of all constructs. Abbreviations in the construct names are based on the sequence position relative to the xHIF-1α transcriptional initiation site, as determined by ribonuclease protection analysis.
HIF(-1658)GFP was made by amplifying a 2492bp fragment from the initial phage DNA isolate using the primers 5'-CCCCCAAGCAGCTAACAACAG and 5'-GCCAAACGAGTGGGGGAGGGAC. Following cloning into pCRII, digestion with EcoRI resulted in a deletion of the 5' end, yielding a final 1958 bp (-1658/+302) fragment that was ligated into pEGFP-1.

HIF(-508)GFP was constructed by amplifying a fragment that extended 808 bp (-508/+302) upstream of the xHIF-1α translational start site using the primers 5'-CTGGAGTTCTAGGAGAAGCTTTGC and 5'-GCCAAACGAGTGGGGGAGGGAC.

HIF(-181)GFP was produced by digesting the xHIF-1α genomic clone with SnaBI and HindIII to excise 1.5 kb from the 5' end of the insert. The remaining overhangs were filled in with Klenow DNA polymerase and the blunt-ends ligated together with T4 DNA ligase, resulting in a 481 bp (-181/+302) insert in pEGFP-1.

HIF(UTR)GFP was constructed by amplifying 279 bp (+23/+302) of the xHIF-1α 5' UTR from the genomic clone using the primers 5'-TTTTGTTTGCCTTGAATCAGGAGT and 5'-GCCAAACGAGTGGGGGAGGGAC.

Ribonuclease Protection Assay

Two probes from different regions of *Xenopus laevis* HIF-1α were employed. The first of these, HIFex1, consists of the region immediately upstream of the xHIF-1α translational start site. pHIFex1 was constructed by PCR amplification of an 808 bp fragment from the genomic clone with the primers 5'-CTGGAGTTCTAGGAGAAGCTTTGC and 5'-
GCCAAACGAGTGGGGGAGGGAC. This fragment was cloned into pBluescriptSK+, linearized with BamHI, and transcribed with T3 RNA polymerase to produce an antisense probe. Linearizing pHIFexI with HindIII and transcribing with T7 RNA polymerase produced a corresponding sense probe. The second xHIF-1α specific probe, HIFex5-6, was constructed from a 206bp fragment that was amplified from exon 5 and 6 of the xHIF-1α cDNA using the primers 5'-

GAACAAATCACAGAGCGAAGTTTC and 5'-

GAGGAATTGGTTCACAGATTACAACC. The resulting fragment was cloned into pCRII in both orientations, linearized with BamHI, and transcribed with T7 RNA polymerase to produce sense and antisense probes.

*Xenopus* elongation factor-1α (EF-1α) or cytoskeletal actin served as an internal control for all assays (Gurdon et al., 1985; Kreig et al., 1989). Because EF-1α is developmentally regulated and not detectable before stage 8, the less abundant cytoskeletal actin gene served as the normalization control for assays where band intensities were quantitated. Both probes were re-subcloned into pBluescriptSK+. A 1782 bp fragment of pSp64 EF-1α/GS17 was excised with PstI, cloned into pBluescriptSK+, linearized with Ncol, and transcribed with T7 RNA polymerase to produce a 185bp EF-1α probe. A 900bp PstI-EcoRI cytoskeletal actin fragment was excised from pSpβ-1, cloned into pBluescriptSK+, linearized with EcoRI, and transcribed with T7 RNA polymerase. These two antisense probes detected protected fragments that were 119bp and 85bp in size, respectively.
Total RNA was isolated from staged *Xenopus* embryos using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Ribonuclease protection assay (RPA) was performed on approximately 20 μg of total RNA using the HIFex1 or HIFex5-6 probe and an internal control using standard methods (Sambrook and Russell, 2001) with the following modifications: transcription of linearized templates (1-2 μg) was carried out at 37°C for 45 minutes in a final volume of 20 μl containing 40 mM Tris (pH 8.0), 8 mM MgCl₂, 2 mM spermidine, 50 mM NaCl, 10 mM DTT, 500 μM r(GAC)TP, 12 μM rUTP, 1.2 U/μl Rnasin, 30 μCi of [³²P]rUTP (800 Ci/mmol, NEN), and 50 U RNA polymerase; an additional 50 U of RNA polymerase was added and the reactions were incubated an additional 45 minutes; hybridizations were performed at 55°C overnight (10-16 hr) in a total volume of 20 μl hybridization mix with 50,000-100,000 cpm of probe; samples were treated with RNase and digested at 37°C for 30 minutes. Protected fragments were resolved on a 6% polyacrylamide-8M urea sequencing gel and visualized by exposing the gel to X-ray film with an intensifying screen at -80°C for 1-4 days. The autoradiographs were digitized into TIF files and subjected to densitometric analysis using the ImageQuant 5.0 software package (Amersham Biosciences). Values were imported into Microsoft Excel and SigmaPlot (SPSS Science) for numerical analysis and data plotting. Background intensities were subtracted from each HIF-1α protected band and normalized to the cytoskeletal actin protected band before comparisons of the relative ratios of intensity at each embryonic stage were determined.
Transgenesis

Transgenic *Xenopus laevis* embryos expressing the xHIF-1α promoter constructs described above were generated using the restriction enzyme mediated integration (REMI) method essentially as described by Kroll and Amaya (1996). The reaction was prepared by mixing 5 μl of NotI linearized plasmid (150-300 ng/μl) with 1.0 μl of sperm nuclei (~4 x 10⁶ nuclei). Following incubation at room temperature for 5 minutes, 2 μl of 100 mM MgCl₂, 0.5 μl of a 1:20 dilution of NotI (1000U/μl), 10 μl of oocyte extract (preheated at 80°C for 8 minutes then centrifuged at 14,000 x g for 5 minutes), and 15 μl of sperm dilution buffer (250 mM sucrose; 75 mM KCl; 0.5 mM spermidine trihydrochloride; 0.2 mM spermine tetrahydrochloride) were added and incubated at room temperature 10 additional minutes. The reaction mix was diluted in sperm dilution buffer to a final concentration of 3-5 sperm nuclei per 5 nl, and injected into dejellied oocytes at a rate of 5 nl/sec using a syringe pump (AH 55-4154, Harvard Apparatus). Normally developing embryos were maintained in 0.1X MMR with gentamycin sulfate (50μg/ml) until stage 33, when they were snap frozen in liquid nitrogen or fixed in 1X MEMFA for further analysis.

Identification of Transgenic Embryos by PCR

To confirm transgene integration in the embryos produced using the REMI method, PCR was performed on genomic DNA using GFP specific primers. Genomic DNA was isolated from individual embryos according to the method of Sive et al. (2001). PCR primers used to amplify GFP were: (forward) 5'-
CAAGCTGACCCCTGAAGTTTCATCTG and (reverse) 5'-
CGGATCTTGAAGTTGACCTTGATC. The reaction mixture for PCR included 10
mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM MgCl₂, 0.2 mM each dNTP, 1μM of
each primer, and 2.5 units of AmpliTaq (PerkinElmer Life Sciences). PCR was
performed as follows: 5 minutes at 95°C followed by 30 cycles of 94°C for 40s,
55°C for 1 minute, and 72°C for 1 minute, followed by a final 7 minutes at 72°C.
PCR results were analyzed on 1.5% agarose gels.

Cell Culture and Transfections

The *Xenopus* renal epithelial cell line A6 (CCL-102; American Type
Culture Collection) was maintained in 75% NCTC-109 medium (BioWhittaker,
Inc.) supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 60°C
for 30 minutes), 100 μg/ml of streptomycin, and 100 U/ml of penicillin, at 26°C in
humidified 5% CO₂. For each experiment, approximately 6 ×10⁵ cells were co-
transfected with the GFP construct of interest and pCMVβ (BD Biosciences)
using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s
instructions. Briefly, 2 μg of DNA was diluted in 250 μl of serum-free medium
and 14 μl of PLUS reagent (Invitrogen) was added. Following a 15 minute
incubation at room temperature, 20 μl of LipofectAMINE diluted in 250 μl serum-
free medium was added to the reaction. This was incubated at room
temperature for 15 minutes and gently added to cultures in 60 mm dishes
containing 4 ml made NCTC-109 medium. After 24 hours, the transfection
medium was replaced with fresh medium and the cells were incubated for an
additional 48 hours as above. To assay cultures for GFP activity, each cell monolayer was digested with 0.25% trypsin solution and lysed using sonic disruption and 50 µl reporter lysis buffer (Promega). Cell lysate fluorescence was measured using a VersaFluor fluorometer (Bio-Rad) equipped with GFP filters. These values were normalized to β-galactosidase activity as measured using the β-galactosidase enzyme assay kit (Promega) according to the manufacturer’s protocols. The SuperANOVA software package was used to perform ANOVA analysis to test for differences among the six treatment groups. Because the variance of pEGFP-C1 was significantly different from other treatments, ANOVA was carried out on log-transformed data. Eight a priori comparisons (orthogonal contrast comparisons) were performed. The critical value for each of the planned comparisons was adjusted to 0.00625 according to the method of Bonferroni to give an experiment-wise significance level of p < 0.05.
RESULTS

Isolation of Xenopus HIF-1α Upstream Regions

A xHIF-1α exon 1 cDNA probe (GenBank accession AY189821) was used to screen a *X. laevis* genomic library at low stringency in order to identify clones containing a HIF-1α promoter region. We screened $2.5 \times 10^6$ plaques and obtained five positive clones. One of these clones was further analyzed by Southern blot using the 5’ xHIF-1α cDNA probe to localize the approximate position of upstream regulatory regions. A 4554 bp EcoRV fragment and a 2386 ScaI fragment that hybridized with the probe were subcloned into pBluescriptSK+ and sequenced using both primer-labeled and dye terminator methods. Subsequent sequence analysis revealed that the sequence contained the 5’ flanking sequence, first exon, and 2020 bases of the first intron of HIF-1α.

Sequencing of additional HIF-1α promoter alleles

Recombinant phage DNA from the four additional positive clones was subjected to Southern blot analysis using the 5’ xHIF-1α cDNA probe to confirm the inserts contained HIF-1α. Both strands of each clone were sequenced using oligonucleotide primers designed against the original EcoRV promoter clone to obtain approximately 3 kb of upstream sequence. A multiple alignment of these sequences revealed two distinct alleles with clearly divergent nucleotide sequences (Fig. 1). xgHIF1a-1, xgHIF1a-2, xgHIF1a-3, and xgHIF1a-5, together
Fig 1. **Schematic representation of nucleotide sequence polymorphisms of xHIF-1α promoter alleles.** *A*, the horizontal line represents the sequence of xgHIF1a-1 (GenBank accession AY262068), used in transgenic experiments. Vertical dashes signify SNPs between this strand and xgHIF1a-2 (GenBank accession AY262066), xgHIF1a-3 (GenBank accession AY262065), and xgHIF1a-5 (GenBank accession AY262067). Single deletions and insertions are symbolized by triangles and diamonds, respectively. A small number above or below the symbols indicates the number of nucleotides inserted or deleted if more than one; +1 indicates the site of transcriptional initiation. *B*, the horizontal line represents the sequence of the more divergent xgHIF1a-4 (GenBank accession AY262064) allele. Symbols represent the differences between this allele and one other isolate, xgHIF1a-1.
share over 99% identity, with 10 single nucleotide polymorphisms (SNPs), 11 deletions, and 1 insertion between them. The sequence of xgHIF1a-4, the remaining clone, has only 95% identity with the other promoter sequences and represents a more divergent xHIF-1α allele; when compared to xgHIF1a-1 alone, xgHIF1a-4 contains 76 SNPs, 8 insertions, and 5 deletions.

**RPA analysis**

Ribonuclease protection assay was utilized to identify the xHIF-1α transcriptional start site and to determine steady state mRNA levels throughout development. An antisense probe consisting of an 808bp xHIF-1α fragment located immediately upstream of the translational start site was hybridized with total RNA isolated from embryos at different developmental stages. This analysis revealed two distinct protected fragments, HIFex1(300) and HIFex1(365), that mapped to approximately 300 bp and 365 bp upstream of the translational start site and displayed developmental regulation (Fig. 2A). In addition, a 206 bp probe spanning exons 5 and 6 of the xHIF-1α gene was employed as a control; this probe protected a fragment (HIFex5-6) of the predicted size in all developmental stages assayed and also showed developmental regulation (Fig. 2B). The relative expression levels of the protected fragments HIFex5-6 and the HIFex1(300) are similar in unfertilized eggs and follow the same trend throughout the developmental stages assayed. A sharp increase in expression is observed which peaks around mid-gastrulation (stage 12), with levels two to three times those present before the midblastula
Fig 2. **Ribonuclease protection analysis of xHIF-1α transcriptional initiation site.** *A,* the 808 bp HIFex1 probe was hybridized with 20 µg of total RNA from embryos of different developmental stages. Two protected fragments were resolved, of approximately 365 bp (upper) and 300 bp (lower) representing putative transcriptional initiation sites. *B,* the HIFex5-6 probe protected a fragment of the expected size (206 bp) in all stages assayed. In both cases, a *Xenopus* cytoskeletal actin antisense probe was used to correct for procedural errors. The protected fragments obtained from both HIFex1 and HIFex5-6 were replicated in at least five independent experiments. Identical protected fragments were observed using *Xenopus* elongation factor-1alpha (EF-1α) as an internal control probe. Numbers above each lane represent the developmental stages assayed: ue = unfertilized egg; 3/4 = early cleavage; 10 = early gastrulation; 12 = mid-gastrulation; 20 = early tailbud; 25 = late tailbud; 33 = hatching; 41, 43 = swimming tadpole.
Fig 3. **Developmental profile of xHIF-1α expression.** A, graph representing the relative expression levels of the three protected fragments observed in the RPAs (parentheses in legend indicate the size in base pairs of the protected band). The levels of HIFex5-6 and HIFex1(300) increase during gastrulation and are followed by a rapid decline to steady state levels post stage 23. HIFex1(365) follows a similar trend, increasing at gastrulation before dropping to undetectable levels by early tailbud stage. Densitometric analysis was used to normalize xHIF-1α to cytoskeletal actin expression (see Experimental Procedures). Data points for each probe represent the mean values obtained from two independent experiments. B, graph showing the ratio of the two protected fragments obtained with the HIFex1 probe. The 365 bp fragment is found exclusively in early embryonic stages before rapidly diminishing and becoming undetectable by stage 23. Similar trends in xHIF-1α expression were observed using EF-1α as a normalization control.
transition (Fig. 3A). This increase is followed by a rapid decline over neurulation (stage 13-20) to a steady level by early tailbud (stage 23). In contrast, the HIFex1(365) protected fragment shows a more modest rise during gastrulation followed by a dramatic drop in expression similar to that observed for the two other fragments (Fig. 3A). However, by stage 23 (early tailbud stage), signal from the HIFex1(365) fragment falls to an undetectable level. The difference in regulation is particularly evident when comparing the ratio of the HIFex1(365) protected fragment to the HIFex1(300) fragment over time (Fig 3B).

In situ hybridization analysis

The spatial expression pattern of xHIF-1α was examined by whole-mount in situ hybridization of hatching (stage 33) Xenopus embryos using a digoxigenin-labeled antisense RNA probe consisting of a 1467 bp xHIF-1α cDNA fragment (Fig. 4). The embryos hybridized with this probe consistently demonstrated a stronger signal than negative control embryos processed in parallel using a complementary sense RNA probe (Fig. 4, A and B). xHIF-1α is expressed ubiquitously at low levels with significantly elevated levels of expression in the head and developing nervous system, as well as in the somites (Fig. 4A). To confirm this expression pattern, embryos hybridized with the antisense probe and their sense counterparts were subjected to histological analysis. A transverse section at the level of the hindbrain shows increased expression in the nervous system, otic vesicles, and somites (Fig. 4, C and D). More posterior sections
Fig 4. **Spatial analysis of endogenous xHIF-1α expression in the Xenopus embryo.** A, representative whole mount *in situ* hybridization of a stage 35 embryo using a digoxigenin-labeled xHIF-1α antisense probe showing a ubiquitous expression pattern. Elevated expression is also seen in the head, somitic tissue, and developing nervous system when compared to stage 35 embryos hybridized with a complementary sense probe (B). The observed expression pattern was confirmed through histological analysis of embryos hybridized with either the xHIF-1α antisense (C, E) or sense (D, F) probe. Scale bars = 1 mm (A and B) or 0.5 mm (C, D, E, F). NO, notochord; NT, neural tube; OV, otic vesicle; SO, somite; arrowheads indicate somitic tissue; an asterisk also indicates the otic vesicle.
show xHIF-1α to be expressed at high levels throughout the neural tube and somitic tissue (Fig. 4, E and F).

In vivo functional characterization of HIF-1α regulatory regions

To determine the upstream elements required for proper expression of HIF-1α, we designed a series of GFP constructs in which the promoter region was progressively deleted from the 5' end (Fig. 5). Using the REMI technique (Kroll and Amaya, 1996), these constructs were inserted into the genome of Xenopus embryos and the spatial pattern of GFP expression was visualized using fluorescence microscopy. For this analysis, only those embryos that were of normal appearance or those exhibiting minor morphological defects were scored. Following observation, PCR was used to confirm integration of the transgene in a total of 37 embryos designated as positive or negative for visual fluorescence. Of this number, 38% expressed GFP at clear visual levels and tested positive for transgene integration. Forty-six percent of the embryos were negative for fluorescence, but did show GFP integration. The remaining 16% were not fluorescent and did not show evidence of GFP integration.

Experiments using HIF(-1658)GFP, containing a fragment of the xHIF-1α promoter extending 1958 bp immediately upstream of the translational start site, produced a fluorescence pattern matching endogenous xHIF-1α RNA expression in 49% of developing embryos (n=49) (Fig. 6A). Similar to the pattern detected with in situ hybridization, a diffuse fluorescence is seen throughout the embryo when compared to GFP-negative controls. In addition, an increased GFP signal
Fig 5. **Summary of 5' upstream region analysis using xHIF-1α GFP constructs in transgenic embryos.** The upstream regions of xHIF-1α in each construct are represented by a line. The number indicated in the parentheses of each construct name corresponds to the position of the first base in the construct relative to the xHIF-1α transcriptional initiation site (+1). White boxes show the 5’ untranslated region, and a shaded box represents the open frame GFP coding sequence. Promoterless pEGFP-1 was used as a negative control. To the right, bars indicate the percentage of positive embryos (i.e. individuals with HIF-1α specific signals out of all embryos scored) for each construct. Numbers next to the percentages indicate the total number of transgenic embryos scored for each construct.
Fig 6. **xHIF-1α upstream sequences direct tissue specific expression of GFP in transgenic Xenopus embryos.** A representative HIF(-1658)GFP transgenic embryo (A) shows elevated levels of GFP expression in the head and eye, neural tube, and somitic tissue when compared to negative control embryos (D) containing a promoterless pEGFP-1 transgene. HIF(-508)GFP and HIF(-181)GFP transgenic embryos (B and C) show a GFP expression pattern identical to the construct containing 2 kb of upstream sequence. E, a representative HIF(UTR)GFP transgenic embryo showing the less uniform pattern of GFP expression obtained using this construct (the posterior end of this embryo is out of the plane of focus and hence appears abnormal, though fluorescence is present throughout axial structures). F, higher magnification of the anterior end of a HIF(-1658)GFP transgenic embryo demonstrates that the neural tube expresses elevated levels of GFP despite being unable to be visualized as a distinct structure in whole mount. G and H, arrowheads indicate the elevated GFP expression pattern in the neural tube and somitic tissue of a HIF(-1658)GFP transgenic (G) compared to the same region in a negative control embryo (H). Scale bars = 1 mm (A, B, C, D, E) or 0.5 mm (F, G, H). ey, eye; gu, gut; nt, neural tube; so, somite.
is observed in the head and eye region (Fig. 6F) and also extends down the length of the neural tube and somitic tissue (Fig. 6G).

To further delineate the upstream regions sufficient to reproduce HIF-1α specific expression, we generated transgenics containing two constructs with successive deletions from the 5' end of the promoter. HIF(-508)GFP and HIF(-181)GFP were both able to drive GFP expression in a pattern that matched endogenous xHIF-1α (Fig. 6B, 6C), although in a smaller percentage of embryos (33% (n=36) and 25% (n=67), respectively). The GFP expression patterns present in these embryos were indistinguishable from those observed in the HIF(-1658)GFP transgenics. An additional construct, HIF(UTR)GFP, contained only the 5' untranslated regions of the xHIF-1α gene. Although its intensity was somewhat variable and more mosaic than the previous constructs, this fragment was also able to produce a HIF-like GFP expression pattern in 14% of the 49 embryos scored (Fig. 6E). A negative control construct, consisting of promoterless pEGFP-1 plasmid, yielded only 4% of embryos (n=43) showing a GFP pattern that resembled endogenous HIF-1α expression.

**In vitro functional analysis of HIF-1α regulatory regions**

In order to establish the regions of the xHIF-1α promoter required for basal transcriptional activity, the *Xenopus* renal epithelial A6 cell line was transfected with each xHIF-1α promoter construct and assayed for GFP activity. pEGFP-C1, containing the CMV promoter, and promoterless pEGFP-1 plasmids were used as positive and negative controls, respectively. All values were
normalized to β-galactosidase activity to provide an internal control for transfection efficiency. HIF(-1658)GFP, HIF(-508)GFP, and HIF(-181)GFP were all able to drive clearly visible GFP expression in transfected cells (Fig. 7). In addition, there was no statistically significant difference in the measured GFP fluorescence levels of each in transfected cells. These results suggest that the 481 bp region flanking the translational start site is sufficient to drive constitutive HIF-1α promoter activity in A6 cells.
Fig 7. **Transient transfection analysis of the xHIF-1α promoter-GFP constructs in Xenopus A6 cells.** xHIF-1α promoter-GFP constructs were co-transfected with pCMVβ into A6 cells and the relative levels of fluorescence measured after 72 hours. All fluorescence values are normalized to β-galactosidase activity and expressed as relative fluorescence units (RFU). Promoterless pEGFP-1 plasmid was used to control for basal autofluorescence and its RFU value was arbitrarily set to 0. pEGFP-C1, containing a CMV promoter, was used as a positive control. Values are the means ± standard error from at least three independent trials and are grouped together using letters to the right of error bars (a, b, or c). Different letters indicate significant differences with an experiment-wise p < 0.01. The same letters indicate values that are not statistically significantly different from each other (p > 0.20 in all cases).
Relative Fluorescence Units (RFU)

- pEGFP-C1
- HIF(-1658)GFP
- HIF(-508)GFP
- HIF(-181)GFP
- HIF(UTR)GFP
- pEGFP-1
DISCUSSION

In order to investigate the transcriptional regulation of HIF-1α during vertebrate development, we report the cloning and functional characterization of the *Xenopus* HIF-1α promoter, the first such investigation in a non-mammalian vertebrate species. To date, the only report of a related non-mammalian promoter is from the nematode, *C. elegans*, which has a functional homologue 76% similar to human HIF-1α (Jiang et al., 2001). An alignment of the 2 kb upstream of the *C. elegans* hif-1 gene shows no significant similarity to any mammalian or *Xenopus* HIF-1α promoter sequence. The previously isolated mouse and human HIF-1α promoters show 70% nucleotide identity over their 5’-flanking and untranslated regions (Iyer et al., 1998; Luo et al., 1997). However, a comparison of the *Xenopus* HIF-1α promoter to the mouse and human promoters reveals no significant alignment (40% and 43%, respectively) over the 700 bp region immediately 5’ to the translational start site of the gene. This lack of sequence similarity is consistent with previous reports that have demonstrated considerable divergence between the *Xenopus* rhodopsin and C/EPB promoters and those of other species (Batni et al., 1996; Kockar et al., 2001; Foka et al., 2001). Yet all three HIF-1α promoters do share certain characteristics; all lack a TATA box and have a transcriptional initiation site located approximately 300 bp upstream of translational start. Moreover, a 14 bp alignment of the bases to either side of the three species’ transcriptional initiation sites reveals a striking
71% identity (Fig. 8). In addition, the region extending ~170 bp upstream of the initiation site is GC-enriched in the three promoters (Xenopus 64%; mouse and human 79%). All have at least two housekeeping E-box regulatory domains present, with the position of one (starting at -417 relative to transcriptional start) shared by Xenopus and mouse. Taken together, these results indicate possible selection for maintaining specific elements of sequence structure present in vertebrate HIF-1α upstream regions.

Sequence comparisons of gene alleles in the pseudotetraploid Xenopus can be informative for identifying conserved promoter elements. Therefore, two kilobases of upstream sequence from both strands of five independently isolated xHIF-1α promoter clones was obtained. An alignment of these five sequences reveals two distinct alleles that share 95% identity, a level of nucleotide divergence that has frequently been reported in other isolated allelic variants of Xenopus promoters (Ghanbari et al., 2001; Graf and Kobel, 1991). Xenopus Slug promoter alleles have two conserved motifs critical for regulation of the gene separated by less conserved regions and interspersed with different repetitive sequence (Vallin et al., 2001). Single nucleotide polymorphisms (SNPs) account for the majority of sequence divergence in the isolated xHIF-1α promoters, though short insertions and deletions are also present. One such five base insertion located at -542 (relative to transcriptional start) in xHIF-1α results in the creation of a CCAAT-enhancer binding protein-β consensus binding sequence. In stark contrast to the distal regions of the Xenopus HIF-1α alleles, the proximal promoter region (-208/+1) in the two alleles shares 100% identity.
Fig 8. **Comparison of HIF-1α transcriptional initiation sites.** A 21 bp alignment centered on the transcriptional initiation sites of the mouse, human, and *Xenopus* HIF-1α promoters. The first 14 bp of the alignment are 71% identical between the species. Extending the alignment to a total of 21 bp maintains 57% identity. The previously reported mouse and human transcriptional initiation sites are underlined. An arrow represents the start of transcription in *Xenopus* as determined by RPA analysis.
human: CTCAGTG — CACA — GTGCTGCCC
mouse: CTCAGTG — CACA — GAGCCTCC
xenopus: CTCGGTGACGCAAGCACTTTTC

71%  57%
+1
This sequence identity is mirrored in the 5' UTR, which contains only a single SNP between the two. This evidence of selective pressure to maintain the proximal promoter region suggests its importance in the regulation of HIF-1α transcription.

In order to fully examine the transcriptional regulation of HIF-1α, we have characterized its expression pattern over the course of *Xenopus* development. A dramatic rise in xHIF-1α mRNA levels is seen during gastrulation just prior to the development of the nervous system. This finding is consistent with mouse *Hif-1a* knockouts that show increased mesenchymal cell death leading to a failure of the neural tube to close and subsequent embryonic death at E10.5 (Iyer et al., 1998).

The time interval surrounding gastrulation represents a critical developmental period during which highly coordinated cell and tissue movements result in extensive rearrangement of the embryo. It has been shown that hypoxic conditions during this period in *Drosophila* result in cell cycle arrest of syncytial divisions (DiGregorio et al., 2001). It is also at this time that the control of embryonic cellular processes is switched from maternal to zygotic regulation in *Xenopus* (Stack and Newport, 1997). HIF-1α is also known to be involved in the regulation of a number of genes involved in various developmental pathways, including transforming growth factor-β3 (Caniggia et al., 2000), insulin-like growth factor-2 (Feldser et al., 1999), and vascular endothelial growth factor (Iyer et al., 1998). While the observed upregulation of xHIF-1α mRNA during gastrulation may represent a transient response to a local hypoxic stimulus within the embryo, it is also consistent with a programmed developmental rise in
expression. Further experiments to characterize the degree of hypoxia present in developing *Xenopus* embryos will address the reasons for this increase in HIF-1α expression.

Several variants of HIF-1α mRNA displaying tissue-specific expression profiles in the adult have been reported in mouse, rat, and human (Chun et al., 2002; Wenger et al., 1997; Luo et al., 1997; Drutel et al., 2000; Gothie et al., 2000). These isoforms are produced through alternative splicing or the use of multiple transcriptional initiation sites. In mouse, two distinct promoters produce alternate first exons, leading to a constitutive or more restricted expression pattern (Wenger et al., 1998). We present evidence for the existence of two HIF-1α RNA species in a non-mammalian organism using ribonuclease protection assay. The different expression patterns of the xHIF-1α isoforms over the course of development suggests a functional difference between the two, as has recently been proposed for another *Xenopus* gene with both a maternal and zygotic form, tumorhead (Wu et al., 2003).

*In situ* hybridization shows endogenous *Xenopus* HIF-1α mRNA to be ubiquitously present throughout the embryo as has been demonstrated in the adult tissues of mouse and human (Wiener et al., 1996; Gradin et al., 1996; Luo et al., 1997; Wenger et al., 1998). To date, there have been a number of reports that HIF-1α mRNA levels are differentially regulated during development. In the mouse, increased HIF-1α mRNA levels were found to be present in areas of the embryo stained with the hypoxia marker pimonidazole (Lee et al., 2001). In addition, chick embryos show marked upregulation of HIF-1α in tissues
consistent with the hypoxic areas in developing mouse embryos (Takahashi et al., 2001; Etchevers, 2003). Our results demonstrate that HIF-1α is spatially regulated within the *Xenopus* embryo, with increased mRNA levels in the developing nervous system and axial tissues following hatching (stage 35). This finding is in agreement with reports from developing mouse embryos, in which increased HIF-1α levels are found in the heart, somites, and brain (Lee et al., 2001).

Transgenic experiments utilizing the REMI technique were used to identify regions of the promoter capable of directing correct spatial expression of HIF-1α in developing *Xenopus* embryos. A limitation of this technique, however, is the difficulty of controlling the amount of GFP expression in a given individual due to variable copy number and the random location of transgene integration into the genome (Liu and Green, 2001). Variability in GFP fluorescence was observed in different transgenic individuals both between and among injection batches. As a result, accurate quantitation of expression levels was difficult among the embryos that tested PCR-positive for transgene integration. This question was addressed by transient transfection of *Xenopus* cell culture to measure the basal promoter activity of each GFP construct. We have found that a 181 bp portion of the *Xenopus* promoter is sufficient to drive constitutive *in vitro* GFP expression. While there is no statistically significant difference in the expression levels produced by HIF(-1658)GFP, HIF(-508)GFP, or HIF(-181)GFP, a rising trend in expression is observed as the length of the promoter increases. This data is consistent with results in mouse, in which 232 bp of the xHIF-1α promoter
yielded only slightly less luciferase reporter gene expression than a 1.4 kb fragment (Wenger et al., 1998). Similar experiments in a human cell line show equivalent levels of luciferase reporter gene expression when driven by a 5 kb and 700 bp portion of the xHIF-1α promoter (Iyer et al., 1997).

The findings from mammalian and *Xenopus* cell culture suggest that correct transcriptional control of HIF-1α can be achieved by a relatively short promoter region. *In vivo* transgenic experiments using deleted xHIF-1α promoter GFP constructs strongly support these conclusions by demonstrating that HIF(-181)GFP can drive reporter expression in transgenic embryos in an endogenous pattern. The notion that HIF-1α requires a relatively short upstream sequence to direct tissue specific expression is further supported by the high degree of sequence conservation in the proximal promoter and 5' untranslated regions in two isolated xHIF-1α alleles.

While HIF-like GFP expression patterns were observed with all HIF constructs, the HIF(-1658)GFP construct yielded the most normal looking embryos that matched the endogenous xHIF-1α pattern. It is possible to conclude that this construct contains additional regulatory elements, serving to enhance the levels of overall transcription or to confine increased expression to specific tissues. Other *Xenopus* promoters have been shown to have distinct modules mediating tissue specific transcription levels (Ryffel and Lingott, 2000; Vallin et al., 2001). Alternately, a longer promoter can lead to a general enhancement of transcription (Wardle et al., 2002). Interestingly, the HIF(UTR)GFP construct was able to drive reporter expression in a low
percentage of the transgenic embryos and also showed activity in transient transfection experiments. Mounting evidence suggests that cis-acting elements in untranslated regions can have a significant role in enhancing the transcription of some genes. This has been reported in a number of mammalian studies, including the human hematopoietic prostaglandin D synthase gene, in which the untranslated first exon showed ~60% of the activity of the entire -1044/+290 region (Fujimori et al., 2000; Cheng et al., 2003; Wang et al., 2002; Landry et al., 2001; Hiroi et al., 2001).

In the present study we set out to characterize the transcriptional regulation of HIF-1α during development. To that end, we have isolated and functionally characterized the xHIF-1α promoter region in *Xenopus laevis*. Using transgenesis, we have shown that 181 bp of the proximal promoter will drive reporter GFP expression in a pattern matching endogenous xHIF-1α mRNA expression. Similar results were obtained using *in vitro* cell culture and we have shown this region to be highly conserved between the two *Xenopus* alleles described above. In addition, mRNA levels were found to increase during gastrulation, indicating an increased need for HIF-1α during this time period. Utilizing the versatile *Xenopus* system to study vertebrate gene expression *in vivo*, we have formed a basis for further examining the role that HIF-1α transcriptional regulation plays during embryonic development.
APPENDIX A

MISCELLANEOUS PLASMID AND PRIMER NOMENCLATURE
• **HIF-1α promoter-GFP constructs.** These constructs consist of *Xenopus* HIF-1α upstream regions ligated into pEGFP-1 vector. They were used to investigate the transcriptional regulation of HIF-1α both *in vivo* with transgenesis and *in vitro* through transient transfection of the A6 cell line.

  - HIF(-1658)GFP
  - HIF(-508)GFP
  - HIF(-181)GFP
  - HIF(UTR)GFP

• **Miscellaneous GFP constructs.** These plasmids were used as positive and negative controls in transgenesis and transient transfections.

  - pEGFP-1—promoterless GFP vector from Clontech.
  - pEGFP-C1—contains the CMV promoter to drive GFP expression (from Clontech).

• **HIF-1α genomic promoter clones.** These are five independent isolates of HIF-1α upstream regions in pBluescriptSK+ that were sequenced and aligned to locate possible promoter alleles.

  - xgHIF1α-1 (GenBank accession AY262068)
  - xgHIF1α-2 (AY262066)
  - xgHIF1α-3 (AY262065)
  - xgHIF1α-4 (AY262064)
  - xgHIF1α-5 (AY262067)

• **Miscellaneous plasmids.**

  - pxHIF1—This clone contains the 1467 bp xHIF-1α cDNA in pBluescriptSK+ used to transcribe *in situ* hybridization probes. This sequence is deposited in GenBank under the accession number AY189821.

  - pxHIFex1—This clone contains the 808 bp immediately upstream of HIF-1α translational start site in pBluescriptSK+. It was used to transcribe the HIFex1 RPA probe.

• **RPA probes.**

  - HIFex1—808 bp radiolabeled RNA probe transcribed from pxHIFex1 used in the identification of xHIF-1α transcriptional initiation site.

  - HIFex5-6—206bp radiolabeled RNA probe from a region spanning exons 5 and 6 of xHIF-1α.

• **RPA protected fragments.**

  - HIFex1(300)—300 bp fragment of the HIFex1 probe representing a HIF-1α form expressed throughout *Xenopus* embryonic development.

  - HIFex1(365)—approximately 365 bp fragment of the HIFex1 representing an alternate HIF-1α form whose expression drops to undetectable levels following gastrulation.

  - HIFex5-6—206 bp fragment of the HIFex5-6 probe used to quantify HIF-1α expression levels over the course of development.
TABLE 1

Summary of oligonucleotide primers employed. An asterisk following base numbering indicates positions that refer to xHIF-1α cDNA sequence. Superscript a following base numbering indicates positions referring to pEGFP-1.

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APPENDIX B

PRIMARY SEQUENCE DATA
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DEFINITION Xenopus laevis hypoxia-inducible factor 1 alpha 5' flanking
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ACCESSION AY262064
VERSION     AY262064
KEYWORDS     .
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        Pipidae; Pipidae; Xenopodinae; Xenopus.
REFERENCE  1 (bases 1 to 2673)
AUTHORS    Sipe, C.W. and Saha, M.S.
TITLE      Cloning and functional characterization of upstream
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        Xenopus laevis
JOURNAL    Unpublished
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AUTHORS    Sipe, C.W. and Saha, M.S.
TITLE      Direct Submission
JOURNAL    Submitted (25-MAR-2003) Biology, College of William and
        Mary, P.O. Box 8795, Williamsburg, VA 23185, USA
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            Pipoidea; Pipidae; encopodinae; Xenopus.
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TITLE      Cloning and functional characterization of upstream
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JOURNAL    Unpublished
REFERENCE  2 (bases 1 to 2747)
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TITLE      Direct Submission
JOURNAL    Submitted (25-MAR-2003) Biology, College of William and
            Mary, P.O. Box 8795, Williamsburg, VA 23185, USA
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REFERENCE   1 (bases 1 to 2825)
AUTHORS     Sipe, C.W. and Saha, M.S.
TITLE       Cloning and functional characterization of upstream Regulatory regions of hypoxia-inducible factor 1 alpha in Xenopus laevis
JOURNAL     Unpublished
REFERENCE   2 (bases 1 to 2825)
AUTHORS     Sipe, C.W. and Saha, M.S.
TITLE       Direct Submission
JOURNAL     Submitted (25-MAR-2003) Biology, College of William and Mary, P.O. Box 8795, Williamsburg, VA 23185, USA
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            301 ccagatacgc gctcccttaaac acaagataac agtcgcctgag tagatctaagg agacagactc
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caacttaacac actgtgccaa ttagaaaaag tttccatttac caccactcct ttgtagtctat
cattttgacca gttctctatc caggtacaaaa tacatatgtt ccgaccaacca ttctttaatt
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satta
LOCUS       AY262068  2498 bp  DNA  linear  VRT 25-MAR-2003
DEFINITION Xenopus laevis hypoxia-inducible factor 1 alpha 5' flanking region (clone xgHIF1a-l) genomic sequence.
ACCESSION  AY262068
VERSION     AY262068
KEYWORDS    
SOURCE      African clawed frog.
ORGANISM    Xenopus laevis
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi; Amphibia; Batrachia; Anura; Mesobatrachia;
            Pipoidea; Pipidae; Xenopodinae; Xenopus.
REFERENCE   1 (bases 1 to 2498)
AUTHORS     Sipe, C.W., Gruber, E.J. and Saha, M.S.
TITLE       Cloning and functional characterization of upstream Regulatory regions of hypoxia-inducible factor 1 alpha in Xenopus laevis
JOURNAL     Unpublished
REFERENCE   2 (bases 1 to 2498)
AUTHORS     Sipe, C.W. and Saha, M.S.
TITLE       Direct Submission
JOURNAL     Submitted (25-MAR-2003) Biology, College of William and Mary, P.O. Box 8795, Williamsburg, VA 23185, USA
FEATURES    Location/Qualifiers
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            /clone="xgHIF1a-l"
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   61  taacacaaga taacagctgc cttggatagc taagaactacg acctcaattg aataatcagg
  121  tccactagg gcaacattag ttacattgac taggagaacc acaagctgct cagaagacag
  181  tccatctctca aagtctctgc ctttctgaa acgcataaa cagcagaaat cacctgagat
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  301  atttataatt gtaagctgaa tatttggtaa atttaagatta aatatattttt attttgtgat
  361  aaaaactgta cagagcctcat atgctagatt ttaattacgg tttgaaaaag caaagagtcca
  421  tcaaggtcag ccctctcaca tggaaaccca gctatcctac acacacccct cctatatttt
  481  acatacataa tatatacttt ttaacgatttt tatttattttt attaagtatta aaatatatttt ttattttttt
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  721  ccactttatt ggttaaaccg cccccctgta tgggtctatg atgctactgt gatcgggtgt
  781  aaagttgtaat cagttccctcc tgcagacttt tttttctcag agaacaacat cccaaccctt
  841  acaatccttt attttctccttc cttctctcattt ccacagcactt cattctgtaa cattctgtaa
  901  ctctcctcct agctactatt atatcctctc taagacgctt gctttccaaa gcaacttcgt tatttctt
  961  actccagatt aggccctcttct ttgaagctat gaaaggttatt aataattttt tatttttttt
 1021  aatgtaagcc ctttttttaga taagacagaa cttatattgc ttttattttc ccaatggtagc
 1081  acgtccccaag attagacacaattttctct cttttcttatccaaa ccaatgcttttcc caatttttta
 1141  aaactccttc cacactgtgca tttattgtat actttgtact tatattattt ttgctaaagtt
 1201  gcattaaccttc gctttttttttc acatggaacc tctttttttt tgttttctag catttttttt
 1261  gctttagcaca atacatctgtc aaaggggcat cttctctgtc ggaacactata ttttctgca
 1321  attttattttt atctgtcagaa tataagacag cttctttcatc gcccccttccc gttttatattt
 1381  taacaaggtt gaaagcaggt gcctttcatg cagacctcag gcgtacttcca cttaacccac
 1441  tgctgcaaat ggaataattttt cttttttttt cttttttttt tttttttttt tttttttttt
 1501  tctcttacca ggttcacattc atagttttttc gccacatcct cttataatattt accagtaacc
 1561  ttggtttcag cactgtatc aatggttttag ctaagtttata gtaaatacaca cttcatgcca
APPENDIX C

PRIMARY TRANSGENIC EMBRYO DATA
Representative gels from PCR genotyping of transgenic embryos. Genomic DNA was isolated from individual embryos and amplified using GFP specific primers. The ~480 bp band present in the above samples indicates a transgenic embryo. The lanes immediately to the left of the ladder are negative controls consisting of genomic DNA from an adult X. laevis.
TABLE 2

Summary of all transgenic experiments using truncated HIF-1α promoter-GFP constructs.

<table>
<thead>
<tr>
<th>Construct Name</th>
<th># Normal Embryos</th>
<th># Exhibiting HIF-1α Fluorescence Pattern</th>
<th>Percentage Exhibiting HIF-1α Fluorescence Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF(-1658)GFP</td>
<td>49</td>
<td>24</td>
<td>49.0%</td>
</tr>
<tr>
<td>HIF(-508)GFP</td>
<td>36</td>
<td>12</td>
<td>33.3%</td>
</tr>
<tr>
<td>HIF(-181)GFP</td>
<td>67</td>
<td>17</td>
<td>25.4%</td>
</tr>
<tr>
<td>HIF(UTR)GFP</td>
<td>49</td>
<td>7</td>
<td>14.3%</td>
</tr>
<tr>
<td>pEGFP-1</td>
<td>42</td>
<td>1</td>
<td>2.4%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>243</td>
<td>61</td>
<td>25.1%</td>
</tr>
</tbody>
</table>
TABLE 3

Summary of PCR genotyping experiments to determine if embryos contain a GFP transgene.

<table>
<thead>
<tr>
<th>Construct Name</th>
<th># Fluorescent Embryos</th>
<th># Non-fluorescent Embryos</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF(-1658)GFP</td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>HIF(-508)GFP</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>HIF(-181)GFP</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>HIF(UTR)GFP</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>pEGFP-1</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>TOTAL</td>
<td>14</td>
<td>23</td>
<td>37</td>
</tr>
</tbody>
</table>
TABLE 4

Summary of PCR genotyping experiments to determine if those embryos exhibiting visual fluorescence contain a GFP transgene.

<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Total # Fluorescent Embryos</th>
<th># Transgenic</th>
<th>% Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF(-1658)GFP</td>
<td>4</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>HIF(-508)GFP</td>
<td>4</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>HIF(-181)GFP</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>HIF(UTR)GFP</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>pEGFP-1</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>TOTAL</td>
<td>14</td>
<td>14</td>
<td>100</td>
</tr>
</tbody>
</table>
TABLE 5

Summary of PCR genotyping experiments to determine if those embryos not exhibiting visual fluorescence contain a GFP transgene

<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Total # Non-fluorescent Embryos</th>
<th># Transgenic</th>
<th>% Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF(-1658)GFP</td>
<td>7</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>HIF(-508)GFP</td>
<td>5</td>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>HIF(-181)GFP</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>HIF(UTR)GFP</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>pEGFP-1</td>
<td>5</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>TOTAL</td>
<td>23</td>
<td>17</td>
<td>74</td>
</tr>
</tbody>
</table>
RNAse Protection Assay
(adapted from Sambrook and Russell, p. 7.65-7.74)

NOTE: Follow all guidelines for working with RNA (i.e. use plug tips, clean gloves, be extremely careful about contamination, etc.).

Probe Synthesis
1. Setup the following transcription reaction in an eppendorf tube at room temperature:
   - 4.0 µl 5X transcription buffer
   - 1.0 µl nuclease-free H2O
   - 2.0 µl 0.1M DTT
   - 4.0 µl ribonucleotides (mix equal amounts of rATP, rCTP, rGTP, and dd H2O)
   - 2.4 µl 100 µM rUTP
   - 2.0 µl linearized DNA template
   - 0.6 µl RNAse inhibitor
   - ***behind shield from here on***
   - 3.0 µl 32P-rUTP (30 µCi)
   - 1.0 µl appropriate RNA polymerase

2. Mix reaction, spin down in picofuge, and incubate at 37°C for 45 minutes.
   (NOTE: this is a good time to prepare the acrylamide gel you will need to run out your probe for purification. See end of protocol for recipe.)

3. Add an additional 1.0 µl RNA polymerase and incubate at 37°C for another 45 minutes.

4. Add 1.0 µl RQ1 DNAse. Mix well, spin one second in picofuge. Incubate at 37°C for 15 minutes.

5. Add 10 µl RPA-formamide loading dye to reactions, boil 3 minutes to denature, and load on gel.

6. Run gel at 2000V until fast dye front is about 2/3 down the gel.

Probe Purification
(turn on 65°C water bath for elution after starting gel run)

1. Carry gel to dark room (305A) and separate with a licor wedge. REMEMBER ALARA - use the upper plate as a shield between you and the gel. Cover the gel completely with saran wrap.

2. Turn off lights and place a piece of film atop saran wrap and below the upper plate (crease one corner of the film for orientation purposes). Make sure to align the edges of the film with the edges of the bottom glass plate. Add some weight to the top plate and expose for 2 minutes.

3. Place the film in a lightsafe container (an old film bag works well) for transport to room 311. Develop the film following the machine instructions posted there.

4. Bring the film back to 305A and place film on bench over a piece of white paper. Place the plates over the film, aligning them in the same manner as step #2.

5. Gather the items you need for excising the bands and bring to room 305A: clean razor blades (for each band), DEPC water, geiger counter, and eppendorf tube containing 400 µl of elution buffer (for each band - recipe at end of protocol).
6. Slide the top plate off the first band, pull saran wrap back, and cut the band out with a razor blade that has been washed with DEPC water. Place the band in the eppendorf tube containing elution buffer. REMEMBER ALARA!

7. Transport the tubes containing bands back to room 301 and vortex vigorously. Make sure the bands are immersed in the buffer and incubate in the 65°C water bath for 3-4 hours.

8. Following elution, transfer eluates to a new labeled eppendorf tube after spinning down briefly in the microfuge to gather gel at the bottom of the tube.

9. Remove 1 μL of eluate and count in the liquid scintillation counter.

10. Add 1 ml cold, 100% ethanol to eluates and place in -80°C on dry ice until solid. (For a 3 day assay, you can stop here if needed) – Turn water bath to 50°C

11. Spin at 4°C for 20 minutes to pellet labeled probe. Remove supernatant to an eppendorf tube and discard in solid radioactive waste (take care to keep appropriate records). Invert tubes on a pad of paper towels and allow to dry 5 minutes. – Turn a heat block to 85°C

12. Resuspend pellets in an amount of hybridization solution (recipe at end of protocol) that will bring probe to around 100K cpm / μL. (For example: if you had 200K cpm before precipitation, use 500 μL of hybridization solution to halve per μL cpm). Remove 1 μL of this final probe solution and count in liquid scintillation counter. If needed, you may add more hybridization solution to bring counts down and recount.

**Hybridization Reactions**

1. Thaw RNA on ice and aliquot X μL of RNA into eppendorf tubes labeled for each hybridization. (check with MSS for RNA mass and volume you should use!)

2. Dry RNA in the speed-vac until supernatant is nearly all gone (~1 μL liquid left). DON’T OVERDRY!

Each hybridization reaction should be 20 μL in volume. You want to add appropriate amount of probe (usually 50-100K cpm - check with MSS!) and enough hybridization solution to bring to 20 μL volume. Use scintillation counts from step #12 above to calculate volume of each needed.

3. Add appropriate amount of hybridization solution to dried-down RNA. It is important to ensure that all RNA is dissolved in hybridization solution. Make sure to wash hybridization solution over the sides of tube multiple times and vortex each tube vigorously for at least 20 seconds.

4. Add appropriate amount of probe to each reaction and mix well. Spin down in picofuge and denature in 85°C heating block for 10 minutes. Save probes overnight by placing in a shielded container in -20°C.

5. Take the entire heating block to 50°C water bath and transfer tubes to bath directly.

6. Allow probe to hybridize overnight (or for at least 10 hours).

**Digestion Reactions**
1. Set heat block to 50°C and transfer eppendorf tubes to it. Add 350 μl digestion buffer (recipe at end of protocol) to each sample (in room 305 behind shield, and still in heat block -- this assures RNase digestion at prescribed stringency).

2. Flick mix and transfer each tube to 37°C (also behind shield). Incubate for 30 minutes. – Thaw proteinase K (10 mg/mL) and tRNA (5 mg/mL) on ice (both in -20°C freezer in 301)

3. Pour acrylamide gel (recipe at end of protocol) using white, narrow sharkstooth comb in inserted to form wells.

4. Stop RNA digestion by adding 20 μl of 10% SDS followed by 10 μl of proteinase K (10 mg/mL), mix and incubate for 15 minutes at 37°C in heating block.

5. Add 400 μl of P/C to each sample, vortex or shake well, and spin for 5 minutes in centrifuge behind shield.

6. During spin, add 5 μl of carrier tRNA (5 mg/mL) to new labeled eppendorf tubes. Transfer the aqueous phase of extraction to these tubes and mix well.

7. Add 1 ml cold, 100% ethanol and place in -80°C freezer on dry ice until solid.

8. Spin at 4°C for 20 minutes. Remove supernatant from each sample to radioactive waste by pouring. – begin water bath boiling

9. Use a pipette tip to completely remove any remaining supernatant. Dry in speedvac for 3-5 minutes until just dry. – prepare gel for running (prepare comb, assemble apparatus, fill with buffer)

**Running Samples**

1. Resuspend samples in 2 μl of TE and 2 μl of RPA-formamide dye. Vortex each sample for 20 seconds to ensure complete resuspension.

2. Prepare ‘probe only’ samples. To 4 μl of RPA-formamide dye add a volume of probe containing around 10K cpm.

3. Place all samples in plastic racks in boiling water bath to denature.

4. Ensure integrity of gel by adding a few microliters of RPA dye to each well. Avoid wells that are clogged or leaking in the final load.

5. Bring samples over to picofuge 3 at a time, spin down briefly, and load into wells. When there are ~6 samples left, turn off hot plate, and add ladder to water bath for denaturation.

6. Load all samples, then the probe only controls, and finally the ladder onto the gel.

7. Run the gel until the slow dye front is ~8 inches from the bottom of the plate (usually between 1.5-2 hours). Ask MSS how long to run gel as it will vary from assay to assay.

8. Carefully separate plates with licor wedge and apply 3MM paper to the gel. Smooth the paper over the gel and give it a minute to affix completely. Peel the paper up, cover the gel with saran wrap, and place on the gel dryer for 2-2.5 hours at 78°C
9. Remove saran wrap. Carry to dark room and load into cassette - gel, film, intensifying screen. Place in -80°C freezer for 6 hours - 2 weeks depending on nature of the expression (ask MSS for advice!).

**RPA Recipes**

**Elution Buffer: (10 ml)**
- 667 µl 7.5M AmOAc
- 1000 µl 0% SDS
- 20 µl 0.5M EDTA (pH 8.0)
bring to 10 ml with SDD H2O

**Hybrization Buffer: (5 ml)**
- 4 ml deionized formamide
- 400 µl 5M NaCl
- 400 µl 0.5M PIPES
- 10 µl 0.5M EDTA
- 190 µl SDD H2O
store at -20°C

**6% Acrylamide Gel**
- 46 g urea
- 40 ml SDD H2O
- 15 ml 40% acrylamide
- 5 ml 10X TBE

**RUNNING BUFFER: 0.5X TBE**

1. Combine ingredients in 250 ml beaker and stir on lowest heat with foil covering top (turn heat off after a few minutes of stirring)
2. Clean glass plates well with DEPC H2O and isopropanol.
3. Prepare 10% APS solution (1 ml SDD H2O and 0.1 g ammonium persulfate).
4. Assemble plates together with spacers in between and cover 3 sides with yellow sequencing tape.
5. Add 700 µl 10% APS and 18 µl TEMED, stir briefly, and pour gel.

**Digestion Mix: (10 ml)**
- 100 µl 1.0M TRIS, pH 7.6
- 100 µl 0.5M EDTA pH 8.0
- 600 µl 5M NaCl
- 40 µl RNAse A (10 mg/ml)
- 1.4 µl RNAse T (100,000 U/ml)
bring to 10 ml with SDD H2O

**RPA-formamide loading dye:**
- 800 µl deionized formamide
- 20 µl 0.5M EDTA
- 90 µl xylene cyanol (10mg/ml)
- 90 µl bromphenol blue (10mg/ml)
Store at 4°C

**RPA-formamide loading dye:**
General Cell Culture Methods

In order to keep cell health at a maximum, flasks of cells must be cared for on a routine basis. Fresh culture media must be added every 3-4 days, and when cells approach confluency, they must be split into several flasks to maintain exponential growth. It is important to maintain aseptic technique whenever working with cells (sterilize everything with 70% ethanol prior to using, never open reagents outside of hood, clean up spills immediately, turn UV light in hood on when done, etc.)

Made culture media for Xenopus A6 cells consists of: 75% NCTC-109, 15% SDD H2O, and 10% heat-inactivated fetal bovine serum. Penicillin (100U/ml) and streptomycin (100 µg/ml) may also be added to control bacterial growth. To make a fresh bottle of made media, add the following to an unopened 100 ml bottle of NCTC-109: 18 ml SDD H2O, 12 ml FBS, and 1.2 ml of pen/strep solution (10,000 U/ml).

A. Changing into fresh media (“feeding the cells”)
To change out culture media, simply draw off the “old” media in the flask being careful to avoid disturb the cell monolayer. Discard this supernatant into an appropriate waste container containing a bleach solution. Add fresh culture media to the container (7 ml for a T25 flask, 5 ml for a 60mm dish) and place in incubator.

B. dPBS
It is important to never wash cell cultures with pure water, instead Dulbecco’s phosphate buffered saline is used (examine chemical bottle carefully, there are two forms of sodium / potassium phosphate!). For a 500 ml volume:

\[
\begin{align*}
0.10 \text{ g KCl} \\
0.10 \text{ g KH}_2\text{HPO}_4 \\
4.00 \text{ g NaCl} \\
1.08 \text{ g Na}_2\text{HPO}_4 \cdot 7 \text{ H}_2\text{O}
\end{align*}
\]

Add the solids to a bottle, bring up to 500 ml with TC water, and autoclave. After the bottle has cooled slightly, close the cap completely and do not open it until inside the hood under sterile conditions. Aliquot into 50 ml falcon tubes for use.

C. Subculturing (“splitting cells”)
This process will digest the cell monolayer off the bottom of the flask for dispersal into new flasks at a lower titer. You will need to set a heating block at 37°C and prewarm anertydorof 2.5% trypsin solution (in 316 freezer) and bottle of culture media (316 fridge) in the incubator.

1. The working concentration of trypsin is 0.25%. In a 15 ml falcon tube, add 1.0 ml of 2.5% trypsin to 9 ml of dPBS and mix well.
2. Pipette off the media from the flask you wish to split and discard into appropriate biowaste container.
3. Add ~7 ml of dPBS to the flask to wash the residual culture and dead cells away. Gently swirl the dPBS around in the flask for a few moments and pipette off into biowaste.
4. Add approximately 2 ml of 0.25% trypsin solution to the flask. Make sure the solution covers the entire flask surface.
5. Incubate the flask at 37°C for 2-3 minutes to separate the cells from the flask. You will be able to see this process macroscopically, but it is helpful to look under the microscope to see the cells “ball up” and come off the bottom of the flask.

You will need to titer your cells according to how you will use them. A good ratio for housekeeping subculture is 1:4, meaning you would split the cells into 4 separate flasks. There is no need to remove the trypsin solution, as the culture media will inactivate it.
6. Mix the cells vigorously by pipetting up and down to break up cell clumps and evenly disperse them. Pipette equal amounts of culture into new flasks (make sure that the cells are well suspended when doing this to make sure each flask gets an equal number of cells).
7. Bring the new cultures up to final volume by adding 7 ml of made culture media and place in the incubator. The cells will settle and reattach overnight.

D. Freezing cells for long-term storage
This procedure is used to store cells over breaks or for other periods of time where routine care is not possible.
1. Trypsinize the cells and add 8 ml of made culture medium. Transfer the cells to a sterile falcon tube.
2. Centrifuge gently (125 x g) for 3 minutes at room temp and remove supernatant into biowaste. Wipe down the outside of the tube with 70% ethanol before returning it to the hood.
3. Mix cell culture freezing medium (Invitrogen) well by inversion of thawed tube. Gently resuspend the cells in 2 ml of cell culture freezing medium by pipetting up and down carefully.
4. Centrifuge for 1 minute at 125 x g and remove supernatant to biowaste.
5. Resuspend the cells in 2 ml of cell culture freezing medium.
6. Aliquot 1 ml into each of two cryotubes.
7. Freeze slowly at -80°C in a heavily insulated styrofoam container overnight. Place cells in long term storage container.

E. Reviving cells
1. Remove cells from freezer and thaw rapidly by agitation in a 37°C waterbath (40-60 seconds).
2. After rinsing tube with 70% ethanol, return to the hood and add cells dropwise to fresh made culture medium in a flask.
3. Incubate at 37°C until cells attach to flask and then change medium to remove trace amounts of DMSO.
GFP Fluorescence and β-Galactosidase Assay of Transfected Cells
(from Promega protocol)

Cell Lysate Preparation
1. Add 4 volumes of water to 1 volume of 5X reporter lysis buffer (RLB – from Promega kit) to produce a 1X stock solution.
2. Remove the growth medium from the cells to be assayed into a 15 ml falcon tube. Add 1 ml 0.025% trypsin solution to the plate and incubate at 37°C for 2 minutes.
3. Wash the solution over the plate to resuspend all cells off the bottom of the plate. Withdraw the trypsin and add it to the falcon tube containing the media from step 2.
4. Wash the plate with 1 ml of dPBS and add to the falcon tube.
5. Spin the falcon tube at 750 x g for 5 minutes to pellet cells. Withdraw all but ~200 μl of the supernatant to liquid waste.
6. Resuspend the cell pellet in the remaining supernatant and transfer to an eppendorf tube. Spin the cells for 5 minutes at 750 x g to pellet.
7. Pipet off the supernatant to liquid waste. Add 500 μl of dPBS to the pellet and resuspend by pipetting up and down. Spin the cells for 5 minutes at 750 x g.
8. Remove all dPBS from the pellet and add 60 μl of 1X RLB. Resuspend the pellet by vortexing vigorously. Sonicate the cells for 10 seconds on the ‘4’ setting.
9. Spin tubes in the picofuge to determine if complete lysis has occurred. If not, sonicate the cells for a further 10 seconds. Repeat this step until lysis is complete.

GFP Activity Assay
NOTE: It is necessary to let the fluorometer warm up for 30 minutes to obtain accurate readings.
1. Fill the fluorometer cuvette with 60 μl of 1X RLB and place in the machine. Set the gain to HIGH and ZERO the machine until it holds steady at ±5.
2. Withdraw 1X RLB from the cuvette and discard. Place 60 μl of test sample into the cuvette. Ensure that no air bubbles are visible in the windows of the cuvette as this will interfere with the light path. Place the cuvette into the fluorometer and allow reading to steady before recording.
3. Withdraw test sample back to eppendorf tube to save for β-gal assay.
4. Wash the cuvette twice with 100 μl of 1X RLB to remove any residual fluorescence before placing new sample in the cuvette. Repeat steps 2-4 for each sample.

β-Galactosidase Assay
1. Thaw 2X assay buffer. If the solution has precipitate, warm at 37°C briefly to dissolve.
2. Dilute the cell lysate with 90 μl of 1X RLB.
3. Add 150 μl of Assay 2X buffer to each tube and mix well by vortexing.
4. Incubate the reactions at 37°C overnight.

5. The following day, stop the reactions by adding 250 µl of 1M Sodium Carbonate. Mix by shaking briefly.

6. IMMEDIATELY read the entire sample’s absorbance at 420 nm on a spectrophotometer to obtain a value for β-galactosidase activity.
**Transgenic Sperm Nuclei Preparation**

These solutions need to be made up prior to beginning the procedure:

<table>
<thead>
<tr>
<th>30ml 2X NPB</th>
<th>Final Conc. In 1X soln</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ml 1.5M sucrose</td>
<td>250mM</td>
</tr>
<tr>
<td>900μl 1.0M HEPES</td>
<td>15mM</td>
</tr>
<tr>
<td>120μl 0.5M EDTA</td>
<td>1.0mM</td>
</tr>
<tr>
<td>300μl 0.1M spermidine</td>
<td>0.5mM</td>
</tr>
<tr>
<td>120μl 0.1M spermine</td>
<td>0.2mM</td>
</tr>
<tr>
<td>60.8μl 1.0M DTT</td>
<td>1.0mM</td>
</tr>
</tbody>
</table>

Bring up to 30 ml with SDDH₂O.

Make 40mls of 1xNPB (for testis washes and spins)

<table>
<thead>
<tr>
<th>10ml 1XNPB + 3%BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml 2X NPB</td>
</tr>
<tr>
<td>2 ml sddH₂O</td>
</tr>
<tr>
<td>3 ml 10%BSA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10mls 1XNPB +0.3%BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5ml 2x NPB</td>
</tr>
<tr>
<td>2.35ml SDDH₂O</td>
</tr>
<tr>
<td>150μl 10%BSA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1ml Storage Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>500μl 2X NPB</td>
</tr>
<tr>
<td>300μl glycerol</td>
</tr>
<tr>
<td>170μl dH₂O</td>
</tr>
<tr>
<td>30μl 10%BSA</td>
</tr>
</tbody>
</table>

**Sperm Dilution Buffer**

<table>
<thead>
<tr>
<th>3.34 ml 1.5M Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5ml 1.0M KCL</td>
</tr>
<tr>
<td>100μl 0.1M spermidine</td>
</tr>
<tr>
<td>40μl 0.1M spermine</td>
</tr>
<tr>
<td>80μl 0.1N NaOH (should bring to pH 7.3-7.5)</td>
</tr>
<tr>
<td>14.94ml dH₂O</td>
</tr>
</tbody>
</table>

store at -20°C in 0.5ml aliquots.

1. Remove testes from a male and rinse 3X in cold 1xMMR and 2x in 1xNPB in petri dishes. With forceps remove as much fat tissue and blood vessels as possible.

2. In a dry petri dish, add a few drops of 1X NPB and homogenize testes with forceps.

3. Resuspend homogenate in 8 ml cold 1X NPB and filter through Nitex mesh (100um) into a 15 ml corex tube.

4. Spin at 3000 RPM for 10 minutes at 4°C in Sorvall HB-4 rotor.

5. Set up Percoll step gradient in a 15 ml corex tube. bottom: 4ml 50% Percoll in 1X NPB; middle: 4ml 24% Percoll in 1X NPB.

6. Gently resuspend the sperm pellet in 3ml 1X NPB. Carefully layer this solution on top of the Percoll gradient.
7. Spin at 3000 RPM for 15 minutes at 4°C in Sorvall HB-4 rotor. This step removes inviable sperm.

8. Remove as much Percoll as possible by pipetting, and carefully resuspend sperm pellet in 8ml cold 1X NPB.

9. Spin at 3000 RPM for 10 minutes at 4°C in Sorvall HB-4 rotor to wash out any residual Percoll.

10. Resuspend sperm pellet in 1ml of room temp 1X NPB. Add 5µl 10mg/ml digitonin (stock 10mg/ml in DMSO stored at -20°C). Incubate at room temp for 5 minutes.

11. Add 10ml cold NPB with 3% BSA (no inhibitors) and spin at 3000 RPM for 10 minutes at 4°C in Sorvall HB-4 rotor.

12. Carefully resuspend pellet in 5mls cold 1X NPB with 0.3%BSA and spin as above.

13. Carefully resuspend sperm nuclei in 100-500ul (depending on concentration) in sperm storage buffer.

14. Count sperm nuclei using hemocytometer. Dilute sperm stock 1:100 in sperm dilution buffer and add 1ul of 1:100 Hoechst stain stock to dye DNA. Follow hemocytometer counting guidelines to determine the number of sperm / nl.

Sperm nuclei can be used 2-3 days if stored at 4°C. For long term storage of sperm nuclei, snap freeze in liquid nitrogen. On injection day, thaw an aliquot on ice to use in transgenic reaction.
Preparation of High Speed Egg Extract

Solutions for Preparations of High-Speed Egg Extracts: (make these up before beginning the procedure)

2.5M KCl (186.4g in 1L SDD H2O)
0.5M MgCl₂ (10.165g in 100ml SDD H2O)
1M CaCl₂ (14.702g in 100ml SDD H2O – filter sterilize and store at 4°C)
1.5M Sucrose (51.35g in 100ml SDD H2O – filter sterilize and store at 4°C)
5M NaCl (146.1g in 500ml SDD H2O)
1M HEPES (26.03g in 100ml SDD H2O -- titrate to pH of 7.7 with 10N KOH)
0.1M EGTA (0.384g in 10ml SDD H2O)

<table>
<thead>
<tr>
<th>20X Extract Buffer Salt Stock (1L)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>800ml 2.5M KCl</td>
<td>2M</td>
</tr>
<tr>
<td>40ml 0.5M MgCl₂</td>
<td>20mM</td>
</tr>
<tr>
<td>2ml 1M CaCl₂</td>
<td>2mM</td>
</tr>
<tr>
<td>SDD H2O to 1L</td>
<td></td>
</tr>
<tr>
<td>Filter sterilize and store at 4°C.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10X MMR (1L)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>200ml 5M NaCl</td>
<td>1M</td>
</tr>
<tr>
<td>8ml 2.5M KCl</td>
<td>20mM</td>
</tr>
<tr>
<td>20ml 0.5M MgCl₂</td>
<td>10mM</td>
</tr>
<tr>
<td>20ml 1M CaCl₂</td>
<td>20mM</td>
</tr>
<tr>
<td>50ml 1M HEPES</td>
<td>50mM</td>
</tr>
<tr>
<td>SDD H2O to 1L</td>
<td></td>
</tr>
<tr>
<td>Filter sterilize and store at 4°C.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1X MMR (2.5L)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>50ml 5M NaCl</td>
<td>100mM</td>
</tr>
<tr>
<td>1ml 5M KCl</td>
<td>2mM</td>
</tr>
<tr>
<td>5ml 0.5M MgCl₂</td>
<td>1mM</td>
</tr>
<tr>
<td>5ml 1M CaCl₂</td>
<td>2mM</td>
</tr>
<tr>
<td>12.5ml 1M HEPES</td>
<td>5mM</td>
</tr>
<tr>
<td>SDD H2O to 2.5L and autoclave</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1X XB Salts (2L)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>100ml 20X XB Stock</td>
<td></td>
</tr>
<tr>
<td>SDD H2O to 2L</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extract Buffer (XB) (500ml)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.66ml 1.5M Sucrose</td>
<td>50mM</td>
</tr>
<tr>
<td>25ml 20X EB Stock</td>
<td>1X</td>
</tr>
<tr>
<td>0.5ml 1M HEPES*</td>
<td>10mM</td>
</tr>
<tr>
<td>SDD H2O to 500ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CSF-XB (100ml)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ml 20X Salts</td>
<td>1X</td>
</tr>
<tr>
<td>200μl 0.5M MgCl₂</td>
<td>1mM</td>
</tr>
<tr>
<td>1ml 1M HEPES</td>
<td>10mM</td>
</tr>
<tr>
<td>3.33ml 1.5M Sucrose</td>
<td>50mM</td>
</tr>
<tr>
<td>5ml 0.1M EGTA</td>
<td>5mM</td>
</tr>
<tr>
<td>SDD H2O to 100ml</td>
<td></td>
</tr>
</tbody>
</table>
Energy Mix (1ml)  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.049g creatine phosphate</td>
<td>150mM</td>
</tr>
<tr>
<td>0.012g ATP</td>
<td>20mM</td>
</tr>
<tr>
<td>40μl MgCl₂</td>
<td>20mM</td>
</tr>
</tbody>
</table>

SDD H₂O to 1ml  
store @ -20°C in 100μl aliquots

2% (w/v) cysteine in 1x XB Salts
Prepare ~600ml within 1 hour of use and titrate to pH 7.8 w/ NaOH.

It is extremely important to prepare all solutions before beginning the next section of the protocol as it is time sensitive.

1. To prime the frogs for ovulation, inject 12 female adult Xenopus frogs with 50 units of HCG into the dorsal lymph sac. Maintain the frogs at room temperature for 24 hours.

2. The day before the extract preparation begins, inject each frog with 800 units of HCG. Maintain each frog in its own container with 4 liters of dechlorinated water.

3. Place the frogs at 15-18°C overnight.

4. The next morning, pool all laid eggs in 1X MMR. Discard any batch that contains noticeably mottled, lysing, or dying cells. In addition, manually expel eggs from each frog and pool into the large egg batch.

   **NOTE:** It is important to have **LARGE** numbers of eggs (enough to fill 3 Beckman tubes to the top after dejellying). Therefore, multiple batches of squeezing eggs may be required...store eggs in High Salt MBS at 18°C until obtaining enough eggs.

   Carry out the following procedures promptly through all steps once initiated. Optimally, begin the high-speed spin within 60 minutes of dejellying the eggs.

5. Remove as much 1X MMR as possible from the eggs and dejelly in 1X XB Salts, containing 2% cysteine. Add a small amount of cysteine and swirl the eggs. Partially replace with fresh cysteine several times during the dejellying process.

6. Wash eggs four times in 100ml of Extract Buffer and then twice in 50ml of CSF-XB containing 1x protease inhibitors.

   **Note:** Completely cover the eggs with solution during each wash.

7. Use a wide-bore Pasteur pipette to transfer eggs to a 14x95mm Beckman ultraclear tubes (Beckman 344060).

   **Note:** These tubes hold about 10ml of solution. You need at least **three** tubes at this point! Try to transfer an equal volume of eggs to each tube. Uneven loading at this stage can make balancing the tubes very difficult later on.

8. Remove as much CSF-XB as possible from each tube and pack the eggs in a clinical centrifuge. Spin for about 60 seconds at 1000rpm (150g) and then an additional 30 seconds at 2000rpm (600g).

9. Remove excess CSF-XB and balance the tubes as well as possible without disturbing the packed eggs.

10. Centrifuge at 10,000 RPM for 10 minutes at 2°C in the ultracentrifuge SW4Ti swinging bucket rotor.
11. Remove tubes from centrifuge jackets and insert an 18-gauge needle through the wall of the centrifuge tube at the base of the cytoplasmic layer. Slowly draw out the cytoplasm and transfer the cytoplasm to a fresh Beckman ultraclear tube on ice.

12. Estimate the volume of extract and add a 1:1000 dilution of protease inhibitor stock solution to the isolated cytoplasm.

13. Recentrifuge the cytoplasm in polypropylene tubes for an additional 10 minutes at 10,000 RPM to clarify, again using the SW4Ti swinging bucket rotor. Collect the clarified cytoplasm as in step 13.

14. Measure the exact volume of the cytoplasm obtained and add 0.05 volume Energy Mix (~175 µl). Transfer the mixture to a 3ml thick-walled polycarbonate ultracentrifuge tube (Beckman #355870 for use with Beckman SW4Ti rotor).

Note: It is critical that each tube is at least half full or it will collapse in the centrifuge.

15. Add CaCl₂ to a final concentration of 0.4mM (~1.4 µl 1M CaCl₂).

Note: This inactivates CSF and pushes the egg extract into interphase.

16. Incubate for 15 minutes at room temperature. Balance the tubes carefully and place in SW4Ti rotor with the rubber adaptor for small tubes in place. Centrifuge at 200,000 x g (this is ~38,000 RPM in ultracentrifuge using SW4Ti rotor) for 1.5 hours at 4°C.

Note: The cytoplasm will fractionate into four layers, top to bottom: lipid, cytosol, membranes/mitochondria, and glycogen/ribosomes.

17. Insert a 17 or 18-gauge needle into the top of the tube, through the lipid layer, and remove the cytosolic layer from each tube (~30-50% total volume). Transfer this fraction to fresh ultracentrifuge tubes.

Note: It may be necessary to collapse the number of tubes down at this point to maintain a concentrated extract.

18. Repeat centrifugation at 200,000 x g for 20 minutes at 4°C.

19. Transfer the final supernatant to 0.5ml microcentrifuge tubes in 20ul aliquots. Snap freeze aliquots in liquid nitrogen and store at −80°C.

20. The quality of egg extract should be tested for activity by combining it with sperm nuclei at room temperature. If extract is active, sperm nuclei should swell visibly when visualized under a microscope (thicken and lengthen) within 10 minutes.
**Transgenic Procedure**

You will want to prepare the following before the actual transgenic day:

1. **Siliconize needles.** Do this before you actually pull the needles. Attach a small piece of Tygon tubing to the end of a capillary (Harvard Apparatus). A pipette tip can now fit into the other end of the tubing. Backfill the needle with Sigmacote until several drops come out of the bottom. Withdraw most of the solution by releasing the plunger of the pipet. Quickly rinse by backfilling the needle twice with 200 µl of SDD water.

2. **Make needles.** Use the vertical needle puller set to 48, 690 (no weights). Once the needle is pulled, you will need to break it at so the bore is 60-80 µm (check size with a microscope and a 0.01 µm slide). Ideally, the needle should have a taper of 12-15 mm with a beveled tip. Do this by clipping the tip with tweezers held at a 45 degree angle to the appropriate length.

3. **Prepare pump.** It is necessary to fill the entire tube and syringe with mineral oil, but bubbles in any part of the system will interfere with the flow. Routine fills can be performed using the following procedure:
   a. Detach end of tubing from injection apparatus.
   b. Fill the plastic collar with mineral oil using a pipet. Make sure that you do not introduce air bubbles into the collar or tubing. Place the end of the tubing below the surface of the oil.
   c. Gently pull back on the plunger (while keeping end in the mineral oil) to refill the tube and syringe.
   d. Use a pipet to add an excess amount of mineral oil to the joint where the tube will be reattached and hook the tubing back up to injection apparatus.

4. **Make injection dishes.** (2.5% molecular grade agarose in 0.1x MMR)
   For 100mls (makes 6-8 dishes):
   - 2.5g agarose
   - 100ml 0.1x MMR

   Completely melt in microwave for ~2 min. Pour into 60mm Falcon dishes until liquid is ~0.5 cm from the top edge. Make injection depressions by placing a small hexagonal weigh boat on the surface of the agarose before it hardens (ensure there are no air bubbles underneath). Wrap dishes in parafilm and store at 4°C.

5. **Quantify plasmid DNA.** Use the GeneQuant machine located in the Bradley lab to determine the concentration of your linearized plasmid DNA. The concentration of DNA added to a transgenic reaction should be ~150-200 ng/µl.

6. **Prepare solutions.** You will need 1X MMR, 0.4X MMR containing 6% Ficoll, 0.1X MMR containing 6% Ficoll, and 0.1X MMR.

   The following procedure is **extremely** time intensive. Make plans to begin early in the morning and have most of the day free for sorting.

   1. Inject frogs (usually 2 to ensure eggs) with 800 units HCG the night before.

   2. Fresh oocytes are collected from the female frog by squeezing. Dejelly in 2.3% cysteine (pH 7.8-8.0) for no more than 5 minutes. Wash in 1X MMR and then transfer to injection dishes filled with 0.4X MMR and 6% Ficoll.

   2. Thaw sperm nuclei and sperm dilution buffer on ice. Heat treat egg extract (straight out of the freezer) at 85°C for 8 minutes. Following this incubation, spin at max speed in microcentrifuge tube for 5 minutes.
3. While spinning egg extract, combine 1μl of sperm nuclei with 5μl of plasmid DNA. Incubate at room temperature for 5 minutes.

4. To the DNA/sperm nuclei mix add 10μl heat-treated egg extract, 0.5μl NotI (1:20 dilution in sdd water) and 2.4μl 100mM MgCl2. Gently mix by pipetting through a cut-off pipette tip. Do not mix vigorously and try to avoid introducing bubbles to the solution as the nuclei are very delicate. Incubate at room temperature for 10-12 minutes.

5. After this incubation, dilute the sperm with 400 μl sperm dilution buffer and 26 μl 100mM MgCl2. Backfill a siliconized needle by attaching a small piece of Tygon tubing to the back end of the injection needle. Insert a pipette tip into the tubing and fill the needle with the diluted, transgenic sperm nuclei. Remove the tubing and attach needle to microinjector.

6. Turn pump on and set to 9.0μl/min. This is to make sure that there is no blockage, and you should see a drop form at the end of the needle. Quickly turn the pump off after seeing that the needle is clear.

7. Set the pump to 0.6 μl/min. Place tip of needle into MMR/Ficoll solution (you will be able to see oil swirls in the Ficoll solution if the needle is flowing).

8. Inject each egg at a 90 degree angle to its surface with a quick piercing motion. After a few minutes, the pigment of injected eggs will contract (this is a good indicator of the injection status of each egg). The nuclei are only stable for < 30 minutes, so you want to move quickly.

9. Following injection, place at 18°C for ~2-3 hours. During this time, the first cell divisions will occur.

10. Sort the eggs in dishes, separating out the cleaving eggs. Place these in 0.1X MMR with 6% Ficoll overnight. 
NOTE: Save the “bad” eggs until the end of sorting so that you can go back and do a quick second sort for eggs you may have missed or were slow to develop. After this last sort, dispose of the eggs. If they haven’t cleaved by now, they aren’t going to!

11. The following morning during gastrulation, transfer embryos to 0.1X MMR and allow to develop until desired stage.
Densitometric RPA Gel Analysis using ImageQuant 5.0
(See ImageQuant 5.0 User’s Guide for more details)

Before starting, read the ImageQuant manual as it has a lot of information about image analysis and background subtraction so you will know how to analyze your images correctly.

A. Image Acquisition

1. Scan the RPA film in on the HP 4470c scanner using the HP PrecisionScan software with the auto-resolution feature turned on. Save the scanned image as an 8-bit grayscale TIFF file.

B. Optimizing Image View and Marking ROIs for Analysis

1. Open the TIFF image in ImageQuant.
2. Open the Gray/Color Adjust window. The histogram plot in the Histogram area of the window contains a statistical view of the image data. Modify the high and low values in the histogram window to achieve maximal contrast and brightness and click the OK button. This will not modify your data, it is only for viewing purposes.
3. To outline a region of interest, use the rectangular selection tool to draw a box around a band of interest. Try to narrow the box to include only the band in which you are interested. Avoid including background in the rectangle.
4. After you have outlined the first band, you can use it as a ‘stamp’ to outline other bands of interest. Click and hold the mouse button to drag the rectangle until it is over the next band of interest and press the ‘v’ key. This will imprint this rectangle on your image.
5. Enclose each band of interest in a rectangle. Make sure that each rectangle is the same size!

C. Analyzing Lanes and Obtaining Values

1. Click and hold the mouse to drag a rectangle over your bands of interest. This will select your objects for analysis.
2. Set the Background Correction method from the Preferences menu. Choose which background method you want to use (see manual for detailed description of each). For RPA, drawing an additional rectangle around blank space to give a background reading works well. In this case, select the Object Average button and, in the pull down box, choose the rectangle number that represents background. Press the Set button to apply this method to all chosen objects.
3. From the Analysis menu choose Volume Report and press the Report button to generate values for each object.
4. A window will come up showing the values obtained for each object. The Volume measurement is the value that corresponds directly to the intensity of the band. If you close the report, ImageQuant will ask if you would like to transfer your data to Microsoft Excel to save it as a spreadsheet.
REFERENCES


VITA

Conor Sipe grew up in Virginia Beach, VA where he attended Frank W. Cox High School. While there he was inspired by his AP Biology teacher to pursue study in the biological sciences. Following graduation in 1996, he attended The College of William and Mary in Virginia and, in 2000, earned a B.S. in Biology. He chose to stay at The College for his master’s degree and study the development of the amphibian *Xenopus laevis* in Dr. Margaret Saha’s laboratory. He currently resides in Williamsburg, VA where he enjoys playing Frisbee and raising his hamster.