1994

The Isolation and Characterization of a Novel G-Protein-Coupled Receptor involved in Angiogenesis

Rebecca Ruth Stucki Miles
College of William & Mary - Arts & Sciences

THE ISOLATION AND CHARACTERIZATION OF A NOVEL G-PROTEIN-COUPLED RECEPTOR INVOLVED IN ANGIOGENESIS

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

Rebecca Ruth Miles

1994

APPROVAL SHEET

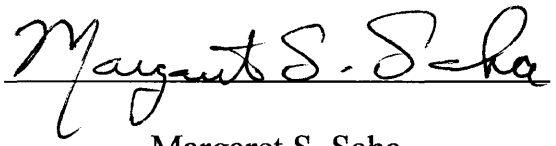
This thesis is submitted in partial fulfillment of

the requirements for the degree of

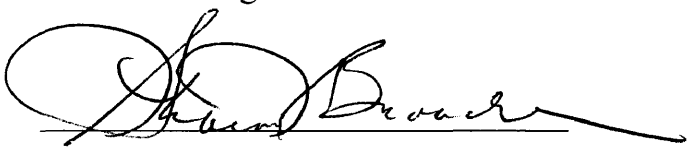
Master of Arts

Rebecca Ruth Miles

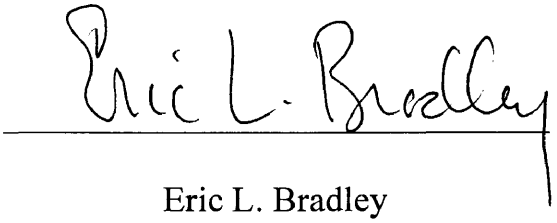
Approved, July 1994



Margaret S. Saha



Sharon T. Broadwater



Eric L. Bradley

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Margaret Saha for the opportunity to pursue a fascinating research project in her laboratory. I feel this experience has been the most valuable component to my graduate work and I am very grateful for the effort, the time and financial resources involved in Dr. Saha's support of this project. I would also like to thank the members of my committee, Drs. Bradley and Broadwater, for their careful consideration of this manuscript and for their personal interest in me as a graduate student. I am very grateful to Dr. Joe Scott and Jewel Thomas for their willingness to assist me in the preparation of the photographic material used in this project. The long hours spent in rooms 300 and 316 would have been much longer without the laughter, support and camaraderie shared by my labmates, Cleve Sinor, Kathy Joubin, Sareena Khosla, Ilsa Kaatari and Jennifer Oakes. And lastly, I am eternally thankful for the unchanging nature of the ones who love and support me most, my husband and my family.

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

The mechanisms of embryonic induction are of central importance to understanding how the basic body plan is established in vertebrate embryos. The identification of signaling pathways involved during inductive processes has enabled significant progress to be made in understanding the molecular basis of embryonic induction. Although many studies have characterized developmental signaling pathways, namely, the growth factor-tyrosine kinase and steroid-hormone receptor pathways, very little work has been done to identify G-protein-coupled receptors during development. This is suprising since they are expressed ubiquitously in adult signaling pathways. In an attempt to identify and characterize candidate receptors expressed during development, a st. 42 swimming tadpole cDNA library was screened for G-protein-coupled receptors. This resulted in the isolation and characterization of a novel member of the G-protein-coupled receptor superfamily that shares 48% homology with a human gene, APJ. Based on its expression in endothelial tissues that give rise to the vasculature, namely, the heart and blood vessels, this gene has been named *XEGR-1* for Xenopus endotheial G-protein-coupled receptor. The further characterization of this class of receptors may lead to the development of therapeutic agents that are able to control unregulated angiogenesis in diseases such as arthritis and tumorigenesis.

THE ISOLATION AND CHARACTERIZATION OF A NOVEL G-PROTEIN-COUPLED RECEPTOR INVOLVED IN ANGIOGENESIS

INTRODUCTION

The central, driving question of developmental biology is how a single cell, namely the fertilized egg, gives rise to a complex, patterned organism. The expression of particular genes at specific times during development causes the differentiation of unique cell types, leading ultimately to the assembly of these cell types into a functioning organism. There are two known mechanisms governing differential gene expression during development: the localization of cytoplasmic determinants and embryonic induction. Cytoplasmic determinants are maternally deposited molecules in the egg that serve to convey some of the earliest information involved in the differentiation of the zygote and are important during early development (reviewed by Dawid and Sargent, 1986; Davidson, 1986). The other mechanism, embryonic induction, involves tissue interactions whereby one group of cells influences the developmental fate of another group of cells (Slack, 1993). Once the zygote begins to undergo cleavage, it is the progressive inductive interactions among dividing cells that orchestrate the developmental events of differentiation, morphogenesis and organogenesis. The elucidation of the mechanisms by which differential gene expression, the "central dogma" of developmental biology, occurs has resulted in significant progress in addressing this question.

Inductive interactions possess two elements: a tissue that produces the inducing signal and a tissue that is capable or "competent" to respond to it. A classic developmental process demonstrating the ability of one group of cells to influence the development of another group of cells by means of a series of attuned tissue interactions is vertebrate lens induction (reviewed by Saha et al., 1992). As the neural tube expands, the optic vesicle emerges from the lateral forebrain and touches the surface ectoderm, causing the ectoderm at this location to thicken and form the lens placode, subsequently giving rise to the lens, which, in turn, induces the cornea. The optic vesicle then invaginates to form the optic cup which will become the retina. Recent studies have shown that a combination of signals arising from the anterior neural plate as well as the optic vesicle are responsible for inducing the lens (Henry and Grainger, 1990). In addition, the period of competence in the responding ectoderm to these lens inducing signals has been shown to be very narrow (Servetnick and Grainger, 1991), only occurring during late gastrulation. These and other studies of embryonic induction have provided the framework for analyzing the mechanisms underlying the embryonic induction.

To understand the nature of inductive interactions during development, it is necessary to look beyond tissue interactions and analyze the mechanisms involved at the molecular level. Perhaps one of the most significant and thoroughly studied examples of embryonic induction at the molecular level is mesoderm induction.

After fertilization, the embryo undergoes a series of rapid mitotic cell divisions, creating a hollow ball of cells called a blastula. Vertebrate embryos at this stage are composed of two cell types: endoderm in the vegetal half of the embryo and what in amphibian embryos is termed the "animal" half. Signals arising from the cells of the vegetal pole induce the cells of the middle zone to become mesoderm. The ectodermal cells of the animal cap are protected from mesoderm-inducing factors by the blastocoel and are not transformed into mesoderm (Smith, 1993). The presence of mesoderm-inducing signals in the vegetal pole of the embryo was first demonstrated by Nieuwkoop (1969). He showed that when animal cap tissue is cultured alone, ectodermal tissue develops; however, when recombinants of vegetal tissue and animal cap tissue were constructed and then cultured together, mesodermal tissues formed. For years after, many investigators searched for the elusive molecules responsible for mesoderm induction. Finally, fibroblast growth factor (FGF) and activin were identified as the ventral and dorsal mesoderm-inducing agents respectively (Kimelman and Kirschner, 1987; Slack *et al.*, 1987; Asashima *et al.*, 1990; Smith *et al.*, 1990).

As discussed above for lens induction, the responding tissue must be competent. Although a tissue can acquire competence in several ways, the most common mechanism is to express a receptor for the inducing molecule during a limited period of time. In order to provide additional proof that fibroblast growth

factor and activin were the natural mesoderm inducing agents, it was necessary to demonstrate simultaneously the expression of activin and FGF receptors in presumptive mesoderm (Gillespie *et al.* 1989; Musci *et al*, 1990) and to show that mesoderm induction could be disrupted by the inhibition of these receptors (Amaya *et al.* 1991). These exciting experiments have done much to establish not only the current understanding of mesoderm induction, but have also elucidated the molecular basis of inductive mechanisms in general.

Given the understanding of inductive mechanisms coupled with an extensive knowledge of adult signaling pathways, a model for inductive interactions and cell - cell communication has emerged. Generally, a molecule (ligand) is secreted from a signaling cell and "travels" to a responding cell expressing the appropriate receptor. The binding of the ligand to its receptor then activates secondary pathways that convey the information to the nucleus and enable the cell to respond appropriately. Many of these ligands have been identified and have been classified into the following groups: peptide growth factors, steroids, peptide hormones, and miscellaneous small molecules. Each of these classes of molecules binds predictably to and activates a specific type of receptor. In particular, several families of growth factors (i.e. fibroblast growth factor, transforming growth factor-beta, and the cytokines) have been characterized that are not only involved in diverse functions in the adult such as

ovulation, bone formation, and wound repair, but also play a critical role in primary and secondary inductions during development. Tissues that are competent to these growth factors have been found to express cell-surface proteins which contain intrinsic tyrosine kinase activity. The structure of the tyrosine kinase receptor and its methods of signal transduction have been worked out in great detail (reviewed by Ullrich and Schlessinger, 1990).

Steroid hormones and their receptors have also been well characterized as an important class of signaling molecules (reviewed by Fuller, 1991; O'Malley, 1990). Estrogens, testosterone and glucocorticoids are examples of important steroids functioning in the adult. A well-studied example of an important steroid molecule involved in development is retinoic acid. This vitamin A derivative plays a critical morphogenetic role in early pattern formation of the embryo. The receptors for retinoic acid belong to a unique family of non-membrane bound receptors. Like all steroids, retinoic acid is lipid-soluble and is able to diffuse directly through the membrane into the cytosol where it binds to its receptor (reviewed by Summerbell and Maden, 1990). The hormone-receptor complex then translocates to the nucleus where it serves as a transcription factor to activate the expression of specific genes.

One of the most thoroughly characterized and perhaps most utilized signaling pathways in the adult organism is mediated through G-protein-coupled

receptors. It has been estimated that nearly 80% of all known receptors are G-protein-coupled receptors (Bockaert, 1991). G-protein-coupled receptors are involved in such functions as the transmission of hormonal signals, odorant reception and vision (Bockaert, 1991). The regulation of this signaling pathway is critical for homeostasis and many diseases, including cancer, can result from their deregulation (Clapham, 1993). The cloning of G-protein-coupled receptors has revealed sequence and structural similarities which have been conserved between organisms such as bacteria, insects, mice and humans, and has led to their placement into a superfamily of receptors (Probst et al., 1992). Analysis of the amino acid sequence predicts a conformation that spans the membrane seven times, usually orienting the amino terminus to the extracellular side and the carboxy-terminus to the cytoplasmic side. These signals are typically transduced when a ligand binds the extracellular domain and induces a conformational change in the receptor, causing the intracellular domain to bind G-proteins which activate second messenger pathways that enable the cell to respond to the signal.

Given that there is a finite number of genes with which to create and maintain an organism (estimates range from 100,000 to 500,000 genes), a common mechanism used to overcome this limitation is to reuse developmental genes in adult systems. This, coupled with the estimation that G-protein- coupled receptors may comprise nearly 1% of the genome (Clapham, 1993), would make it highly

probable that this class of receptors is expressed during development. However, although more than 100 G-protein-coupled receptors in the adult have been cloned and characterized, very little work has been done to demonstrate a role for them during development.

The goal of this project was to identify and characterize G-protein-coupled receptors that are expressed during the development of Xenopus laevis. Xenopus was chosen as an experimental model in this study for the following reasons: 1), Xenopus embryos can be readily collected for experiments because single matings between male and female frogs generate thousands of embryos and 2), the large size of the embryos make them amenable to surgical manipulations, tissue culture and biochemistry, even at early developmental stages when other vertebrate embryos cannot be easily manipulated. In order to identify novel G-protein-coupled receptors, a cDNA library was screened with a polymerase chain reaction (PCR) fragment amplified with degenerate primers to the third and sixth transmembrane regions. Using this approach, a novel G-protein-coupled receptor cDNA was isolated from a stage 42 cDNA library that is expressed from neurula to swimming tadpole stages in tissues that appear to be involved in vasculogenesis and angiogenesis, namely, ventral mesoderm, angioblasts, heart and blood vessels. The results of this study characterize the sequence and expression pattern of this clone, which has been named *XEGR-1* for Xenopus endothelial G-protein-coupled

receptor; it also describes some of the tissue interactions that may be important in regulating its expression during development.

EXPERIMENTAL PROCEDURES

Animals

Sexually mature Xenopus laevis frogs were purchased from Xenopus I (Ann Arbor, MI). Embryos were obtained from matings induced by subcutaneous injections of human chorionic gonadotropin (hCG) (Sigma) at concentrations of 1000 U for females frogs and 750 U for male frogs. Embryos were cultured at 14-16° C in 0.1 X normal amphibian media (NAM) containing 50 ug/ml gentamicin sulfate. All embryos used in subsequent experiments were staged according to Nieuwkoop and Faber (1967).

Low Stringency Homology Screening

In order to identify novel G-protein-coupled receptors involved in vertebrate development, a cDNA library constructed in lambda ZAP II (Stratagene) from stage 42 swimming tadpoles (Saha and Grainger, 1992) was screened at low stringency with a ^{32}P-random-primed-labeled 0.5 kb PCR fragment. The PCR fragment (a gift from Roger Cone, Oregon Health Sciences University) was originally amplified from a Xenopus laevis melanophore library

Approximately 20 ug of the PCR fragment, designated R1 by Cone, was received already cloned into the vector pBS (Stratagene). In order to generate a sufficient amount of DNA for screening procedures, R1 was transformed into Escherichia coli DH5 alpha cells which were made competent for transformation by incubation for 30 minutes in a buffer containing 13% glycerol, 10 mM Tris-HCl, 100 mM $CaCl_2$, and 50 mM $MgCl_2$. To select for those cells which were successfully transformed with R1, the cells were grown on agar plates containing 50 ug/ml ampicillin. Plasmid DNA was then isolated from an overnight culture of a successful transformant using a standard protocol of alkaline lysis and PEG precipitation (Sambrook et al., 1989). Linear R1 was then prepared by excision from pBS with restriction enzymes EcoR1 and Sal I. The resulting fragment from this digestion was isolated by gel electrophoresis and purified using GeneClean (Bio101).

A probe was synthesized using Promega Prime-a-Gene labeling system. that is based on the method developed by Feinberg and Vogelstein (1983) in which random hexamers are used to prime single-stranded linear DNA templates for DNA synthesis in vitro. The reaction was conducted according to the manufacturer's protocol using approximately 5 ug of R1. The amount of probe synthesized in the reaction was estimated according to the ratio of incorporated counts to total counts. Total counts were determined by spotting a DE-81 filter

(Whatman) with 0.5 ul of sample and measuring counts per minute (cpm) with a Geiger counter. The filter was then washed twice with 10 mL of 0.5 M Na_2HPO_4 (pH 7.5) and once with 10 mL of 95% ethanol and measured again for cpm to determine the counts which had been incorporated into the probe and retained on the filter. The ratio of the total to the incorporated counts was used to determine the amount of radioactivity that was incorporated into the probe. The probe was extracted once with phenol/chloroform and precipitated in 0.3 M sodium acetate and ethanol and stored at -80° C until needed.

For primary screening of the stage 42 cDNA library, agar plates were prepared at a density of 50,000 to 60,000 plaque forming units (pfu) per 150 mm petri plate of confluent E. coli BB4 cells according to a standard protocol (Sambrook et al., 1989). Using 150 mm nylon membranes, duplicate lifts were made on six plates. In order to expose the phage DNA to the probe, the phage coat was broken open by pressurizing the filters in the autoclave at 10 psi and 100°C for one minute. The phage DNA was then cross-linked to the filters by UV-irradiating at 1.2 x 10^4 joules/cm^2 for 120 s (Fisherlinker).

The filters were pre-hybridized at standard low stringency conditions in a buffer solution containing 1 M NaCl, 1% SDS, 10 mM Tris-HCl (pH 7.5), 100 ug/ml salmon sperm DNA, and 30% formamide at 42° C for 6h. The filters were then hybridized in the same buffer solution containing the labeled probe (10^7

cpm/mL) overnight at 42° C. The filters were washed once in 2X SSC, 1% SDS, for 10 minutes at room temperature, twice for 30 minutes at room temperature, and then at 50° C until counts reached a level of 200-400 cpm. The filters were exposed to X-ray film (Fuji) with an intensifying screen overnight at -80° C and then developed and analyzed for the presence of positive signals from both duplicate filters. Films showing positive clones were realigned with the original plates and the area around the positive plaque was bored out and the phage particles were eluted in 1 mL of storage media overnight (Sambrook, et al., 1989).

In order to purify the plaque of interest from the suspension of phage particles eluted from the agar plug of the primary screen, this subpopulation of phage was plated out at a density of approximately 1,000 pfu per 90 mm plate of confluent BB4 cells and re-screened with the probe. Preparation of the filters, hybridization and washing conditions, exposure to film and isolation of positive clones was identical to the conditions described above for the primary screen.

To obtain a single positive plaque, the phage from the secondary plug were plated out at a density of approximately 25 pfu per 90 mm plate of confluent BB4 cells and re-screened with the probe in the manner described above. Four single plaques, corresponding to positive signals, were bored out and eluted overnight in 500 ul of storage media. These were designated xR1a, xR1b, xR1c and xR1d.

Having obtained a plaque-pure suspension of phage particles, excision of the pBluescript plasmid containing the cloned cDNA insert from the lambda ZAP vector (Stratagene) was performed with minor modifications according to the manufacturer's protocol. BB4 cells containing the excised pBluescript plasmid were selected by growing on agar plates containing 50 ug/mL ampicillin. Using a standard protocol of alkaline lysis and PEG precipitation (Sambrook, et al., 1989), plasmid DNA was then isolated from overnight cultures that had been inoculated with a colony demonstrating ampicillin resistance.

Sequence Analysis

Dideoxy sequencing of the clones (xR1a, xR1b, xR1c, xR1d) identified in the homology screen was performed using Sequenase Version 2.0 (United States Biochemical) according to the manufacturer's protocol, a method based upon the chain termination technique developed by Sanger et al. (1977). Universal and reverse primers (United States Biochemical) which recognize pBluescript sequences flanking the insert were used to obtain sequence from the extreme 5' and 3' ends of these clones. Sequence analysis demonstrated that these clones were identical. All further manipulations were performed on xR1a, which was renamed *XEGR-1*. Templates for sequencing were created by subcloning restriction fragments generated from digests of *XEGR-1* with Hind III, Kpn I, Xba

I, Hae III and Alu I. Restriction fragments were isolated by gel electrophoresis, purified with GeneClean (Bio 101) and ligated into pBluescript with T4 ligase (Promega) according to Sambrook et al. (1989). Sequence was also obtained by designing 16-20 mer oligonucleotide primers to internal sequences of *XEGR-1* (Table 1). Sequence alignments were generated using the FASTA and GCG programs provided by the University of Virginia.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from whole embryos following a standard protocol (Sambrook et al., 1989) and separated through a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane. The nucleic acid was cross-linked to the membrane by UV-irradiating at 1.2×10^4 joules/cm^2 for 120 s (Fisherlinker) and prehybridized for six hours at 42° C in a buffer containing 50% formamide, 1 M NaCl, 1% SDS, 10 mM Tris-HCl (pH 7.5), and 100 ug/mL salmon sperm. The blot was then hybridized in the same buffer containing a ^{32}P-radiolabeled probe synthesized from the full length clone identified in the homology screen (as described previously) overnight at 42° C. Filters were washed twice at room temperature in 2X SSC and 1% SDS for 20 minutes each and then at 60° C until counts reached a level of 200-400 cpm. The filters were then exposed to X-ray film (Fuji) with an intensifying screen at -80° C overnight.

Table 1. Primer design. In order to obtain complete sequence from both strands, 16-20 mer oligonucleotides were designed to bind to internal sites of *XEGR-1*. Their nucleotide sequence and the position at which they bind *XEGR-1* are indicated.

TABLE 1

PRIMER	SEQUENCE	POSITION
I	5' GACTTACACCAGTCAC 3'	1950
II	5' ATATGGAAAGGCAGCC 3'	810
III	5' GTGTCCTTGGAGGACTTT 3'	990
IV	5' GTGGGGACAGAGACTTTGTT 3'	1140
V	5' GGTAATCTAGCACTGGCT 3'	250
VI	5' TTACTGCTTCATCGGTGG 3'	700
VII	5' AGCCCTATGGGAGTTTTC 3'	1740

Whole-mount in situ Hybridization

In order to visualize the expression pattern of *XEGR-1*, whole-mount in situ hybridization was performed essentially as described by Harland (1991). Embryos used in this procedure were prepared by removing the gelatinous coat with a 2-5 minute rinse in a solution containing seven pellets of sodium hydroxide and 2 g of cysteine / 100 mL of sterile water, followed by three rinses in 0.1 X NAM (Slack, 1984). Embryos were harvested at different stages by removing the vitelline membrane and fixing in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for one to two hours. Embryos were then stored in 100% methanol at -20° C until needed. The antisense probe was transcribed with T7 polymerase (Stratagene) on *XEGR-1* which had been linearized with the restriction enzyme Bam HI. The most effective antisense probes were synthesized when omitting the DNAse treatment and hydrolysis steps recommended in Harland's procedure. Following hybridization, embryos were fixed overnight in MEMFA and then stored in 1 X PBS at 4 degrees Celsius until further use. Stained embryos were prepared for photography by clearing the tissue with a 2:1 solution of benzyl benzoate and benzyl alcohol. Embryos were prepared for sectioning by dehydration in serial dilutions of ethanol and xylene followed by embedding in

Paraplast. Sections were made on a microtome at a width of 8 um and mounted on slides treated with Mayers albumin in a mixture of 1:1 xylene and Permount.

Embryo Manipulations

In order to investigate the regional expression of *XEGR-1* , embryos at stage 11.5 (mid-gastrula) and stage 14 (early neurula) were dissected into dorsal and ventral pieces and then further subdivided into anterior, middle and posterior regions (as described in Saha and Grainger, 1992), yielding a total of six pieces. All manipulations were performed with fine glass needles under a dissecting microscope and the resulting explants were cultured in 3/8 NAM until sibling embryos reached stage 32. Explants and sibling embryos (used as controls) were harvested and fixed in MEMFA in preparation for in situ hybridization.

RESULTS

Isolation of *XEGR-1*

Low stringency screening of a stage 42 cDNA library with a GCR-specific probe (as described in experimental procedures) resulted in the isolation of four positive clones. Restriction digests of these clones with Eco R1, Xba I and Kpn I revealed that they were all approximately 2.2 kb in length and generated similar restriction fragments, indicating that all four clones were probably the same. Northern blot analysis with the *XEGR-1* cDNA clone revealed a single major transcript approximately 3.0-3.5 kb in size (results not shown) present in total RNA extracted from gastrula, neurula and tailbud stages.

Sequence Analysis and Comparison

The determination of sequence for the extreme 5' and 3' ends of the clones identified in the homology screen confirmed that all four were identical to each other. The xR1a clone, renamed *XEGR-1*, was sequenced completely on both strands. Sequence across the entire length of this clone was generated by assembling partial sequences (as described in experimental procedures) from both strands. Figure 1 shows the nucleotide sequence and the translation of the

Figure 1. Nucleotide sequence of *XEGR-1* and the deduced amino acid sequence. The nucleotide sequence of the *XEGR-1* cDNA clone is presented as well as a translation of the assumed open reading frame. The initiating codon, ATG, is bolded and the Kozak consensus sequence (ACAACCATGG), located immediately upstream from the start site, is underlined. The putative transmembrane regions have been underlined and potential phosphorylation sites on the cytoplasmic domain have been marked with an asterisk.

```
   1 CGGGACTTTCTATTGCTTCACACAGCCTTCAAAGATGGAAACAGAAGGCT
  51 TGAGCCTATCTATCAACACGACTATATACGGAAATGAGACTGGACTACAA
 101 CCATGCGATGAAACAGACTGGGATTTCTCCTATCTCTGCTACCTGTCTTT
         M  K  Q  T  G  I  S  P  I  S  A  T  C  L  L
 151 TACATGATCGTGTTTGTCCTTGGACTCTCAGGGAATGGAGTGGTCATCTT
       H  D  R  V  C  P  W  T  L  R  E  W  S  G  H  L
 201 TACAGATGGAAGTCCAAGCCAAAGCGGAGATCTGCAGACACCTACATAGG
     Y  R  W  K  S  K  P  K  R  R  S  A  D  T  Y  I  G
 251 TAATCTAGCACTGGCTGACCTGGCCTTTGTGGTAACACTGCCTCTATGGG
       N  L  A  L  A  D  L  A  F  V  V  T  L  P  L  W  A
 301 CCACATACACTGCTCTAGGCTTTCACTGGCCCTTTGGTTCTGCACTGTGC
         T  Y  T  A  L  G  F  H  W  P  F  G  S  A  L  C
 351 AAGCTCAGCAGCTATGTGGTCTTGCTTAACATGTTTGCCAGTGTTTTTTG
     K  L  S  S  Y  L  V  L  L  N  M  F  A  S  V  F  C
 401 CCTGACCTGCCTCAGTTTTGATCGGTACCTGGCCATTGTCCATTCGCTTT
       L  T  C  L  S  F  D  R  Y  L  A  I  V  H  S  L  S
 451 CCAGTGCTAAACTTCGCTCCCGCTCCTCCATCATTGTATCCTTGGCTGTT
         S  A  K  L  R  S  R  S  S  I  I  V  S  L  A  V
 501 ATTTGGCTCTTCTCAGGGCTTTTAGCACTCCCAAGTCTGATTCTGCGTGA
       I  W  L  F  S  G  L  L  A  L  P  S  L  I  L  R  D
 551 CACACGTGTAGAAGGCAATAACACTATCTGTGACCTGGACTTCAGTGGTG
         T  R  V  E  G  N  N  T  I  C  D  L  D  F  S  G  V
 601 TTTCAAGCAAGGAGAATGAAAATTTCTGGATCGGGGGGCTAAGCATTCTT
         S  S  K  E  N  E  N  F  W  I  G  G  L  S  I  L
 651 ACCACAGTTCCAGGATTCCTGCTGCCCCTGCTCCTTATGACCATCTTTTA
       T  T  V  P  G  F  L  L  P  L  L  L  M  T  I  F  Y
 701 CTGCTTCATCGGTGGCAAGGTGACCATGCATTTCCAAAACCTAAAGAAGG
       C  F  I  G  G  K  V  T  M  H  F  Q  N  L  K  K  E
 751 AAGAACAGAAGAAAAAGAGGCTTCTTAAGATTATTATTACGCTGGTTGTA
         E  Q  K  K  K  R  L  L  K  I  I  I  T  L  V  V
 801 GTGTTTGCTATCTGCTGGCTGCCTTTCCACATTCTGAAAACCATTCACTT
     V  F  A  I  C  W  L  P  F  H  I  L  K  T  I  H  F
 851 TCTAGACCTCATGGGCTTCCTGGAACTTTCTTGCTCTACACAAAACATCA
       L  D  L  M  G  F  L  E  L  S  C  S  T  Q  N  I  I
 901 TTGTCAGCCTGCACCCCTATGCCACCTGCTTGGCATACGTTAATAGCTGC
         V  S  L  H  P  Y  A  T  C  L  A  Y  V  N  S  C
 951 TTAAACCCTTTCCTCTATGCCTTCTTTGACTTGCGATTTCGCTCCCAATG
       L  N  P  F  L  Y  A  F  F  D  L  R  F  R  S  Q  C
1001 TTTTTTTTTTTTTGGTTTCAAAAAAGTCCTCCAAGGACACCTCAGCAACA
       F  F  F  F  G  F  K  K  V  L  Q  G  H  L  S* N  T
1051 CATCTTCCAGTTTAAGTGCACAGACTCAAAAATCTGAAATTCACTGTGTA
         S* S* S* L  S* A  Q  T* Q  K  S* E  I  H  C  V
1101 GCCACAAAGGTATAATTGAGTAAAGGGACATTTGATGGGGTGTGGGGACA
     A  T* K  V  end
1151 GAGACTTTGTTCTAAAGAATTTTTGGGTTAATTGAATGTTATCTCTTGTT
1201 ACTGGTCAAAAGGGAAGGTCATCTGCTGATTACAGAACAGGGACTTGCAC
1251 AGTCTACTGGATCGTATCACTGTCTGTAAAATGCCCCCACTTAGTGGAGA
1301 TCTGGCTTTTCCTGAGTAACAGACAAACCTTAGTGAATAAGACAATCTTG
1351 CTCAGTCCCCTCTCCAGAAAACAGGAGAGCTGGTTGGACAGGGTGGGAGA
1401 AACTGTTTACTGAATTTTGTTATTGATGCAAGAGGGAACTTGGTATGACA
1451 TGGGTTAACATATGCTGTATATTGCTGGGTTTCTTCTTTGTATTTGCTAA
1501 GGATTTTTGCTTTCCTGTAAATATTTTGCTTGTTAAAGCACTTGTATAAT
1551 TTCACACTTTTAACACATTGTTTAAAGGTAATTTTGGAATGTAATGTAAT
1601 TGAAATGTTATTTAGCCAATTCACTGCTTTGTTATTATTACACGGACATT
1651 AAGGTGAACTGGTTAGAAGCAGTTAAAATTAACAACTGTCTTGAAACCCG
1701 TAGTTGATAAAGAAGCAAAGAATCATCACAGTGTAGGGGTTAATGTAGAT
1751 TTCCTTTTAAGCCCTATGGGAGTTTTCCTGAACTACTACTTAAAGTATGC
1801 CCAAACAGCCTGTGACTGGCTGAGGATTCTGGGAATTGTAGTTTCGATTA
1851 TACTCTGGGGTATGCAGGTTTGCCACCAAAACTCTAAAGGGATAAAGATG
1901 TGCAGAAAGGGAAATCTATTGGATATGACAAACTGAAAACTGCAAACTAT
1951 TAACCAAAGATAAGGACTACTGAATGGCTGATATTTTGGTGACTGGTGTA
2001 AGTCATTGCTTCTTATCTATATGTACCATGCTCTAGATATGCGTGTATAT
2051 ATATATATATATATATATATATATATATATATATATATATATATATATAT
2101 ATATATATAAAATATTCCCTTGGAAAGGGGGGATCATTGATAAAACTGTG
2151 TTGTTTGATGCAATAAAAATTTTGGACTATGAAAAAAAAAAAAAAAAAA
2201 AA
```

Figure 1. Sequence of *XEGR-1*.

Figure 2. Amino acid sequence comparisons with *XEGR-1*. The protein sequences of *XEGR-1* with human APJ and Xenopus angiotensin II type one receptors are compared. A period below a character indicates that a position is well conserved,while an asterisk below a character shows that a postion is perfectly conserved.

```
XEGR-1     M       KQTG ISPISATCLLHDRVCPWTLREWSGHL    YR
APJ        M       EEEGGDFDNYYGA  NQSECEYTDWKSSGALIPAIYMLVFLLG
XLAT1      MLSNISAGENSEVEKIVVKCSKSMGMHNYIF     ITIPIIYSTIFVVG
           *        ...   .                ..            *

                       WKSKPKRRSADTYIGNLALADLAFVVTLPLWATYTALG
           TTGNGLVLWTVFRSSREKRRSADIFIASLAVADLTFVVTLPLWATYTYRD
           VFGNSLVVIVIYSYMKMKTMAS VFLMNLALSDLCFVITLPLWAVYTAMH
                         . * ... ... .**..** **.******.**

           FHWPFGSALCKLSSYLVLLNMFASVFCLTCLSFDRYLAIVHSLSSAKLRS
           YDWPFGTFFCKLSSYLIFVNMYASVFCLTGLSFDRYLAIVRPVANARLRL
           YHWPFGDLLCKIASTAITLNLYTTVFLLTCLSIDRYSAIVHPMKSRIRRT
           ..****. .**..*   . .*....** ** **.*** ***... .   *

           RSSIIVSLAVIWLFSGLLALPSLILRDT RVEGNNTI CDLDFSGVSSKE
           RVSGAVATAVLWVLAALLAMPVMVLRTTGDLENTTKVQCYMDYSMVATVS
           VMVARLTCVGIWLAVFLASLPSVIYRQIFIFPDTNQTVCALVY     HS
                .. . .*.   * ..* .. * .    ... . * . .       .

           NENFWIGGLSILTTVPGFLLPLLLMTIFYCFIGGKVTMHFQNLKKEE QK
           SEWAWEVGLGVSSTTVGFVVPFTIMLTCYFFIAQTIAGHFRKERIEGLRK
           GHIYFMVGMSLVKNIVGFFIPFVIILTSYTLIGKTLKEVYRAQRA    R
           ..  .  *... ... ** .*. .. . * .*. ..   ..  .     .

           KKRLLKIIITLVVVFAICWLPFHILKTIHFLDLMGFLELSCSTQNIIVSL
           RRRLLSIIVVLVVTFALCWMPYHLVKTLYMLG  SLLHWPCDFDLFLMNI
           NDDIFKMIVAVVLLFFFCWIPHQVFTFLDVLIQMDVIQ NCKMYDIVDTG
           .  ....*...*. * .**.* .. . .  *   . ..  *.   .. .

           HPYATCLAYVNSCLNPFLYAFFDLRFRSQCFFFF                G
           FPYCYCISYVNSCLNPFLYAFFDPRFRQACTSMLCCGQSRCAGTSHSSSG
           MPITICIAYFNSCLNPFLYGFFGKKFRKHFLQLIKYIPPK  MRTHASVN
            *   *..* *********.**. .**     ..                .

           FKKVL  QGHLSNTSSSLS   AQTQKSEIHCVATKV
           EKSASYSSGHSQGPGPNMGKGGEQMHEKSIPYSQETLVVD
           TKSSTVSQ RLSDTKCASNKIALWIFDIEEHC       K
            *.      .  ..  . .       . . ..
```

Figure 2. Amino Acid Sequence Comparisons with *XEGR-1*.

assumed open reading frame encoding a putative protein of 335 amino acids. The ATG start codon is designated by bold letters and the canonical Kozak consensus sequence located immediately upstream from the initiating methionine is underlined (Kozak, 1986). The locations of the transmembrane regions which have been predicted by a hydropathicity analysis are also denoted. Potential phosphorylation sites on the cytoplasmic domain have been indicated by an asterisk. Comparison with primate, rodent, mammalian and vertebrate sequence libraries in Genebank revealed that a human gene, APJ, has the highest sequence similarity, being 48% homologous at the amino acid level. APJ is a novel G-protein-coupled receptor with unknown function that appears to share similarities with angiotensin II type one receptor (O'Dowd et al., 1993). The ligand for the angiotensin receptor, angiotensin II, is known to mediate its signal through two receptor subtypes, AT1 and AT2, which have been identified using sequence homologies and pharmacological differences (Mukoyama, et al., 1993). Figure 2 compares the protein sequences of APJ and Xenopus angiotensin II type one receptors to *XEGR-1*.

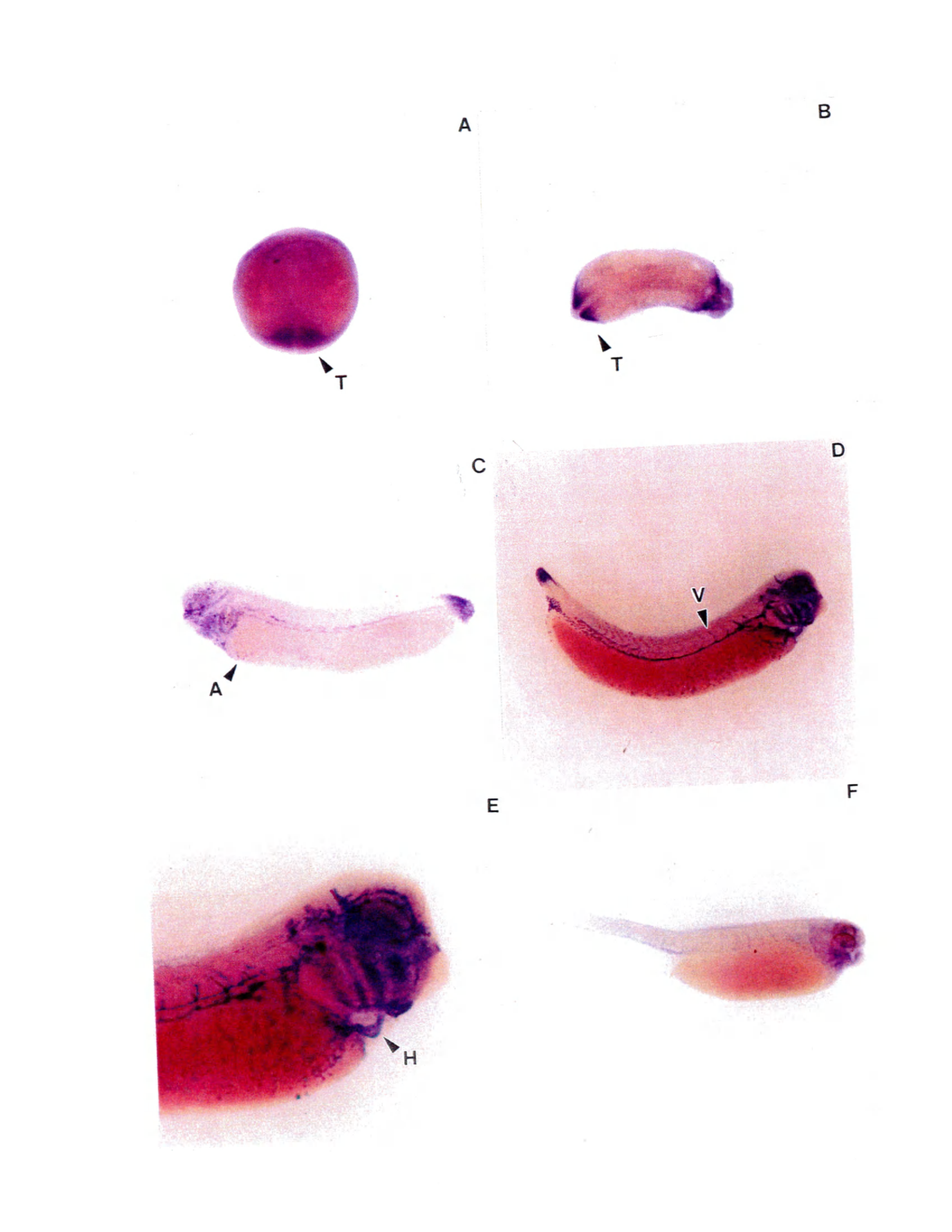
Expression Pattern of *XEGR-1*

Whole-mount in situ hybridization was used to demonstrate the spatial as well as the temporal expression of *XEGR-1*. *XEGR-1* is first clearly detectable in

early neurula stages (st. 14-16) as a diffuse stain in the most posterior region of the neural plate which is fated to become the tail (De Robertis, 1993) (Fig. 3A). In later neurula stages (st. 17-19), this diffuse staining in the presumptive tail becomes more intense and localized (Fig. 3B). During this period, a diffuse stain also appears in the ventral mesoderm in the anterior region of the embryo. As the embryo nears tail bud stages (st. 24-28), the diffuse stain in the ventral mesoderm is transformed into punctate staining that appears in discrete cells (Fig. 3C). As the tail emerges during stages 29-34, staining continues to condense in the very tip of the tail while angioblasts appear to coalesce into structures that appear to be the rudiments of a vascular system. Specifically, signal can be seen concentrated in the heart region, in blood vessels throughout the head, between the somites, and in bilateral vessels running alongside the notochord (Figs. 3D,E). A marked decrease in the amount of visible stain in the vasculature and in the tail occurs during stages 36-42, with signal essentially disappearing by swimming tadpole stages (st. 45) (Fig. 3F).

In order to more closely examine the expression of *XEGR-1* during development, embryos which had undergone in situ hybridization were sectioned transversely along the entire anterior-posterior axis. The analysis of the stain in these sections confirmed the patterns that appeared in the whole embryo. The vascular structures could now be examined in relation to their anatomical location.

Figure 3. Whole-mount in situ hybridization with *XEGR-1*. Whole-mount in situ hybridization was performed on Xenopus embryos at different developmental stages. (A) Early neurula (st. 14), dorsal view. The arrow indicates the presumptive tail (**T**)area. (B) Late neurula (st. 20), dorsal view. The arrow is pointing to the tail region. (C) Early tail bud (st. 28), lateral view. Angioblasts (**A**) in the ventral region of the embryo are indicated. (D) Hatching (st. 34), lateral view. The blood vessels (**V**) running below the notochord and between the somites are marked. (E) Hatching (st. 34), close up of the head region. The endocardium of the heart (**H**)is clearly visible in this embryo. (F) Swimming tadpole (st. 42), lateral view.


A
B
T
T
C
D
A
V
E
F
H

Using an anatomical textbook as a guide (Lehman, 1977), an attempt was made to identify some of these structures (Fig. 4). The diffuse stain seen in the posterior neural plate of whole embryos (st. 14-16) was not visible in cross section at the very early stages; however, by stage 18, a strong signal was apparent in the presumptive tail region as well as the anterior ventral mesoderm (Figs. 4A, B). During pre-tailbud and tailbud stages, localized stain appeared in regions which give rise to the endocardium of the heart (Figs. 4C,D) and specific blood vessels identified as the carotids, pericardinal veins, lateral arteries, and the hepatic vein (Fig. 4E). Of particular interest was a discrete stripe of stain that appeared on either side of the neural tube, between the eye and the ear, during stages 32-36 (Fig. 4F) which does not appear to correspond to any anatomical structure illustrated in Lehman's text.

Regional Expression of *XEGR-1* in Explants

To begin to address the question of which tissue interactions regulate *XEGR-1*, a preliminary set of explant experiments was initiated to define the regional contributions of the ventral and dorsal mesoderm to vasculogenesis. Embryos at stage 11.5 (mid-gastrula) and stage 14 (early neurula) were dissected into dorsal and ventral pieces (Figs. 5,6) and then further subdivided into anterior, middle and posterior pieces and then cultured in vitro until hatching stages (st. 32-

Figure 4. Histological analysis of *XEGR-1* mRNA expression. Following completion of the in situ hybridization procedure, embryos were sectioned transversely along the anterior-posterior axis and analyzed for stain. (A) A neurula (st.18) embryo, sectioned through the ventral anterior region. (B) Section through the tail of a st. 20 embryo. (C) Early tailbud embryos (st. 24) sectioned through the heart (**H**) region. (D) Hatching embryo (st. 34) sectioned through the heart. Diffuse stain in the area that will give rise to the carotid arteries is apparent as well as discrete staining of the endocardium (**E**). (E) Cross-section through the gut region of a st. 32 embryo showing stain in the pericardinal veins (**PV**) that run bilaterally along the embryo. (F) Stain on either side of the neural tube is present in this tailbud embryo (st. 32).

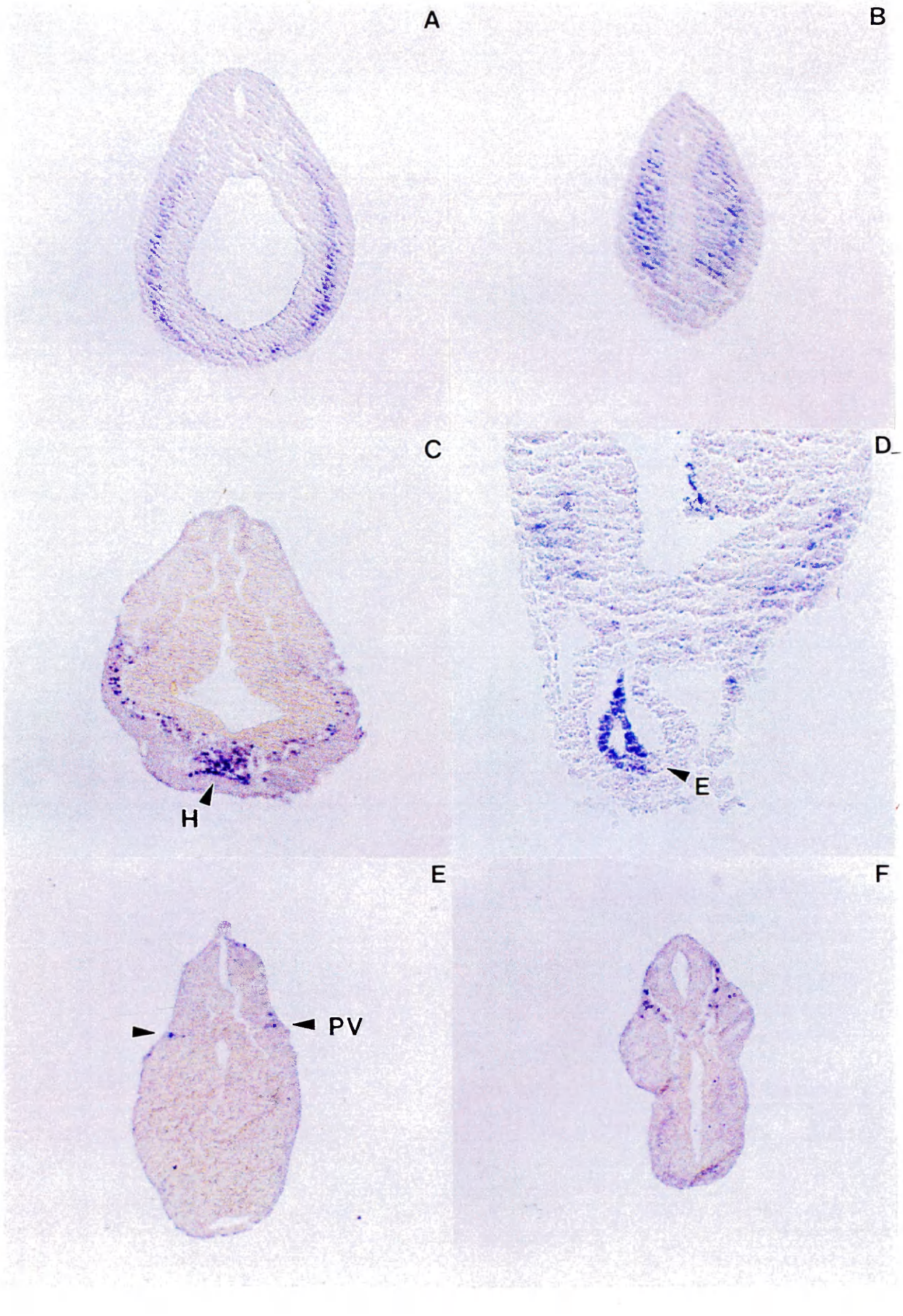

A
B
C
H
D
E
E
PV
F

Figure 5. Schematic diagram of the surgical operations performed on stage 11.5 embryos. Gastrula stage embryos were divided into dorsal and ventral regions, and then further subdivided into anterior, middle and posterior pieces (yielding a total of six pieces). The side of the embryo with the longest distance from the edge of the blastopore to the animal cap was considered to be the dorsal region while the animal cap and the blastopore were considered to be anterior and posterior respectively.

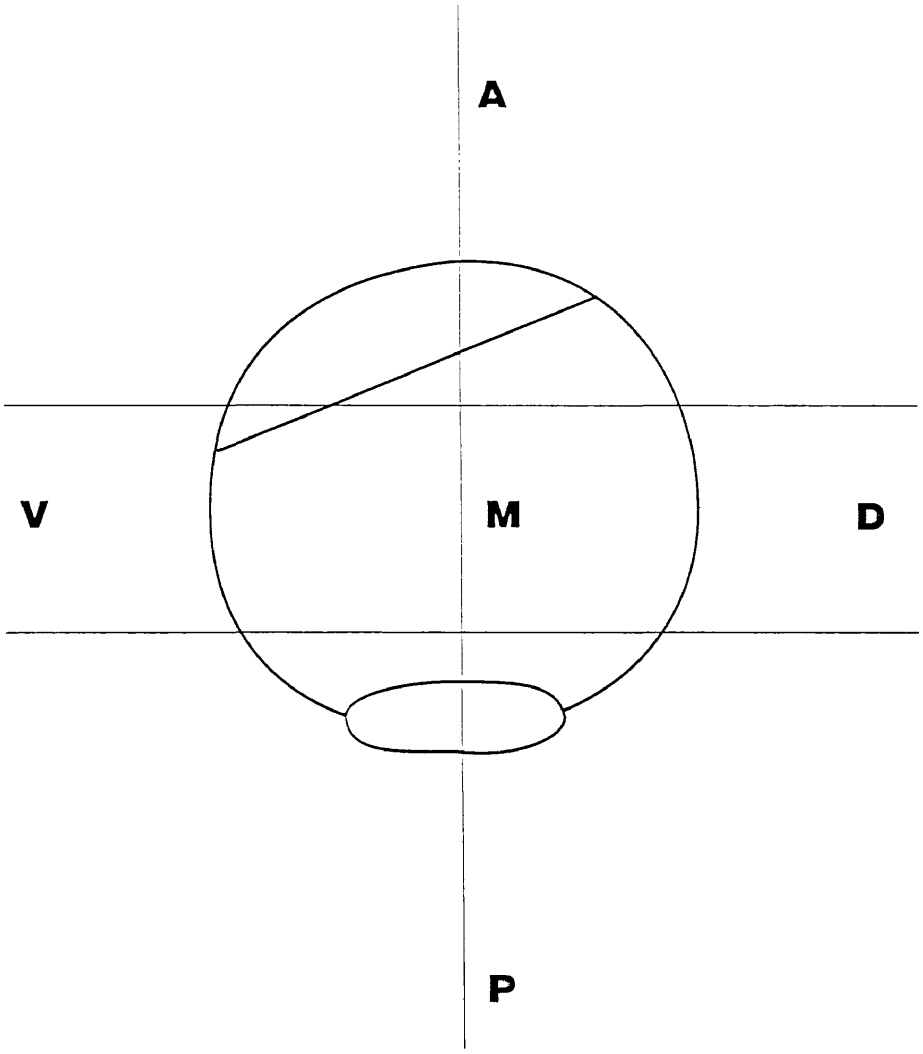
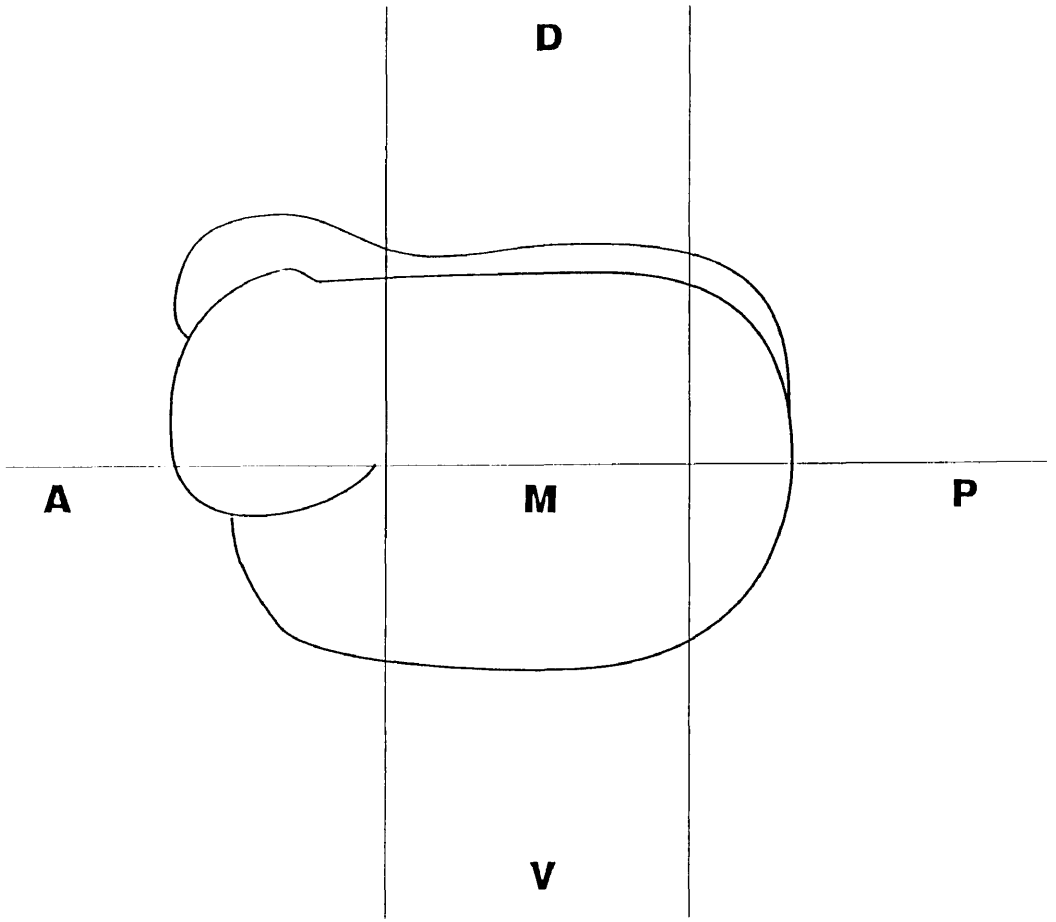

A
V
M
D
P

Figure 6. Schematic diagram of the surgical operations performed on stage 14 embryos. Neurula stage embryos were divided into dorsal (neural plate, notochord and somites) and ventral (endoderm and ventral mesoderm) regions, and then further subdivided into anterior, middle and posterior pieces (yielding a total of six pieces).


D
A
M
P
V

34). These explants were then subjected to in situ hybridization with an *XEGR-1* antisense mRNA probe. By assaying for the expression of *XEGR-1* in various regions of the embryo, it is possible to determine when this gene is "specified," that is, the point at which a piece of tissue excised from the embryo and cultured in vitro is able to express a gene which it would have otherwise expressed if left intact in vivo (Slack, 1991). In effect, this type of experiment examines when a piece of tissue is capable of expressing a given gene in the absence of further inductive interactions with surrounding tissues. It is important to distinguish clearly between the time at which a gene is expressed (as it would be expressed normally in vivo) and the time at which it is specified (the time at which a piece of tissue can be removed and still express the gene) (Saha, 1993). The results of this experiment are summarized in Table 2. Analysis of the ventral explants from stage 11.5 embryos shows an overall pattern of stain which is diffusely localized on one side of the explant. Of the three ventral regions, the anterior and middle pieces exhibited the most staining (Figs. 7D,E) while the posterior piece was generally negative (Fig. 7F). The signal seen in the dorsal explants revealed a different overall pattern of expression from the ventral explants and could be described as more punctate and discrete. Once again, the anterior and middle pieces of the dorsal mesoderm displayed significantly more staining than the posterior piece (Figs. 7A-C).

Table 2. Summary results of the regional expression of *XEGR-1* in explants taken from stage 11.5 and 14 embryos. The numbers in the table indicate the number of explants that expressed *XEGR-1* (positive) and the number of explants that did not (negative). (VA) ventral anterior, (VM) ventral middle, (VP) ventral posterior, (DA) dorsal anterior, (DM) dorsal middle, (DP) dorsal posterior.

TABLE 2

EXPLANT	CONTROL	POSITIVE	NEGATIVE	PERCENT POSITIVE
11.5 VA	positive	7	5	58%
VM	positive	9	6	60%
VP	positive	2	8	25%
DA	positive	8	5	62%
DM	positive	13	2	87%
DP	positive	2	9	18%
14.5 VA	positive	21	0	100%
VM	positive	12	8	60%
VP	positive	4	2	67%
DA	positive	18	0	100%
DM	positive	15	0	100%
DP	positive	17	0	100%

The pattern of stain was also examined in the dorsal/ventral explants that were removed at later stages, namely, neural plate stage embryos (st.14). *XEGR-1* is expressed in these explants in a manner which mimics the pattern seen in the corresponding regions of whole embryos. Ventral-anterior explants stained in a pattern similar to that seen in the heart forming region (Fig. 8D), ventral-middle pieces had a punctate line of stain similar to the bilateral stripe seen below the notochord in whole embryos (Fig. 8E), and ventral-posterior pieces exhibited a small degree of punctate stain like the signal seen at the base of the tail in whole embryos (Fig. 8F). Dorsal-anterior explants closely resembled a normal head and stained strongly throughout (Fig. 8A), while dorsal-middle pieces displayed both diffuse staining in the somites and punctate stain in a line beneath the notochord (Fig. 8B). Dorsal-posterior explants exhibited stain concentrated in the tip of the tail-like structure in a manner very similar to that seen previously in the tail of whole embryos (Fig. 8C).

Figure 7. Expression of *XEGR-1* in stage 11.5 explants. Gastrula stage embryos were divided into dorsal and ventral regions, subdivided into anterior, middle and dorsal pieces and then cultured in vitro until control embryos had reached stage 32-34. In situ hybridization was performed with mRNA antisense probes to *XEGR-1* on explants from all six regions. (A) Discrete points of signal can be seen in these dorsal-anterior explants. (B) Areas of intense stain are present in the explants from the dorsal middle region. (C) Dorsal-posterior explants showed virtually no stain. The ventral-anterior (D) and the ventral-middle(E) pieces showed an overall pattern of stain which is diffusely localized to one side of the explant, while (F) the ventral-posterior explant was generally lacking any detectable signal.

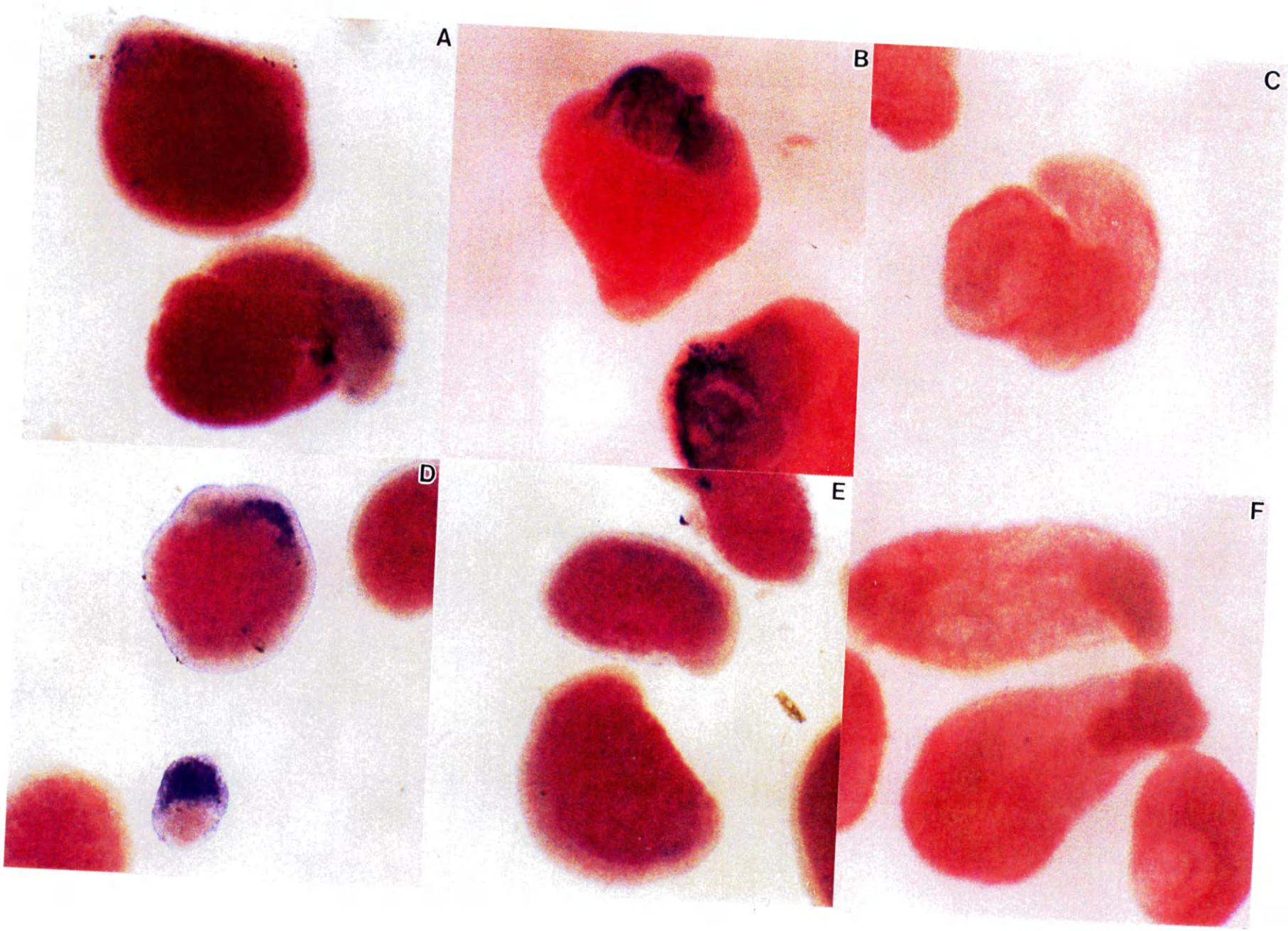
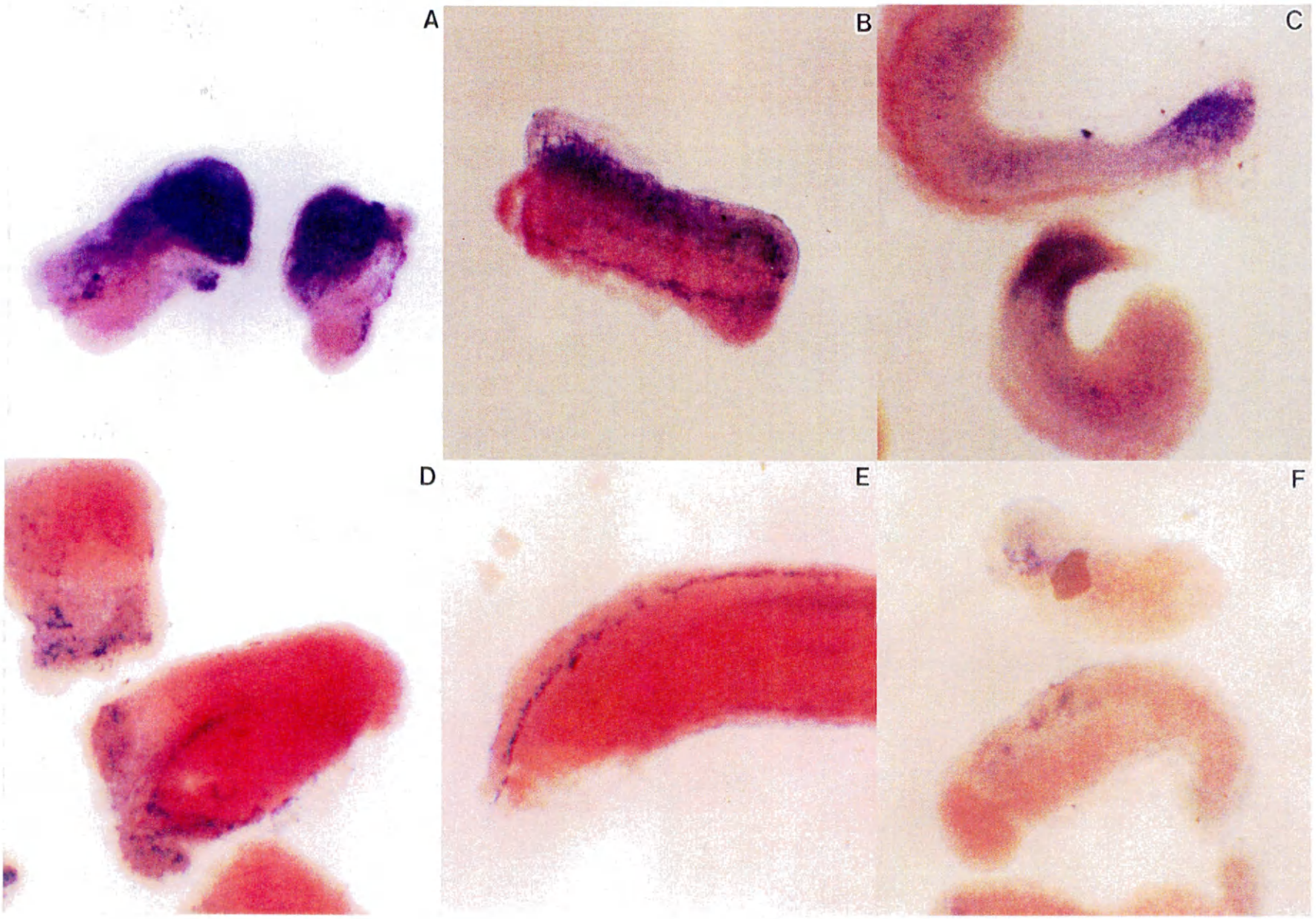

A
B
C
D
E
F

Figure 8. Expression of *XEGR-1* in stage 14 explants. Early neurula stage embryos were divided into dorsal and ventral regions, subdivided into anterior, middle and posterior pieces and then cultured in vitro until control embryos reached a stage of 32-34. In situ hybridization was performed with mRNA antisense probes to *XEGR-1* on explants from all six regions. Overall, the expression pattern seen in these explants, mimicked the pattern seen in vivo. (A) Dorsal-anterior pieces closely resembled a normal head and stained strongly throughout, while dorsal-middle pieces (B) displayed both diffuse staining in the somites and punctate stain in a line beneath the notochord. Dorsal-posterior explants (C) exhibited stain concentrated in the tip of the tail in a manner very similar to that seen in the tail of whole embryos. Ventral-anterior explants (D) stained in a pattern similar to that seen in the heart forming region, while ventral-middle pieces (E) had a punctate line a stain similar to the bilateral stripe in whole embryos. Ventral-posterior pieces (F) were also consistent with the in vivo pattern.


A
B
C
D
E
F

DISCUSSION

XEGR-1 is a Novel Member of the G-protein-coupled Receptor Superfamily

Sequence analysis of *XEGR-1* demonstrates that it shares sequence and structural characteristics which define the GCR superfamily, and that it is related to a host of angiotensin, somatostatin, neuropeptide and IL-8 receptors. However, *XEGR-1* is most closely related to a human gene, called APJ (O'Dowd, et al, 1993), sharing 48% homology at the amino acid level. APJ is a novel gene that is angiotensin receptor-like. Comparison of *XEGR-1* with the known angiotensin receptor cloned in Xenopus shows only 30 % homology at the amino acid level, suggesting that *XEGR-1* is a novel G-protein coupled receptor.

Angiotensin II mediates it signal through two subtypes of angiotensin receptors, AT1 and AT2, which have been identified using sequence homologies and pharmacological differences (Chiu, et al., 1989). The function of the AT1 receptor in controlling blood pressure in the adult is well characterized, whereas the function for AT2 remains undefined. The abundant expression of AT2 receptors in embryonic tissues and fetal brain (Tsutsumi, et al., 1991) in addition to their expression in skin wounds and vascular injury indicates that AT2 may be involved in growth and development (Mukoyama, et al., 1993).

It is not clear from sequence comparisons whether *XEGR-1* represents the AT2 receptor subtype in Xenopus. In rats, there is only 34% homology between AT1 and AT2 receptor subtypes. Given this relative dissimilarity between AT subtypes in rat, the possibility exists that we have cloned the AT2 receptor subtype in Xenopus since *XEGR-1* is 30% homologous to Xenopus AT1. However, comparisons of *XEGR-1* to other AT2 receptors show only 30% homology. The pharmacological analysis of *XEGR-1* with angiotensin II and its receptor antagonists in addition to further comparison with additional genes should lead to a firmer identification of *XEGR-1*.

Potential Involvement of G-protein-coupled Receptors in Vasculogenesis and Angiogenesis

The establishment of a circulatory system is of primary importance to a developing embryo as it divides and utilizes the finite store of energy provided by the yolk. Studies of chick/quail chimeras have identified two mechanisms that are responsible for assembling the heart and network of blood vessels that will nourish the growing embryo. The first mechanism, called vasculogenesis, involves the in situ differentiation of angioblasts from mesoderm. These endothelial precursors then migrate to specific locations in the embryo and fuse together into primitive vascular cords that subsequently develop into mature blood vessels. Structures

such as the heart and vessels at the endodermal/ mesodermal boundary are formed as a result of vasculogenesis. Angiogenesis is the second mechanism by which new blood vessels are formed. This process generates new vessels from pre-existing ones by sprouting vascular branches that will extend into new regions of tissue (Poole and Coffin, 1989; Coffin and Poole, 1991).

Many studies have attempted to define which of these processes are used to vascularize different regions of the embryo (reviewed by Noden, 1989, 1992). With the development of monoclonal and polyclonal antibodies that recognize endothelial tissue in quail embryos such as QH-1 and MB-1 (Pardanaud et al., 1987, Labastie et al., 1988) the question of endothelial cell precursor (angioblast) origin and migration has been examined. By grafting different types of quail mesoderm into chick tissue and identifying the presence of immunopositive cells, it has been possible to differentiate which grafts in the host tissue contain cells that give rise to vascular endothelium (Noden, 1989; Poole and Coffin, 1989). The results of these studies indicate that several embryonic tissues contain endogenous angioblasts. Only prechordal mesoderm, notochord, neural crest, brain and spinal cord are not capable of forming vascular endothelium. It is therefore thought that angioblasts must migrate into these tissues.

Although these methods in chick and quail models have established a basic fate map of endothelial precursors that give rise to the vasculature, they have not

led to the identification of either the tissue interactions nor the signaling pathways that are responsible for the intial induction and differentiation of angioblasts and their subsequent assembly into vascular structures.

Some progress has been made in the understanding of the molecular events involved in vasculogenesis and angiogenesis with the identification of vascular endothelial growth factor (VEGF) (Ferrara and Henzel, 1989; Gospodarowicz, et al., 1989) and its receptor, *flk-1*, a member of the tyrosine kinase receptor superfamily. Studies have demonstrated that the expression of VEGF and *flk-1* is co-localized in endothelial cells throughout mouse development, including very early expression in the blood islands of day 8.5-10.5 embryos (Millauer, et al., 1993). This suggests that the growth factor-tyrosine kinase pathway may serve as one of the primary signaling systems in vasculogenesis and angiogenesis.

Further clues to the molecular players involved in these processes have been presented in several miscellaneous studies that have suggested a possible role for G-protein-coupled receptors in vasculogenesis and angiogenesis. One study demonstrated a potential role for angiotensin II (which mediates its signal through a G-protein-coupled receptor) in regulating angiogenesis in the bovine corpus luteum (Stirling et al., 1990). Another interesting study showed that a novel G-protein-coupled receptor called edg-1 was induced by the tumor promoter phorbol 12-myristate 13-acetate (PMA) in endothelial cells and caused their differentiation

into capillary-like structures (Hla and Maciag, 1990). A third study demonstrated that a molecule involved in the neovascularization of tumors called angiogenin was able to activate phospholipase C, leading to a brief rise in 1,2-diacyglycerol and inositol triphosphate (Moore and Riordan, 1990). The activation of these second messenger molecules is typically mediated by G-protein-coupled receptors. A study conducted by Bauer et al. (1992), used a human vascular endothelial cell line as a model for angiogenesis. By blocking the normal process of tube formation seen in these cells with pertussis toxin, a possible role for G-proteins in angiogenesis was demonstrated. Lastly, the identification and characterization of *XEGR-1* in this study offers further evidence for the involvement of G-protein-coupled receptor signaling pathways in vasculogenesis and angiogenesis.

The temporal and spatial expression of *XEGR-1* during early neurula stages through swimming tadpole stages in tissues that give rise to the cardiovascular system (i.e. ventral mesoderm, and the developing heart and blood vessels) suggests that it may be a suitable molecular marker for studying the induction and differentiation of endothelial precursors in Xenopus. With the identification of additional molecular markers specific for endothelial precursors along with an increasing knowledge of the early inductive interactions in amphibian cardiovascular development (Sater and Jacobson, 1990), Xenopus could be an alternative to chick and quail models. For unlike chick, Xenopus laevis tisues can

be isolated and manipulated from the earliest stages of development, making Xenopus well suited for an analysis of the early tissue interactions leading to the assembly of the cardiovascular system.

XEGR-1 as a Marker for Amphibian Endothelial Precursors

In order to begin to examine the tissue interactions involved in the differentiation of amphibian endothelium, a preliminary round of in situ hybriziations was initiated using *XEGR-1* as an endothelial marker. In this experiment, embryos at stage 11.5 and stage 14 were divided into dorsal and ventral pieces and then subdivided into anterior, middle and posterior pieces (Figs. 5,6) for the purpose of observing which regions were capable of giving rise to endothelial cells that express *XEGR-1*. Several interesting observations were made from this initial investigation.

First, the expression pattern observed in all six regions of the stage 14 explants was very similar to the pattern seen in vivo. This may suggest that for the most part, angioblasts throughout the embryo are "determined," that is, they have undergone the tissue interactions necessary for these angioblasts to form normally patterned vascular tissue. Both the ventral and dorsal pieces have already acquired some endothelial identity by this stage in development and are able to regulate the expression of *XEGR-1*. The expression of *XEGR-1* observed in stage 11.5 explants

was, however, very different from the pattern seen in the corresponding regions of the stage 14 explants. Both the anterior and middle pieces of the ventral and dorsal explants showed areas of expression while the posterior pieces were completely unable to express *XEGR-1*. The absence of *XEGR-1* expression in the presumptive tail explants was unexpected given the strong signal seen in the tail region throughout the course of development. It will be necessary to refine these experiments to determine if additional tissue interactions are necessary to regulate the expression of *XEGR-1* in this region during gastrula stages.

Although the overall expression pattern detected in stage 11.5 explants generally did not resemble the in vivo pattern, there were a few interesting similarities. The expression of *XEGR-1* in ventral anterior and ventral middle explants was somewhat more diffuse and usually localized to one side of the explant. This type of diffuse stain was observed in whole-mounts in the ventral mesoderm during tailbud stages. In contrast, the stain in dorsal anterior and and dorsal middle pieces at stage 11.5 appeared to be more punctate and present in discrete groups of cells. This pattern of punctate stain was also observable in regions of dorsal mesoderm in whole-mounts. It is apparent from these comparisons of stage 11.5 explants that the expression of *XEGR-1* in dorsal explants and in the ventral explants is different in composition. This difference

may suggest that the mesodermal tissue in the dorsal region of the embryo may have endothelial properties different from that in the ventral region.

Lastly, the expression of *XEGR-1* in stage 14 dorsal anterior explants was unexpected. The tissue in this explant is largely composed of prechordal mesoderm, neural crest and brain; tissues which previous studies in chick have determined to be unable to give rise to endothelial precursors. The removal of the dorsal anterior area from surrounding tissue at this early stage should have prevented any migration of endothelial cells into this area. The expression of *XEGR-1* in these explants suggests that either endothelial migration is occuring earlier than stage 14 or that prechordal mesoderm and neural crest are capable of vasculogenesis.

Future Directions

There are several experiments that could be pursued at this point in the investigation of *XEGR-1* to determine its role in angiogenesis. First, it will be necessary to refine the explant experiments carried out in this study. Virtually nothing is known regarding the specific tissue interactions responsible for angiogenesis and vasculogenesis. Once the explant experiments have defined the regional tissue interactions needed to regulate the expression of *XEGR-1*, it would be possible to do in vitro recombinants of several tissues to define and confirm

what inductive interactions are taking place. Experiments of this kind should not only provide an understanding of the tissue interactions involved in regulating the expression of *XEGR-1* but also help define which regions of the mesoderm differentitate into the respective components of the cardiovascular system.

Second, to determine if there is any relationship between the expression of *XEGR-1* and angiotensin receptors, the expression pattern of *XEGR-1* could be compared with the expression pattern of the angiotensin II receptor that has already been cloned in Xenopus (Bergsma, et al., 1993). Whole-mount in situ hybridizations have not been done on this receptor and it would be interesting to see if the spatial as well as the temporal expression of these receptors overlap. In addition, it would also be intriguing to clone the Xenopus homologs for VEGF and *flk-1* and compare their expression patterns with that of *XEGR-1*.

Third, the signaling pathway used by *XEGR-1* could be determined by identification of its ligand and the second messenger system to which it is coupled. The first candidate ligand to be examined should be angiotensin II. Another approach to identifying possible ligands would be to express the receptor in a bacterial strain or baculoviral system. *XEGR-1* could be attached to a column and assayed for molecules which bind to it. Candidate ligands could then be radiolabeled and used in binding assays with *XEGR-1*. A review of the current literature on angiogenic molecules revealed that angiogenin, an agent essential for

neovascularization in tumors, may act through an unidentified membrane protein that activates endothelial phospholipase C and causes a subsequent increase in 1,2-diacyglycerol (DAG) (Moore and Riordan, 1990). The production of these second messenger molecules indicates the activation of a G-protein-coupled pathway. Given this information, it would be interesting to examine the binding capabilities of angiogenin to *XEGR-1*. Once the ligand has been identified, it would then be possible to analyze the second messenger pathway activated by *XEGR-1*. This analysis could be accomplished by stimulating endothelial cells in vitro with the ligand and then assaying for an increase in the levels of second messenger molecules like cAMP, 1,2 diacylglycerol or calcium ions.

Finally, in order to begin to understand the function of *XEGR-1* in angiogenesis/vasculogenesis. It would be possible to observe the effects of ectopically expressing *XEGR-1* in embryos by injecting transcripts into Xenopus oocytes which then become distributed to each of the dividing cells in the embryo. If *XEGR-1* is a critical control molecule early in the pathway leading to the determination of angioblasts, then one may expect misplaced or additional vascular structures. A genetic approach to understanding function would also be intriguing; given the difficulty of inactivating genes in Xenopus, the cloning of the homologs of *XEGR-1* in mice where gene inactivation experiments are now possible would be desirable.

Angiogenesis is important not only in development, but also in adult functions such as reproduction and wound repair. Angiogenesis in the adult is tightly controlled because any deregulation of this process can lead to a host of diseases, namely, arthritis, neovascularization of the eye during later stages of diabetes and tumorigenesis (Folkman and Shing, 1992). Further investigation of molecules like *XEGR-1* in signaling pathways involved in angiogensis and vasculogenesis may lead to the development of therapeutic agents that may enhance angiogenesis during would repair and arrest it during arthritis and carcinogenesis.

REFERENCES

Amaya, E., Musci, T. and Kirschner, M. (1991) Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. Cell, 66: 257-270.

Asahima, M., Nakano, H., Uchiyama, H., Sugino, H., Nakamura, T., Eto, Y., Ejima, D., Nishimatsu, S-I., Ueno, N.and Kinoshita, K. (1991) Presence of activin (erythroid differentiation factor) in unfertilized eggs and blastulae of Xenopus laevis. Proceedings of the National Academy of Sciences USA, 88: 6511-6514.

Bauer, J., Margolis, M., Schreiner, C., Edgell, C., Azizkhan, J., Lazarowski, E. and Juliano, R. (1992) In vitro model of angiogenesis using a human endothelium-derived permanent cell line: contributions of induced gene expression, G-proteins, and integrins. Journal of Cellular Physiology, 153: 437-449.

Bergsma, D., Ellis, C., Nuthulaganti, P., Nambi, P., Scaife, K., Kumar, K. and Aiyar, N. (1993) Isolation and expression of a novel angiotensin II receptor from Xenopus laevis heart. Molecular Pharmacology, 44: 277-284.

Bockaert, J. (1991) G proteins and G-protein-coupled receptors: structure, function and interactions. Current Opinion in Neurobiology, 1: 32-42.

Breier, G., Albrecht, U., Sterrer, S. and Risau, W. (1992) Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development, 114: 521-532.

Clapham, D. (1993) Mutations in G-protein-linked receptors: novel insights on disease. Cell, 75, 1237-1239.

Coffin, J. and Poole, T. (1991) Endothelial cell origin and migration in embryonic heart and cranial blood bessel development. The Anatomical Record, 231: 383-395.

Davidson, E. (1986) Gene activity in early development. 3rd ed. Academic Press, Orlando, Florida.

Dawid, I. and Sargent, T. (1986) Molecular embryology in amphibians: new approaches to old questions. Trends in Genetics, 2: 47-50.

Dawid, I., Taira, M., Good, P. and Rebagliati, M. (1992) The role of growth factors in embryonic induction in Xenopus laevis. Molecular Reproduction and Development, 32:136-144.

Feinberg, A. and Vogelstein, B. (1983) Analytical Biochemistry, 132:6.

Ferrara, N. and Henzel, W. (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochemistry Biophysics Research Communication, 161: 851-858.

Flamme, I. and Risau, W. (1992) Induction of vasculogenesis and hematopoiesis in vitro. Development, 116: 435-439.

Folkman, J. and Shing, Y. (1992) Angiogenesis. The Journal of Biological Chemistry, 267: (16) 10931-10934.

Fuller, P. (1991) The steroid receptor superfamily: mechanisms of diversity. The FASEB Journal, 5: 3092-3099.

Gillespie,L., Paterno, G. and Slack, J. (1989) Analysis of competence: receptor for fibroblast growth in early Xenopus embryos. Development, 106: 203-208.

Gospodarowicz, D., Abraham, J. and Schilling, J. (1989) Isolation and characterization of a vascular endothelial mitogen produced by pituitary -derived folliculo stellate cells. Proceedings of the National Academy of Science USA, 86: 7311-7315.

Harland, R. (1991) In situ hybridization: an improved method for Xenopus embryos. Methods in Cell Biology, 36: 685-695.

Henry, J.J. and Grainger, R. (1990) Early tissue interactions leading to embryonic leading to embryonic lens formation in Xenopus laevis. Developmental Biology, 141: 149-163.

Hille, B. (1992) G protein-coupled mechanisms and nervous signaling. Neuron, 9: 187-195.

Hla, T. and Maciag, T. (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-

protein-coupled receptors. The Journal of Biological Chemistry, 265 (16): 9308-9313.

Kimelman, D. and Kirschner, M. (1987) Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell, 51: 569-577.

Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell, 44: 283-292.

Labastie, M., Poole, B., Peault, M. and Le Douarin, M. (1986) Surface marker for menopoietic and endothelial cell lineages in quail that is defined by a monoclonal antibody. Proceedings of the National Academy of Sciences USA, 83: 9016-9020.

Lehman, H.E. (1977) Chordate Development, Hunter Publishing Company, University of North Carolina-Chapel Hill.

Maden, M. and Holder, N. (1991) The involvement of retinoic acid in the development of the vertebrate central nervous system. Development supplement 2, 87-94.

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Marinez, M., Moller, N.P.H., Risau, W.and Ullrich, A. (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell, 72: 835-846.

Moore, F. and Riordan, J. (1990) Angiogenin activates phopholipase C and elicits a rapid incorporation of fatty acid into choleterol esters in vascular smooth muscle cells. Biochemistry, 29: 228-233.

Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R. and Dzau, V. (1993) Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. Journal of Biological Chemistry, 268: 24539-24542.

Musci, T., Amaya, E., Kirschner, M. (1990) Regulation of the fibroblast growth factor receptor in early Xenopus embryos. Proceedings of the National Academy of Sciences USA, 87:8365-8369.

Nieuwkoop, P.D. and Faber, J. (1967) Normal table of Xenopus Laevis (Daudin). Amsterdam: North Holland Pub. Co.

Nieuwkoop, P.D. (1969) The formation of mesoderm inurodelean amphibians. Induction by the endoderm. Roux's Archives of Developmental Biology, 162: 341-373.

Noden, D.M. (1989) Embryonic origins and assembly of blood vessels. American Review of Respiratory Disease, 140:1097-1103.

Noden, D.M. (1990) Origins and assembly of avian embryonic blood vessels. Annals of the New York Academy of Science, 558: 236-249.

O'Dowd, D., Heiber, M., Chan, A., Heng, H., Tsui, L., Kennedy, J., Shi, X., Petronis, A., George, S. and Nguyen, T. (1993) A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. Gene, 136 (102): 355-360.

O'Malley, B. (1990) The steroid receptor superfamily: more excitement predicted for the future. Molecular Endocrinology, 4 (3): 363-369.

Parndanaud, L., Altman, C., Kitos, F., Dieterlen-Lelievre and Buck, C. (1987) Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development, 100: 339-349.

Poole, T. and Coffin, D. (1989) Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. The Journal of Experimental Zoology, 251: 224-231.

Probst, W., Snyder, L., Schuster, D., Brosius, J., Sealfon, S. (1992) Sequence alignment of the G-protein-coupled receptor superfamily. DNA and Cell Biology 11 (1): 1-20.

Saha, M. (1993) Determination of the dorsal-ventral axis in Xenopus. National Institutes of Health grant, submitted October 1, 1993.

Saha, M. and Grainger, R. (1992) A labile period in the determination of the anterior-posterior axis during early neural development in Xenopus. Neuron, 8: 1003-1014.

Saha, M., Servetnick, M. and Grainger, R. (1992) Vertebrate eye development. Current Opions in Genetics and Development, 2: 582-588.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. and Coulson, A. (1977) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA, 74: 5463.

Sater, A. and Jacobson, A. (1990) The role of the dorsal lip in the induction of heart mesoderm in Xenopus laevis. Development, 108: 461-470.

Savard, P. (1992) Body axis determination during early development in amphibians. Biochemistry and Cell Biology, 70: 875-891.

Servetnick, M. and Grainger, R. (1991) Changes in neural and lends competence in Xenopus octoderm evidence for and autonomous timer. Developmental Biology, 112:177-188.

Slack, J.M.W. (1984) Regional biosynthetic markers in the early amphibian embryo. Journal of Experimental Embryology and Morphology, 26:67-79.

Slack, J.W.M. (1987) Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature, 326: 197-200.

Slack, J.M.W. (1991) From Egg to Embryo, 2nd ed., Cambridge: Cambridge University Press.

Slack, J.M.W. (1993) Embryonic induction. Mechanisms of Development, 41: 91-107.

Smith, J., Price, B., Van Nimmen, K., Huylebroeck D. (1990) Identification of a potent Xenopus mesoderm-inducing factor as a homologue of activin A. Nature, 345:729-731.

Smith, J. (1993) Mesoderm-inducing factors in early vertebrate development. The EMBO Journal, 12 (12): 4463-4470.

Stirling, D., Magness, R., Stone, R., Waterman, M. and Simpson, E. (1990) Angiotensin II inhibits luteinizing hormone-stimulated cholesterol side chain cleavage expression and stimulates basic fibroblase growth factor expression in

bovine luteal cells in primary culture. The Journal of Biological Chemistry, 265 (1): 5-8.

Summerbell, D. and Maden, M. (1990) Retinoic acid, a developmental signalling molecule. Trends in Neuroscience, 13 (4): 142-147.

Tsutsumi, K. and Saavedra, J. (1991) Diffierential development of angiotensin II receptor subtypes in the rat brain. Endocrinology, 128 (1): 630-632.

Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. Cell, 61: 203-212.

Wahli, W. and Martinex, E. (1991) Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. The FASEB Journal, 5: 2243-2249.

Whitebread, S., Mele, M., Kamber, B. and de Gasparo, M. (1989) Preliminary biochemical characterization of two angiotensin II receptor subtypes. Biochmeistry Biophysics Research Communications, 163: 284-291.

Yamaguchi, T., Dumont, D., Conlon, R., Breitman, M. and Rossant, J. (1993) *flk-1*, an *flt* related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development, 118: 489-498.

VITA

Rebecca Ruth Stucki Miles

Rebecca was born on June 20, 1968 in Monroe, Wisconsin. She graduated valedictorian from Wyoming Park High School in Wyoming, Michigan, June 1986. She then attended Indiana Wesleyan University where she graduated summa cum laude with a Bachelor's degree in Biology and Political Science, May 1990. Subsequently, Rebecca worked on a breast cancer project at the University of Chicago as a research assistant until August of 1992 when she entered the College of William and Mary as a graduate teaching assistant in the Department of Biology.