CHARACTERIZATION OF A NOVEL Gli5 GENE DURING EMBRYONIC DEVELOPMENT IN XENOPUS LAEVIS

MING MAI

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Characterization of a Novel *Gli5* Gene during Embryonic Development in *Xenopus laevis*

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Abstract

Human *Gli* oncogene gene was first isolated from its amplification in a glioblastoma cell line, DM-259 MG. Because *Gli* gene is the vertebrate homolog of the *Drosophila* segment polarity gene, *Ci* (*cubitus interruptus*), isolation of the *Xenopus Gli* gene and analysis of its function in embryonic development were performed. Using RT-PCR and 5' RACE-PCR, 2.1 Kb of the 5' end of *Xenopus Gli5* gene was cloned and sequenced. Sequence comparison found that region one and the zinc finger motif of *Xenopus Gli5* were very similar to other Glis. In region one and the zinc finger region, the amino acid identities between XGli5 and Chicken Gli2/4, XGli4, XGli3, XGli1, Human Gli3, HGli1 are 98.6%, 100%, 78.5%, 78.5%, 81,5%, 65.7% and 92%, 97%, 95%, 91.3%, 94%, 87.3% respectively. In addition to these two conserved regions, the GF (gain of function) region and the region located between GF region and region one are also conserved among Gli family. But, outside of these conserved regions the homology is less conserved.

From Northern Hybridization and RT-PCR analysis, it was found that although *Xenopus Gli5* was expressed at all stages of embryonic development, the peak of expression was at stage 8. This expression pattern is different from that of known *Xenopus Gli* genes in embryonic development. Using affinity purified XGli5 antibodies, through competitive Western blotting analysis, the XGli5 protein expression was detected in stage 28 embryos as a 190 KDa band.

A XGli5 fusion protein was made in the pGEX.KT vector. GST+Gli5 protein was purified by GST affinity chromatography. The fusion protein was used to test the specificity of XGli5 polyclonal antibodies and whether *Xenopus* Gli5 can bind the same specific DNA sequence as those in other Gli protein. According to the results of electrophoretic mobility shift assays (EMSA), XGli5 can specifically bind the same Gli binding sequence, 5′-GACCACCCA- 3′ as other Glis. Therefore, it is likely that XGli5 is a transcription factor as other Glis which can regulate transcription by binding specific promoter. To explore the relation between *XGli5* expression and FGF induction, animal pole explants from *Xenopus* embryos were incubated with FGF (100ng/ml) for different lengths of time and then total RNA was extracted. The level of *Xenopus Gli5* gene expression was examined by RT-PCR. Preliminary results showed that FGF induced *Gli5* expression in explants.

Overall, a new number of *XGli* gene family, *XGli5* was isolated. The full length of *XGli5* cDNA is 8.5 Kb and encodes a 190 Kda protein. Because XGli5 can also bind the core Gli binding sequence, XGli5 may function as a transcription factor. In the early *Xenopus* embryonic development, FGF, the mesoderm induction signal, can also induce *XGli5* expression.

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List of abbreviations and Symbols Used

Bp	Basepair (s)
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
DEPC	Diethylpyrocarbonate
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GST	Glutathione S-transferase
HCG	Human chorionic gonadotropin
ICR	Internal control region
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobases
KDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MMLV	Moloney-Murine Leukemia Virus Reverse transcriptase
MOPS	3-[N-morpholino]propanesulfonic acid
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RACE	Rapid amplification cDNA end
RT-PCR	Reverse transcription followed by PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	SRC homology domain 2
TDT	Terminal Deoxynucleotidyl Transferase
TEMED	N,N,N',N'- tetramethylethylenediamine
TGFβ	Transforming growth factor β
UTR	Untranslated region
XGli5	*Xenopus Gli5*

XGli-1kb	*Xenopus Gli5* fragment amplified by XF4 and Zb2 primers

Introduction

1.1 Embryonic development of *Xenopus laevis*

1.1.1 The importance of research on embryonic development

A primary objective of developmental biology is to explore the mechanisms of embryonic cell interaction and differentiation in order to understand how tissues and organs are formed. Obviously, there are many genes involved in the regulation of embryonic development, the most complicated process in an organism. Interestingly, many of the genes that can regulate embryonic cell growth, division, and differentiation are also involved in carcinogenesis in the adult. Therefore, it is very important to perform research on embryonic development in order to investigate the function of these oncogenes. For example, recently, people identified a gene which has a strong correlation with Gorlin syndrome (or nevoid basal cell carcinoma syndrome) (Hahn et al., 1996). This Gorlin gene is located at chromosome 9q22 and shows 67% similarity to the *Patched* gene of *Drosophila* at the DNA level. Hahnís group found six frameshift and nonsense mutations in this gene in affected patients (Hahn et al., 1996). Interestingly, *Patched* is one of the segment polarity genes in *Drosophila* embryonic development and is

involved in the HH (hedgehog) signal pathway to regulate normal *Drosophila* embryonic development (Von Ohlen et al., 1997). Recently, Dahmane *et al* found that activation of another gene in the hedgehog signal pathway, *Gli1*, can also induce basal cell carcinoma (BCC) formation (Dahmane et al., 1997). Therefore, the more gene regulation mechanisms in embryogensis are revealed, the more an opportunity is offered to increase our understanding of tumorigenesis.

1.1.2 Characterization and events of embryonic development of *Xenopus laevis*

The availability of good animal models is very important for research in developmental biology. The fruit fly, *Drosophila melanogaster*, is a powerful system to study the genes that regulate embryonic development (Ingham, 1988). In vertebrates, a few animal models are available. In the widely used mouse embryo, *in vitro* manipulation of embryo is difficult (Hogan et al., 1994). Because of characteristics of *in vitro* fertilization, the *Xenopus* embryo is a useful system for evaluating the function of genes whose products are important in regulating crucial developmental events in embryogenesis.

1.1.2.1 The advantage of *Xenopus laevis* system

Xenopus laevis is an ideal animal for developmental studies for several reasons.

A). *Xenopus* eggs are large and embryonic development is external. This makes it easily manipulated by such techniques as microinjection of specific mRNAs in order to evaluate their expression, distribution and function (Gurdon et al., 1971; Gurdon and Melton, 1981).

B). We can easily obtain a large number of eggs (>1000) by injection of HCG (Human Chorionic Gonadotropin) into female frogs. These artificially fertilized eggs develop synchronously and can be used for biochemical extraction and purification of developmentally important molecules.

C). The morphology of *Xenopus laevis*, the characteristics of embryo anatomy and histology, and the fate map and cell lineages in the early embryo are all well defined (Dale and Slack, 1987; Moody, 1987). All of this descriptive knowledge of the *Xenopus* embryo is very helpful for the analysis of molecular mechanism of embryonic development.

D). The *Xenopus* oocyte and embryo has been used as an *in vivo* translation system for identifying and investigating many proteins after injection of their mRNAs. Because *Xenopus* embryos have been shown to faithfully translate heterologous mRNA (Lane, 1983; Soreq, 1990), this *in vivo* translation system can be used

to investigate the function of genes whose products are suspected to be important for regulating developmental events during embryogenesis.

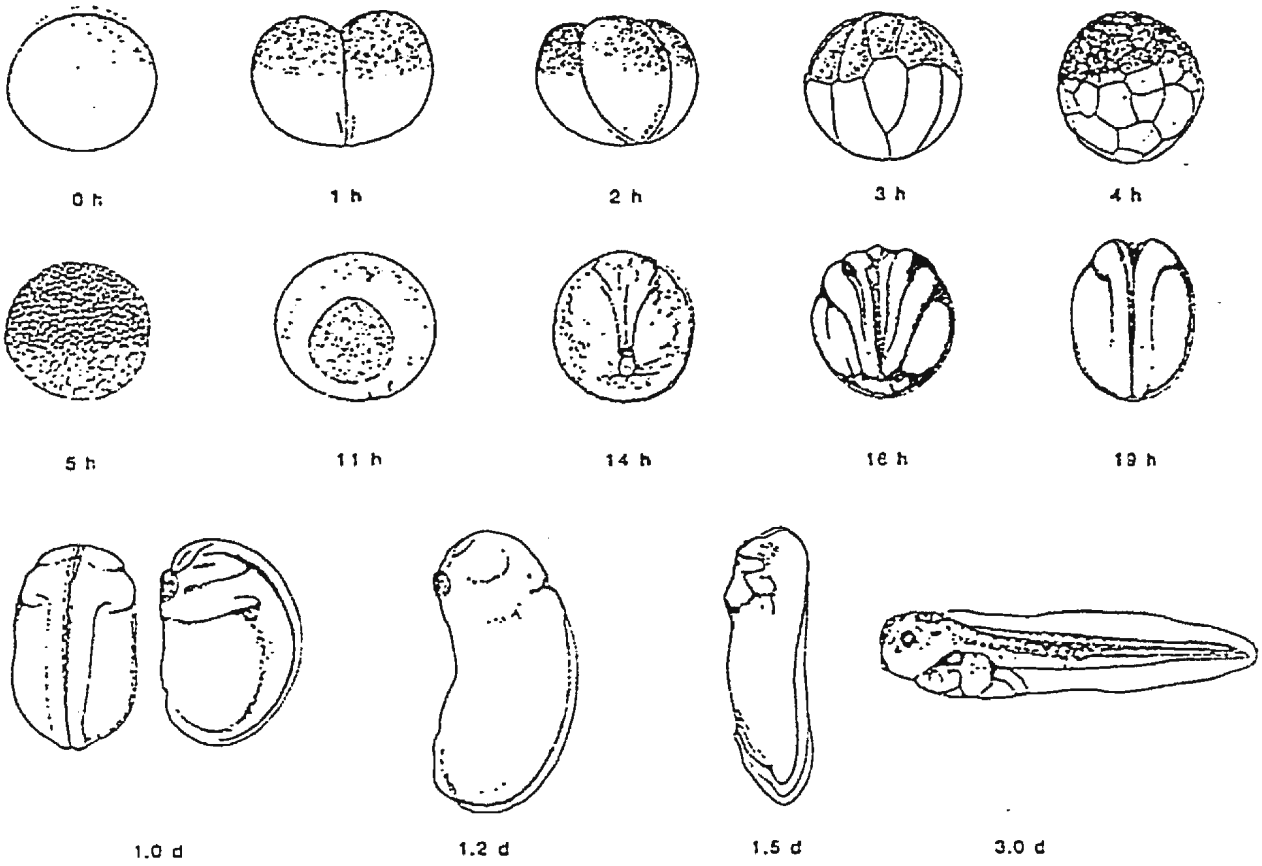
1.1.2.2 The events of *Xenopus* embryonic development

The unfertilized egg of *Xenopus laevis* has an animal hemisphere and a vegetal hemisphere. The materials located in two hemispheres are quite different. The animal hemisphere is darkly pigmented, with small and sparse yolk granules, but the vegetal hemisphere has little pigment with many large yolk platelets and mitochondria near the vegetal cortex (Bragg, 1939; Tourte, et al., 1984; Danilchik and Gerhart, 1987). Meanwhile, some maternal RNAs and proteins have unique distribution in animal or vegetal hemisphere and these asymmetrical distribution implies that they may play as cytoplasmic determinants to determine fate of embryogenesis (Rebagliati et al., 1985; Litvin and King, 1988).

The process of *Xenopus* embryogenesis is well known (Keller, 1975 and 1976). Briefly, eggs lie in the transparent vitelline membrane surrounded by the jelly coat. About one hour after the sperm enters the animal hemisphere, the cortex of the egg rotates 30° toward the point of sperm entry, relative to the interior of the

embryo (Vincent et al., 1986). Microtubules (MTs) serve as tracks for cortical rotation because many agents such as UV-irradiation and colchicine etc. which depolymerize MTs also inhibit cortical rotation (Manes et al., 1978; Manes and Elinson, 1980; Elinson and Rowning, 1988). This rotation leads to the establishment of the vegetal dorsalizing region, the Nieuwkoop center which is required for dorsal development (Gerhart et al., 1989). The next important event is cleavage, in which the single large egg cell is subdivided into two identical smaller cells. The first cleavage bisects the animal and vegetal hemisphere and separates the fertilized egg approximately into left and right cell (Hubrecht-Laboratorium, 1967). The second division also begins at the animal hemisphere and is perpendicular to the first division. It separates the embryo into dorsal and ventral halves (Fig.1.1). The third cleavage is in the equatorial plane and separates the embryo into animal and vegetal parts. This division is not equal and vegetal cells are larger than animal cells. As cleavage continues, a cavity called a blastocoel forms in the centre of the animal hemisphere. At this stage, the embryo is called the blastula. After 12 divisions, the embryo is in the stage of mid-blastula transition (MBT) in which cell division synchrony is lost, rate of cell division slows, and zygotic gene

Fig.1.1. Different stages of *Xenopus* embryonic development which are measured in hours (h) and days (d) after fertilization (Adopted from Nieuwkoop and Faber staging table, 1975)



transcription starts (Shiokawa et al., 1979; Newport & Kirschner, 1982). The next important developmental stage is called gastrulation, in which extensive morphogenetic movements caused by the rearrangement of blastula cells occurs.

Gastrulation begins when dorsal blastula cells below the equator start to migrate internally and the archenteron of the embryo develops by the invagination of these cells (Hubrecht-Laboratorium, 1967). The force of embryonic cell migration is mainly provided by a belt of tissue around the equator of the embryo called the marginal zone (Keller, 1981). The opening of the archenteron at the vegetal region is called the blastopore. As the embryo develops, when the marginal cells reach the edge of blastopore (dorsal lip of blastopore), they turn inward and migrate along inner surface of archenteron. The animal part of cells stretch to cover the entire surface of the sphere and the inside archenteron enlarges as the blastocoel is obliterated. Eventually, as the process of gastrulation continues, a three layer embryo is created. The endoderm is the innermost layer which is mainly formed from the vegetal cells. The outermost layer, which is set up by the movement of the animal hemisphere cells, is the ectoderm. The layer between the endoderm and ectoderm is the

mesoderm which is from marginal zone cells. In other words, the three classical germ layers, ectoderm, mesoderm and endoderm, have achieved their final trilaminar arrangement (Nieuwkoop, 1969).

Neurulation is the next stage of development. The dorsal ectoderm is induced to form the central neural system. Following further embryonic development, the neural plate becomes visible. Then, the neural folds rise and join together to organize the neural tube and neural crest. After neurulation, the embryo begins organogenesis which is based on interactions among the three embryonic layers. Endoderm produces the digestive system and organs of appendage such as liver and pancreas. Mesoderm produces cardiac and skeletal muscle, notochord, bone and cartilage, connective tissue, kidney, blood and mesenchyme. Ectoderm gives rise to the nervous system and epidermis (Hubrecht-Laboratorium, 1967).

1.1.3. Mesoderm induction and dorsoventral patterning

One of the most important concepts of development is embryonic induction where a group of embryonic cells influence adjacent cells and further change their status and function during development. According to the pioneering work of Org (1967), Nieuwkoop (1969), and Slack and Smith (1983), mesoderm induction begins at the

early blastula-stage embryo. Vegetal cells pass mesoderm induction signals to the cells above them and induce them to be ventral mesoderm. Dorsal vegetal cells (Nieuwkoop center) can induce cells above them to be dorsal mesoderm called Spermann organizer, in recognition of the finding that, when this region is transplanted to the ventral side of an early gastrula embryo, it is able to induce an ectopic axis (Spemann and Mangold, 1924). The other vegetal cells induce the marginal cells above them to become the lateral and ventral mesoderm. The third signal, which is emitted at the grastrulation stage originates from the organizer and can induce the marginal mesodermal cells adjacent to them to be intermediate mesodermal tissues. Although this induction model and other modifications (Kimelman et al., 1992; Sive, 1993; Watable et al., 1995) can explain the phenomena of dorsoventral patterning, uncovering molecular interactions in this event can help us to understand the mechanism of early embryonic development.

1.1.4. Molecular basis of mesoderm induction

1.1.4.1. β-catenin: Wnt signal pathway involved in dorsalization

β-catenin is bi-functional protein, which can be a cell membrane-associated protein necessary for cadherin-mediated adhesion (Ozawa et al., 1989) and a vertebrate homology of the *Drosophila* protein, Armadillo, involved

in the Wingless signal pathway (Peifer et al., 1992). In *Xenopus*, it accumulates preferentially at the dorsal side throughout early cleavage (Schneider et al., 1996) and the *Xenopus* embryo develops without dorsal structure by depletion of maternal β-catenin (Heasman et al., 1994). This implies that β-catenin is necessary for dorsalization of *Xenopus* embryogenesis. Now, it is clear that the basic pathway of β-catenin is involved in the Wnt signal pathway (McMahon and Moon 1989; Christian et al., 1992). Xwnt protein can bind the membrane protein, *Xenopus* Frizzled (Xfz), the putative Xwnt receptor (Yang-Snyder et al., 1996). The Wnt signal activates Disheveled (Dsh) protein which can repress the Glycogan Sythase Kinase 3β (GSK-3β) activity (Wagner et al., 1997). GSK-3β encodes a Ser/Thr protein kinase and phosphorylates β-catenin on the amino-terminal serine and threonine residues and thereby targets it for destruction by the ubiquitin-proteasome pathway (Aberle et al., 1997). Recently, the dorsalization effects of lithium have been shown to be due to its effects in the Xwnt/β-catenin pathway. Specifically, lithium inhibits the GSK-3β which in turn causes the accumulation of β-catenin (Klein et al., 1996; Stambolic et al., 1996; Hedgepeth et al., 1997). It is clear that β-catenin bind to GSK-3β through

a protein, Axin and conductin (Behrens et al., 1998; Kishida et al., 1998), which help the formation of complex of these proteins. Repression of GSK-3β the endogenous β-catenin will accumulate and enter the nucleus where it modulates transcription in concert with TCFs (member of the family of transcriptional factors, variably referred to as T-cell factor (TCF), lymphoid enhancer (LEF), or pangolin) (Molenaar et al., 1996) and activate gene transcription such as Siamois which is involved in the dorsal inductions (Lemaire et al., 1995).

1.1.4.2. TGF molecules in mesoderm induction

Vg1 is a maternally expressed member of the transforming growth factor-β superfamily, located to the vegetal part of *Xenopus* egg and cleavage embryos (Rebagliati et al., 1985; Weeks and Melton, 1987). While the active form of Vg1 protein cannot be detected in the early *Xenopus* embryo, artifically processed Vg1 can be used to rescue UV- ventralized embryos completely (Thomsen and Melton, 1993). This implied that location of Vg1 in a dorsal cell is important in dorsal axis formation. A truncated, dominant inhibitory activin type II receptor fully inhibits the mesoderm-inducing activity of processed Vg1 in animal part, suggesting that inhibition of endogenous mesoderm formation by this

mutant receptor may be due to inhibition of Vg1 signaling (Klein and Melton, 1994).

Activin is another member of TGF family and can induce dorsal mesodermal tissues (Thomsen et al., 1990). For example, a 1.5- fold increasing activin results in alteration of induction pattern from homogeneous muscle to notochord formation (Green et al., 1992). It has been proposed that an endogeneous gradient of activin could direct mesodermal patterning (Green and Smith, 1990). However, overexpression of Follistatin, a natural activin inhibitor, does not block mesoderm induction (Schulte-Merker et al., 1994). This result raises questions about the role of activin in endogenous mesoderm induction.

Bone morphogenetic proteins (BMPs), capable of stimulating bone formation, are maternally expressed in *Xenopus* embryos (Koster et al, 1991). BMP4 can change the fate of dorsal mesoderm into ventral mesodermal tissues, including blood and mesenchyme (Dale, et al., 1992). In the *Xenopus* embryo, BMPs inhibit development of dorsoanterior structures, consistent with a ventralizing effect on mesoderm (Dale, et al., 1992). Overexpression of BMPs in the dorsal marginal zone by localized RNA injections causes upregulation and ectopic expression of ventral specific marker genes and repression of dorsal genes (Clement et al., 1995; Schmidt et al., 1995). Therefore, production of ventral fates

within the ectodermal and mesodermal germ layers is dependent on the activity of BMPs. Inhibition of BMPs is, however, possibly sufficient to promote dorsal cell fates. The experiments showed that Chordin, Noggin and Follistatin are antagonists of endogenous BMPs and are expressed in the dorsal marginal zone in order to inhibit the ventral promoting BMPs and thereby allow dorsal development (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997).

1.1.4.3 Organizer proteins

Xenopus embryonic cells have the capacity to differentiate as dorsal structures but this capacity is subject to widespread inhibition (Gerhart, 1996). In the dorsal cells, the function of organizer is to emit the proteins to release this inhibition and protect to be ventralized. As mention above, BMPs, especially BMP4, plays an important role in this inhibition (Steinbeisser et al., 1995). There are three major organizer proteins, Chordin, Noggin, and Follistatin that antagonize BMP4 and allow the cells differentiate as dorsal fates.

Chordin is a secreted protein activated by the homeodomain-containing transcription factors Goosecoid and Xnot2 (Sasai et al., 1994) It is detected in the dorsal marginal zone about an hour before gastrulation. As gastrulation begins chordin is seen only in the dorsal

blastopore lip and further located in the prechordal plate and notochord. Chordin can induce a secondary axis when microinjected into the ventral sides of *Xenopus* blastula by blocking the action of BMP4. It is now known that both chordin and noggin directly bind and inactivate BMP4, thus preventing its ventralizing function (De Robertis and Sasai, 1996; Piccolo et al., 1996; Sasai et al., 1996; Zimmerman et al., 1996). Noggin is another organizer protein. The *Noggin* gene was first isolated from lithium treated gastrulae (Smith and Harland 1991, 1992). Injection of *noggin* mRNA into UV-irradiated embryos completely rescues the dorsal axis and allows the formation of a complete embryo. It seems that Noggin is an excellent candidate for mediating some of the functions of the organizer. Noggin can accomplish two major functions of the organizer. It induces neural tissue from the dorsal ectoderm, and it dorsalizes the mesoderm cells that will contribute to the ventral mesoderm (Smith 1993; Lamb et al., 1993), which is supported by the fact that BMP4 is inactivated by Noggin.

Follistatin, the activin-binding protein, is located in the dorsal blastopore lip and later becomes restricted to the notochord (Hemmati-Brivanlou and Melton, 1994). It was shown that Follistatin can inhibit activities of BMP7 (Yamashida et al., 1995). Because BMP7 is needed for the activation of BMP4, by inhibiting BMP7 (De Robertis and

Sasai, 1996), Follistatin also has a function in preventing ventralization of the mesoderm.

1.1.4.4. FGF

FGF (fibroblast growth factor) is the first molecule demonstrated to induce mesoderm in animal pole explants (Slack, et al., 1987; Kimelman and Kirschner, 1987). Maternal bFGF and eFGF are present at the stage of development when mesoderm induction and play a role in mesoderm induction is believed to occur (Slack and Isaacs, 1989). Generally, FGF is a ventral mesodermal inducer, based on the results that FGF induces more ventral tissues (such as mesenchyme and mesothelium) in animal cap explants compared to the tissues induced by high concentrations of activin (including most- dorsal mesoderm such as notochord) (Godsave and Slack, 1989; Green et al., 1990). However, FGF may be involved in dorsal mesoderm formation because of the evidence that injection of bFGF mRNA in animal explant can induce dorsal mesoderm (Kimelman and Maas, 1992). This FGF induced dorsal mesoderm is modified by Xwnt-8, which is maternally located at dorsal ventral part of embryo (Christian et al., 1992). Ectopically expressed FGF cooperating with X-wnt8 specifies dorsal mesoderm (Kiemlman and Maas, 1992; Christian et al., 1992). Embryos expressing a dominant negative FGF receptor fail

to express several immediate early mesoderm markers such as Brachyury and Xpo and the embryo develops with severe mesoderm defects, strongly suggesting that FGF receptor signaling is required for primary mesoderm induction (Amaya et al., 1993). Studies have revealed several signal components downstream of FGF receptors in mesoderm induction. FGF signal transduction is involved in the MAP kinase (mitogen-activated protein kinase) cascade (Gotoh and Nishida, 1996). Overexpression of dominant negative Ras or Raf-1 constructs blocks mesoderm induction by activin and FGF, and active Ras or Raf-1 can induce mesoderm in animal caps (Whiteman and Melton, 1992). Binding of Ras and Raf-1 is thought to contribute to the activation of kinase activity of Raf-1 (Vojtek, et al., 1993) and Raf-1 phosphorylation and activation of MAPKK cascade is required and sufficient for mesoderm induction in animal cap by activin and FGF (Gotoh et al., 1995). The observation that a low but detectable level of MAPK activity exists during early embryogenesis, which in animal caps can be blocked by blocking FGF signaling, further supports the involvement of FGF activated MAPK in mesoderm induction (LaBonne et al., 1995). It is not known how MAPK functions in the mesoderm induction. It is possible that MAPK activates transcription factors, which may be the FGF signal early response gene such as Xbra (Smith et al, 1991). Recently, one of FGF signal early response genes, er-1 was isolated and it may have an

important function in FGF signal transduction in *Xenopus* mesoderm induction (Paterno et al., 1997).

1.2. Zinc finger proteins and *Xenopus* embryonic development

1.2.1 Transcription factors

The process of transcription requires a certain interaction between specific DNA sequences, RNA polymerase, and some proteins which are called transcription factors. Some transcription factors which directly interact with RNA polymerase in the promoter region (upstream of transcription initiation site) to determine basal transcription level are called basal transcription factors. Basal transcription factors are further classified into three types according to different RNA polymerases that they associate with, *e.g.* TFIID (RNA polyermase II associated transcription factor D) and TFIIIA (RNA polymerase III associated transcription factor A). The other transcription factors are called transcriptional activators or repressors which bind the regulatory regions (e.g. enhancers) in order to interact with the basal transcriptional complex (directly or indirectly) and regulate basal level of transcription (Roberts et al., 1995). Many transcription factors are zinc finger proteins.

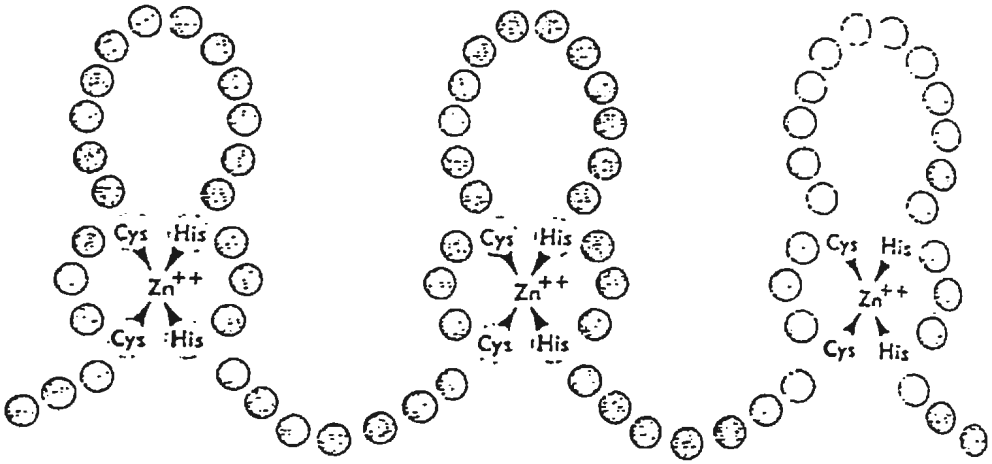
1.2.2 Zinc finger proteins

Zinc finger proteins were first found in TFIII A (Evans and Hollenberg, 1988), a 5S rRNA gene-specific transcription factor from *Xenopus laevis*. This protein contains nine tandem repeats of a sequence element referred to as the C2H2 zinc finger since it contains highly conserved pairs of cysteine and histidine residues (Miller *et al.*, 1985), each of which fold about a zinc atom (Fig.1.2) (Diakun *et al.*, 1986). The consensus sequence of a single finger is as follows:

Cys-X 2-4-Cys-X3-Phe-X5-Leu-X2-His-X3-His (X- any amino acid)

There are 28 amino acids in a typical zinc finger repeat. Conserved residues can be expected to be either structurally important or involved in specific binding to nucleic acids. In addition to the four highly conserved Zinc coordinating residues, other similarly conserved positions are the two hydrophobic residues Phe and Leu in the loop region, and the two amino acids following the second conserved Cys. A moderately conserved stretch of six additional residues is found in the region linking the second conserved His of one finger element with the first conserved Cys of the next one. This is termed the H/C-link (Schuh et al., 1986). The results of NMR

Fig. 1.2. Zinc finger Model. The conservative two cysteine and two histidine residues fold and form finger structure. The zinc is held in a tetrahedral structure of two cysteine and two histidine (Adopted from Lewin, 1994)



spectroscopy of zinc finger proteins suggest a structure that agrees quite well with the model predicted (Miller et al., 1985; Berg, 1988; Lee et al., 1989).

There is another type of zinc finger, for example, steroid receptors, which does not have the C2H2 structure(Carson-Jurica et al., 1990). However, in the zinc finger of steroid receptors, only four cysteines are conserved. Therefore, this type of finger is called the C2/C2 finger. The consensus zinc finger sequence of steroid receptors is as follows.

Cys-X2-Cys-X1-3--Cys-X2-Cys (X- any amino acid)

These features of the zinc finger provide very important systematic criteria with which to distinguish different zinc finger proteins. The other conserved structures of zinc finger protein include the FAX (Knochel et al., 1989) and KRAB (Bellefroid et al., 1991) domains which define structurally distinct subfamilies of zinc finger proteins. These are not reviewed here. The biochemical aspects of zinc finger proteins focus on the binding of nucleic acids and regulation of transcription. Most zinc finger proteins bind specific DNA sequences. However, some Zinc finger proteins have the ability to bind RNA or both DNA and RNA such as dsRBP-ZFa or TFIIIA in *Xenopus* (Finerty and Bass, 1997; Theunissen et al., 1992). To explore the zinc finger DNA or RNA binding

ability, many techniques such as footprinting, chemical modification protection, and mutagenesis analysis etc. are adopted (Wolffe 1991). The principle behind zinc finger protein interaction with DNA is that the zinc finger cluster is composed of multiple modules which match with a similar modular arrangement of a 3bp tandem repeat DNA recognition element (Nardelli et al., 1991).

Further, Pavletich and Pabo used crystallographic techniques to describe the specific interaction between zinc finger proteins and target DNA. In both human Gli and Zif zinc finger proteins they found that the N-terminal part of the zinc finger forms a β-sheet and the C-terminal part forms a α-helix, which stretches fit one turn of the major groove of DNA (Pavletich and Pabo, 1993; Pavletich and Pabo 1991).

Generally, zinc finger proteins such as TFIIIA regulate transcription by binding to a specific target sequence. When the zinc finger region binds the DNA or RNA target sequence, its C-terminus itself as a activation domain interacts with other transcription factors and RNA polymerase to form a complex to initiate transcription (Smith et al., 1984). There are three features of zinc finger proteins in transcriptional regulation: 1) either as positive or negative regulators of transcription; 2) act in a concentration-dependent manner; 3)overall

transcriptional regulation depends on promoter context and combinatorial effects with other transcription factors (El-Baradi, 1991; Klug and Rhodes, 1987).

1.2.3 Zinc finger proteins and *Xenopus* development

Since zinc finger proteins (ZFPs) represent more than 1000 structurally distinct nucleic acid binding proteins in vertebrate genome, their function in *Xenopus* embryonic development have not been studied extensively (Bellefroid et al., 1997). Many *Xenopus* ZFPs were isolated according to the conserved sequence of *Drosophila* ZFPs. The features of ZFPs in *Xenopus* members are similar to their invertebrate counterparts (Bellefroid et al., 1997).

In *Xenopus* embryonic development, most ZFPs are involved in neural tissue development and differentiation. Xsna, a ZFP and a homologue of *Drosophila* gene snail, was isolated in 1990 (Sargent and Bennett, 1990). Its expression is zygotically in all early mesoderm. Furthermore, Xsna is expressed ectodermally in the band of cells that surrounds the prospective neural crest and the anterior neural fold from stage 11 (Essex et al., 1993). Since Xsna and another Xsna related ZFP, Xslu, can be induced by mesoderm from the dorsal or lateral marginal zone but not

from the ventral marginal zone and induction of neural crest starts at stage 10 (Mayor et al., 1995), it is clear that both Xsna and Xslu are involved in the early neural crest induction (Mayor et al., 1995). When truncated FGF receptor was expressed ectopically in order to block FGF signaling, Xslu expression was inhibited. However, inhibition of Xslu expression is not the consequence of neural plate inhibition. This indicated that Xslu did not directly determine neural plate induction (Mayor et al., 1997). Recently, it was found that chordin can induce a new *Xenopus* ZFP, Zic-r1 (Mizuseki et al., 1998). It is believed that Zic-r1 acts in the pathway to initiate neural induction and connects the neural inducer with the downstream proneural genes (Mizuseki et al., 1998).

Xenopus ZFPs also have functions in neuronal differentiation. X-MyT1, one of *Xenopus* ZFPs, was found as an early marker of primary neurogenesis (Bellefroid et al., 1996). Overexpression of X-MyT1 by microinjection of X-MyT1 mRNA into two cell *Xenopus* embryo can promote ectopic neuronal differentiation and escape lateral inhibition. Repression of X-MyT1 function inhibits normal neurogenesis as well as ectopic neurogenesis caused by overexpression of X-NGNR-1. It is believed that X-MyT1 is an essential element in the cascade of events that allow

cells to escape lateral inhibition and enter the pathway that leads to terminal neuronal differentiation (Bellefroid et al., 1996).

Not all *Xenopus* ZFPs are involved in neural development. A nanos-like zinc finger protein in *Xenopus*, Xcat-2, was found in the vegetal cortical region in stage VI full-grown oocytes (Mosquera et al., 1993). As a maternally expressed gene, *Xcat-2* has its highest transcript levels in oocytes and falls to barely detectable levels by gastrulation. The localized and maternally restricted expression of *Xcat-2* RNA suggests a role for its protein in setting up regional differences in gene expression that occurs early in development (Moseqera et al., 1993). XCNBP is another example of *Xenopus* ZFP that maybe not involved in neural tissue development (Flink et al., 1998). At the early gastrula stage, XCNBP is expressed in all three germ layers. Because XCNBP can interact with both DNA and RNA, it is suggested that XCNBP functions in transcriptional and translational regulations in a wide variety of tissues during *Xenopus* development (Flink, et al., 1998).

One of most important ZFPs in *Xenopus* embryonic development are those belonging to the *Gli* gene family, homologues of the *Drosophila* segment polarity gene,

Cubitus interruptus (*ci*). I will discuss them in detail as follows.

1.3 *Gli* gene family and its function as related to development

1.3.1 The *Gli* gene family

The Human *Gli1* gene was discovered in 1987 by Vogelsteinís laboratory (Kinzler et al., 1987). They adopted a denaturation-renaturation gel technique (Roninson, 1983) which revealed overexpression of the *Gli1* gene in the glioblastoma cell line D-259 MG. Subsequently, this *Gli1* gene was located at chromosome 12q 13.3-14.1 (Kinzler et al., 1987; Arheden et al., 1989) and was shown to amplify in cells from other human glioblastomas and in some liposarcomas (Roberts et al., 1989). Another important feature of the *Gli1* gene is that it has been shown to transform primary rodent cells in conjunction with adenovirus E1A *in vitro*. This supports the belief that the *Gli1* gene can function as an oncogene and can play a role in neoplastic transformation when amplified in primary human tumors (Ruppert et al., 1991).

After finding the *Gli1* gene, Vogelsteinís group isolated other *Gli*-related genes by screening a genomic DNA library with the zinc finger region of the *Gli1* gene as a probe (Ruppert et al., 1988). In the six positive clones, there were two *Gli* related genes which showed about 85% similarity to the human *Gli1* gene. They were

defined as *Gli2* and *Gli3*. The other four clones demonstrated amino acid similarity to *Gli1* only in the H/C link region but the predicted amino acid sequences were more similar to the *Kruppel* family. These four clones were named HKR 1 to 4 (human kruppel-related genes). At the same time, mouse *Gli1* genes were cloned and characterized (Vortkamp et al., 1992; Walterhouse, 1993). In 1994, Hui et al reported the isolation of a mouse *Gli2* gene by screening mouse embryonic cDNA and genomic libraries. Afterwards, full length of mouse Gli2 and Gli3 clones were isolated (Thien et al., 1996; Hughes et al., 1997). In 1996, chick Gli and Gli3 were also isolated from the embryo (Marigo et al., 1996).

Gli genes not only exist in vertebrates but also find their counterparts in invertebrates, such as the segment polarity gene *ci* in *Drosophila* and *Tra-1* in *C. elegans* (Orenic et al., 1990; Zarkower and Hodgkin, 1992). It is not like vertebrate *Gli* gene family, which have at least 3 members (Ruppert et al., 1988; Hui et al., 1994; Tanimura et al., 1998). Only one *Gli* gene was found in each invertebrate species (Orenic et al., 1990; Zarkower and Hodgkin, 1992).

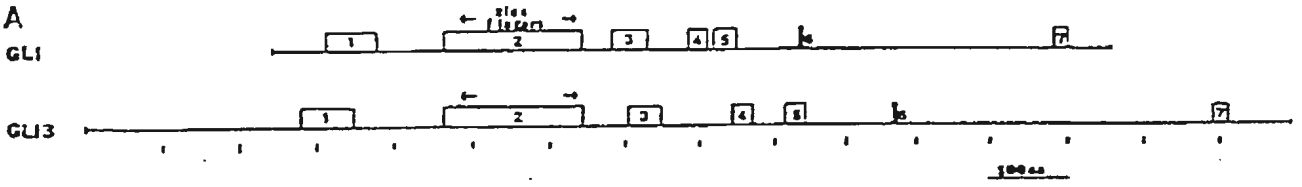
1.3.2 Structural characteristics of *Gli* gene family

The first cloned human *Gli* gene called *Gli1* has a 3318 bp open reading frame which encodes a 1106 amino acid non-interrupted 118 Kda protein (Kinzler et al., 1988). Structural analysis indicates that the zinc finger is the most common characteristic of all of the zinc finger Gli proteins. The essential function of zinc fingers is to bind a target nucleic acid fragment (Wolffe, 1991). Analysis of the Gli1 and other family members revealed that Gli proteins are similar to the *Kruppel* zinc finger protein family whose prototype is the *Drosophila* embryonic *kruppel* family which has a zinc finger consensus sequence (Schuh et al., 1986). One characteristic of Kruppel is that the C-terminal histidine from one finger is linked to the next finger with the sequence HTGEKP. Human Gli1 has high similarity with kruppel only within the zinc finger region and the similarity does not extend beyond this. Ruppert *et al.*(1988) further analyzed the Gli-kruppel family and divided this family into two subgroups. One subgroup, called the Gli-like subgroup, has the consensus finger amino acid sequence, [Y/F]XCH3GCX3[F/Y]X5LX2HX3-4H[T/S]GEKP. The other subgroup, called kruppel-like subgroup, has the amino acid sequence, [Y/F]XCH2CH3FX5LX2HXRXHTGEKP. According to these

consensus sequences, it is easy to distinguish between the Gli and kruppel proteins.

After the discovery of more members of the Gli gene family, such as Gli2 and Gli3, sequence comparison uncovered even more information. In 1990, Ruppert et al. found 7 conserved regions between Gli1 and Gli3 including the zinc finger region (Figure 1.3). These conserved regions may represent domains within Gli1 and Gli3 which mediate common functions in the Gli gene family. In addition to these 7 regions, compared with mouse Gli2 and Gli3, another homologous region was found between region 1 and 2 with 54% amino acid identity (Hughes et al., 1997). Although there is a high degree of similarity within these regions between Gli1 and Gli3, outside of these conserved regions, the amino acid sequence is quite different. Structural analysis of *Xenopus* Gli-3 and Gli-4 revealed three novel, closely spaced conserved sequences, RRXS, located between region 4 and 6. The RRXS is a perfect consensus sequence for a cAMP-dependent protein kinase (PKA) phosphorylation site (Marine et al., 1997). The carboxy-terminal conserved region 7 which is composed of negatively charged acidic amino acid residues may function as a transcriptional activating domain since such acidic regions are one of the characteristics of

Fig. 1.3. Alignment of human Gli protein sequences. (A) comparison of homology between hGli1 and hGli3 proteins. Similar regions are depicted as boxes. Numbered from amino terminus to carboxyl terminus (B) Tabulation of data (Adopted from Ruppert *et al.*, 1990)



B

REGION	GLI(aa)	GLI3(aa)	IDENTITIES/TOTAL (%)
1	62-135	279-348	45/68 (66%)
2	218-400	462-645	152/183 (83%)
fingers	237-392	482-637	137/156 (88%)
3	439-485	704-748	25/47 (53%)
4	539-565	844-871	19/27 (70%)
5	574-604	914-943	17/30 (57%)
6	689-695	1066-1072	7/7 (100%)
7	1037-1055	1494-1512	11/19 (58%)

transcriptional activating domains (Fig.1.3) (Giniger and Ptashne, 1987 ; Ruppert et al., 1990). Indeed, a carboxyl-terminal region, which includes 69 amino acids between 1020-1091 of human Gli1, was identified as a Gli1 transcriptional activation domain by deletion and nested deletion of human Gli1 gene and cotransfection with reporter gene to Hela cells (Yoon et al., 1998).

1.3.3 Function of *Gli* gene family

1.3.3.1 Gli, the transcription factor

After Vogelstein's laboratory cloned and characterized members of the human *Gli* gene family (Kinzler et al., 1987; Ruppert et al., 1988; and Ruppert et al., 1990), other researchers have tried to elucidate the function of Gli.

Because Gli proteins contain zinc finger motifs, the first experiments attempted to show that they were DNA binding transcription factors. Kinzler and Vogelstein (1990) used a novel approach for the identification of binding sites of Gli proteins (Kinzler and Vogelstein, 1990). Total sonicated human DNA (200-300 bp) was linked with catch-linkers and bound to a Gli1 recombinant fusion protein. After centrifugation and digestion with proteinase K, the recovered DNA was amplified by using PCR with catch-linker primers. They found three Gli1 binding sites, all of which have a 9 bp consensus

sequence, 5'-GACCACCCA- 3'. This work demonstrated that Gli protein can bind to DNA in a sequence specific manner. Alexandre *et al* provided the evidence that *Drosophila* Gli homolog, Ci can function as a transcriptional activator in yeast (Alexandre et al., 1995). Similarly, Von Ohlen et al., found that Ci is not only a sequence-specific DNA binding protein but also drives transcription from the *wingless* promoter in the transiently transfected cells (Von Ohlen et al., 1997). Furthermore, mutagenesis of putative Ci binding sites in the *wingless* promoter region greatly reduces Ci binding, and that the reduced binding correlates with greater than 90% reduction in transcriptional activation (Von Ohlen et al., 1997). As their invertebrate counterpart, the Gli family of vertebrates also function as transcriptional activators. A Gli binding consensus sequence was found in the essential region of the minimal HNF-3β (Hepatocyte Nuclear Factor-3β) floor plate enhancer (Sasaki et al., 1997). With co-transfection of human Gli1 and reporter gene with Gli binding consensus sequence, it was found that Gli1 activates but Gli3 represses reporter gene expression (Saski et al., 1997). Similar to human Gli3, *Xenopus* Gli-3 and Gli-4 also showed the transcription repression of reporter gene with co-transfection XGli3 or XGli4 and CAT reporter gene with Gli binding consensus

sequence (Marine et al., 1997). Recently, the isoforms of human Gli2 were cloned and their activities enhance TAX (a viral transactivator protein) dependent transcription in human T-cell Leukemia virus type-1 (Tanimura et al., 1998). Taken together, the Gli gene family encodes transcriptional activators that binds the enhancer with Gli binding consensus sequence to regulate gene expression. Gli1 and Gli2 are transcriptional activator but Gli3 and Gli4 are transcriptional repressors.

1.3.3.2 Gli mediates hedgehog signal transduction pathway

1.3.3.2.1 Ci function in hedeghog signal pathway

Drosophila homologue of Gli, *ci (cubitus interruptus)* is necessary for normal *Drosophila* embryonic development. Mutation of *ci* causes interruptions in wing veins four and five due to inappropriate expression of the Ci product in the posterior compartment of imaginal disks (Slusarski et al., 1995). It was found that Ci was involved in the hedgehog signaling pathway (Alexandre et al., 1995; Dominguez et al., 1995). Specifically, hedgehog binds its receptor, patched, preventing its normal inhibition of smoothened (Smo) (van den Heuvel and Ingham, 1996). This allows Smo to signal through the positive actions of Fused (Fu) and Ci, which regulate target genes (Ruiz I Altaba, 1997). This process is

repressed by Costal2 (cos2) and protein kinase A (PKA)(Robbins et al., 1997, Li et al, 1995). The function of Ci is regulated by proteolysis, with which the full length of 155 Kda Ci will be cleaved into N- termini 75 Kda protein (Aza-Blanc et al., 1997). The smaller form of N-termini Ci will translocate to the nucleus where it represses Hh and Ptc (Aza-Blanc et al., 1997). This 75 Kda Ci shows homogeneous distribution throughout the anterior compartment and is negatively regulated by a feedback loop of Hh signal such as Patched (Hepker et al., 1997), insuring that only anterior cells close to Hh-expressing posterior cells at the boundary will not process Ci and will not repress transcription of target genes. In the A-P boundary region, 155 Kda Ci is modified by Hh signal to produce small amount of a nuclear form Ci that will transactivate target gene such as *Decapentaplegic (Dpp)*, *Patched*, and *Wingless* (Aza-Blanc et al., 1997). In this way, Ci mediates the Hh signal and maintains wingless expression in the anterior cells along the A-P compartment boundary (Hepker et al., 1997).

1.3.3.2.2. Vertebrate Glis in Hh signal transduction pathway

Unlike *Drosophila* Ci, vertebrate Glis have at least 3 family members which have different roles in embryonic development (Ruiz I Altaba 1997). Gli is involved in

neural differentiation and limb development in which Hh signal is required (Ruiz I Altaba, 1997). In early neural development, *Xenopus* Gli1 is expressed in midline cells at the time that they are induced to become floor plate, located immediately overlying the Sonic hedgehog (Shh)-expressing notochord. Afterwards, Gli1 is expressed in immediately adjacent cells that appear to become ventral neurons (Lee et al., 1997). In mouse, Gli1 is also expressed in regions close to Shh-expressing cells such as floor plate (Sasaki et al., 1997). However, Gli2 and Gli3 are broadly expressed throughout the neural plate in both frog and mice (Lee et al., 1997; Sasaki et al., 1997). Therefore, Gli1 is the major mediator of the Shh signal. Ectopic Shh signaling induces Gli1 but represses Gli3 (Marigo et al., 1996; Lee et al., 1997) and misexpression of Gli1, but not Gli3, can mimic the effect of ectopic Shh signaling in the induction of various markers and ventral cell types in the neural tube in frogs and mice (Hynes et al., 1997; Lee et al., 1997), suggesting that Gli1 functions as transcriptional activator in Shh signaling pathway. Gli3, in contrast, has been proposed to be a repressor of Shh, since loss of Gli3 in mouse Xt mutant results in the ectopic expression of Shh (Masuya et al., 1997). As for Gli2, it is more complicated. In mice, Gli2 functions as a transcriptional

activator, directly or indirectly, regulating the expression of Shh-responsive genes (Ding et al., 1998). The Gli2 mutant mice show the diminished Shh signaling and lack of floor plate differentiation. However, motor neurons can develop without Gli2 and in the absence of floor plate induction (Ding et al., 1998). In *Xenopus*, only Gli1 can induce the differentiation of floor plate cells and Gli2 and Gli3 repress the ectopic induction of floor plate cells by Gli1 and endogenous floor plate differentiation (Ruiz I Altaba, 1998). Gli2 seems to directly mediate induction of motor neurons by Shh (Ruiz I Altaba 1998). Although the role of Gli2 in neural differentiation is not completely understood, Gli2 definitely mediates Shh signal pathway.

In limb development, Gli proteins are also associated with Shh signal. All three Glis are expressed in the posterior region where Shh expression is restricted (Buscher and Ruther, 1998). The *Extra toes* (Xt) mouse, which possesses a null mutation of *Gli3*, exhibits craniofacial defects as well as a preaxial polydactyly (Johnson, 1967; Schimmang et al., 1992; Hui and Joyner, 1993). In this *Xt* mouse, Shh is expressed ectopically in the anterior region of the limb because the function of Gli3 is lost (Buscher et al., 1997). However, mouse Gli1 expression is anteriorly up-regulated adjacent to

the ectopic Shh expression domain and is consistent with an up-regulation of Gli1 by Shh in *Hx* mouse (Hemimelic-extra toes mutant mouse) limb buds and chicken wing buds in which Shh is ectopically expressed (Buscher and Ruther, 1998; Platt et al., 1997; Marigo et al., 1996). This indicated that Gli1 is an activator of the Shh signal to increase Shh target gene expression and Gli3 is a repressor of the Shh signal to restrict Shh in the posterior region of the limb (Buscher and Ruther, 1998). The relationship between Gli2 and Shh signal is not clear but Gli2 strongly associates with limb development because disruption of the *Gli2* gene leads to craniofacial and skeletal alterations, which reduced limb length (Mo et al., 1997).

In addition to the involvement of Shh-Gli in neural and limb development, Shh-Gli signaling affects organ development, and differentiation including gastrointestinal development, lung development, spermatogenesis, tooth development, and skin tumorigenesis (Yang et al., 1998; Grindley et al., 1997; Persengiev et al., 1997; Hardcastle et al., 1998; Dahmane et al., 1997). For example, in mouse lung development, three Glis are expressed in distinct but overlapping domains in pseudoglandular stage. Overexpression of Shh in lung results in increased levels of Patched and Gli1.

Although Gli3 is not up-regulated by the Shh signal, it is important for lung development because the *Gli3XtJ* embryo in which both alleles of Gli3 are deleted has a stereotypic pattern of abnormalities in lung morphogenesis (Grindley et al., 1997).

1.4 Research summary

Because *Xenopus* embryos have many advantages as an experimental model, I chose *Xenopus* embryos in order to investigate gene expression and regulation during *Xenopus* embryonic development and to reveal new function of the gene. Since many embryonic regulatory genes are involved in tumorigenesis, it is essential to investigate the functions of the Gli in embryogenesis in order to better understand tumorigenesis. Therefore, I chose to characterize the *Gli* gene during *Xenopus* embryonic development as my research project. The strategy I used was first to clone a conserved region of *XGli* gene by RT-PCR. Then, with 5′- RACE-PCR, the 5' end of *XGli* was cloned. To obtain the full length this *Xenopus Gli* gene, 3′ RACE-PCR was tried. A cloned *Xenopus Gli* fragment was used as a probe to do temporal and spatial expression work during the embryonic development. In order to investigate XGli protein expression, it is necessary to have a high titer Gli antibody. Polyclonal Gli antiserum was made by

injection of a synthetic peptide into rabbits and collection of Gli antiserum. Western blotting was then done to show Gli protein expression levels at different developmental stages. Immunostaining was necessary to show Gli protein global distribution in *Xenopus* embryos. It was known that the Gli protein functions as a putative transcription activator, so I attended to show that this XGli protein functions as a potential transcriptional activator by binding DNA in a sequence specific way to regulate downstream gene expression. To understand the function of XGli in early embryonic development and the relation between Gli and mesoderm inducers such as FGF, I investigated XGli expression in different FGF induced *Xenopus* embryonic animal explants.

Materials and Methods

2.1 Materials

All restriction endonucleases were purchased from Bethesda Research Laboratories (Gibco-BRL, Life technology Inc.), New England Biolabs Inc., Boehringer Mannheim Canada, Pharmacia Fine Chemical, and Promega.

T4 DNA ligase, Reverse transcriptase, Calf Intestinal Alkaline Phosphatase (CIAP) and Terminal Deoxnuclootidyl Transferase (TDT) were purchased from GIBCO-BRL.

Kodak XAR-5 X-ray film was obtain from Eastman Kodak Co.

The α-P32 dATP, α-p32 dCTP, γ-p32-ATP, UTP and S35 dATP were purchased from Dupont and Amersham.

The random labeling kit was from Gibco-BRL.

The Sequence Version 2.0 was supplied by United States Biochemical Corporation.

Cycle Sequence Kits were purchased from Promega and CDL.

The pBluescript KS clone vector was the product of Stratagene.

2.2 Methods

2.2.1 Embryo manipulation

Xenopus laevis, both adult males and females, were supplied by a commercial company (Xenopus Inc. Ann Arbour Mich.). Eggs were induced by *in vitro* fertilization and obtained using our lab's method (Ryan and Gillespie, 1994). Briefly, the adult female was injected human chorionic gonadotropin (HCG) and the mature eggs were manually stripped from the female frogs and fertilized with sperm that came from macerated male *Xenopus* testes. Then, 2.5% w/v cysteine hydrochloride pH 7.8-8.1 (Sigma) was used to remove the jelly coats of the eggs. The dejellied eggs were cultured in 1/20 NAM (Appendix) solution at room temperature until they developed to the required stage.

2.2.2 RNA extraction

2.2.2.1 Large scale preparation of RNA from *Xenopus laevis* embryos

The LiCl method was chosen to prepare total RNA from different developmental stages (Lichtenstein et al., 1975). When the embryos developed to the required stage, total RNA was extracted from about 200 embryos. These embryos were washed with DEPC treated dH2O (see abbreviation) and then transferred to a precooled

Table 1. *Xenopus* Embryonic developmental stages (Hubecht-Laboratorum, 1967)

Stage	Criteria of development	Time after fertilization at 22 °C
Stage 1	One cell stage, shortly after fertilization	0 hour
Stage 6	Advanced 32 cell stage	3 hours
Stage 8	Blastula stage	5 hours
Stage 11	Gastrula, house-shoe shaped blastopore stage	11.3/4 hours
Stage 16	Middle neural fold stage	14 hours
Stage 22	early organogenesis	24 hours
Stage 31	hatched tadpole, late organogenesis	36 hours
Stage 36	early tadpole	48 hours
Stage 41	late tadpole	72 hours

Dounce homogenizer with 5 ml of embryonic RNA extraction buffer (Appendix). Subsequently, the homogenizer was kept on ice and embryos were homogenized with 10 times strokes of the pestle. The whole extraction solution was sonicated 3 times to shear genomic DNA at 30% of maximum power for 30 seconds each time and left on ice overnight. Then, after the extraction solution was centrifuged at 12,000 g for 30 minutes, the RNA pellet was resuspended in 4 ml of 0.3 M NaOAc (pH5.2) with 0.5% SDS and extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture (24:24:1). The

aqueous layer was extracted with an equal volume of chloroform. Then, the aqueous layer was precipitated with 3X 100% ice cold ethanol and incubated at -20°C overnight. The RNA was recovered by centrifugation at 15,000 g for 30 minutes. The pellets were washed with 2 ml of 70% ethanol, dried, dissolved in 100 µl of DEPC dH2O, and stored in -70°C freezer.

2.2.2.2 Embryo explant total RNA extraction

This method can be used for a small amounts of total RNA extraction. Five animal explants of *Xenopus* embryo were solubilized in 200 µl NETS solution (Appendix) and an equal volume of phenol:chloroform:iso-amyl alcohol mixture (24:24:1). After centrifugation, the aqueous phase was removed and placed in a new tube and 1 ml 100% ice cool ethanol with 20 µl of 3 M sodium acetate pH 5.2 was added. Total RNA was recovered by centrifuging at 12,000 g for 15 minutes at 4°C. The total RNA pellet was resuspended in 50 µl of DNase buffer (Appendix) and incubated at 37°C 20 minutes. After digestion, the mixture was extracted with phenol twice and precipitated with 100% ethanol and 1/10 volume of 3 M NaoAC pH 5.2. After washing with 70% ethanol, RNA was dissolved in 50 µl DEPC dH2O and stored in -70°C freezer.

2.2.3 PCR cloning of the *Xenopus Gli* gene

2.2.3.1 RT-PCR to amplify conserved region of *Xgli* fragment

10μg of total RNA and 100 ng of random primer (Gibco-BRL) were first denatured at 70°C for 10 minutes and immediately transferred on ice. Then, 0.5 μg/ μl total RNA (with primer), 5 ng/μl random primer (Gibco-BRL), 10 mM DTT, 1 mM deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100 and 200 unit of MMLV (Moloney-murine leukemia virus) reverse transcriptase (Gibco-BRL) was added in total 20 μl reaction. The reaction tube was incubated at 37°C water bath for 120 minutes and then stopped by heating the reaction at 75°C for 10 minutes.

PCR (polymerase chain reaction) then was performed. In total 25 μl, in addition to 1 μl of cDNA template, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM MgCl2, 100 μg/ml gelatin, 200 μM deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.25 μM ZB2 and XF4 primers (Table 2), and 2 unit Taq polymerase (Promega) were added. The PCR

Table 2. The primers for 2.1 Kb of *XGli5*

Name of primer	position or usage	sequence (from 5' to 3')
ZB2	2062-2083 (R)*	-gtgcacagttttgacgtgttt-
XF4	1124-1143 (F)*	-ttcgacctgcagacaatgatc-
G-tail	For 5'-RACE (F)	-gagaattcgtcgacatcgag$(c)_{17}$-
R202-183	1306-1324 (R)	-caaatgaactcaggctgcg-
G-RACE	Adapter of G-tail (F)	-gagaattcgtcgacatcgag-
R1	1198-1218 (R)	-ccgtaggagccgctggcagc-
XGli F 375-396	375-396 (F)	-cctgtcccatagcagaagattc-
XGli R 946-966	946-966 (R)	-gtatgcaattgcatgacttgc-
3' -Tail	For 3' -RACE (R)	-gagaattcgtcgtcgacatcga$(t)_{17}$-
3'- RACE	Adapter of 3' Tail (R)	-gagaattcgtcgtcgacatcga-
Gli 421-5'	1543-1563 (F)	-agccctccggaccgacctgac-
XF1	5' end of human *Gli3* (F)	-tatccgtgctggtggtgctg(a/g)tg-
XF2	3' end of human *Gli3* (R)	-gtggagaactctattgtgaa(a/g)tg-

* R- Reverse direction; * F- Forward direction

cycle was as follows.

1 cycle	95°C 2 minutes
30 cycles	94°C 30 second
	55°C 1 minute
	72°C 1 minute and 30 seconds
1 cycle	72°C 15 minutes

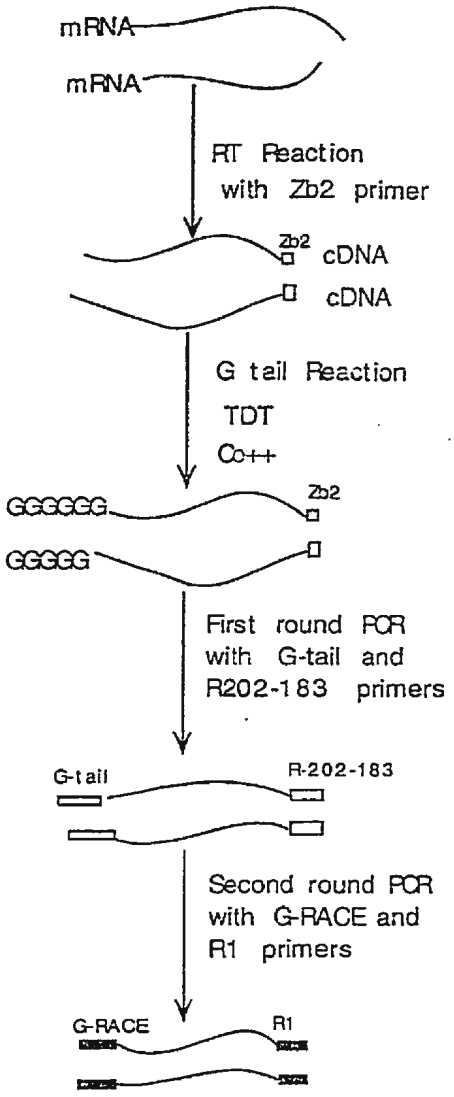
The PCR products were cloned in pBluescript II (KS+) vector and sequenced by USB sequenase Version 2.0. kit.

2.2.3.2 5' RACE-PCR

In order to clone the 5' end of this fragment, I performed 5' RACE (Rapid Amplification cDNA End)- PCR. Initially, the cDNA was prepared with 10 µg RNA. The reverse transcription was performed as above, except that instead of using random primer I used the gene specific primer, ZB2. I developed a two step amplification method to do RACE-PCR (Fig. 2.1). First, TDT (see abbreviation) was used to add dGTP at the 3'-OH end of cDNA. Briefly, in 25 µl of reaction volume, in addition to 5 µl of the first strand cDNA as template, final concentration of 4 µm dGTP, 100 mM potassium cacodylate, pH 7.2, 1 mM CoCl2, 0.2 mM DTT, and 20 unit TDT (Gibco-RRL) was added. The reaction was performed in 37°C waterbath for 30 minutes. According to Gibco-BRL TDT protocol, a range of 15-35 G nucleotide were added to the 3í-OH end of the cDNA pool. Secondly, the first step of PCR reaction was performed with one pair of primers; G-Tail and R202-183 (Table 2). DNA polymerases for first step PCR reaction were the combination DNA polymerases: *pfu* exo+ (with exonuclease activity, Stratagene) and Deep vent exo- (without exonuclease activity, New England Lab) with the ratio of Deep vent:pfu (1:1/100). PCR cycles are as follows.

program Cycle	95°C	35 second
	54°C	1 minute

Fig. 2.1. Procedure for 5' RACE PCR
Zb2, R202-183, and R1 are gene specific primers. R202-183 is the nest primer of Zb2. R1 is the nest primer of R202-183. G-tail and G-Race are the universal primers. G-Race is the nest primer of G-tail.



72°C 2 minutes
35 cycles and 72°C 15 minutes

10 µl of PCR product was loaded on a 1% agarose gel for 30 minute electrophoresis at 5 volts/cm. The gel was stained with 0.5 % ethidium bromide and observed under UV light. In this first step PCR, I obtained PCR products which ranged from 300 bp to 2 kb and appeared as a smear. The remained PCR product was diluted 1 to 1000-10,000. 1 µl of diluted PCR product was used as the template for a second round of nest PCR. The primers for second PCR amplification are G-RACE and R1 (Table 2). The components of PCR reaction were the same as RT-PCR which was described above excepts primers. The second PCR program was as follows.

program cycle	95°C	45 seconds
	58°C	1 minutes
	72°C	1.5 minutes

35 cycles and 72°C 15 minutes.

2.2.3.3 3' RACE-PCR

10 µg of RNA was used to synthesize cDNA with 3'-Tail primer (Table 2). 1 µl of 20 µl cDNA was used as template to perform long RT-PCR. The primers were the 3'-RACE primer (adapter of 3'-Tail) and gene specific primer, *Gli* 421-5'. In order to obtain long and high fidelity 3' end, combination DNA polymerase (0.02 unit of Vent exo+ and 2

unit of Vent exo-) was used in 50 µl of PCR reaction. The programmed cycles are as follows.

program cycles	94°C	30 Seconds
	55°C	1 minute
	72°C	5 minutes
35 cycles and	72°C for	15 minutes

PCR products were observed under UV light after 1% agarose electrophoresis. In order to identify the PCR band belongs to the *XGLi*. By using the first PCR product as template, I performed the second PCR with Zb2 and *Gli* 421-5 nest primers.

2.2.3.4 *XGli* 3' end PCR amplification from cDNA libraries

To obtain 3' end, *Xenopus* stage 1 and 8 cDNA libraries were used as a template to perform PCR. With library primer T7 and *Gli* gene specific primer, *Gli* 421-5, PCR cycles were as follows.

35 cycles	95°C	30 second
	55°C	1 minutes
	72°C	5 minutes
1 cycle	72°C for	15 minutes

PCR products were observed under UV light after 1% agarose electrophoresis.

2.2.3.5 LA (long and accurate) PCR to amplify full length of this *Xenopus Gli*

Because northern hybridization analysis has revealed that this Gli cDNA is longer than 8 Kb, LA-PCR was used in order to obtain full length of this *Gli* cDNA. With conserved region primers, XF1 and XF2 (Table 2) which were designed according to the human *Gli* 3' and 5' end conserved regions and cDNA synthesized by oligo dT, PCR was performed with different combination DNA polymerases: Vent exo- /Vent exo+, pfu exo-/Vent exo+ and *Klentaq*/Vent exo+. The hot start antibody was also used in LA-PCR as the Clontech protocol described (Clontech catalogue, 1995). PCR cycles were as follows.

1 cycle	95 °C	3 minutes
25 cycles	95 °C	1 minute
	55 °C	1 minutes
	72 °C	10 minutes
1 cycle	72 °C	15 minutes

The PCR product was electrophoresed on 1% agarose gel and observed under UV light.

2.2.4 PCR product purification and electrophoresis

2.2.4.1 gel electrophoresis

To separate different lengths of PCR products or DNA fragments, 0.8%- 2% agarose gel is used to separate DNA fragments larger than 200 bp. If the fragment is smaller than 200 bp, 5% acrlymaide is adopted.

2.2.4.2 PCR product purification

PCR product was electrophoresed on a 0.8% agarose gel and after staining with 0.5 mg/ml ethidium bromide, the PCR amplified DNA band was cut out under low intensity of UV light. The slice of gel that contained the DNA band was placed in a microspin tube (Millipore) or the DNA purification tube which was made in our laboratory. Our microspin tube includes two tubes, an inner small one and an outer big one. The bottom of the inner small tube has a filter which can let DNA pass through but not gel. After the tube was spun in a microcentrifuge at 8000 rpm for 20 minutes, the eluted liquid at the bottom of the big tube was collected and precipitated with 2.5 volume of ethanol. The DNA pellet was then dissolved in a certain amount of dH2O or TE buffer (Appendix). The DNA concentration was determined by absorbance of UV light at 260nm using the spectrophotometer (Beckman-LKB).

2.2.5 Subcloning of DNA fragments and PCR products

2.2.5.1 DNA fragment subcloning

To subclone the enzyme digested DNA fragment, the Bluescript II KS vector (Stratagene) was used to prepare sticky and blunt end ligations. For the sticky end ligation, the linearized vector was treated with CIAP (see abbreviation). Specifically, 20 µl reaction included

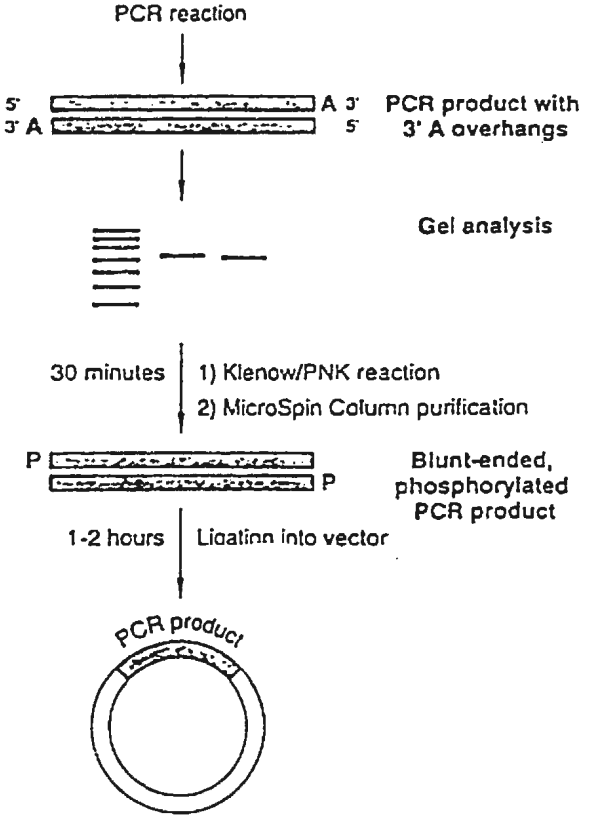
5 µg linearized vector, 1 unit CIAP, 10 mM Tris-HCl pH 8.5, and 0.1 mM EDTA. The mixture was incubated at 37°C 30 minutes and then heated 75°C for 15 minutes to inactivate the CIAP. After phenol/chloroform extraction and ethanol precipitation, the vector was ready to be used for ligation. Total 10 µl ligation included 100 ng vector, 100 ng DNA insert, 1 mM ATP, 50 mM Tris-HCl pH 7.6, 10 mM MgCl2, 1 mM DTT, 5% PEG-8000 (w/v), and 1 unit of T4 DNA ligase (Gibco-BRL). The reaction was incubated at 14°C for 16 hours. After transformation, bacteria colonies were found on agar plates and the colonies with insert screened by PCR with T3 and T7 primers on polylinker of the vector.

For blunt end ligation, the only difference from the sticky end ligation is that the vector and DNA fragment require Klenow and T4 polynucleotide kinase (PNK) enzyme treatment to fill the overhangs. After DNA fragment or vector digestion with restriction enzyme, 10 unit of Klenow was added in 20 µl reaction volume which included 4 µg DNA fragment or vector, 50 mM Tris-HCl pH 8.0, 10 mM MgCl2, and 50 mM NaCl and incubated at room temperature for 20 minutes. Klenow was then heat inactivated by subjecting it to 75°C for 10 minutes.

2.2.5.2 PCR product cloning

Compared to restriction enzyme digested DNA fragments, PCR products are more difficult to clone because most of PCR products have 3' A overhang which is hard to clone into the blunt end vector. I modified Pharmica PCR cloning method (Pharmica, 1994)(Fig. 2.2). In detail, KS bluescript vector was digested by EcoRV to linearize the plasmid with a blunt end and treated with CIAP to remove the 5í terminal phosphate groups. Then, 1 µl of 0.5 mM ATP, 1 µl of 5 unit of Klenow, and 1 µl of 10 unit of T4 polynucleotide kinase were directly added in the 25 µl PCR products and incubated at room temperature for 30 minutes. The treated PCR products were electrophoresed on a 0.8% agarose gel. The DNA band was purified from the gel by microspin column (Millipore). The ligation mix included 50 ng treated vector, 100 ng of microspin purified PCR products, 50 mM Tris-HCl pH 7.6, 10 mM MgCl2, 1 mM ATP, 1 mM DTT, 5% PEG-8000, and 2 unit T4 ligase (Gibco-BRL). The reaction was carried out at 14°C for 16 hours. After transformation, the colonies were screened by PCR. An alternative method that I used to clone the PCR product was the Invitrogen Kit, having a special 3' end T overhang in the vector, TA-clone vector. Since Taq DNA polymerase usually adds

Fig.2.2. PCR products cloning procedure (Adopted from Pharmica catalog,1994)



one A at 3' end of PCR products, they are easily cloned in a TA-clone vector which has a complementary T overhang (Invitrogen, 1995).

2.2.6 Plasmid preparation and DNA sequencing

2.2.6.1 plasmid preparation

The alkali lysis method (Sambrook et al., 1989) was performed to prepare plasmids in 500 ml of bacteria culture. The crude plasmids were purified with CsCl gradient ultracentrifugation (Sambrook et al., 1989).

2.2.6.2 DNA sequencing

After pure recombinant plasmids were obtained, the insert DNA fragment sequencing was done by UBI Sequenase version 2.0 kit or Cedarlane Laboratories Cycle Sequencing kit as described in the manufacturer's instructions.

2.2.7 Northern Hybridization

30 µg of the total RNA samples (stages 1, 6, 8, 11, 16, 22, 31, and 41) and RNA marker(Gibco-BRL) were prepared for Northern hybridization. RNA electrophoresis was performed in 1.2 % formaldehyde denatured agarose gel for 3-4 hours at 120 Volts (Ausubel et al., 1994).

The capillary method was adopted to transfer gel to nylon membrane (Amersham) (Ausubel et al., 1994). The nylon membrane was then dried in the air and the RNA was fixed by baking it at 80°C for 2 hours or UV crosslinked using a Stratagene Crosslinker operating at 1120 engs/cm. Before performing hybridization, the transfer efficiency was monitored using a standard RNA marker which was on the same membrane according to Molecular Cloning(Sambrook et al., 1989). The membrane bearing RNA was prehybridized with hybridization buffer (Appendix) at 60°C for 2 hours. Then, old buffer was removed and fresh hybridization buffer was added to the hybridization bag. XGli-1Kb fragment was cut out of off the plasmid and used to prepare DNA probe by random labeling system according to the manufacturer's instructions (Gibco-BRL). As for hybridization, 25 ng DNA probe with $2X10^8$ cpm/μg specific activity was denatured by heating at 95°C for 5 minutes and immediately added to the hybridization buffer and incubated for 20 hours at 60°C. Then, the blot was washed twice with 1XSSC, 0.1% SDS at room temperature for 15 minutes each; once with 0.5XSSC, 0.1% SDS at 55°C; once with 0.1XSSC and 0.5% SDS at 55°C for 15 minutes. After washing, the blot was exposed on X-ray film at -70°C for overnight to two days with intensifying screens. The mRNA expression level was measured by a densitometer, GelScan

XL 2.1 using auto/adiograms within the liner range of exposure. The XGli-1Kb probed blot was stripped off by 100°C 0.5% SDS for 10 minutes and reprobed with *Xenopus* Histone 4 probe which was prepared by the Histone 4 plasmid directly radiolabeling with random primer (the histone plasmid was received from Dr. Ken Kao).

2.2.8 GST Gli5 fusion protein expression and purification

2.2.8.1 GST+Gli5 recombinant vector construction

The pGEX.KT vector (Pharmacia) was used to express XGli-1Kb fragment containing the five zinc finger motifs. In order to maintain the reading frame of this fragment, 10 µg of pGEX.KT vector was digested by EcoRI and treated with Klenow to fill in the overhang. Dephosphorylation of the 5' phosphate of the vector was done by adding 1 unit of CIAP at 37°C 30 minutes. 10 µg of XGli-1Kb fragment in pBluescript (KS+) was digested with SmaI and HindIII and filled in 3′ end with Klenow. Blunt end ligation was performed by adding 2:1 molar ratio of fragment and vector at 14°C for 20 hours. Afterwards, the blunt end ligation reaction mixture was used to transform competent bacteria cell, JM109 by the heat shock method (Sambrook et al., 1989).

The recombinant colonies were selected by PCR with primer on the pGEX.KT vector and gene specific primer. The orientation and reading frame of recombinant colonies were determined by sequencing the 5' vector polylinker flanking region and the inserted XGli-1Kb using the UBI sequenase Version 2.0 kit.

2.2.8.2 Induction of GST+Gli5 fusion protein expression

Correct clone from above was isolated and grown in 10 ml of 2XYT-G medium (Appendix) with 100 µg/ml ampicillin at 37°C for 12-15 hours with agitation. The culture medium was diluted 1:10 with LB medium and incubation with shaking at 37°C until OD_{600nm} reached 1-2. 1 mM of the final concentration of IPTG was added to the cultures to induce synthesis of the GST/XGli-1Kb fusion protein. Cultures were incubated for an additional 4 hours at 37°C. The cells were collected by centrifugation 2000g for 10 minutes at 4°C. The pellets were then washed with 5 ml of 1XPBS (Appendix) and resuspended in 500 µl of ice cold 1XPBS with 1 mM PMSF. The suspension was sonicated on ice for 15 seconds at 30% maximum power and a final concentration of 1% Triton X-100 was added and gently mixed. Then, the suspension solution was centrifuged at 10,000 g for 10 minutes. After the Triton X-100 soluble

supernatant was transferred to a fresh tube, the insolubilized cell pellet was treated with the following procedure to increase solubilization.

a). The pellet was dissolved in MT-PBS buffer (150 mM NaCl, 16 mM Na2HPO4, and 4 mM NaH_2PO_4 pH7.3).

b). Centrifuged at 7000 g for 10 minutes and the pellet resuspended in denaturation buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 8 mM DTT, 6 M Urea).

c). After vigorous vortexing and recentrifugation, 200 ul of supernatant was dialyzed in 1 litre of reconstitution buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM DTT, and 20% glycerol) overnight at 4°C.

2.2.8.3 Purification of GST+Gli5 fusion Protein and SDS-PAGE

1.33 ml of glutathione sepharose 4B (Pharmcia) was used to purify fusion protein. The glutathione sepharose 4B was washed with 1XPBS three times, and 1 ml of 1XPBS was added to make 50% of slurry of glutathione sepharose 4B. About 20 µl of each fusion protein sample was added to 20 µl of 50% slurry glutathione sepharose 4B and mixed for 5 minutes. 100 µl of 1XPBS was then used to wash the glutathione sepharose 4B three times. After 1XPBS was removed, 10 µl of glutathione elution buffer (10mM reduced glutathione in 50 mM Tris-HCl pH 8.0) was added and incubated at room temperature for 5 minutes. The tube

was centrifuged 1000g for 5 minutes to sediment the sepharose beads and the supernatants were transferred to a fresh tube for SDS-PAGE.

2 µl of 6XSDS loading buffer(300mM Tris.Cl, 12% SDS, 600mM DTT, 0.6% bromophenol blue, and 60% glycerol) (Sambrook et al., 1989) and 10 µl of the fusion protein sample was boiled for 5 minutes before loading on the gel. Proteins were electrophoresed on a 12% SDS-PAGE gel (Sambrook et al., 1989). The gel was stained with 0.25% Commassie blue (0.25 g Commassie blue R250 in 90 ml of methnanol:H_2O (1:1) and 10 ml of glacial acetic acid) and destained with destaining solution (200 ml methanol, 60 ml of glacid acetic acid, and 740 ml of dH2O) (Sambrook et al., 1989).

2.2.9 Gel Mobility Shift

To investigate if this XGli zinc finger region also bind the same DNA sequence as other Glis, the following sequence primers were used to do gel mobility shift assays.

Forward primer	5'-TAGACCACCCAGG-3'
Reverse primer	5'- CCTGGGTGGTCTA-3'

The forward and reverse primers were diluted to 200ng/µl from 2µg/µl. The two primers were annealed by heating 65°C and slow cooling to 37°C for 10 minutes in

primer annealing buffer (Appendix). The primers were radiolabeled with T4 Polynucleotide Kinase (GIBCO-BRL) and γ-^{32}P-ATP (4500Ci/mmol)(Amersham) as described in Molecular Cloning (Sambrook et al., 1989). 15 µg of fusion protein was incubated with binding mixture (10,000cpm DNA probe, 4 ug poly(dI-dC) (Pharmacia), 300 µg/ml BSA, 1X binding buffer which included 12% glycerol, 12 mM Hepes (pH 7.9), 4 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, and 1 mM DTT) for 30 minutes at 32°C. A series of increasing concentrations of unlabelled annealled oligo nucleotide, 50 ng, 100 ng, and 500 ng were added to the binding reaction to compete with radiolabelled probe as a specific binding control. In order to show the protein specific binding, 1µl of affinity purified XGli polyclonal antibody (see preparation below) was added to the binding reaction either before or after the fusion protein was added. The protein-DNA binding complexes were electrophoresed on a 4% low ionic strength, nondenaturing polyacrylamide gel(Ausubel et al., 1994). Before loading the samples, this polyacrylamide gel was prerun at 4°C for 1 hour at 100 volts. Then samples were loaded and run at 150 volts for 2-3 hours. When running was complete, the gel was dried and exposed to X-ray film with intensifying screens.

2.2.10 Synthetic peptide and polyclonal antibody preparation, purification, and characterization

2.2.10.1 Synthetic peptide

The synthetic peptide was chosen in the region that is just outside of zinc finger conservated region of XGli and has 17 amino acids: cys-arg-asn-lys-val-lys-thr-glu-glu-glu-val-leu-gln-arg-asp-arg-ser. This peptide was synthesized by Research Genetics Incorporation (Huntsville, AL USA).

2.2.10.2 Immunization of Rabbits

New Zealand white rabbits (female) were chosen for preparation of polyclonal antibody. Before injection of the synthetic peptide, a blood sample was taken from each rabbit for preimmune serum. The synthetic peptide was conjugated with keyhole limpet hemocyanin(PIERCE). Then, 200 ug of the peptide conjugate was injected following the schedule below. After the clot removed from the blood, the collected antiserum was kept in -20 °C freezer.

2.2.10.3 Purification of anti-XGli5 serum

The affinity purification method was chosen to purify the Gli antibody. First, Gli synthetic peptide was used

Table 3. Schedule of Rabbit immunization

Days	Working events
0	preimmune serum bleeding
2	IM injection
16	IM injection
26	Subcutaneous injection
36	subcutaneous injection
46	Blood collection
50	Blood collection

to make an affinity column. The Gli synthetic peptide has a terminal cysteine residue which can be used for conjugation to the Sulfolink coupling gel (PIERCE). 3 mg of Gli peptide was used to make the affinity column based on the protocol from PIERCE.

The Gli anti serum was purified by passing through the Gli affinity column(PIERCE). According to PIERCE's protocol, after 3 ml of Gli antiserum bind the column, 8 ml of elution buffer (PIERCE) is added and collected in 8 tubes. Tubes 3, 4, 5, containing the antibodies, were collected and the samples were concentrated using a Centricon 30 (Amicon Inc.). The final concentration of

the pure Gli polyclonal antibody was determined by OD at 280nm.

2.2.10.4 Characterization of XGli5 polyclonal antibody

In addition to ELISA measurements (Ausubel et al., 1994) the XGli fusion protein was used in combination with Western blotting to evaluate the affinity and specificity of the polyclonal antibody. A dilution series of XGli fusion protein (5 ug, 0.5 ug, 0.05 ug, and 0.005 ug) were electrophoresed on a 12% polyacrylamide gel and transferred onto PVDF membranes (Millipore) to perform a western blotting procedure according to Molecular Cloning (Sambrook et al., 1989). The membrane was incubated in 1:300 diluted XGli antibody and preimmune antiserum overnight and then in 1: 3000 diluted secondary antibody (Sheep anti rabbit IgG conjugated to alkaline phosphatase) (Gibco-BRL) for one hour. Alkaline phosphatase was detected with the NTB and BCIP reagent (Gibco-BRL) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl, and 0.1% Tween).

2.2.11 Protein expression in the *Xenopus laevis* embryo

2.2.11.1 *Xenopus* embryo protein extraction

About 100 embryos from different developmental stages were homogenized with a precooled Dounce homogenizer in 5 ml of Tris buffer (pH6.7) with 0.4 mM PMSF. An equal

volume of Freon (1,1,2- trichlorotrifluoroethane) (Sigma) was added to this homogenized solution and vortexed. The mixture was spun at 10,000 rpm in microcentrifuge for 10 minutes. The aqueous layer was transferred to a clean tube and 5 volumes of ice cold acetone were added to precipitate the protein. It was then dissolved in 200 µl of dH2O and stored at -70°C.

2.2.11.2 Competitive western blotting

About 50 µg of proteins from different developmental stages was collected as determined by Bio-Rad protein assay kit(Bio-Rad). Each sample was loaded twice and electrophoresed on an 8% SDS-PAGE gel. After electrophoretic transferring, the PVDF membrane which bears duplicate samples was cut into two pieces. One blot was incubated with purified Gli polyclonal antibody (1:300 dilution) overnight and the 1:3000 diluted second antibody (sheep anti rabbit IgG conjugated to alkaline phosphatase) for one and half hour. The color staining was performed as described below. The other identical blot was treated with same concentration of Gli antibody (1:300) as well as 100 µg/ml XGli fusion protein which functions as the competitor of XGli protein by binding Gli antibody. After overnight incubation, the membrane was incubated with the 1:3000 dilution second antibody

(sheep anti rabbit IgG conjugated to alkaline phosphatase) for 1.5 hours and then stained with 33 µl of BCIP and 66 µl of NTB in 10 ml of alkaline phosphatase buffer.

Results

3.1 cDNA Cloning *Xenopus Gli* fragment

3.1.1 cDNA Cloning 5' end of *XGli* fragment

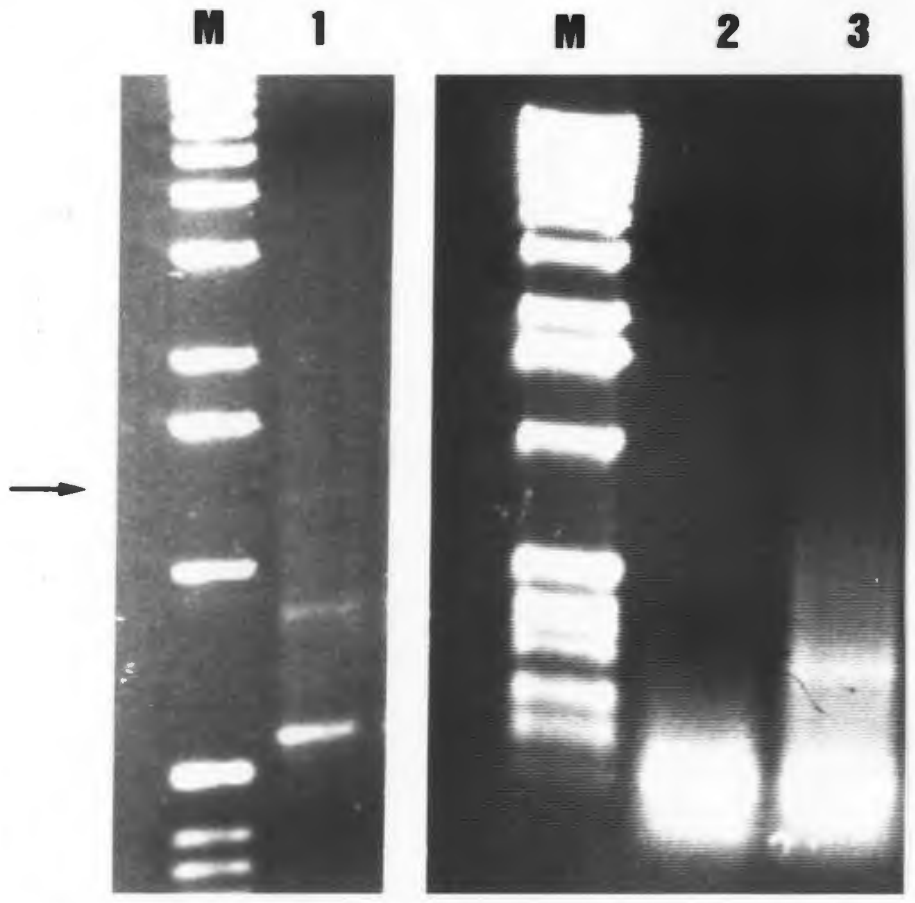
3.1.1.1 RT-PCR to clone a conserved region of *XGli* cDNA

The RT-PCR primers, XF4 and Zb2 were designed according to the conserved regions of human *Gli3* cDNA. XF4 is located in the middle of region I and Zb2 is located in the C-terminus of the zinc finger motifs (Table 2). *Xenopus* stage 11 embryonic cDNA was amplified by XF4 and Zb2 primers. One 960 bp PCR product was obtained, isolated, and cloned in the pBluescript II KS vector. The nucleotide sequence of this fragment showed high similarity to the human Gli family. Because this fragment is about 1 Kb (960bp), it was called the *XGli*-1kb.

3.1.1.2 5' RACE -PCR

In order to isolate the complete coding region of this *Xenopus Gli* cDNA, Rapid Amplification 5' or 3' cDNA End (5' or 3' RACE) was performed. I first tried several RACE-PCR methods and did not obtain a clear amplified band (Frohman *et al.*, 1990; Liu *et al.*, 1993).

Fig.3.1. Two step amplification of 5' RACE PCR lane 2, 3 shows the first step amplification,2: PCR with Taq enzyme; 3:PCR with combination enzyme (pfu exo+/vent exo-).lane 1 represents the second step amplification. Arrowhead shows the 1.2Kb amplified band.M:DNA molecular marker.



I then modified these 5' RACE methods and created a two round PCR amplification method. With this two round PCR amplification, three bands which were about 500 bp, 600 bp, and 1,200 bp were obtained (Fig.3.1). All of them were cloned in pBluescript II KS vector. After I sequenced these three clones, they have identical sequence at the 3' end. Analysis of the longest 1.2 Kb fragment sequence revealed that there was a 90 bp overlapping sequence between the newly amplified 1.2 Kb and *XGli*-1kb fragment (Fig.3.2). Furthermore, there was a Kozak consensus sequence near the 5' end of the 1.2 Kb fragment (Kozak, 1986). Therefore, this total 2Kb fragment is 5′ part of this *XGli* cDNA.

3.1.2 Cloning 3' end of *XGli* fragment

3.1.2.1 3' RACE- PCR

Using the following primers: 3' RACE (adapter of 3' Tail) and Gli 421 (Table 2), multiple faint PCR amplified bands from 1 kb to 6 Kb were obtained. After the second PCR was performed with internal primers: Zb2 and Gli 421-5'(Table 2), no any specific PCR amplified bands were found.

3.1.2.2 3' PCR amplification from cDNA libraries

Two libraries from stage 1 and 8 cDNA of *Xenopus* embryos were used as templates for amplification of the

3' end of *XGli*. But, no specific amplified DNA bands were observed.

3.1.3 Amplification of full length of *XGli5* cDNA with LA-PCR

According to the human *Gli* cDNA sequence, two 5' and 3' end primers, XF1 and XF2 were designed. LA-PCR (long and accurate PCR)(Barnes, 1994) was performed to obtain full length cDNA corresponding to my cloned *XGli* fragemnt. I tried three kinds of thermal stable enzyme combination for the LA-PCR. The combinations of Vent^{exo+}/Vent^{exo-} and vent^{exo+}/pfu^{exo-} were unsuccessful in obtaining any DNA amplification. However, with *KlenTaq*/vent^{exo+}, one 13 Kb fragment was amplified, but this fragment did not produce the expected size DNA band with internal *Gli* specific primers in a second round of PCR.

Above all, I successfully cloned about 2.1 Kb of *XGli* 5′ end but was unable to clone the 3′ end of this *XGli*. Because many important studies can be done by using this 5′of *Xgli* fragment. I focused the rest of my work on the 5′ end of this *XGli*.

3.2 Comparison of XGli5 fragment with other Glis

From the predicted amino acid sequence (Fig. 3.2), this 2.1 Kb cDNA, which encoded 596 amino acids, had five zinc finger regions which are very similar to the zinc finger regions of Gli proteins (Fig. 3.3). In addition to the zinc finger regions, the 2.1 Kb cDNA includes other

conserved regions such as region 1 and GF region (gain of function region)(Bono et al., 1995).

Compared with known Xenopus Gli1, Gli3, Gli4, this XGli is not identical to any of the three known XGlis. The amino acid identity between this XGli and XGli1, 3, and 4 are 34.5%, 60.1%, and 60.5% respectively. Since it does not belong to XGli1, XGli3, and XGli4, I suspect it may be XGli2. I compared the amino acid sequence between this XGli and human Gli2. It did not show significant homology (Fig. 3.3). Therefore, I believe that this Gli fragment is a new member of Gli family in Xenopus. I call this fragment XGli5 in order to distinguish XGli1, XGli3, and XGli4.

All members of the Gli family encode a highly conserved stretch of amino acids which are predicted to form a tertiary structure known as zinc fingers. The structure of XGli5 predicts the formation of the 5 zinc fingers in that region (Fig. 3.3). When zinc finger motifs of different Gli proteins are compared, although they can bind the same DNA sequence there are a few amino acid differences in the zinc finger region. The amino acid identities between XGli5 and Chicken Gli2/4, XGli4, XGli3, XGli1, human Gli3, Gli2, Gli1 and mouse Gli3 are 92%, 97%, 95%, 91.3%, 94%, 92%, 87.3%, and 94% respectively.(Fig. 3.3). As for other invertebrate Gli

Fig. 3.2 Nucleotide sequence and deduced amino acid sequence of the *Xenopus Gli5* cDNA 5' end. The sequence from nucleotide number 1122 to 2082, called *XGli*-1kb was cloned by degenerate RT-PCR with XF4 and Zb2. The sequence from nucleotide number 1 to number 1219 was cloned by 5' RACE PCR. The overlap region is from nucleotide number 1219 to 1122. The bold C and H are the Cys and His in C2H2 structure of each zinc finger. The arrows show the conserved region 1 between nucleotide number 1047-1256. The GF region is between nucleotide number 605 to 681

```
1       CCCGGGGGGATACACTAGTTCTAGAGCGACCGCCCCCCCCCCCCCGGACCATCTCACTCCA
61      CTCAGCGAGCGAGGAGACHAAAGGGGACAGGGAATCCAAAACTTTTTTTCCAACTGCTCC
121     CCGTCACCCTCTGCGCCCGGAAACTCCGGACTGTGCGGCCCAGGAACCTCTGACTCTGCG
181     AACCCTAAACTCCGGAGTGTGCAACTGGCACTTCTCACCATGGGCCCCGAAAGTGATTCA
241     GACCCCTTAGAGATGGATCTATAAAGTCAGTGTAAAACAGGAGAGGACCCCGTTATGGAG
                                                              M  E
301     CACCCGAAAACTCTTGCTGAACTGCTCCATCAATTGCTCCAGGGTATCCGACCCCATCCC
         H  P  K  T  L  A  E  L  L  H  Q  L  L  Q  G  I  R  P  H  P

361     GCTGATGGGAGCTTCCTGTCCCATAGCAGAAGATTCTCCACTCCGGAATATGAGTCAGTG
         A  D  W  S  F  L  S  H  S  R  R  F  S  T  P  E  Y  E  S  V

421     ACATGGAGAATTCTGGACCAATCAGGACTGCGCACAAGAAGGAAGCCAAGAAGTTCCATT
         T  W  R  I  L  D  Q  S  G  L  R  T  R  R  K  P  R  S  S  I

481     CTGGTTGGAAATGGATTCCCAGATCCTGGAAAGAAATTGGGAGGACTGAGCAATTCACAA
         L  V  G  N  G  F  P  D  P  G  K  K  L  G  G  L  S  N  S  Q

541     GGAGCTTCTGCTCCTCTCTTCCCTGCCTTCCACTCTCCCTTCCCCATTGAAATGCGCCAC
         G  A  S  A  P  L  F  P  A  F  H  S  P  F  P  I  E  M  R  H

601     CAAGAGGGAAGATACCATTATGACACACATGGCATTCACACTCTCCATGGCCCCCGACTC
         Q  E  G  R  Y  H  Y  D  T  H  G  I  H  T  L  H  G  P  R  L
         GF region →
661     CTCAGTGGCAGCCCTGTGATTTCAGACATTTCTTTAATCCGCCTCTCCCCACACTCTCTG
         L  S  G  S  P  V  I  S  D  I  S  L  I  R  L  S  P  H  S  L
               GF region  ←
721     GGTACAACAGAGTTTGGTCATGCCCATCATTATGGCAGCTCCCACATGGAGCATTACCTC
         G  T  T  E  F  G  H  A  H  H  Y  G  S  S  H  M  E  H  Y  L

781     CGTTCTGTGCACAACAGCCCCACCCTGTCTAGTATCTCTGCAGCCCGTGGGCTCAGCCCA
         R  S  V  H  N  S  P  T  L  S  M  I  S  A  A  R  G  L  S  P

841     GGCAGAAGTGGCCATGAGCACTTAAGAGAAAGGGGTATCTATGGTTTAGCACCTCCGCCC
         G  R  S  G  H  E  H  L  R  E  R  G  I  Y  G  L  A  P  P  P

901     CCTGGTACCACCCCTCCAGAATACTGCCACCAAATGGCCTTCCTGGCAAGTCATGCAATT
         P  G  T  T  P  P  E  Y  C  H  Q  M  A  F  L  A  S  H  A  S

961     GCATACGGGGAGCTCCTGGTGCAGAGCGCAGCAGCTGGAAATACGTCGCACCTTCACGAT
         A  Y  G  E  L  L  V  Q  S  A  A  A  G  N  T  S  H  L  H  D
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1021    TACCTCACCCCCATGGATGTATCAAGATTCTCCAGCCCCCGAGTTACACCGAGATTAAGC
         Y  L  T  P  M  D  V  S  R  F  S  S  P  R  V  T  P  R  L  S
                                  → Region 1
1081    CGGAAACGGGCCCTTTCCATCTCTCCTCTGTCCGATGCCAGCATTGACCTGCAGACCATG
         R  K  R  A  L  S  I  S  P  L  S  D  A  S  I  D  L  Q  T  M

1141    ATCCGCACCTCCCCCAACTCGCTGGTTGCTTACATCAACAACTCTCGGAGCAGCTCAGCT
         I  R  T  S  P  N  S  L  V  A  Y  I  N  N  S  R  S  S  S  A

1201    GCCAGCGGCTCCTACGGGCACCTTTCTGCAGGCGCAATAAGCCCAGCGTTTAGTTTCCCC
         A  S  G  S  Y  G  H  L  S  A  G  A  I  S  P  A  F  S  F  P
                                                    Region 1 ←
1261    CATCCCATAAACCCTGTAGCCTATCAGCAGCTCCTGAGCCAGCAGCGCAGCCTGAGTTCA
         H  P  I  N  P  V  A  Y  Q  Q  L  L  S  Q  Q  R  S  L  S  S

1321    TTTGGACACACCCCTTTGCTCCATCCGTCGCCGACGTTTGCATCCCGCCAACAGGGATTA
         F  G  H  T  P  L  L  H  P  S  P  Y  F  A  S  R  Q  Q  G  V

1381    CTCACATCCGCCAATCCAGCTCCTCCCAGCAACAACAACAACCCTGACTCCATCCTGAAC
         L  T  S  A  N  P  A  P  P  S  N  N  N  N  P  D  S  I  L  N

1441    AAAGTGAGCAGTGAGTCTACGGTGAGCAGTACAGTCAATCAGGTGATACACAAGCGCAAC
         K  V  S  S  E  S  Y  V  S  S  Y  V  N  Q  V  I  H  K  R  N

1501    AAAGTGAAGACCGAAGAAGAGGTCCTTCAGCGCGACTCCCCCAGCCCTCCGGACCGACCT
         K  V  K  Y  E  E  E  V  L  Q  R  D  S  P  S  P  P  D  R  P

1561    GACCACCTGAAGGAGGATTTGGATAAGGACGAGTGCAAACAGGAGCCCGACATTATATAC
         D  H  L  K  E  D  L  D  K  D  E  C  K  Q  E  P  D  I  I  Y

1621    GAGACCAACTGCCACTGGGACGGCTGCAGCAAGGAGTTTGATACCCAGGATCAGCTCGTA
         E  Y  N  C  H  W  D  G  C  S  K  E  F  D  T  Q  D  Q  L  V

1681    CATCACATCAATAATGACCATATCCATGGGGAGAAAAAGGAGTTTGTGTGTCGCTGGCAA
         H  H  I  N  N  D  H  I  H  G  E  K  K  E  F  V  C  R  W  Q
         2
1741    GAATGCTCCAGGGAGCAGAAACCATTCAAGGCACAGTACATGCTGGTGGTTCATATGAGA
         E  C  S  R  E  Q  K  P  F  K  A  Q  Y  M  L  V  V  H  M  R

1801    AGGCATACGGGGGAGAAACCACATAAATGCACCTTTGAAGGTTGTTTCAAGGCCTATTCC
         R  H  T  G  E  K  P  H  K  C  T  F  E  G  C  F  K  A  Y  S
         3
1861    AGGCTGGAGAATCTGAAGACACAGTTGCGTTCACACACCGGGGAGAAGTCGTACGTCTGT
         R  L  E  N  L  K  T  H  L  R  S  H  T  G  E  K  S  Y  V  C
         4
1921    GAGCACGAGGGCTGCAATAAGGCATTTTCCAATGCATCTGATCGAGCCAAACATCAGAAC
         E  H  E  G  C  N  K  A  F  S  N  A  S  D  R  A  K  H  E  N

1981    AGAACCCATTCCAATGAGAAACCTTACATCTGTAAGATCCCAGGGTGCACAAAGCGTTAC
         R  T  H  S  N  E  K  P  Y  I  C  K  I  P  G  C  T  K  R  Y
         5
2041    ACGGACCCCAGCTCTCTCAGAAAACACGTCAAAACTGTGCAC
         T  D  P  S  S  L  R  K  H  V  K  T  V  H
```
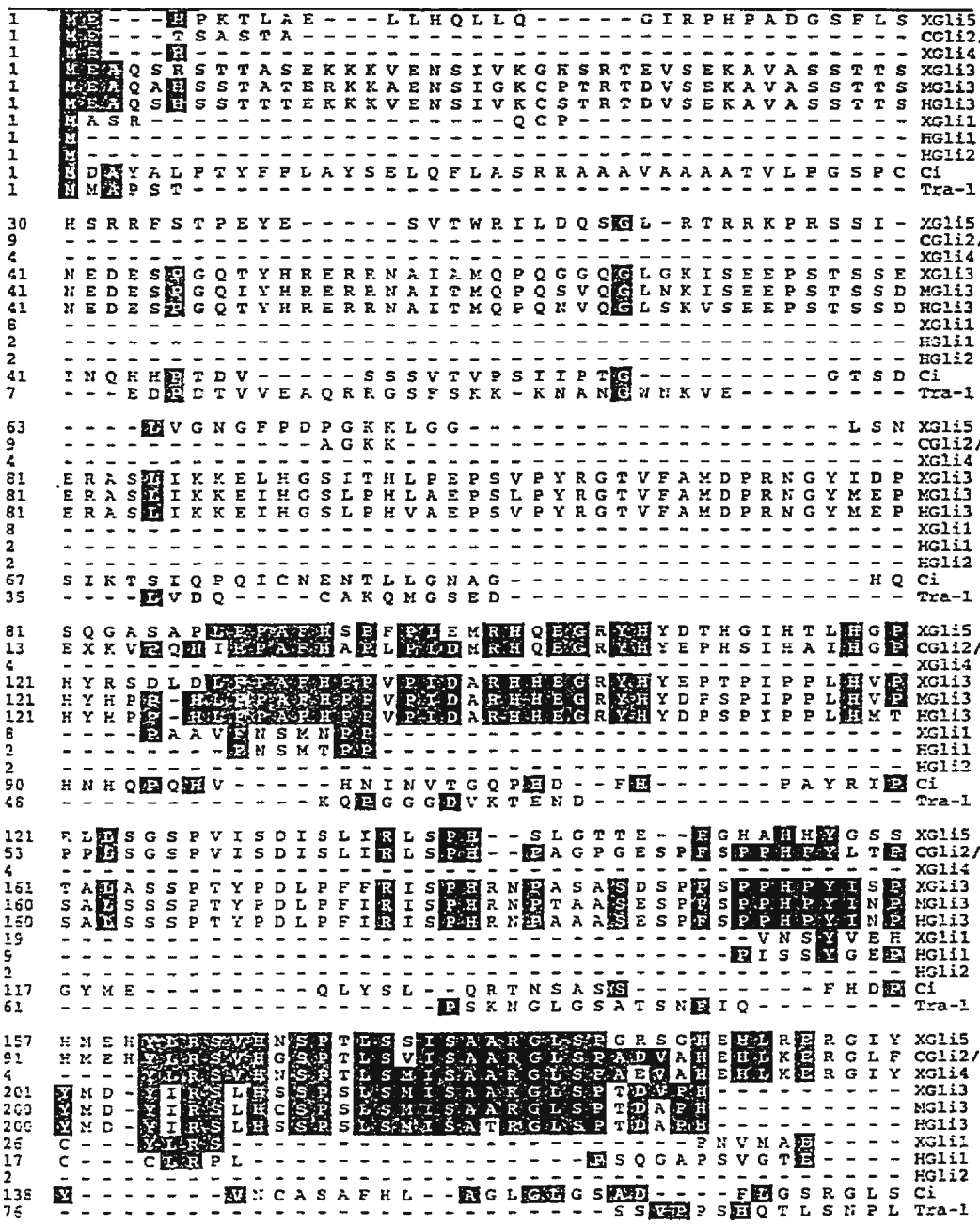
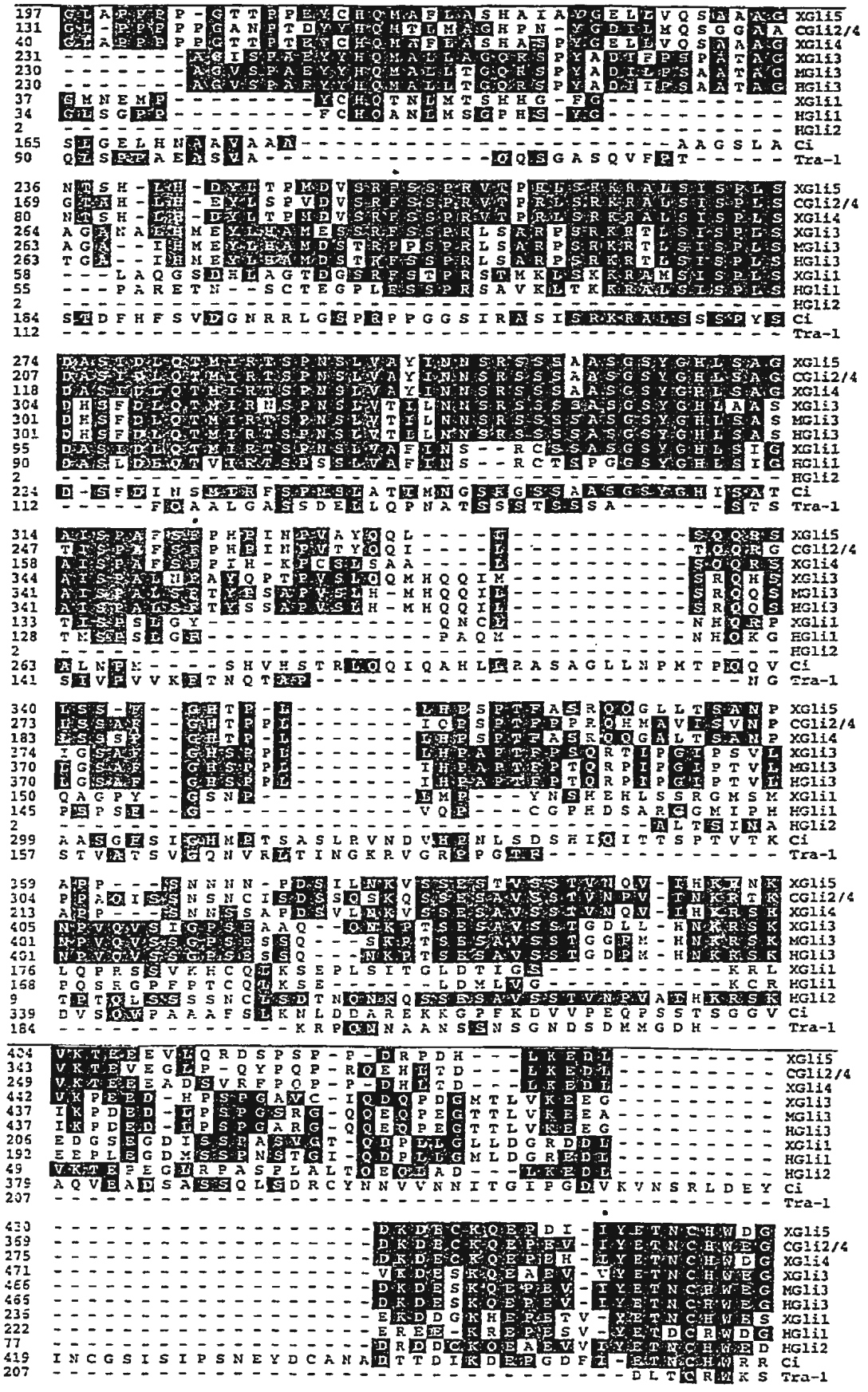

Fig. 3.3. Alignment of N-terminal amino acid sequence of *Xenopus* Gli5 and chicken Gli 2/4, *Xenopus* Gli-4, *Xenopus* Gli-3, Mouse Gli3, Human Gli3, Human Gli2, *Xenopus* Gli1, Human Gli1, *Drosophila* Ci, *C. elegans* Tra-1 with DNA star software. Black box indicates amino acid identity regions. Dash indicates not homolog regions. Region 1 of XGli5 is from amino acid 252 to 321. Zinc finger region of XGli5 is from amino acid 441 to 596.





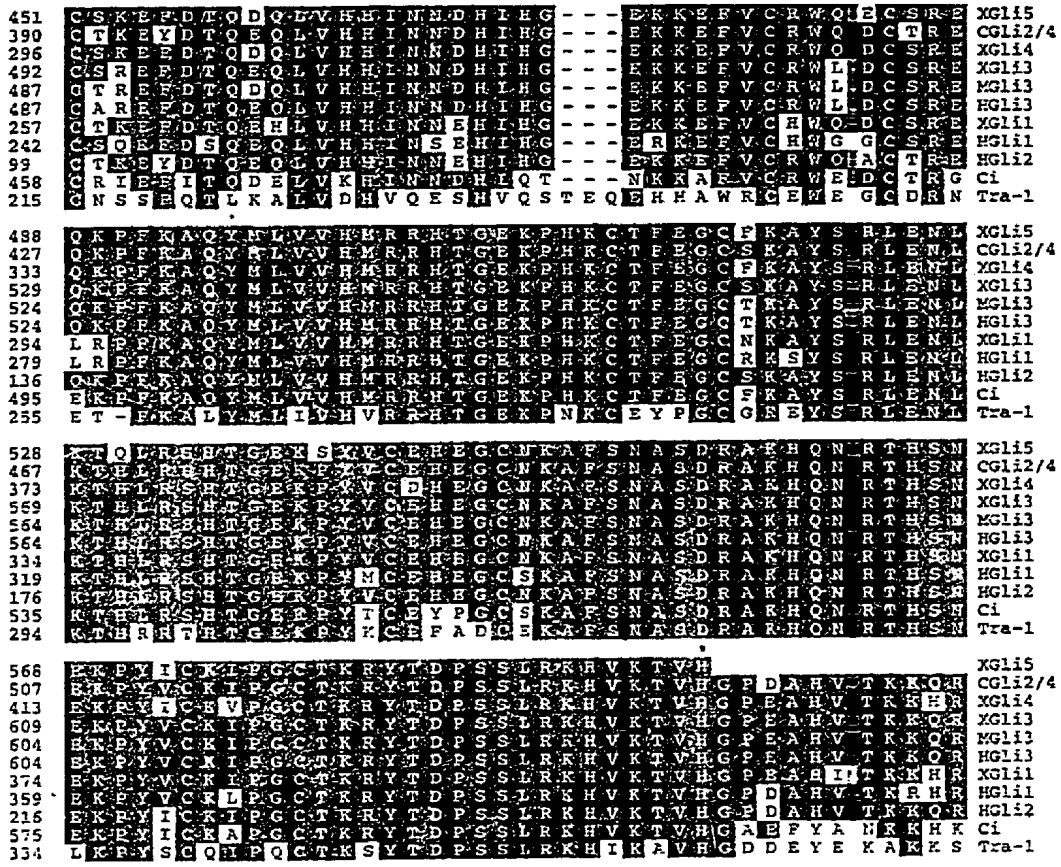


Fig. 3.4. Comparison of GF region of amino acid sequence among C. elegans, human Gli3 and *Xenopus* GLI3, Chicken Gli2/4 and XGli5. Two arrowheads show the GF region in *C.elegans*. Asterisks represents conserved amino acids in the GF region. Dots represents the amino acids identity between the Gli3 and *Xenopus* Gli.

GF region comparison

```
                    ..... . ..  .. ........   .  ..    ...
XGli5       QGASAPLFPAFHSPFPIEMRHQEGRYHYDTHGIHTLHGPPLLSSGSPVI
CGli2/4     XKVPQHIFPAFHAPLPIDMRHQEGRYHYEPHSIHAIHGPP-PLSGSPVIS
XGli3       YRSDLDLFPAFHPPVPIDARHHEGRYHYEPTPIPPLHVPT-ALASSPTYP
HGli3       HYHPPHLFPAFHPPVPIDARHHEGRYHYDPSPIPPLHMTSALSSSPTYP
CB TRA1     ADDQETKLKDDLEKEASTYKSSSATDRFEQSSHPPSHQTVENPIKLEPP
CE TRA1     DKQPGGGDVKTENDPSKNGLGSSATSNFIQSSVPPSHQTLSNPLQLSPP
                                 →   *  **  * *    ←
```

proteins such as Ci and Tra-1, although they still can bind the same target DNA sequence as that of Gli family, they have lower similarity in zinc finger region and identities of amino acids between XGli5 and Ci and Tra-1 are is 86% and 64% respectively (Fig. 3.3).

As for region 1 which includes 70 amino acids at the N-terminus of the zinc finger motifs (Fig.3.3), the amino acid identities between XGli5 and CGli2/4, XGli4, XGli3, XGli1, Human Gli3, Gli1 are 98.6%, 100%, 78.5%, 78.5%, 81.5%, and 65.7%. Human Gli2 does not have region 1 homology sequence.

At the Gli N-terminus there is a 16 amino acid conserved region, GF region which is defined by Bono et al. (Bono et al., 1995). Although human Gli3 has some similarity with Tra-1 in the GF region (Bono et al., 1995), it is difficult to find conserved amino acids between XGli5 and Tra-1 (Fig.3.4). Only one amino acid, Histidine, did not change among the Tra-1, HGli3, XGli3 and XGli5. However, there are some similarities in the boundary of GF region between XGli5 and Gli3 (Fig.3.4). According to my results (Fig. 3.3), between region 1 and the GF region there is a new 27 amino acid conserved region, which is only conserved among the Gli3, Gli4, and Gli5 and Gli1 and Gli2 do not have this homology region. The amino acid identities between XGli5 and CGLi2/4,

XGli4, XGli3, MGli3, and HGli3 within this conserved region are 77.7%, 81.5%, 66.7%, 66.7%, and 63%.

3.3 *XGli5* mRNA temporal expression during development

3.3.1 Northern hybridization analysis of *XGli5* at different stages

To understand the function of XGli5, the expression pattern of this gene was investigated during early *Xenopus* embryonic development. In order to cover the entire period of embryonic development, total RNA was isolated from embryos at stage 1, 6, 8, 11, 16, 31, and 41. First, northern analysis showed one major band at the position about 1.5 cm to the top of the blot (Fig.3.5). After comparing with RNA marker that was stained with methylene blue, this major band was predicted to be 8.5Kb (Fig.3.5). At stage 8, another faint band below the major band was observed. This band may represent another member of *Gli* gene family or a degradation product of full length 8.5 Kb of *XGli5* mRNA. The same blot was washed and reprobed with *Xenopus* histone 4 cDNA, a loading control, and showed an intense band that corresponded to a size of 400bp (Fig.3.5). When compared with known histone 4 mRNA levels at different developmental stages, I found that before stage 8, *XGli5* mRNA levels was low. At stage 8, *XGli5* mRNA levels rapidly increased and then decreased to lowest levels after stage 11. At stage 16, at which the neural fold is formed, *XGli5* mRNA levels increased again.

Fig. 3.5. Northern hybridization of *Xenopus Gli5* gene during the different stages of embryonic development. 1, 2, 3, 4, 5, 6, and 7 show the stage 1, 6, 8, 11, 16, 31, and 41. The arrowhead showed that *Gli3* was about 8.5Kb and H4 represents the *Xenopus* Histone 4 gene which is about 400bp.

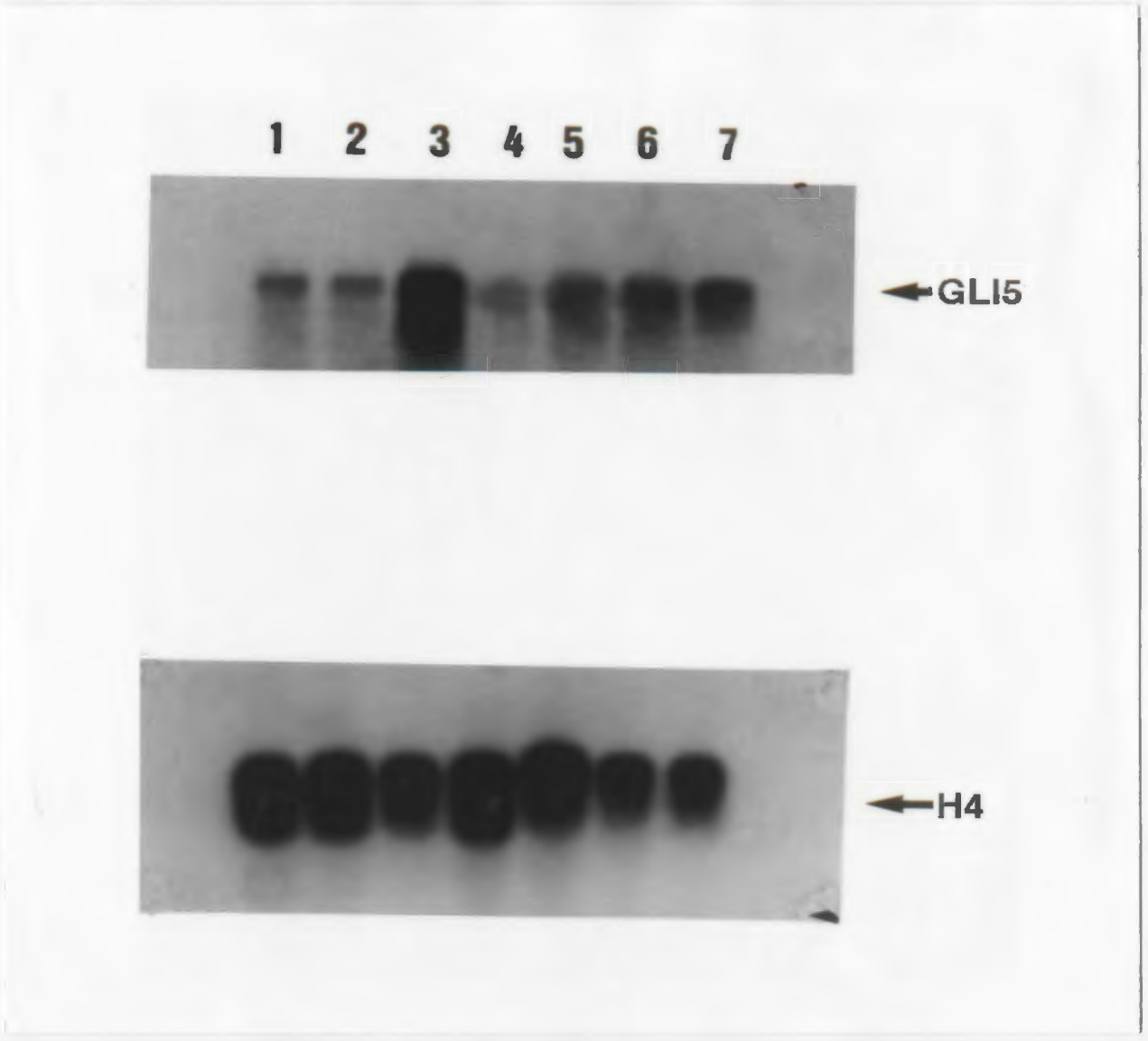
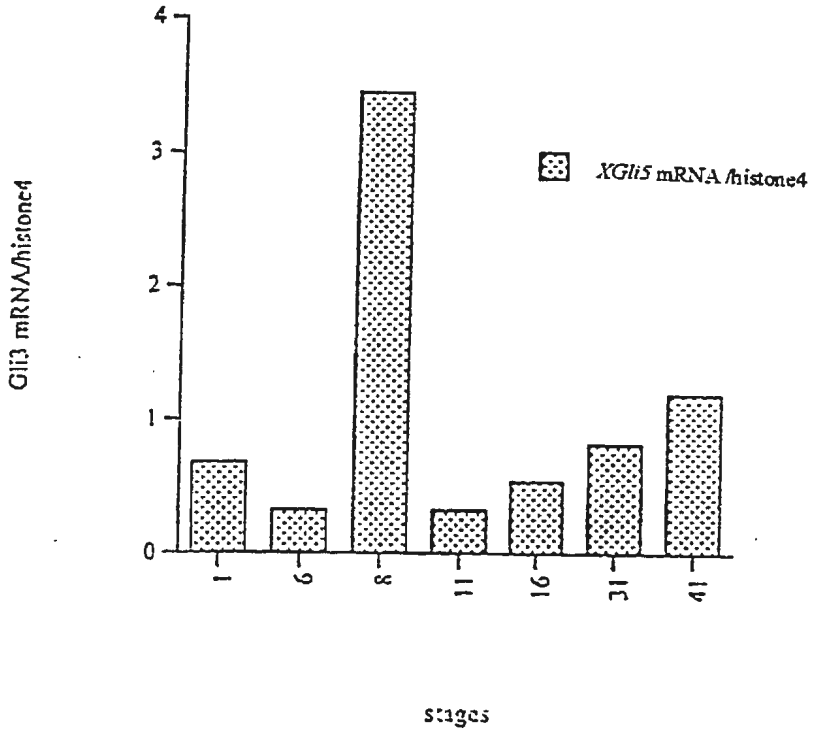


Fig.3.6 Relative expression level of *Xenopus Gli5* at the different stages of embryonic development.



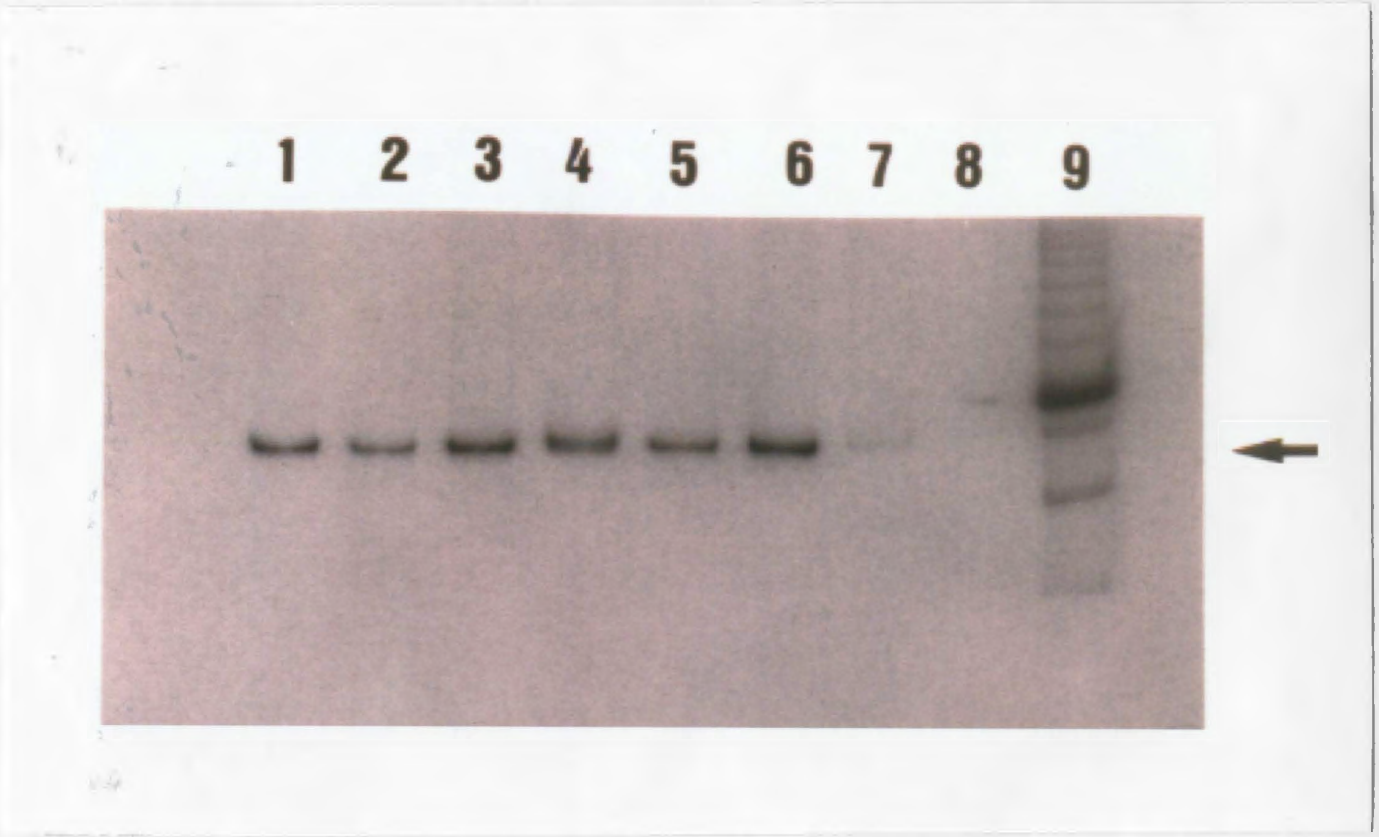
Afterwards, *XGli5* mRNA levels increased consistently until stage 41 (Fig.3.5).

I measured the relative densities of *XGli5* and Histone 4 probed Northern blots using densitometer. This analysis revealed that steady state mRNA levels of *XGli5* at stage 8 was 4 fold higher than the levels of mRNA at the stage 1 (Fig. 3.6). At stage 11, *XGli5* mRNA levels are the lowest one which is half mRNA level of stage 1. After stage 11, *XGli5* mRNA levels begin to increase and by stage 41, the levels of *XGli5* mRNA are 0.7 fold more than that of stage1.

3.3.2 RT-PCR of different stages of *Xenopus* development

The probe used for Northern hybridization was from a *XGli*-1kb DNA fragment in which there are several conserved regions. The result of Northern blot may represent not only specific *XGli5* but also mRNAs of other *Gli* family members. In order to identify *XGli5* expression specifically, *XGli5* cDNA sequence derived from the 5i non conserved region was chosen to synthesize *XGli5* cDNA specific primers and to perform RT-PCR. I chose stage 1, 6, 8, 11, 22, and 36 to represent early *Xenopus* embryonic development as in northern analysis. In Fig. 3.7, there was a clearly amplified band around 550 bp that corresponded to the predicted size of *XGli* PCR product in

Fig.3.7. RT-PCR at different stages of embryonic development. Lane 1, 2, 3, 4, 5, and 6 represent *XGli5* mRNA expression at stage 1, 6, 8, 11, 16, and 33 respectively. Lane 7 is the plasmid DNA as positive control (< 10 copies of plasmid).Lane 8 is the PCR negative control. Lane 9 represents the Molecular weight Marker



all of the different developmental stages. This also proved that *XGli5* mRNA expression starts from stage 1 and extends to stage 36. Furthermore, it was found that the density of RT-PCR amplified band at stage 8 and 36 is higher than that of other developmental stages (Fig.3.7), although this RT-PCR was not quantitative. On the basis of results of northern hybridization analysis and RT-PCR, *XGli5* mRNA expression initiated from stage 1 and continued to stage 41. At stage 8, *XGli5* mRNA expression reached the highest levels, decreased down to the lowest levels at stage 11 and then gradually increased its expression from stage 16 to 41.

3.4 Regulation of *XGli5* mRNA expression by FGF signal

To know whether *XGli5* is involved in the FGF mesoderm signal pathway, I treated animal pole explants with FGF and monitored changes in the steady state levels of *XGli5*. Animal pole explants were cut out from early stage 8 of *Xenopus* embryos and incubated in 100ng/ml FGF-NAM solution (100ng/ml FGF in 1X NAM solution, appendix) for various times at room temperature (Ryan and Gillespie, 1994). RT-PCR was performed with *XGli5* gene specific primers. The highest level of *XGli5* mRNA expression was detected after 30 minutes of FGF treatment. *XGli5*

expression decreased from 60 to 240 minutes of incubation (Fig.3.8). After 12 hours incubation, *XGli5* mRNA expression gradually increased (Fig.3.8). On the other hand, a low levels of *XGli5* mRNA expression was detected in control explants (0 minute FGF incubation) (Fig. 3.8). This may correspond to maternal *XGli5* mRNA in the explants. This experiment was repeated and I obtained the same results.

3.5 Preparation of XGli5 fusion protein and expression in *E.coli*

A 960bp *XGli*-1kb fragment including the zinc finger motifs was cloned into the prokaryotic fusion protein vector pGEX.KT (Smith and Johnson, 1988). After IPTG induction, bacterial lysates were analyzed on SDS-PAGE (Fig. 3.9). A single 58 KDa band was found following protein purification by affinity chromatography with glutathione sepharose 4B resin. The majority of this XGli fusion protein was found in the insoluble fraction (Fig. 3.9). After insoluble fraction was denatured with 6M Urea buffer and then neutralized, high yield of soluble GST+XGli fusion protein was obtained (Fig.3.9).

3.6 Preparation, purification, and analysis of XGli5 polyclonal antibody

A Gli5 polyclonal antibody was prepared by injecting a Gli5 synthetic peptide (N-CRNKVKTEEEVLQRDRS-C) into rabbits. This peptide was designed to be specific to

Fig. 3.8 FGF induced the XGli5 gene expression. lanes 1-8 represent time course of incubation in FGF solution. 1-8 show the 0 minutes, 30 minutes, 60 minutes, 120 minutes, 240 minutes, 480 minutes, 12 hours, and 24 hours incubation respectively. The arrow shows the amplified band.

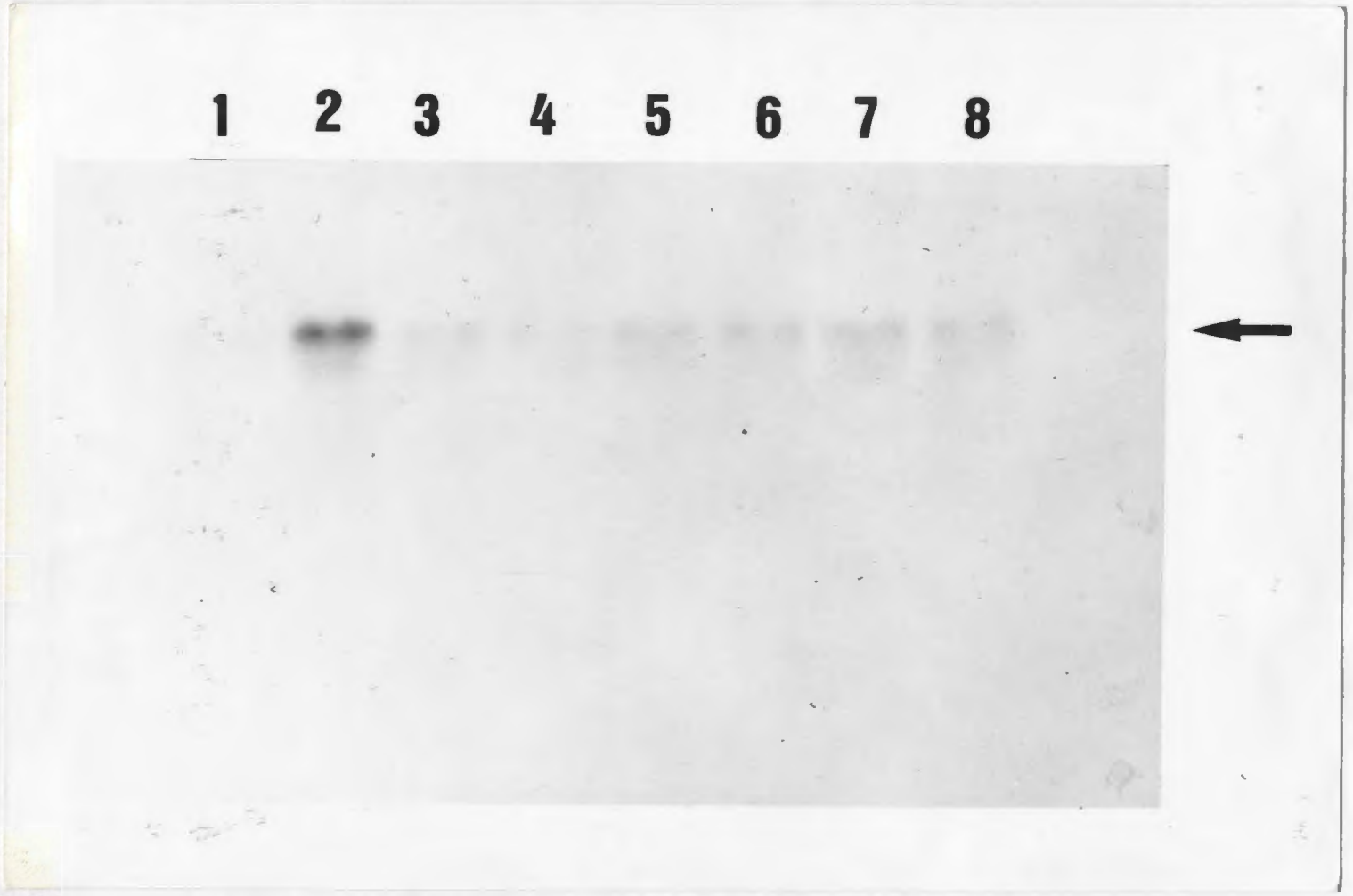
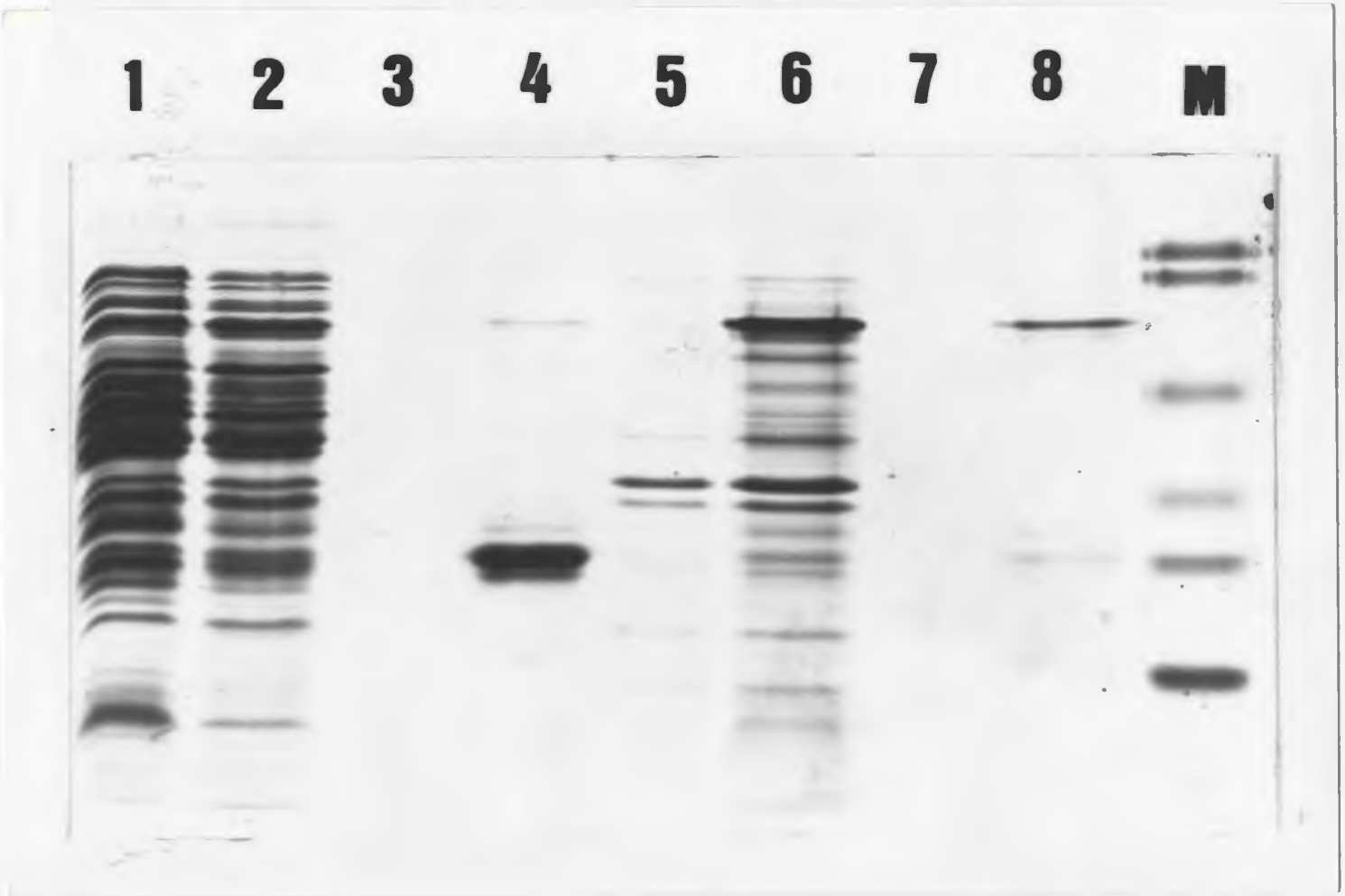


Fig. 3.9. *Xenopus* Gli5 GST fusion protein production. 1, total bacterial protein from bacteria culture without IPTG; 2, total soluble bacterial protein from bacteria culture with IPTG; 3, soluble bacterial protein which was from bacteria culture without IPTG purified by glutathione sepharose; 4, soluble protein which was from bacteria culture with IPTG purified by glutathione beads; 5, total insoluble protein from bacteria culture without IPTG; 6, total insoluble protein from bacteria culture with IPTG; 7, insoluble protein from bacteria culture without IPTG was purified by glutathione sepharose 4B.; 8, insoluble protein from bacteria culture with IPTG was purified by glutathione sepharose 4B. M. Low molecular weight marker.



XGli5 since it is outside of the zinc finger conserved regions of the Gli family. Through a series of injections, antisera were obtained. High titer and specific antiserum is necessary to do protein research. Hence, the specificity of XGli5 antiserum was first tested by Elisa with XGli5 synthetic peptide as a substrate. To determine whether these antisera can specifically recognize XGli5 protein, XGli5 fusion protein Western blotting was performed. About 0.5 ug of fusion protein was used in Western blotting using different preimmune and immune XGli5 peptide antiserum. Two of three rabbit antisera stained a positive band at 1 to 3000 dilution (Fig.3.10). Therefore, it was confirmed that two XGli5 antisera were capable to recognize XGli5. The positive XGli5 antiserum were performed further affinity purification to obtain specific XGli5 IgG. XGli5 synthetic peptide was used to make an affinity column and the XGli5 antiserum was purified by passing through this column. About 2.5 mg of purified XGli5 antibody was obtained by affinity purification.

3.7. XGli5 protein expression

50 µg of protein isolated at different developmental stages were electrophoresed on SDS-PAGE and transferred on the PVDF membranes in order to perform Western

Fig. 3.10. XGli5 fusion protein Western blotting for identifying the specificity of different XGli5 antisera. M is low molecular weight marker. 1 is the first Gli5 antiserum; 2 is the first preimmune serum; 3 is the second Gli5 antiserum; 4 is the second preimmune serum.

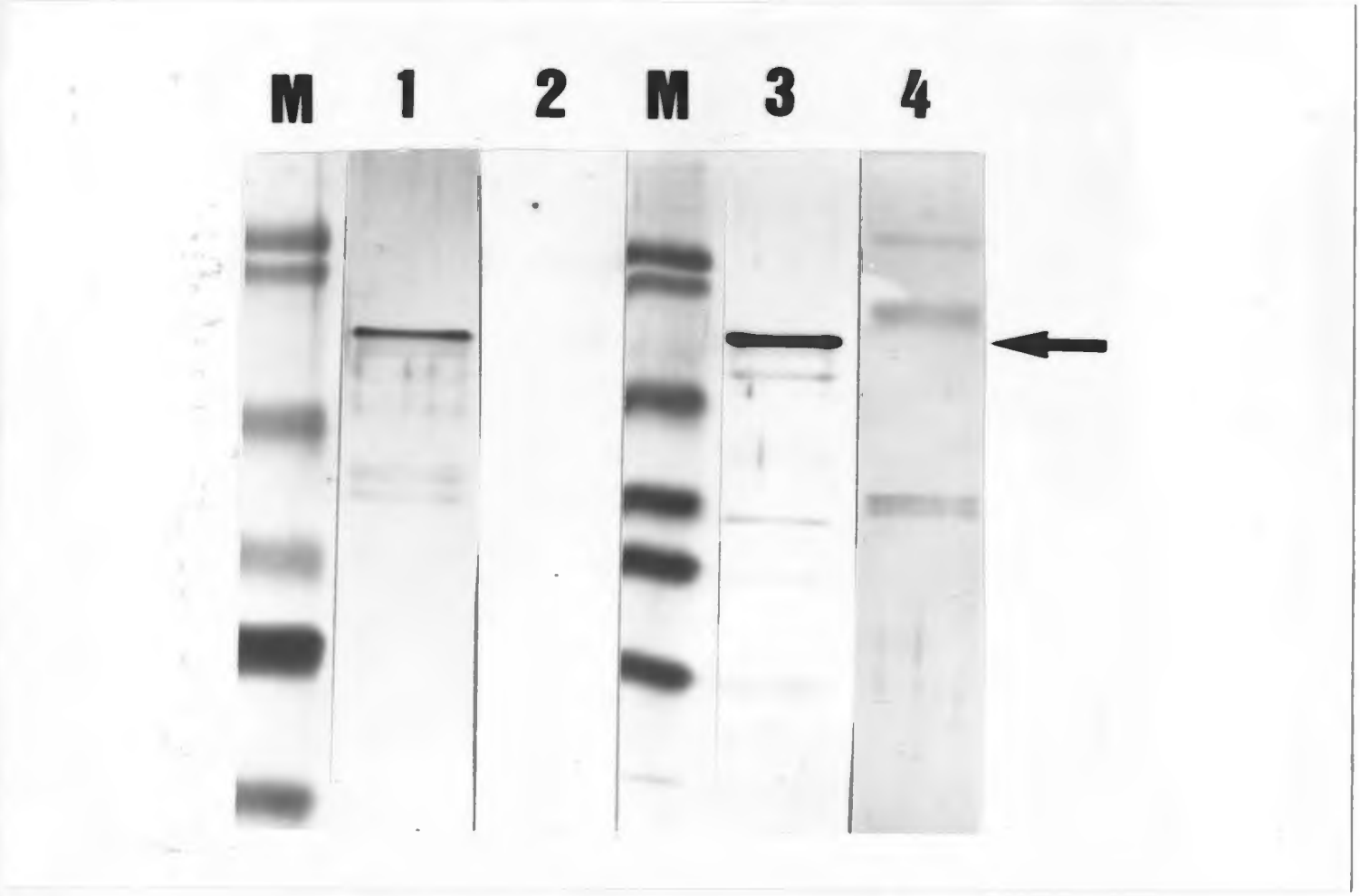
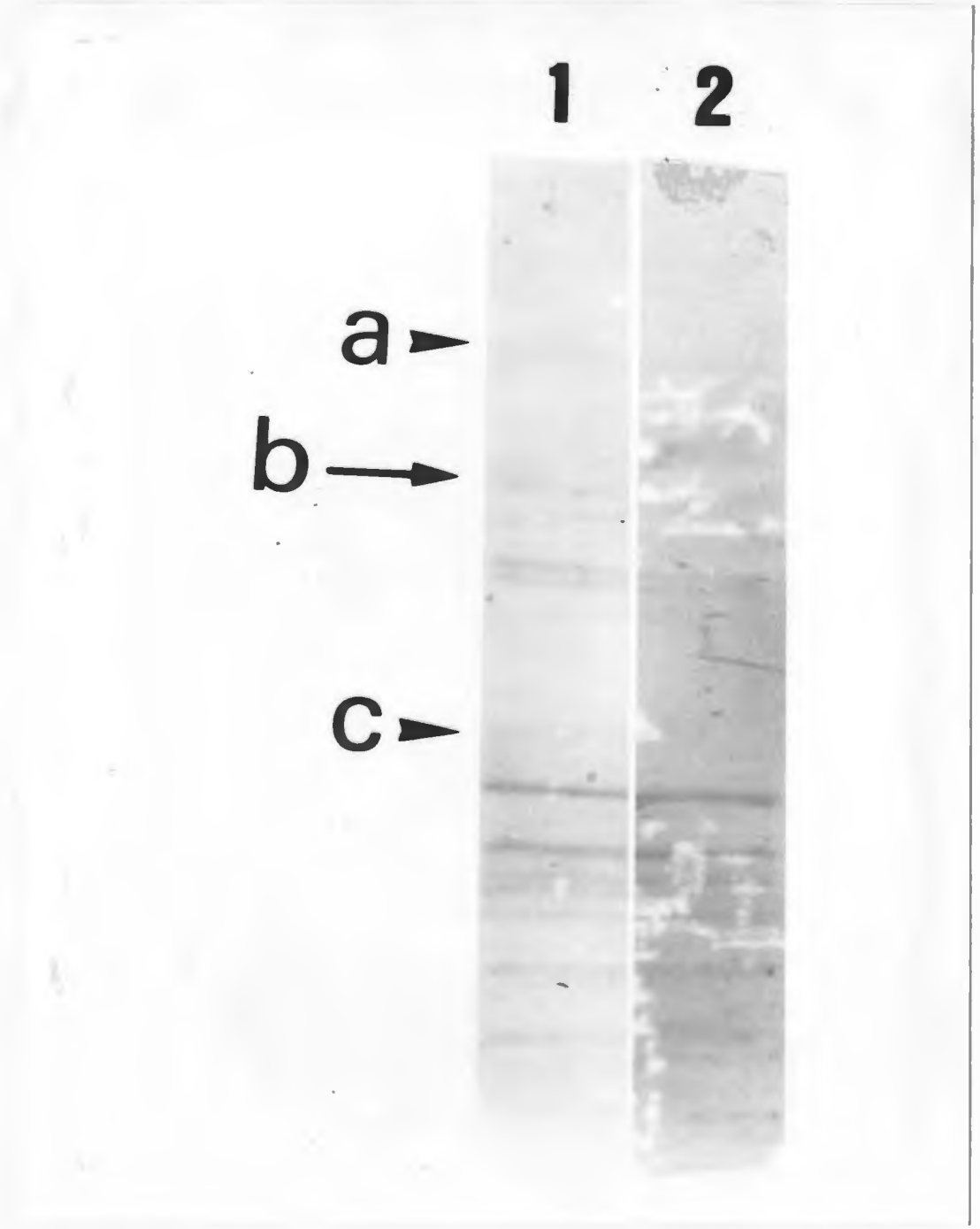


Fig.3.11. XGli5 protein size and expression. stage 28 total protein was used to make the blot. 1, showing the normal immune blot; 2, is the competitive western blot with XGli5 fusion protein added in the primary antibody incubation reaction. There are three competed bands labeled a,b,c. a is about 350 kda; b is about 190 kda and c is about 90 Kda.



blotting immunodetection (Sambrook et al., 1989) with affinity purified XGli5 antiserum. The results were not satisfactory because multiple bands were detected on each lane masking the XGli5 protein with a high background.

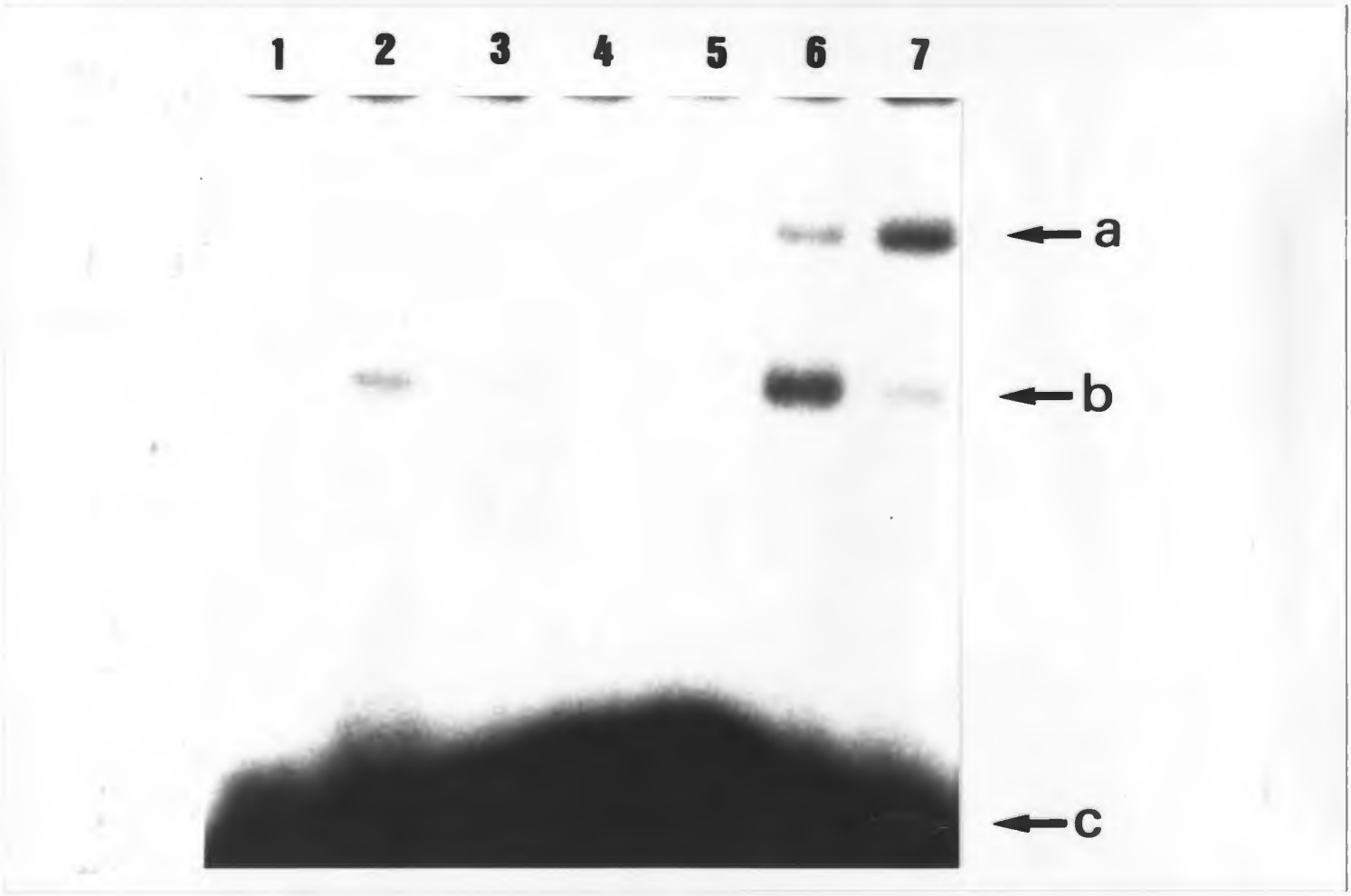
In order to discriminate between the Gli5 protein and other cross-reacting proteins in *Xenopus* embryo, a competition experiment was performed by competitive Western blotting. The result showed clearly different bands at stage 28 embryo on the corresponding lanes of two blots (Fig.3.11). Compared with two blots, there were three different bands: a, b, and c which were 350 Kda, 190 Kda, and 90 Kda respectively (Fig.3.11). In lane 1 (reaction blot) of the three bands, band b was the most intense one and in lane 2 (competition blot) band b disappeared completely after the antibody incubated with blot and XGli5 fusion protein. However, band a and c were still detectable. This implies that band b represents XGli5 protein which is about 190 Kda. I also attempted to look at the spatial pattern of XGli5 RNA and protein expression using whole mount *in situ* hybridization with method from Smith and Harland (Smith and Harland, 1992) but was not successful.

3.8. XGli5 DNA Binding Activity

Electrophoretic Gel Mobility Shift Assay (EMSA) was performed to investigate possible XGli5 DNA binding

activity. The human Gli binding sequence, a 13-mer sense and antisense oligos (including 9-mer consensus), was used as a probe. In the presence of the fusion protein GST+XGli, a slower migrating band was shown as compared with the labelled probe alone (Fig.3.12). This shifted band indicated a specific protein-DNA interaction between GST-XGli5 and the labelled probe. Competition with increasing concentrations of unlabelled probe reduced or eliminated the shifted band. This illustrated that the XGli5 interacted with DNA in a sequence-specific manner (Fig. 3.12). In order to prove XGli5 binding activity, the anti-XGli5 antibody was added to the reaction. XGli5 protein bound to the DNA probe as well as to the antibody. The DNA-XGli5-anti-XGli5 antibody complex migrated slower than the XGli5-DNA complex alone and formed what is called a super shift band (Ausubel et al., 1995). A super shifted band was observed when the anti-XGli5 antibody was added. A more intense super shifted band was observed when the anti-XGli5 antibody was added before incubation (Fig. 3.12). Based on these assays, I have demonstrated that the XGli5 protein can bind a specific DNA sequence, 5'-GACCACCCA- 3' which is identical to the binding sequence of other Gli and Ci or Tra-1.

Fig. 3.12. Gel mobility shift shows that XGli5 protein can bind the specific DNA sequence. A. supershifted band; b. Shifted band; c. Free probe. 1. Labeled double strand oligo; 2, XGli5 fusion protein bind with labelled probe; 3-5, unlabelled probe competition with labelled probe; 6-7, XGli5 antibody was added in the EMSA reaction. 6. antibody was added after EMSA reaction; 7. antibody was added before EMSA reaction.



Discussion

4.1 Identification of XGli5

4.1.1 Sequence comparison to show that the fragment I cloned is a new member of *Xenopus* Gli family

With RT-PCR and 5' RACE-PCR, I have cloned a 2.1 Kb cDNA fragment from *Xenopus* embryos. After this fragment was sequenced by the Sanger dideoxynucleotide triphosphate termination method, the amino acid sequence was deduced. One of important structural characterization of this fragment is the five zinc finger motifs, which fits the structure of Gli zinc finger motifs: [Y/F]XCH3GCX3[F/Y]X5LX2HX3-4H[T/S]GEKP. Therefore, this fragment encodes a gli-type zinc finger protein.

Using the BLAST algorithms (Altschul et al., 1990) to search Genbank, It was found that this fragment was very similar to the Gli family. A relative high score value introduced in BLAST represents the similarity measures of two compared sequences (Altschul et al., 1990). If one sequence has higher value of high score than that of another sequence, this sequence is more similar to the compared sequence than that of another sequence. In XGli5 BLAST research, the high score comparison reveals that *Gallus gallus* zinc finger transcription factors (gli2/4) hits the highest score, 821. *Xenopus* Gli-4 is 809; Mouse Gli3 is 641; Human Gli3 is 635; *Xenopus* Gli-3 is 632. Human Gli1 and Gli2 are next with low scores: 440 and

390. According to the structural characterization and BLAST search result, this *Xenopus* fragment belongs to the Gli family and is most similar to Chicken Gli2/4. Because it does not match any known *Xenopus* Gli sequences, this fragment is a potentially new member of Gli family in *Xenopus*. I call it XGli5.

According to alignment results between this new member, XGli5 and other Gli family members, this *Xenopus* fragment contains four conserved regions which include zinc finger motifs (amino acid 444-596), region 1 (amino acids 252-321), new conserved region (amino acids 161-187) and GF region (amino acids 107-128). In the zinc finger region, sequence comparison of XGli5 with different species of Gli proteins shows that this *Xenopus* fragment has high similarity with the frog, mouse, and human Gli family (> 92% amino acid identity) except human Gli1 and has less similarity with Ci and Tra-1 (<86% amino acid identity). This is further evidence that this *Xenopus* fragment is likely to be a member of the Gli family.

As for the other conserved regions, such as region 1, the new conserved region, and GF region, sequence comparison reveals that this *Xenopus* fragment is similar to other members of Gli family but does not have identical amino acid sequence. According to BLAST alignment, XGli5 is very similar to XGli4. Both XGli5 and

XGli4 have identical region ones and they are very conserved in the zinc finger motifs. But, the XGli5 has an additional 150 amino acids at the N-terminus, which includes the GF region. There are two questions about this new Gli fragment. First, Is it an isoform of XGli4? Second, are there more Gli family members in *Xenopus*? By comparing XGli5 and XGli4, it is possible that by alternative splicing, XGli4 skips several exons at the 5′ end. However, in addition to having more than 150 amino acids at the N-terminus, many amino acids are different at the gap between region 1 and zinc finger motif. Within the newly identified 27 amino acid conserved region, there are 5 amino acid differences between XGli5 and XGli4. As for the most conserved zinc finger motif, there are still some amino acid variations. This illustrates that XGli5 is similar to XGli4 but they are different Gli members. According to BLAST search and homolog comparison, XGli5 is not Gli2, which does not have GF and region 1. Although there are some amino acid differences between XGli5 and chicken gli2/4, a BLAST search of XGli5 reveals that chicken gli2/4 has the highest score. The chicken gli2/4 has four conserved regions (GF region, new conserved region, region 1, and zinc finger motif), which are also present in the XGli5. Moreover, these four regions are very conserved between XGli5 and Chicken

gli2/4. It is possible that XGli5 belongs to a member of chicken gli2/4 in *Xenopus*.

Since I only cloned 2.1 Kb, an additional 6 Kb sequence remains to be identified. Therefore, it is hard to conclude that this fragment represents chicken gli2/4 homologue in *Xenopus*. At present, it is not important to know whether it is *Xenopus* gli2/4 or not. All the current information shows that the XGli5 has high similarity to the Gli family and is also different from all known *Xenopus* Gli proteins. This implies that XGli5 is a new member in *Xenopus* Gli family.

4.1.2 Evidence from Northern and Western blotting analysis

The temporal expression of this *Xenopus* Gli5 mRNA was examined by Northern hybridization analysis. The full-length *Xenopus Gli5* transcripts was shown to 8.5 Kb. Based on the results of Western blotting, the molecular weight of XGli5 is about 190 Kda. However, based on the full length cDNA clone, XGli3 is about 9.4 Kb though there is no Northern hybridization data and the predicted size of the XGli3 protein is 173 Kda (Marine et al., 1997). Full length of XGli4 is 4.4 Kb which encodes a 150 Kda protein (Marine et al., 1997). According to RT-PCR, XGli3 and XGli4 mRNA expression begins at stage 11.5 and stage 9 (Marine et al., 1997). Based on Northern analysis

and RT-PCR, XGli5 mRNA expression begins from stage 1 to stage 41. Therefore, taken together, the different size of its cDNA and its expression pattern suggests that XGli5 may represent a new member of the *Xenopus* Gli family.

4.2 Characteristics of the XGli5 fragment

4.2.1 Characteristics of the GF region

The GF region was first described in Tra-1 of *C. elegans* by Bono et al.(Bono et al., 1995). Tra-1 is the terminal gene in a genetic signal transduction cascade involved in sex differentiation (Hunter and Wood, 1990). With high Tra-1 activity, XX *C. elegans* embryos will develop as females (Bono et al., 1995). However, some XO *C. elegans* are affected by Tra-1 mutations that results in Tra-1 gain-of-function and instead of males, these XO *C. elegans* become females. Through mutation analysis, this gain of function mutation is located near the N-terminus of Tra-1 protein at the position of amino acids 73-88 which is called the GF region (Bono et al., 1995). Bono *et al.* suggested that the GF region in the Tra-1 was a target site for negative regulation of Tra-1 protein expression and this regulation was probably through protein-protein interactions.

Since human Gli3 has a region similar to the GF region, it was predicted that human Gli3 gene post-translational regulation may also involve a process of GF region protein-protein interaction to regulate downstream gene expression (Bono et al., 1995). However, in the XGli5 amino acid sequence, I did not find a region similar to GF region of Tra-1. But, at the N-terminal flanking region of the predicted GF region, there is more than 70% amino acid similarity between XGli5 and Gli2/4 or Gli3 (Fig. 3.4). Likewise for the vertebrate Gli family, the GF region may be shifted to the N-terminal flanking region which might also be involved in the regulation of interactions between Gli and downstream target proteins.

In order to demonstrate this GF region's function of XGli5 in *Xenopus* embryonic development, one might adopt a dominant negative mutant approach. Dominant negative mutants are those in which the mutation dominantly affects the phenotype by producing a defective protein or RNA molecule that interferes with the function of the normal gene product in the same cell. Specifically, a mutated or deleted GF region form of XGli5 would be cloned into an expression vector. The RNA transcribed from the vector could then be microinjected into *Xenopus* fertilized egg and through overexpression of this truncated XGli5 and observation of the *Xenopus* embryo

phenotype, the function of GF region of XGli5 might be identified. Secondly, since the GF region of Tra-1 is a potential protein-protein interaction region to regulate sex differentiation (Bono et al., 1995), it will be possible to demonstrate the function of the GF region by the yeast two hybrid system, which is based on the fact that many eukaryotic trans-acting transcription factors are composed of physically separable, functionally independent domains (Clontech protocol, 1997). Such regulators often contain a DNA binding domain that bind to a specific enhancer sequence, referred to as an upstream activation site (UAS) in yeast and one or more activation domains which can direct to transcribe the gene downstream of the UAS. In yeast GAL4 protein, the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cells, the DNA binding domain and activation domain peptides do not directly interact with each other and cannot activate the responsive genes. However, if these two domains are brought into close physical proximity by interaction of two fusion proteins, the transcriptional activation function of GAL-4 will be restored (Allen et al., 1995). Therefore, with GAL-4 two hybrid system, it will be possible to determine if

protein-protein interactions exist in the GF region of XGli5.

4.2.2 Characteristics of the zinc finger motifs

In *Xenopus*, zinc finger motifs are located at the C-terminal end of the 2.1 Kb fragment of XGli5. This motif includes five repeats of sequence element in which the position of two cysteines and two histidines is very conserved. Each sequence element can facilitate folding into a finger-like structure. Comparisons with different Gli-related proteins, such as, XGli5, XGli3, XGli4, XGli1, human Gli3, *Drosophila* Ci, and *C. elegans* Tra-1 reveal that all of them have five tandem repeated elements which are organized into the five zinc fingers which in turn can bind DNA in a sequence-specific manner(Kinzler and Vogelstein, 1990; Zarkower and Hodgkin, 1993; Vortkamp et al, 1996). Based on zinc finger region sequence comparisons btween these proteins (Fig. 3.3), zinc finger 1 is less conserved than others. Finger 4 and 5 are the most conserved with all of amino acids in the fingers identical. Finger 2 and 3 are in between. That the different fingers have variable evolutionary conservation may imply that each finger has its own characteristic for binding target DNA molecules. In fact, conserved fingers are likely to be crucial for

DNA binding. This explanation was well supported by crystal structure analysis of the human Gli1 DNA binding complex (Pavletich and Pabo, 1993). They observed human Gli1 and DNA complex crystal structure at a resolution of 2.6 Å and found that finger 1 which forms a β-sheet, contact finger 2 but does not bind to DNA. Fingers 2 to 5 fit the major groove and wrap around the DNA and form a full helical turn. Surprisingly, only fingers 4 and 5 make extensive base contact in 9 bp DNA region that is conserved in all of the known Gli binding sites (Vortkamp et al., 1996). Thus, based on the crystal structure analysis, more variable amino acids found in finger 1 of XGli5 suggest that it has different function in regulation DNA binding through extensive contact with finger 2. No amino acid differences in finger 4 and 5 indicate that their amino acid sequence is necessary for the zinc fingers to bind to an identical 9 base pair core sequence. However, as we know, Tra-1 also binds the core sequence (Zarkower and Hodgkin, 1993) but four and five amino acid variations have been found in fingers 4 and 5, respectively. It may be that these amino acids are not important in determining DNA binding and its secondary structure also fits the structure of the 9 basepair core sequence. However, the explanation needs further

experimental evidence such as the X-ray crystal structure analysis of Tra-1 DNA binding complex etc..

In order to demonstrate that XGli5 is the same as other Gli proteins which bind an identical 9 basepair core sequence, I made XGli5-GST fusion protein which includes the five zinc finger motif and performed EMSA (Electric Mobility Shift Assays) using the core sequence of Gli binding sequence. It was found that XGli5 also specifically binds the 9 basepair core sequence.

Although most of Gli proteins can bind the same core sequence, obviously they cannot have the same function. For example, human Gli1 is an oncogene which was found in glioblastoma cell line DM-259 (Kinzler et al., 1987) while human Gli3 is involved in limb and craniofacial development(Vortkamp et al., 1991 & 1992). Similarly, Tra-1 is the terminal sex differentiation gene in *C. elegans*. Why do the Gli family, which bind the same core sequence, have a variety of functions? There are several possible explanations. First, although Gli1 can bind the same core sequence with fingers 4 and 5 (Pavletich and Pabo, 1993), a variety of amino acid differences have been found in fingers 1, 2 and 3 of various Gli proteins. These variations may affect specific DNA binding, and in turn may affect the function of these Gli proteins. Second, in addition to the zinc finger DNA binding

motifs, other functional domains, such as the acidic activation domain or GF region, also play a role in Gliís function. If there are acidic activation domain variations among the different Gli proteins, it may result in different transcriptional activation among these Gli proteins, regardless of identical DNA binding sequences. Indeed, Gli1 can induce transcriptional activation through the C-terminal 18 amino acid conserved region which is highly similar to the α-helical herpes simplex viral protein 16 activation domain (Sasaki et al., 19997; Yoon et al., 1998). Gli3, however, functions as a transcriptional repressor(Lee et al., 1997; Marine et al., 1997) even though Gli3 also includes the C-terminal activation domain. Yoon et al.(1998) believed that the N-terminus GF region in Gli3 may function as a repressor domain. Therefore, with the GF region, Gli3 is a transcriptional repressor and without the GF region, like Gli1, trans-activation will be restored (Yoon et al., 1998). Third, although amino acid sequences of the functional domains of Gli proteins are same, they may have different functions in different tissues or at different developmental stages. Indeed, the same protein may regulate different functions in different tissues at different developmental stages (Akimaru et al., 1997). Specifically, by binding to different coactivators, which

are a group of transcription factors, various Gli proteins can interact with the transcriptional activators and GTFs (general transcription factors) to determine tissue specific expression. For example, the *Drosophila* transcription factor, dCBP, functions as a coactivator of Ci and interaction between Ci and dCBP is necessary to activate Ci target genes such as Patched in the hh signaling pathway (Akimaru et al., 1997).

Because we only obtained 2.1 Kb of 5í *XGli5* cDNA, the 3í of *XGli5* cDNA may have some structural characteristics which remain unknown. In order to elucidate the function of XGli5, one must clone the carboxyl terminus of XGli5 and then perform a function analysis of the full length XGli5. In order to examine XGli5 as a transcriptional activator or repressor, one might transfect *XGli5* into a yeast heterologous system to test its transcription activation activity as carried out during the functional analysis of Ci in *Drosophila* (Alexandre et al., 1996). Briefly, yeast cells are co-transformed with plasmids containing the HIS3 reporter gene which is under control by consensus Gli binding site and the XGli5 cloned downstream of the galactose-inducible promoter. Control and co-transformed cells are tested for their ability to grow on a yeast medium lacking histidine in the presence or absence of galactose. XGli5's transcriptional

activation ability will be observed when the yeast can grow in a galactose rich restriction medium lacking histidine.

4.3 FGF induces XGli5 expression in early embryonic development

4.3.1 XGli5 involved in FGF signal pathway

Based on my Northern hybridization and RT-PCR results, XGli5 messages can be detected in very early embryonic developmental stages such as stage 1 and are present throughout development to stage 8. At early stages, transcription of *XGli5* may not start and these *XGli5* mRNAs are likely the maternal mRNA accumulated during oogenesis because in *Xenopus* there is little or no transcription before MBT (mid-blastula transition) stage (Newport & Kirschner, 1982; Shiokawa et al., 1979) and protein synthesis is directed by mRNA accumulated in oogenesis (Slack, 1991). After stage 8, while the *Xenopus* embryo is in the stage of mid-blastula transition (MBT), *XGli5* mRNA transcription starts and rapidly reaches steady state levels. Concomitantly, mesoderm induction begins when the mesoderm induction factors are emitted and induce animal cells to form a mesodermal germ layer located around the equator of the embryo at blastula stage(Slack, 1991). Many experiments have implied that

peptide growth factors and their receptors are involved in this process (Klein and Melton, 1994 and Kessler and Melton, 1994). One of them is FGF which is capable of inducing mesoderm in *Xenopus* embryonic development (Slack et al., 1987; Amaya et al., 1991). Since there is a temporal correlation between high expression of XGli5 and mesoderm induction, XGli5 may be involved in mesoderm development and induced by FGF. In order to further confirm this presumption I examined the effect on FGF induction of XGli5. Early stage 8 animal caps, incubated in FGF for various times, were extracted for RNA which was used to perform RT-PCR with *XGli5* specific primers. It was found that after a half hour of FGF incubation, the *XGli5* mRNA reached the highest level which matched with the result of Northern hybridization. This implies that at early stage 8, FGF induction begins and the zygotic genes, such as *XGli5*, are induced by FGF signals and are highly expressed at the time of mid-blastula transition (MBT). Furthermore, it is possible that *XGli5* might be one of the FGF downstream response genes and is involved in the FGF signal transduction pathway during *Xenopus* embryonic development. Although no direct experiments prove that *XGli5* is involved in the FGF signal, there is some evidence that suggests the relationship between Gli family and FGF signaling. First,

the *Gli3* gene mutation is directly involved in the limb and craniofacial abnormalities (Vortkamp et al., 1991 & 1992). In mouse, *Gli3* mutation was directly related to extra-toes formation (Schimmang et al., 1992 and Hui and Joyner, 1993). This evidence strongly suggests that the *Gli3* gene is somehow involved in limb and craniofacial development. Meanwhile, in the *extra-toes* (*Xt*) mutant mouse, FGF showed a stronger signal in the most anterior region in limb bud and can upregulate the *Gli3* gene in Xt/Xt mouse (Buscher et al., 1997; Masuya et al., 1995). Further, Cohen et al. (1995) reported a remarkable finding that beads soaked in FGF implanted in the presumptive flank of a chick embryo was capable of inducing the formation of a normal limb. This also suggested that an endogenous, localized source of FGF from within the mesoderm of the flank may initiate the proliferation of the mesenchyme in normal limb development. Second, like the human craniofacial disease known as *Greig Cephalopolysyndactyly* syndrome caused by mutation or deletion of the human *Gli3* gene, many other craniofacial syndromes are associated with mutation of the FGF receptor (Mulvihill *et al.*, 1995). No matter which step is mutated, it will result in abnormal craniofacial development. Based on this, it is possible that the FGF signal transduction pathway is also involved

in Gli3. Third, *Drosophila* Gli (Ci) is mediated by the hedgehog (HH) signal to maintain wingless level in the anterior compartment along the AP axis (Mohler and Vani, 1992; Alexandre et al., 1995; Dominguez et al., 1996; Von Ohlen et al., 1997). HH-Ci signaling is also conserved in vertebrates. Like invertebrate counterparts, vertebrate Gli3 and Ptc are regulated by Ihh (Indian hedgehog, one of the hedgehog family members, with biological properties similar to sonic hedgehog) signaling to regulate the growth rate of cartilage (Vortkamp et al., 1996). In mouse limb development, Shh and Gli3 are located in posterior and anterior region of the limb respectively (Buscher et al., 1997). *The extra toes* (Xt) mutant mouse, which has a null Gli3 mutation, expressed Shh in the posterior as well as anterior of limb. But, in the Xt limb bud, FGF is misexpressed in relation to the ectopic Shh expression domain (Buscher et al., 1997). On the other hand, in vertebrate limb development, there is a positive feedback loop between HH and FGF signals. FGF is induced by the Shh signal and Shh expression is maintained by FGF (Laufer et al., 1994; Perrimon et al.,1995). Such a feedback loop allows the coordination of mesodermal outgrowth and patterning (Laufer et al., 1994). Thus, FGF and Gli are tightly linked by the HH signal in development.

Based on my evidence, given that *XGli5* mRNA level was induced by FGF, it can be safely hypothesized that XGli5 is involved in the FGF signal transduction pathways to regulate downstream gene expression to determine the pattern of embryonic development.

4.3.2 XGli5 in early mesodermal development

Assuming that my hypothesis (XGli5 is involved in the FGF signal) is correct and given that be high expression of XGli5 is detected at the window of MBT, XGli5 may be involved in mesoderm induction through the FGF signaling pathway. Although more direct evidence is needed, some experiments can link Gli to mesodermal development. First, mouse and *Xenopus Gli* mRNAs are located in mesoderm and ectoderm (Hui et al., 1994; Marine et al., 1997). This expression pattern of *Gli* mRNAs implies that Gli may play a role in mesodermal development. Second, in *Drosophila,* Ci directly regulates Wg (Wingless) transcriptional activation by binding its promoter (Von Ohlen et al., 1997). If Ci-Wg regulation is conserved in vertebrates, Gli might regulate Wg vertebrate homolog, Wnt signals. As we know, FGF can induce ventrolateral mesoderm (Godsave and Slack, 1989). Injection of Xwnt-8 mRNA can induce complete dorsal axis formation (Sokol et

al., 1991). It has been known that Xwnt-8 is a FGF signal modifier that can cause FGF induced animal cells to make a dorsal mesoderm (Christian et al., 1992). XGli5 might mediate this modification because FGF induces XGli5 that may in turn regulate Wnt transcription. But, it requires further study to determine how the XGli5 might regulate the function of Xwnt-8. Third, FGF induces *XGli5* expression in mesodermal induction and is related to the Hedgehog signal pathway. Based on my result, FGF can induce XGli5 in the animal cap assay and after 30 mins of FGF induction, *XGli5* mRNA reaches a steady-state level. With the same method, after FGF treated 30 mins of animal explants, FGF failed to induce Shh expression (Takabatake et al., 1996). Why in mesoderm induction, can FGF induce XGli5 but not Shh? Functional analysis of Gli1 and Gli3 indicates that Gli1 as a transcriptional activator to activate downstream gene such as HNF-3β (Sasaki et al., 1997) and Gli3 as a transcriptional repressor to repress gene expression such as Shh in floor plate cells in neural development (Ruiz I Altaba, 1998). Yoon et al.(1998) predicted that although the Gli C-terminus of VP-16 like amino acid sequence is the transcriptional activation domain, the GF region at the N-terminus of Gli maybe the repressor domain. Because XGli5 also has the GF region, when FGF induces XGli5 expression the

accumulation of XGli5 in mesoderm may repress Shh expression. Therefore, it is possible that FGF cannot induce Shh expression in mesoderm induction.

Although there is a connection between XGli5 and FGF mesoderm induction, In order to identify the function of XGli5 in mesoderm, several crucial experiments will be performed. First, whole mount *in situ* hybridization should be performed to locate the mRNA of *XGli5* in *Xenopus* embryos although XGli3 and XGli4 are found in mesoderm (Marine et al., 1997). I used a 1 Kb of *XGli5* cDNA fragment as a probe to perform whole mount *in situ* hybridization but it did not clearly show an expression pattern of the *XGli5* mRNA.

Second, I have found that like other Gli members, the five zinc finger motif of XGli5 protein binds DNA in a sequence specific manner. To understand function of XGli5 as a transcriptional activator or repressor, cotransfection of XGli5 and a reporter gene in which the Gli binding consensus sequence is added to the promoter region of the reporter gene should be done. According to reporter protein levels, I will predict whether the XGli5 is a transcriptional activator or repressor as Yoon et al (1998) identified human Gli1 as a transcriptional activator.

The final targets of FGF signaling are those genes that can immediately respond FGF. Recently, a novel FGF early response gene, er-1, which encodes protein as a transcriptional factor has been identified and may play an important role in mediation of FGF signal in early mesoderm induction (Paterno et al., 1997). To know whether XGli5 is aslo an early response gene, Cycloheximide could be used to inhibit *de novo* protein synthesis before FGF induction in animal caps and XGli5 RT-PCR will be performed. To illustrate that FGF can directly induce XGli5 in mesoderm induction, FGF receptor dominant negative constructs would be made and mRNA from this dominant negative FGF receptor will be injected into fertilized eggs. Comparison of the *XGli5* mRNA levels of wild type and dominant negative FGF receptor embryos should demonstrate whether XGli5 is directly involved in the FGF signaling pathway.

Third, in *Drosophila*, Ci directly regulates *Wingless* (*wg*) transcriptional activation by binding its promoter (Von Ohlen et al., 1997). If the *ci-wg* regulation is conserved in vertebrates, What is the function of XGli5 in Xwnt-8 signal pathway? Xwnt-8 can modify FGF signal and change FGF induced ventral mesoderm to dorsal mesoderm (Chrintian, 1992). I would perform dominant

negative XGli5 experiment to see whether it will alter Xwnt-8 and FGF mesoderm induction pattern.

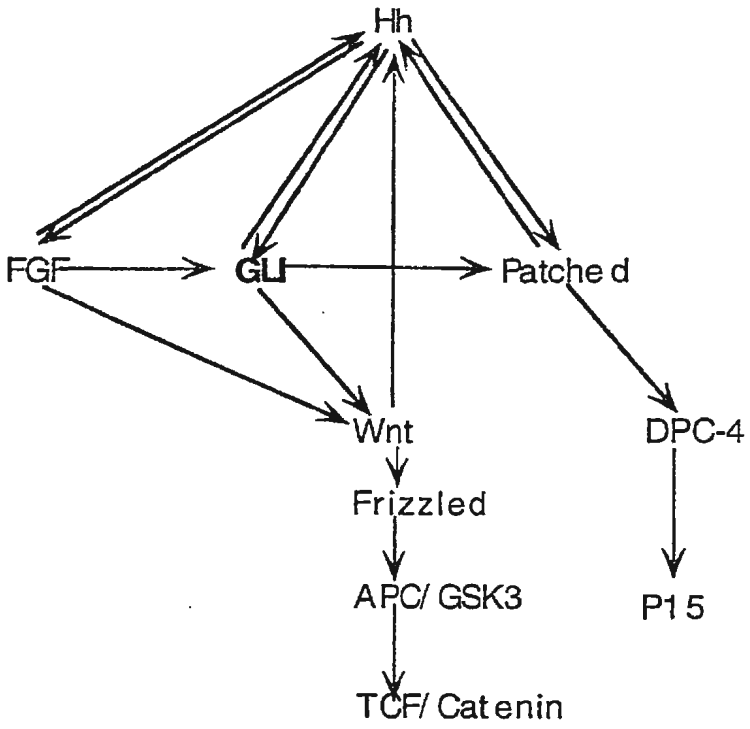
In summary, I cloned a 2.1 Kb 5' of the *XGli5* cDNA, a putatively new member of Gli family, which encodes a 190 Kda protein with two functional domains: the five zinc finger motif and GF region. XGli5 GST fusion protein with zinc finger motifs can specifically bind the DNA sequence 5'-gaccaccca- 3' which is identical with the core DNA binding sequences of other Gli proteins. The GF region, located at the N-terminus of XGli5, may regulate a downstream gene as a negative repressor. During embryonic development, *XGli5* mRNA accumulates to a steady-state level follwing MBT. Since FGF is a typical mesoderm inducer and can induce *XGli5* expression at early embryonic development, it is possible that XGli5 is involved in mesoderm induction by FGF.

Signal transduction in early *Xenopus* embryonic development is very complicated. Although many genes and gene families have been identified and partial function of these genes are understood, more questions have emerged and need to be answered. The Gli family is one such group. According to current research, a predicted signaling pathway of Gli-related gene interactions in embryonic development of vertebrates is as following Fig. 4.1.

4.4 Future directions

1. Clone the remaining 3í 6 Kb of *XGli5* and obtain the full length *XGli5* gene.
2. Using *in situ* hybridization to determine *XGli5* expression and distribution.
3. To test the function of this gene, we will make several dominant-negative mutant constructs using different functional regions of the gene, and microinject these into embryos in order to observe alteration in the phenotype of embryos.

Fig.4.1. Predicted signaling pathway of Gli related genes in embryonic development. Arrow head indicates the signal direction



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Appendix 1. Table of NAM(Normal Amphibian Medium)

Salt	100 ml 1XNAM/20	1 litre 10XNAM
NaCl	-	65g
KCl	-	1.5g
Ca(N03)2.4H20	-	2.4g
MgSO4.7H2)	-	2.5g
NaBicarb	1	-
EDTA(0.5M,pH8.0)	-	2 ml
DH2O	93.75 ml	887 ml
HEPES(1M pH 7.5)	-	100 ml
10X NAM salt	5 ml	-
Gentamycin	0.25g	-

Appendix 2 Table of LB medium and LB agar

LB Medium (PH 7.5)		LB agar	
Bacto-trypton	1%		
Bacto-yeast extract	0.5%	Agar	1.5g
NaCl	1.5%	LB medium	100ml

Appendix 3. Table of 2 XYT-G medium

Reagent	Final concentration(g/l)
Tryptone	16
Yeast extract	10
NaCl	5
Glucose	20

Appendix 4 Buffers and solution

1. 1X PBS

   0.01 M NaHPO4
   0.01 M NaHPO4
   0.15 M NaClpH 7.5

2. 10X MOPS

   0.2 M MOPS pH 7.5
   0.05 M Sodium acetate
   0.01 M EDTA

3. 10X TBE

   1 M Tris-HCl pH 8.0
   0.9 M Boric acid
   0.02 EDTA

4. 20X SSC

   3 M NaCl
   0.3 M Naeitrate pH 7.2

5. 100X Denhardtís

   2% BSA,
   2% Ficoll
   2% Polyvinglpyrrolidon

6. DNAase Buffer

40 mM Tris-HCl pH 8.0
10 mM NaCl
6 mM MgCl2
1 mM CaCl2

7. Gel Mobility shift Assay primer annealing buffer

40 mM Tris-HCl pH 7.5,
20 mM MgCl2
50 mM NaCl

8. Loading Buffer

50% Glycerol
1 mM EDTA
0.25% Bromophenol blue
0.25% Xylene cyanol FF

9. NETS solution

5 M NaCl
0.5 M EDTA
1 M Tris-HCl pH 7.5
10% SDS

10. TE Buffer

10 mM Tris-HCl
1 mM EDTA

11. *Xenopus* embryonic RNA extraction buffer

3 M LiCl, 6M Urea
10 mM NaoAC pH 7.5
10.1% SDS
0.5% 2-mercaptoethanol v/v)