Aus dem Institut für Entwicklungsgenetik
des GSF-Forschungszentrums für Umwelt und Gesundheit, GmbH
Direktor: Prof. Dr. Wolfgang Wurst

Anfertigung unter der Leitung von Prof. Dr. Jochen Graw

Vorgelegt über den Lehrstuhl für Molekulare Tierzucht und Biotechnologie
Der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München
Vorstand: Prof. Dr. Eckhard Wolf

Untersuchung und Charakterisierung des *Hic2*-Gens der Maus

Inaugural-Dissertation
Zur Erlangung der tiermedizinischen Doktorwürde
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

von

Aleksandra Terzic

aus Sarajevo/Bosnia und Herzegowina

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I dedicate this work to my parents

1. Introduction

1.1 Promoter CpG island hypermethylation and gene expression

Approximately 50% of mammalian gene promoters are associated with one or more CpG islands (Ioshikhes and Zhang, 2000).
Clusters of CpG dinucleotides or "CpG islands" (Gardiner-Garden M. and Frommer M., 1987) are present in the promoter and exonic regions of approximately to 50% of mammalian genes.

By contrast, other regions of the mammalian genome contain few CpG dinucleotides and these are largely methylated (Larsen et al., 1992). The decreased occurrence of CpG is best explained by the fact that methylated cytosines are mutational hotspots leading to CpG depletion (Coulondre et al., 1978). A large number of experiments have shown that methylation of promoter CpG islands plays an important role in gene silencing (Bird, 2002), genomic imprinting, X-chromosome inactivation, the silencing of intragenomic parasites, and carcinogenesis (Baylin et al., 1998).

The extent of aberrant promoter hypermethylation and its association with loss of gene function in cancer suggests that CpG island methylation is an important mechanism in inactivating tumor suppressor genes (TSGs) (Narayan et al., 2003).

For tumor suppressor genes, promoter hypermethylation is very important and can change alone ever designate true tumor suppressor gene function (Baylin at al., 2001). Aberrant promoter methylation changes that occur in cancer are associated with transcriptional repression and loss of function of the gene by interrupting the binding of proteins involved in transcription activator complex (Jones and Baylin, 2002). There are a lot of the genes which are affected by promoter CpG island methylation in aging and / or cancer such as *BRCA1*, *HIC1*, *MYOD1*, *p57, PAX6*, *WT1*.

1.2 POZ-Zincfinger Proteins

The C_2H_2 zinc finger domain defines a family of genes that has been expanded independently in each of the major eukaryotic lineages including yeast, worm, fly, and human (Lander et al., 2001 and Venter et al., 2001). In humans, the result of the expansion has been remarkable, in that more than 700 C_2H_2 zinc finger domain-containing genes are predicted to exist, making it the second most populous family in the human genome (Lander et al., 2001). The C_2H_2 zinc finger is a small peptide domain with a secondary structure stabilized by a zinc ion bound to the conserved cysteine and histidine residues of the finger (Pavletich and Pabo, 1991). The primary role of these regulators is to bind to specific DNA segments via their zinc fingers and interact with other cellular factors to control the transcription of target genes. Such conserved modules found at the N-terminus of C_2H_2 zinc finger transcription factors include the BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/poxvirus and Zinc finger) (Bardwell and Treisman, 1994).

The C_2H_2 zinc fingers represent one of the most common types of DNA-binding domains. This large family of transcription factors can be further divided in to functional subclasses sharing a divergent and highly conserved module (Collins et al., 2001). One such module is the BTB/POZ (for bric a brac, tramtrack, Broad-Complex/poxviruses and zinc fingers) domain an approximately 120 amino acid domain identified in various species from yeast to human. It occurs at the N-terminus of developmentally regulated transcription factors containing C_2H_2 zinc fingers as well as in actin-binding proteins (Bardwell 1994; Albagli et al.,1995). The BTB/POZ domain has even been found in a new subfamily of Rho proteins conserved from *Dictyostelium* to mammals (Dhordain et al., 1995).

The PLZF BTB/POZ domain (Ahmad et al., 1998), encompass most of the residues involved in the dimerization of this domain are well conserved.

The BTB/POZ is an evolutionarily conserved domain found in most organisms, including human, mouse, fly, and yeast (Lander et al., 2001 and Venter et al., 2001). This domain is capable of mediating homo- and heterodimerization and several BTB/POZ-containing proteins silence gene expression by distinct recruitment mechanisms. For example, the BTB/POZ-proteins BCL6 (B cell lymphoma) and PLZF (promyelocytic leukemia zinc finger) repress transcription by recruiting SMRT (silencing mediator for retinoid and thyroid hormone receptors)/N-CoR (nuclear receptor corepressor)-mSin3A-histone deacetylase

complexes (David et al., 1998; Dhordain et al., 1998; Huynh and Bardwell 1998, and Wong and Privalsky, 1998), whereas *HIC-1* (hypermethylated in cancer) mediates transcriptional repression by both histone deacetylase-dependent and independent mechanisms that involve binding to the corepressor, CtBP (C-terminal binding protein) (Deltour et al., 1999 ; 2002). Several human BTB/POZ and C_2H_2 zinc fingers proteins have been implicated in malignant diseases. BC-6 (B-cell lymphoma-6) and PLZF (promyelocytic leukemia zinc finger) are implicated in translocations associated respectively with diffuse large cell lymphomas and promyelocytic leukemias (Albagli et al., 1995).

How important are zinc finger proteins?

Zn fingers proteins seem to regulate central nervous system development, may function between DNA-RNA hybrids, and may be oncoproteins in leukemogenesis. BTB proteins are transcriptional regulators found at the N-terminus of 5-10% C_2H_2 Zinc finger. There is a large number of human BTB genes which vary in the different number of zinc fingers: *PLZF* (9ZnF), *BCL-6* (6ZnF), *HIC-1* (5ZnF), *MIZ-1* (13ZnF), *KIAA0352* (9ZnF), *RP58* (4ZnF), *hcKROX* (4ZnF).

1.3 *HIC1* (Hypermethylated in cancer 1 gene) gene, human *HIC2* homologue

HIC1 encodes a protein with five Krüppel-like C_2H_2 zinc fingers in the C terminus and a protein-protein interaction domain called the BTB/POZ domain at the N terminus. Many proteins with BTB/POZ domains and zinc fingers are transcriptional repressors, such as *Drosophila* Tramtrack and human BCL6 and PLZF, which are associated with translocations in some leukemia (Albagli et al., 1995, Bardwell et al., 1994). *HIC1* (hypermethylated in cancer) is a candidate tumor suppressor gene identified because of its association with a "CpG" island at 17p13.3 that is aberrantly hypermethylated and transcriptionaly inactivated in several common types of human cancer (Makos-Wales et al., 1995) and lost in the Miller-Dieker syndrome (Carter et al., 2000).

HIC1 significantly decreases the clonogenic survival of various cancer cell lines, and its expression is up regulated by p53 through a functional p53 binding site in its 5' flanking region (Guerardel et al., 2001, Makos-Wales et al., 1995). HIC1 is a transcriptional repressor

(Deltour 1999) as is its avian homologue, FBP-B (F1 binding protein B), (Liu et al., 1994). Both the HIC1 and FBP-B BTB/POZ domains are autonomous transcriptional repression

domains but, in sharp contrast with those of BCL-6 and PLZF, they are unable to recruit SMRT/NCoR-mSin3A-HDAC complexes and are insensitive to trichostatin A (TSA), a specific inhibitor of HDACs (Deltour, 1999).

1.4 *Hic1* a mouse gene

The *Hic1* gene encodes a zinc-finger protein with a poxvirus and zinc-finger (POZ) domain and has been mapped to mouse chromosome 11 in a region exhibiting conserved synteny to human chromosome 17. The deduced *Hic1* protein consists of 892 amino acids including a POZ domain and five C_2H_2 zinc fingers at the C terminus. During embryonic development, *Hic1* is expressed in mesenchymes of the sclerotome, lateral body wall, limb and cranio-facial regions surrounding the outgrow of peripheral nerves during their differentiation. During fetal development, *Hic1* additionally is expressed in mesenchymes apposed to precartilaginous condensations, at many interfaces to budding epithelia of inner organs, and weakly in muscles.

Activation of *Hic1* expression was observed in the embryonic anlagen of many tissues displaying anomalies in Miller-Dieker syndrome (MDS) patients (Grimm et al., 1999).Together with perinatal death and a reduction in overall size, *HIC1*$^{-/-}$ mouse embryos have other developmental anomalies resembling those found in MDS patients (Carter et al., 2000), and parts of the *HIC1* expression territories overlap with regions exhibiting abnormalities in MDS patients (Grimm et al., 1999).

1.5 *HIC2* (Hypermethylated in cancer 2) a Human Homologue of the Putative Tumor Suppressor Gene *HIC1*

By sequencing clones obtained from a size-fractionated fetal brain cDNA library, Kikuno et al. (1999) cloned HIC2, which they designated KIAA1020.Deltour et al. (2001) extended the sequence of KIAA1020 in the 5-prime direction by comparison with a genomic clone and by RT-PCR of a cerebellum cDNA library. The deduced full-length HIC2 protein contains 615 amino acids. It has an N-terminal BTB/POZ domain, a conserved GLDLSKK/R polypeptide, and 5 C-terminal Kruppel-type C2H2 fingers, 4 of which are grouped and are separated by a typical 7- to 8-amino acid link. HIC2 shares more than 80% homology with HIC1 through the BTB/POZ and zinc finger domains, and both proteins have identical GLDLSKK/R motifs. This motif is highly related to the consensus motif PxDLSxK/R firs identified in the C-terminal part of the E1A protein (Schaeper et al., 1995), later in numerous transcriptional repressors in *Drosophila* (Nibu et al., 1998) and vertebrates (Criqui et al., 1999) able to bind to the CtBP (C-terminal Binding Protein) corepressor (Deltour et al., 2002). It encodes a hypermethylated in cancer 2 like family member.

By transient transfection in COS7 cells, Deltour et al. (2001) found that HIC2 colocalize with HIC1 onto nuclear dots. Their BTB/POZ domains heterodimerize and repressed transcription from a reporter plasmid. Inhibitor studies indicated that HIC1 and HIC2 do not recruit HDAC (a histone deacetylase) containing complexes. The central GLDLSKK/R motif was found to interact with C-terminal-binding protein. The HIC2 gene contains 2 exons and spans about 6.1 kb. The promoter region contains a putative TATA box. By genomic sequence analysis, the *HIC2* gene was localised to chromosome 22q11.2. (Deltour et al., 2001).

RT-PCR ELISA revealed highest expression in cerebellum, with intermediate levels in several other brain regions; no expression was detected in substantia nigra. In whole tissues, expression was highest in liver, followed by heart, brain, lung, kidney, testis, ovary, fetal liver, and fetal brain. Little or no expression was detected in skeletal muscle, pancreas, and spleen.

1.6 *γFBP* the avian homologue of the *HIC1* and *HIC2* gene

Database searches with the murine Hic1 protein sequence revealed similarities to the chicken γF-crystallin binding protein (γFBP). Similar to Hic1 and HIC1, γFBP encodes also a protein with a POZ domain and five zinc fingers (Grimm et al., 1999).

γ*FBP* was expressed during lens differentiation and functionally implicated in the regulation of the γ*F-Crystallin* (*Crygf*) gene (Liu et al. 1994). They have isolated three chicken cDNAs that encode proteins capable of binding specifically to the Gamma F-1-binding motif. These three cDNAs represent differential splicing products from a single gene, γFBP. The protein isoforms encoded by two of these cDNAs differ in their ability to modulate the activity of promoters containing the gamma F-1-binding motif. Among them, γFBP-B functions as a transcriptional repressor in lens cells, and its expression is developmentally regulated during lens development, suggesting a role for this isoform in the spatial regulation of γF-crystallin gene expression.

The expression of the different mRNA transcripts is differentially regulated in various tissues. The three distinct cDNAs presumably correspond to differentially spliced products of a single gene, since the original cDNA isolated. (Liu et al. 1994).

Isoforms are in a tissue-specific maner : γ*FBP-A* highly in kidney and lung, γ*FBP-B* higher in the lens, retina, stomach, extremely low in heart, muscle and lung, γ*FBP-C* weakly in heart, kidney and lens.

Also is known the case of *HIC1* and γ*FBP-B, HIC1* appears unique because it contains a 13-aa insertion acquired late in evolution, because it is not found in its avian homologue, γ*FBP-B*, a transcriptional repressor of the γ*F*-crystallin gene. This insertion, located in a conserved region involved in the dimerization and scaffolding of the BTB/POZ domain, mainly affects slightly the ability of the *HIC1* and γ*FBP-B* to homo- and heterodimerize *in vivo*, both the *HIC1* and γ*FBP-B* domains behave as autonomous transcriptional repression domains (Deltour et al., 1999). During somatic differentiation, FBP expression becomes restricted to the sclerotome (Liu et al., 1994).

1.7 *HIC1* and the Miller-Dieker syndrome

Most genetic disorders are the result of a mutation in a single gene. However, one of the most difficult problems ahead is to find out how genes contribute to diseases that have a complex pattern of inheritance, such as in the cases of diabetes, asthma, cancer and mental illness.

Grimm et al., 1999, observed activation of mouse *Hic1* expression in the embryonic anlagen of many tissues displaying anomalies in Miller-Dieker syndrome (MDS) patients. Besides lissencephaly, MDS patients exhibit facial dysmorphism and frequently additional birth defects, e.g., anomalies of the heart, kidney, gastrointestinal tract, and the limbs.

Thus, *HIC1* activity may correlate with the defective development of the nose, jaws, extremities, gastrointestinal tract, and kidney in MDS patients. The location of *HIC1* in the Miller-Dieker syndrome critical deletion region on 17p13.3 makes it a candidate gene for involvement in the MDS gene deletion syndrome.

To study the function of murine *Hic1* in development, Carter et al., 2000, created *Hic1*-deficient mice. They found that these animals died perinatal and exhibited varying combinations of gross developmental defects throughout the second half of development, including acrania, exencephaly, cleft palate, limb anomalies, and omphalocele. These abnormalities demonstrated a role for *Hic1* in the development of structures affected in the Miller-Dieker syndrome, and provided functional evidence to strengthen its candidacy as a gene involved in that disorder.

1.8 Gene map locus 22q11.21

There are some of interesting genetic disorders which are located on the human chromosome 22, near the location of the *HIC2* gene. One of them is 22q11 deletion and chronic myeloid leukemia.

22q11 deletion is associated with Velo-Cardio-Facial Syndrome (VCFS, Sprintzen Syndrome), DiGeorge Sequence, CATCH 22, Conotruncal Anomaly Face syndrome (CAFS) (Japan) and sometimes seen in Opitz G/BBB syndrome, CHARGE Association and Cayler-Cardio-Facial Syndrome.

Findings provide evidence that people with a 22q11 deletion have disrupted brain development which may involve abnormal neural crest cell migration, (Van Amelsvoort et al., 2001).
Velo-cardio-facial syndrome (VCFS) is a common genetic disorder associated with variably sized deletions at chromosome 22q11 (Scambler et al., 1992). Characteristic physical features include cleft lip and/or palate, coronotruncal heart defects, ear anomalies and facial features such as a prominent nasal bridge (Shprintzen et al., 1978). Neurodevelopment abnormalities include dysarthric speech and ocular abnormalities, (Golding-Kushner et al, 1985; Mansour et al, 1987; Swillen et al, 1997). Qualitative neuroimaging studies in VCFS reported cerebellar atrophy, agenesis of the corpus callosum, white matter hyperintensities (WMHIs), cavum septum pellucidum/vergae and cerebral atrophy (Mitnick et al., 1994; Lynch et al., 1995; Chow et al., 1999). 22q11- presents several features, some of these may include: eye problems, scoliosis, low muscle tone, microcephaly, cleft palate, immune system problems, gastro-intestinal problems.

Chronic myeloid leukemia is fundamentally a genetic disorder, specifically a somatic cell genetic disorder, which has environmental causes such as irradiation and chemical (e.g., benzene) exposure (Jacobs, 1989).
Prakash and Yunis (1984) located the breakpoints in CML to subbands 22q11.21 and 9q34.1. Although the position of the breakpoint in chromosome 9 is quite variable, the breakpoint in chromosome 22 is clustered in an area called bcr for breakpoint cluster region.

Deletion on chromosome 22q11 is associated with brain abnormalities that are most likely neurodevelopment (Van Amelsvoort et al., 2001)

1.9 Tumor suppressor gene

A tumor suppressor gene is a gene that reduces the probability that a cell in a multicellular organism will turn into a tumor cell. A mutation or deletion of such a gene will increase the probability of a tumor. Tumor suppressor genes, or more precisely, the proteins they code for, have a dampening or repressive effect on the regulation of the cell cycle.

This is basically done by the tumor suppression genes/proteins in three ways:

1. Repression of genes they are essential for continuing of the cell cycle. If these genes are not expressed, the cell cycle will not continue, effectively inhibiting cell division.
2. Coupling the cell cycle to DNA damage. As long as there is damaged DNA in the cell, it should not divide. If the damage can be repaired, the cell cycle can continue.
3. If the damage can not be repaired, the cell should initiate apoptosis, the programmed cell death, to remove the threat it poses for the greater good of the organism.

The first tumor suppressor protein discovered was the pRb protein in human retinoblastoma. An important tumor suppressor is the p53 gene (Wikipedia, the free encyclopedia).

Tumor suppressor gene protein product inhibits mitosis. They behave as recessives; that is, as long as the cell contains one normal allele, tumor suppression continues. (Oncogenes, by contrast, behave as dominants; one defective allele can predispose the cell to tumor formation). Because tumor suppressor genes are recessive, cells that contain one normal and one mutated gene that is, are heterozygous still behave normally. However, there are several mechanisms which can cause a cell to lose its normal gene and thus be predisposed to develop into a tumor.

All of these result in a "loss of heterozygosity" or "LOH". Mechanisms of LOH:

1. Deletion of the normal allele; the chromosome arm containing the normal allele; the entire chromosome containing the normal allele (resulting in aneuploidy).
2. Loss of the chromosome containing the normal allele followed by duplication of the chromosome containing the mutated allele.
3. Mitotic recombination.
4. Gene conversion

Kimball's Biology Pages http://users.rcn.com/jkimball.ma.ultranet/BiologyPages

Tumor suppressor genes normally function to inhibit the cell growth and to prevent the development of tumors. Some tumor suppressor genes are localized on human chromosome 22:
EP300, involved with some colorectal, breast and pancreatic cancers, located on 22q13.2, *NF2*- neurofibromatosis type 2 genes, involved in bilateral acoustic neuromas, meningiomas, schwannomas, located on 22q12.2, *SMARCB1*, involved with malignant rhabdoid tumors, located on 22q11.23

P53 is the prototypic tumor suppressor gene that is well suited as a molecular link between the causes of cancer, i.e., carcinogenic chemical and physical agents and certain viruses, and the development of clinical cancer. The *p53* tumor suppressor gene is mutated in the majority of human cancers. Genetic analysis of human cancer is providing clues to the etiology of these diverse tumors and to the functions of the *p53* gene. Some of the mutations in the p53 gene reject endogenous causes of cancer, whereas others are characteristic of carcinogens found in our environment (Curtis H. 1996). The *p53* gene activity stops the formation of tumors. Mutations in p53 are found in most tumor types. In the cell, *p53* protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2). A function of the tumor suppressor *p53* becomes evident when the gene is mutated or has been eliminated. Gene *p53* is highly conserved and present in "normal" mammalian cells and moreover, even in cells of birds, fishes and amphibian, (Hess and Brander, 1996).

The Wilms tumor suppressor gene ***WT1*** is implicated in the ontogeny of genito-urinary abnormalities, including Denys-Drash syndrome and Wilms tumor of the kidney. *WT1* encodes *Krüppel*-type zinc finger proteins that can regulate the expression of several growth-related genes, apparently by binding to specific DNA sites located within 5' untranslated leader regions as well as 5' promoter sequences. WT1 like the *Krüppel*–type zinc finger protein TFIIIA, regulate gene expression by both transcriptional and posttranscriptional mechanisms (Caricasole et al., 1996). Expression of the Wilms' tumor gene *Wt1* in the epicardium is critical for normal heart development and has a role in the growth of coronary vessels after myocardial infarction (Scholz et al., 2003, Abstract). WT1 was detected in more advanced estrogenreceptor-negative tumors. In this highly malignant subset, the tumor suppressor protein p53, which can physically interact with WT1, was also sometimes detected. Alternative splicing of the WT1 mRNA may regulate gene targeting of the WT1

protein through changes either in its regulatory or zinc-finger domains (Silberstein et al., 1997). The Wilms' tumor gene *Wt1* is known for its important functions during genitourinary and mesothelial formation. *Wt1* is necessary for neuronal development in the vertebrate retina (Wagner et al., 2002).

HIC1 (hypermethylated in cancer 1) a candidate tumor suppressor gene is hypermethylated and silenced in a large number of solid tumors and his methylation seems to be a progression event in hematopoetic neoplasms (Issa J.P. et al., 1997). *HIC1* is a candidate tumor suppressor gene since it significantly decreases the clonogenic survival of various cancer cell lines, and its expression is up regulated by p53 through a functional p53 binding site in its 5' flanking region (Guerardel et al., 2001 and Makos-Wales et al., 1995). Chen et al., (2003) showed that mice disrupted in the germline for only 1 allele of *Hic1* developed many different spontaneous malignant tumors, including a predominance of epithelial cancers in males and lymphomas and sarcomas in females. Complete loss of *Hic1* function in the heterozygous mice seemed to involve dense methylation of the promoter of the remaining wild type allele. They concluded that *HIC1* is a candidate tumor suppressor gene for which loss of function in both mouse and human cancers is associated only with epigenetic modifications.

1.10 Aim of the work

The aim of this work was the characterization of the *Hic2* gene; partial isolation, localization, promoter analysis and embryonic expression of the novel mouse gene *Hic2,* the homologue of humane gene *HIC2*, a candidate for tumor suppressor gene and a way to represent the effect of the outcome of results to science following an animal model, which could give a more light in mechanism of some human diseases.

2 Materials and Methods

2.1 Materials

There were next tools and materials used:

2.1.1 Products

Table 2.1: Tools

Tools	Producer
Agarose-Gelelectrophoresis	GIBCO BRL, Karlsruhe, Germany
Analysis balance	Chyo balance Corp, Japan
Analysis balance (Mettler PL 1200)	Utting, Germany
Bacterial incubator	WT-Binder, Bottmingen, Schweiz
Bacterial shaker	Infors, München, Germany
Bio photometer	Eppendorf, Hamburg, Germany
Cell culture incubator	Heraeus, Hanau, Germany
Cell culture sterile desk	Gelaire, Opera, Italy
Refrigerated centrifuge (Universal 16R)	Hettich, Tuttlingen, Germany
Refrigerated centrifuge (Sorvall RC-5B)	Sorvall, Bad Homburg, Germany
Electrophoresis power supply (ST 304)	Gibco, BRL, France
Hybridisation oven	Hybaid, United Kingdom
Incubator (WTE Binder)	Tuttingen, Germany
Light microscope (Axiolab)	Zeiss, Oberkochen, Germany
Light microscope (Axiovert 35)	Zeiss, Oberkochen, Germany
Light microscope (Axioplan 2)	Zeiss, Oberkochen, Germany
Light microscope (Orthoplan)	Leitz, Wetzlar, Germany
Luminometer (Autolumat LB953)	Berthold, Bundoora, Australia
Microtome (Jung RM 2055)	Leica, Nussloch, Germany
PCR-thermal cycler (Omnigene)	Hybaid, United Kingdom
PCR- thermal cycler (PTC-225)	Biozym, Germany
PCR- thermal cycler (Perkin Elmer 9600)	Norwalh, USA
PCR- thermal cycler (T3)	Biometra, Germany

Table 2.1: Tools

Tools	Producer
pH-Meter (Calimatic 5436)	Bachhofer, Germany
Shaker-incubator (CH-4103)	Bottmingen, Germany
Spectrophotometer (UltrospecIII®)	Amersham, Germany
Stereo microscope (Stemi SVII)	Zeiss, Oberkochen, Germany
Stereo microscope (MZ6)	Leica, Beinsheim, Germany
Stereo microscope (MZ APO)	Leica, Beinsheim, Germany
Thermo mixer (5436)	Eppendorf, Hamburg, Germany
Thermostat 5320	Eppendorf, Hamburg, Germany
Vortex-2genie	Scientific Industries USA

Table 2.2: Cell culture material

Cell culture material	Producer
10 cm Tissue Culture Plate	Becton Dickinson, Heidelberg
6 cm Tissue Culture Plate	Becton Dickinson, Heidelberg
22 mm Tissue Culture Plate (12 wells)	Becton Dickinson, Heidelberg
15 mm Tissue Culture Plate (24 wells)	Nunc, Wiesbaden
15 ml Tubes	Nunc, Wiesbaden
50 ml Tubes	Nunc, Wiesbaden
2 ml Collection tube	Eppendorf, Hamburg
1, 5 ml Collection tube	Eppendorf, Hamburg

In all cell culture experiment was used HEK-293 (human, embryonic, kidney) cell line.

Table 2.3: Bacterial strains

Strain	genetic back ground
DH5α	*deo*R, *end*A1, *gyr*A96, *hsd*R17 ($r^-_k m^+_k$), *rec*A1, *sup*E44, *thi*-1, Δ(*lac*ZY A-*arg*FV169), Φ80*δlac*ZΔM15; (Stratagene)
TopF10'	F' ($lacI^q$, Tn 10 (tet^r)), *mcr*A, Δ(*mrr-hsd*RMS-*mcr*CB)Φ80*lac*Z ΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7696, *gal*U, *gal*K, *rps*L(Str^r), *end*A1, *nup*G; (Invitrogen)
XL1-Blue	MRF'Δ(*mcr*A) 183, Δ(*mcr*CB-*hsd*SMR-*mrr*) 173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*[F' *pro*AB, $laqI^qZ$ΔM15, Tn 10 (tet^r)]; (Stratagene)

All bacterial strains are from the Lab strains *E.coli* K12

Table 2.4: Chemicals for cell culture

Chemicals	Producer
Agarose (low and high melt)	Biozym, Hess. Oldendorf
Ampicilin	Sigma, Deisenhofen, Germany
DNA Molecular weight standard 3 and 8	MBI Ferments, St.Leon-Rot
DNA Ladder 1 kb	MBI Ferments, St.Leon-Rot
dNTP- Set	Pharmacia, Freiburg, Germany
Dual-Luciferase™ Reporter assay System	Promega, Mannheim, Germany
Dulbecco's Eagle-medium	GIBCO BRL, Karlsruhe, Germany
Ethidiumbromide	Peqlab Erlangen, Germany
Foetal calve serum	GIBCO BRL, Karlsruhe
Glycogen	Roche, Mannheim, Germany
IPTG	Peqlab Biotechnologie, Erlangen
NucleoSpin® Extract 2 in 1	Macherey-Nagel, Düren
NucleoSpin® Plasmid	Marcherey-Nagel, Düren
PCR-TOPO Cloning Kit	Invitrogen, Karlsruhe, Germany
Penicillin/Streptomycin	GIBCO BRL, Karlsruhe, Germany
Profection Mammalian Transfection Systems	Promega, Mannheim, Germany

Quiagen DNA Quick Purification Kit	Quiagen, Hilden, Germany
Quiagen Plasmid Maxi Kit	Quiagen, Hilden, Germany
Rapid Ligation Kit	MBI fermentas, St.Leon-Rot
Trypsin/EDTA (10 X), liquid	GIBCO BRL, Karlsruhe
X-gal	Peqlab Biotechnologie, Erlangen

Table 2.5: Plasmids

Plasmid	Producer
pcDNA3.1	Invitrogen, Karlsruhe
pCR.II-TOPO	Invitrogen, Karlsruhe
pRL-SV 40	Promega, Mannheim
pPLLucII	Y. Kamachi (see references)

Table 2.6: Enzymes

Enzyme	Producer
Alkaline Shrimp-Phosphatase	Roche, Mannheim
Klenow- Fragment	MBI fermentas, St. Leon-Rot
Proteinase E	Roth, Karlsruhe
Proteinase K	Roche, Mannheim
Restrictionsendonucleasen	MBI fermentas, St. Leon-Rot
T4-Ligase	MBI fermentas, St. Leon-Rot
Taq-DNA-Polymerase	GIBCO BRL, Karlsruhe

Table 2.7: Solutions for cultivating of *E.coli*

Solution	Ingredients
LB-Medium	1% Bacto-Tryton
	0.5% Bacto-Yeast extracts
	1% NaCl
	With NaOH pH to 7.0 and autoclaved
LB Agar	15 g/l Difco-Agar
	Autoclaved
SOC-Medium	2% Bacto-Trypton
	0.5% Bacto-Yeast extracts
	10 mM NaCl
	2.5 mM KCl
X-Gal	20 mM Glucose (sterile filtrated)
	20 mg/ml X-Gal in Dimethylformamide
	Light protected and stored at –20°C
IPTG	200mg/ml in water, (sterile filtrated)
	Stored at –20°C
Ampicilin	10 mg/ml in water
Kanamycin	10 mg/ml in water

For selection with antibiotics, was used “Selections medium” with 100 µg/ml Ampicilin or Kanamycin. For blue-white selection was added 40 µl X-Gal and 4 µl IPTG

Table 2.8: Solutions for agar electrophoresis

Solution	Ingredients
6 X DNA Loading buffer	30% Glycerol
	0.2% Bromphenolblue
	0.2% Xylencyanol
	Diluted in Tris/HCl (pH 7.5)
TE-Buffer (Tris/EDTA)	10 mM Tris/HCl (pH7.0)
	1 mM EDTA
5 X TBE (Tris/Broat)	0.45 M Tris

	0.45 M Boric acid
	10 mM EDTA
Ethidiumbromide stock solution	10 mg/ml Ethidiumbromide in water
	Light protected stored at 4 °C

Table 2.9: Solutions for Acrylamide electrophoresis

Solutions	Ingredients
Bis-Acrylamide	48.3g Acrylamide
	Or 160 ml of 30% Acrylamide solution
	1.7g Bis-Acrylamide
	Or 85 ml of 2% Bis-Acrylamide solution
10 x TBE	0.9 M TRIS
	0.9 M Boric acid
	0.25 M EDTA
	75 ml Glycerol
Solution was filtrated and stored at 4°C	
For 100 ml of stock Bis-Acrylamide solution was added 36 µl of TEMED and 1 ml of 10% ammonium perisulfate and polymerised at room temperature for 1 hour.	

Table 2.10: Composition of FA (formaldehyde) gel buffers

Buffer	Ingredients
10 X FA gel buffer MOPS	200 mM 3-[N-morpholino] propanesulfonic acid
	50 mM sodium acetate
	10 mM EDTA
	pH to 7.0 with NaOH
1 X FA gel running buffer	100 ml 10 X FA gel buffer
	20 ml 37% formaldehyde
	880 ml DEPC water
5 X RNA loading buffer	16 µl saturated bromphenol blue solution
	80 µl 500 mM EDTA, pH 8.0
	720 µl 37% formaldehyde
	2 ml 100% glycerol

	3084 µl formamide
	4 ml 10 X FA gel buffer
	Added DEPC water to 10 ml
	Stored at 4°C

Table 2.11: Solutions for transfection

Solution	Ingredients	
Polyfect	from Quiagen Transfection Kit	
PBS-Buffer, 10X	80 g	NaCl
	11.44 g	Na_2HPO_4
	2 g	KCl
	2 g	KH_2PO4
Dissolved in 1 liter of sterile, deionised water		
The pH of 1X PBS was 7.4		
DMEM Medium	+ 5 ml	Penicillin/Streptomycin
	+ 50 ml	FKS (foethal calf serum)
DMEM Medium	without serum, proteins and antibiotic	

Table 2.12: Other buffers

Buffer	Ingredients	
PCR Buffer	100 mM	Tris/HCl pH 8.3
	15, 17.5 or 20 mM	$MgCl_2$
	500 mM	KCl
	0.1 %	Glycerine
TEN Buffer	10 mM	Tris/HCl pH 7.5
	1 mM	EDTA
	0.1 N	NaOH
1% SDS	1 g	in 100 ml water

Table 2.13: Chemicals for Northern Blot

Solution	Ingredients
10 x MOPS	0.4 M Morpholinopropanesulfonic acid
	0.1 M Na-acetate-3 x H2O
	10 mM EDTA
pH adjusted to 7.2 with NaOH; stored dark in fridge	
[500 ml: 41.9 g MOPS, 6.8 g NaAc, 10 ml 0.5 M EDTA]	
Loading Buffer	1 x MOPS
	18.5 % Formaldehyde
	50 % Formamide
	4 % Ficoll400
	Bromphenolblue
[For 1 ml of loading buffer : 100 µl 10 x MOPS, 500 µl Formamide, 185 µl Formaldehyde, 40 mg Ficoll400, Bromphenolblue, 215 µl H2O]	
Prehybridization-buffer	5 x SSC
	50 % Formamide
	5 x Denhardt's-1 % SDS
	100 µg/ml Salmon sperm DNA
[For 100 ml of prehybridization buffer: 25 ml 20 x SSC, 50 ml Formamide, 5 ml 100 x Denhardt's, 1 g SDS, 1 ml 10 mg/ml DNA]	
Hybridization-buffer	
Prehybridization buffer with 5 % Dextransulphate (Na-salt, MW 500,000, 50 % stock-solution) and without non-homologous DNA	
100 x Denhardt's solution for 500 ml	10 g Ficoll 400
	10 g polyvinylpyrrolidone MW 360000
	10 g BSA fraction
20 x SSC	3 M NaCl
	0.3 M Na-citrate
[For 1 l of 20x SSC: 175.3 g NaCl, 88.2 g NaCitrate]	

Strip-solution	5 mM Tris/HCl pH 8
	0.2 mM EDTA
	0.05 % Na-pyrophosphate
	0.1 X Denhardt's solution

[500 ml: 2.5 ml 1 M Tris, 200 µl 0.5 M EDTA, 5 ml 5 % NaPP, 1 ml 50 x Denhardt's]

Table 2.14: Buffer solutions for Midi DNA isolation (Commercial)

Buffer	Intergradient
S1:	50 mM Tris/HCl, 10 mM EDTA, 100 µg Rnase A / ml, pH 8.0
S2:	200 mM NaOH, 1% SDS
S3:	2.80 M Kac, pH 5.1
N2:	100 mM Tris, 15%EtOH and 900 mM KCl
	Adjusted with H_3PO_4 to pH 6.3, 0.15% Triton X-100
N3:	Tris, 15% EtOH and 1150 mM KCl adjusted with H_3PO_4 to pH 6.3
N5:	100mM Tris, 15% EtOH and 1000 mM KCl adjusted to pH 8.5

Table 2.15: Solutions for Mouse Tail Genomic DNA isolation

Solution	End concentration	Stock
TNE	10 mM Tris pH 8.0	1 M
(Tris/NaCl/EDTA)	100 mM NaCl	5 M
	1 mM EDTA	0.5 M
SDS	1 %	10 %
Proteinase K	10 mg/ml	100 mg/ml
Protease E	10 mg/ml	100 mg/ml
Phenol/Chloroform/Isoamylalc.	25:24:1	BIMOL, Hamburg
TE	10 mM Tris pH 8.0	1 M
	1 mM EDTA	0.5 M

Table 2.16: Chemicals for RNA probes

Chemicals	Producer
DEPC (Diethyl Pyrocarbonate)	Sigma, St. Louis, USA
DIG RNA Labelling Mix	Roche, Mannheim, Germany
DNAse RNase free	Roche, Mannheim, Germany
RNAse Inhibitor	MBI fermentas, Vilnius Lithuania
RNA polymerase SP6	Roche, Mannheim, Germany
RNA polymerase T3	Roche, Mannheim, Germany
RNA polymerase T7	Roche, Mannheim, Germany
t RNA, (10 µg/µl)	Roche, Mannheim, Germany

All solutions used before and for hybridization were absolutely RNAse-free, used DEPC-H_2O for preparation of RNA-se free solutions, autoclaved for 60 min; DEPC treatment of Tris buffer was twice autoclaved.

Table 2.17: Solutions for in situ hybridisation

Solution	Ingredients
DEPC-H_2O	0.01% DEPC (50 µl/500 ml)
PBS (Phosphate-Buffered Saline)	30 ml NaCl 5M
	15 ml Na-Phosphate Buffer 1M, pH 7.3
	Added to 1l with DEPC water
4% PFA/PBS	4 g PFA (Para formaldehyde)
	100 ml DEPC/PBS
A few drops NaOH 10N; adjusted pH to 6-7 with HCl, used indicator paper	
PBT	PBS
	0.1% Tween 20
tRNA	10 µg/µl in DEPC water
Phenolized 2 x and stored as aliquots at –20°C	
Proteinase K	2 or 20 µg/ml in Proteinase K Buffer
Proteinase K Buffer	10ml 1M Tris/HCl pH 7
	1ml 0.5 M EDTA
	Added to 0.5 l with water

PBT/Glycine	PBS
	Glycine 2 mg/ml
RIPA	2.5 ml SDS 10%
	15 ml NaCl 5 M
	5 ml NP40
	25 ml Deoxycholate 10%
	1 ml EDTA 0.5 M
	25 ml 1 M Tris/HCl pH 8.0
	Added to 500 ml DEPC water
4%PFA/0.2% glutaraldehyde/ PBT	4%PFA/PBT pH 6-7
	400 µl glutaraldehyde 25%
	Adjusted pH to 6-7 with HCl
	Added to 50 ml PBT
Deionised formamide, (Roth)	
20 x SSC	17.53 g NaCl
	8.82 g sodium citrate
	Dissolved in 80 ml DEPC water
	Adjusted to pH 7 with HCl
	Adjusted to 100 ml and autoclaved
Heparin (Sigma)	100 mg/ml in 4 x SSC DEPC
Citric acid	1 M in DEPC water
Hybridisation buffer	5 ml Deionised formamide
	2.5 ml 20 x SSC
	5 µl heparin
	10 µl Tween20
	2.5 ml DEPC water
	Adjusted to pH6 with 1 M citric acid
SSC/Formamide/Tween20	5 ml 20 x SSC
	25 ml FA
	50 µl Tween20
	Added to 50 ml with water
10 x TBS (Tris buffered saline)	8 g NaCl
	0.2 g KCl

	25 ml Tris 1 M, pH 7.5
Added to 100 ml with water	
TBST	added 0.1% Tween20 to aliquot before use
Rnase A Buffer	1ml NaCl 5 M
	100 µl Tris HCl 1 M, pH 7.5
	10 µl Tween20
	8.89 ml water
RNase A (Sigma)	Dissolve RNA-se A at a conc. of 10
	µg /µl in 0.01 M NaAc pH 5.2
Heated to 100°C for 15 min.; Cooled slowly at RT	
Adjusted pH by adding 0.1 volumes of 1 M Tris/HCl pH 7.4	
Stored as aliquots at –20°C	
MAB (Maleic acid buffer)	11.6 g maleic acid 0.1 M
	8.8 g NaCl 0.15 M
	Added 800 ml water
	Adjusted with solid NaOH to pH 7.5
	Added water to 1l
MABT	MAB + 0.1%Tween20

Blocking stock solution

Blocking reagent (Roche) was dissolved in MAB to a final concentration of 10% with shaking and heating either on a heating block or in a microwave oven. This stock solution is autoclaved and stored as aliquots at –20°C subsequently or at 4°C, add 0,1%Tw20 on day of usage

Dig Antibodies coupled to AP	(Roche)
TBST*/Levamisol	TBST
	2 mM Levamisol
*Tris buffered saline-Tween20	
Alkaline Phosphatase Buffer	1 ml NaCl 5M
	2.5 ml MgCl 1M
	50 µl Tween20
	5 ml Tris 1M, pH 9.5
	To 50 ml with water

Staining solution	
BM purple AP substrate	Added 2 mM Levamisole
	Added 0.5-1% Tween20
Centrifuged, pellet was not used	

For embedding of the embryos Histowax (Cambridge Instruments, Nussloch) was used. Paraffin sections of embryos were mounted on SuperFrost® Plus Microscope Slides, (precleaned, ready to use). BM Purple was used to stain paraffin sections and whole embryos. After staining, the slides were embedded with Kaiser's Glycerol gelatine (Merck) and covered with Cover glasses from Marienfeld, Germany

Table 2.18: Primers list

Name	Sequens	Lab number
BACTL l*	5'- GGCTGTATTCCCCTCCATCGTGG- 3'	XO32599
BACTL r*	5'- GCTCATTGTAGAAGGTGTGGTGCCAG- 3'	XO32599
Hic2-3.1	5'- CCACCATGTTCAGTGAGCAAGGAAG - 3'	29340
Hic2-5.1	5'- AAGGTCTGAAGTTTCAGAGGGTGGAGG - 3'	29339
Hic2-Seq1	5'- CCTGTCCCCAGTCCCTTCAC - 3'	32415
Hic2-Seq2	5'- ACGCATGTGCTCTGTGAGGCGGTAC - 3'	32633
Hic2-seq3	5'- TGGCAGGAACCCTTAGATCCTGTGGC - 3'	32634
Hic2kleinT3-1	5'- TGAGCCCACTGAGGTTCCACACTC - 3'	33006
Hic2-511-R1	5'- ATACGATGCACTGGGGCCACC - 3'	33007
F08seq1	5'- CCAGCCACATGCGGCAACAC - 3'	33854
F08seq2	5'- CTCTGACACATGCAGCTCCCGG - 3'	33855
F08seq3	5'- GGAGCTGCATGTGTCAGAGGTT - 3'	33985
F08seq4	5'- ATCGGAGGACCGAAGGAGCTAA - 3'	34159
F08seq5	5'- CCGATCAGCCAAACGTCTCTTC - 3'	34296
F08seq6	5'- CGTCCTCAAGCTGCTCTTGTG - 3'	34445
F08seq7	5'- CCACTTAAACCGTATAGCCAACAA - 3'	34576
Hic2-F16-ns	5'- CTTCAGTTTGCGGCGACAGAGG - 3'	35645
Hic2-klns-2	5'- ACCCTGCCGCTTACCGGATAC - 3'	36182
F16-07-n	5'- TCGAGCCTGGATGACAGAGGC - 3'	36623
F16-45-n	5'- GACCGTGTCCTCTCATTTCCTGC - 3'	36624
Neu_seq	5'– TCTGATTCCCAGGATCTGATGCC – 3'	37039
F16-45-n1	5'– GACCGTGCCTCTCATTTCCTGCC – 3'	37040

F16-45-n2	5‘- GACCGTGCTCTCTCATTTCCTGCC- 3‘	37353
Hic2-NB-3	5‘- TGCACTGCAAAGACACAAGCACAC- 3‘	37400
Hic2-NB-5	5‘- GCCAAAGACCCGTGGGAGC - 3‘	37401
Hic2-ISS1-3	5‘- GGCCCAGAGGGCTCTTTAGTTGC - 3‘	37402
Hic2-ISS2-3	5‘- CACGAGAGCAAGAGTACCGATGGC - 3‘	37404
Hic2-ISS2-5	5‘- GGAACAGTCAGGGTGACCCATTG - 3‘	37403
Hic2-F16-ns3	5‘- TGGCCCACAACCTCTGTTCTATCC- 3‘	37473
PCR-ns-n	5‘– GGCACCACCTGTGGGCA- 3‘	37571
PCR-ns-c	5‘- AGGTTCTCTTTGTGACCTCTGACC - 3‘	37572
Hic2-ns‘-4	5‘- GAGACAGGATCTCCCTTTGGAACC - 3‘	38091
Hic2ML	5’- CAGGTATGAAAGAAGTCATTCACAG - 3’	40713
Hic2MR	5’- TTAGGTATTTAGCTCATTTACATTTCC – 3’	40714
EX-2- L	5‘- ATGGAGCTTCCCAGCCA- 3‘	41914
EX-2-R1	5‘- TGAACTTGCCTCCACAGAGC - 3‘	41915
EX-2-R2	5‘- CTGCTCCAGGCACCACTT - 3‘	41916
EX-2-L-1	5‘- AGCACTCTACTCACTGCTGCC -3‘	41957
EX-2-L-2	5‘- GTGCTGGTGGAGGTGGC - 3‘	41958
EX-2-R-1	5‘- CAGGCAGGGCTCAGAATC- 3‘	41959
Son1-R	5‘- CCACTACTCCCATTGGTGCTGG- 3‘	42030
Son1-L	5‘– GCAAACTGAAGCGAGCTGGC - 3‘	42029
Son2-L	5‘- CTGAGTGACAGCCAGCGAGAGTC- 3‘	42031
Son2-R	5‘- CTCTGCCCACTGTCCTCACTCC- 3‘	42032
Son3-Ra	5‘- CCACTCTGCCCACTGTCCTCAC - 3‘	42033
Son3-Rb	5‘- TGAATGCCGGAGGCTCTTGC - 3‘	42034
IEx1-1D	5’- GCAGCTAGGTGGCATTATGTAG - 3’	44117
Prom-L-1	5’ – CAGTAGAGGCAGATTGGCTC - 3’	44116
IEx1-3L	5’- CAGAAGAGCCCTGTTGCC - 3’	42576
IEx1-5L	5’- CCCTGCTTGTTCCAGGG - 3’	42577
IEx1-6L	5’- CCAGGTCTGGTCACATTCC - 3’	42579
IEx1-7L	5’- GAGGTCCTGAAGAACCATGCT - 3’	43773

The primers were synthesised from UTZ Linzner from the AG BIODV/IEG, GSF or MWG BIOTECH AG Company (Ebersberg, Germany). * MWG number.

2.2 Methods

2.2.1 Polymerase Chain Reaction PCR

2.2.1.1 PCR

The purpose of a PCR is to make a huge number of pieces of DNA (Don et al., 1991). This is necessary to have enough starting template for sequencing or cloning. This was done with an automated cycler (Perkin Elmer, Stratagene, Biozym), which heated and cooled the tubes with reaction mixture in a very short time. The cycling reactions were:

1. Denaturation
2. Annealing
3. Extension

Denaturation was done at 94 -95°C for 1-2 minutes. During this step the double strand melts to single stranded DNA (all enzymatic reactions were stopped).

Annealing was done between 49-60°C. Extension was at 72°C, which is an optimal temperature for the Taq DNA polymerase and synthesis of double strands.

Components for PCR-pool were: DNA (2 µl recombinant plasmid DNA, 1µl genomic DNA), 0.2 mM dNTP, 15-20 mM $MgCl_2$ 10 X Taq-Buffer, 1 U Taq-Polymerase in one volume from 20 µl. It was possible to add some additives like DMSO (2-10%) and Q-solution, especially for template DNA with a high GC content.

Cycle	Reaction	Time	Temperature	Repeats
1	Denaturation	1-2 min	95°C	1 X
2	Denaturation	0.5-1 min	95°C	35-40 X
	Annealing	0.5-1 min	49-60°C	
	Extension	0.5-2 min	72°C	
3	Extension	10-15 min	72°C	1 X

After PCR, the products were assessed by Agarose gel electrophoresis. Amplification products were derived from the gel, extracted and used as samples for sequencing and for cloning.

2.2.1.2 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is the sensitive technique for m-RNA detection. The technique consists of two parts:

1. synthesis of cDNA from RNA by reverse transcription (RT)
2. amplification of a specific cDNA by polymerase chain reaction (PCR)

First consideration for synthesis of cDNA was RNA isolation, which should be of high quality and free of genomic DNA contamination. RT-PCR begins with reverse transcription reaction used an RNA template, primer (random or oligo dT), rNTPs, buffer and a reverse transcriptase.

Generating full-length first-strand cDNA from mRNA was done with Ready To Go™ T-Primed First-Strand Kit. This kit has been designed to provide all the reagents necessary to generate full-length first strand cDNA from mRNA template using an oligo (dT) Primer containing a *NotI* restriction site. For cDNA synthesis it was used about 5 µg of total RNA. The sample was brought to a volume of 33µl in RNase-free micro centrifuge tube using DEPC-treated water, and heated to 65°C for 5 minutes just like Reaction Mix too. Both were transferred to a 37°C for 5 min. RNA solution was transferred to the First strand Reaction Mix and was not mixed. It was incubated at 37°C for 5 min and mixed gently and centrifuged 1 min at 13000 rpm. After was incubated at 37°C for 60 minutes. The completed first-strand reaction was ready for PCR amplification.

RNA was from different tissues and embryonic stages: 9.5, 10.5, 12.5, 13.5, 15.5, 17.5 and new born pups. Tissues used were from brain, eye, liver, kidney, retina, heart, and lung.

Components for RT- PCR-pool were: cDNA 1-2 µl, 0.2 mM dNTP, 17.5-22 mM $MgCl_2$ 10 X Taq-Buffer, 1 U Taq-Polymerase in one volume from 20 µl. It was added Q-solution.

Cycle	Reaction	Time	Temperature	Repeats
1	Denaturation	1-2 min	95°C	1 X
2	Denaturation	0.5-1 min	95°C	35-40 X
	Annealing	0.5-1 min	56-60°C	
	Extension	0.5-2 min	72°C	
3	Extension	10-15 min	72°C	1 X

After PCR reaction the products were assessed by Agarose gel electrophoresis. Some of them were derived from the gel and used as template for probes for RNA In situ hybridisation or for cloning in special vector for cDNA.

2.2.2 Gel Electrophoresis

2.2.2.1 Acrylamide gel electrophoresis

Acrylamide gels were prepared for analysis of PCR and digested products.
Mixed stock solution containing Acrylamide: Bis-Acrylamide was prepared with 48.3 g of Acrylamide or 160 ml of 30% Acrylamide solution, 1.7g of Bis-Acrylamide or 85 ml of 2% Bis-Acrylamide solution, 50 ml of 10 x TBE which contained 0.9 M Trizma base, 0.9 M Boric acid, 0.25 M EDTA, 75 ml of Glycerol and added water to 500ml. Solution was filtrated and stored at 4°C 100ml of the stock solution was taken and 36 µl of TEMED and 1ml of 10% ammonium perisulfate was added and polymerised at room temperature for 1 hour between two glass plates.
In 1 X TBE buffer at 80 V gel was runned about 4 hours.

2.2.2.2 Agarose gel electrophoresis

Agarose gels were prepared for analysis of PCR and digested products. The gel was polymerised at room temperature for 15 minutes. For PCR product extraction small DNA Low Melt Agarose was used. Agarose gel was used for test of isolated RNA and prepared *In-situ probes.* In that case Ethidiumbromide was added inside, 2 µl in 100 ml gel-pool; voltage was 200V for 15-25 minutes.

2.2.2.3 Preparation of denaturing- agarose gels (RNA)

The integrity and size distribution of total RNA was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining. 28S and 23S ribosomal RNA bands were presented at approximately twice the amounts of the 18S and 16S RNA. Gel was prepared with Agarose, and runned in running buffer. RNA sample was prepared with 40µl of RNA and 10 µl of 5 X loading buffer.

2.2.3 Computer Sequence analysis

For sequences analyses different programs were used

Program	analysis
Vector NTI	primers
Contig-express	Contig, new molecules with DNA, cDNA
NCBI	EST-s analysis
ORF Finder (NCBI)	open reading frame
Genescan	exon-intron position
WEBGENE	CpG islands
CGAP	virtual northern blot
SWISSPROT-EURASIP	multiple alignments

Sequencing reaction

ABI 3100 genetic Analyzer for sequencing was used. This Analyser is based on capillary electrophoresis and requires careful preparation of sequencing samples for optimum results.
Template for sequencing was prepared in this order:
PCR reaction was done with genomic DNA (100 ng/ µl); PCR product was extracted from the gel per Macherey-Nagel NucleoSpin extract kit.

Components: 1-2 µl PCR products
1 µl Sequencing-Primer (10pmol/µl)
2 µl Big Dye Kit
5-6 µl water

Completely volume 10 µl

Cycle	Reaction	Time	Temperature	Repeats
1	Denaturation	10 sec	98°C	1 X
	Denaturation	10 sec	94°C	
2	Annealing	5 sec	50°C	30X
	Extension	4 min	60°C	
3	Extension	4 min	60°C	1 X

Precipitation: 10 µl PCR product
8 µl water
32 µl EtOH (RT; 95%)

Vortexed, leaved for 15 min at RT, centrifuged 15 min at 13000 rpm/min(Rotor 12154-H, Sigma 3K18) precipitate twice washed with 100 µl of 70% EtOH, and again twice centrifuged for 15 min at 13000 rpm/min. Precipitate dried in speed vac for 20 min.
For resuspending of template DNA was used DEPC water.
Pellets eluted in 100 µl HPLC water, 15-20 µl added to ABI 3100 Sequencer

Template	Template quantity	Primer quantity	Total
PCR product			
100-200bp	1-3 ng	3.2 pmol	12µl
200-500bp	3-10 ng	3.2 pmol	12µl
500-1000bp	5-20 ng	3.2 pmol	12µl

Almost all templates were sequenced by "Sequisrve" Company from Vaterstetten and some of them in the GAC-GSF self-made.

The GSS division of GenBank

The GSS division of GenBank is similar to the EST division, with the exception that most of the sequences are genomic in origin, rather than cDNA (mRNA). It should be noted that two classes (exon trapped products and gene trapped products) may be derived via a cDNA intermediate. Care should be taken when analyzing sequences from either of these classes, as a splicing event could have occurred and the sequence represented in the record may be interrupted when compared to genomic sequence. The GSS division contains (but is not limited to) the following types of data: random "single pass read" genome survey sequences, cosmid/BAC/YAC end sequences, exon trapped genomic sequences, Alu PCR sequences and transposon-tagged sequences.

2.2.4 Nucleic Acids Isolation

2.2.4.1 Mouse Tail Genomic DNA Isolation

Tail samples (1cm) were placed in 1.5 ml micro centrifuge tube and stored at -80°C to minimize cross-contamination. All work was done on the ice. 500 µl TEN buffer, 50 µl 1% SDS, 25 µl Pronase E and 7.5 µl Proteinase K were added to the tails, and incubated over night at 37°C with mixing. The solid parts were after centrifugation on the bottom, supernatant was transferred in a new tube. It was added 500 µl of Phenol-Chloroform-Isoamyl Alcohol (PCI, 25:24:1), mixed well by repeted inversion. It was centrifuged at full speed (14000 rpm/min) for 5 minutes. Then was transferred the resultant aqueous phase to a fresh tube. To Sample –containing tube was added 950 µl 100% EtOH, it was formed a visible DNA precipitate which was recovered by lifting the DNA with a yellow pipette tip and partial suction from a pipettor, transferred to a 1.5 ml tube containing 70% EtOH. It was centrifuged at 14000 rpm/min and dried in speed vac. DNA was resolubilizated in 500 µl TEN buffer and stored at -20°C.

2.2.4.2 Plasmid DNA isolation

Midi DNA preparation was done with Nucleobond ax 100 kit. The bacterial culture was centrifuged at 4000 rpm/min for 5 min at 4°C. Pellet was resuspended in 4 ml of buffer S1. It was added 4 ml of buffer S2, inverted gently 6-8 times and incubated at room temperature for 5 min. Then it was added 4 ml buffer S3, gently inverted 6-8 times and incubated on ice for 5 minutes. Lysate was centrifuged at 14000 rpm/min(R 12154-H, Sigma 3K18) at 4 °C for 25 minutes. In the mean time cartridges were equilibrated with 2.5 ml buffer N2. Clear lysate was loaded onto equilibrated cartridges and washed with 2 x 5 ml of buffer N3. It was eluted with 5 ml of buffer N5 and precipitated with 0.7-0.8 volume of isopropanol (3.6 ml), centrifuged for 30 min at 14000 rpm/min at 4°C, washed with 70% EtOH, preequilibrated at 4°C and centrifuged at 14000 rpm/min for 10 minutes at 4°C. The pellet was dried at room temperature about 10 minutes, resolubilizated in water and stored at –20°C.

Mini DNA preparation was done with NucleoSpin Plasmid kit. The bacterial culture was centrifuged at 4000 rpm/min for 1min. Supernatant was removed. It was added 250 µl of A1 buffer, and resuspended by vigorous vortexing. Than it was added 250 µl of A2 buffer and inverted 6-8 times, incubated at room temperature for a maximum of 5 minutes, added 300 µl of A3 buffer, inverted 6-8 times and centrifuged 10 minutes at 13000 rpm/min at room temperature. NucleoSpin® Plasmid column was placed in a 2 ml collecting tube; supernatant

was loaded on the column and centrifuged at 13000 rpm/min for 1 min. After it was added 600 µl of A4 buffer, centrifuged for 1 min at 13000 rpm/min. It was dried wail reinserted column into the 2 ml collecting tube, and centrifuged for 2 min at 13000 rpm/min. Finally membrane was incubated 1 min at room temperature and diluted in 50 µl of AE buffer during the centrifuge for 1 min at 13000 rpm/min. (Stored at 4°C and –20 °C).

The concentration and quality of a sample of DNA were measured with a UV spectrophotometer. A solution contained 50 µg/ml of double strand DNA had an absorbancy (optical destiny) of 1.0 at wave length of 260 nm.

DNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. Clean DNA has an $OD_{260/280}$ between 1.8 and 2.0.

2.2.5 RNA Isolation

2.2.5.1 Isolation of total RNA from animal tissues with RNeasy® Mini kit

Different embryos development stages and tissues were used. Embryos were 9.5, 10.5, 12.5, 13.5, 14.5, 17.5 and tissue was from new born mouse pups: brain, eye, lung, skin, kidney, heart. The tissue were prepared sterile from the body, placed in RNase -free tubes and stored at dry ice, just like embryos to avoid the Ribonucleases (RNases). Ribonucleases are very stable and active enzymes. Since RNases are difficult to inactivate and only minute amounts are sufficient to destroy RNA, all plasticware or glassware were used with first eliminating possible RNase contamination (autoclaved). Starting material was up to 20-30 mg. Tissue was placed in vessel for homogenizer, 350-600 µl RTL Buffer was added and homogenised with homogenizer at room temperature. In RTL Buffer β-ME (2-Mercaptoethanol) was added before use (β-ME must be added to buffer RTL and buffer RLC before use. β-ME is toxic. RNeasy® Mini Handbook, Second Edition, Qiagen). Lysate was centrifuged for 3 min at 13000 rpm Supernatant was used and added 1 volume 350-600µl of 70% EtOH to the cleared lysate and mixed well by pipetting 700µl of the sample including any precipitate was applied in mini spin column sitting in a 2 ml collection tube and centrifuged for 1 min. Flow-trough was discarded and added 350µl of RW-1 Buffer then centrifuged 30 seconds at 13000 rpm. After it was added DNAse I incubation mix (10µl DNAse I + 70µl RDD Buffer). Incubation was at room temperature for 15 min and after was added 350µl RW1 Buffer and centrifuged 30 sec at 13000 rpm. Column was placed in the new 2-ml collection tube, and washed 2 x with 500µl RPE Buffer for 2 min at 13000 rpm. Empty column was placed in the new collection tube and centrifuged for 1 min. RNeasy column was transferred into a new 1.5 ml

Collection tube and eluted with 30-50µl of Rnase-free water and centrifuged 1 min at 13000 rpm. This RNA was stored at –80°C, and is stable for a longer time.

The concentration and purity of RNA was determinate by measuring the absorbency (optical density) at 260 nm (A_{260}) and 280 nm (A_{280}). An absorbency of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 = 1 = 40 µg/ml). The measurements were in water diluted 2:198, and purity was given trough the ratio between the absorbency values at 260 and 280 nm. RNA was checked at 1% agarose gel for 10 min at 200V.

Calculations involved in RNA quantitation were:

Volume of RNA sample =100 µl

Dilution = 10 µl of RNA sample + 490 µl water (1:50)

A_{260} = 0.23; measured in water A_{260} value of 1 was equal to 40 µg/ml of RNA.

The ratio between the readings taken at 260 nm and 280 nm (A_{260}/ A_{280}) provided an estimate of the purity of RNA, ranging from 1.5-1.9 is for different types of water, ratio of 1.8-2.1 for 10 mM Tris/HCl, and pH 7.5.

2.2.5.2 Isolation of total RNA with RNAzol^{TM} B

Different embryos development stages and tissues were used. Embryos were 9.5, 10.5, 12.5, 13.5, 14.5 and tissues were from new born mouse pups: brain, eye, lung, skin, kidney, heart. The tissue were prepared sterile from the body, putted in RNAse free tubes and stored at dry ice, just like embryos to avoid the Ribonucleases (RNases). Ribonucleases are very stable and active enzymes. Since RNases are difficult to inactivate and only minute amounts are sufficient to destroy RNA, all plasticware or glassware were used with first eliminating possible RNase contamination. Starting material was up to 20-30 mg. Tissue was placed in vessel for homogenizer, 100- 500 µl of RNAzolTM solution was added. All steps were done on the ice. 1/10 volume of the Chloroform was added, 15 sec vortexed and 15 min incubated on ice, centrifuged for 15 min 13000 rpm at 4°C. Supernatant stored in new Rnase free cap and added 1 vol. Isopropanol, incubated 15 min at 4°C and centrifuged 13000 rpm at 4°C. Precipitate was dried under the vacuum for 2-3 minutes; 50 µl Rnase-free water was used for elution and incubated for 15 min at 60°C.

The concentration and purity of RNA was determinate by measuring the absorbency (optical density) at 260 nm (A_{260}) and 280 nm (A_{280}). An absorbency of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 = 1 = 40µg/ml) .The measurements were in water diluted

2:198, and purity was given trough the ratio between the absorbency values at 260 and 280 nm. RNA was checked at 1% agarose gel for 10 min at 200V.

DNAse treatment was done with 3 µg/µl of RNA, added 1µl RQ1 DNAse (Promega) and 1 µl RQ1 10 x buffer, and incubated for 30 min at 37°C, after was added 1µl RQ1 DNAse stop solution and incubated for 15 min at 65°C.Total RNA was stored at -80°C. Under these conditions no degradation of RNA was found.

2.2.5.3 Generating full-length first-strand cDNA from an mRNA

It was done with Ready To Go™ T-Primed First-Strand Kit. This kit has been designed to provide all the reagents necessary to generate full-length first strand cDNA from an mRNA template using an oligo (dT) Primer containing a *NotI* restriction site. It was used undegraded mRNA (gel check) for cDNA synthesis, about 5 µg of total RNA. The sample was brought to a volume of 33µl in an RNase-free micro centrifuge tube using DEPC-treated water, and heated to 65°C for 5 minutes just like Reaction Mix too. Both were transferred to a 37°C for 5 min. RNA solution was transferred to the First strand Reaction Mix and was not mixed. It was incubated at 37°C for 5 min and mixed gently and centrifuged 1 min at 13000 rpm. After was incubated at 37°C for 60 minutes. The completed first-strand reaction was ready for PCR amplification.

RTG-Mix components:

- dATP, dCTP, dGTP, dTTP, FPLC pure
- Murine Reverse Transcriptase
- RNA guard (porcine)
- RNase/DNase-free BSA
- *NotI*-d $(T)_{18}$ Primer

(5‘-d[$ACTGGAAGAATTCGCGGCCGCAGGAAT_{18}$]-3‘)
PCR check was with β-actin primers:
BACTL l 5‘- GGCTGTATTCCCCTCCATCGTGG- 3‘
BACTL r 5‘- GCTCATTGTAGAAGGTGTGGTGCCAG- 3‘
95°C 1‘
95°C 35“
56°C 35“ 40 x
72°C 35“

2.2.6 In situ hybridization

In situ hybridisation (ISH) is the detection of a target DNA or RNA sequence in a tissue section using a labelled nucleic acid probe. It is still the only hybridisation technique which allows cellular and subcellular localisation of the target. The basic technique utilizes the fact that DNA and RNA will undergo hydrogen bonding to complimentary sequences of DNA or RNA. Specific DNA or RNA fragments may be readily identified within cells after hybridisation in-situ with appropriate DNA/RNA probes. During hybridisation at the cellular level, a labelled specific DNA or RNA probe locates onto complementary cellular target DNA/RNA. This probe is then detected with a suitably labelled antibody (Montgomery, 2002). In situ hybridization experiments on whole mount embryos and paraffin sections were performed.

2.2.6.1 Non radioactive *In situ* hybridisation on paraffin sections

Fixatives and fixation

Animals must be sacrificed with as little stress as possible and the tissue fixed quickly to avoid the contamination of the tissue with RNases on skin and laboratory instruments (Simmons et al., 1989). Tissue samples used in this experiment were collected from C3H mouse stem. After removal from the animal, tissues were 'fixed' to preserve the living structure as closely as possible. The ideal fixative prevents tissue autodigestion (autolysis); inhibits bacterial or fungal growth (preserves); makes the tissue resistant to damage during subsequent processing, embedding and sectioning stages; is isotonic with the tissue and therefore does not alter tissue volume; does not distort any part of the tissue structure; does not dissolve tissue components and is not detrimental to the tissue component being studied. Fixatives for ISH should ideally retain mRNA within the tissue whilst preserving tissue morphology, but should not significantly raise background. At the same time they should allow probes to penetrate the tissue and hybridise with the maximum number of target molecules.

Formaldehyde is the most common fixative in routine use. It is easy to prepare and stock solutions made up in phosphate buffered saline keep for months at room temperature. Commercial formaldehyde solutions contain ~40% w/v (~12M) formaldehyde in water and 10 - 15% methanol. Formaldehyde is very water soluble and exists at low concentrations (4%) mainly as the monomer. At high concentrations (40%) it gradually polymerises and

precipitates as paraformaldehyde. Formaldehyde causes small changes in tissue volume during fixation, especially when dissolved in phosphate buffer, and the resulting morphology is very good for light microscopy. It penetrates tissue rapidly initially, but large tissue pieces fixed by immersion in formaldehyde solutions may not be fully fixed for days (Montgomery, 2002).Paraformaldehyde is the most common fixative used for ISH. Tissue fixation is fast enough to prevent degradation of mRNA, but it is also easy to control the degree of fixation. Unlike formaldehyde, paraformaldehyde-containing fixative solutions have a short shelf life and need to be made up fresh each time. Paraformaldehyde is sold as a powder, which polymerises further with age. Old paraformaldehyde powder is difficult to dissolve and will not adequately retain mRNA within the sections during ISH. Glutaraldehyde is a very strong cross-linking di-aldehyde fixative. It is used for electron microscopy and for ISH with oligonucleotide probes (4% w/v glutaraldehyde and 20% w/v ethylene glycol in phosphate buffered saline).

Paraffin-embedding

Tissues are first dehydrated through graded alcohols and then 'cleared' with an antemedium, xylene. Paraffin wax and ethanol were immiscible, whereas antemedia were miscible with both compounds. After clearing, the tissues were infiltrated with paraffin wax. A range of waxes are available, whose melting temperatures are proportional to their chain length and in the range 42 – 60°C. Thin sections, ie ~ 2 mm and thicker, can be cut from tissues infiltrated wax with a melting point of 56 – 58°C.

Sectioning

Series of sections were cutted at 5 - 6 mm, picked up with forceps and floated first onto the surface of a cold water bath. Individual paraffin sections from the ribbon, were collected onto non-coated microscope slides and transferred to the 42°C water bath, and then recovered onto SuperFrost microscope slides. Sections onto the slides were drayed at 37°C.

Probe Selection

The first step of *in situ* hybridization is selection of a probe. There are several different probes that can be used, each with advantages and disadvantages. These probe types are double stranded DNA (dsDNA), single stranded DNA (ssDNA), single stranded complimentary RNA (sscRNA), and synthetic oligonucleotide. Which probes work for ISH has to be determined empirically (Montgomery, 2002). In this study RNA probes were used. One problem with

RNA probes are that they can have higher levels of non-specific binding to tissue components, which can lead to higher background and lower penetration of the probe into the tissue.

Probe Generation

There are a number of methods that can be used in order to generate a labelled probe for *in situ* hybridization. The generation of RNA probes utilizes in vitro transcription. In this method, the sequence is cloned into a vector containing phage transcription promoters that can initiate transcription in the presence of the corresponding RNA polymerase. If two different phage transcription promoters are placed on both sides of the polylinker cloning sites of the vector in opposite orientations, it is possible to selectively transcribe sense and anti-sense RNA probes. Transcribing only one strand at a time prevents reannealing of complimentary strands during hybridization (Montgomery, 2002). By inclusion of either labeled or unlabeled nucleotides, it is possible to make labeled or unlabeled probes that can be used for different control procedures used in *in situ* hybridization. Single-stranded RNA - complementary RNA (cRNA) or riboprobes can be synthesised in vitro using one of the several commercially available phagemids. These contain promoters for the highly specific bacteriophage DNA-dependant RNA polymerases from the Salmonella bacteriophage SP6, and the E. coli. Bacteriophages T3 and T7. The three polymerases all have similar biochemical properties and consist of a single polypeptide chain. Each is highly specific for its own promoter and does not use other bacteriophage, plasmid, bacterial or eukaryotic promoters at a significant rate. For ISH, subclone an insert in the range 500 - 1000 bp in length. This is large enough to be sensitive, but small enough to penetrate tissue without prior hydrolysis.

Probe Labels

For *In situ* hybridisation RNA probes were labelled with Dioxigenin-rUTP (Boehringer Mannheim) were synthesized from linearized cDNA templates according to the manufacturer's instructions. *Hic2* probes were linearized with *BamHI* and *XhoI* , and synthesized with T7 and Sp6 RNA polymerase. Control *En1 (engrailed)* probe was obtained from M. Panyhuesen, linearized with *BamHI* and *HindIII*, and synthesised with T7 and T3 RNA polymerase.

Prehybridization of slides

Slides were dewaxed in xylol 1 X 5 min and 2 X 10 min. Then dehydrated trough EtOH series: 95%, 90%, 80%, 70%, 50%, and 30% each for 2 minutes. Washed in PBS 2 X 5 min; 4%PFA in PBS for 30 min at room temperature; PBS 2 X 5 min. Treated with Proteinase K (2μg/ml) in Proteinase K buffer approx. 3 minutes. After that washed 2 x 5 min with PBS; 4%PFA for 30 min; PBS 2 X 5 min; 2XSSC 2 X 2 min; Tris/Glycine buffer for 30 min or longer. All steps prior to and during hybridization were conducted under RNase-free conditions. Sections were deparaffinized with xylene (5 min X 3 times), and rehydrated through descending ethanol concentrations [100% (5 min X 3 times), 90%, 80%, 70% and 50% (5 min each) and PBS (10 min). The sections were treated with Proteinase K (2 μg/ml in PBS), pH 7.4 at room temperature (RT, about 20°C) for 15 min. The slides were then immersed in 0.2% (w/v) Glycine in PBS (10 min) and PBS (3 min X 2 times). The sections were acetylated for 20 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (TEA), pH 8.0 and the slides were washed twice in 4 X SSC (10 min each). The 1 X SSC solutions consisted of 0.15 M sodium chloride and 0.015 M trisodium citrate. Following incubation with 50% (v/v) deioned formamide/2 X SSC solution at 42°C for 30 min, the sections were incubated with hybridization solution which contained approximately 0.5 μg/ml DIG-labelled probes in hybridization buffer (50% deioned formamide, 10% (w/v) dextran sulphate, 1 X Denhardt's solution and 200 μg/ml tRNA in 0.6 M NaCl; 10 mM Tris-HCl, pH7.4; 1 mM EDTA, pH8.0). On each slide 100 μl hybridization solution were applied. Slides were then incubated in a humidified chamber at 42°C for 16 h.

Hybridization

Hybridization is performed by placing a small amount of solution containing the hybridization probe on a cover slip, which is then placed on the slide containing tissue sections to incubate overnight. It is important to work out all bubbles from under the cover slip. Hybridisation mix contained next components: 50% Formamide, 5XSSC, heparin (2 mg/ml), 0.1% Tween20, 100 μg/ml denatured tRNA and denatured probe (3 min at 80°C) diluted 1:50 or 1:100, 5% dextransulphate. Slides were taken out of the Tris/Glycine buffer one after other, fluid was removed with paper. The labelled probe is resuspended in hybridisation buffer and then hybridised under a coverslips, with the dehydrated tissue sections it was needed 120 μl hybridisation mix per slide, and slides were covered with coverslips and placed horizontally in plastic boxes (Haereus). Boxes were putted in tissue soaked with 5 X SSC and in foil (wet chamber effect) and placed at the oven bottom and incubated at 65°C over night.

Removal of the unbound probe

The next day, washes are serially applied to the slides to remove the probe that is not bound to target DNA/RNA. This removed the free probe, dextran and blocking agents. The coverclips were floated off in 5 X SSC at RT, washed in 0.5 x SSC/20% FA 4 X 30 min at 65°C for 1 hr with changed of washing solutions and cooled down to 37°C. Washed in NTE buffer for 15 min at 37°C; NTE + 10 µg/ml RNAse A, for 30 min at 37°C; NTE for 15 min at 37°C; in 0.5 X SSc/20%FA for 2 hours at 65°C; in 2 X SSC 2 X 15 min at RT. Stored in 1% blocking solution in MABT until the antibody was added. After hybridisation, probes were recognized by an anti-digoxigenin antibody conjugated with alkaline Phosphatase. Dig antibody was diluted 1:5000 in 1 % blocking solution and shacked at 4°C for 1-3 hours. Per slide was added 120 µl of 1% blocking solution + antibodies. Slides were covered with cover slips and placed horizontally in plastic boxes. Boxes were putted in tissue soaked with 5 X SSC and in foil (wet chamber effect) and putted at 4°C over night.

Removal of unbound antibody

The coverslips are removed in TBST and the sections then washed in TBST for 1 hr and in NTMT 2-3 X for 10 min at room temperature. This removed unbound antibodies and blocking agents.

Detection of the staining

Staining was done with BM purple AP substrate. That is a chromogenic substrate for alkaline Phosphatase (AP) designed for precipitating enzyme immunoassay. It develops a permanent, dark purple band or spot at the AP binding site (Montgomery, 2002).

Before use BM purple AP was centrifuged at 2500 rpm (Rotor 12154-H, Sigma 3K18) for 7 minutes and added 2 mM Levamisol and 0.1% Tween 20. On each slide 120 µl staining solution were applied. Then the slides were incubated in a humidified chamber overnight at 4°C.

After finishing the staining slides were washed 2 x 15 min in NTMT and refixed in 4%PFA/PBS for 5 min. Finally, slides were 2-3 times with Millipore-water washed. Kaiser's Glycerol gelatine was warmed up in a water bath at 50-55°C until it was liquid. Fluid from the slides was removed. On each slide 500 µl Kaiser's Glycerol gelatine were applied and covered with a cover slip.

2.2.6.2 Whole mount *In situ* hybridisation

ISHs of whole mount embryos (WISHs) were performed according to Spörle and Schughart. The embryos were prepared in PBS, fixed in 4%PFA/PBS over night at 4°C, washed twice 30 minutes on ice with PBS, dehydrated on ice through 25%, 50%, 75% and 2 x 100 MetOH/PBS/DEPC, 10 minutes each and stored at –20°C.

Pre-hybridisation

Embryos were rehydrated on ice through 75%, 50%, 35% MetOH/PBS, and 10 minutes each. Washed on ice 2 x 10 minutes with PBT, bleached with 6% H2O2 in PBT for 30 minutes, after that washed again 3 x 10 minutes with PBT. Treated with Proteinase K (1µg/ml) in Proteinase K buffer approx. 5 minutes. After that washed 2 x 5 min with PBT/glycine, 2 x 5 min with PBT, 3 x 10 min with RIPA buffer, 3 x 5 min with PBT. Fixed exactly 20 min with 4%PFA/0, 2%glutaraldehyde in PBT on ice, washed 3 x 5 min with PBT and sorted in Cryo vials because of very small hybe volume. Washed at RT in hybe-buffer/PBT (1:1) 10min, hybe-buffer 10 min and incubated with hybe-buffer at 65°C for 3 hrs.

Hybridization

Probes were denaturatied at 80°C for 3 min, hybed embryos in prewarmed 65°C hybe-buffer with tRNA (100µg/ml, 1:50-1:100 diluted DIG labelled probe (0,1-1µg/ml), putted over night in rotating oven at 65°C.

Removing of the unbounded probe

Started with wash 2 x 30 min at 65°C with hybe buffer, 5 min with 1:1 hybe buffer/RNAse solution, incubated 2 X 30 min at 37°C with RNAse solution contained 100 µl RNAse A. After that incubated in 1:1 RNAse solution/SSC/FA/Tween20 for 5 min at 37°C. Embryos were heated in SSC/FA/Tween20 from room temperature to 65°C and washed 4-5 hours at same temperature with SSC/FA/Tween20.Then were cooled down, washed 10 min at RT in 1:1 SSC/FA/tween20/TBST; 3 X 10 min at RT with 1 X TBST; 2 X 10 min at RT in MABT; incubated for 1 hr at RT in 10% blocking solution/MABT. At the same time were preadsorbed Dig antibodies at 4°C for 1 hr in 1% blocking solution/MABT, diluted 1:5000. Embryos were incubated I antibody solution at 4°C over night, while shacked.

Remove unbound antibody

Embryos were washed 3 X 5 min at RT with TBST and whole day with TBST at RT. Were left in TBST on shaker 3-4 days; TBST was exchanged every two days.

Staining

Staining started with wash step: 3 X 20 min with alkaline Phosphatase buffer pH 9.5, stained were in staining solution with 0.1% Tween20 and 2 mM Levamisol ; Solution was centrifuged before use 7 min at 2500 rpm and changed before was turned from yellow to purple. Embryos were developed at 4°C in the dark. Staining was followed with dissecting microscope; embryos were washed 3 X 20 min in alkaline phosphates buffer, fixed in 4%PFA/PBS over night and stored in same solution for several months.

2.2.6.3 Staging of Embryos

Because of differences in the time of implantation and in individual rates of development, embryos develop at variable rates after fertilization. In fact, at any particular day of gestation the embryos of each litter may exhibit considerable variability in their apparent morphological state of development. The Theiler (Theiler, K., 1972 and Butler and Juurlink, 1987) staging systems can be used to reduce this developmental variation. These staging systems assign embryos to one of several specifically defined stages of development. For the gestational ages referred to in Table 1 below, the morning on which the vaginal plug is found is counted as day one (E1) of gestation. The characteristic external features that can be used to assign an embryo to a specific Theiler or Carnegie stage of development are compiled from Theiler (1972), from Butler and Juurlink (1987) and from personal observations. Note that up to Theiler stage 19 each stage covers a half day of gestation, but the subsequent stages each cover about one day of gestation. The Carnegie system can only be used to stage embryos during the embryonic period of development (up to ~E17.5 for rats and E16 for mice), whereas the Theiler stages cover the whole period of intrauterine development.

		Gestational Age	
Theiler Stage	Carnegie Stage	Mouse	Rat
14	11	E10	E11.5
15	12	E10.5	E12
16	13	E11	E12.5
17	14	E11.5	E13
18	15	E12	E13.5
19	16	E12.5	E14
20	17	E13	E14.5
	18	E13.5	E15
21	19	E14	E15.5
	20	E14.5	E16

22	21	E15	E16.5
	22	E15.5	E17
23	23	E16	E17.5
24	N/A	E17	E18.5
25	N/A	E18	E19.5

Mice were obtained from the GSF animal facilities. 102 x C3H mice were used for the supply of all tissues and the preparation of embryos.

2.2.7 Northern Blot

RNA transfer to the membrane

Electrophoresis

After RNeasy Mini Kit total tissues RNA preparation the same was tested on the special Agarose-Formaldehyde gel. Gel box was cleaned with NaOH and SDS overnight, and rinsed with DEPC-water. For 1.2% gel in 150 ml was used 1.8 g agarose, 15 ml 10X MOPS buffer, 130.5 ml DEPC-water and 7.6 ml Formaldehyde.

Sample preparation

Every slot contained about 20 µg of total RNA from liver, brain, kidney, testes, lung, and whole mouse embryos RNA stage 12.5 and 13.5. RNA brought to the equal volume with DEPC-water, added the same volume loading buffer and 0.5 µl EtBr (0.5 µg/µl), heated for 5 min at 90 °C, cooled on ice.

Gel run

17 hours at 25V in Loading Buffer (1X MOPS/EDTA) the gel runned
0.24-9.5 kb RNA Ladder was used for size control.

Northern transfer of RNA

After 17 hours gel was soaked 3 times 5 min in distilled water (to remove Formaldehyde) and photographed with ruler beside it in 0.05 M NaOH/ 1X SSC washed, and for 20 minutes in 10 X SSC.
Capillary blot with 10 x SSC transfer buffer was seated up:

2 wet Whatman - gel – membrane (Hybond ^{TM}-N+ Membrane (Amersham Pharmacia biotech), optimised for nucleic acid transfer and was soaked in water for a few seconds. - 2 wet Whatman - 2 dry Whatman - paper towel - glasplate - weight. It was 16-24 h with transferred with changes of the paper towel. Lanes were marked, membrane removed, washed briefly in 2 x SSC. Membrane was placed on wet Whatman paper and UV-crosslink damp (auto crosslink setting, 254 nm, Stratagene, Stratalinker) and baked at 80 °C for 1-2 h.

Hybridization

For 1-4 h membrane was prehybrizated at 42 °C with 5-10 ml prehybridization buffer. The 0.5 kb EcoRI-NotI insert of the CloneID 337300 was labelled with [[α]-^{32}P] dCTP (Amersham). Radioactive labeled probe heated for 3 min at 95 °C, cooled on ice. Prehybridization buffer was discarded; hybridization buffer and probe added and incubated ON at 42 °C. Washed 1 x 15 min with 2 x SSC at RT, 2 x SSC, 0.1 % SDS at 65 °C, and with 0.1 x SSC, 0.1 x SDS at 65 °C (optional). Wet membrane was exposed under saran wrap (-80 °C).

Stripping and re-hybridization

For 30 min to 3 h membrane was washed in strip solution at 75 - 85 °C until no radioactivity can be detected on the membrane. Air dried and stored at RT for re-hybridization (up to 10 times) follows the hybridization protocol.

2.2.8 Promoter Analysis

The construct of the *Hic2* promoter analysis included:

1. Promoter PCR analysis
2. Promoter in the pCR.IITOPO Vector
3. Promoter in pPLLucII Vector
4. Transfection of the promoter deletions and reporter gene assay

2.2.8.1. PCR amplification of promoter sequences

About 1.2 kb of promoter sequence was divided in 5 deletions. First step was PCR reaction.

Cycle	Reaction	Time	Temperature	Repeats
1	Denaturation	1 min	95°C	1 X
2	Denaturation	35 sec	95°C	40 X
	Annealing	35 sec	46°C	
	Extension	35 sec	72°C	
3	Extension	10 min	72°C	1 X

Products were excised from the gel and extracted with NucleoSpin® Extract 2 in 1. For each 100 mg agarose gel it was added 300 µl NT1 buffer, was incubated at 50°C for 10 minutes and centrifuged 1 min at 13000 rpm (Rotor 12154-H, Sigma 3K18). Than it was added 500 µl buffer NT2 and again centrifuged for 1 min at the same speed like by step before. After it was added 600 µl buffer NT3 and centrifuged for 1 min at full speed; added 200 µl buffer NT3 and centrifuged for 2 min at full speed. Last step was elution with 30 µl elution buffer NE. The PCR products were cloned Forward primers contain an *Xho*I site for cloning, whereas the reverse PCR primer has a *BamH*I restriction site.

2.2.8.2 Promoter in the pCR.II-TOPO vector

Forward primers contain an *Xho*I site for cloning, whereas the reverse PCR primer has a *BamH*I restriction site.

It was taken 4µl of the extracted DNA and 1µl of salt solution and mixed and incubated at room temperature for 5 min. Transformed with DH5α cells. Ligated and incubated at ice for 30 min, applied heat shock at 42°C for 45 seconds and putted back on the ice. Added 250 µl of LB medium and incubated at 37°C on a shaker for 1 hour. Incubated mix was putted on the LB-IPTG-X-GAL plates, and incubated overnight at 37C°.Next day were picked white colonies and incubated in shaker at 37°C for 1 hour.

The positive samples from the promoter pools were cultured overnight at 37°C with LB medium + Ampicilin. Next day performed plasmid extraction only from the cultures that has become turbid followed the protocol of the kit.

2.2.8.3 Promoter in the pPLLucII Vector

The pPLLucII Vector empty (Y.Kamachi) was prepared with *SalI/HindIII* restriction sides. The PCR products were cloned into the cloning site of the pPLLucII reporter vector using *Sal*I/*BamH*I and *Hind*III restriction enzymes. For fragments in 5'-3' orientation was used 1 µg of Vector (2 µl) was used, 4 µl Y^+ buffer, 0.5 µl *SalI,* 0.5 µl *HindIII* and 13 µl water; incubated for 60 minutes at 37°C, heat inactivated for 15 min at 65°C and dephosphoryled with 3 µl Dephosphorilatyon buffer, 2 µl water and 5 µl Alkaline Shrimp-Phosphatase. Incubated for 10 min at 37°C, heat inactivated for 15 at 65°C. From the pCRII-TOPO Vector insert was released with *HindIII/XhoI* restriction enzymes. PCR products were excised from the gel and extracted with NucleoSpin® Extract 2 in 1 and eluted in 35 µl water, added 2 µl pPLLucII Vector (empty), 4 µl Rapid Ligation Buffer, and 1 µl T4 Ligase; incubated at room temperature for 60 minutes and stored at 4°C over night and transformed with DH5α cells, Placed on the LB-IPTG-X-GAL plates, and incubated overnight at 37C°. Next day were picked white colonies and incubated in shaker at 37°C for 1 hour. The positive samples from the promoter pools were cultured overnight at 37°C with LB medium + Ampicilin. Plasmid extraction was performed only from the cultures that have become turbid.

2.2.8.4 Transfection and reporter gene assay

For Luciferase (*Luc*) reporter assay, HEK-293 fibroblast cells were cultivated in 96-well plates for 24 h and transfected by PolyFect Transfection Reagent (Qiagen) using 0.7 µg plasmid-DNA. PolyFect Reagent is a solution of specifically designed activated-dendrimers; consist of dendrimer molecules of a defined spherical architecture with branches radiating from a central core. The branches terminate at charged amino groups, which can interact with negatively charged phosphate groups of nucleic acids. PolyFect Reagent assembles DNA into compact structures. This reagent is provided as a ready-to-use solution. The DNA mix in transfection reaction is compounded by 0.5 µg reporter vector, containing a sequence 5' of *Hic2* fused to the *Luc* reporter gene 0.1 µg effector (parental plasmid pcDNA3.1 as negative control) and 0.1 µg pRL-SV40 for transfection control. Cells were harvested 48 h after transfection and cellular extracts were assayed with the Dual-Luciferase Reporter Assay System (Promega).

Vector	End conc.
pPLLucII	0.63 µg
pcDNA3.1	0.18 µg
pRLSV40	0.09 µg

The cells were divided to the 96-well Plate, dilution was 1:3. It was taken 2 ml from the cell suspension, added 8 ml DMEM Medium to the end volume 100 µl pro well. The transfection pool was done with pPLLucII Construct 3 x 0.21µg in the DNA pool (Promoter construct 1-5), pcDNA3.1 3 x 0.06 µg in DNA pool, pRLSV40 3 x 0.03 µg in DNA and mixed. PolyFect 3 x 1 µl and DMEM (without all) added to 30 µl in the PolyFect pool and mixed. PolyFect - pool was added to DNA-pool and incubated for 10 minutes at room temperature. To the cells in the wells were added 50 µl from the fresh DMEM Medium (with all), after was added fresh DMEM Medium (with all) and already 80 µl pro well, incubated 40-48 hours at 37°C under 5% CO_2.

Vector	0	1	2	3	4	5	End conc.
pPLLucII	23	17.5	4	6.3	8	10.2	0.63 µg
pcDNA3.1	4.6	4.6	4.6	4.6	4.6	4.6	0.18 µg
pRLSV40	3.6	3.6	3.6	3.6	3.6	3.6	0.09 µg
DMEM	13.8	19.3	32.8	30.5	28.8	26.6	to 45 µl

2.2.8.5 Measurements

For this part of experiment Dual-Luciferase ® Reporter Assay was used, which contains an optimised Lysis reagent for preparing cell lysate and all of the reagents necessary for the sequential assay of firefly and *Renilla* Luciferase, and Plate-reading Luminometer. The term "dual reporter" refers to the simultaneous expression and measurement of two invidual reporter enzymes within a single system.

Passive Lysis Buffer is supplied as a 5X concentrate. Reagent was prepared like 1X concentration by adding 1 volume of 5X PLB to 4 volumes of distilled water. The 5X PLB was stored at -20°C. From the cultured cells was removed the growth medium and applied 20 µl of 1X PBL. The culture plate was placed on an orbital shaker and shacked for 10 minutes at room temperature. After that culture plate was stored at -80C° for 20 minutes, and for 20 minutes at room temperature. Luciferase Assay reagent II (LAR II) was prepared by

resuspending the provided lyophilized Luciferase Assay Substrate in 10 ml of supplied Luciferase Assay Buffer II, mixed and stored at -20C°. Stop & Glo® Substrate was supplied in a 50X concentration. It was added 1 volume of 50X Stop & Glo® Substrate to 50 volumes of Stop & Glo® Buffer.

Luminometer (Ultrospec III) contained injector and measurement part, was connected with computer. Measurement program was called "Simplicity". The first step was injector lines cleaning. Position 1 (Firefly) to Mpl (Micro plate), 500µl 10X, position 2 (Renilla) to Mpl (Micro plate), 500µl 10X, together were 3 wash steps, 1X with H_2O, 1X with 70% EtOH, 1X H_2O. Next step was air soak. The injector lines were putted outside of the solutions, and soaked air (empty). Pos. 1 (Firefly) to Mpl (Micro plate) 300 µl 3X, Pos. 2 (Renilla) to Mpl (Micro plate) 300 µl 3X, all time injector lines were on the air. In the next step were soaked LAR II and Stop & Glo. Wash solution were changed with measurement solutions. Pos. 1 (firefly-LAR II) to MPL (Micro plate) 200 µl 3X, Pos. 2 (Renilla- Stop & Glo) to Mpl (Micro plate) 200 µl 3X. On the wash plate was made control was it really sucked. For measurement A1 corner from the micro plate was putted in the right up corner.

Protocols: RD Raw Data, Dual: Pos. 1 Firefly, Pos. 2 Renilla, And FK Fast Kinetics
Injector was cleaned again. Measurement solutions were sucked back to the bottle. Pos. 1 (Firefly) to bottle 300 µl 3X, Pos. 2 (Renilla) to bottle 300 µl 3X. The last step was done like a first one, Position 1 (Firefly) to Mpl (Micro plate), 500µl 10X, position 2 (Renilla) to Mpl (Micro plate), 500µl 10X, together were 3 wash steps, 1X with H_2O, 1X with 70% EtOH, 1X H_2O. The injector lines were putted in the water and Luminometer was switched off.

Calculations were done with Microsoft Excel program in three steps:

1. Middle value from the zero value
2. Relative Luciferase activity
3. Normalisation

For relative Luciferase activity was used next formula:
RLA= (measurement n* - middle value) [Firefly] / (measurement n*-middle value) [Renilla]

Normalisation:

N value = Relative Luciferase Activity n*/ middle value** (relative Luciferase activity”0”)

* were own measured results (1-3)

** (relative Luciferase activity”0”)

The calculations for graphics were done with Sigmaplot program which made a graphics already.

3. Results

3.1 Isolation of the mouse *Hic2* gene

Searching the cDNA-Bank from Clontech with a 0.9 kb *EcoRI/NdeI Hic1*-fragment, a new clone λC5-1-1 was found. The clone C5-1-1 had one 2kb long insert. The DNA sequences from C5-1-1 and *Hic1* were identical but just in the 3'part of the sequence which coded zinc finger domain and in the 3'UTR of the Hic1 and γFBP proteins. Screening of the EST database (dbEST) with the C5-1-1 sequence detected one EST clone 2.9kb (AA118503, I.M.A.G.E. clone ID 555891). This clone is generated from mouse paws E13.5 cDNA bank and began with zinc finger domain followed a 3' untranslated region (Fig.3.1A). The new isolated cDNA was named *Hic2*, and presented a paraloge of *Hic1*. The sequence C5-1-1 is in the Gene Bank under number AF117382 (Grimm C., 1999, Dissertation).

Does *Hic2* contain important domains such as GDLSK and BTB/POZ, like its paralog *Hic1* ?

The open reading frame in the 5' part of the C5-1-1 sequence was not evident it was not possible to get 5' of the sequence and promoter region. In the GSS-Database were identified E16R and F08, two mouse genomic clones, ordered by Utah Genome Centre.

The F08 clone defined 3'sequences, began with a zinc finger domain, and coded the stop codon and poly-A tail.

The E16R clone defined 5'sequences, began with a part of 3'UTR, followed by the zinc finger domain and encoded two domains GLDLSK/K and POZ/BTB. The long E16R sequence was compared with Celera data base and a big genomic clone about 500 000 bp was identified (Fig.3.1B). This clone contained the complete *Hic2* gene sequence. Parallel with sequencing it was possible to use Ensemble Mouse data base which already showed position of the H*ic2* transcript in the Mouse genome.

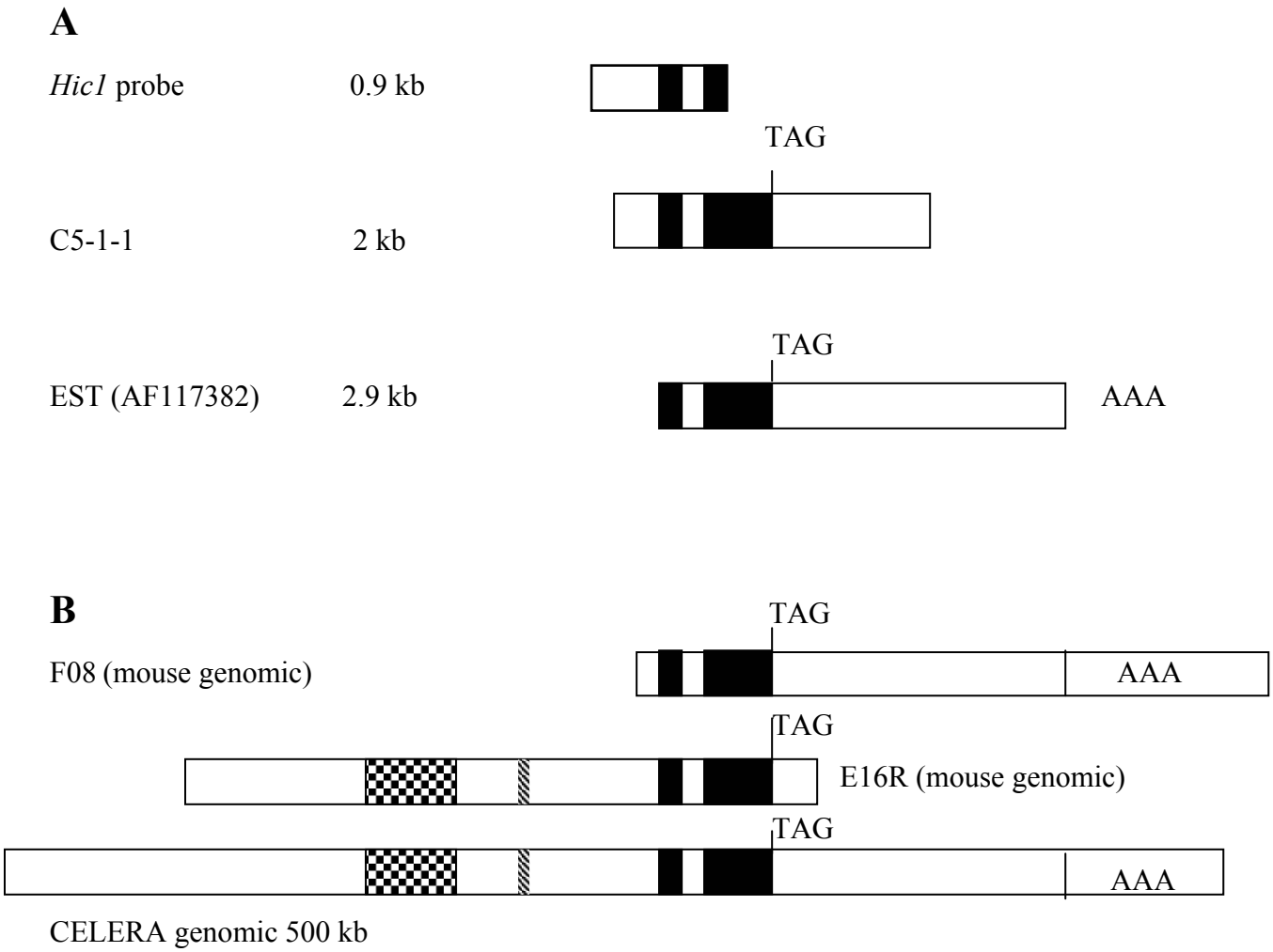
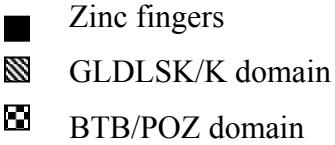


Figure 3.1. *Hic2* cDNA clones. A) *Hic1* probe isolated C5-1-1 *Hic2* clone and in the database identified *Hic2*-EST clone (Grimm C.) B) New *Hic1* probe positive clones. The UGC1M0225E16R and UUGCIM0327F08 clones helped with identity the *Hic2* genomic structure and were used to localize *Hic2* to chromosome 16.



3.2. Genomic organisation of the *Hic2* locus

Database searches with genomic *Hic2* sequence revealed similarities with human *HIC2* gene, some members of KIAA1020 and DKFZp434F0616 protein, as well as human *HIC-3* gene. The search did not show high identity with any of the mouse gene sequence (Fig.3.2).

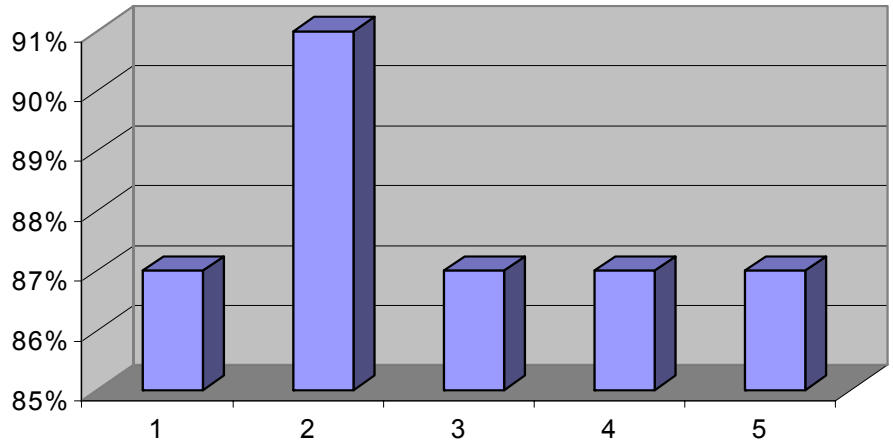


Figure3.2 Nucleic acid sequence homologies between *Hic2* and some genes showed the highest percent identity with human mRNA for *HRG22 (HIC2)* protein. 1) Homo sapiens *HIC1*-related gene on Chromosome 22, 2) *Homo sapiens* mRNA for *HRG22 (HIC2)* protein, 3) *Homo sapiens* mRNA DKFZp434F0616, 4) *Homo sapiens* mRNA for KIAA1020 protein and 5) *Homo sapiens HIC-3* mRNA

In *silico* analysis demonstrated that the complete *HIC2 (HRG22)* coding sequence and the large 3' untranslated sequence were collinear with a unique genomic region. The *Hic1* coding sequence and the large 3' untranslated sequence were not completely collinear with the *Hic2* coding sequence (Fig.3.3). More identities in genomic locus showed *Hic2* with its human orthologues *HIC2.*

Using several gene prediction programs (Genescan, Webgene), the highest score for a splice acceptor site in the 5' *Hic2* genomic sequence highlighted a sequence located 40 bp upstream of the putative BTB/POZ initiation codon (Fig.3.3 and 3.4).

The 5' boundary of the *Hic2* exon that was named exon 2 (Fig. 3.3), was fully validated by the identification in the database of a mouse mammary tumor tissue EST BI654062 and the human kidney EST BE501479.

Analysis of the *Hic2* genomic sequence upstream of exon 1, WebGene program predicted a putative TATA box (position 5807), pattern (Fig. 3.4).

In the 5' end of the *Hic2* exon 2 the WebGene program predicted 2 CpG islands at the positions 4099 to 4342, and 5285 to 5580. This fact could play a role in promoter activity and hypermethylisation. The first CpG island (4099-4342) is located in the 5' of exon 2, and the second one (5285-5580) in the 3' part of *Hic2* intron (Fig. 3.3).

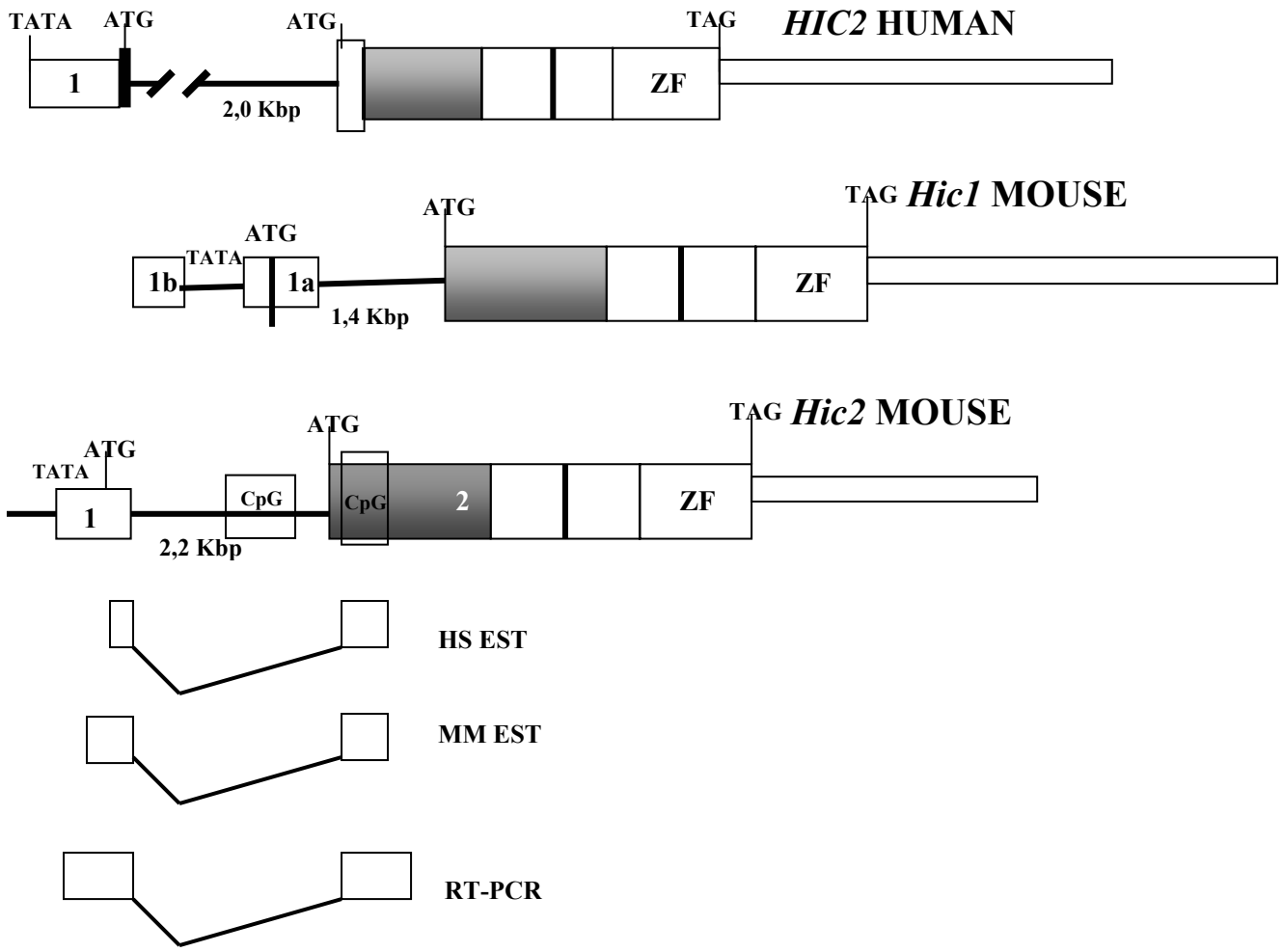


Figure 3.3 Schematic genomic structure of the *Hic2* locus and comparison with the related *HIC2* and *Hic1* locus. The structure of the *Hic2* locus has been deduced from the partial cDNA clone C5-1-1, mouse ESTs clone BB659883, generated from lung E13; BQ965108, generated from undifferentiated limb; BI654062 generated from mammary tumor tissue, 5 month old mouse. Human EST clone BE501479 generated from kidney, and the nucleotide sequence of a RT-PCR product. Comparison of the genomic *Hic2* and the cDNA sequences revealed two exons, separated by a 2kb intron.

The exon-intron boundaries are in good agreement with the splice site consensus sequence. In comparison with the human *HIC2,* the beginning of the exon 2 sequence are almost identical. Position 2584 in mouse sequence is one C and in human the same position is G; the codon in the human sequence is GCG for alanin, while in the mouse the triplet is CCG for prolin. In the mouse sequence the position 2625 is A, and in the human sequence C. The codon triplets are CCC and a CCG, respectively, both are for prolin. The WebGene program predicted potential splice signal, for donor site position 2588 CAG…GTGGTG and for acceptor site position 397 GCTTTGCAG…TATA (Fig. 3.4).

Exon 2

	W	C	A	W	A	G	R	G	D	M	G	P	D	M	E
***HIC2*…tgcccacag**	GTG-GTGCGCGTGGGCAGGGCGCGGGGACATGGGGCCCGACATGGAG														
***Hic2*…tgcccacag**	GTG-GTGCCCGTGGGCAGGGCACAGGACATGGGGCCAGACATGGAG														

Figure 3.4 5' boundary of the human *HIC2* and mouse *Hic2* exons 2. The splice donor sites are underlined. The noncoding and intronic sequences are shown as lowercase letters, the coding sequences are indicated as capital letters.

To address the functionality of TATA box as a putative promoter, RT-PCR was done using normal mouse brain (newborn) cDNA, since this organ yielded the highest level of *Hic2* expression. One nested sense primer (E1-L) located just downstream of the putative TATA box, and one nested antisense primer (EX2-M-R1) in the *Hic2* exon 2 yielded a fragment of the expected size (Fig. 3.5A).

Northern analysis of total RNA from kidney of new born mice revealed a single transcript about 1.9 kb (Fig. 3.5 B) suggesting that the complete transcribed region of *Hic2* including the entire coding region was identified.

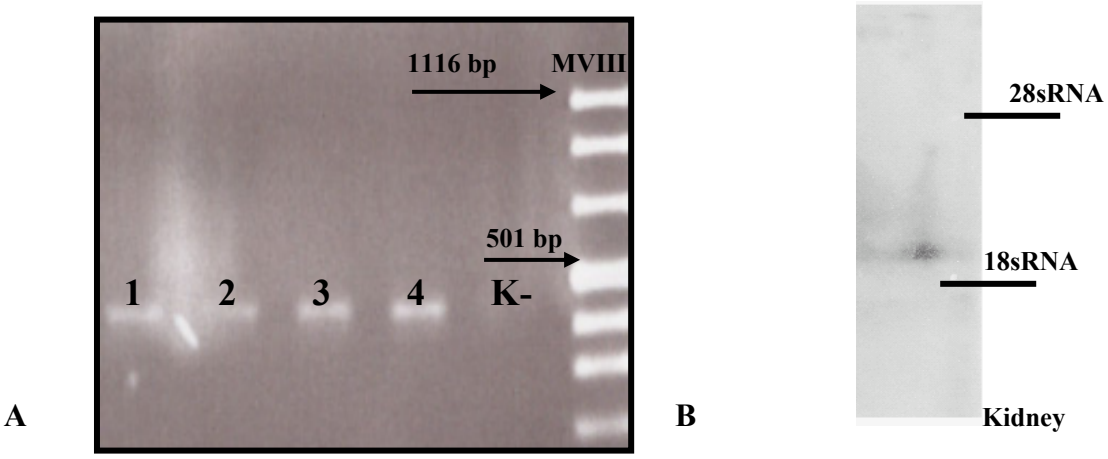


Fig. 3.5

A) RT-PCR with nested primers demonstrated that this band corresponded to the correct splicing of exon 1 sequences to exon 2. The primers yielded a fragment of the expected size (450bp). 1 and 2 for E 12.5 cDNA, 4 and 5 for brain (new borne mouse) cDNA

B) Northern analysis showed a single transcript about 1.9 kb, the total RNA (tRNA) was isolated from the mouse kidney (new borne).

The Hic2 transcript is located on mouse chromosome 16 position 16000001-17000000 complement 727777 to 729582, join the complement 727777 to 729582. A transcript which is similar to *Homo sapiens* Hypothetical protein [DKFZp434H177.1] is located 5' to the Hic2 on the chromosome 16 16000001-17000000, complement 707799 to713403. There genes are located in the 3'flanking region from Hic2: an SDFZ like protein at position 16.16000001-17000000 complement 749673 to 751535, an MIR stromal cell-derived f2-like at complement 600878 to 602681, and an NAS hypothetical protein at complement 609377 to 614455.

The deduced Hic2 protein consists of 601 amino acids including a POZ domain and five C2H2 zinc fingers at the C terminus (Fig. 3.9). Following the POZ domain, Hic2 contains a cluster of basic amino acids, RRKLKRAKK (Fig. 3.6). Coding exon (2) containing a long ORF yielding 617 amino acids protein (Fig. 3.6).

Hic2 genomic sequence

```
   1 CGAATTGGGC CCTCTAGATG CATGCTCGAG CGGCCGCCAG TGTGATGGAT ATCTGCAGAA
  61 TTCGCCCTTG CAGCTAGGTG GCATTATGTA GCCTTTAAAG CACATTGTAA ATGTCCCTTG
 121 CCTTTAATCC CAGCACTCCC AGAGGCAGGT GGGTATCTGT AAGTTCGAGG CCAGCCTGCT
 181 CTATGTAAGA GGGTTCCTGT CGAAAGGAAA GGAAAGGAAA GGAAAGGAAA GGAAAGGAAA
                                    GKLF                 EVI1
 241 GGAAAGGAAA GGAAAGGAAA GGAAAGGAAA AGAAAGGAAA AGAAAGGAAA GGAAAGGAAA
 301 GGATACAGGG AGGGGGCATC TTAGGAGGCT AAAGGCAAGA CACAGCTGGC TGTCAGGAGG
           SP1,WT1                                  AP4
 361 CTAGGGCAAG GCTGGCCATA TTTACATAAC AAAAGGTCAA AACCAGGAGC CACTTGTGGA
               p53
 421 AGGGCTATTT ATAAGAGCCA ATCTGCCTCT ACTGCCTCTT TCCCCTTCTT TCCTTGGCAA
                      CCAT box
 481 CAGGGCTCTT CTGTCTACAG GCTCAACTCA GCACAACAAC CAACAGCAAC CGGGTGAGGG
 541 GTGCTCTCTG CCGCCATCAC TCTGTGTTCT ACAAGAGGAG GGATCTTGAG GTTCTGGATC
 601 CCTGTACTCC ATACAGAACG GCAGCCTGGT CTCCCCTGGA ACAAGCAGGG GTAGGAGGAG
 661 CCTGCAGCAG GCAAGAATCC ATCTCTGACA GCCCTTCCCC CACACCATCC CTAGGGCCAG
                                      WT1,ZBP-89
 721 AGGACAGGTC CTTCAGGAAT GTGACCAGAC CTGGAAGGGC GAATTCCAGC ACATTTACAA
 781 TGTGCTTTAA AGGCTACATA ATGCCACCTA GCTGCTCATA TCTAAAACAA GGTCTCTCTT
          TATA
 841 TTTTTGGTGT GTGTGTATGT GTGTCTCATT TTTTTTCCTC TTGCTTTGTT GCCTCAGGTG
 901 TTTGTGAACC TCTTTGGGAG TAGAGACCCC CCCACCCCTT GTTCCTCTCT TCTCCTCCGT
 961 CCCTCACTGA TGCCTGCTTT TTTTTCTCCA GGTAACCAGG CTTTGATGCA CCCTCAGGAA
1021 GTCTGTGCAC TCGAGGAGGC CACCCCCCGC CGGCTGCTGC TCACATGGTT TCTGGGCCCC
1081 TGGCACTCCG GTAGGCCTGG GAGAGGAAGA ATCACCACTG GTTTCCAGTC CTTGGGGCTT
1141 CCTGGGTCTC TCATACAGCT TTGCAGTATA cattctgtcc tggccctcag aattcaggtt
1201 gctatatgtg cacagtgcag gcaggcagca tgtggggtgt ctaggacttg tggcccaggc
1261 cttggccaca tatagctttt cttttatttc tcttggtagg cctgaggtac cagctattat
1321 tgctggcaga gctggcactg ccattgcctt ggggctgata tatagggcct gtgtggaaat
1381 cataccttag gctctttgtt tctacttagc atgtggagaa gccttggttt tcctcatctc
1441 cagccactca gtatcccact gatgtgcttc agctgttgtg ggcaaggggc aggcaggcag
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1561 tcacagtgtg ctgggttatt agcttccttc tctgaacagg gcagattgtt agttttacag
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2401 cagcagttat gggagggagg atcagtattt gggtcaggac taggagttag caaatgagaa
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2701 tgctatacca aaagtccccc atagccaccc acccccattt tttttccttt ttatctttta
2761 aattttaacc ttttagttta aaaaaatttg tgtgtgtgtg tgtgtgtgtg tgtgtgtaag
2821 tgtgggaaga ccagtcccgt ggggtgggtt tacgcctgga ctggccagct gccagtcatt
2881 tttgtaggtt ctctttgtga cctctgacct gtgaatgact tctttcatac ctgaaagagt
2941 aacaaaggaa aattcaaacc ccattaacat ttctagattt atttttatgt atgtgagtgc
3001 tttgcctgca tgtatataac tgcttgagtg cctgctgctt gcagagttaa taagagggca
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3061 tcagatcctg ggaatcagac ttacagatga tttgaagaac agcaagtgca cttagccaat
3121 aagccctcta cctagcctat acaccttggg ttttgcttgg cacctgtatt tgtcatgacc
3181 tctttttcca tgtgagtagt tggcaggaaa tgagaggaca cggtcttata cataatgaaa
3241 tctgtcctgt acactgtgcc tgaggagggt ggaatccggt gggctcagta cataccctgt

3301 gccaggcctt cgtgcaggtt gcaggttaac tatctatcCT GCCTGTTCTT GCCCACAGGT
                                                  L  P  V  L  A  H  R
3361 GGTGCCCGTG GGCAGGGCAC AGGGACATGG GGCCAGACAT GGAGCTTCCC AGCCATTCAA
    W  C  P  W  A  G  H  R  D  M  G  P  D  M  E  L  P  S  H  S  K
3421 AACAGCTCCT GCTACAGCTG AACCAGCAGA GGGCCAAGGG CTTCCTGTGT GACGTCATCA
     Q  L  L  L  Q  L  N  Q  Q  R  A  K  G  F  L  C  D  V  I  I
3481 TCATGGTGGA GAACTCTATC TTCCGGGCCC ACAAGAATGT CCTGGCTGCT AGCAGCATCT
     M  V  E  N  S  I  F  R  A  H  K  N  V  L  A  A  S  S  I
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     Y  F  K  S  L  V  L  H  D  N  L  I  N  L  D  T  D  M  V  S
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     I  Y  T  G  S  T  V  F  Q  Q  I  L  D  F  K  L  L  P  S  D
3661 AGCCATCTGA GCCAAACTTC AGCACTCTAC TCACTGCTGC CAGCTACCTC CAGCTGCCTG
     Q  P  S  E  P  N  F  S  T  L  L  T  A  A  S  Y  L  Q  L  P
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     E  L  A  A  L  C  R  R  K  L  K  R  A  G  K  P  F  G  P  G
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     R  V  G  T  A  G  I  G  R  P  T  R  S  Q  R  L  S  T  A  S
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     V  I  Q  A  R  F  P  G  L  V  D  V  R  K  G  H  P  A  P  Q
3901 GCTCCCTCA GGCCAAAGGC TCAGATGATG AGCTTTTTCT GGGCACCTCC ACTCAGGAGA
     E  L  P  Q  A  K  G  S  D  D  E  L  F  L  G  T  S  T  Q  E
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     S  T  S  T  N  G  S  S  G  G  C  E  Q  E  L  G  L  D  L  S
4081 AGAAGAGCCC ACCCTTACCT CCTACAACCC CTGGCCCCCA CCTCACACCC GAGGACCCAG
      K  K  S  P  P  L  P  P  T  T  P  G  P  H  L  T  P  E  D  P
4141 CCCAGCTGAG TGACAGCCAG CGAGAGTCAC CCGCACCCAC ATCGACCTCT GCCCTGCCTG
     A  Q  L  S  D  S  Q  R  E  S  P  A  P  T  S  T  S  A  L  P
4201 TCGGCAATAG TGCCTCATTT GTTGAGCTGG GGGCCACTCC TGAGGAGCCC ATGGATGTGG
     V  G  N  S  A  S  F  V  E  L  G  A  T  P  E  E  P  M  D  V
4261 AAGGGGCTGA GGAAAACCAC CTGAGTCTGC TGGAAGGACA GGGTGGGCAG CCTCGCAAGA
     E  G  A  E  E  N  H  L  S  L  L  E  G  Q  G  G  Q  P  R  K
4321 GCCTCCGGCA TTCAGCCCGC AAAAAAGATT GGAACAAAAA GGAACCTGTG GCTGGGTCCC
     S  L  R  H  S  A  R  K  K  D  W  N  K  K  E  P  V  A  G  S
4381 CCTTTGATAG GAGAGAAACA* GGATCTAAGG GTTCCTGCCC AGGGGAAGAA GTGAAGGGA
     P  F  D  R  R  E  T  G  S  K  G  S  C  P  G  E  E  G  E  G
4441 CTGGGGACAG GGTTCCCAAT GGCGTTCTGG CTAGCAGTGC TGGTGGAGGT GGCCCCAGTG
     T  G  D  R  V  P  N  G  V  L  A  S  S  A  G  G  G  G  P  S
4501 CATCGTATGG GGAGCAGTCA TTCCCCTGCA AGGAGGAGGA GGAGAATGGC AAAGATGGGA
     A  S  Y  G  E  Q  S  F  P  C  K  E  E  E  E  N  G  K  D  G
4561 GTGAGGACAG TGGGCAGAGT GGGAGTGAGG GGGGCAGCGG CCATACTGGC GCCCACTATG
     S  E  D  S  G  Q  S  G  S  E  G  G  S  G  H  T  G  A  H  Y
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     V  Y  R  Q  E  G  Y  E  T  V  S  Y  G  D  N  V  Y  V  C  I
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     P  C  A  K  G  F  P  S  S  E  Q  L  N  A  H  V  E  T  H  T
4741 AGGAGGAGTT ATTCATCAAG GAAGAAGGGG CTTATGAGAC CGGCAGTGGG GGTGCCGAGG
     E  E  E  L  F  I  K  E  E  G  A  Y  E  T  G  S  G  G  A  E
4801 AGGAGGCTGA GGACCTGTCA ACACCTAGTG CAGCCTACAC AGCTGACTCT CGTCCTTTCA
     E  E  A  E  D  L  S  T  P  S  A  A  Y  T  A  D  S  R  P  F

4861 AGTGCTCAGT TTGTGAGAAG ACCTACAAGG ACCCAGCCAC ACTGCGGCAA CACGAGAAGA
     K  C  S  V  C  E  K  T  Y  K  D  P  A  T  L  R  Q  H  E  K
4921 CACACTGGTT GACCCGTCCG TTCCCCTGCA ACATTTGTGG AAAGATGTTC ACACAGCGAG
     T  H  W  L  T  R  P  F  P  C  N  I  C  G  K  M  F  T  Q  R
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4981 GCACTATGAC ACGCCACATG CGGAGCCACC TGGGCCTGAA GCCCTTTGCC TGTGATGAGT
     G  T  M  T  R  H  M  R  S  H  L  G  L  K  P  F  A  C  D  E
5041 GTGGTATGCG TTTCACTCGC CAGTACCGCC TCACAGAGCA CATGCGTGTG CACTCAGGTG
     C  G  M  R  F  T  R  Q  Y  R  L  T  E  H  M  R  V  H  S  G
5101 AGAAGCCCTA TGAGTGCCAG CTCTGTGGAG GCAAGTTCAC CCAACAGCGC AACCTCATCA
      E  K  P  Y  E  C  Q  L  C  G  G  K  F  T  Q  Q  R  N  L  I
5161 GCCACCTGCG CATGCACACT TCCCCTTCCT AGaagccaaa GACCCGTGGG AGCCCTCTAC
     S  H  L  R  M  H  T  S  P  S
5221 AGACTTGCCA CTTGGGAACC CACGGAAGCA TGGTGGGAGG GTTGAGCCCC AAAGTCTGAC
5281 ATGAGGCTCC TGGCAGCCCC TCTGGCCCAG CCTCCTCACG CCCAGAGCTT TAATCAACAG
5341 TATACCAAGG GAAGCCAAGA GAGAAGCTGA CCAGTCTAGC CCTATGCATG TGGTGCTGGT
5401 GACTATTCTA CCTCCCTGGG CCCACTGGGC TTTCCAGCTC CCTGAAGGAG CTGCTGAAGA
5461 GCCGGTGGGA GTCCATCATG TGGGTGCCAG TGGGGCCTGG TTGGTTCCTT TCCTGCAGGT
5521 TGGGCTGGGC CACAGGGTTT CCTGTGGCCA AAAGTGGTGC CTGGAGCAGG CCTAGCTGGC
5581 AGGGACCTCG GCTGTGTGTG TGCATGTGTG CTTGTGTCTT TGCAGTGCAG TGTGACTCTG
5641 GTGTTGGGGT GCTGGTGCTG GGTGGCAGTT CCTTTCTCTG TGAGCTGGGG TGGCAACTGC
5701 TAGCTGGCAC TGGAACAGTC AGGGTGACCC ATTGCCTACC TCTCTGCAAC TAAAGAGCCC
5761 TCTGGGCCTA TATTGCTGTT ATTCCTACTG AGCTGTTCCT ATTTCTTTTT TACAGTTTTT
5821 AAAAATTCTT TTTTAAAACC AAAATGAACA ATATTTTCCT CCTGCCTCTT TGGGGTTGAG
5881 CCTGGGTCTG CAGACTGAAT GTAACCGGGG CAGCTGCCCC TCCCTATCAT TTTCCCTAGT
5941 CCTGGTGCTT GGGCATGGTT GAACCTGCCT CTCCAAGGCC CAGAAGCCCT TCTTGCCCAA
6001 AGGCTTCCCT GATTCTGAGC CCTGCCTGCC TGGGCTTCCT CAGGAGAATA TGCTTTTCCA
6061 TAATTTCCAA GGAATGTCCC TTGTTAGAGG TACTTATTTT TATTGAGAAC CACACCCTCC
6121 CCCATATACC TTTTTGAGAT GTGGAACTGG AAGGTCTGAA GTTTCAGAGG GTGGAGGGGA
6181 GGCTAGATTC GGTGCCAGAG CCATCGGTAC TCTTGCTCTC GTGTGCACAC
6301 GCGCGCGCAT GTGCGTGTGT GTTGCTTATG CACAGATGCT TCACTTCCTT
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Figure 3.6 *Hic2* genomic DNA and deduced amino acid sequence. The deduced amino acid sequence is shown below the nucleotide sequence of the DNA. The BTB/POZ domain is blue coloured, the C-terminal zinc fingers are shown in bold and underlined. The cluster of basic amino acids (R R K L K R A G K) is underlined and GLDLSK is shown in bold. The TATA box is red coloured. The recognition sites for known transcriptions factors in the 5'UTR are underlined and green coloured. Possible start codons (ATG) are underlined, stop codon (TAG) is red coloured, position of the known cDNA sequence (Grimm), position 4440 is marked with *.

The coding sequences, exons and 3'UTR are indicated as capital letters, intron sequences are shown as lower case letters. Repetitive TG and TC sequence in 3'UTR (Microsatellite) is underlined. PolyA-tail is underlined. Abbreviations : GKLF, Gut-enriched Krüppel-like factor, EVI1, Ecotropic viral integration site 1 encoded factor, SP1, Stimulating protein1, ubiquitous zinc finger transcription factor, WT1, Wilms Tumor Suppressor, AP4, Activator protein 4, p53, Tumor suppressor p53, CCAT box, cellular and viral CCAT box, ZBP-89, Zinc finger transcription factor 89.

3.3 Deduced amino acid sequence and domain structure

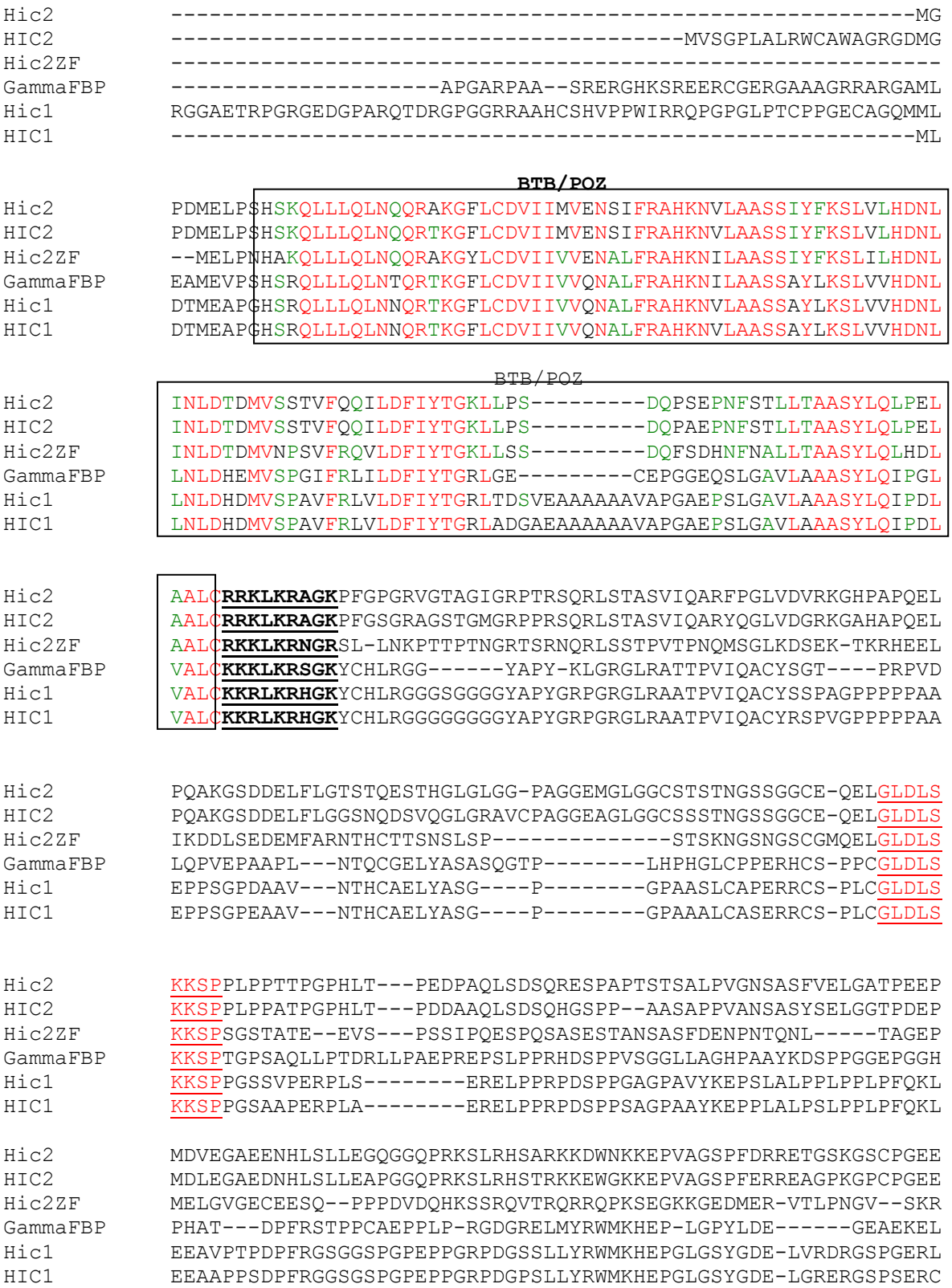
The deduced Hic2 protein consists of 601 amino acids including a POZ domain and five C2H2 zinc fingers at the C terminus (Fig. 3.7). Following the POZ domain, Hic2 contains a cluster of basic amino acids, RRKLKRAKK (Fig. 3.6).
Database searches with mouse Hic2 protein sequence revealed similarities to the human HIC2 protein, chicken γF-crystallin binding protein (γFBP), mouse Hic1 and human HIC1 protein (Fig. 3.7). Similar to *Hic2* and *HIC2*, γFBP encodes also a protein with a BTB/POZ domain, five zinc fingers and a short, conserved GLDLSK/K motif. Comparison among Hic1, HIC1 and γFBP proteins showed more or less identity , to better appreciate the relationship among the Hic2 and Hic1 proteins from various species a multiple alignment using the CLUSTAL program was generated (Fig.3.7).

Multiple alignment analysis highlighted two regions of strong conservation between Hic2 and Hic1 to the BTB/POZ and Zinc fingers domains (Fig. 3.7 and 3.8).
Regarding the BTB/POZ domain, the first 87 residues are well conserved. The 3' part of the BTB/POZ domain is less conserved .

A short GLDLSK polypeptide located in the central region between BTB/POZ and zinc finger domain is perfectly conserved (Fig.3.7 and 3.8). The GLDLSK sequence identity between Hic2 and human HIC2, zebrafish HRG22, chicken γFBP-B, mouse Hic1 and human HIC1 protein was 100%.

The zinc fingers domain was more highly conserved than the central region. It is split into five parts; the more upstream zinc finger 1, and the downstream zinc fingers 1-4. This C-terminal end appears globally more highly conserved 70%. The upstream zinc finger motif is present and well conserved. The four C-terminal C_2H_2 zinc fingers are separated by a typical 7- to 8-amino-acid conserved link, the H/C link found in *Krüppel*-like zinc fingers and are thus likely to be involved in sequence-specific DNA-binding.

Multiple alignment



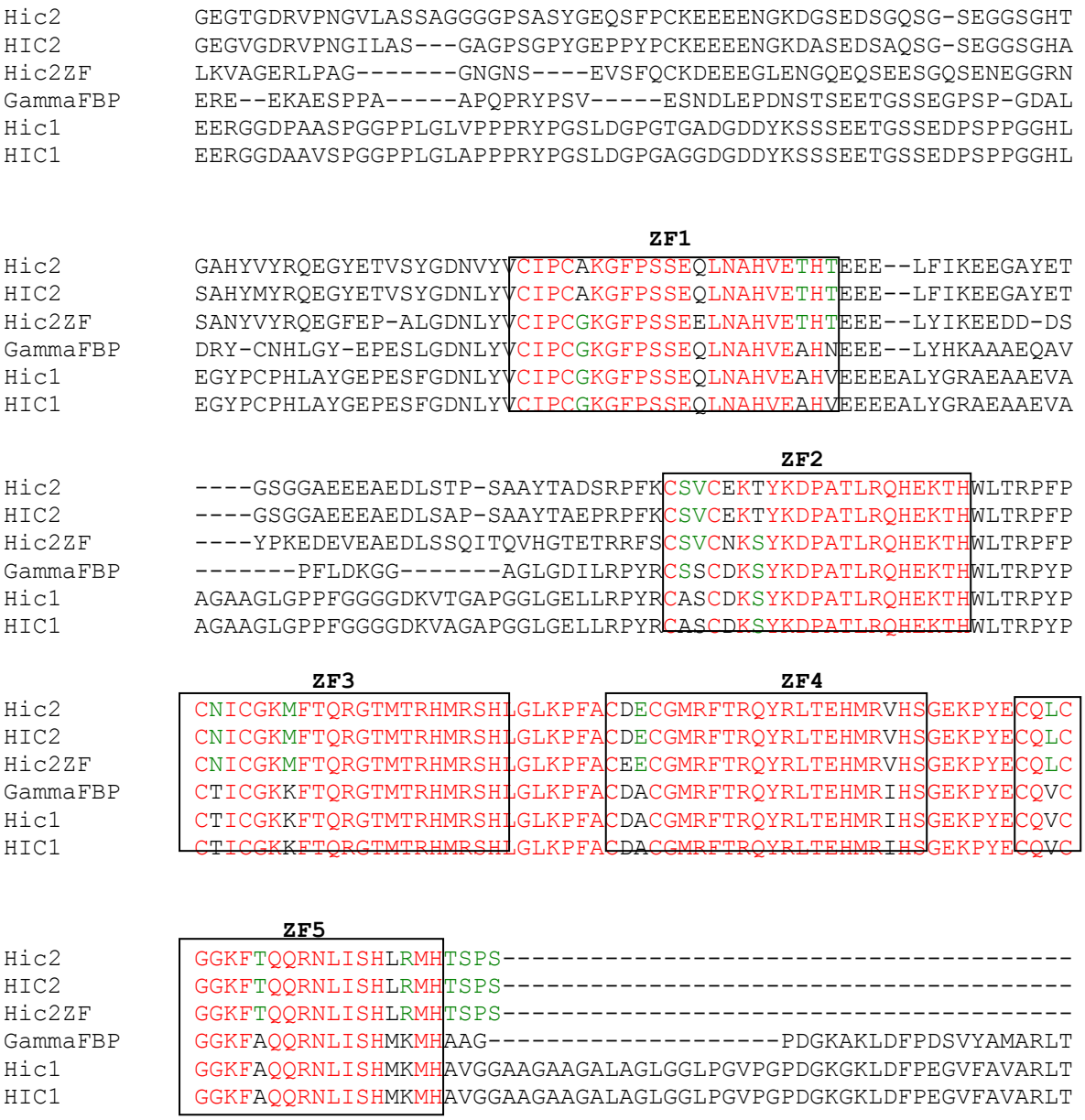


Figure 3.7 Comparison of mouse Hic2 related proteins. Multiple alignment of mouse Hic2, human HIC2, chicken γFBP, zebrafish HRG22, mouse Hic1 and human HIC1 proteins. Sequences were aligned with Swissprot Expassy CLUSTAL protein alignment program. The BTB/POZ and zinc finger domains are boxed. The RRKLKRAGK cluster of basic amino acids is underlined, the GLDLSK polypeptide is red coloured, the upstream zinc finger ZF 1 is boxed, like the downstream zinc fingers ZF2, ZF3, ZF4 and ZF 5.

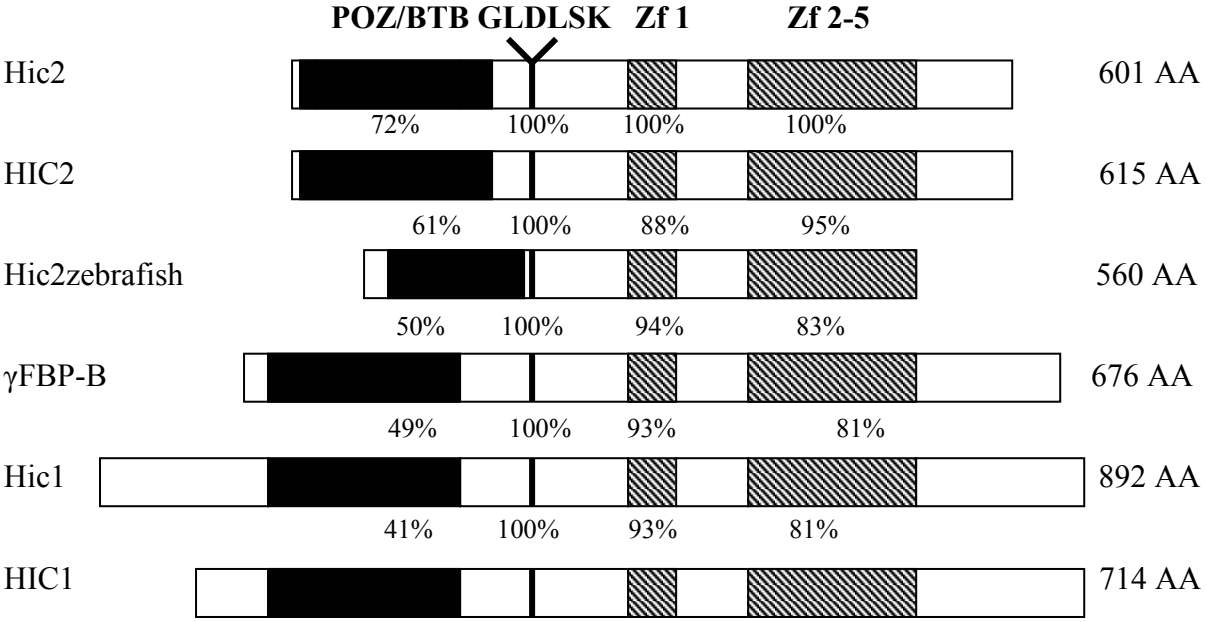


Figure 3.8 Domain structure. Schematic representation of the predicted mouse Hic2, human HIC2, chicken γFBP, zebrafish HRG22, mouse Hic1 and human HIC1 proteins.

Degrees of conservation (% of identity) obtained with the NCBI BLAST search program are indicated. Sequence accession numbers are for mouse Hic2 protein NP 849253, AF 117382, human HRG22 (HIC2) protein XP 036937, chicken γFBP-B protein Q 90850, zebrafish HRG22 (HIC2) protein NP 878289, mouse Hic1 protein NP 006488 and human HIC1 protein NP 006488. The Hic2 BTB/POZ domain sequence showed 72% identity with human HIC2, 61% identity with zebrafish Hic2, 49% identity with chicken γFBP-B protein, 49% identity with mouse Hic1 and 41% identity with human HIC1 sequence. The Hic2 protein zinc finger 1 (ZF1) sequence showed 100% identity with human HIC2, 88% identity with zebrafish Hic2, 94% identity with γFBP-B protein, and 93% identity with mouse Hic1 and human HIC1. The Hic2 protein zinc finger 2 (ZF2) sequence showed 100% identity with human HIC2, 90% identity with zebrafish Hic2, 81% IDENTITY WITH γFBP-B protein, 77% identity with mouse Hic1 and human HIC1. The Hic2 protein zinc finger 3 (ZF3) sequence showed 100% identity with human HIC2, 90% identity with zebrafish Hic2, 81% identity with γFBP-B protein, 77% identity with mouse Hic1 and human HIC1.The Hic2 protein zinc finger 4 (ZF4) sequence showed 100% identity with human HIC2 and zebrafish Hic2 and 90% identity with γFBP-B protein, mouse Hic1 and human HIC1. The Hic2 protein zinc finger 5 (ZF5) sequence showed 100%

identity with human HIC2 and zebrafish Hic2 and 80% identity with γFBP-B protein, mouse Hic1 and human HIC1 protein.

Following the structure of conserved domains it is clear that is a more homology between mouse Hic2 and human HIC2 including the zebrafish Hic2 sequence, and on the other side there is a more homology between mouse Hic1 and human HIC1.

That's why we can say there are two protein subgroups in the same family. Chicken γFBP-B showed more homology with Hic1 subgroup (Fig.3.9).

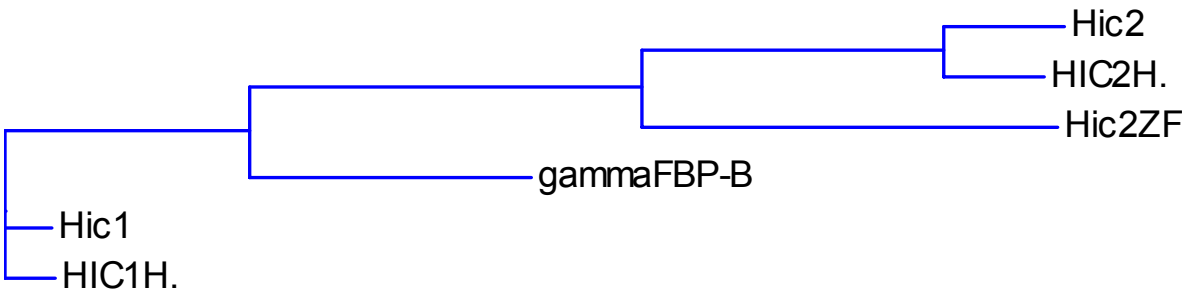


Figure 3.9 The phenogram of protein family members. A branching diagram that links entities by estimates of overall similarity. There are two sub group of the family members Hic1 and human HIC1, Hic 2 and human HIC2 including the zebrafish Hic2 protein.

In the sequence identities γFBP-B showed more identity with sub group Hic1/HIC1. Till now there are known various species (human, mouse, zebrafish, chicken) with the same protein family, which are coding the same, important, conserved domains.

3.4 Characterization of the *Hic2* promoter

3.4.1 Binding sites and deletions constructs in the *Hic2* promoter

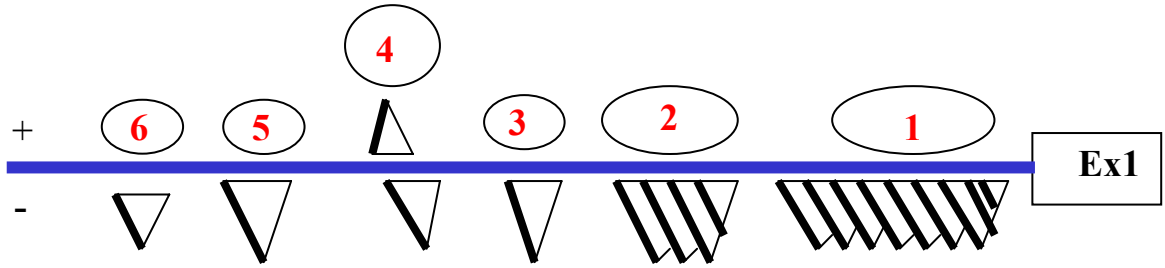
The computer program *PromoterInspector* (http://genomatix.gsf.de) is designed for location of regulatory regions within long genomic sequences. However this program can not predict all promoters.

For the location of potential binding sites but in short sequence stretches with known regulatory function, the *MatInspector Professional* (http://genomatix.gsf.de) was used.

This program predicted a few binding sites for gene regulation: Gut-enriched Krüppel-like factor, Stimulating protein 1 (SP1), Ecotropic viral integration site 1, Zinc finger transcription factor ZBP-89, AREB6 (Atp1a1 regulatory element binding factor 6), Wilms Tumor Suppressor, p53 and glucocorticoid receptor, C2H2 zinc finger protein binds glucocorticoid dependent to GREs (Fig.3.10A).

Therefore a 1.2 kb fragment directly upstream of the *Hic2* coding was cloned, in front of a *Luc* reporter gene (Y.Kamachi, 1993), (Fig. 3.10B).

A



B

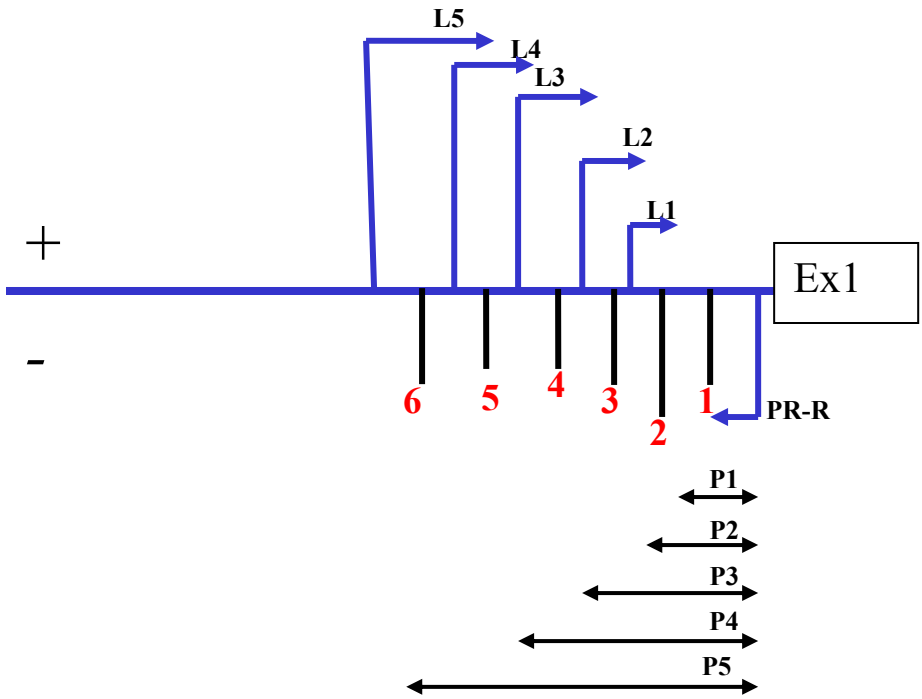


Figure 3.10 Binding sites and deletion constructs in the *Hic2* promoter.

A) Binding sites predicted with MatInspector program. 1) Gut-enriched Krüppel-like factor and Ecotropic viral integration site 1 encoded factor; 2) stimulating protein 1 SP1, ubiquitous zinc finger transcription factor and Wilms Tumor Suppressor and tumor suppressor p53 binding site; 3) cellular and viral CCAAT box; 4) Zinc finger transcription factor ZBP-89 and Wilms Tumor Suppressor; 5) AREB6 (Atp1a1 regulatory element binding factor 6); 6) Glucocorticoid receptor, C2H2 zinc finger protein binds glucocorticoid dependent to GREs.

B) Deletions constructs of the Murine *Hic2* promoter. PCR primers used for amplification of the genomic sequence are given in TABLE 2.14. 1-6 positions of binding sites, PR-R for reverse primer, L1-L5 for forward primers, P1-P4 for promoter fragments with deletion and P5 for total fragment without deletion. P1 385 bp, P2 424 bp, P3 518 bp and P4 685 bp. Total fragment without deletion P5 1167 bp.

Hic2 promoter sequences were PCR amplified (Fig.3.11 A); Forward primers contain an *Xho*I site for cloning, whereas the reverse PCR primer has a *BamH*I restriction site.

These were cloned in to the pCR.IITOPO Vector, and recovered from the vector with *BamHI/XhoI* digestion (Fig.3.11 B).

The fragments were cloned into the cloning site of the pPLLucII reporter vector (Y.Kamachi, 1993), using *SalI/BamHI* and *HindIII* restriction enzymes (Fig.3.11 C).

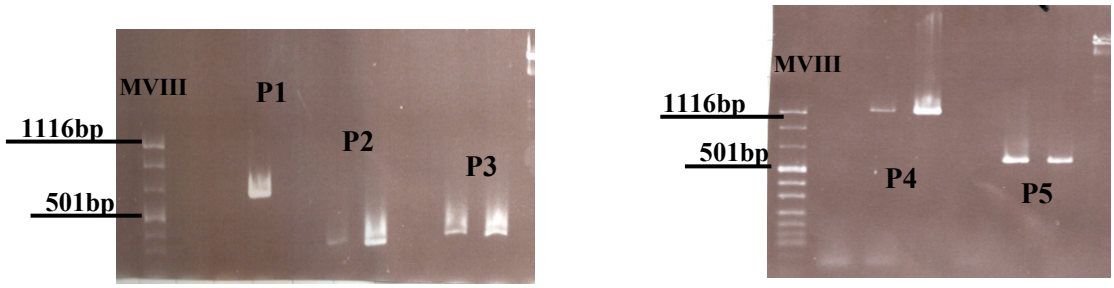


Figure 3.11 A) The *Hic2* promoter deletions PCR products. All five deletions were done with same forward primer IEX1-1D, reverse primers are located between the binding sites. 1) deletion 4 (primers IEX1-1D/IEX16L) 685bp, 2) deletion 1 (primers IEX1-1D/PromL1) 385 bp, 3) deletion 2 (primers IEX1-1D/IEX1-3L) 424 bp, 4) total fragment without deletion (primers IEX1-1D/IEX1-7L) 1167bp, 5) deletion 4 (primers IEX1-1D/IEX1-6L) 685 bp.

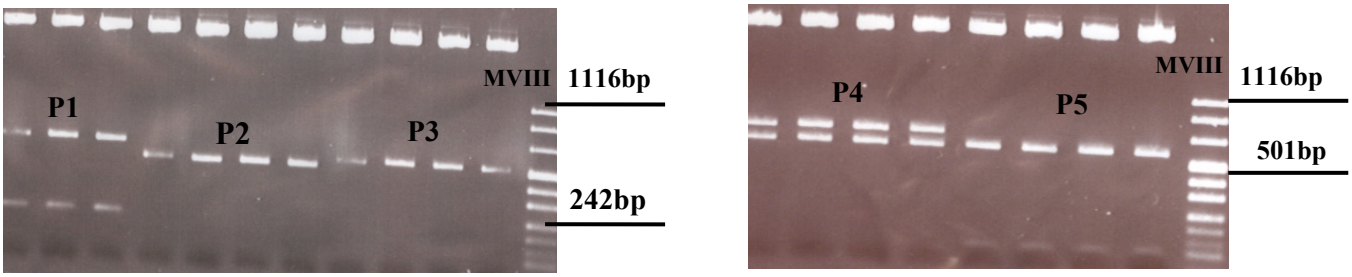


Figure 3.11B. The PCR products were cloned and recovered from the pCR.IITOPO Vector with *BamHI/XhoI* enzymes.

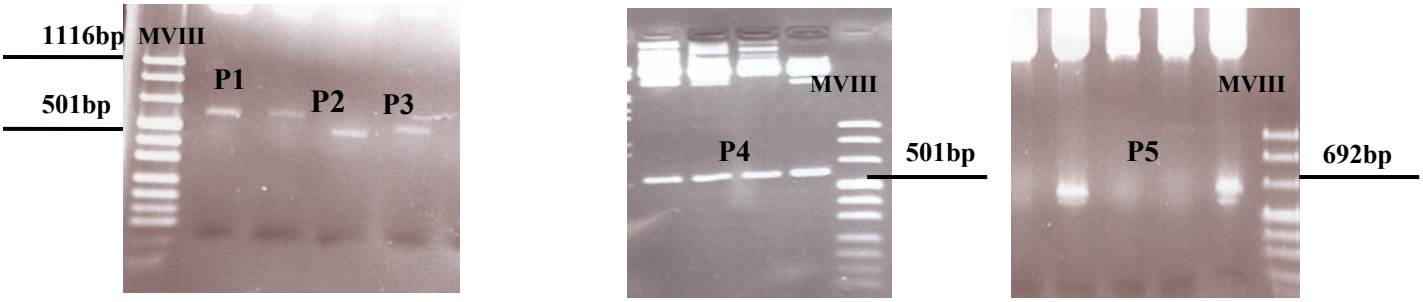


Fig.3. 11 C. *BamHI/HindIII* digestion from pPLLucII. II Vector

For transfection experiments, the HEK-293 (human embryonic kidney cells) cell line was used. This cell line can be used as a basic transfection system (Lengler and Graw, 2001).

3.4.2 *Hic2* promoter activity

Fragments within the 1.2 kb fragment (385bp, 424bp, 581bp, 685bp and 1167bp), result in a different promoter activity (Fig.3.12). The transfection of the *Hic2* promoter constructs in HEK-293 fibroblast cell line was done three times under the same conditions.

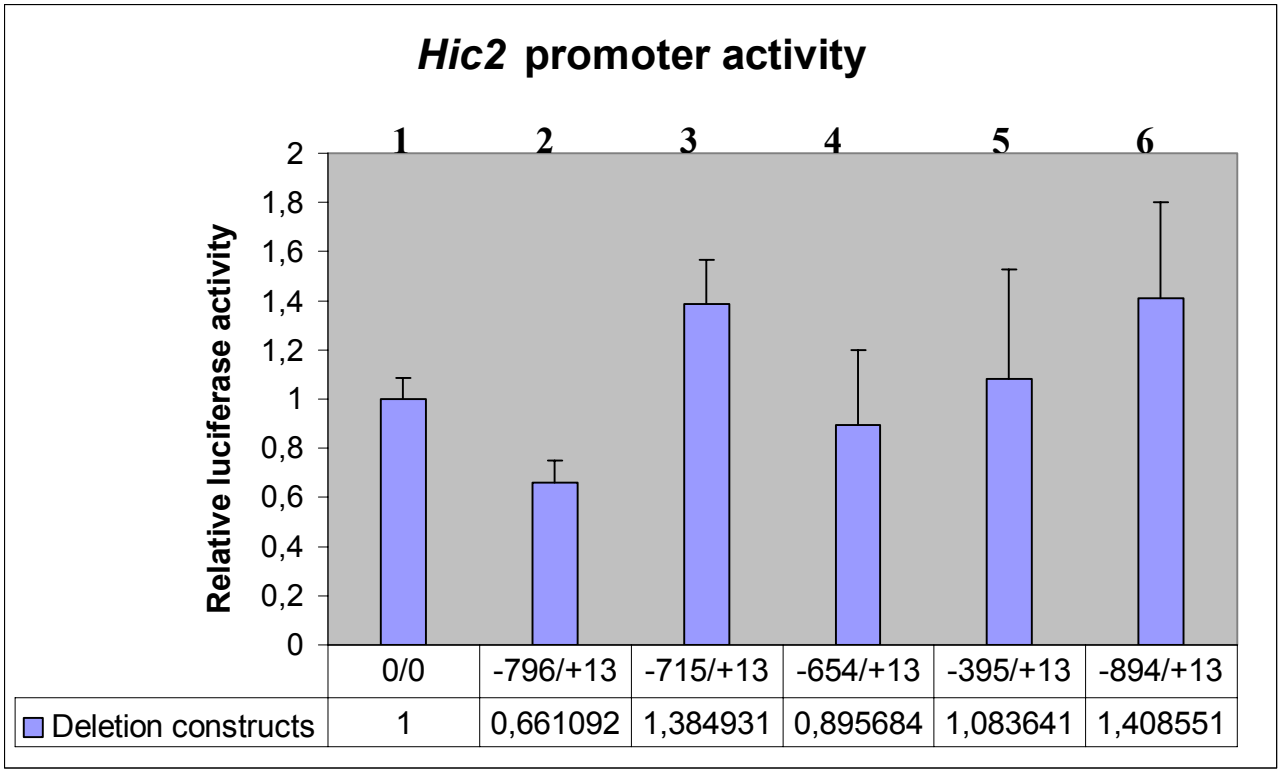


Figure 3.12 Function of *Hic2* promoter fragments. The peak activity of the *Hic2* promoter is located in construct number five (-894/+13, total fragment without deletion), the next higher activity is in the deletion number two (-715/+13). The relative Luciferase activity for column 1 control 0/0 is 1, for fragment 1 is 0.6611, for fragment 2 is 1.3849, for fragment 3 is 0.8957, for fragment 4 is 1.0836 and for total fragment column 5 is 1.4086.

Cutting the promoter sequence only 385 bp of its 3' end showed the lower activity but its activity drops down to the level of the promoter-less reporter plasmid. It is obvious that the 365bp fragment number one has the lowest activity (-796/+13). This deletion is just upstream from the TATA box and coding nine Gut-enriched Krüppel-like factor binding sites and nine Ecotropic viral integration site 1 encoded factor binding sites.

The fragment number 2 (-715/+13) which included proximal binding sites in 3' of the promoter (GEKL and Evil1) and 4 binding sites from stimulating protein 1 SP1, ubiquitous zinc finger transcription factor and Wilms Tumor Suppressor and tumor suppressor p53 binding site, showed increased promoter activity.

This deletion showed already after deletion five the highest activity in the cell culture. But we can not exclude binding factors from the first deletion. Obviously alone in the construct (deletion1), binding factors are not active but with binding sites from Wilms Tumor Suppressor and tumor suppressor p53 binding site, which should play a big role in the *Hic2* activation, (tumor suppressor function), activation is higher. This part (deletion 2) could be essential for the function of the *Hic2* gene.

The fragment number 3 (-654/+13) showed decreased activity. This construct include the binding sites from deletion 1 and cellular and viral CCAAT box. This transcription factor (cellular and viral CCAAT box) should have a suppressor effect in this deletion construct.

Fragment number 4 (-395/+13) showed high activity. This construct included binding sites from deletion one to three and for Zinc finger transcription factor ZBP-89 and Wilms Tumor Suppressor and AREB6 (Atp1a1 regulatory element binding factor 6). It showed a direct connection between those binding factors and promoter activity.

The total fragment without deletion (5), (-894/+13), included all binding sites and the Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs which is specific just for this construct and showed the highest activity.

Because of the binding sites in the of the promoter (Zinc finger transcription factors, Wilms Tumor Suppressor and tumor suppressor p53 binding site, involved in tumor- and cancerogenesis) this part could be essential for the function of the *Hic2* promoter.

3.5 Expression of the mouse *Hic2* mRNA

The expression of the mouse *Hic2* was investigated by *in situ hybridisations* (ISHs) of whole mount embryos and on paraffin sections.
As templates for riboprobes, the 576bp and 449bp, *Hic2* mRNA clone AF117382, and 300bp cDNA clone from the Exon 2 were used. No obvious differences were observed in the expression patterns detected by riboprobes generated from both templates.
No expression was observed in the earliest stages investigated, Theiler stages (TS) 12-14 (day 8-9 p.c.).
At later stages, *Hic2* expression was detected in restricted territories of brain, *sinus centralis*, olfactory bulb, *canallis centralis medullae spinalis*, embryonic ectoderm (neuroepithelium of neural tube), small intestine.
RT-PCR analysis showed tissue-specific expression of the *Hic2* probe. Expression was strong in adult organs, especially in the brain.

3.5.1 The whole mount In-situ hybridisation (WMISH)

The RNA In *situ* hybridisation was done on whole mount embryos. As positive control for whole mount in situ hybridisation an *En1* probe was used. This probe is characteristically expressed in the brain in the earlier stages of mouse development (TS13), and in the limbs and somites in later stages (TS 14-18) (Fig. 3.13).
In mouse embryo the expression of this gene was first detected at E8 in overlapping bands of the anterior neural folds.
By E12, the gene was expressed in a ring of cells in the central nervous system at midbrain/hindbrain junction *En-1* was also expressed in two lateral stripes extending the length of the hindbrain and spinal cord in the developing vertebral column and in the tail and limb buds. These data are consistent with *En-1* gene playing a role early in development in defining spatial domains in the CNS (Joyner and Martin, 1987).

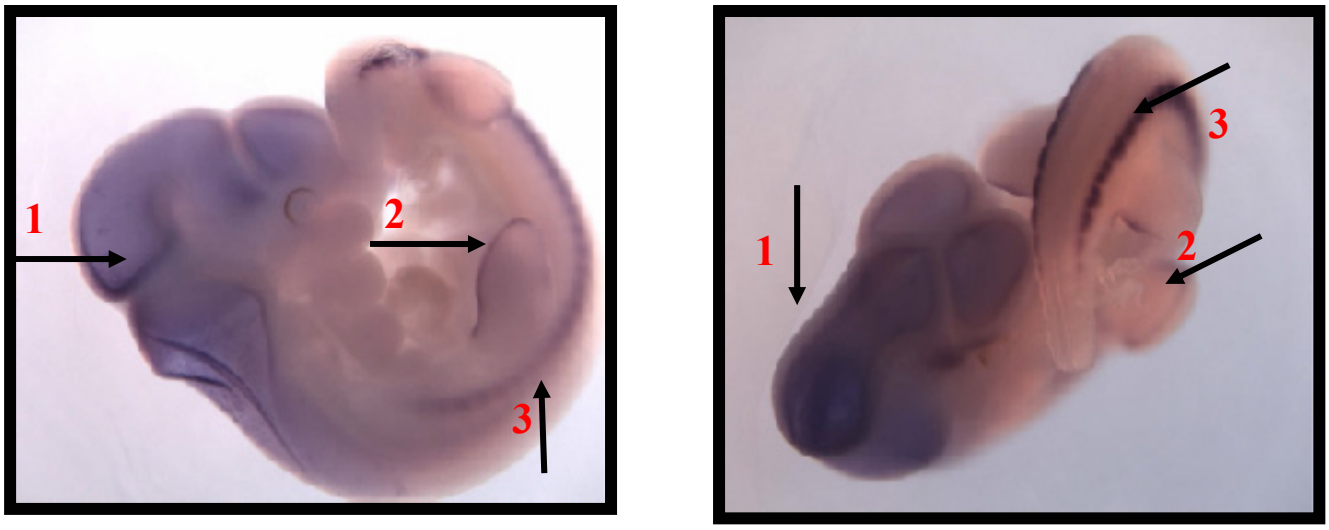


Figure 3.13 *En-1* probe with characteristic patterns.
The embryos are in the Theiler stage (TS) 17, gestational age E11.5. 1) for brain, 2) for distal part of the limbs, 3) somite derivate and distal arch.

3.5.2. *Hic2* is expressed in the central and peripheral nervous system

From Figure 3.14 , it is evident that *Hic2* is expressed in a variety of tissue and organs like different parts of the brain, embryonic ectoderm (neural plate, neural channel and neural tube), in the limbic region of the brain, lateral ventrical of the olfactory lobe (lobe of the brain that rests on the lower surface of a temporal lobe and projects forward from the anterior lower part of each cerebral hemisphere, that is continuous anteriorly with the olfactory nerve, that consists of an olfactory bulb, an olfactory tract, and an olfactory trigone, and canallis centralis of the spinal cord (Fig. 3.14).

A

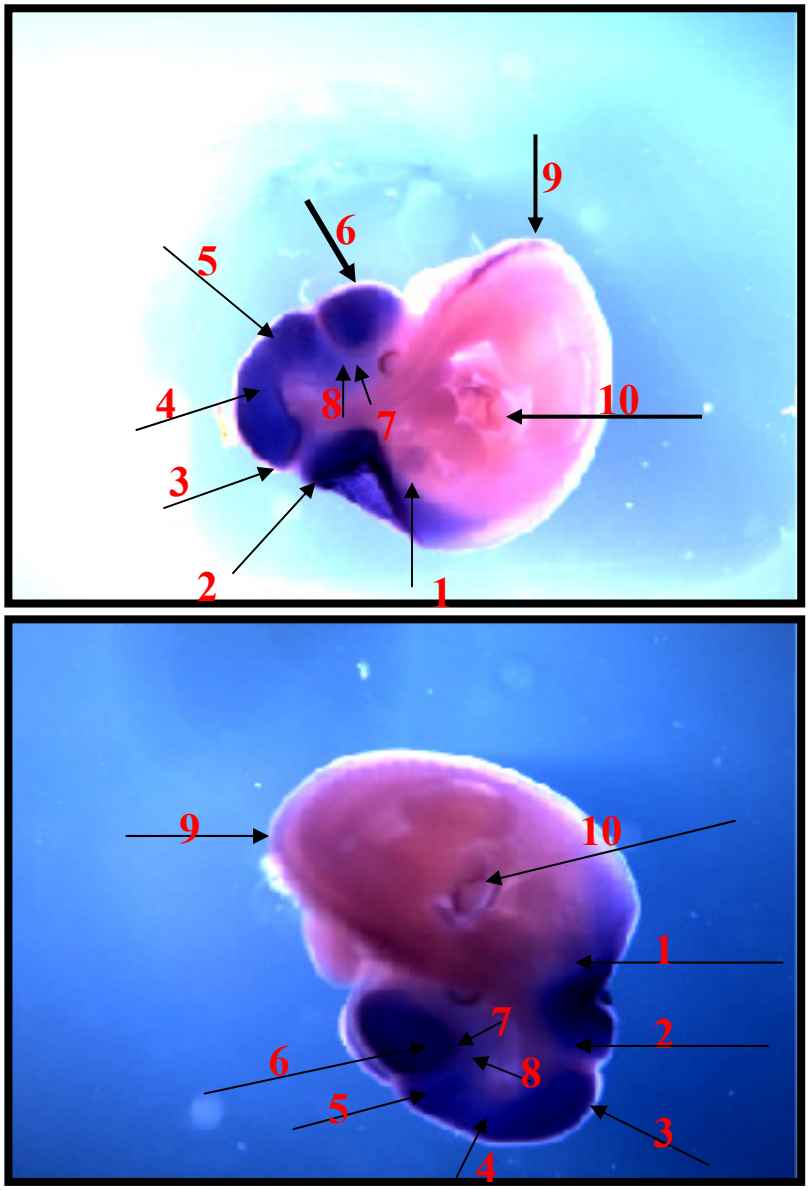


Figure 3.14 The embryos are Theiler stage (TS) 17, gestational age E11.5. A) *Hic2* is expressed in (1) sinus centralis, (2) myelencephalon, (3) metencephalon, (4) mesencephalon, (5) diencephalons, (6) telencephalon, (7) infundibulum, (8) pons, (9) canallis centralis medullae spinalis, and (10) small intestine. *Hic2* expression is high present in the nervous system. (A) lateral views, (B) dorso-lateral views. Proximal but suprarostral part of the brain showed the highest level of expression. Cranio-apical part is separated from the median part of the head in the region near the infundibulum and pons. This area which is more medially located showed no expression. Caudal part of spinal cord (9) in the ectoderm from the region of gluteal musculature showed expression.

B

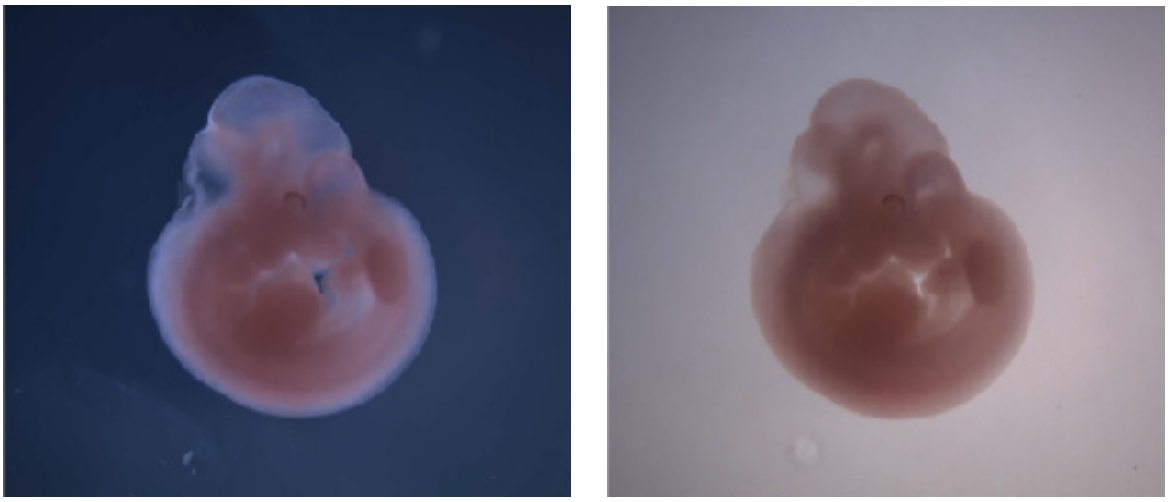


Figure 3.14 B) Sense *Hic2* probe

3.5.3 *Hic2* is expressed in the limbic region of the brain

Transverse sections of the head TS24 (E17.5), showed *Hic2* expression in the olfactory bulb. (Fig.3.15).The olfactory bulb is located in the limbic region of the brain. Each olfactory bulb is located on either side of the nasal passage at the base of the brain. They lie on a perforated, thin layer of bone that separates the nasal cavity from the brain.

The function of the olfactory bulb is to relay sensory signals to the olfactory tract. The olfactory tract contains axons from some of the neurons in the olfactory bulb. Some of those axons go directly to the phylogenetically oldest part of the cerebral cortex where they synapse, others travel through the anterior commissure to the contra lateral (opposite side) olfactory bulb.

Hic2 expression in olfactory bulbs

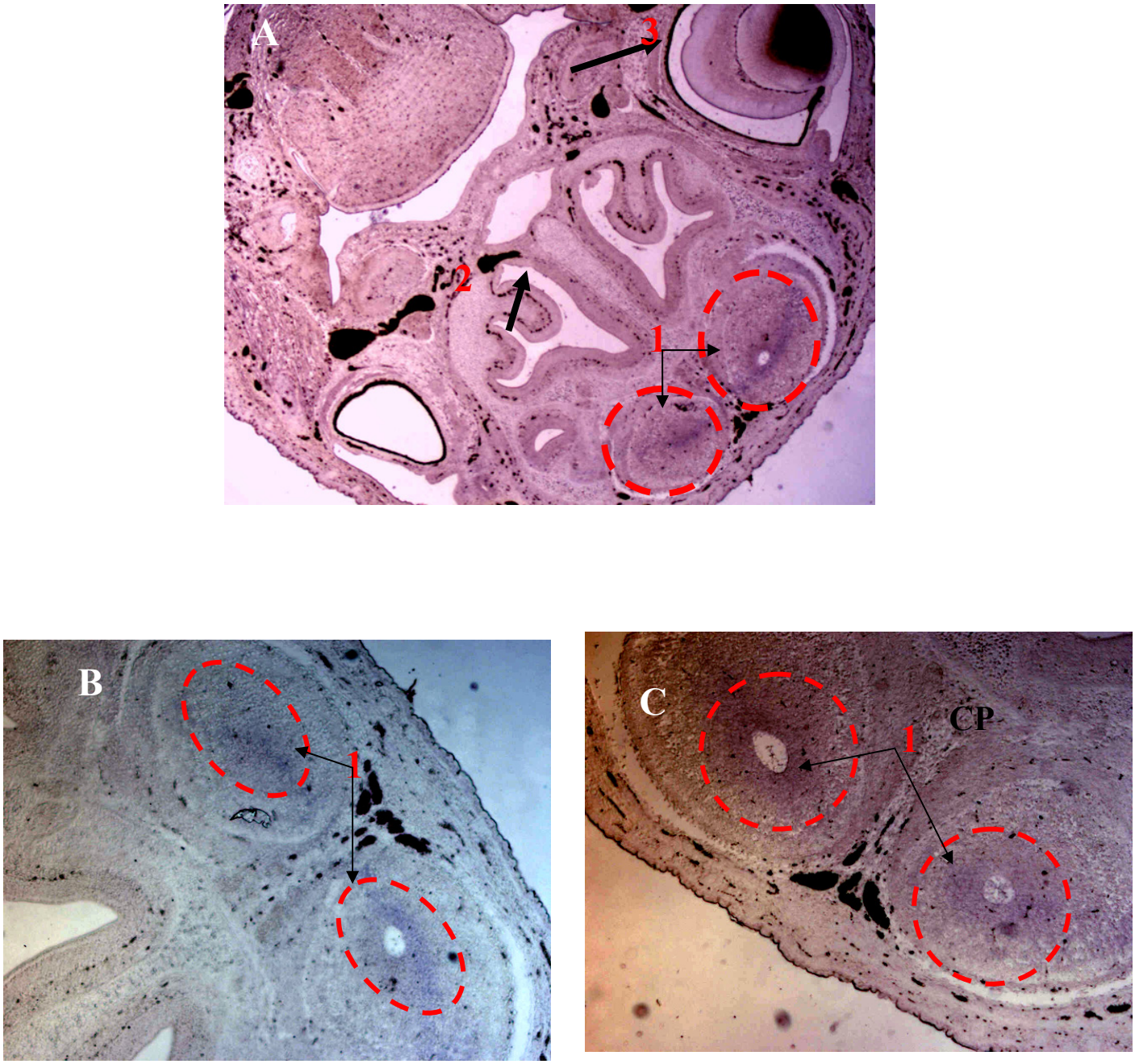


Figure 3.15 Transverse section (Theiler stage 24, E17.5). Expression is located in the olfactory bulbs (A) 1 ringed in red, 2 for nasal cavity and 3 for eye. (B) and (C) for higher magnification, 1 expression in the olfactory bulbs, (CP) cartilage primordium of the nasal septum, (ON) olfactory nerves, (NC) nasal capsule.

3.5.4 *Hic2* is expressed in the embryonic ectoderm

In whole body sagittal sections at TS 19, (gestational age E12.5) *Hic2* expression in the ectoderm was detected.

The ectoderm produces the nervous system, the neural plate, the neural channel and the neural tube. *Hic2* is expressed in suprabasal layers of the caudal part of the neuroepithelium in a part characteristic for gluteal muscle development (Fig.3.16).

The mouse embryo comprises three tissue layers, two extra-embryonic, the visceral endoderm and the extra-embryonic ectoderm, and one embryonic, the epiblast. At E5.5 a small subset of visceral endodermal cells, the anterior visceral endoderm (AVE), becomes morphologically distinct from the rest of the visceral endoderm and expresses a characteristic set of molecular markers. These cells are initially located at the distal tip of the embryo and then move up unilaterally to the prospective anterior converting the proximal-distal axis of the embryo into an anterior-posterior axis (Rodriguez et al., 2002).

During gastrulation in vertebrates the cells of the embryonic ectoderm give rise to epidermal progenitors in the ventral side and neural progenitors in the dorsal side (Chang et al., 1998).

Hic2 expression in embryonic ectoderm

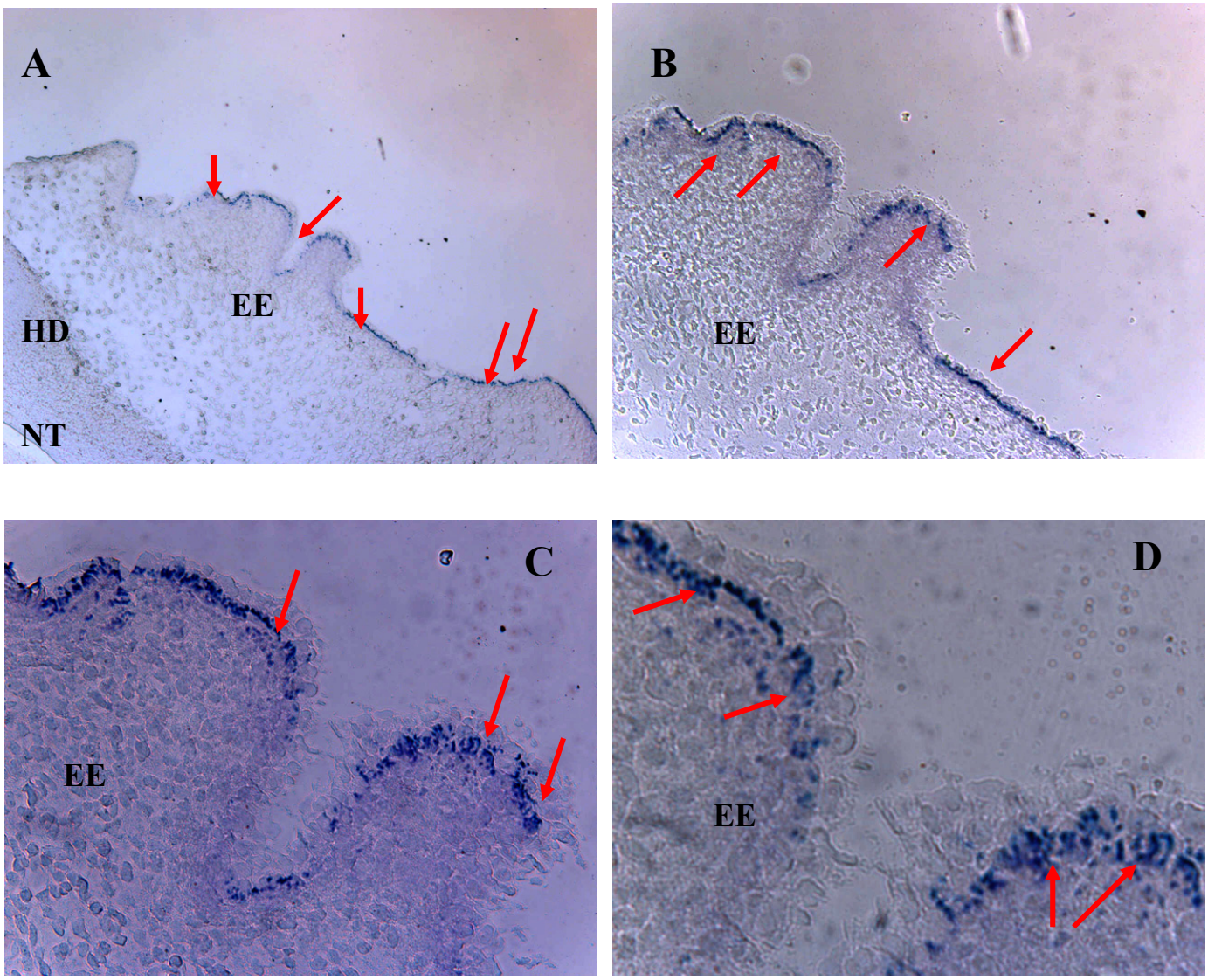


Figure 3.17 Sagittal sections of the embryos Theiler stage 19, Carnegie stage 16, gestational stage E12.5.

Caudal part of the body, region near the tail and gluteal musculature. (A) and (B) for lower magnification, (C) and (D) for higher magnification. EE embryonic ectoderm, HD horda dorsalis Expression is obvious in granular layer of the ectoderm.

3.5.5 *Hic2* is expressed in small intestine

The duodenum is the first part of the small intestine. It is located between the stomach and the jejunum. The jejunum and ileum are suspended from the posterior abdominal wall by the mesentery of the small intestines. The jejunum occupies a central position in the abdominal cavity, below the liver and the stomach, and behind the transverse mesocolon, the transverse colon and the greater omentum. The ileum is the posterior or lower part of the small intestine. The *Hic2* is expressed in the small intestine, duodenum, ileum and jejunum (Fig. 3.18).

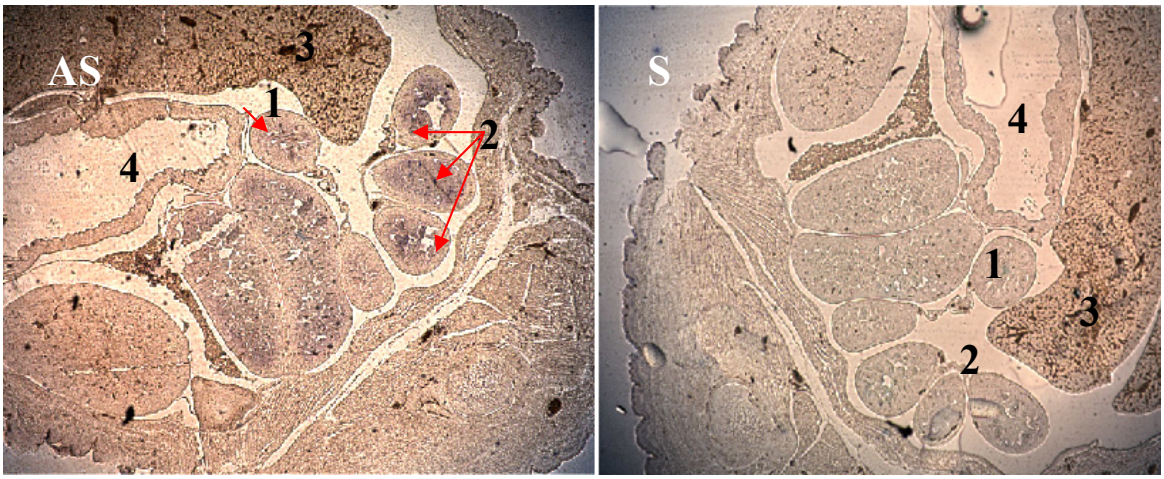


Figure 3.18. Sagittal section abdomen TS24,(gestational age E17.5). (AS) for anti sense probe with expression. (S) for control sense probe. 1. Lumen of first part of duodenum, 2.small intestine, 3. Liver, no expression found 4. Lumen of bladder (orientation).

3.5.6 *Hic2* is not expressed in eye

RT-PCR analysis showed *Hic2* expression in the eye TS24, E17.5 (Fig.3.19). That was a reason to test the gene at different developmental stages.

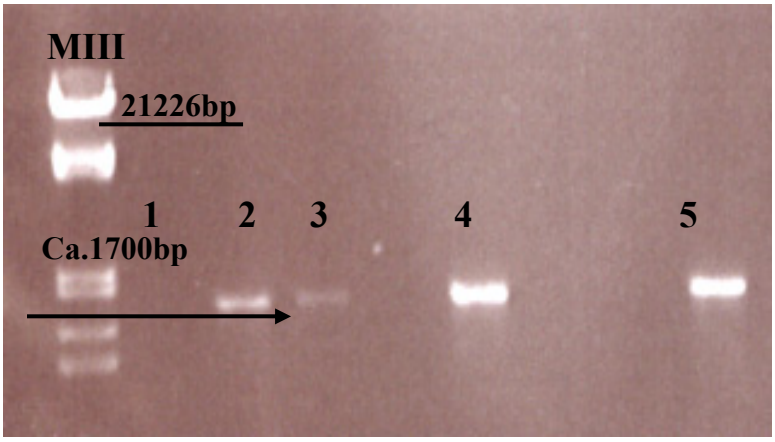


Figure 3.19 RT-PCR results : 1) K-, 2)E12.5 head, 3) E12.5 body 4) E13.5 whole body and 5) E17.5 eye. The expression in the eye was found just per RT-PCR analysis; ISH analysis on the section of the eye in different embryonic development stages was negative.

Eye specific control probes with specific expression patterns were included: *CrygD* probe which is expressed in the lens, *Pax6* expressed in neural part of the retina and *Pax2* expressed in nervus opticus of the eye (Fig.3.20).

CrygD is specifically expressed in the eye lens (Lengler and Graw, 2001).

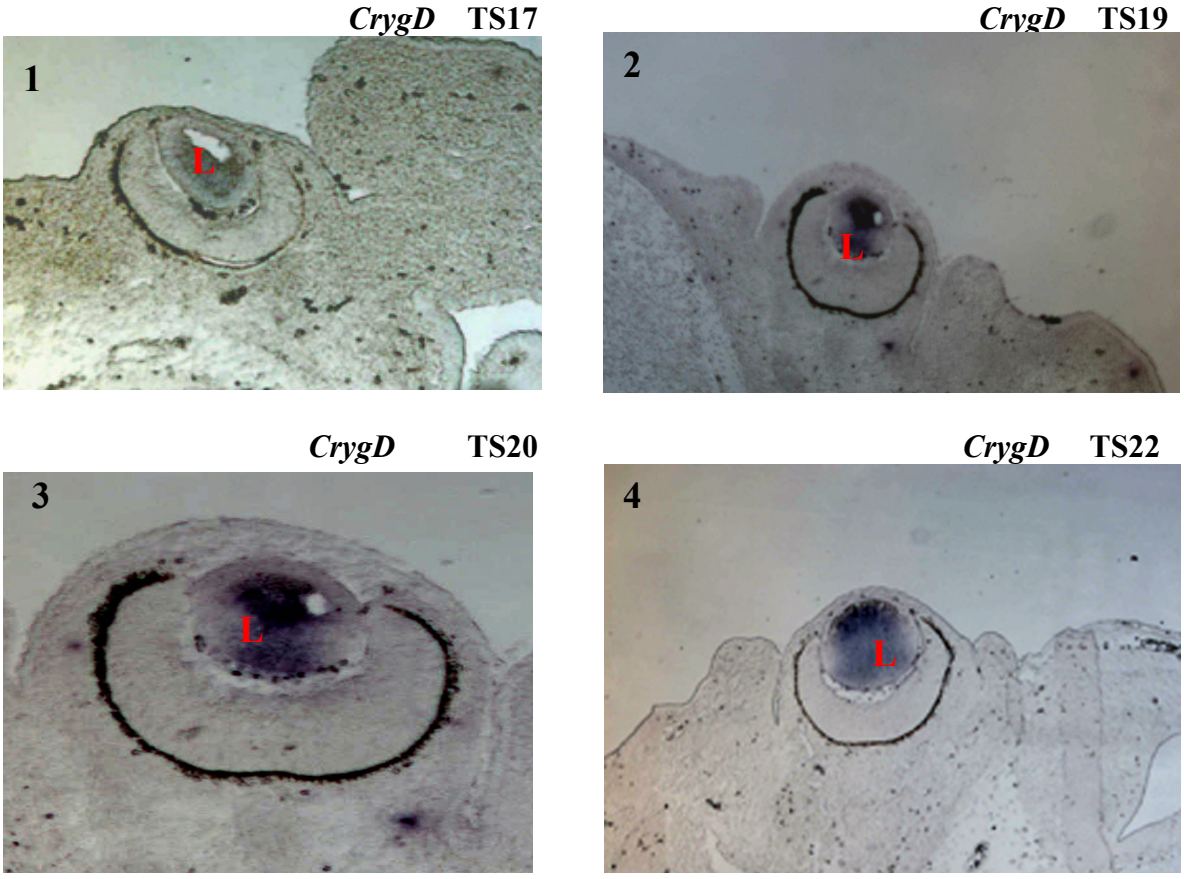
Pax6 expression predominate in lens placode and corneal and conjunctival epithelia, in lens placode, optic vesicle and CNS, and only weakly in corneal and conjunctival epithelia (Xu et al., 1999).

In the eye, Pax2 expression is restricted to the ventral half of the optic cup and stalk and later to the optic disc and nerve (Norens et al., 1990).

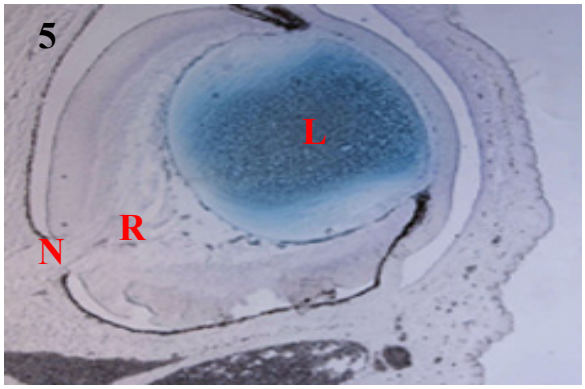
Expression of the mouse *Hic2* gene in lens at embryonic, or fetal stages by means of *in-Situ* hybridisations of whole embryos and on paraffin sections was not detected (Fig.3.21).

Thus, it is not likely that the Murine *Hic2* gene is implicated in lens differentiation.

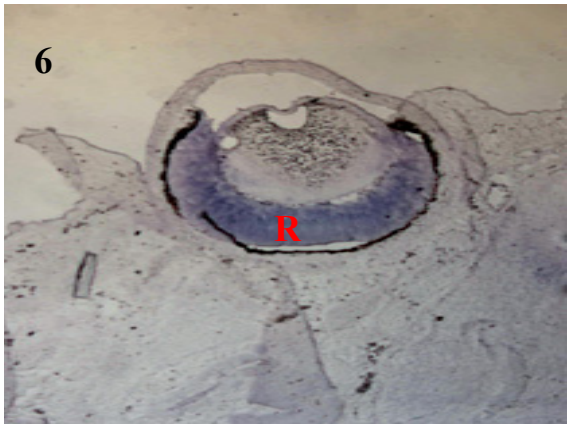
A



CrygD **TS24**



Pax6 **TS 24**



Pax2 **TS24**

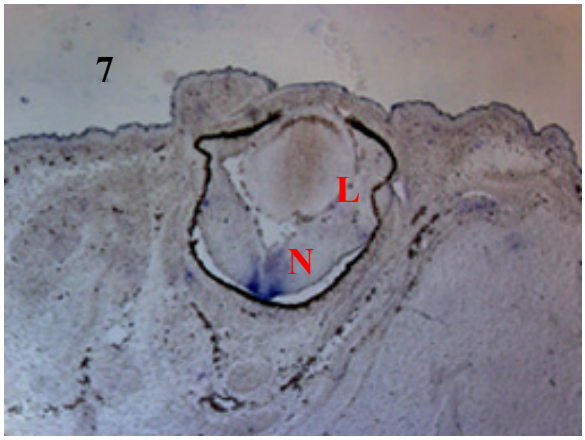


Figure 3.20 *In situ* hybridisation to assay *CrygD*, *Pax6* and *Pax2*: 1) E11.5, 2) E12.5, 3) E13.5, 4) E14.5 and 5) E17.5 , 6)E15.5 *Pax6* probe and 7) E15.5 *Pax2* probe L lens, R retina and N nervus opticus.

CrygD, Pax6 and *Pax2* were used because of the specific expressionpattern. L lens, R retina and N is for nervus opticus.

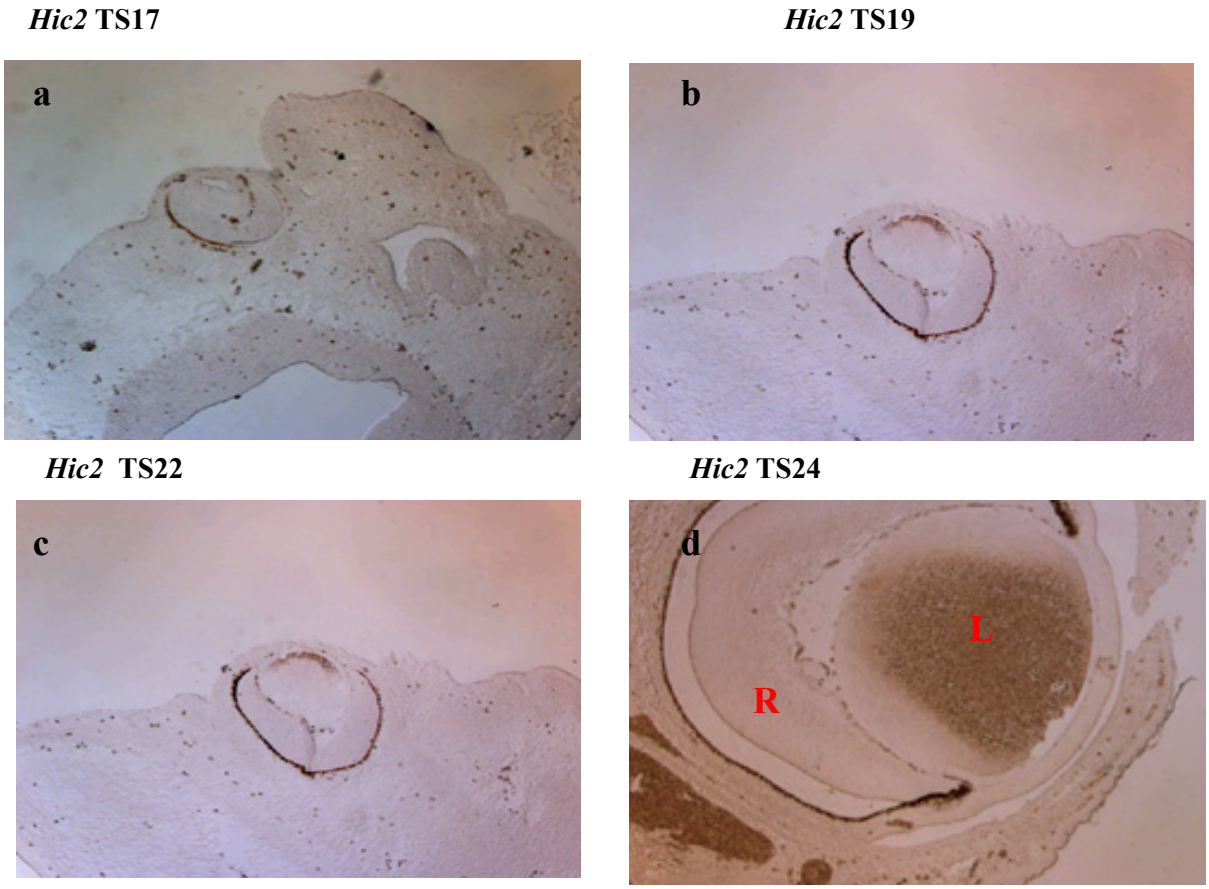


Figure 3.21 In situ hybridisation to assay *Hic2* expression. 1) E11.5, 2) E13.5, 3) E15.5, 4) E17.5. *Hic2* probe from 3'UTR with expression in the brain and intestine gave no results in the eye. c) and d) showing a dark field, but that is no expression. During the in situ hybridisation Proteinase K made some more digestion in the lens cells. There is no difference between sense and antisense slides.

4. Discussion

In this study, a new BTB/POZ transcription factor was identified by sequencing and bioinformatics analyses, and validated and extended by genomic cloning and RT-PCR analysis. Sequence analysis of the murine *Hic2* gene revealed that it represents the murine orthologues of the previously described human *HIC2* gene (Deltour et al., 1999).

The genomic organisations of human and mouse *Hic2* are highly similar. They have strong sequence homologies (>80%) in their BTB/POZ and Zinc finger domains as well as in short peptide in their middle region (GLDLSK/K). *HIC2* defines a subgroup of BTB/POZ domains unable to recur HDACs-containing (histone deacetylase) complexes (Deltour et al., 2001). The embryonic expression of *Hic2* is in line with its human homologue *HIC2* gene. The expression in brain, embryonic ectoderm (neural plate, neural channel and neural tube), and lateral ventricul of olfactory lobe, showed that the gene seems to be involved in development of central and periphery nervous system.

4.1 *Hic2* Promoter and its binding sites

The promoter was not predicted by the computer program *PromoterInspector*. It might be concluded that his activity is already low (second class promoter) but promoter activity might not be correlated with gene expression but could depend on with promoter hypermethylation which is often correlated with gene silencing. One hypothesis which could be tested new promoter activity to a consider gene function and determine if a hypermethylated gene is reactivated by demethylation. The next step should be transfection from the same deletion constructs in different cell lines (cancer and normal).

MatInspector predicted some of the binding sites which could play a different role in *Hic2* promoter activity and gene expression.

Transcription factors are modular proteins from functionally discrete domains and can be classified on the structure of the domain that binds DNA (Collins et al., 2001). Almost the entire *Hic2* promoter binds sites GKLF1, Sp1, ZBP-89, AREB6, (Fig.4.1) coding the zinc finger domain and are involved in tumor and cancer genesis.

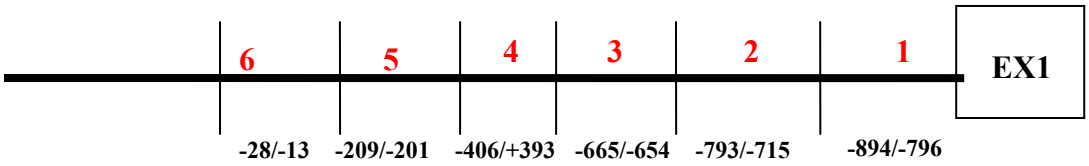


Figure 4.1 Positions of the some binding sites in distal part of *Hic2* promoter. 1) Gutenriched Krüppel like factor, 2) Stimulating protein 1, 3) CCAAT box, 4) Zinc finger transcription factor ZBP-89, Wilms tumor suppressor, p53, 5) Atp1a1 regulatory element binding factor, 6) Glucocorticoid receptor

The *Hic2* share the same expression pattern with some of the transcription factors in its promoter: With Gut-enriched Krüppel-like factor (GKLF) it is epithelia cells of the gastrointestinal tract (Shields et al., 1996; Gareth-Sinha et al., 1996); With Ecotropic viral integration site 1 (Evil1) it is the peripheral nervous system (Hiroshi et al., 1999).
ZBP-89 factor is interesting because of its expression which is known to be increased in gastric carcinoma cells (Ye et al., 1999).

Nine Gut-enriched Krüppel-like factor binding sites are predicted between nucleotides -894 to -881, -884 to -871, -874 to -861, - 864 to -851, -841 to –854 to -841, -844 to -831, -834 to -821, -819 to -806, and - 809 to -796. The gut-enriched Krüppel-like factor (GKLF) is a recently identified eukaryotic transcription factor that contains three C_2H_2 zinc fingers. The amino acid sequence of the zinc finger portion of GKLF is closely related to several Krüppel proteins, including the lung Krüppel-like factor (LKLF), the erythroid Krüppel-like factor (EKLF) and the basic transcription element binding protein 2 (BTEB2);(Shields and Yang 1997). Expression of GKLF is enriched in epithelia cells of the gastrointestinal tract and in the epidermal layer of the skin (Shields et al., 1996; Gareth-Sinha et al., 1996).

Nine Ecotropic viral integration site 1 encoded factor binding sites are predicted between nucleotides -894 to -878, -882 to -868, -872 to -858, -862 to - 848, -852 to - 838, -837 to -823, -827 to -813, -817 to -803, and -807 to -796. The Ecotropic viral integration site 1 *Evil1* gene is thought to function as a transcription factor and a proto-oncogene for leukemia. Expression of the *Evil1* gene was found in the renal tubules in the corticomedullary junction of the kidney and the cytoplasm of the developing oocytes in adult mice, fetal expression has been reported in urinary system, limb buds, heart, nasal pits, and bronchial epithelium. Additional

expression was observed in neural crest-derived cells associated with the peripheral nervous system and embryonic mesoderm (Hiroshi et al., 1999).

One Stimulating protein 1 SP1, ubiquitous zinc finger transcription factor is predicted between nucleotides -793 to -781. Transcription factor SP1 is located in the nucleus of a mammalian cell and importantly related to expression of many cellular genes (Kuwahara et al., 2000). Sp1 is a ubiquitous, 778-amino acid transcription factor that recognizes GC-rich sequences present in many promoters (Azizkhan et al., 1993, Black et al., 1996). Although Sp1 has been viewed as a constitutive transcriptional activator which acts as a basal factor for TATAA-less promoters, an increasing number of studies indicate that Sp1-dependent transcription is regulated in response to a variety of signals. Sp1 expression is restricted to the brain (Adrian et al., 1999). Sp1-like proteins and Krüppel-like factors (KLFs) are highly related zinc-finger proteins that are important components of the eukaryotic cellular transcriptional machinery (Kaczynski et al., 2003).

One cellular and viral CCAAT box is predicted between nucleotides -665 to -654. Cellular and viral CCAAT box is a promoter element common to all major histocompatibility complex class II genes (Dorn et al., 1987).

One Zinc finger transcription factor ZBP-89 is predicted between nucleotides -406 to -395. Zinc-finger binding protein (ZBP-89, also known as BFCOL1, BERF-1 and ZNF-148) is a Krüppel-type zinc-finger transcription factor that binds to the same GC-rich sequences as Sp1. ZBP-89 expression is known to be increased in gastric carcinoma cells (Ye et al., 1999). Two zinc finger transcription families ZBP-89 and Sp1 regulate the gastrin promoter (Juanita, 1984).

One AREB6 (Atp1a1 regulatory element binding factor 6) is predicted between nucleotides –209 to –201. The transcription factor AREB6 contains a homeodomain flankend by two clusters of Krüppel type C_2H_2. AREB6 binds to the E-box consensus sequence, CACCTGT, either the N- or the C-terminal zinc finger cluster (Ikeda et al., 1998). AREB6 is known to regulate the expression of the Na, K-ATPase α 1 subunit, interleukin 2 and δ-crystallin genes (Ikeda K., Kawakami K. 1995).

One Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs is predicted between nucleotides -13 to -28. The glucocorticoid receptor (GR) belongs to the nuclear receptor (NRs) family. Most members of this family display an identical structural organization with an amino-terminal region A/B followed by a DNA-binding domain (DBD) consisting two zinc fingers (Moras et Gronemeyer, 1998). Activation of target genes relies in most cases on the ability of the GR to bind to specific DNA sequences named glucocorticoid responsive elements (GREs) (Mangelsdorf et al., 1995). Transcriptional repression of GREs has been linked to cell death (Rogatsky et al., 1999).

All those transcriptions factors should play a role in the gene expression and promoter activity.

4.2 Conserved domains

The POZ domain and the zinc-finger region of the Murine *Hic2*, the human *HIC2* and the chicken Gamma proteins are highly identical, thus suggesting that these proteins might exert similar functions. Comparison of the Murine *Hic2* expression and that of a related gene in chicken γ, revealed some similarities, but also some differences.
In comparison with other members of the HIC1 and HIC2 family (amino acid sequence identities), (Fig. 9), Hic2 showed more homology with HIC2 family members (human and zebra fish). *Hic1* and *Hic2* belong to the same BTB/POZ domain family, share the same domains (GLDLSK/K and Zinc finger 1-5) but expressions patterns are absolutely different.

4.2.1 Hic2 BTB/POZ domain

The BTB/POZ domain showed the highest identity with human and zebrafish homologue, and lowest with human HIC1 homologue. The POZ domain acts as a specific protein-protein interaction domain. This motif, found in a growing number of zinc finger proteins, can inhibit DNA binding and mediate dimerization (Bradwell et al., 1994).

The characteristics of the family of genes to which *Hic2* belongs are quite consistent with a role for the gene in developmental processes. Members of the POZ domain family of zinc-finger transcription factors all possess a structure similar to that of *Hic2*, with highly

conserved N-terminal POZ domain and variable number of Krüppel –type zinc fingers towards the C-terminus (Bardwell and Treisman, 1994, Alibagli et al., 1995).

The full- length Hic2 and Hic1 proteins share several functional properties and that's why is for suspecting that their BTB/POZ domain heterodimerize. Heterodimer formation should show possible other DNA-binding specify and bind the other cofactors like homodimer. The BTB/POZ domain is not restricted to DNA-binding proteins or to other transcription factors but is likely to be a more general one. The BTB/POZ domain is involved in homodimer or heterodimer formation as well as oligomerization (Deltour et al., 2001).

4.2.2 GLDLSKK/R polypeptide

A short GLDLSKK/R polypeptide located in the central region between BTB/POZ domain and zinc fingers is perfectly conserved (Part 3 Fig.9 and 10). In this short polypeptide sequence Hic2 showed 100% identity with other Hic1 and Hic2 family members.
This motif is highly related to the consensus PxDLSxK/R first identified in the C-terminal part of the E1A protein (Shaeper et al., 1992), and later in numerous transcriptional repressors in *Drosophila* and in vertebrates able to bind CtBP (C-terminal Binding Protein) corepressor. The invariant praline residue found in virtually all CtBP binding proteins is replaced by a Glycine residue in the Hic2 and Hic1 proteins (Part 3 Fig. 9 and 10). Founding of GLDLSKK/R motif in a region which is not well-conserved between Hic2 and Hic1 proteins from various species suggests that it can fulfil an important functional role.

4.2.3 *Hic2* zinc finger domain

In comparison with the five the zinc finger domains , Hic2 protein showed some differences with its mouse ortolog Hic1 and chicken γFBP protein(Part 3 Fig.10).
The zinc finger 1-4 protein sequences showed more similarity between Hic1 and γFBP which seems to belong to the same protein subgroup of the Hic1 and Hic2 family members (Figure 4.2).

```
                    ZF1                         ZF2
Hic2          CIPCAKGFPSSEQLNAHVETH CSVCEKTYKDPATLRQHEKTH
GammaFBP      CIPCGKGFPSSEQLNAHVEAH CSSCDKSYKDPATLRQHEKTH
Hic1          CIPCGKGFPSSEQLNAHVEAH CASCDKSYKDPATLRQHEKTH

                    ZF3                         ZF4
Hic2          CNICGKMFTQRGTMTRHMRSHL CDECGMRFTRQYRLTEHMRVHS
GammaFBP      CTICGKKFTQRGTMTRHMRSHL CDACGMRFTRQYRLTEHMRIHS
Hic1          CTICGKKFTQRGTMTRHMRSHL CDACGMRFTRQYRLTEHMRIHS

                   ZF5
Hic2           CQLCGGKFTQQRNLISHLRMH
GammaFBP       CQVCGGKFAQQRNLISHMKMH
Hic1           CQVCGGKFAQQRNLISHMKMH
```

Figure 4.2 comparison of the zinc finger protein sequences between Hic2, Hic1 and γFBP. In the zinc finger 1 (ZF1) sequence it is two amino acids, for Hic2 it is one alanine (A), and for Hic1 and γFBP it is one glycine (G), one threonine (T) for Hic2 and one alanine (A) for Hic1 and γFBP. In the zinc finger 2 (ZF2) sequence there are four amino acids, for Hic2 and γFBP it is one serine (S), and for Hic1 one alanine (A), For Hic2 is one valine (V) and for Hic1 and γFBP serine (S), Hic2 coded one glutamic acid (E) and Hic1 and γFBP one asparatic acid (D) and finally for Hic2 is one threonine (T) and for Hic1 and γFBP serine (S).

The sequences from first two zinc fingers showed more similarity between Hic1 and γFBP.

In the zinc finger 3 (ZF3) sequence it are two amino acids, for Hic2 one asparagine (N), and for Hic1 and γFBP one threonine (T), one methionine (M) for Hic2 and one lysine (K) for Hic1 and γFBP. Zinc finger four (ZF4) sequence showed two amino acids differences, for Hic2 is glutamic acid (E) and for Hic1 and γFBP one alanine (A), for Hic2 one valine (V) and for Hic1 and γFBP one isoleucine (I) on the same place. The last zinc finger five (ZF5) sequence showed differences in four amino acids. For Hic2 it is one threonine (T), and for Hic1 and γFBP is one alanine (A), than one leucine (L) in the case of Hic2 and one methionine (M) by Hic1 and γFBP, and at least one arginine (R) for Hic2 and one lysine (K) for Hic1 and γFBP

Chicken has not γ-crystalline but the novel zinc finger proteins γFBP interact with the mouse γF-crystallin promoter and are expressed in the sclerotome during early somitogenesis. The expression of the murine γF-crystallin gene is most active at the beginning of lens fiber cell differentiation and that the level of expression gradually decreases during lens development. The increase in the levels of γFBP-B transcript and the transinhibitory effect of γFBP on promoters containing the γF-binding motiv, together with the gradual decline of γF-crystallin

gene expression during lens development, suggest that γFBP-B may play a physiological role in the regulation of γF-crystallin gene expression (Liu et al., 1994).

Becouse of the high similarity between Hic2 and γFBP zinc finger protein sequences it is possible that Hic2 bind on the γF-crystallin-promoter. The *Hic2* expression in this work was not found on the eye (Fig. 3.21 B), seams to be that γF-crystallin *in vivo* not suppose to be target gene.

4.3 Comparison of the *Hic2* and *Hic 1* expression patterns

During embryonic development, *Hic1* is expressed in the mesenchyme of the sclerotome, lateral body wall, limb and cranio-facial regions surrounding the outgrowing peripheral nerves during their differentiation. During fetal development, *Hic1* additionally is expressed in mesenchymes apposed to precartilaginous condensations, at many interfaces to budding epithelia of inner organs, and weakly in muscles. *Hic1* expression was observed in the embryonic anlagen of many tissues displaying anomalies in Miller-Dieker syndrome (MDS) patients (Grimm et al., 1999).

Hic2 and *Hic1* genes share a three highly conserved domains but the expression pattern is different just like an exon-intron structure. *Hic1* is expressed in somite derivatives and body wall of mouse embryos at TS17-20, in somite derivatives, epaxial and hypaxial mesenchymes, firstly, of the sclerotomes and later, also of the lateral body wall.

Epaxial and hypaxial *Hic1* expressions in these territories are sharply delineated against the non-expressing mesenchyme of the intercalated, central somite derivatives. *Hic1* is also expressed in the limb anlagen, in cranio-facial regions and in mesenchymes associated with spinal ganglia, in mesenchymes surrounding peripheral nerves and limb anlagen at TS19-23, in mesenchymes surrounding cranial peripheral nerves, epithelial of the nose salivary glands and caudal pharyngeal pouches. *Hic1* is also expressed in the mesenchymes of a variety of inner organs, e.g. adrenal gland and metanephric kidney. During early cornea development, *Hic1* is transiently expressed in a ring-shaped mesenchyme (Grimm et al., 1999).

Because of the *Hic2* expression in *canallis centralis medullae spinalis*, embryonic ectoderm (neural plate, neural channel and neural tube), and the lateral ventricle of the olfactory lobe, showed that gene seems to be involved in development of central and periphery nervous system (Fig. 15).

Hic1 expressing mesenchymal embed the developing peripheral nerves) suggesting a role for *Hic1* in the specification of perineural mesenchymes from the earliest stages of peripheral nervous system (PNS) development (Grimm et al., 1999).

Hic1 and *Hic2* might share expression territories in the sclerotome, head mesenchyme, vertebral arch and rib associated mesenchymes and both could have a similar function in peripheral nervous system. *Hic2* is expressed in the limbic region of the brain olfactory lobe (Fig. 16). There might be share expression territories in the head where *Hic1* is activated in the embryonic anlagen of some tissues affected in MDS (Miller Diecker syndrome) patients. *Hic2* showed expression in the small intestine caecum, duodenum, jejunum (Fig.3.18).

4.4 *Hic2* and its avian homologue γFBP

γ*FBP* and *Hic2* might share expression territories in the sclerotome, head mesenchyme, urogenital ridge, intestine, vertebral arch and rib associated mesenchymes. However, any expression of the Murine *Hic2* gene in lenses at embryonic, fetal stages by means of ISHs of whole embryos and on paraffin sections was not detected (Fig. 3.21 B). Thus, it is not likely that the Murine *Hic2* gene is implicated in lens differentiation.

4.5 *Hic2* exonic CpG islands

The increased stringency also substantially reduced the number of exonic CpG islands. The biological functions of these islands are not well understood, but CpG islands located in nonpromoter regions can play significant roles in gene regulation, they also seem to be frequent targets for *de novo* methylation in cancer and aging. Therefore, although the increased stringency preferentially locates CpG islands in the 5′ regions of genes, it may also result in the loss of smaller regions of DNA from the data set that may be functionally important in gene control (Takai and Jones, 2002).

HIC1 (hypermethylated in cancer) is a candidate tumor suppressor gene identified because of its association with a “CpG” island at 17p13.3 that is aberrantly hypermethylated and transcriptionaly inactivated in several common types of human cancer (Makos-Wales et al., 1995).

By regulating the expression of a large number of genes that have GC-rich promoters may take part in virtually all facts of cellular function, including cell proliferation, apoptosis, differentiation, and neoplastic transformation (Kaczynski et al., 2003).

The role of promoter hypermethylation, an epigenetic alteration, has been associated with the silencing of tumor suppressor genes in human cancer; *HIC1* promoter methylation was frequently seen in association with microsatellite instability in carcinoma of cervix uteri. Promoter methylation was associated with gene silencing in cervical cancer (CC) cell lines. *HIC1* (18.3%) genes were less frequently methylated in primary tumors.

The *HIC1* gene was methylated in 5 of 8 cell lines. Three of the methylated cell lines (C-4I, SiHa, and ME-180) and one of the three unmethiylated cell lines (MS751) showed down regulated expression. Treatment with 5-Aza-2' deoxycytidine or n-butyrate alone or the combination of both activated gene expression in all the cell lines, whether or not promoter had detectable methylation (Narayan et al., 2003).

4.6 *Hic2* a candidate for tumor suppressor gene?

For a better understanding of the potential role of *Hic2* as a possible candidate for tumor suppressor gene two binding sites with tumor suppressor genes associated with human tumors are important, p53 (numerous cancers) and Wilms tumor suppressor *WT1*(kidney). Two Wilms Tumor Suppressor binding sites are predicted between nucleotides +405 to +393 and -790 to – 778. One tumor suppressor p 53 is predicted between nucleotides –734 to -715. Are they direct involved in *Hic2* activity could be next important examination of the promoter region.

SAGE Brain Anatomic Viewer Results, The Cancer Genome Anatomy Project (CGAP) showed that *HIC2* involved in different carcinoma of the brain which are located in the cerebrum and cerebellum: oligodendroglioma, glioblastoma, grade II astrocytoma, grade I astrocytoma, ependymoblastoma, ependymoma (Fig.4.3).

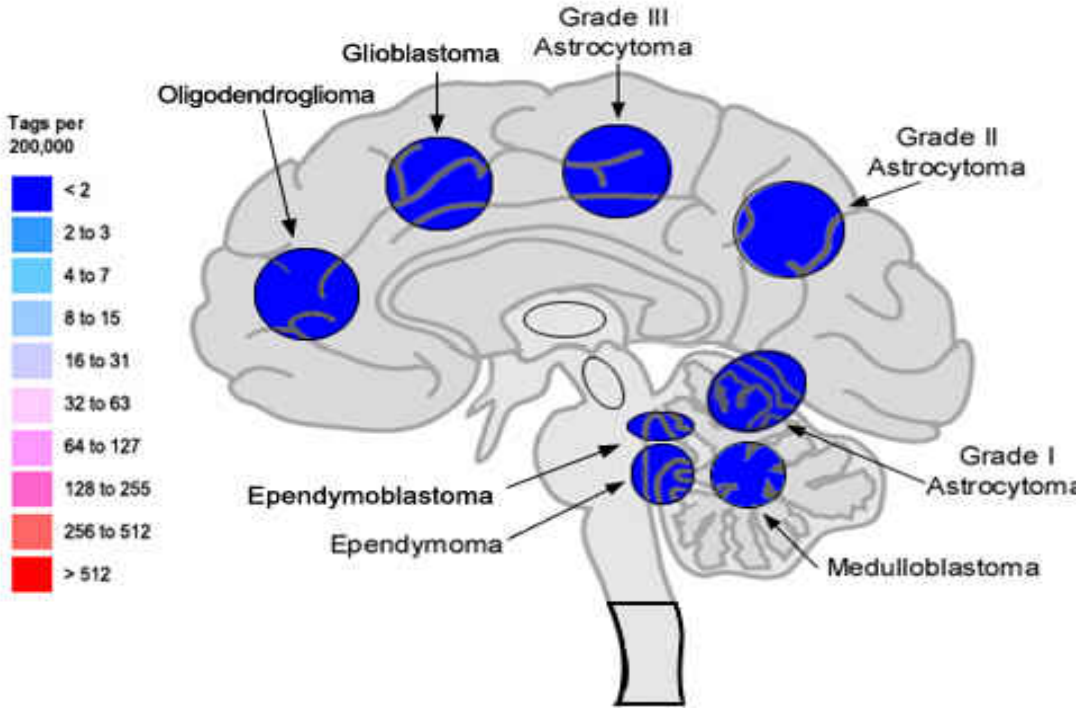


Figure 4.3 SAGE Brain Anatomic Viewer Results, The Cancer Genome Anatomy Project (CGAP) results during the analysis of cancer tissue and cell lines. Different carcinoma types located in the cerebrum and cerebellum, (agenda is on the left side).

Vertebrate DNA is generally depleted in the dinucleotide CpG; it has recently been shown that some vertebrate genes contain CpG islands, regions of DNA with a high G+C content and a high frequency of CpG dinucleotide relative to the bulk genome. Possible functions of CpG islands in transcriptional and post-transcriptional regulation of gene expression were related to theories for the maintenance of CpG islands as "methylation-free zones" in germline DNA (Gardiner, Fromer 1987).

For example the *HIC1* (hypermethylated in cancer) is a candidate tumor suppressor gene identified because of its association with a “CpG”island at 17p13.3 that is aberrantly hypermethylated and transcriptionaly inactivated in several common types of human cancer (Makos-Wales et al., 1995).

By regulating the expression of a large number of genes that have GC-rich promoters may take part in virtually all facts of cellular function, including cell proliferation, apoptosis, differentiation, and neoplastic transformation (Kaczynski et al., 2003).

Database searches identified on chromosome 22q11.2 in a region subject to translocation and also known as BCRL-2 (Breakpoint Cluster Region-Like 2), Mitellman break point, in this region are mapped some of the genes involved in tumor and cancer genesis (Dunham et al., 1999).

5. Summary

5.1 Analysis and characterization of the mouse *Hic2* gene

Like *Hic1* and γFBP (chicken), *Hic2* cDNA coded five Krüppel-type C_2H_2 zinc finger domain. Through the sequencing and comparation with different protein sequences from the homolog proteins from different species (HIC1, Hic1, HIC2, γFBP, and Hypothetical protein), Hic2 protein shares more than 80% homology with HIC1 through the BTB/POZ and zinc finger domains, and both proteins have identical GLDLSKK/R motifs.

A new part of *Hic2* gene coding, exon two, and new exon one deduced full-length Hic2 protein contains 601 amino acids. *Hic2* gene has two Exons (240 and 1842 bp), with one Intron (2182 bp).

The location of the gene is mouse chromosome 16 (B1, UniGene Cluster Mm.103787 Mus musculus). The human gene *HIC2* maps to chromosome 22q11.2, and is a homolog of the *HIC1* candidate tumor suppressor gene located at 17p 13.3. (Deltour et al. 2001).

Upstream from the TATA box MatInspector predicted different transcription binding sites. Between them Wilms Tumor Suppressor and p53 are most interesting transcription sites for the *Hic2* gene. That's why the *Hic2* promoter activity was checked.

A 1.2 kb promoter fragment of *Hic2* has been characterized in a gene reporter assay system. The peak activity of the *Hic2* promoter is associated with the total fragment, the next higher activity is in the fragment which included Wilms Tumor Suppressor and p53 transcription sites.

The expression of the mouse *Hic2* was investigated by *in situ hybridisations* (ISHs) of whole mount embryos and paraffin sections. *Hic2* expression was detected in restricted territories of the brain, sinus centralis, olfactory bulb, *canallis centralis medullae spinalis*, embryonic ectoderm (neuroepithelium of neural tube), and small intestine.

Because of its expression in the central nervous system, and mapping position of its human homolog *HIC2, Hic2* could be involved in some syndromes. Patients with a 22q11 deletion have disrupted brain development which may involve abnormal neural crest cell migration, (Van Amelsvoort et al., 2001). It is now recognized that the 22q11.2 deletion syndrome encompasses the phenotypes previously described as DiGeorge syndrome (DGS) and velocardiofacial syndrome (Shprintzen syndrome),(Thomas and Graham 1997).

5. Zusammenfassung

Die cDNA des *Hic2*-Gens kodiert wie *Hic1* und *γFBP* (Huhn) ebenfalls fünf C_2H_2 Zink-Finger des Krüppel-Typs. Im Vergleich der ermittelten *Hic2*-Sequenz mit verschiedenen Proteinsequenzen homologer Proteine aus verschiedenen Spezies (*HIC1*, *Hic1*, *HIC2*, *γFBP*, sowie ein hypothetisches Protein) zeigt *Hic2* über 80% Homologie zu HIC1 im Bereich der BTB/POZ- und Zink-Finger-Domänen. Darüber hinaus besitzen beide Proteine identische GLDLSKK/R-Motive.

Innerhalb dieser Arbeit wurde ein neuer Bereich des ersten und zweiten kodierenden Exons von *Hic2* charakterisiert. Die kodierende Sequenz wurde vollständig bestimmt. Das vollständig abgeleitete Hic2-Protein besteht aus 601 Aminosäuren. Das *Hic2*-Gen enthält zwei Exons (240 bp und 1842 bp) und ein Intron von 2182 bp.

Hic2 ist auf dem Maus-Chromosom 16 lokalisiert (B1, UniGene Cluster Mn. 103787 Mus musculus). Das menschliche *HIC2*-Gen liegt auf dem Chromosom 22q11.2. Es ist zu *HIC1*, einem auf 17p 13.3 lokalisierten Tumor-Suppressor Kandidaten-Gen, homolog (Deltour et al. 2001).
Stromaufwärts der TATA-Box wurden durch MatInspector verschiedene Tranksriptions-Bindestellen identifiziert. Hierbei stellen der Wilms Tumor-Suppressor sowie p53 die interessantesten Transkriptionsstellen des *Hic2*-Gens dar. Aus diesem Grund wurde die *Hic2*-Promotoraktivität überprüft. Ein 1.2 kb großes Promotorfragment von *Hic2* wurde in einem Gen-Reporter Nachweissystem charakterisiert. Die größte Promotoraktivität zeigte sich mit dem vollständigen, undeletierten Fragment, gefolgt von der Aktivität des Fragments zwei, welches die Wilms Tumor-Suppressor- und p53-Transkriptionsstellen enthält. Dies deutete auf eine mögliche funktionelle Bedeutung dieser Bereiche für die *Hic2*-Expression hin.

Die Expression des *Hic2*-Gens der Maus wurde mit Hilfe von *in situ Hybridisierungen* (ISHs) auf ganzen Embryonen und Paraffin-Schnitten untersucht. Eine *Hic2*-Expression wurde in abgegrenzten Regionen des Gehirns, sinus centralis, Riechkolbens, canallis centralis medullae spinalis, embryonalen Ektoderms (Neuroepithelium des Neuralrohrs), und des Dünndarms detektiert. Aufgrund seiner Expression im zentralen Nervensystem sowie der durch Kartierung bestimmten Lokalisation des homologen menschlichen *HIC2* könnte *Hic2* an einigen durch Deletionen verursachte Syndrome beteiligt sein. Einige Befunde lieferten

Beweise, dass Personen mit einer 22q11-Deletion unter einer unvollständigen Gehirnentwicklung leiden, die eine abnormale Wanderung von Zellen der Neuralleiste beinhalten könnte (Van Amelsvoort et al., 2001). Mittlerweile ist bekannt, dass das 22q11.2 Deletionssyndrom mit den früher beschriebenen Phänotypen des DiGeorge-Syndroms (DGS) sowie des velokardiofaziales Syndroms (Shprintzen-Syndrom) in Verbindung steht (Thomas and Graham 1997).

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8. Curriculum vitae

Name:	**Aleksandra**
First name:	**Terzic**
Birthday:	16.12.1970
Birthplace:	Sarajevo, Bosnia and Herzegovina
Father:	Radul Terzic
Mother:	Gabrijela Terzic birth Vujinovic
1976-1984	Primary school
1984-1988	Secondary school
1988-1992	Study of the Veterinary medicine, University Sarajevo
1994-1996	Veterinary technician in GSF-Institute for genetic
1997-1999	Study of the Veterinary medicine, University Sarajevo
2000-2003:	Pursuing Ph.D from Ludwig- Maximilians University, Munich, **GSF**-National Research Center for Environment and Health, Neuherberg, Germany in the Institute of Developmental Genetics at the laboratory of Molecular Eye Development under the supervision of **Prof.Dr. Jochen Graw .**

9. Apendix

Abbreviations

AA	amino acid
AP	alkaline Phosphatase
ATP	adenosinetriphosphate
Bp	base pare
BTB	Protein-protein-interactions-Domain, For Broad complex, tramtrack, Bric a brac, too POZ or ZIN
°C	Celsius degree
cDNA	complementary DNA
CTP	Cytosinetriphosphate
dATP	Desoxyadenosinetriphosphate
dCTP	Desoxycytosinetriphosphate
DEPC	Diethylpyrocarbonate
dGTP	Desoxyguanosinetriphosphate
Dig	Dioxigenin (Steroid from *Digitalis purpurea*)
DNA	Desoxyribonucleicacid
DTT	Dithioerythriol
dTTP	Desoxythymidintriphosphate
dNTP	Desoxyribonukleosidtriphosphate
ddNTP	Didesoxyribonukleosidtriphosphate
dpc	days post coitum
EDTA	Ethylendiamintetraacetate
EST	Expressed sequence tag
FA	Formamide
G	Gramm
GTP	Guanosinetriphosphate
IDG	Institute for development genetic
ISH	*in situ* Hybridisation
IPTG	Isopropyl-β-D-thio-Galactopyranosid
K	kilo
Kb	kilobasepare
L	liter
LB	Luria-Broth
m	meter
M	mol
MAB	Maleic acid buffer
MDS	Miller-Dieker-Syndrome
ME	Mercaptoethanol
min	minutes
N.	nervus
NTP	Nucleosidtriphosphate
^{32}P	Phosphorisotope
PAA	Polyacrilamide
PBS	Phosphate buffered saline
p.c.	post coitum
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PN	Phosphat-Nonident P40 buffer
POZ	Protein-protein interaction domain, for Poxvirus or zinc
RNA	Ribonucleicacid
rpm	rotations per minute
SDS	Sodium dodecyl sulphate
sec	seconds
SSC	Sodium chloride-sodium citrate
TAE	Tris-Acetate-EDTA buffer
TBE	Tris-Borat-EDTA buffer
TBS	Tris buffered saline
TE	Tris-EDTA
TS	Theiler stadium
Tris	Tri(hydroxymethyl)aminomethan
U	Unit
UTP	Uraciltriphosphate

Some gene names abbreviations:

En1	Engrailed 1
Evil1	Ecotropic viral integration site 1
γFBP	γF-crystalline binding protein
HIC1	Hypermethilated in Cancer 1
Hic2	Hypermethilated in Cancer 2
OVCA2	Ovarian Cancer 2
WT1	Wilms tumor suppressor

LUX IN TENEBRIS LUCET