3 Materials and Methods

3.1 Materials

3.1.1 Laboratory equipment

• Balance	Mettler-Toledo GmbH, Giessen
• Cell electroporator	Gene Pulser II, Bio-Rad Laboratories GmbH, München
• Centrifuge	Super T21, RC-2B, Sorvall GmbH, Bad Homburg Avanti J20, Beckmann Coulter GmbH, Krefeld 5417C, Eppendorf AG, Hamburg
• Filmprocessor	Curix, Agfa-Gevaert, NV, Mortsel, Belgium
• Freezer, -80°C	Forma, ThermoQuest Analytische Systeme GmbH, Egelsbach
• Gel electrophoresis equipment	Amersham Pharmacia Biotech Europe GmbH, Freiburg
• Gel documentation for ethidiumbromide-stained agarose gels	Herolab GmbH, Wiesloch
• Heating block	Grant Instruments, Cambridge, UK
• Incubator	Heraeus Instruments GmbH, Wiesloch
• Incubator shaker	New Brunswick scientific GmbH, Nürtingen
• Microdispenser	Hydra, Robbins scientific, Dunn GmbH, Asbach
• Microtitre plate filling machine	Genetix, Christchurch, Dorset, UK
• Packing machine for microtitre plate blocks	Lady Pack (shrink wrap), Pactur, Bologna, Italy Tippy Pack (binding machine), Spot, Manfred Pütz GmbH, Freiburg
• Pipettes, adjustable	Abimed Analysen Technik GmbH, Langenfeld
• Pipettes, adjustable, multichannel	Dunn Labortechnik, GmbH, Asbach Corning Costar, Acton, MA, USA Eppendorf AG, Hamburg
• PhosphorImager	Molecular Dynamics GmbH, Krefeld
• Plasmid isolation robot	PI-100Σ Kurabo Industries Ltd. Osaka, Japan
• Power supply	Bio-Rad Laboratories GmbH, München
• Robot for colony picking an spotting	Developed in-house
• Shaker	Rocky, Fröbel Labortechnik, Wasserburg
• Spectrophotometer	Shimadzu Deutschland GmbH, Duisburg
• Thermocycler	PTC100, PTC200, PTC225, MJ Research, Inc.; Watertown, USA
• Ultrasonic homogeniser	Branson Ultrasonic, Danbury, CT, USA
• UV crosslinker	Stratagene, La Jolla, CA, USA
• Vortex	Vortex Genie 2, Bender und Hobein AG, Zürich, Switzerland

3.1.2 Chemicals and enzymes

• Agarose	Invitrogen/Life Technologies, Karlsruhe
• Agarose, low melting point	FMC Seaplaque GTG, Biozym, Hessisch Ohlendorf
• Ampicillin	Sigma, Taufkirchen
• ATP	Amersham Pharmacia Biotech Europe GmbH, Freiburg
• Bacto Agar	Difco, Becton Dickinson, Sparks, MD; USA
• Bacto trypton	Difco, Becton Dickinson, Sparks, MD; USA
• Bacto yeast extract	Difco, Becton Dickinson, Sparks, MD; USA
• Betain, anhydrous	Fluka, Taufkirchen
• β-Mercapto-ethanol	Sigma Taufkirchen
• Biotin-16-dUTP	Boehringer Mannheim
• Casamino acids	Difco, Becton Dickinson, Sparks, MD; USA
• dATP, dCTP, dGTP, dTTP, sodium salt	Amersham Pharmacia Biotech Europe GmbH, Freiburg
• [α-^{32}P]dCTP, [α-^{33}P]dCTP, [γ-^{32}P]ATP, [γ-^{33}P]ATP	Amersham Pharmacia Biotech Europe GmbH, Freiburg
• pd(NTP)$_6$, random hexamer primer, Na salt	Amersham Pharmacia Biotech Europe GmbH, Freiburg
• DNA molecular weight standards	MBI Fermentas, St. Leon-Roth Promega GmbH, Mannheim
• DNA Polymerase I (E. coli), Large (Klenow) Fragment	United States Biochemical, Cleveland, OH, USA, New England Biolabs GmbH, Schwalbach/Taunus
• DMEM, 10 x	Invitrogen/Life technologies, Karlsruhe
• DTT	Serva, Heidelberg
• EDTA (Titriplex® III)	Merck, Darmstadt
• Ethidium Bromide, 1% solution	Fluka, Taufkirchen
• FBS	Invitrogen/Life Technologies, Karlsruhe
• Gentamycin	Invitrogen/Life Technologies, Karlsruhe
• Glutamax	Invitrogen/Life Technologies, Karlsruhe
• HEPES	Sigma, Taufkirchen
• IPTG	Sigma, Taufkirchen
• Kanamycin	Sigma, Taufkirchen
• Lysozyme	Boehringer Mannheim GmbH
• LB medium	Bio 101, Vista, CA, USA
• Lyticase	Sigma, Taufkirchen
• Phenol	Roti-Phenol, Carl Roth GmbH & Co. KG, Karlsruhe
• PMSF	Sigma, Taufkirchen
• Pronase	Boehringer Mannheim GmbH
• Proteinase K	Roche Diagnostics, Mannheim
• Restriction Enzymes	MBI Fermentas, St. Leon-Roth New England Biolabs GmbH, Schwalbach/Taunus
• Phosphatase, calf intestinal	Boehringer Mannheim GmbH
• Phosphatase, shrimp alkaline	Amersham Pharmacia Biotech Europe GmbH, Freiburg

• Sybr DX DNA Blot stain	Molecular probes, Leiden, NL
• T4 DNA Ligase	New England Biolabs GmbH, Schwalbach/Taunus
• T4 Polynucleotide Kinase	New England Biolabs GmbH, Schwalbach/Taunus
• X-gal	Sigma, Taufkirchen
• TRIS	Merck, Darmstadt
• Yeast nitrogen Base	Difco, Becton Dickinson, Sparks, MD; USA
• 2xYT broth agar	Bio 101, Vista, CA, USA
• 2xYT broth	Bio 101, Vista, CA, USA

Salts, acids, bases and solvents not mentioned in the table were *pro analysi* quality from Merck (Darmstadt) or Sigma (Taufkirchen)

3.1.3 Oligonucleotides

IRS-PCR primer:

MMA	AGA AYR TGC AAA CTC CAC ACA GA
MMAdeg	AGA AYR TGC AAA CTC VAC AYA RA
MMAsh	RRG AGA AYR TGC AAA CTC VAC A
MMA2	GGA GAA CAT GCA AAC TCC ACA
MMA3	AGA ACA TGC AAA CTC CAC ACA GA
MMB	CCT GGA GRA AAC CCA CRC ARA CA
MMBrev	TGT YTG YGT GGG TTT YCT CCA GG
MMBrev-2	YGT TKG YGT GGG TTT CCT CC
C1	ACT GCG CCA CTG YGT CGC C
C2	GAA CAT GCA AAC TCC ACA CAG
C3	ATG TTT CCC AGW GAT GGG TTG
C4	CAT TCC GCT GTG GYG ACC C
Angel2	TTT CAG TTT TGG GTG AAC TAT CC
ArielleA	CTC CAG AAG AAA AAA TAT TAT
ArielleB	ATA TKA TKT RCT GTC ATC ATG

Vector Oligonucleotides:

M13-forward	GCT ATT ACG CCA GCT GGC GAA AGG GGG ATG TG
M13-reverse	CCC CAG GCT TTA CAC TTT ATG CTT CCG GCT CG
5/86	GCA CGC GTA CGT AAG CTT GGA TCC TCT AG
3/86	CCG GTC CGG AAT TCC CGG GT
Sport 11mer	GCA CGC GTA CG

AFLP primer:

AGA	AAC CCT CAC TAA AGT ACC GA
BGA	AAC CCT CAC TAA AGA TCC GA
adBam1=adAcc1	AAC CCT CAC TAA A
adBam2	GAT CTT TAG TGA GGG TT
adAcc2	GTA CTT TAG TGA GGG TT
EcoRI-5’	CTC GTA GAC TGC GTA CC
EcoRI-3’	AAT TGG TAC GCA GTC TAC

Eco	GAC TGC GTA CCA ATT C
Eco$(N)_{1-3}$*	GAC TGC GTA CCA ATT C$(N)_{1-3}$
MseI-5'	GAC GAT GAG TCC TGA G
MseI-3'	TAC TCA GGA CTC AT
Mse	GAT GAG TCC TGA GTA A
Mse$(N)_{1-3}$*	GAT GAG TCC TGA GTA A$(N)_{1-3}$

* core Eco or Mse primer with a 1-3 base extension.

Oligonucleotides were synthesised using the phosphoamidit solid phase method (Caruthers et al., 1987). Synthesis was carried out in house by the service group if the MPI for Molecular Genetics, by MWG, Munich by TIB Molbiol, Berlin or by Invitrogen/Life Technologies, Karlsruhe.

Oligonucleotides used for radiation hybrid mapping and for hybridisation against the PAC-library are listed in chapters 7.2 and 7.3.

3.1.4 Kits

Clone Amp pAMP10 System for Rapid nondirectional cloning of Amplification products	Invitrogen/Life Technologies, Karlsruhe
PCR DIG probe synthesis kit	Boehringer Mannheim
Qiaprep spin miniprep kit	Qiagen GmbH, Hilden
Qiaex II Gel Extraction System	Qiagen GmbH, Hilden

3.1.5 Other materials

3MM Blotting paper	Whatmann GmbH, Göttingen
Agar Plates	Bio Assay Dish, Nunc Gmbh & Co. KG, Wiesbaden
Dialysis membranes	Mixed cellulose ester, pore size 0.025 µm, Millipore GmbH, Eschborn
Microtitre pates, 384 well	Genetix, Christchurch, Dorset, UK
Microtitre plates 96 well	Greiner Labortechnik GmbH, Frickenhausen
Hybond N^+ Nylon membranes, 222x222 mm^2	Amersham Pharmacia Biotech Europe GmbH, Freiburg
PCR plates, 96 well	Abgene, Hamburg
PCR plates, 384 well	Abgene, Hamburg PerkinElmer Life Science, Boston, MA, USA
Sterile filters	Cellulose nitrate membrane, pore size 0.2 µm, Nalgene, Hamburg
Thin layer chromatography sheets	PEI polygram, Machery-Nagel, Düren
Polypropylene tubes 15ml and 50 ml, sterile	Greiner Labortechnik GmbH, Frickenhausen
Replicators, 96 and 384 pin	Genetix, Christchurch, Dorset, UK

3.1.6 Nucleic acids

Zebrafish T51 Radiation Hybrid panel DNA (Kwok et al., 1998) (Geisler et al., 1999) was purchased from Research Genetics, Huntsville, AL, USA. Zebrafish LN54 Radiation Hybrid panel DNA (Hukriede et al., 1999) was a kind gift from Marc Ekker, Loeb Institute for Medical Research, University of Ottawa, Ontario, Canada. Zebrafish Tü and WIK strain genomic DNA, as well as TÜxWIK backcross panel DNA was a kind gift from Pascal Haffter and Robert Geisler from the Max-Planck-Institute for Developmental Biology, Tübingen, Germany. Zebrafish AB and India strain genomic DNA was a kind gift of Marc Fishman, Massachusetts General Hospital, Charlestown MA, USA. Zebrafish SJD strain genomic DNA was a kind gift from Stephen Johnson, Washington University School of Medicine, St. Louis, MO, USA

3.1.7 E. coli strains, cell lines and zebrafish strains

E coli strains:

DH10B:
F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(ara, leu)7697 *gal*U *gal*K λ^- *rps*L *nup*G (Invitrogen/Life Technologies, Karlsruhe)
DH10B was used as a host strain for the IRS marker library. It is also the host strain for the zebrafish PAC library (Amemiya et al., 1999)

XL1-Blue:
*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F'*pro*AB *lac*I^qZΔM15 Tn10 (Tet^r)] (Stratagene)

Zebrafish strains:

The Tü zebrafish wildtype strain originates from a pet shop and was inbred by sibling matings for several generations. The Tübingen mutagenesis screen has been carried out using this strain (Haffter et al., 1996).
The WIK line derives from wild catch in India. WIKxTü crosses are used for mapping of mutant genes at the MPI for Developmental Biology in Tübingen (Rauch et al., 1997).
The AB line (Chakrabarti et al., 1983) was bred for many generations at the University of Oregon, Eugene. The Boston mutagenesis screen was carried out using this strain (Driever et al., 1996).
The India line (IN) originated from a collection of wild fish from the north-east of India in 1990. AbxIndia crosses were used to construct the Boston zebrafish microsatellite-based genetic map (Knapik et al., 1996).
The C32 strain (Streisinger et al., 1981) is a clonal line generated by early pressure treatment (Figure 2).
The SJD strain (Nechiporuk et al., 1999) was generated from the India (Darjeeling) line by early pressure treatment.

Zebrafish Cell lines:

ZF4 is an embryonic fibroblast line from zebrafish. It has been characterised and shown to contain more than 90 chromosomes (Driever and Rangini, 1993).

ZF9 was derived from fin amputation of adult zebrafish AB strain. It was used to construct two radiation hybrid panels (Kwok et al., 1998) (Hukriede et al., 1999).
LFF was derived from zebrafish embryos. LFF and ZF4 lines were used to generate somatic cell lines (Chevrette et al., 1997).

3.1.8 Libraries

Zebrafish genomic libraries:

Library name	Library type	Number of clones	Average insert size	Genome coverage	Reference
MGH_y932	YAC	19,000	470 kb	5.2x	(Zhong et al., 1998)
HACHy914	YAC	34,560	240 kb	4.8x	(Amemiya et al., 1999)
BUSMP706	PAC	104,064	115 kb	7x	(Amemiya and Zon, 1999)

Zebrafish cDNA libraries

Library name	Stage/tissue	Remarks	Creator
ICRFp524	Late somitogenesis		M. Clark
MPMGp567	Shield stage		M. Clark
MPMGp532	Adult liver		M. Clark
MPMGp609		Normalised and rearrayed ICRFp524 and MPMGp532	M. Clark
MPMGp637		Normalised and rearrayed MPMGp567	M. Clark

All zebrafish cDNA libraries used during this work were created by M Clark, MPI for Molecular Genetics, Berlin. Clones can be obtained from the resource centre of the German genome project (RZPD http://www.rzpd.de/)

3.1.9 Buffers and Solutions

AL1
50mM glucose
10 mM EDTA
25 mM Tris/HCl pH 8.0

AL2
0,2 M NaOH
1% SDS

AL3
3M KOAc pH 5.5
For 100 ml:

	60 ml 5 M potassium acetate 11.5 ml glacial acetic acid 28.5 ml H_2O
Antibiotics (1000x)	50 mg/ml Ampicillin 30 mg/ml Kanamycin
CIA	96% v/v Chloroform 4% v/v Isoamyl alcohol
Denaturing Solution	0.5 M NaOH 1.5 M NaCl
dNTPs	100 mM stock solutions of dATP, dCTP, dGTP and d TTP in Tris, pH 7.5 were mixed in equal amounts to result in a stock solution of 25 mM of each nucleotide.
Hybridisation buffer	Church buffer (modified) 5 % SDS 0.25 M Na_2PO_4, pH 7.2 1 mM EDTA Ssarc buffer 4 x SSC (600 mM NaCl, 60 mM Na_3-citrat) 7.5% sodium N-lauryl sarcosinate 1mM EDTA
10 x Ligation buffer:	50 mM Tris-HCl (pH 7.5) 10 mM MgCl2 10 mM dithiotreiol 1 mM ATP 25 µg/ml BSA
Neutralising buffer	1 M Tris/HCl pH 7.5 1.5 M NaCl
10 x PCR buffer (standard)	750 Tris-HCl pH9 200 mM $(NH_4)_2SO_4$ 15 mM $MgCl_2$ 0.1% w/v Tween 1.5 mM Cresol red
Pronase stock solution	20mg/ml in H_2O, store at –20°C
Proteinase K stock solution	20mg/ml in H_2O, store at –20°C
20 x SSC	3 M NaCl 0.3 M Na_3-citrate pH 7.5

TAE buffer	40 mM Tris-acetate, pH 8.0 1 mM EDTA for 50 x stock solution (per liter): 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8)
TBE buffer	0.5 x (working solution) 45 mM Tris-borate 1 mM EDTA for 5 x stock solution 54 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8)
TE buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA autoclaved

3.1.10 Culture Media

Agar plates	15 g Agar/l liquid medium
DMEM	1 x DMEM 0.375 % w/v $NaHCO_3$ calibrate pH to 7.3 with NaOH 10 % v/v FBS (heated to 55°C for 20 min for complement inactivation) 1 x Glutamax 1 mM Hepes, pH 7.2 10 µg/ml Gentamycin (50 mg/ml)
Freezing medium: (Cell culture)	DMEM w/o Hepes 20 % FBS 10 % DMSO
10x HMFM (for freezing of bacteria)	<u>Part 1</u> 0.9 g $MgSO_4 \cdot 7H_2O$ 4.5 g Na_3-citrate$\cdot 2H_2O$ 9 g $(NH_4)_2SO_4$ 440 g Glycerol H_2O ad 800 ml, autoclave <u>Part 2</u> 18 g KH_2PO_4 47 g K_2HPO_4 H_2O ad 200 ml, adjust to pH 7.0, autoclave, add parts 1 and 2 together

LB Medium	bacto-tryptone 10 g bacto-yeast extract 5g NaCl 10 g H_2O ad 1 l, autoclave
SOB	20 g/l tryptone 5 g/l yeast extract 10 mM NaCl 10mM KCl autoclave
SOC	20 g/l tryptone 5 g/l yeast extract 10 mM NaCl 10 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose autoclave without glucose, add 40 % sterile glucose
YAC broth, selective	20 g Glucose 14 g Casamino acids 1 ml 100x Tyrosine HCl 5 ml 200x adenine HCl H_2O ad 900 ml, autoclave Add 100 ml YNB For freezing, add 10% v/v Glycerol
YAC broth Agar	YAC broth + 15 g/l Bacto Agar
YNB	67 g Yeast Nitrogen base without amino acids in 1 l H_2O, filter sterilise
2 x YT	16 g bacto-tryptone 10 g bacto-yeast extract 5 g NaCl H_2O ad 1 l, autoclave

3.2 Methods

3.2.1 Isolation of genomic DNA from tissue or adult fish

(Westerfield, 1995)

Anaesthetised fish were frozen in liquid nitrogen and ground to powder using a precooled mortar and pestle. The powder was carefully spread on the surface of DNA extraction buffer (10 mM Tris HCl pH 8, 100 mM EDTA pH 8, 0.5% SDS, 200 µg/ml Proteinase K; about 10 ml per 1 g of material). If trypsinised cell culture is used as starting material, it is directly suspended in extraction buffer, omitting the grinding step. The suspension was swirled to get homogeneous and incubated over night at 55°C. The cell lysate was extracted twice with equilibrated phenol and once with phenol:chloroform:isoamylalkohol (25:24:1).

Ammonium acetate is added to a final concentration of 2 M: the solution is overlaid with 2 volumes of ethanol by slowly letting it run down the side of the tube. The tube is swirled gently until the solution is thoroughly mixed. The precipitated DNA is removed using a Pasteur pipette, of which the end has been sealed over a Bunsen burner and bent into a U-shape. The DNA is washed by transferring the pipette tip into a tube containing 70 % ethanol for about 5 min. The DNA is air dried for 5 min and resuspended in 5-10 ml TE with 100 µg/ml (DNAse free) RNAse A. The DNA is allowed to resuspend (which can take several hours) and RNAse digestion is performed for up to one hour at 37°C. The DNA solution is extracted once with phenol:chloroform:isoamylalkohol (25:24:1), and DNA is precipitated as in the previous step. DNA is washed in 70% Ethanol and resuspended in 0.5-2 ml TE. DNA concentration is determined by measuring OD at 260 nm in a spectrophotometer (1 OD is equivalent to 50 µg/ml).

3.2.2 Plasmid preparation

(Birnboim and Doly, 1979)

To isolate small amounts of high copy number plasmids for analytical purposes, 1.5 ml of an overnight culture were centrifuged. The supernatant was discarded, and the precipitated bacteria were resuspended in 100 µl AL1 supplemented with RNAse A (100 µg/ml). 200 µl AL2 are added and the solution is incubated 5 min. on room temperature. 150 µl AL3 are added, the solution is mixed gently but carefully and incubated on ice for 10 min. Precipitated chromosomal DNA, Proteins and SDS is sedimented by centrifugation at high speed. The clear supernatant is transferred to a fresh tube. DNA is precipitated by adding 2 volumes of ethanol at room temperature and incubation for 2 min. The DNA is pelleted by 5 minutes of centrifugation at high speed, the supernatant is discarded, and the DNA pellet is washed using 70% ethanol. After air drying the pellet for 10 min the DNA is redisolved in 20-50 µl TE. The typical yield of high copy number plasmids prepared by this method is about 3-5 µg DNA per ml of original bacterial culture.
For low copy number cloning systems, e.g. PACs, the starting material was 35 ml of a bacterial overnight culture, and the amount of lysis buffers is adjusted to be able to resuspend the larger amount of bacteria. Large-scale PAC preparation was done in a robot using essentially the same protocol.
For obtaining plasmid DNA of high purity, Qiagen silica columns were used according to the instructions of the manufacturer.

3.2.3 Lysis of yeast cells

In order to obtain YAC DNA for PCR, yeast cells have to be lysed. This is carried out according to the following protocol: Yeast cells are grown in 200 µl YAC broth for 2-3 days at 30°C in microtitre plates. The cells are pelleted by centifugation, resuspended in 100 µl TE, pelleted again, and resuspended in 20 µl TE containing 10 mM DTT and 20 U lyticase (Sigma). The mixture was incubated for 45 minutes at 30°C. 60 µl of 140 mM NaOH were added and incubation was continued for 7 minutes at room temperature. Finally, 60 µl of 1M Tris pH8 were added.

3.2.4 DNA precipitation

(Sambrook et al., 1989)

DNA in solution is precipitated by adding 1/10 volume 3 M NaAc, pH 5.2 and 2.5 volumes of ethanol. If the coprecipitation of dNTPs has to be reduced to a minimum, NaAc is

replaced by 1/5 volume of a 10 M stock solution ammonium acetate (Ammonium ions can inhibit T4 polynucleotide kinase!). If the solution contains SDS, which should not be coprecipitated, 0.2 M NaCl is used. DNA is pelleted by centrifugation at high speed, washed using 70% ethanol, and resuspended in TE.

3.2.5 Restriction digest

Restriction digests were done using up to 5 µg DNA and 5-10 units of restriction endonuclease. Reaction was performed using the buffer recommended by the manufacturer at the appropriate temperature for 1h to over night. A fraction of the digestion is checked on a agarose gel using undigested DNA as a marker. Restriction enzyme is inactivated if possible by incubation at 65°C for 30 min. If necessary, phenol:chloroform extraction and DNA precipitation is carried out.

3.2.6 Dephosphorylation of DNA 5'-ends

(Sambrook et al., 1989)

To prevent self-ligation of DNA, the 5'-phosphate residues were removed using calf intestinal phosphatase (CIP). 1 unit phosphatase is needed for 100 pmoles of protruding 5'-termini and for 2 pmol blunt or recessed termini. Dephosphorylation was done in a 50-100 µl reaction in CIP buffer for 30-60 min at 37°C.

10x CIP buffer: 10 mM $ZnCl_2$
10 mM $MgCl_2$
100 mM Tris-HCl pH 8.3

To test if the reaction was successful, a control ligation is performed using DNA treated and untreated with CIP, respectively. The phosphatase is inactivated by adding 5 mM EDTA, 0.5% SDS, 100 µg/ml proteinase K and incubation for 30 min at 55°C. The solution is extracted with phenol/chloroform, and DNA is recovered by ethanol precipitation. Alternatively, shrimp alkaline phosphatase (SAP) is used instead of CIP. This enzyme can be completely inactivated by heating.

3.2.7 Ligation of restriction fragments

To ligate DNA fragments into cloning vectors, vector and insert DNA were mixed in a molar ratio of 1:1 to 1:3 in a concentration of 0.1 to 1 µM of 5'-termini (corresponding to 33–333 ng /µl for DNA of 1 kb length). The reaction was performed in ligation buffer, usually with 400 NEB units of T4 DNA ligase (equivalent to 6 Weiss units) in 10 µl total volume. Incubation was at 16°C over night. Before electroporation, the ligation reaction is dialysed against 0.5 x TE. This is done by transferring the ligation reaction on a Millipore filter disc (VSWP, mixed cellulose ester, pore size 0.025 µm, 13mm diameter); which swims on the surface of a 0.5 x TE solution, with the shiny side on top, and leaving it for 15 min.

3.2.8 Preparation of E. coli cells for electroporation

Electroporation is applied to introduce DNA into eukaryotic and bacterial cells. The process of transforming E. coli by electroporation involves exposing a dense suspension of cells and DNA to a brief (3 to 6 ms), high voltage electrical pulse (Dower et al., 1988) (Calvin and Hanawalt, 1988).

E. coli cells were made competent for transformation by electroporation by growth in low-salt medium and washing in 10% glycerol. One liter of SOB-medium with the appropriate antibiotic prewarmed to 37°C was inoculated with 1 ml of an *E. coli* overnight culture and grown to an OD_{600} of 0.6-0.8 at 37°C. The cells were harvested by centrifugation at 5,000 rpm (2,600 g) in a GS3 rotor for 10 min, resuspended in 1 litre ice-cold, autoclaved 10% (v/v) glycerol, and centrifuged and resuspended again in the same manner. After a third centrifugation, cells were resuspended in 40 ml 10% (v/v) glycerol, transferred to 50 ml polypropylene tubes, centrifuged again and resuspended in a minimal volume 10% (v/v) glycerol (usually 0.5-1 ml). Aliquots were transferred in 1.5 ml tubes, shock frozen in a dry ice/ethanol mixture and stored at –80°C.

3.2.9 Transformation

Cells were thawed on ice, and 25 µl were mixed with 1-2 µl ligated plasmid. The cells were electroporated in electroporation cuvettes of 1 mm gap size according to the manufacturer of the electroporation apparatus. 1ml SOC was added to the cells immediately after application of the pulse, and the suspension was shaken at 37°C for 1 h. Determination of the titre was done by diluting 2 aliquots (e.g. 0.5 and 5 µl) to 100 µl and plating the dilution on LB-agarplates containing the appropriate antibiotic. The rest of the transformation reaction can be stored without significant loss of titre for e few days at 4°C , or is supplemented with HMFM, frozen and stored at –80°C.

3.2.10 Blue/white selection

Blue/white selection for presence of insert DNA was carried out by adding 625 µM IPTG and 125 µM X-gal to the nutrient agar before pouring the plates. Alternatively 30 µl of a 1 M IPTG solution and 20 µl of a 5% X-gal (or bluo-gal) solution (in DMF) are spread evenly on an agar plate before spreading the bacteria.

3.2.11 Picking and handling of libraries

Transformed cells were plated at a density of 3000-5000 colonies/plate onto 22x22 cm 2xYT agar plates (Genetix) containing the appropriate antibiotic and were grown overnight at 37°C. A picking robot was used to automatically pick colonies and inoculate 384-well microtitre plates filled with 2xYT medium containing HMFM and the appropriate antibiotic. The CCD camera of the robot takes 48 digital pictures from the plate, which are analysed by labview software. Colonies within a certain size limit and with a defined ratio of horizontal to vertical diameter were selected for picking. The clones are picked by a 96-pin picking device, with each pin picking an individual clone. The 96 clones are then transferred to a 96 or 384-well microtitre plate. The picking device (or gadget) is sterilised in an ethanol bath before the next 96 clones are picked. More than 3000 clones can be picked this way in one hour. Bacteria are grown in microtitre plates at 37°C overnight and replicated into new microtitre plates using 384-pin replicating tools. All copies are stored frozen at –80°C.

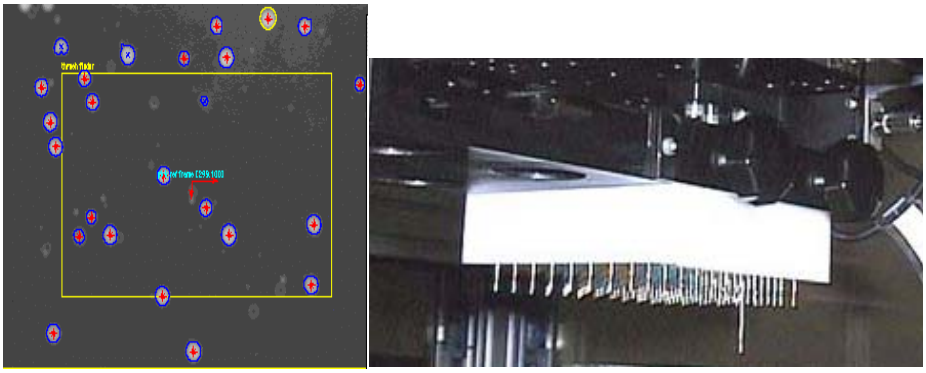

Figure 7

Automated colony picking by a picking robot. Left: Image analysis using Labview software. Only colonies marked by a red cross are selected for picking. Right: picking head with a protruding pin.

3.2.12 Spotting of bacterial colonies onto nylon membranes

(Nizetic et al., 1991)

A picked library is spotted onto 22x22 cm nylon membranes by a robot using a 384-pin spotting gadget. Spotting in a 5x5-block spotting pattern results in a densities of 57,600 spots, with 27,600 colonies spotted in duplicate and an ink guide dot at the centre of each block. During spotting, the robot transfers small amounts (ca. 0.1 µl) of culture medium onto Hybond N^+ nylon membranes, which are on top of blotting paper prewetted in medium. At the end of the spotting procedure, membranes are placed on agar containing the appropriate antibiotics, and grown for 16 h at 37°C.
Membranes are put (colony face up) on top of 3MM Whatman paper soaked in denaturing solution for 4 min at room temperature, followed by 4 min in a steaming water bath at 94°C. Filters are neutralised by putting them for 4 min on Whatman paper soaked with neutralising solution. Proteins and cell debris is removed by submerging the filters in a solution containing 50 mM Tris-HCl pH 8.5, 50 mM EDTA, 100 mM NaCl, 1% w/v Na-lauryl-sarcosine and 250 µg/ml proteinase K and incubated without agitation for 20 min at 37°C. the membrane is laid on top of dry blotting papers to let it dry and DNA is UV-crosslinked. As an alternative to proteinase K treatment, filters can be incubated in 50 mg/ml pronase, 50 mM Tris-HCl, 1% w/v Na-lauryl-sarcosine.
A small number of colonies (up to 384) can be spotted on 7x11 cm membranes by hand using a replicating device. The membranes are further processed as the robotically spotted membranes.

3.2.13 Spotting of DNA onto nylon membranes

PCR products stored in 384-well microtitre plates are robotically spotted onto Hybond N^+ nylon membranes prewetted in 0.4 M NaOH. Each PCR product is spotted in duplicate in a 5x5-block spotting pattern (57,600 spots per membrane, or 27,600 PCR products). At the centre of each 5x5 block either concentrated genomic salmon sperm DNA or ink is spotted as a guide spot. To increase the amount of DNA in each spot, up to ten rounds of spotting are used for each PCR product, transferring a total of 10 to 100 ng of DNA per spot. After spotting, the membranes are neutralised in 1 M Tris-HCl (pH 7.4)/1.5 M NaCl, dried, and the DNA crosslinked to the nylon membrane by UV irradiation. Such membranes can be used 10 times and more.

If the number of PCR products to be spotted is small (up to 384) they can be spotted on 7x11 cm membranes using a 96 channel dispensing system (Hydra, Robbins scientific). The filters are processed as the robotically spotted filters.

3.2.14 Capillary blotting of DNA onto nylon membranes ("Southern Blotting")
(Southern, 1975)

Southern blotting is used to transfer DNA fragments from agarose gels onto nylon membranes. An agarose gel with the sample DNA and a molecular weight marker is run, stained with ethidiumbromide and photographed with a ruler placed at its side to later identify the size of detected bands. If fragments > 10 kb have to be transferred, DNA is nicked by in 0.125 M HCl solution for 10 minutes. The gel is rinsed with H_2O, transferred to denaturing solution and incubated for 30 min. The gel is placed in neutralising solution and incubated for 30 min. A capillary blot is set up as described (Sambrook et al., 1989) using 20xSSC as transfer buffer. After blotting overnight, the transfer apparatus is carefully dismantled. Before separating the gel and membrane, the membrane is marked to allow identification of the gel lanes with a pencil. The DNA is fixed to the membrane by UV crosslinking.
Alternatively, the step involving neutralisation of the gel may be omitted, and transfer is done using denaturing solution as transfer buffer. According to Sambrook et al., 1989, alkali transfer might increase background in hybridisations.

3.2.15 Labelling by random hexamer priming
(Feinberg and Vogelstein, 1983)

This method is used to label linear DNA fragments to high specific activity. It is based on a DNA polymerisation reaction primed by random hexanucleotides binding to the template. Ca. 50 ng of template DNA are diluted in a volume o 12 µl, denatured for 5 min at 94°C and chilled on ice. 18 µl of labelling solution 3 µl of nonradioactive 100µM nucleotide mix (dATP, dGTP, dTTP), 15 µg acetylated BSA and 5 units Klenow enzyme, 3 µl [α-^{32}P] dCTP (3000 Ci/mmol, 10 µCi/µl) or 3 µl [α-^{33}P] dCTP (2500 Ci/mmol, 10 µCi/µl) are added. The labelling reaction is incubated for 1 h at 37°C or overnight at room temperature.
Labelling solution: 50 µl Hepes buffer (1M Hepes pH 6.6)
50 µl TM buffer (250 mM Tris-HCl pH 8, 25 mM $MgCl_2$
50 mM β-mercaptoethanol)
14 µl hexanucleotides (45 A_{260} units/ml in TE)

This adds up to a total reaction volume of 40 µl, with the following concentrations: 1.25 ng/µl template DNA (equivalent to 1.9nM 1 kb DNA fragments), 0.4 µg/µl BSA, 7.5 µM of each nonradioactive dNTP, 250 nM of labelled dCTP (equivalent to 30 µCi), 44 ng/µl hexanucleotides, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol. The pH is kept at 6.6, because the exonuclease activity of the Klenow polymerase is inhibited at acidic pH.
The amount of radioactive dCTP and Klenow polymerase was varied depending on the experiment. If hybridisations of 22x22 membranes were done, the amounts were chosen as outlined above. If hybridisation of 7x11 or 7x22 membranes were done in 15 ml polypropylene tubes, 5 µCi isotope and 2.5 units Klenow polymerase were used.
Unincorporated nucleotides can be removed using gel chromatography in a Sephadex G50 column. Alternatively DNA is precipitated by adding 10 µl 10 M NH_4Ac, 3 µl 10 mg/ml tRNA, 2 µl 0.5M EDTA and 100µl ethanol. The mixture is centrifuged for 15 min, the supernatant is removed, and the pellet is resuspended in TE. Mostly, the removal of

unincorporated nucleotides was omitted. Before hybridisation, the DNA is denatured by adding 1M NaOH or applying heat. Alternatively, the labelled DNA is competitively reannealed using an excess of unlabelled zebrafish DNA to block repetitive DNA (Baxendale et al., 1991). This is done by adding 1 µg/µl sheared or sonicated zebrafish genomic DNA and 0.12 M Na_2HPO_4. The DNA is denatured at 94°C for 5 min, reannealed at 65°C for 2 h and added to the prehybridised filter.

3.2.16 Hybridisation of DNA probes labelled by random priming.

Filters are prehybridised at 65°C for at least 30 min in Church buffer. Denatured or competed probe is added and hybridisation is performed at 65°C for at least 16 hours. Filters are washed once in 2x SSC/0.1% SDS at 65°C for 20 minutes and once in 0.1x SSC/0.1% SDS at 65°C for 40 minutes. After the second wash, filters are rinsed shortly in TE or 1 mM EDTA.
Up to four 22x22 cm filters separated by nylon meshes are hybridised in glass bottles in 20 ml Church buffer. If 7x11 cm or 7x22 cm filters or Southern blots are used, hybridisation is done in 15 ml Church buffer in a 15 ml polypropylene tube. If IRS-PCR products are used as probes, hybridisation and washing temperature is raised to 70°C to reduce background.

3.2.17 Labelling of oligonucleotides by polynucleotide kinase

For oligonucleotide fingerprinting, where a short (10 bp) oligonucleotide is hybridised to high density spotted PCR products, the following protocol is used for labelling:
30 pmol of oligonucleotide, 20 pmol of [γ-^{33}P]-ATP (2500 Ci/mmol, equivalent to 50 µCi), 20 units T4 polynucleotide kinase are mixed in 30 µl of the buffer recommended by NEB and incubated for 30 min at 37°C. Incorporation of the isotope is tested by PEA thin layer chromatography, with 0.75 M KH_2PO_4 as run buffer.
For hybridisation of 35mer oligonucleotides, the protocol was modified: 5 pmol of oligonucleotide, 3 pmol of [γ-^{32}P]-ATP (5000 Ci/mmol, equivalent to 15 µCi), 20 units T4 polynucleotide kinase are mixed in 30 µl of the buffer recommended by NEB and incubated for 45 min at 37°C. Before adding to the hybridisation buffer, the probe is denatured by adding 0.3 M NaOH.

3.2.18 Labelling of "overgo" probes
(Cai et al., 1998)

Overgo probes are created by annealing two oligonucleotides having an overlapping complementary sequence. Single strand overhangs are filled in with ^{32}P labelled dATP and dCTP, resulting in a double stranded probe with high specific activity (Figure 31).
Overgo probes are designed using an overgo design program accessible through the World Wide Web (http://genome.wustl.edu/gsc/overgo/overgo.html). Usually an overgo pair consisted of two 24mer oligonucleotides with a 8 bp overlap. For labelling, 10 pmol of each oligonucleotide were mixed with 90 mM Hepes, pH 6.6, 5mM MgCl2, 7.5 mM DTT, 50 mM dGTP, 50 mM dTTP, 2 µCi [α-32P] dCTP (3000 Ci/mmol, equivalent to 66 nM end concentration), 2 µCi [α-32P] dATP dCTP (3000 Ci/mmol, equivalent to 66 nM end concentration) and 2 units of Klenow polymerase Total reaction volume is 10 µl. Labelling reactions are carried out for 2 h at room temperature. If desired, unincorporated nucleotides are removed using Sephadex G-50 spin columns or ammonium acetate precipitation. Probes are denatured at 94°C for five minutes or by addition of 0.3 M NaOH.

3.2.19 Hybridisation of oligonucleotides

For oligonucleotide fingerprinting, the PCR membranes are prehybridised in 1x Ssarc buffer for at least 20 min at 4°C in tubes. The prehybridisation solution is discarded, the labelled probe is mixed with 15 ml 1x Ssarc buffer, and the hybridisation mix is poured in the hybridisation tubes. This results in a probe concentration of 1.3 nM if it is assumed that all nucleotides have been incorporated. Hybridisation is carried out at 4°C for 3-16 h. Membranes are subsequently washed in a large volume of 1x Ssarc for 20-40 min at 4°C, dried briefly on blotting papers, wrapped in saran wrap, and exposed on phosphor storage screens over night. Bound oligonucleotides are removed by washing membranes in 0.1x Ssarc at 65°C for 10-30 minutes. Membranes can be used for at least 20 hybridisation-stripping cycles without significant loss of signal.
For hybridisation of 35mer oligonucleotides or overgo probes on PAC library filters, the filters (separated by nylon meshes) are prehybridised in Church buffer for 30 minutes at 55°C in tubes. The prehybridisation solution is discarded, the labelled and denatured probe is mixed with 15 ml of Church buffer prewarmed to 55°C, and the hybridisation mix is poured into the hybridisation tubes. This results in a probe concentration of 200 pM if it is assumed that all nucleotides have been incorporated. Hybridisation is carried out at 55°C overnight. The hybridisation solution is poured off, and the filters are washed in 50 ml washing buffer (0.5M Na2PO4, pH 7.2, 0.1% SDS, 1mM EDTA) in the tubes for 20 min at 55°C. Subsequently, the filters are transferred to pizza boxes, the meshes are removed and the filters are washed in preheated washing buffer at 55°C while gently agitating. The membranes are dried briefly on blotting papers, wrapped in Saran wrap, and exposed on phosphor storage screens over night. Bound oligonucleotides are removed by washing membranes in 1x TE/0.1% SDS for 20 minutes at 65°C, and rinsing in TE. Membranes are stored in TE. Colony filters seem to be not as stable as PCR filters, because the signals are decreasing when the filter is used more than 5 times.

3.2.20 Generation of amplified restriction fragments

A: DNA cut with two enzymes and ligated to labelled oligonucleotides
(Vos et al., 1995)

Restriction and ligation

0.5 µg of genomic DNA is cut with 5 units EcoRI and 5 units MseI in 40 µl 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA for 1 h at 37°C. Next, 10 µl of a solution containing 5 pmol EcoRI adapters, 50 pmol MseI adapters, 1 Weiss unit T4 DNA ligase, 1 mM ATP in 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5mM DTT, 50 ng/µl BSA was added and the incubation was continued for 3 h at 37°C. The adapter sequence has been chosen, that the restriction recognition site is not restored when adapters are ligated to restriction fragments. Ligation can therefore be carried out in the presence of restriction enzyme to avoid concatemerisation of restriction fragments. Double stranded adapters were prepared by mixing equimolar amounts of both adapters, heating to 95°C, and cooling to room temperature. After ligation, the reaction mixture was diluted to 500 µl with 10 mM Tris-HCl, 0.1 mM EDTA pH 8, and stored at -20°C.

Amplification

The PCR reaction was carried out in two steps: The first step was performed with unlabelled EcoRI and MseI primers having a single selective nucleotide extension. 20 µl PCR reactions were carried out using 6 pmol of both primers, 5 µl template DNA (5ng), 0.4 units taq polymerase, 10 mM Tri-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of all our dNTPs. The amplification reaction was performed for 20 cycles with the following cycle profile: A 30 s DNA denaturation step at 94°C, a 1 min annealing step at 56°C, and a 1 min extension step at 72°C. After this preamplification step, the reaction mixture was diluted 10-fold with 10 mM Tris-HCl, 0.1 mM EDTA pH 8. A second amplification step was performed, using 1 pmol (labelled if appropriate) EcoRI primer and 5 pmol unlabelled MseI primer, both having two or three selective nucleotide extensions. 5µl of the diluted PCR product of the preamplification were used as template. Secondary PCR was performed for 36 cycles with the following cycle profile: A 30 s DNA denaturation step at 94°C, a 1 min annealing step, and a 1 min extension step at 72°C. The annealing temperature in the first cycle was 65°C, was subsequently reduced each cycle by 0.7°C for the next 12 cycles and was continued at 56°C for the remaining 23 cycles.

B: Alternative AFLP protocol using genomic DNA cut with one enzyme
(Himmelbauer et al., 1998)

As an alternative to the protocol of Vos *et al*., a method using just one infrequently cutting restriction enzyme was employed. Genomic zebrafish DNA (1µg) was digested in a volume of 30 µl overnight at 37°C with 20 units of BamHI. After heat inactivation of the restriction enzyme, the DNA was precipitated, and dissolved in 10 µl TE. Non-phosphorylated, double stranded adapters were made by mixing equimolar amounts of RBam24 and RBam12 primers, heating to 95°C and cooling to room temperature. Ligation of adapters was performed overnight at 16°C in a volume of 15 µl using 1 µg restricted genomic DNA, 1 pmol double stranded adapters, 400 NEB units T4 DNA ligase in the buffer recommended by the supplier. Ligation reactions were subsequently diluted to 50 µl with TE and stored at –20°C. Amplification was carried out on 2 µl of the diluted ligation reaction using primer RBam with or without selective nucleotide extensions. The reaction was performed in 1 x PCR buffer with 200 µM of each dNTP in a volume of 60 µl. The reaction was incubated at 72°C for 10 min to fill in the recessing 3'-termini. Then 200 pmol of primer was added, and after an initial denaturation of 3 min at 94°C, samples were amplified in 35 cycles of 94°C, 30 s; 60°C, 30 s; 72°C, 3 min; following the last cycle, an additional extension step of 10 min at 72°C was added.

3.2.21 Addition of T-overhangs in cloning vectors
(Marchuk et al., 1991)

To clone PCR products, one can take advantage of the fact, that *taq* polymerase adds a adenosyl residue to the 3'-prime end of PCR products by using cut cloning vectors having a 3'-T overhang. This overhang can be generated by using 10 µg of a cloning vector, which has been cut at its cloning side with an enzyme producing blunt ends (e.g. EcoRV). The DNA is diluted in 50 µl PCR buffer, containing 2mM dTTP and 1 unit taq polymerase per µg plasmid DNA. The reaction mix is incubated for 2 h at 72°C. The absence of any other nucleotides in the reaction results in the addition of a single thymidine at the 3'-end of each fragment. After phenol extraction and precipitation, the DNA is ready for cloning.

3.2.22 Cloning of PCR products using the pAMP10 system

The library of zebrafish IRS-PCR products was constructed by using the Cloneamp® pAMP10 system for rapid nondirectional cloning of amplification products (Invitrogen/Life Technologies, Karlsruhe, Figure 8). PCR was done using a primer carrying a $(CUA)_4$ extension at its 5'-end. 10-50 ng PCR product, 25 ng vector DNA and 1 unit Uracil DNA glycosylase were incubated in a volume of 20 µl of 50 mM KCl, 20 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$ at 37°C for 30 min. The annealing mixture was dialysed against ice cold 0.5 x TE using Millipore filters (see 3.2.7) and used for transformation of E. coli DH10B cells.

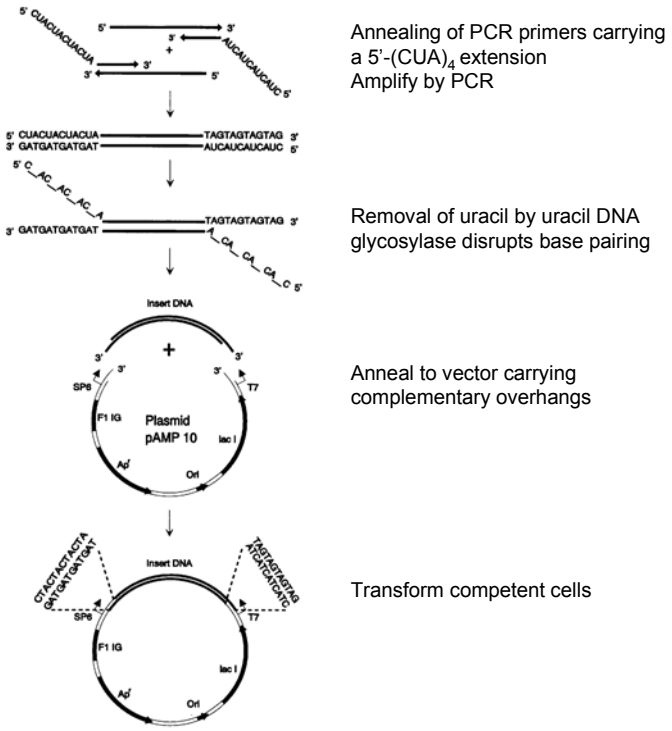

Figure 8

Principle of the Cloneamp system

3.2.23 IRS-PCR

IRS PCR was performed in 20-120 µl reaction volume, using 25-100 ng genomic, radiation hybrid, or clone pool DNA. The reaction was done in standard PCR buffer, supplemented with 0.5 mM $MgCl_2$, 200 µM of each dNTP, 2µM IRS-primer and 2-3 units taq polymerase per 100 µl reaction. After an initial denaturation step of 1 min at 94°C, 30 cycles were carried out with 30 seconds at 94°C; 60 seconds at 55°C; 3.5 min at 72°C. Following the last cycle, an additional extension step of 10 min at 72°C was added.
If IRS-PCR was performed on PAC-clones, template DNA was provided by dumping a pipette tip or a replicator into the bacterial culture and transferring it into the prepared PCR mix. If YAC clones were used as a template, yeast cells were lysed. The lysate was diluted, and 1-5 µl lysate were used as PCR template. PCR was performed using 35 cycles.
PAC and YAC IRS-PCR products were cut out of a low melting point agarose gel under weak UV light. The agarose plugs were melted following addition of 50 µl H_2O, and 12 µl of this mixture was used for random primed labelling.

3.2.24 Amplification of plasmid inserts

Plasmid inserts were PCR amplified for use as hybridisation probe, for spotting or for sequencing. In large-scale applications, PCR was done in 30 µl in a 384 well plate or in 50 µl in a 96 well plate. PCR was performed in 1 x PCR buffer, with 0.1 mM primer, 200 mM of each dNTP, 1 M betain, 2-3 units taq. As vector specific primers, M13 forward or 5/86 were used to bind to one side of the insert, and M13 reverse or 3/86 were used to bind to the other site of the insert. Ready PCR mix was inoculated with a bacterial suspension using a replicator. PCR was performed for 30 cycles with the following cycle profile: 20 seconds denaturation at 94°C, and 210 seconds annealing and extension at 65°C.

3.2.25 DNA Sequencing

DNA sequencing was carried out using the didesoxy nucleotide chain-termination method (Sanger et al., 1977) with dye terminator labelling on purified plasmids or PCR products. Either vector specific universal primers or sequence specific primers were used. For sequencing of 3'-ends of cDNAs, a $(T)_{23}N$ primer was used, which binds to the polyA tail. Sequences were read on ABI 377 and 3700 automatic sequencers. Automated base calling was performed by the program Phred (Ewing et al., 1998).

3.2.26 Radiation hybrid mapping of ESTs

Radiation hybrid and control DNA was dispensed in 384 well microtitre plates by using a Hydra microdispenser (Robbins Scientific). Each well contained 5 µl of a 5ng/µl (10 ng/µl) DNA solution of the T51 panel (LN54 panel). PCR was performed in a 10 µl volume containing 1x PCR buffer, 0.5 µM of each forward and reverse primer, 200 µM of each dNTP, 1 M betain and 1 U *taq* polymerase, 1x PCR buffer. The reaction was done in a PTC 225 tetrad thermocycler (MJ Research), with initial denaturation at 94°C for 30 s, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Each marker was assayed twice. PCR products were run on 2% agarose gels and photographed. Results were scored using a web based scoring interface and entered into an Oracle database (M. Kramer, pers. communication). If discrepancies were detected between duplicate assays, PCR was performed for a third time. T51 radiation hybrid vectors were submitted online to the MPI for Developmental Biology (http://wwwmap.tuebingen.mpg.de/), where the markers were integrated in the existing Radiation hybrid maps. Accordingly, LN54 radiation hybrid vectors were submitted to the NIH (http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi).

3.2.27 Computational tools and bioinformatics

Computational analysis like sequence database search, alignment, map construction, image analysis were done mainly on a DEC alpha computer (Digital Equipment Corporation) running the OSF1 Unix operating system. Bioinformatics software packages mainly used were the Staden package http://www.mrc-lmb.cam.ac.uk/pubseq/ (Staden et al., 2000), GCG http://www.gcg.com/ (Devereux et al., 1984) and EMBOSS http://www.uk.embnet.org/Software/EMBOSS/index.html (Rice et al., 2000). Oligonucleotide primer design was done using the Staden package (for hybridisation oligonucleotides) or Primer3 http://www-genome.wi.mit.edu/genome_software/other/primer3.htm.l Physical map construction has been done using the program wprobeorder (Mott et al., 1993). Hybridisation data were stored in an Oracle database.
Masking of repeats and low complexity sequences was performed by using RepeatMasker (http://repeatmasker.genome.washington.edu/).

Filtering, processing and visualisation of BLAST results was done using MSPcrunch and Blixem (Sonnhammer and Durbin, 1994).
Scripts for automating processes were written in the Unix shell scripting language or Perl. More details about software use are described in the results section.