

3. Materials

3.1 Laboratory equipment

- Blotting device, semi-dry, for protein gels Hoefer TE 70, Amersham Pharmacia Biotech Europe GmbH, Freiburg
- Cell electroporator Gene Pulser II, Bio-Rad Laboratories GmbH, München
- Centrifuges Super T21, RC-2B, Sorvall GmbH, Bad Homburg
1-13, Sigma Laborzentrifugen GmbH, Osterode am Harz
- Densitometer scanner for Coomassie-stained protein gels Personal Densitometer, Molecular Dynamics GmbH, Krefeld
- Fluorescence video documentation for high-density filters PXL CCD video camera, Photometrix, Tucson, Arizona USA
IQLab software, Scientific Analytics, Vienna, Virginia USA
- Freezer, -80°C Forma, ThermoQuest Analytische Systeme GmbH, Egelsbach
- Gel electrophoresis equipment Hoefer SE 200, Amersham Pharmacia Biotech Europe GmbH, Freiburg
- Gel-documentation for ethidiumbromide-stained agarose gels Herolab GmbH, Wiesloch
- Incubator Heraeus Instruments GmbH, Hanau
- Incubator shaker New Brunswick Scientific GmbH, Nürtingen
- Mass spectrometer Reflex II MALDI-TOF, Bruker Franzen Analytik GmbH, Bremen
- Microtitre plate filling machine Genetix, Christchurch, Dorset, UK
- Multiple Gel Caster for SDS-polyacrylamide gels Hoefer SE 215, Amersham Pharmacia Biotech Europe GmbH, Freiburg
- Packing machines for microtitre plate blocks Lady Pack (shrink wrap), Pactur, Bologna, Italy
Tippy Pack (binding machine), Spot, Manfred Pütz GmbH, Kerpen
- PCR-machine PTC100, MJ Research, Inc., Watertown, USA
- Pipettes, adjustable Abimed Analysen Technik GmbH, Langenfeld
- Pipettes, adjustable, 8-channel Eppendorf, Köln
- PhosphorImager SI Molecular Dynamics GmbH, Krefeld
- Power supply Bio-Rad Laboratories GmbH, München
- Robot for colony picking and spotting Genetix, Christchurch, Dorset, UK
- Shaker for filters Rocky, Fröbel Labortechnik, Wasserburg
- Spectrophotometer Shimadzu Deutschland GmbH, Duisburg
- Ultrasonic homogeniser Branson Ultrasonic, Danbury, CT, USA

- Vortex Vortex Genie 2-Mixer, Bender und Hobein AG, Zürich, Switzerland

3.2 Chemicals, nucleotides, antibodies and enzymes

- 29% acrylamide, 0.8% bisacrylamide Rotiphorese Gel 30, Carl Roth GmbH, Karlsruhe
- ammonium persulfate BIO-RAD Laboratories GmbH, München
- ampicillin Sigma, Deisenhofen
- anti-human HSP90 α antibody, mouse monoclonal anti-Hsp90, Transduction Laboratories, Lexington, Kentucky, USA
- anti-mouse IgG, AP conjugated Pierce, KMF Laborchemie Handels GmbH, St. Augustin
- anti-mouse IgG, AP conjugated Boehringer Mannheim GmbH
- ATP Boehringer Mannheim GmbH
- attophos JBL Scientific, San Luis Obispo, USA
- betaine Sigma, Deisenhofen
- L-cysteine Sigma, Deisenhofen
- dATP, dCTP, dGTP, dTTP, lithium salts Boehringer Mannheim GmbH
- [α -³²P]dCTP Amersham Pharmacia Biotech Europe GmbH, Freiburg
- DEPC Sigma, Deisenhofen
- DNA Polymerase I (*E. coli*), Large (Klenow) Fragment New England Biolabs GmbH, Schwalbach/Taunus
- DTT Serva, Heidelberg
- EDTA Merck, Darmstadt
- ethidium bromide, 1% solution Fluka
- D-+-glucose monohydrate Merck, Darmstadt
- glycerate 3-phosphate, tricycloammonium salt Sigma, Deisenhofen
- glycerol Merck, Darmstadt
- glycogen from mussels Boehringer Mannheim GmbH
- guanidine hydrochloride Sigma, Deisenhofen
- iodoacetamid Aldrich
- IPTG Sigma, Deisenhofen
- imidazole Sigma, Deisenhofen
- kanamycin Sigma, Deisenhofen
- lysozyme Boehringer Mannheim GmbH
- myokinase, 2,000 units/mg, rabbit muscle Sigma, Deisenhofen
- β -NADH, disodium salt Sigma, Deisenhofen
- n-octyl β -D-glucopyranoside O-9882, Sigma
- oligonucleotides MWG-Biotech, Ebersberg
- phosphoenolpyruvate Sigma, Deisenhofen
- 3-phosphoglycerate kinase Boehringer Mannheim GmbH
- pronase Boehringer Mannheim GmbH

- pyruvate kinase/L-lactate dehydrogenase Sigma, Deisenhofen
- RGS-His antibody, mouse monoclonal Qiagen GmbH, Hilden
- TCEP-HCl Pierce, KMF Laborchemie Handels GmbH, St. Augustin
- sarkosyl (sodium N-lauroylsarcosine) Sigma, Deisenhofen
- Shrimp alkaline phosphatase Amersham Pharmacia Biotech Europe GmbH, Freiburg
- sodium pyrophosphate Sigma, Deisenhofen
- TEMED Life Technologies GmbH, Karlsruhe
- Triton X-100 Sigma, Deisenhofen
- TRIzol Life Technologies GmbH, Karlsruhe
- trypsin, sequencing grade, modified V511A, Promega
- Tryptone, Bacto Difco Laboratories, Detroit, USA
- Tween 20 Sigma, Deisenhofen
- urea Merck, Darmstadt
- 4-vinylpyridine Aldrich
- yeast extract Difco Laboratories, Detroit, USA
- 2×YT Broth Agar BIO 101, Vista, CA, USA
- 2×YT Broth BIO 101, Vista, CA, USA

Anorganic salts, acids and bases and alcohols were *pro analysi* quality from Merck, Darmstadt. Restriction enzymes and T4-DNA-Ligase were from New England Biolabs GmbH, Schwalbach/Taunus. A preparation of Taq DNA polymerase was a gift of Uwe Radelof, MPIMG Berlin.

3.3 Oligonucleotides

Oligo-nucleotide	Sequence (5' to 3')	Annealing-Temperature
pQE65	TGA GCG GAT AAC AAT TTC ACA CAG	65°C
pQE276	GGC AAC CGA GCG TTC TGA AC	65°C
SPORT 3/86	CCG GTC CGG AAT TCC CGG GT	65°C
SPORT 5/86	GCA CGC GTA CGT AAG CTT GGA TCC TCT AGA	65°C
M13-Forward	GAC GTT GTA AAA CGA CGG CCA G	55°C
M13-Reverse	CAC AGG AAA CAG CTA TGA CC	55°C
NotI primer-adapter	p-GAC TAG TTC TAG ATC GCG AGC GGC CGC CC (T) ₁₅	
SaII adapter top strand	TCG ACC CAC GCG TCC G	
SaII adapter bottom strand	p-CGG ACG CGT GGG	

3.4 Kits

- cDNA cloning kit
Superscript Plasmid System, Life Technologies GmbH, Karlsruhe
- mRNA isolation kit
PolyAtract mRNA isolation system III, Promega GmbH, Mannheim

3.5 Other materials

- 3MM Blotting paper
Whatman GmbH, Göttingen
- Agar plates
Bio Assay Dish, Nunc GmbH & Co. KG, Wiesbaden
- Chroma Spin columns
Clontech Laboratories, Palo Alto, CA, USA
- Filter plates, 96-well
MADV N 65, Millipore GmbH, Eschborn
- Glass microfibre filters, 2.5 cm
GF/C, Whatman GmbH, Göttingen
- Microtitre plates, 384-well
Genetix, Christchurch, Dorset, UK
- Ni-NTA agarose
Qiagen GmbH, Hilden
- Ni-NTA silica magnetic beads
Qiagen GmbH, Hilden
- Nylon filters, 222 × 222 mm²
Hybond-N⁺, Amersham Pharmacia Biotech Europe GmbH, Freiburg
- Protein size standard
Boehringer Mannheim GmbH
- PVDF filters, 222 × 222 mm²
Immobilon P, Millipore GmbH, Eschborn
Hybond-PVDF, Amersham Pharmacia Biotech Europe GmbH, Freiburg
- QIAquick columns
Qiagen GmbH, Hilden
- Replicators, 384-pin
Genetix, Christchurch, Dorset, UK
- Size standard, DNA
1 kb-ladder, Life Technologies GmbH, Karlsruhe
- Size standard, protein
low range, Boehringer Mannheim GmbH

3.6 Buffers and media

AP buffer

1 mM MgCl₂
100 mM Tris-HCl, pH 9.5

Attophos stock solution

2.4 M diethanolamine
5 mM attophos
0.23 mM MgCl₂
set pH to 9.2 with HCl
sterilised by filtration through a 0.2 µm pore size filter.

Buffer A

6 M guanidine hydrochloride
0.1 M NaH₂PO₄
0.01 M Tris
set pH to 8.0 with HCl

Buffer B

8 M urea
0.1 M NaH₂PO₄
0.01 M Tris
set pH to 8.0 with HCl

Buffer C

as Buffer B, pH 6.3

Church buffer (modified)

5% SDS
0.25 M Na₂HPO₄, pH 7.2
1 mM EDTA

Coomassie blue staining solution

1.25 g Coomassie Brilliant Blue G 250 (Serva) are dissolved in 225 ml technical grade ethanol. 225 ml distilled water and 50 ml acetic acid were added. The mixture was stirred for 2 h and filtered through a folded filter (No. 595, Schleicher & Schuell, Dassel).

Denaturing solution

0.5 M NaOH
1.5 M NaCl

10 x Freezing Mix

4 mM MgSO₄
15 mM Na₃-citrate
68 mM (NH₄)₂SO₄
36% glycerol
0.13 M KH₂PO₄
0.27 M K₂HPO₄
(pH 7.0), autoclaved

40% (w/v) glucose

400 g D-+-glucose monohydrate were dissolved in distilled water to 1 litre and sterilised by filtration through a 0.2 µm pore size filter.

Lysis Buffer

50 mM Tris-HCl, pH 8.0
0.3 M NaCl
0.1 mM EDTA

Matrix Solution

1% (w/v) HCCA (α-cyano-4-hydroxycinnamic acid)
50% acetonitrile
0.1% TFA

Neutralising solution

1 M Tris-HCl, pH 7.4

1.5 M NaCl

10 x PCR Buffer

0,5 M KCl

1% Tween 20

15 mM MgCl₂

0.5 M Tris-HCl, pH 8.8

sterilised by filtration through 0.2 µm pore size filter

Pronase solution

50 mM Tris-HCl, pH 8.5

50 mM EDTA

100 mM NaCl

1% sarkosyl

50 mg/ml Pronase

SB

12 g/l Bacto-tryptone

24 g/l yeast extract

0.4% (v/v) glycerol

17 mM KH₂PO₄

72 mM K₂HPO₄

A 20× potassium phosphate solution and a 20/19× solution of the remaining ingredients are autoclaved separately, and then mixed in a 1:19 ratio.

4 x SDS loading buffer

0.2 M Tris-HCl pH 6.8

8% SDS

40% (w/v) glycerol

0.004% bromophenol blue

0.1 M DTT was added separately to protein samples.

SOB

20 g/l tryptone

5 g/l yeast extract

10 mM NaCl

10 mM KCl

autoclaved

SOC

20 g/l tryptone

5 g/l yeast extract

10 mM NaCl

10 mM KCl

10 mM MgCl₂

10 mM MgSO₄

20 mM glucose

autoclave without glucose, add 40% glucose sterilised by filtration

20 x SSC

3 M NaCl

0.3 M Na₃-citrate

pH 7.0

TAE buffer

40 mM Tris-acetate, pH 8.0

1 mM EDTA

TBE buffer

90 mM Tris-borate, pH 8.0

1 mM EDTA

TBS

10 mM Tris-HCl, pH 7.5

150 mM NaCl

TBST-T

20 mM Tris-HCl pH 7.5

0.5 M NaCl

0.1% (v/v) Tween 20

0.5% (v/v) Triton X-100

TE buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA

autoclaved

TEN buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA

25 mM NaCl

autoclaved

2×YT Agar

46 g premixed 2×YT-Broth Agar (16 g tryptone, 10 g yeast extract, 5 g NaCl, 15 g agar) were dissolved in distilled water to 1 litre and sterilised by autoclaving at 120°C for 20 min.

2×YT-Broth

31 g premixed 2×YT-Broth (16 g tryptone, 10 g yeast extract, 5 g NaCl) were dissolved in distilled water to 1 litre and sterilised by autoclaving at 120°C for 20 min.

3.7 Strains

***E. coli* SCS1**

(Stratagene)

hsdR17($r_K^- m_K^+$) *recA1 endA1 gyrA96 thi-1 relA1 supE44*

4. Methods

4.1 Plasmid constructs

pQE-30 (Qiagen) is a pBR322-based expression vector that carries a phage T5 promoter and two *lac* operators for IPTG-inducible recombinant protein expression. pQE30NST was constructed from pQE-30 as follows. In the first step, pQE-30N was generated by inserting a synthetic oligonucleotide carrying a *Bgl*II and a *Not*I site into the unique *Pst*I site of pQE-30. In subsequent steps, an oligonucleotide carrying an SP6 promoter was inserted between the *Bam*HI and the *Sal*I site of pQE-30N, followed by insertion of a second oligonucleotide carrying a T7 promoter between the *Hind*III and the *Not*I site. The resulting vector, pQE30NST, can be used for cloning of cDNAs with *Sal*I and *Not*I overhangs. The insert can be transcribed *in vitro* in sense direction using SP6 RNA polymerase and in antisense direction using T7 RNA polymerase. Figure 1 shows a map of pQE30NST (GenBank accession number AF074376).

The helper plasmid pSE111 was obtained from Eberhard Scherzinger (unpublished results). pSE111 was constructed in two steps from pSBETc, a pACYC177-based expression vector that carries the *argU* gene, a kanamycin resistance gene and a T7 RNA polymerase promoter site for recombinant protein expression (92). (i) An *Xmn*I-*Eco*RV fragment, nucleotide position 2,041–2,521, was excised from pSBETc to remove the T7 promoter region. (ii) A 1.2 kbp *Eco*RI fragment containing the *lacI*^Q gene was excised from plasmid pVH1 (93) and inserted into the unique *Eco*RI site of the plasmid resulting from step (i). Plasmids of 5.1 kbp with *lacI*^Q inserts in both possible orientations were obtained; in pSE111 transcription of the *lacI*^Q gene was clockwise in the published pSBETc map (92).

4.2 PCR and DNA sequencing

The polymerase chain reaction (PCR) can generate a large number of copies from even the smallest amounts of DNA (94). This was enabled by the isolation of a thermostable DNA polymerase from *Thermus aquaticus*. During the PCR, DNA is denatured at high temperature,

specific oligonucleotide primers are annealed and elongated at lower temperatures in a cyclic manner.

A typical PCR contained 0.25 μM of each primer, 65 μM of each dNTP, 1 \times PCR-Buffer, 1 ng plasmid template, 7.4 U/ μl Taq DNA polymerase. For PCR amplification of cDNA clones in the vector pQE30NST, the primers pQE65 and pQE276 were used. In the PCR reaction, a 2 min denaturation step at 94°C was followed by 30 cycles of

10 sec. at 94°C

10 sec. at 65°C

5 min at 72°C.

PCR products were analysed by 1.4% agarose gel electrophoresis, purified using QIAquick PCR purification spin columns and quantified photometrically.

Cycle sequencing (95) is a combination of the Sanger sequencing method (96) with PCR. The sequencing template is regenerated by cycling denaturation, therefore less template is needed. As an alternative to radioactivity, fluorescent dyes are used to label primers (97) or dideoxynucleotide chain-terminators (98). By using four different fluorescent dyes with different emission wave-lengths, reactions for the four bases can be combined in one lane of a sequencing gel. PCR products were sequenced by dye-terminator cycle sequencing using the pQE65 primer and automated ABI sequencers (Perkin Elmer) by the service department of our institute.

4.3 Antibody affinity purification

Rabbit anti-GAPDH serum was obtained from Eberhard Scherzinger and was affinity purified according to Gu et al. (99). His₆-GAPDH fusion protein was expressed in *E. coli* in a 400 ml culture and cells were lysed in 10 ml Buffer A. His₆-GAPDH was bound to 1.2 ml Ni-NTA agarose (Qiagen) by shaking for 1 h at room temperature. Using a column, the agarose beads were washed with Buffer C until the A₂₈₀ of the flow-through was below 0.02. Chaotropic salts were removed by washing with 150 mM NaCl, 50 mM Tris, pH 7.4. 1 ml anti-GAPDH serum was added to the beads. The suspension was shaken for 1 h in the column. The column was washed with 5 ml of (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and 5 ml of (2 M NaCl, 50 mM Tris-HCl, pH 7.4). 0.6 ml elution buffer (4 M MgCl₂, 10 mM Tris-HCl, pH 6.0) was added and incubated 15 min. Antibodies were eluted in 0.5 ml fractions. The first 5 fractions

were pooled and dialysed against PBS overnight at 4°C. The purified antibody detected GAPDH in an ELISA, and a maximum signal was observed at a dilution of 1:500.

4.4 cDNA library construction and arraying

4.4.1 Total RNA preparation

Total RNA from adult kidney liver and human fetal brain was isolated according to Chomczynski and Sacchi (100). With this method, cell homogenates are directly added to a monophasic acidic phenol guanidine isothiocyanate solution. Upon adding chloroform, the RNA remains in the aqueous phase, while – because of the low pH – proteins and DNA enter the organic and the interphase. Total RNA is recovered by isopropanol precipitation.

To avoid RNase contamination, disposable plastic ware was used whenever possible. Glassware was baked at 200°C before use. RNase-free water was prepared by stirring two times distilled water with 0.01% (v/v) DEPC overnight, followed by autoclaving twice to remove the DEPC.

Tissues were homogenised by grinding under liquid nitrogen and directly transferred to 10 ml per gram tissue of TRIzol, a monophasic phenol guanidine isothiocyanate solution, in a Dounce homogeniser. The mixture was homogenised and passed once through a 23 gauge needle to shear genomic DNA. 0.2 ml chloroform per ml of TRIzol were added. The mixture was shaken vigorously, incubated at room temperature for 5 min, and centrifuged for 30 min at 6,000 rpm in an SS34 rotor in 30 ml Corex glass centrifugation tubes to separate the aqueous and inorganic phases. The aqueous, upper phase was recovered, and total RNA was precipitated with one volume of isopropanol. The mixture was incubated on ice for 10 min, and centrifuged for 10 min at 15,000 rpm in an SS34 rotor in plastic tubes, that had been treated with 5 N NaOH overnight to inactivate RNases. The large, white pellet was washed once with 70% ethanol, centrifuged again for 5 min, and dissolved in 200 µl DEPC-treated water. Total RNA was stored at -80°C.

4.4.2 Selection of polyadenylated (poly(A)⁺) RNA

poly(A)⁺ RNA was separated from the remainder of total RNA, which is largely rRNA and tRNA. Total RNA was denatured by heating to expose the poly(A) (polyadenylated) tails, and annealed to biotinylated oligo(dT) probes. The annealed nucleic acids were bound to

streptavidin conjugated paramagnetic particles, which were restrained by a magnet during buffer changes. The particles were washed with a moderate ionic strength buffer, and poly(A)⁺ RNA was eluted with water.

The FastATrackIII kit (Promega) contains RNase free water, biotinylated oligo(dT) Probe, 20×SSC and streptavidin paramagnetic particles (SA-PMP). 0.7 mg (mouse kidney) or 0.4 mg (human fetal brain) of total RNA in 0.5 ml RNase free water was heated to 65°C for 10 min. 3 µl of the biotinylated oligo(dT) Probe and 13 µl of 20×SSC was added and the mixture was cooled to room temperature. One tube with SA-PMP particles was placed in a magnetic stand (a tube holder with a magnet), and the liquid phase was removed. The particles were washed four times with 0.3 ml 0.5×SSC by gently flicking the bottom of the tube until all of the particles were resuspended. After the final wash, as much of the aqueous phase as possible was removed without disturbing the SA-PMP particles. To elute poly(A)⁺ RNA, the particles were resuspended in 250 µl RNase-free water. The SA-PMP particles were magnetically captured, the eluted poly(A)⁺ RNA was recovered and precipitated by adding 0.15 volume of 3 M sodium acetate and 3 volumes of ethanol. The mixture was incubated on ice for 10 min and centrifuged for 20 min at top speed in a cooled microcentrifuge. The pellet was washed once with 70% ethanol, centrifuged again, and dissolved in 10 µl water. RNA concentrations in the washing fractions and the eluate were determined by UV absorbance measurement at 260 nm in a 5 µl microcuvette.

4.4.3 cDNA synthesis

cDNA libraries were prepared by oligo(dT) priming (101) using a Superscript Plasmid System kit. mRNA was reverse transcribed by Superscript II RT, an enzyme engineered from Moloney Murine Leukemia Virus RT (102), using a primer with a *NotI* restriction site followed by (T)₁₅. RNA in RNA-DNA duplexes was partially digested with RNase H to prime second strand cDNA synthesis by *E. coli* DNA polymerase I. DNA ligase was used to fuse different second strand molecules created on the same template. Double stranded cDNA was made blunt end with T4 DNA polymerase, followed by ligation of *SalI* adapters. By digestion with *NotI*, cDNA with 5'-*SalI* and 3'-*NotI* overhangs was created. This allowed directional cloning between vector *SalI* and *NotI* sites. *NotI* recognises an 8-bp sequence, therefore only very few recognition sites are present in random DNA sequences. cDNA was size fractionated by gel filtration, and ligated with the vector pQE30NST, followed by transformation of *E. coli* cells.

(w/v) TCA, 1% (w/v) sodium pyrophosphate, followed by washing for 2 min in 50 ml of 95% ethanol at room temperature, and drying at room temperature. This filter carried only the incorporated radioactivity and was used to determine the yield of first strand cDNA. First strand yield was calculated from the amount of nucleotides in the reaction (40 nmole) the amount of nucleotides in 1 μg of single stranded DNA (3.03 nmole) and the ratio of the measured activities in counts per minute (cpm), as measured in a scintillation counter.

$$\text{yield} = \frac{\text{incorporated activity}}{\text{total activity}} \frac{40 \text{ nmole}}{3.03 \text{ nmole} \mu\text{g}^{-1}} = \frac{\text{incorporated activity}}{\text{total activity}} 13.2 \mu\text{g}$$

The specific activity of double stranded cDNA was calculated by dividing the whole activity incorporated (= 50 · incorporated activity measured on second filter) by twice the single strand yield.

In X days, the specific activity decays by $2^{-X/14.3}$, because the half life of ^{32}P is 14.3 days.

The remaining 30 μl of the first strand aliquot were ethanol precipitated and analysed by gel electrophoresis (see 4.4.4).

Second strand synthesis, generation of *SalI*, *NotI* overhangs

Second cDNA strands were synthesised by adding the following to the remaining 18 μl first strand reaction:

- 93 μl DEPC-treated water
- 30 μl 5 \times second strand buffer
- 3 μl 10 mM dNTP mix
- 1 μl *E. coli* DNA ligase
- 4 μl *E. coli* DNA polymerase I
- 1 μl *E. coli* RNase H

The mixture was incubated for 2 h at 16°C. To generate blunt ends, 2 μl of T4 DNA polymerase was added and incubated for 5 min at 16°C. The reaction was placed on ice and extracted once with 150 μl phenol:chloroform:isoamylalcohol (25:24:1). cDNA was precipitated by adding 0.5 volume of 7.5 M ammonium acetate, followed by 2.4 volumes of ethanol (-20°C) and centrifugation at room temperature at top speed for 20 min in a microcentrifuge. The pellet was washed once in 75% ethanol (-20°C), centrifuged again for 5 min and was dissolved in 25 μl DEPC-treated water. *SalI* adapters were ligated to the cDNA

by adding 10 μ l 5 \times T4 DNA ligase buffer, 10 μ l *SalI* adapters, 5 μ l T4 DNA ligase and incubation for 16 h at 16°C, followed by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation as described above. In the next step cDNA was digested with *NotI*. The DNA pellet was dissolved in 41 μ l DEPC-treated water, 5 μ l React 3 buffer and 4 μ l *NotI* were added and incubated for 2 h at 37°C, followed by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation as described above.

Size fractionation

Size fractionation of cDNA is important for several reasons. Residual adapters are present in large molar excess and can impede vector ligation to cDNA by ligating to the *SalI* termini of the pre-digested vector. Additionally, the fragments released from the cDNA by *NotI* digestion have *SalI* termini at one end and *NotI* termini at the other, and can contaminate the library with apparently “empty” clones. Size fractionation also reduces the tendency of smaller (<500 bp) inserts to predominate the library. These smaller cDNAs can arise for several reasons:

If the mRNA preparation is not size-selected, partially degraded mRNAs are selected on the oligo(dT) cellulose columns along with longer mRNAs. These will be reverse transcribed into small cDNAs.

If extreme care is not taken to prevent RNase contamination during first strand synthesis, degradation can occur when the mRNA is manipulated. Some mRNAs contain regions that are not readily reverse transcribed, and the reverse transcriptase is not able to synthesise complete first strands. Column chromatography is a simple method of producing size-fractionated cDNA, free of adapters and other low molecular weight DNAs. The Superscript Plasmid System contains prepacked, disposable 1 ml Sephacryl S-500 HR columns that remove cDNAs <500 bp and size-fractionate cDNAs >500 bp, thus facilitating construction of libraries from fractions enriched for larger cDNA. Individual size fractions contain cDNA ranging from 500 bp to several kilobasepair size. The average cDNA size gradually decreases with increasing fraction number.

The cDNA pellet was dissolved in 100 μ l TEN. Excess liquid (20% ethanol) was allowed to drain from the column, followed by washing with 3.2 ml TEN. 100 μ l cDNA was added to the column and fraction 1 was collected. cDNA was eluted with TEN. A 100 μ l fraction, followed by single-drop fractions (~35 μ l) were collected. Radioactivity was measured by scintillation counting.

4.4.4 Gel electrophoresis of first strand cDNA

First strand cDNA was analysed by denaturing agarose gel electrophoresis. 1 µg size standard (1 kb -ladder) was labelled for 10 min at room temperature in a 20 µl reaction containing 50 µM each of dATP, dTTP, dGTP, 5 µCi [α -³²P]dCTP, 5 units *E. coli* DNA Polymerase I, Large (Klenow) Fragment with 3'→5' exonuclease activity, 10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 7.5 mM DTT, followed by heat inactivation at 75°C for 20 min and ethanol precipitation. 0.5 µl of the labelled size standard and the ethanol-precipitated first strand sample were dissolved in 10 µl 1× alkaline agarose gel sample buffer [30 mM NaOH, 1 mM EDTA, 10% (v/v) glycerol, 0.01% bromophenol blue]. The agarose gel [1.4% (w/v)] was cast in 30 mM NaCl, 2 mM EDTA and was equilibrated for 2 to 3 h in alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA) before loading the samples. Electrophoresis was for 16 h at 15 V. The gel was dehydrated under vacuum, exposed to a phosphor imager screen for 2 h and an image was generated on a phosphor imager.

4.4.5 Vector digestion

pQE30NST DNA was digested with *SalI* and *NotI*, followed by *BglII* to reduce background from re-circulised vector molecules. 20 µg pQE30NST were digested in 100 µl with 80 units *SalI*, 0.2 mg/ml BSA, 1× NEB 2 buffer (New England Biolabs) for 3 hours at 37°C. 40 units *NotI* and 20 units *SalI* were added, and digestion proceeded overnight. 10 units *BglII* and 20 units *NotI* were added, and the incubation was continued for 5 h. 10 µl of the reaction was diluted to 90 µl in TE buffer and purified with a Chroma Spin 1000 TE column to remove protein, buffer salts and short DNA fragments released during digestion.

4.4.6 Ligation

10 ng cDNA was ligated to 50 ng *SalI*, *NotI* cut pQE30NST in 20 µl containing 1× T4 ligase buffer and 1 unit T4 DNA ligase for 3 h at room temperature, followed by ethanol precipitation with 1 µl of 20 µg/µl glycogen from mussels, 11 µl 7.5 M ammonium acetate and 80 µl ethanol (-20°C). The pellet was washed in 75% ethanol and resuspended in 5 µl water.

4.4.7 Preparing *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 (103,104).

Cells of *E. coli* SCS1 carrying the helper plasmid pSE111 were made competent for transformation by electroporation by growth in low-salt media and washing in 10% glycerol. 1 litre SOB with 15 µg/ml kanamycin was inoculated with 10 ml of an overnight culture of SCS1/pSE111 and grown to an OD₆₀₀ 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 at 4°C 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, the cells were resuspended in a minimal volume of 10% glycerol and aliquots were frozen in liquid nitrogen and stored at -80°C.

4.4.8 Transformation

Cells were thawed on ice, and 40 µl were mixed with 2 µl of ligated cDNA. The cells were electroporated in electroporation cuvettes of 0.1 cm gap size according to the manufacturer of the electroporation apparatus. 1 ml of SOC was added to the cells immediately after application of the pulse, and the suspension was shaken at 37°C for 1 h. Freezing mix was added and the mixture was frozen in liquid nitrogen and stored at -80°C.

4.4.9 Colony Picking

Transformed cells were plated at a density of 3,000 clones/plate onto square 23×23 cm² 2×YT agar plates containing 100 µg/ml ampicillin, 15 µg/ml kanamycin and 2% glucose and were grown at 37°C overnight. Using a picking robot (105), colonies were picked into 384-well microtitre plates filled with 2×YT medium containing 100 µg/ml ampicillin, 15 µg/ml kanamycin, 2% glucose and Freezing Mix. Bacteria were grown in the microtitre wells at 37°C overnight and replicated into new microtitre plates using 384-pin replicating tools. All copies were stored frozen at -80°C.

4.5 High-density filters for protein and DNA detection

High-density filters were prepared by robot spotting, as described (76,105), at a density of 27,648 clones per filter in a duplicate pattern surrounding ink guide dots. Bacterial colonies were gridded onto Nylon membrane filters for DNA analysis and polyvinylidene difluoride (PVDF) membrane filters for protein analysis (filter format 222 mm × 222 mm). Filters were placed onto square 2×YT agar plates containing 100 µg/ml ampicillin, 15 µg/ml kanamycin and 2% glucose.

4.5.1 DNA filters

DNA filters were processed as described by Hoheisel et al. (106). Colonies were grown to a size of approximately 1.5 mm diameter. Blotting paper (23×23 cm²) was soaked in Denaturing Solution and nylon filters were placed on top for 4 min. The filters were transferred to a fresh blotting paper soaked in Denaturing Solution. The sandwich was placed on a glass plate sitting above water level in a 95°C water bath. Filters were exposed to steam for 4 min, followed by neutralisation on blotting paper soaked in Neutralising Solution for at least 4 min. Filters were submerged in 600 ml Pronase solution (37°C) and were incubated for 30–40 min. Pronase solution was replaced after using five times. Filters were dried for two days at room temperature, and stored between blotting paper sheets at room temperature.

4.5.2 Protein filters

Colonies on filters for protein analysis were grown overnight at 30°C to a size of approximately 1 mm diameter, and were then transferred to agar plates supplemented with 1 mM IPTG to induce protein expression for 3 h at 37°C. Colonies were lysed by transfer of the filters on blotting paper soaked in Denaturing Solution for 10 min, twice for 5 min on Neutralising Solution and finally on 2×SSC for 15 min. Filters were air-dried and stored at room temperature.

4.6 DNA hybridisation screening of high-density filters

DNA hybridisations using digoxigenin-labelled PCR probes and Attophos alkaline phosphatase substrate (JBL Scientific, San Luis Obispo) were performed as described (107).

Dephosphorylation of Attophos yields a fluorescent product. Digoxigenin-labelled hybridisation probes were prepared by PCR-amplification.

30 μ l PCR reaction were set up containing:

- 1 \times PCR buffer
- 60 μ M of each dNTP
- 0.25 μ M of each primer
- 3 μ M DIG-11-dUTP
- 7.5 U/ μ l Taq DNA polymerase

Template was added to the reaction by transfer of bacteria containing the template plasmid with a tooth-pick from liquid cultures. Alternatively, 1 ng plasmid DNA was used as template. The PCR program was a 1 min denaturation step at 94°C, followed by 30 cycles of 10 sec. 94°C, 10 sec. 65°C (primers SPORT 3/86 and SPORT 5/86) or 55°C (primers M13-Forward and M13-Reverse) and 3 min 72°C.

Filters were pre-hybridised in Church buffer at 65°C for 1 h. The DIG-labelled PCR product was denatured by adding 10 μ l 0.5 M NaOH and immediately transferred to 30 ml Church buffer (65°C). The probe was added to the filter in a plastic bag. The bag was sealed while avoiding to trap any air, and placed in a plastic dish on a rocking shaker inside an incubator at 65°C overnight. A large plastic bag filled half with water was placed on top of the hybridisation bag. The next morning, filters were washed in 1 litre 2 \times SSC, 0.1% SDS at room temperature for 1 h, followed by 1 litre 0.1 \times SSC, 0.1% SDS at 65°C for 1 h. Upon blocking in 500 ml 5% low fat milk powder in PBS, the filters were exposed to AP-conjugated anti-DIG Fab fragments, diluted 1:5,000 in 5% low fat milk powder in PBS for 1 h. Filters were washed twice for 20 min in 500 ml PBS, followed by two washes for 10 min in AP Buffer. Filters were incubated in 1 mM Attophos in AP buffer for 4 h. The fluorescent Attophos dephosphorylation product was detected on the filters by illumination with long-wave UV light. Images were taken by a CCD camera (PXL CCD video camera, Photometrix) controlled by the program IQLab (Scientific Analytics). Positive clones were scored using either of the programs Xdigitse (written by Huw Griffith) or WinClone (written by Markus Kietzmann). Both programs display images of high-density filters. Positive signals are scored by the user and a list of positive clones is generated.

4.7 Antibody screening of high-density filters

Dry protein filters were soaked in ethanol and bacterial debris was wiped off with paper towels in TBST-T. The filters were washed twice for 10 min in TBST-T, followed by two brief washes in TBS and a 10 min wash in TBS. The filters were blocked for 1 h in blocking buffer (3% non-fat, dry milk powder in TBS) and incubated overnight with 50 ng/ml anti-HSP90 α antibody or the anti-GAPDH antibody, diluted 1:5,000. The filters were washed twice for 10 min in TBST-T, followed by two brief washes in TBS and a 10 min wash in TBS. They were then incubated with alkaline phosphatase-conjugated secondary antibody (anti-mouse IgG-AP or anti-rabbit IgG-AP) for 1 h. Having washed three times for 10 min in TBST-T, once in TBS and once in alkaline AP buffer, the filters were incubated in 0.25 mM Attophos in AP buffer for 5 min. Images were taken as described under 4.6.

4.8 Protein expression in *E. coli*

Protein expression in bacteria containing cDNA inserts in the pQE30NST vector was performed as followed. 10 ml of an overnight culture was added to 900 ml SB medium containing 100 μ g/ml ampicillin and 15 μ g/ml kanamycin. The culture was shaken at 37°C until an OD₆₀₀ of 0.8 was reached. IPTG was added to a final concentration of 1 mM. The culture was shaken for 3.5 h at 37°C and cooled to 4°C on ice. Cells were harvested by centrifugation at 2,100 g for 10 min, resuspended in 100 ml (50 mM NaH₂PO₄, pH 8.0, 0.3 M NaCl) and centrifuged again. The cell pellet was used directly or stored frozen at -80°C.

4.9 SDS-PAGE

SDS-PAGE and protein staining was performed according to Laemmli (108). SDS-polyacrylamide gels were prepared in batches of 12 in a multiple gel caster. Separation and stacking gel were prepared as follows:

Separation gel

0.1% SDS

14.5% acrylamide

0.4% bisacrylamide

0.38 M Tris-HCl, pH 8.8

0.1% APS

polymerisation is started by adding

0.03% (v/v) TEMED

Stacking gel

0.1% SDS

3.8% acrylamide

0.11% bisacrylamide

0.125 M Tris-HCl, pH 6.8

0.1% APS

polymerisation is started by adding

0.1% (v/v) TEMED

Electrophoresis was run at 80 V until the bromophenol blue in the SDS-loading buffer entered the separation gel, then the voltage was increased to 180 V. The run was stopped when the bromophenol blue reached the bottom of the gel. The gel was shaken in Coomassie Blue Staining Solution for 30 min and destained in 20% methanol, 10% acetic acid.

For SDS-PAGE of whole cellular proteins, 11 μ l 4 \times SDS loading buffer and 3 μ l 1 M DTT were added to 30 μ l of an *E. coli* cell suspension of 10 OD₆₀₀. The mixture was incubated for 5 min at 50°C and 2 min at 100°C, and centrifuged for 5 min at top speed in a microcentrifuge. 8 μ l of the supernatant was loaded on the gel.

4.10 Metal chelate affinity purification

4.10.1 Purification under denaturing conditions

Proteins were denatured before purification if formation of inclusion bodies was observed. Cells were resuspended in 5 ml per gram wet weight of Buffer A containing 6 M guanidine hydrochloride and lysed by stirring for 15 min. The lysate was cleared by centrifugation at 10,000 g for 30 min. Ni-NTA agarose was added according to the expression strength (binding capacity 5–10 mg/ml resin) and mixed by shaking for 1 h. The mixture was poured into a column, washed with Buffer B and then Buffer C, until the A₂₈₀ of the flow-through was below 0.01. Proteins were eluted with a 4:1 mixture of Buffer C and 0.5 M EDTA.

4.10.2 Purification under native conditions

For purification of soluble proteins, cells were lysed in 3 ml per gram wet weight of Lysis Buffer containing 0.25 mg/ml lysozyme on ice for 30 min. If the protein remained insoluble,

Lysis Buffer containing 1.5% sarkosyl was used, according to Frangioni and Neel (109). This often lead to solubilisation, but the protein was not always eluted from the Ni-NTA column after removal of detergents. DNA was sheared with an ultrasonic homogeniser for 3×1 min at 50% power on ice. The lysate was cleared by centrifugation at 10,000 g for 30 min. Because ionic detergents may interfere with Ni-NTA binding, 3% (v/v) Triton X-100 was added to lysates containing sarkosyl. This non-ionic detergent is capable of sequestering sarkosyl (109) and does not interfere with Ni-NTA binding. Ni-NTA agarose was added according to expression strength, and mixed by shaking at 4°C for 1 h. The mixture was transferred to a column which was washed with ten bed volumes of Lysis Buffer containing 20 mM imidazole. Protein was eluted in Lysis Buffer containing 250 mM imidazole and was dialysed against TBS at 4°C overnight.

4.11 Tryptic digest

After induction of protein expression, bacterial cells were pelleted and lysed in 10 ml Buffer A per gram wet cell weight.

200 µl lysate were incubated with 25 µl of a 50% Ni-NTA magnetic agarose bead suspension (Qiagen) for 1 h at room temperature. The beads were washed three times with Buffer C. Disulphide bonds were reduced in 100 µl 0.1 M tris(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl) in Buffer A for 1 h at room temperature plus 1 h at 50°C. The beads were washed once in Buffer A and thiol groups were alkylated with either 0.1 M iodacetamid or 0.1 M 4-vinylpyridine in Buffer A at room temperature. The beads were washed four times with 50 mM NH₄HCO₃, followed by adding 11 µl of 40 ng/µl trypsin, 1% n-octyl-β-D-glucopyranoside in 50 mM NH₄HCO₃ and incubation over night at room temperature. After removal of the beads, 0.5 µl were mixed with 0.5 µl of matrix solution on a steel target and measured on a Bruker Reflex 2 MALDI-TOF instrument with delayed extraction. Alternatively, peptides were eluted by adding 150 µl 1% TFA to the beads. The eluate was dried under vacuum and the peptides were resuspended in 5 µl of 50% acetonitrile, 1% trifluoroacetic acid (TFA).

4.12 Protein expression and purification in microtitre plates

4.12.1 Protein expression

96-well microtitre plates with 2 ml cavities (StoreBlock, Zinsser, No. 3219009) were filled with 100 μ l SB medium supplemented with 100 μ g/ml ampicillin and 15 μ g/ml kanamycin. Cultures were inoculated with bacteria from 384-well plates that had been stored at -80°C . For inoculation, replicating devices carrying 96 steel pins, length 6 cm, were used. After overnight growth at 37°C with vigorous shaking, 900 μ l of medium SB prewarmed to 37°C were added to the cultures, and incubation was continued for 1 h. For induction of protein expression, IPTG was added to a final concentration of 1 mM, and incubation continued for 4 h. The OD_{600} of eight cultures was measured. Cells were harvested by centrifugation at 1,900 g (3,400 rpm) for 10 min, washed by resuspension in Lysis Buffer, centrifuged for 5 min and were either used immediately or stored at -80°C .

4.12.2 SDS-PAGE of whole cellular proteins

For electrophoresis of whole cellular proteins, bacteria were resuspended in 100 μ l of SDS loading buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, 0.05 M Tris-HCl pH 6.8, 0.1 M DTT) and incubated at 100°C for 2 min. Lysates were centrifuged for 20 min at 1,900 g, and 8 μ l were loaded immediately on 15% SDS-polyacrylamide gels.

4.12.3 Metal chelate affinity purification

For metal affinity purification of His₆-tag proteins, cells were lysed by resuspension in 150 μ l Buffer A. 15 μ l of a 50% glass powder suspension (Glasmehl 280, Kurt Merker GmbH, Kelheim) were added to the lysates to help formation of a compact pellet with the bacterial debris in the subsequent centrifugation at 1,900 rpm for 15 min. Supernatants were filtered through a 96-well filter plate with a non-protein binding 0.65 μ m pore size PVDF membrane (Durapore MADV N 65, Millipore) on a vacuum filtration manifold (Multiscreen, Millipore). Filtrates were collected in a fresh filter plate, and 25 μ l 50% Ni-NTA agarose was added. The plate was sealed with tape, and proteins were bound by shaking for 3 h at 300 rpm. The agarose beads were washed three times by resuspending in Buffer C, shaking for 5 min, and removing of liquid on the vacuum filtration manifold. Finally, proteins were eluted by shaking

for 10 min in 25 μ l of Buffer C containing 0.1 M EDTA. Eluates were filtered and collected into a new microtitre plate, and 5 μ l were analysed by 15% SDS-PAGE.

4.12.4 Solubility of expression products

Cells were resuspended in 100 μ l Lysis Buffer and lysed by addition of 30 μ l 2.2 mg/ml lysozyme and incubation for 1 h on ice. 20 μ l of 50% glass powder was added, and lysates were centrifuged for 15 min at 1,900 g. The pellets were removed with toothpicks, and lysates were filtered through a MADV N 65 filter plate (Millipore). 8 μ l were analysed by 15% SDS-PAGE.

4.13 Enzyme assays

4.13.1 GAPDH assay

This assay was described by Heinz and Freimüller (110).

Reagents

- 50 mM triethanolamine pH 7.5
- 14 mM β -NADH (10 mg/ml)
- 0.5 M $MgSO_4$
- 0.2 M ATP in 25 mM HEPES pH 7.5
- 93 mM glycerate 3-phosphate, tricycloammonium salt
- 114 mM L-cysteine (20 mg/ml, prepared daily)
- 3-phosphoglycerate kinase from yeast (10 mg/ml, 450 units/mg), crystalline suspension in 3.2 M ammonium sulphate (Boehringer Mannheim)

The following reagents were added to a cuvette of 10 mm light path: 500 μ l triethanolamine, 172 μ l distilled water, 12.5 μ l β -NADH, 10 μ l $MgSO_4$, 4 μ l ATP, 25 μ l glycerate 3-phosphate, 25 μ l L-cysteine, 1.5 μ l 3-phosphoglycerate kinase. GAPDH was added in a volume of 1.5–3 μ l and the change of absorbance at 340 nm per minute was measured with a spectrophotometer. Activity was calculated using the absorbance coefficient of NADH at 340 nm of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. Specific activity was based on GAPDH concentration determination by calculation of the absorbance coefficient from the protein sequence and measurement of light absorption at 280 nm.

4.13.2 Calmodulin assay

The assay of Chock and Huang (111) for calmodulin is based on the activation of 3',5'-cyclic nucleotide phosphodiesterase, and involves the following reactions:

3',5'-cyclic nucleotide phosphodiesterase: $\text{cAMP} + \text{H}_2\text{O} = \text{AMP}$

myokinase: $\text{AMP} + \text{ATP} = 2 \text{ADP}$

pyruvate kinase: $2 \text{ADP} + 2 \text{phosphoenolpyruvate} = 2 \text{ATP} + 2 \text{pyruvate}$

L-lactate dehydrogenase: $2 \text{pyruvate} + 2 \text{NADH} = 2 \text{lactate} + 2 \text{NAD}^+$

Reagents

- reaction buffer
 - 0.1 M HEPES pH 8.0
 - 20 mM MgCl_2
 - 1 mM CaCl_2
 - 0.1 M KCl
- 0.1 M phosphoenolpyruvate (PEP)
- 0.2 M ATP
- 14 mM β -NADH (10 mg/ml)
- 0.1 M cAMP
- The nucleotides and PEP were dissolved in 25 mM HEPES, pH 7.5.
- Pyruvate kinase/L-lactate dehydrogenase (PK/LDH) PK 740 units/ml, LDH 1,030 units/ml (Sigma)
- Myokinase 2,000 units/mg, 1.6 mg/ml (Sigma)
- 3',5'-cyclic nucleotide phosphodiesterase (PDE), activator deficient, from porcine brain, 0.5 units/ml (Sigma)
- Working assay mix
 - 4 ml H_2O
 - 5 ml reaction buffer
 - 0.3 ml PEP
 - 25 μl ATP
 - 200 μl NADH

The working assay mix has to be protected from light and may be stored frozen at -20°C for several weeks.

To 700 μl working assay mix in a cuvette 7 μl myokinase, 7 μl PK/LDH, 20 μl PDE and 2 μl calmodulin, 0.016–1.6 mg/ml was added. Basal PDE activity was measured without calmodulin. The mixture is incubated for 20 min to allow the formation of the PDE-calmodulin complex. The reaction was started by addition of 20 μl cAMP and the decrease of absorption at 340 nm per minute was measured in a spectrophotometer.

To demonstrate Ca^{2+} dependence of the reaction, 7.5 μl 0.5 M EGTA was added to sequester Ca^{2+} . 7.5 μl 1 M CaCl_2 was added to reverse this effect.

In this assay, 2 mole NADH are oxidised to NAD^+ for 1 mole cAMP hydrolysed to AMP (see reaction schema in 5.3.2). Therefore, the extinction coefficient of NADH is multiplied by a factor of 2. One unit PDE hydrolyses 1 μmole cAMP to AMP per minute.

$$\text{activity} = \Delta c V_{\text{assay}} = \Delta A (\epsilon \cdot d)^{-1} V_{\text{assay}}$$

$$\epsilon = 2 \epsilon_{(\text{NADH}, 340 \text{ nm})} = 12.44 \text{ mM}^{-1} \text{ cm}^{-1}$$

$$V_{\text{assay}} = 756 \mu\text{l}, d = 1 \text{ cm} \Rightarrow \text{activity} = 61 \Delta A \mu\text{mole}$$

Calmodulin concentrations were determined according to Bradford (112). Lysozyme was used as the standard.