D. Materials and Methods.

D.1. General.

D.1.1. Reagents and materials.

Chemicals were supplied by:

Acetic acid	Merck
Acrylamid / Bis	BioRad
Agar	Gibco BRL
Agarose ultrapure	BioRad
Ampicillin (sodium salt)	Sigma
Bacto-agar	Difco
Bacto-pepton	Difco
Bacto-trypton	Difco
Bacto-yeast extract	Difco
Bromphenolblue	Merck
Casaminoacid	Difco
N,N-Dimethylformamid (DMF)	Sigma
Dithiothreitol (DTT)	Merck
DMEM-Medium	Gibco BRL
Ethanol absolut	Merck
Ethidiumbromide (10 mg / ml)	Sigma
Ethylendiamintetracetic acid (EDTA)	Sigma
Fetal bovine serum (FBS)	Gibco BRL
Gentamicin	Gibco BRL
D-Glucose-Monohydrate	Merck
Glycerol	Merck
GST-agarose	Sigma
Hepes	Sigma
DAPI	Sigma

Isopropylthio-ß-D-thiogalactopyranoside	Sigma
Lipofectin	Gibco BRL
ß-Mercaptoethanol	Merck
Morpholinopropanesulfonic acid (MOPS)	Merck
Ni-NTA Agarose	Qiagen
N-octyl-ß-D-Glucopyranoside	Sigma
Nonylphenoxy Polyethoxy Ethanol (NP-40)	Merck
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Ponceau S	Sigma
Protease inhibitors	Boehringer Mannheim
Sodium azide	Sigma
Sodium dodecyl sulfate (SDS)	BioRad
TEMED	BioRad
Thiamine-Hydrochloride	Sigma
Trizma Base	Sigma
Trypsin	Boehringer Mannheim

Materials used were purchased from:

Agarose gel chamber	Pharmacia Biotech
Analytical balance	Mettler
Axiophot fluorescence microscope	Zeiss
Axioplan 2 LSM 510 confocal microscope	Zeiss
Cellulose acetate membrane filters (OE 66, 0.2 µm)	Schleicher & Schuell
Centrifuges:	
Centrifuge 5415 C	Eppendorf
Sorvall Superspeed RC2-B	Sorvall
Beckman J6-NC	Beckman
Centrifuge rotors:	
SS 34 (Sorvall Superspeed RC2-B)	Sorvall
GSA rotor (Sorvall Superspeed RC2-B)	Sorvall
JS 4.2 (Beckman J6-NC)	Beckman

Dialysis filter (0.025 μm)	Millipore
Dialysis membranes MWCO 10,000 MW	Pierce
Disposable cell scraper	Costar
Electron microscope	Philips 400
Electroporation cuvette (0.1 cm electrode gap, 50)	BioRad
Fluorescence microscope	Zeiss
Incubator	Heraeus
Leighton tubes	Costar
Light microscope CK2	Olympus
pH-Meter (Ika-Combimag RTC)	Radiometer Copenhagen
Microscope Slides	Menzel-Gläser
Nitrocellulose (0.2 μm)	Schleicher & Schnell
Sarastro Phoibos 1000 confocal microscope	MolecularDynamics
Slide-A-Lyzer Cassette	Pierce
Spectrophotometer (DU-62)	Beckman
Thermocycler (PCR) PTC-100	Biozym
Tissue culture flask	Becton Dickinson
Ultracentrifuge:	
Beckman L8-M	Beckman
Ultracentrifuge rotor:	
VT1 65 (Beckman L8-M)	Beckman
Videocamera	Herolab
Vivaspin 4 (cut-off filter 10,000)	Vivascience
Whatman Paper 3MM	Whatman
X-ray films	Kodak

D.1.2. Enzymes.

Restriction endonucleases and T4 DNA polymerase were supplied by New England Biolabs (NEB) and digests were performed as recommended by the manufacturer. T4 DNA ligase, protease inhibitors, lysozyme and trypsin (modified sequencing grade) were purchased from Boehringer Mannheim and factor Xa was acquired from Pharmacia Biotech. Shrimp alkaline phophatase was purchased from

Amersham LIFE SCIENCE. USB supplied the *E. coli* DNA polymerase large fragment (Klenow) and the *Pfu* DNA polymerase was purchased from Stratagene. RNase A and DNase I were purchased from Sigma Chemicals Co.

D.1.3. DNA sizes.

For agarose gels electrophoresis the 1 kb DNA ladder, PhiX174 RF DNA Hind III digest, lambda DNA-BstE II digest, supplied by Gibco BRL and New England Biolabs, were used.

D.1.4. General solutions.

1x PBS (pH 7.4):	Na Cl	140 mM
	KCl	2.6 mM
	Na_2HPO_4	10 mM
	KH_2PO_4	1.7 mM
	ddH_2O	*ad* 1 l
1x TE:	Tris-HCl (pH 8.0)	10 mM
	EDTA	1 mM
1x TAE:	Tris acetate (pH 8.0)	40 mM
	EDTA	10 mM

D.1.5. Antibiotics (stock solutions).

Kanamycin: 30 mg / ml in 50% (v / v) ethanol, stored at -20 °C.

Ampicillin: 50 mg / ml in 50% (v / v) ethanol, stored at -20 °C.

D.1.6. Bacterial media.

Luria-Bertani Medium (LB):	Bacto-tryptone	10 g
	Bacto-yeast extract	5 g
	NaCl	10 g
	ddH_2O	*ad* 1l

Adjusted to pH 7.0 with NaOH.

Autoclaved 20 min at 120°C.

2x YT Medium:	Bacto-tryptone	16 g
	Bacto-yeast extract	10 g
	NaCl	5 g
	ddH_2O	*ad* 1l

Adjusted to pH 7.0 with NaOH.

Autoclaved 20 minutes at 120°C.

SOC Medium:	Bacto-tryptone	20 g
	Bacto-yeast extract	5 g
	NaCl	0.5 g
	ddH_2O	*ad* 1l

TY Medium: LB medium supplemented with:

MOPS-KOH (pH 7.9)	20 mM
Glucose	0.2% (w / v)
Thiamine / HCl	10 µg / ml
Casamino acids	0.1% (w / v)

Adjusted to pH 7.0 with NaOH.

Autoclaved 20 min at 120°C.

Agar Plates 15 g bacto-agar / litre of medium

D.1.7. Bacterial strains.

E. coli XL1-Blue Stratagene

[*F´:: Tn10 proA*$^+$*B*$^+$ *lacI*^{q} *D(lacZ)M15/recA1 endA1 gyrA96 (Nal*^{r}*) thi hsdR17 (*r_K^- m_K^+*) supE44 relA1 lac*]

E. coli SCS1 Stratagene

[*F*$^-$*, endA1, hsdR17(*r^-_K*,* m^+_K*), supE44, thi-1,* l^-*, recA1, gyrA96, relA1*]

E. coli DH10B GibcoBRL

[F– mcrA, D(mrr-hsdRMS-mcrBC), j80dlacZDM15, DlacX74, deoR, recA1, end A1, araD139, D(ara, leu)7697, galU, galK, l-, rpsL, nupG]

E. coli SURE Stratagene

e14-(mcrA), D(mcrCB-hsdSMR-mrr)171, endA1, supE44, thi-1, gyrA96, relA1, lac, recB, recJ, sbcC, umuC:Tn5 (kan^r); uvrC, [F´ proAB, lacI^{q}ZDM15, Tn10,(tet^r)]

D.1.8. Eucaryotic cell lines.

COS-7: Fibroblast-like kidney cells (monkey). Transformed with an origin defective mutant of SV40 virus (Celis, J.E. 1994).

SHSY5Y: Neuroblastoma cells (human). Metastasis to supraorbital area. (Celis, J.E. 1994).

Lymphoblastoid cells: B-lymphocytes obtained from a MJD patient and an healthy individual. Transformed with Epstein-Barr virus (a gift from Josef Priller, Charite', Berlin).

Sf9: *Spodoptera frugiperda* pupal ovarian cell line (PharMingen).

D.1.9. Antibodies.

Anti-GAPDH polyclonal	Büssow, *et al.* 1998
Anti-Histones monoclonal	Chemicon International
Anti-HD1 polyclonal	Scherzinger, *et al.* 1997
Anti-MJD (CT1) polyclonal	this work
Anti-MJD (NT1) polyclonal	this work

FITC-conjugated anti-rabbit antibody	Dako
Cy3-conjugated anti-rabbit antibody	Jackson Laboratories

D.1.10. Kits.

Bio-Rad Protein assay	Bio-Rad
ECL	Amersham
QIAEX II Gel Extraction Kit	Qiagen
QIAGEN Plasmid Maxi Kit	Qiagen
QIAprep Spin Plasmid Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
QuickChange Site Directed Mutagenesis Kit	Stratagene

D.1.11. Vectors.

Name	Size	Host	Resistence	Reference
pBluescript SK (-)	3.0 kb	*E.coli*,	Amp^r	Stratagene (La Jolla, CA, USA).
pQE32N	3.4 kb	*E.coli*,	Amp^r	Büssow, *et al.* 1998.
pGEX-5X-1	4.9 kb	*E.coli*,	Amp^r	Pharmacia.
HMJD1a	~5 kb	*E. coli*,	Amp^r	O. Riess, Ruhr-Univ., Bochum.
pBSK-MJD-46	~4 kb	*E. coli*,	Amp^r	F.Laccone, Göttingen University.
pBSK-MJD-69	~4 kb	*E. coli*,	Amp^r	F.Laccone, Göttingen University.
pBSK-MJD-74	~4 kb	*E. coli*,	Amp^r	F.Laccone, Göttingen University.
pBSK-MJD->150	~4.3 kb	*E. coli*,	Amp^r	F.Laccone, Göttingen University.
pSG5	4.1 kb	COS-7,	Amp^r	Green, *et al.* 1988.
pCMX	4.0 kb	COS-7,	Amp^r	Kakizuka, *et al.* 1991.
pEGFP-C1	4.7 kb	COS-7,	Km^r	Clontech.
pBTM117c	9.5 kb	Yeast,	Amp^r	Wanker, *et al.* 1997.

D.1.12. *MJD1* cDNA and oligo(deoxyribo)nucleotides.

MJD-*Bam* / *Sal* 5`-CGGGATCCAGTCGACGATGGAGTCCATCTTCCACG -3`
*Bam*H I *Sal* I

MJD-*Sal* I 5`-ACGCGTCGACGGAAGCTGACCAACTCCTGC-3`
Sal I

MJD-*Not* I 5`-ATAGTTTAGCGGCCGCTTATGTCAGATAAAGTGTGAAGGT-3`
Not I

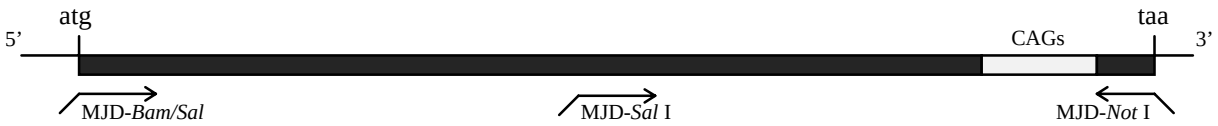

Figure 1. Schematic representation of *MJD1* cDNA. The CAG repeats are represented by a clear box The start (atg) and stop codon (taa) are indicated above the schematic map. The position of the oligo(deoxyribo)nucleotides is represented by arrows.

D.1.13. Construction of plasmids.

pQE-MJD1: 1.1 kb *MJD1* cDNA with 25 CAGs was amplified from the plasmid HMJD1a (a generous gift from O. Riess, Ruhr-University, Bochum) by PCR using the primers MJD-*Bam/Sal* and MJD-*Not*. The fragment was cloned in the pQE32N vector between the *Bam*H I and *Not* I restriction sites. With this vector ataxin-3 is expressed as a fusion protein with an N-terminal 6xHis-tag.

pQE-MJDCT: 0.6 kb of the 3'-terminal end of the *MJD1* cDNA was obtained by DNA amplification of the HMJD1a plasmid using the primers MJD-*Sal* I and MJD-*Not* I. The PCR product was cloned into the polylinker of the pQE32N vector between the *Sal* I and *Not* I restriction sites. In this construct the C-terminal portion of ataxin-3 is expressed with an N-terminal 6xHis-tag as fusion protein.

pQE-MJDNT: This construct was created by deleting a 0.5 kb *Pst* I-*Hind* III fragment from the pQE-MJD1 plasmid and religating after filling in the ends with T4 DNA polymerase. In this construct the N-terminal half of ataxin-3 protein is expressed as a 6xHis-tag fusion protein.

pGEX-MJD1: The 1.1 kb *Sal* I-*Not* I *MJD1* cDNA insert with 25 CAGs was excised from the pQE-MJD1 plasmid and subcloned into pGEX-5X-1 using the same restriction sites. In this construct the *MJD1* cDNA is fused to a GST-tag.

pSG5-MJD1: A 1.1 kb fragment containing the *MJD1* cDNA with 25 CAGs was excised out from the pQE-MJD1 plasmid using two flanking *Bam*H I restriction sites and subcloned in the unique *Bgl* II site of the pSG5 vector. The orientation of the insert was checked by restriction analysis.

pEGFP-MJD1: This plasmid was created by excising out the *MJD1* cDNA from the plasmid pQE-MJD1 using *Sac* I and *Hind* III restriction sites. Subcloning of the resulting fragment into *Sac* I and *Hind* III restriction sites of pEGFP-C1 resulted in the pEGFP-MJD1 plasmid. In this construct the *MJD1* cDNA is expressed together with the gene of the green fluorescent protein (GFP) which is located upstream the *MJD1* cDNA.

pSG5-MJD1NLS-: In this construct three nucleotides (C, G, G) at position 879, 884, 889 of the *MJD1* cDNA were replaced with other three nucleotides (G, C, T) respectively, by site-specific mutagenesis.

pGEX-MJD-42,-64,-71,->150: The *BamH I-Not* I *MJD1* cDNA inserts with 42, 64, 71, and >150 CAGs were excised out from the plasmids pBSK-MJD-42,-69,-74,->150 (a generous gift from F. Laccone, Göttingen University) and subcloned into *Bam*H I and *Not* I cleaved pGEX-5X-1. Using this vector all ataxin-3 proteins are expressed as fusion proteins with glutathione S-transferase.

pSG5-MJD-42,-74,-131,-165: These constructs were created by subcloning the *MJD1* cDNAs from the plasmid pBSK-MJD-42,-69,-74,->150 into the pSG5 vector using *Bam*H I and *Not* I restriction sites. The different numbers of CAGs in the resulting constructs are due to expansions and deletions of the CAG repeat which occur during the propagation of the plasmids in the bacterial hosts (Wells, *et al*. 1996).

D.2. DNA manipulation.

D.2.1. DNA preparation.

Alkaline lysis method.

This method, originally described by Birnboim, *et al*. 1979, yielded between 5 and 10 µg of plasmid DNA per 10 ml of bacterial overnight culture. A single colony of bacteria was used to inoculate 10 ml of LB medium, containing the appropriate antibiotic. The cell culture was incubated overnight with shaking at 37°C and 3 ml of the suspention were pelleted. The cells were resuspended in 100 µl of AL1 buffer:

Glucose	50 mM
Tris-Cl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

and incubated at room temperature for 5 min. Then 200 µl of AL2 lysis buffer:

NaOH	0.2 N
SDS	1% (w / v)

were added and the tube was inverted gently three times. After 5 min on ice, 150 µl of AL3 buffer:

Potassium acetate	3 M
Glacial acetic acid	11.5 % (v / v)

was added and the tube inverted and vortexed for 10 sec. After 10 min on ice, the sample was centrifuged for 20 min at 15,000 g and the supernatant was transferred to a fresh Eppendorf tube for phenol-chloroform-isoamylalcohol extraction. An equal volume of phenol-chloroform was added to the tube, vortexed and centrifuged for 3 min at 15,000 g. The upper aqueous layer was transferred to a separate tube and the extraction repeated with another volume of phenol-chloroform followed by an equal volume of chloroform. The upper aqueous layer was then transferred to a tube containing 1 / 100 total volume of RNase A (10 mg / ml) and incubated at room temperature for 30 min. To this 2.5 volumes (approximately 1 ml) of ice-cold ethanol and 1 / 10 volume of 3 M sodium acetate (pH 5.2) were added and after 5 min at room temperature the DNA was pelleted at 15,000 g for 20 min, washed with 70% (v / v) ethanol and resuspended in 50 µl of 1x TE buffer.

QIAprep Spin Plasmid Kit (Qiagen).

This kit was mainly used for the preparation of plasmid DNA which had to be sequenced or for cloning experiments. It yielded approximately 10 µg of DNA from a 3 ml overnight culture. The DNA preparation was performed as recommended by the manufacturer.

D.2.2 Plasmid maxipreparation.

The plasmid Maxi Kit from Qiagen was used for large scale preparation of plasmid DNA. It yielded approximately 100-500 µg of DNA from a 500 ml overnight culture. The DNA preparation was performed as suggested by the manufacturer.

D.2.3. DNA purification by CsCl gradient.

In order to obtain plasmid DNA ready for eucaryotic cell transfection, I have purified it by CsCl gradient centrifugation (Maniatis, *et al.* 1989). 200 ml of bacteria were grown for 6-7 hours and centrifuged at 4,000 rpm for 10 min. The pellet was resuspended in 8 ml of buffer I:

Tris-HCl (pH 8.0)	25 mM
Sucrose	50 mM
EDTA	10 mM
Lysozyme	0.5 mg / ml

and kept at room temperature for 15 min. After adding 16 ml of buffer II:

SDS	1% (v / v)
NaOH	0.2 N

the cell lysate was incubated 5 min on ice and finally 8 ml of cold buffer III:

Potassium acetate	3 M
Acetic acid	2 M

were added to the cell lysate. After 15 min on ice the lysate was centrifuged for 15 min at 4,000 rpm and the supernatant filtered through cheese cloth. The filtrate was precipitated with 0.6 volume of isopropanol and left on ice for 30 min. After centrifugation at 4,000 rpm for 15 min, the pellet was lyophilised and resuspended in the gradient buffer:

TE (1x)	3.5 ml
Tris base (1 M)	22 µl
EDTA (0.5 M)	27 µl
Cesium chloride	4.4 g
Ethidiumbromide (10 mg / ml)	100 µl.

The DNA solution was filled into a VTi 65 ultracentrifuge tube and equilibrated carefully with the counterbalance tube. The top was sealed and the tubes were put into the VTi 65 rotor. Centrifugation was at 47,500 rpm for 20 hours at 20°C. The

band of the plasmid DNA, visible under long wave UV light (366 nm), was removed with a syringe. The ethidiumbromide was eliminated by mixing 3-4 times the DNA solution with isopropanol saturated with NaCl. The upper phase containing the ethidiumbromide was discarded whereas the lower phase with the DNA was kept and precipitated with 3 volumes of 70% (v / v) ethanol for 30 min at -20°C.
After centrifugation at 12,000 rpm for 30 min the pellet was washed with 80% (v / v) ethanol, lyophilised and resuspended in 400 µl of TE. A treatment with RNase A (0.1 mg / ml) for 30 min and with proteinase K (1 mg / ml) for 1 hour at 37°C was made to eliminate RNA and protein contaminants. After two extractions with phenol-chloroform and a third extraction with chloroform-isoamylalcohol, the DNA was precipitated again by adding 1 / 10 volume 3M sodium acetate, 2 volumes ethanol and kept at -20°C for 30 min. After centrifugation at 12,000 rpm for 30 min the pellet was lyophilised and resuspended in 500 µl TE. The A_{260} was determined and the DNA concentration was calculated using the following formula:

A_{260} = [DNA (µg / ml)] / 50

D.2.4. Agarose gel electrophoresis.

Gels were prepared in 1x TAE buffer using 0.7-2% (w / v) agarose, depending on the size of the DNA fragments that had to be separated. Gels were run at 2-5 V / cm in a horizontal electrophoresis chamber. To visualise the DNA under U.V. light (254 nm), 10 µg / ml of ethidium bromide was added to the gels.
Before loading, the DNA samples were mixed with loading buffer:

Bromophenol blue	0.25% (w / v)
Xylen-cyanol	0.25% (w / v)
Glycerol	30% (v / v)
EDTA	6 mM

D.2.5. Polymerase chain reaction (PCR).

PCR reactions were performed in a thermocycler (Perkin Elmer genAmp 9600). A 50 µl reaction mixture was set up with the buffer supplied with the Stratagene *Pfu* DNA polymerase, 200 µM of each dATP, dTTP, dCTP, dGTP, 100 ng of each oligo(deoxyribo)nucleotide primer, approximately 10 ng of the DNA to be amplified and 0.5u of *Pfu* DNA polymerase. The cycling conditions depended upon the melting temperature of the oligo(deoxyribo)nucleotides, the length of the fragment to be amplified and the complexity of the DNA starting source.
In the experiments to be reported the following standard PCR program was used:

1. 94°C - 4 min
2. 94°C - 30 sec
3. (x)°C - 30 sec
4. 72°C - 1 min
5. 72°C - 10 min
6. 4°C - overnight

Steps 2 to 4 were repeated 30 times. The annealing temperature (T_a) for each primer was calculated with the GC rule, considering the number of GC and AT nucleotides :

$$Ta = [(A + T) \times 2] + [(G + C) \times 4]$$

The final annealing temperature was calculated from the average of the annealing temperature of the two primers.

D.2.6. DNA digestion.

Approximately 1 µg of a DNA minipreparation was used for restriction enzyme digestion. The reaction mixture (20 µl) was set up as follows:

DNA	5 µl (~ 1 µg)
Buffer (10x)	2 µl
[BSA (0.1 mg / ml)	2 µl]
Enzyme A	0.5 µl (1-20 U / µl)
[Enzyme B	0.5 µl (1-20 U / µl)]
ddH2O	*ad* 20 µl

and the mixture was incubated at 37°C for 1 hour. Restriction digests were analysed on a 0.7 % (w / v) agarose gel.

D.2.7. Polyacrylamide gel electrophoresis of DNA.

In order to analyse size differences of small DNA fragments (100-500 bp), a vertical 5% (v / v) polyacrylamide gel was prepared as follows:

Acrylamide 38% (w / v) -Bisacrylamide 2% (w / v) solution	5 ml
5x TBE	8 ml
Ammonium Persulfate 10% (w / v)	210 ml
ddH_2O	27 ml
TEMED	42 ml

The samples were run at 10-15 V / cm in TBE buffer. After the run, the gel was stained for 15 min with 1 mg / ml ethidiumbromide in TBE buffer and the DNA was visualised under short wave U.V. light (254 nm).

D.2.8. DNA purification by agarose gel electrophoresis.

DNA fragments were separated by agarose gel electrophoresis and the desired fragments were excised under long wave U.V. light (366 nm). Purification of the fragments from agarose was achieved using the QIAEX II Gel Extraction Kit (Qiagen) following the manufacturer's suggestions.

D.2.9. Subcloning of restriction fragments.

Vector DNA was digested for 1 h at 37°C with restriction enzymes compatible with the restriction fragment to be inserted. Vectors for directional cloning were

purified on agarose gels as described above. To remove the 5'phosphate groups, all vectors were treated with Shrimps alkaline phosphatase (Amersham LIFE SCIENCE). Incubations were carried out with 1U of phosphatase *per* 100 pmol DNA ends in the recommended buffer at 37°C for 1 hour. The enzyme was inactivated by heating at 68°C for 15 min and removed by using the QIAquick PCR purification kit (Qiagen).

D.2.10. DNA ligation.

Ligation was performed with 0.25 units T4 DNA ligase/µg of DNA vector with the recommended buffer (1 mM ATP) at 15°C overnight. For directional cloning an equimolar insert / vector ratio was used. For blunt-end ligations, 2.5 units T4 DNA ligase/µg of DNA were used. A ligation control with the DNA vector alone was performed in parallel to check the efficiency of the vector dephosphorylation.

D.2.11. DNA precipitation.

DNA was precipitated by adding:

1 volume of 3 M sodium acetate
2 volumes of 100 % (v/v) ethanol

to the DNA solution and keeping the sample at -20°C for 2 hours. After centrifugation (Eppendorf 5415) at 14,000 rpm for 30 min the DNA pellet was washed once with ice-cold 70 % (v/v) ethanol. The precipitate was air dried and resuspended in a small volume of 1x TE.

D.2.12. Preparation of electrocompetent bacteria.

1 litre of 2xYT broth was inoculated with 10 ml of an overnight culture of *E. coli* cells and incubated with vigorous shaking at 37°C until OD_{600} of 0.5-0.7. The cells were cooled on ice for 15 min and centrifuged at 4°C for 15 min at 2,000 g. After removing the supernatant the cell pellet was resuspended in 1 litre of cold 10% (v/v)

glycerol and the cells pelleted again. The cells were washed with 0.5 litre of 10% (v/v) glycerol, followed by 20 ml and finally resuspended in 2 ml of 10% (v/v) glycerol (approximately 3 x 10^{10} cells/ml). The cells were then frozen on dry ice and stored at -70°C in 40 µl aliquots.

D.2.13. Electroporation.

The electrocompetent cells were thawed on ice and mixed with 1-2 µl of the ligation mixture or approximately 10 ng of plasmid DNA to be transformed and incubated on ice for 1 min. The Bio-Rad Gene Pulsar apparatus was set at 25 µF, 2.5 kV and 200 Ohm. The cell/DNA mixture was transferred to a precooled 0.1 cm electroporation cuvette which was then placed between the contacts in the chamber and after the pulse the cells were resuspended in 1 ml of SOC medium. After incubation at 37°C for 1 hour the cells were plated on selective agar plates.

D.2.14. Antibiotic selection and analysis of plasmid DNA.

Only the *E. coli* cells which contain the recircularized plasmid DNA (vector + insert) carrying the antibiotic resistance gene (Amp^r or Km^r) are able to survive on the selective plates containing ampicillin or kanamycin and form colonies.
The plasmid DNA contained in these colonies was extracted by using the QIAprep Spin Plasmid Kit and examined for the presence of the insert by digestion with the same restriction enzyme that had been used for cloning. The orientation of the insert was determined by cutting the DNA at a restriction site inside and outside the insert and comparing the size of the released fragment with that calculated from the restriction map.

D.2.15. Storage of clones.

E. coli clones were stored in 1 x HMFM at -70 °C.

10x HMFM: 0.45% (w / v) $C_6H_5Na_3O_7$
0.9% (w / v) $(NH_4)_2SO_4$
1.8% (w / v) KH_2PO_4
6.3% (w / v) K_2HPO_4
44% (w / v) glycerol
0.36% (w / v) $MgSO_4$

For each DNA preparation the frozen culture was streaked onto agarose plates and single colonies were isolated.

D.2.16. Preparation of GST-fusion proteins.

10 ml TY overnight culture of a single colony containing the expression plasmid of interest was inoculated in 200 ml TY medium. After 1-2 hours of growing at 37°C (OD_{600} = 0.7), the expression of the protein was induced for 3 hours with 2 mM IPTG. After chilling the culture on ice, the cells were harvested by centrifugation at 4,000 rpm (Sorvall Superspeed RC2-B; GSA rotor) for 15 min at 4°C and then washed with cold wash buffer:

Tris-HCl (pH 7.5)	40 mM
NaCl	0.15 M
EDTA	1 mM

and recentrifuged for 10 min at 6,000 rpm. They were resuspended in 20 ml of buffer 1:

NaH_2PO_4	50 mM
Tris Base	5 mM
NaCl	0.15 M
EDTA	1 mM
PMSF	1 mM

adjusted to pH 8.0 with NaOH.

Lysozyme was added to a final concentration of 0.5 mg / ml. The cell suspension was incubated on ice for 45 min. The cells were sonicated on ice for 2 x 45 sec at 200-300 Watt and Triton X-100 was added to a final concentration of 0.1 % (v / v).
After the lysis, the cells were centrifuged for 30 min at 15,000 rpm (Sorvall Superspeed RC2-B, SS34 rotor) at 4°C and the pellet was discarded. The supernatant was incubated with stirring on ice for 60 min at 4°C with 5 ml of a 50% (w / v) slurry of GST-agarose (Sigma), previously equilibrated in buffer
1. The GST-agarose was spin down shortly and washed once with 40 ml of cold buffer 1 containing 0.1% (v / v) Triton X-100 and twice with 40 ml buffer
1. The fusion protein was then eluted from the GST-agarose with buffer 1 containing 15 mM reduced glutathione.

D.2.17. Dialysis.

The purified fusion protein was dialysed twice for 6 hours at 4°C against 1 litre of dialysis buffer:

Tris HCl (pH 7.5)	20 mM
glycerol	8.7 % (v / v)
NaCl	0.15 M
EDTA	0.1 mM

using a 10,000 MWCO dialysis membrane (Pierce).

D.2.18. Concentration of protein solutions.

To increase the concentration of a protein solution a Vivaspin 4 spin column was used. The solution was placed in a 15 ml tube with the appropriate cut-off filter (10,000 molecular weight) and spun at 5,000 x g for 5-15 min. Once the desired concentration was achieved, the protein solution was recovered from the spin column using a small volume pipette.

D.2.19. Protein determination.

Protein concentration was determined using the Bio-Rad Protein assay. A dilution (1:10 to 1:100) of the protein solution to be analysed was mixed with the Bio-Rad protein assay buffer (4:1) and the A_{595} of the mix was measured. The absorbance of the samples was compared to the absorbance of BSA which was used as a standard. When the purified protein was free from any nucleic acids contaminant, the protein concentration was also estimated by determining the absorption at 280 nm (A_{280}). The protein concentration was calculated using the rule:

[P (mg / ml)]= (OD_{280}) x (dilution factor) / 1.25 (Sambrook, *et al.* 1989)

D.2.20. SDS Polyacrylamide gel electrophoresis.

About 10-20 µg of protein was analysed on a 15% (w / v) polyacrylamide gel containing 0.1% (w / v) SDS.

Acrylamide 30% (w / v) / bisacrylamide 0.8% (w / v) solution	10 ml
Tris HCl (pH 8.8) 1 M	5 ml
SDS 10% (w / v)	0.2 ml
ammonium persulfate 10% (v / v)	0.2 ml
TEMED	8 µl
ddH_2O	4.6 ml

The protein samples were prepared as follows:

Protein	(x)
DTT	100 mM
Sample buffer	1x
ddH_2O	*ad* 50 µl

and boiled for 5 min.

After boiling the samples were centrifuged at 12,000 rpm for 2 min and loaded on to the bottom of the sample wells. The run was performed at 10 V / cm until the bromophenol blue dye reached the bottom of the resolving gel.

D.2.21. Coomassie staining.

Proteins separated by SDS-polyacrylamide gels were stained with Coomassie blue. The gel was fixed in:

Methanol	45% (v / v)
Acetic acid	10% (v / v)

for 15 min and was stained for 1 hour in:

Methanol	50% (v / v)
Acetic acid	9.2% (v / v)
Serva blue R	0.175% (w / v).

Gels were destained overnight in:

Methanol	20% (v / v)
Acetic acid	7% (v / v)

and after destaining, the gel was stored in 7.5 % (v / v) acetic acid.

D.2.22. Western blot analysis.

Proteins separated by SDS-polyacrylamide gels can be blotted to a nitrocellulose membrane filter for the detection with appropriate antibodies.
The SDS-PAGE gel was transferred to the membrane using 1x transfer blot buffer:

Tris HCl (pH 8.0)	48 mM
Glycine	39 mM
SDS	0.037% (w / v)
Methanol	20% (v / v)

for 90 min at 40 V / cm in an apparatus for electroblotting.
The nitrocellulose filter was removed from the apparatus and stained with Ponceau S. When the bands of the protein were visible the filter was rinsed with ddH_2O and

dried on a Whatman 3MM paper. The bands of the molecular-weight standards were marked with a pencil.

The filter was blocked for 30 min in blocking buffer:

Skimmed milk	3% (w / v)
PBS	1x
Triton X-100	0.05% (v / v)
NaN_3	0.02% (w / v)

and was then incubated for 1-2 hours with primary antibody diluted into blocking buffer.

After binding of the primary antibody, the filter was washed with blocking buffer 4 times for 5 min and then incubated with the secondary antibody in the same buffer for 1 hour. The filter was again washed 4 times for 5 minutes and the signal was developed using either alkaline phosphatase or the peroxidase-based methods.

In the first case, the nitrocellulose filter was transferred from the final wash to a tray containing the alkaline phosphatase buffer:

Tris HCl (pH 9.5)	100 mM
NaCl	100 mM
$MgCl_2$	5 mM

and incubated 3 times for 5 min. A mix of 66 µl of NBT (nitro blue tetrazolium) and 33 µl of BICP (5-bromo-4-chloro-3-indolyl phosphate) in 10 ml of alkaline phosphatase buffer was added to the nitrocellulose to start the reaction of the signal. When the bands were of the desired intensity (about 10-20 min), the reaction was stopped by adding a few ml of the stopping buffer:

EDTA (pH 8.0)	0.5 M
PBS	1x

For the development of the blots with the peroxidase method, the ECL kit of Amersham was used. 1 ml of buffer A was mixed with 1 ml of buffer B as recommended by the manufacturer and the mix was placed on the nitrocellulose filter. The filter, covered with a Saran wrap, was exposed to an X-ray film (Kodak)

for different times (5, 10, 30 sec, 1, 2, 5 min) until the bands were clearly visible on the developed film.

D.2.23. Proteolytic cleavage of GST fusion proteins.

The GST-ataxin-3 fusion proteins were digested with bovine factor Xa or with modified trypsin in buffer P:

NaCl	150 mM
EDTA	0.1 mM
Tris HCl (pH 8.0)	20 mM
$CaCl_2$	2 mM.

Incubations with trypsin were performed at 37°C for 3 to 16 hours. The digest with factor Xa was performed at 25°C for 16 hours. The digestion was stopped by adding SDS to a final concentration of 2% (w / v) and heating the samples for 3 min at 98°C.

D.2.24. Site-Directed Mutagenesis (Stratagene Kit).

To introduce point mutations into DNA, the QuikChange™ Site-Directed Mutagenesis Kit of Stratagene has been used. A supercoiled, double-stranded DNA (dsDNA) vector with the insert to be mutagenized and two synthetic oligonucleotide primers containing the desired point mutation were used (Kunkel, 1985). The two oligo(deoxyribo)nucleotides, each complementary to the strands of the vector were extended by PCR using turbo *Pfu* DNA polymerase and a new plasmid containing the point mutation was generated. The product was treated with *Dpn* I endonuclease which cuts methylated and hemimethylated DNA (target: 5' -G^{m6}ATC- 3'). The parental DNA template was cut because almost all DNA isolated from *E. coli* is *dam* methylated whereas the new plasmid containing the mutation was resistant to *Dpn* I digestion.

The DNA containing the desired mutations was transformed into Epicurian coli (*E. coli* XL1-Blue supercompetent cells) for amplification of the plasmid.

As control a pWhitescript™ 4.5-kb plasmid was used. This control plasmid contains a stop codon (TAA) instead of a glutamine codon in the beta-galactosidase gene and

Epicurian coli (*E. coli* XL1-Blue) transformed with this control plasmid do not form blue colonies on LB-ampicillin agar plates containing IPTG and X-gal. The oligonucleotide control primers which carry the reverse point mutation generated a new pWhitescript plasmid with the glutamine codon (CAA) in the β-galactosidase gene and replaced the TAA stop-codon. The XL1-Blue cells containing this plasmid form blue colonies on LB / IPTG / X-gal plates.

D.2.25. Cellulose acetate filter assay (CAFA).

Transfected COS-7 cells, washed with cold PBS, were centrifuged at 2,000 rpm for 10 min and resuspended in 200-500 µl lysis buffer:

Tris HCl (pH 8.8)	50 mM
NaCl	100 mM
$MgCl_2$	5 mM
NP-40	0.5% (v / v)
EDTA	1 mM

with protease inhibitors (2 mM PMSF, 10 µg / ml leupeptin, 10 µg / ml pepstatin, 1 µg / ml aprotinin, 50 µg / ml antipain).

After a 30 min incubation, the cells were vortexted for 3 min and centrifuged again for 5 min at 14,000 rpm at 4°C.

The supernatant was aliquoted and used for Western blot analysis and filter tests whereas the pellet was taken up in 100 µl of DNase buffer:

Tris HCl (pH 8.0)	20 mM
$MgCl_2$	15 mM

and DNase I was added to a final concentration of 0.5 mg / ml. After incubation for 1 hour at 37°C, aliquots were taken and analysed with the filter assay and Western blotting, whereas to the rest of the samples

8 µl	1 M Tris-HCl (pH 8.0)
1 µl	1% (w / v) SDS
1 µl	0.1 M $CaCl_2$

were added and the mixture incubated with Trypsin (0.25 mg/ml) at 37°C for 4-5 hours or overnight.

Aliquots of 3 to 20 µl of the reaction mixture were taken, diluted into 100 µl of:

PBS	1x
SDS	1% (w/v).
DTT	50 mM

and boiled for 5 min. The samples were loaded onto the dot-blot apparatus with the cellulose acetate membrane placed in the dot-blot apparatus. When all samples had passed the filter, the membrane was washed twice with 0.1% (w/v) SDS and probed with the appropriate antibodies.

D.2.26. Antisera production.

In order to obtain polyclonal antibodies, a healthy rabbit was immunised with the purified protein (Maniatis, *et al.* 1989). A blood sample was taken prior to the immunisation and used as non-immune serum (NIS). After a primary intradermal injection of 100-200 µg of the immunogen, emulsified in Freund`s complete adjuvant, a second and third injection of the same amount of immunogen was made at intervals of 3 and 2 weeks, respectively. 7-10 days after the last injection, a blood sample of 50 ml was taken and was allowed to clot at room temperature. With a Pasteur pipette the clot was ringed and in the next hours it retracted, leaving the antiserum. The antiserum was centrifuged at 4000 rpm (Sorvall superspeed RC2-B, SS34 rotor) for 10 min at room temperature, aliquoted and stored at -70°C.

D.3. Cell culture and cell biology.

D.3.1. Cell recovery.

The vials containing cells were recovered from liquid nitrogen and thawed immediately in a 37°C water bath. After thawing, cells were transferred carefully to a sterile tube on ice. Drop by drop an equal volume of growth medium was added and the suspension shaken gently.
The cells were transferred to a culture vessel appropriate for the total number of viable cells and a suitable volume of growth medium was added to the container.
After an incubation at 37°C for 3 hours, the cells were observed under the microscope and when they had stuck to the surface of the vessel, the growth medium was changed before returning them to the incubator.

D.3.2. Cell maintenance.

The cells were grown continuously in monolayers in Dulbecco's modified Eagle medium DMEM (Gibco BRL) supplemented with 5% (v/v) (COS-7) and 12% (v/v) (neuroblastoma) foetal bovine serum (FBS) and containing penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were maintained in a 37°C incubator with 5% CO_2.

D.3.3. Subculturing.

The cells were subcultured at 72 hours intervals removing the old growth medium and adding an appropriate amount of trypsin [e.g. 4 ml of 0.25% (w/v) per 250 ml flask]. The fluid was washed around gently and discarded. More trypsin was added and discarded. The cell sheet was examined at frequent intervals. When the cell sheet was sufficiently dispersed, an appropriate amount of growth medium (10 ml per 250 ml flask) was added and the cells were resuspended carefully. Two third

of the cells were discarded and to the residual 20 ml of cells additional fresh growth medium was added.

D.3.4. Storage.

The cells were transferred to a sterile container suitable for spinning and were centrifuged at 1,000 rpm for 10 min. The supernatant was discarded and the cells were resuspended gently in cold 90% (v / v) foetal bovine serum (FBS) and 10% (v / v) DMSO to a final concentration of 1.5 x 10^7 cell / ml. 1-1.5 ml of cells were transferred to ampoules which were kept 1 hour at -20°C, overnight at -70°C and finally placed in liquid nitrogen.

D.3.5. COS-7 transfections with plasmid DNA.

COS-7 cells were plated to 30% confluence in 9 cm dishes and transfected with 10 µg of plasmid DNA and 10 µg carrier DNA (Bluescript) by the calcium phosphate co-precipitation technique (Gorman, *et al.* 1984**)** as follows: 30 µl of 2 M $CaCl_2$ were mixed to 210 µl of the 1x TE DNA solution (DNA plasmid + DNA carrier) and vortexed. Finally 240 µl of 2x HBS:

NaCl	280 mM
HEPES	50 mM
Na_2HPO_4	1.5 mM

(pH adjusted to 7.05 with NaOH)

were added to the DNA mix and the sample was left for 30 min to allow the formation of the precipitate. 1 hour before adding the precipitate to the COS-7 cells, the DMEM medium was changed. The cells were exposed to the precipitate 15-20 hours and then the medium was replaced again.

D.3.6. COS-7 expression analysis.

36-48 hours post transfection, the cells were washed once with cold PBS and then harvested from the dishes using a cell scraper. After centrifugation for 10 min at 2,000 rpm the pellet was resuspended in a small volume of lysis buffer:

Tris HCl (pH 8.8)	50 mM
NaCl	100 mM
$MgCl_2$	5 mM
NP 40	0.5 % (v / v)
EDTA	1 mM

with protease inhibitors (2 mM PMSF, 10 µg / ml leupeptin, 10 µg / ml pepstatin, 1 µg / ml aprotinin, 50 µg / ml antipain).
The lysate was analysed by SDS-PAGE and Western blotting.

D.3.7. Immunofluorescence.

Transfected and untransfected COS-7 cells were grown in Leighton tubes (Costar) and prepared for immunofluorescence detection. 36-48 hours after the transfection the cells were fixed in:

PBS	1x
paraformaldehyde	2% (w / v)

for 4 min at room temperature, permeabilized with:

PBS	1x
Triton X-100	0.1% (v / v)

for 15 min and washed extensively with PBS. The cells were then incubated for 1 hour with the primary antibody at the appropriate dilutions, followed by washing 3 times for 10 min with PBS 0.1% (v / v) Triton X-100. The incubation with the secondary Cy3-conjugated polyclonal or monoclonal antibodies (Jackson Laboratories) was performed for 1 hour. After extensive washing with PBS and 0.1%

(v / v) Triton X-100, the nuclei were counterstained with DAPI (Sigma). The samples were observed with the fluorescence microscope Axiophot-2 (Zeiss). Two alternative methods of fixation (methanol and methanol / acetone 1:1) were tested to confirm the results obtained with the paraformaldehyde method.

D.3.8. Confocal laser scanning microscopy (CLSM) of neuroblastoma cells.

For the subcellular localisation of the ataxin-3 protein in neuroblastoma cells by CLSM, whole cells, *in situ* preparations or isolated nuclei were fixed in:

PBS	1x
paraformaldehyde	4% (w / v)

permeabilized with:

PBS	1x
Triton X-100	0.2% (v / v)

and incubated with the primary antibodies at appropriate dilutions. The secondary antibody used was a FITC-conjugated anti-rabbit antibody (Dako). The observations were carried out using a Sarastro Phoibos 1000 confocal microscope (Molecular Dynamics, Sunnyvale, CA). In brief, for image acquisition, the samples were excited with the 488 nm line of the argon-ion laser attenuated at 10% with the neutral density filter. The emission signal was observed through a dichroic mirror (500 nm) followed by a long pass filter (530 nm). The spatial projections were reconstructed by using a ImageSpace software (Molecular Dynamics, Sunnyvale, CA) running on a workstation Indigo (Silicon Graphics, Mountain View, CA).

D.3.9. Biochemical subcellular fractionation.

Highly pure nuclei were obtained by hypotonic shock in combination with non ionic detergent (Lee, *et al.* 1988). COS-7 and neuroblastoma cells were washed in cold PBS and centrifuged at 2,000 rpm for 5 min at 4°C. The cell pellet was resuspended in a same volume of cold buffer A:

Hepes (pH 7.9)	10 mM
$MgCl_2$	1.5 mM
KCl	10 mM
DTT	0.5 mM

and protease inhibitors were added (2 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, 50 μg/ml antipain). After 5-10 min of swelling, the cells were sheared by 5 passages through a 25-gauge needle. Nuclei were recovered by centrifugation at 3,000 rpm for 1-2 min, resuspended again with buffer A and observed with a phase contrast optical microscope. The cytoplasmic fraction was obtained from the supernatant of the nuclear pellet after recentrifugation at 12,000 rpm for 20 sec to avoid the risk of contamination with nuclear debris.

D.3.10. *In situ* matrix preparation.

The sample preparation was carried out as reported by Fey, *et al.* (1984) and Maraldi, *et al.* (1993). Adherent cells were permeabilized for 10 min at room temperature with TSM buffer

Tris-HCl (pH 7.4)	10 mM
Mg Cl_2	5 mM
NaCl	150 mM

containing 1% (v/v) NP-40 and 2 mM Na-tetrathionate. After washing in TSM buffer, the samples were incubated with 30 U/ml DNase I in TSM for 1 hour at room temperature. After washing in TSM, the cells were extracted twice with 2M NaCl in TSM for 5 min each. Finally the samples were washed in PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 20 min at 4°C. All buffers contained the protease

inhibitors (2 mM PMSF, 10 µg / ml leupeptin, 10 µg / ml pepstatin, 1 µg / ml aprotinin, 50 µg / ml antipain).

D.3.11. Database searches.

Amino acids motif searches was carried out with the PSORT program (Nakai, 1991; Nakai, *at al.* 1992) available on the Internet at the www page: http: / / psort.nibb.ac.jp:8800 / .