3. METHODS

3.1 General methods

3.1.1 Isolation of chromosomal DNA from *T. thermophilus*

The method described here was adapted from the one described by Marmur in 1961 for *E. coli* cells.
100 mg of *T. thermophilus* cells were suspended in 2 ml of 150 mM NaCl, 100 mM EDTA, pH 8.0. The cell suspension was incubated for 30 min at 37°C with lysozyme (final concentration 1 mg/ml). After adding SDS (final concentration 1% w/v) the mix was incubated for 10 min at 60°C. The sample was cooled down in ice and extracted twice with one volume of phenol and once with one volume of chloroform plus 200 mM NaCl. The DNA and the RNA were precipitated by adding two volumes of ethanol 100% and kept for 1 hour at -20°C. The precipitate was collected by centrifugation at 10000 rpm (Sorvall SS-34) for 30 min and washed once with ethanol 70%. The washed pellet was air-dried at room temperature. The air-dried pellet was resuspended in 2 ml of 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 (TE-buffer). To eliminate the RNA the sample was incubated for 1 hr at 37°C with RNase A (DNase free, final concentration 1 mg/ml). The sample was then incubated 1 hr at 37°C with 1 mg/ml of proteinase K, to degrade the RNase A, extracted again with phenol and chloroform and precipitated with ethanol. The DNA was resuspended in TE-buffer and the concentration measured spectrophotometrically.

3.1.2 Preparation of electrocompetent cells (Dower et al., 1988)

A single colony of the desired bacterial strain was grown over-night, at 37°C, in 5 ml of low salt LB-medium (salt concentration of 5 g/l instead of 10 g/l, see section 2.4). 0.5 ml of the over-night culture was used to inoculate 500 ml of low salt LB-medium and the cells were grown until the optical density (OD) at 560 nm was 0.7. The culture was placed in ice for 15 min and the cells were harvested by centrifugation at 5000 rpm for 15 min in a GSA-rotor (Sorvall). The pellet was washed twice using each time 500 ml of a sterilised solution of 10% v/v glycerol in water. The cells were resuspended in 10 ml of 10% v/v glycerol solution, centrifuged once more and resuspended in 1.5 ml of 10% v/v glycerol solution. The resuspended cells were portioned in 40 µl aliquots and immediately shock-frozen in liquid nitrogen and stored at -80°C.
When growing BL21(DE3)pLysS cells chloramphenicol (final concentration of 24 mg/l) was added to the medium.

3.1.3 Transformation of the electrocompetent cells

The electrocompetent cells were thawed in ice and placed in an electrocuvette with 0.1 to 50 ng of the transforming plasmid in water. The pulse (25 µF, 1.8 kV, 200 Ω) was given using a Gene Pulser (Bio-Rad). The transformed cells were incubate 1 hr at 37°C in 1 ml LB-medium and then an aliquot of 100-150 µl was plated on a LB-agar plate supplied with the appropriate antibiotics for selection.

3.2 DNA methods

3.2.1 Determination of the concentration of oligonucleotides, DNA and RNA

The concentration of oligonucleotides, DNA and RNA was determined by absorption measurement in a Beckman DU 530 spectrophotometer. The measurements were performed at 260 nm and 280 nm. The reading at 260 nm allows the calculation of the concentration of nucleic acid in the sample, whereas the ratio A_{260}/A_{280} is an index of the purity of the DNA and RNA preparation.

The conversion factors used for the quantification of DNA and RNA were:

Double stranded DNA	1 unit (A_{260}) = 50 µg
Single stranded DNA or RNA (more than 100 bases)	1 unit (A_{260}) = 40 µg
Single stranded DNA (less than 25 bases)	1 unit (A_{260}) = 20 µg
Single stranded DNA (30-80 bases)	1 unit (A_{260}) = 30 µg

A_{260}/A_{280} = 1.8 for pure DNA*
A_{260}/A_{280} = 2.0 for pure RNA*

*These coefficients are valid for high molecular weight species. In the case of oligonucleotides (less than 100 bases) the base composition can have a significant effect in the extinction coefficient. This means that the A_{260}/A_{280} ratio will change with the sequence.

3.2.2 Polymerase Chain Reaction (PCR)

The amplification of genomic or plasmid DNA containing the IF3 gene and the insertion of restriction sites in the sequence were carried out by PCR (Mullis et al., 1986). The reactions was done in a GeneAmp PCR System 2400 (Perkin Elmer).

PCR from chromosomal DNA. The PCR reaction was performed starting from 250-750 ng of chromosomal DNA, isolated as described in section 3.1.1, 20 pmol of each oligonucleotide, 350 µM of dNTPs mix and 2.6 unit of *Tag* or *Pfu* DNA polymerase. The

reaction was started with a DNA denaturation step (2 min at 94°C), and then repeating a certain number of times the following cycles:

	Reaction Time	Temperature	Number of Cycles
Denaturation	10 sec	94°C	
Hybridisation	30 sec	variable temp. *	10 x
Polymerisation	45 sec	68°C	
Denaturation	10 sec	94°C	
Hybridisation	30 sec	variable temp. *	20 x
Polymerisation	45 sec	68°C	

*The hybridisation temperature depends from the primers and the DNA sequence to be amplified. When restriction sites were added to the sequence, different hybridisation temperatures were used for each set of cycles.

The reaction was ended with a polymerisation step (10 min at 68°C).

PCR from plasmid DNA. The reaction was carried out in the same way with the difference that only 10-50 ng of plasmid DNA were necessary as a template.

3.2.3 Isolation of plasmid from *E. coli* cells

Plasmid was isolated from cells using the Jet Star Plasmid Kit (Genomed) based on anion exchange chromatography. XL1-Blue cells from an over-night culture were pelleted by centrifugation at 6000 rpm, for 10 min and resuspended in buffer E1. The cells were lysated by incubation for 5 min at room temperature in solution E2 (alkaline lysis). The lysis reaction was neutralised with the buffer E3. After addition of the E3 buffer a white precipitate (denature proteins) appeared and it was removed by centrifugation at 15000 g, for 10 min, at 20°C. The clear supernatant was applied to Jet Star columns equilibrated with buffer E4 and let run by gravity flow. The column was washed twice with buffer E5. The plasmid was eluted with buffer E6 and precipitated using 0.7 volumes of isopropanol. The sample was kept for 30 min at -20°C and then centrifuged for 30 min at 10000 g. The pellet was washed with EtOH 70% v/v and centrifuged again. The pellet was air dried for 10 min and the plasmid DNA dissolved in a suitable volume of buffer E7 or water.

Buffer E1 (cell resuspension): 50 mM Tris-HCl pH 8.0
10 mM EDTA
100 µg/ml RNAse A

Solution E2 (cell lysis): 200 mM NaOH
1.0 % SDS (w/v)

Buffer E3 (neutralisation):	3.2 M CH_3COOK-CH_3COOH pH 5.5
Buffer E4 (column equilibration):	600 mM NaCl 100 mM CH_3COONa-CH_3COOH pH 5.0 0.15 % TritonX-100
Buffer E5 (column washing):	800 mM NaCl 100 mM CH_3COONa-CH_3COOH pH 5.0
Buffer E6 (DNA elution):	1.25 M NaCl 100 mM Tris-HCl pH 8.5
Buffer E7 (storing buffer):	10 mM Tris-HCl pH 8.5

3.2.4 DNA extraction from agarose gel

The DNA extraction from agarose gel was performed using the QIAquick Gel Extraction Kit (Quiagen). The principle is based on the high binding affinity of DNA to a silica-gel membrane in the presence of high salt, while contaminants pass through the column (Vogelstein and Gillspie, 1979). The DNA band cut from the gel was incubated with three volumes of high-salt concentration buffer, for 10 min at 50°C. One volume of isopropanol was added to the solution, mixed, applied to the column and centrifuged for 1 min at 10000 g. The column was washed with low salt concentration buffer containing 80% ethanol and the DNA was eluted with 10 mM Tris-HCl pH 8.5 buffer.

3.2.5 DNA digestion with restriction enzymes

DNA samples were incubated with the desired restriction enzymes at a ratio of 1-5 units enzyme per µg of DNA for at least one hour. The temperature was the optimum one for each restriction enzyme. For PCR products the incubation was performed over-night. Double digestion was performed only if the two enzymes were active in the same buffer, otherwise a sequential digestion was performed. The DNA purification after the digestion was normally performed using a column to purify PCR products (PCR Purification Kit, Quiagen). Five volumes of a high salt concentration buffer were added to the digested DNA mix. The solution was then loaded into a silica-membrane column and centrifuged for 1 min at 10000 g. DNA adsorbs to the column in the presence of high salt concentration, while contaminants like enzymes and short oligonucleotides, pass through the column. The column was then washed with low salt concentration buffer containing 80% ethanol and the DNA was eluted with 10 mM Tris-HCl pH 8.5 buffer.

3.2.6 Ligation of DNA fragments into a cloning vector

To insert the DNA sequence corresponding to the desired gene into the appropriate expression vector, 30-50 fmol of the vector (cut with the appropriated restriction enzymes) were incubated with two-six fold molar excess of the DNA fragment in ligation buffer and with one unit of T4-ligase over-night, at 16°C.

Ligation buffer:	50 mM Tris-HCl, pH 7.6
	10 mM $MgCl_2$
	1 mM DTE
	50 µg/µl BSA
	1 mM ATP

3.3 Ribosome methods

3.3.1 Large scale preparation of *Thermus thermophilus* HB8 cells

Large-scale cultures of *T. thermophilus* for ribosome preparation were performed in a 100 litres Bioengineering Fermentor. Hundred litres of sterile *Thermus* medium, were inoculated with 2.5 l of an over-night culture of the *T. thermophilus* HB8, and allowed to grow at 75°C. The bacterial growth was followed by determination of the optical density at 650 nm. When the culture reached the absorbance of 3.0 A_{650}/ml the cells were harvested using a continuous flow centrifuge (Padberg, model 41) operating at 2000 rpm. The cell pellets were shock frozen in liquid nitrogen and stored at -80°C. The yield of the process was of 0.9-1.1 g of wet cells per litre of medium.

Thermus-Medium pH 7.5	12 g/l casamino-acids, 4 g/l yeast extract, 2 g/l NaCl, 1 ml/l NaOH 5 N, 630 µl/l BES, 1 g/l glucose

Basal Element Solution (BES) for 500 ml final volume:

$MgCl_2 \cdot 6H_2O$	12.5 g
$CaCl_2 \cdot 2H_2O$	3.31 g
$MnSO_4 \cdot H_20$	650 mg
$ZnCl_2$	118 mg
H_3BO_3	250 mg
$FeCl_3 \cdot 6H_2O$	2.5 g
$CuSO_4 \cdot 5H_2O$	11.73 mg
$Na_2MoO_4 \cdot 2H_2O$	12.5 mg
$CoNO_3 \cdot 6H_2O$	25 mg
$NiNO_3 \cdot 6H_2O$	10 mg
Pyridoxal hydrochloride	40 mg
Folic acid	5 mg
H_2SO_4 conc.	0.25 ml (fin. conc. 1 mM)

Thiamin hydrochloride	50 mg
Rivoflavin	20 mg
Nicotinamide	40 mg
4-Aminobenzoic acid	40 mg
D-(+)-Biotin	5 mg
Cyanocobalamin	0.2 mg
Calcium-D-pantothenate	40 mg
DL-α-lipoic acid	10 mg
Inositol (Myo-Inositol)	100 mg
Coline chloride	25 mg
Orotic acid	25 mg
Spermidine hydrochloride	50 mg

3.3.2 Isolation of 70S ribosome from *T. thermophilus*

200 g of cells were suspended in 300 ml of buffer A and incubated with lysozyme (final concentration 0.5 µg/ml) for 30 min, in ice. The cells were disrupted passing twice the cell suspension in a French Press operating at 600 bar. The cell lysate was incubated with DNase (final concentration 0.15 µg/ml) for 30 min, in ice. The mix was centrifuged in GSA rotor (Sorvall) at 13000 rpm, for 20 min. The supernatant was mixed with 3.5 volumes of sucrose cushion solution and centrifuged in a Ti45 rotor (Beckmann) at 19000 rpm, 3 hours, to remove the cell fragments still present in the solution. 30 ml of the supernatant were then overlaid in 60 Ti tubes with 5 ml of sucrose cushion solution and centrifuged at 42000 rpm for 22 hours at 4°C. The pellet, containing the ribosomes, was re-suspended in a total volume of 30 ml of buffer B and the OD were measured. The typical yield was around 70-80 OD of crude 70S ribosome per gram of cells.

Buffer A	10 mM	Hepes-NaOH pH 7.8
	30 mM	$MgCl_2$
	150 mM	NH_4Cl_2
	6 mM	β-mercapthoethanol
Buffer B	10 mM	Hepes-NaOH pH 7.8
	30 mM	$MgCl_2$
	75 mM	NH_4Cl_2
	6 mM	β-mercapthoethanol
Sucrose cushion	(37.5%) 1.1 M	Sucrose
	30 mM	$MgCl_2$
	10 mM	Mg Acetate
	75 mM	NH_4Cl_2
	50 mM	Tris-HCl pH 7.5
	6 mM	β-mercapthoethanol

3.3.3. Preparative isolation of 30S and 50S subunits

The 30S and 50S ribosomal subunits were isolated by zonal centrifugation (Eikenberry et al., 1970) using a linear sucrose gradient from 0 to 40% in 10 mM Hepes pH 7.8, 100 mM NH_4Cl, 1 mM $MgCl_2$, 6 mM β-mercapthoethanol. For every zonal run 3000-6000 OD of 70S ribosome were used. The centrifugation was performed using a Beckmann zonal rotor Ti15 at 22000 rpm for 17 hours at 4°C. The gradient was pumped out using a solution containing 50% of table sugar in water. After the zonal centrifugation two pools were made with the fractions containing the 30S and 50S subunits respectively. The 30S and 50S subunits were pelleted in 45 Ti rotors (Beckmann) at 35000 rpm, for 22 hours at 4°C. The pellets were re-suspended in 3 ml of 10 mM Hepes pH 7.8, 60 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercapthoethanol and centrifuged in a SS34 rotor (Sorvall) at 7000 rpm for 15 min at 4°C, to eliminate the aggregated material. The concentration of the 30S and 50S subunit was determined by its absorbance at 260 nm and the suspension was divided in small aliquots, frozen in liquid nitrogen and stored at -80°C. The typical yield starting from 5000 OD of crude 70S was 1000 OD and 1200 OD of pure 30S and 50S subunits, respectively.

3.3.4 Analytical sucrose gradient centrifugation

Sucrose gradient centrifugation is a technique that allows the separation of complexes based on the sedimentation coefficient (S). This technique can be used to separate the 70S, 50S and 30S ribosomal subunits.
A sucrose gradient 5-20% or 10-30% (w/v) was prepared in a Ultra-Clear tubes (Beckmann). The reaction mix was overlaid on the gradient and centrifugation was performed in SW60, SW40 or SW27 rotors (Beckmann). When the SW60 was used it was possible to load up to 1 OD of ribosome, for each tube, and the centrifugation was performed at 37000 rpm for 3 hours. When the SW40 was used it was possible to load up to 10 OD and the centrifugation was performed at 22000 rpm for 16 hours. Finally when the SW27 was used it was possible to load up to 80 OD and the centrifugation was performed as described for the SW40. After centrifugation the gradient was fractionated while monitoring the OD at 260 nm.

3.3.5 Sucrose cushion centrifugation

This technique was used to separate ribosomal complexes from the excess of small molecules or ligands that were used to prepare the complexes.
A polycarbonate centrifuge tube (Beckmann) was a third filled with a 37% w/v buffered sucrose solution and the reaction mix was loaded on top of this solution. The mix was centrifuged in a TL100 Ultracentrifuge (Beckmann) at 90000 rpm, for 3 hours or in an L7-55 Ultracentrifuge (Beckmann) at 42000 rpm, for 16 hours. After centrifugation the pellet, containing the ribosome complexes, was re-suspended in the appropriate buffer and used for further experiments.

3.4 c-DNA methods

3.4.1 Phosphorilation of DNA oligonucleotides

The phosphorilation of the 5'-OH group of synthetic DNA oligonucleotides was performed incubating 3-6 nmol of oligonucleotide with ATP (final concentration 3 µM), 300-500 U of T4 kinase in phosphorilation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA and 0.1 mM ADP), for 1 hour at 37°C. The phosporilated oligonucleotides were purified using the BIO-Spin columns, BioRad (see section 3.4.3).

3.4.2 Radioactive labelling of the 5'-OH group of the oligonucleotide

The labelling of the 5'-OH group of oligonucleotides with [γ-^{32}P] ATP was done incubating 50 pmol of oligonucleotide with 30 µCi of [γ-^{32}P]ATP, 10 U of T4 kinase in phosphorilation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA and 0.1 mM ADP), for 30 min, at 37°C. The phosphorilated oligonucleotides were purified using either one of the two techniques described in the following section.

3.4.3 Purification of oligonucleotides

The purification of the oligonucleotides was done either through extraction from acrylamide-urea gels or through Bio-Spin Chromatography Columns (BIO-RAD).

Extraction from acrylamide-urea gels. Samples containing radioactive labelled oligonucleotides were loaded in a urea-acrylamide gel 22% in TBE buffer. The gel was run at 600 volts for 2 hours. An autoradiography of the gel was obtained immediately after finishing the electrophoresis by exposing the gel to X-ray films. A superimposition of the film to the gel allowed the determination of the position of the oligonucleotide on the gel. The band corresponding to the oligonucleotide was cut using a scalpel and the fragment of gel was broken and incubated with 200 µl of extraction buffer (10 mM Tris-HCl pH 8.5, 10 mM NaCl) and 200 µl of phenol over-night, at 4°C, with constant shaking. The sample was spin down and the phenol phase was extracted three times with 150 µl of extraction buffer. The aqueous phases were pooled and extracted once with 200 µl of chloroform. The DNA was precipitated adding to the aqueous phase 1/10 volume of 1 M $MgCl_2$ and 2.5 volumes 100% EtOH and let for 1 hour at -80°C. The sample was centrifuged and the pellet suspended in 40 µl of water.

Acrylamide-urea gel	42% w/v urea
	22% w/v acrylamide

TBE buffer	108 g/l Tris-base
	55 g/l Boric acid
	200 mM EDTA pH 8.0

Bio-Spin Chromatography columns purification. Columns of Bio-Gel P6 gel were equilibrated in 1 ml of SSC buffer (150 mM NaCl, 17.5 mM Na Citrate pH 7.0, 0.02% Na Azide). The exclusion size limit using these columns is 5 bases. The packing buffer was removed from the columns by centrifugation and the sample was loaded and eluted with a further centrifugation step. Molecules smaller than the column exclusion limit were retained by the column, while the labelled oligonucleotides were readily eluted by the centrifugation step. The yield was at least 50%, even for very short oligonucleotides (i.e. 8 bases).

3.4.4 Binding of DNA oligonucleotides to the T30S subunit

A ten molar excess of [^{32}P]-labelled oligonucleotide was incubated with T30S subunits for 30 minutes at 40°C and then cooled to room temperature slowly. The reaction mixture was loaded onto a sucrose gradient 5-20% in 10 mM Hepes, 10 mM $MgCl_2$, 60 mM NH_4Cl and centrifuged at 19000 rpm for 15 hours in a Beckman SW40 rotor.
The absorbance at 260 nm and the [^{32}P] radioactivity of the collected fractions were monitored. The amount of oligo bound to the T30S was calculated from the amount of radioactivity per pmol of 30S (calculated from the absorbance at 260 nm) in each fraction that migrates together with the T30S peak on the gradient. The unbound oligo was found at the top of the gradient.

3.4.5 Labelling of TAMM with [^{35}S]-cysteine

In this study we used TAMM (tetrakis-acetoxy-mercury-methane, **Error! Reference source not found.**), a mercury cluster, to label the SH group of modified nucleotides or of cysteine residues (see section 4.1.1 and 4.2.3).
To avoid inter-molecular cross-linking, three of the four mercury atoms in the TAMM molecule should be blocked. Therefore, before labelling the oligonucleotides, TAMM was incubated with a three molar excess of [^{35}S]-cysteine in 1% v/v acetic acid for 15 min, at room temperature. The use of [^{35}S]-cysteine as blocking reagent allows in addition of the blocking, the possibility to follow the extent of binding of the TAMM modified oligonucleotides or proteins to the T30S subunits.

3.4.6 Labelling of the oligonucleotides with 3[^{35}S]-Cys·TAMM

The modified oligonucleotides (see section 4.1.1) were phosphorylated using T4 polynucleotide kinase prior to the labelling with TAMM (see section 3.4.1).
A 1.3 molar excess of the 3[^{35}S]-Cys·TAMM complex was used for each 6-Thio-dG contained in the oligonucleotide sequence. The labelling was performed incubating the

3[^{35}S]-Cys·TAMM with the oligonecleotides for 15 min, at room temperature. The unbound 3[^{35}S]-Cys·TAMM complex was separated from the 3[^{35}S]-Cys·TAMM·oligonucleotide by filtration through a NAP-5 Sephadex G-25 column (Pharmacia).
The binding of the 3[^{35}S]-Cys·TAMM to the 6-Thio-dG from the oligonucleotides was follow by the disappearance of an absorption peak at 340 nm of the oligonucleotides containing 6-thio-dG, as well as by the co-migration of [^{35}S]-cysteine with the oligonucleotide peak after NAP-5 column.

3.4.7 Binding of the [^{35}S]-Cys·TAMM·oligonucleotide to the T30S

A ten molar excess of the 3[^{35}S]-Cys·TAMM labelled oligonucleotide was incubated with T30S subunits for 30 minutes at 40°C and then let to reach room temperature slowly. The reaction mixture was loaded onto a sucrose gradient 5-20% w/v in 10 mM Hepes pH 7.8, 60 mM NH_4Cl, 10 mM $MgCl_2$, and centrifuged in a Beckman SW40 rotor at 22000 rpm, for 10 hours at 10°C.
The absorbance at 260 nm and the [^{35}S] radioactivity of the collected fractions were monitored. The amount of oligonucleotide bound to the T30S was calculated from the amount of radioactivity that migrated together with the 30S peak on the gradient (see section 3.4.4). The unbound oligonucleotide was found at the top of the gradient.

3.4.8 Formation of the 30S·TAMM·oligonucleotide complex

The 30S·TAMM·oligonucleotide complex was formed by incubating ten-fold molar excess of TAMM·oligonucleotide with T30S for 30 minutes at 40°C in 10 mM Hepes pH 7.8, 10 mM $MgCl_2$, 60 mM NH_4Cl. The sample was loaded on top of a sucrose cushion (37.5% w/v) and centrifuged in a Beckman TL-100 at 90000 rpm, for 3 hours, at 10°C to eliminate the excess of TAMM·oligonucleotide. The pellet containing the 30S·TAMM·oligonucleotide complex was suspended in 10 mM Hepes pH 7.8, 10 mM $MgCl_2$, 60 mM NH_4Cl, dialysed overnight against the same buffer and the concentration measured at 260 nm. Aliquots were stored at room temperature, since low temperature caused precipitation of the TAMM·oligonucleotide complex.

3.5 Protein methods

3.5.1 Overproduction of proteins

The recombinant proteins used in this study were overproduced in *E. coli* BL21(DE3)pLysS cells, which were transformed with the pET11a vector containing the desired DNA fragment. The pET system is based on the T7 promoter-driven system originally developed by Studier and colleagues (Studier et al., 1990).
For protein production, a recombinant plasmid is transferred to host *E. coli* strains containing a chromosomal copy of the gene for T7 RNA polymerase. These hosts are

lysogens of bacteriophage DE3, a lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter and the gene for T7 RNA polymerase. This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). Addition of IPTG to a growing culture of the lysogen induces T7 polymerase, which in turn transcribes the target DNA in the plasmid. The *E. coli* BL21(DE3)pLysS cells contain the pLysS plasmid that is a pET-compatible plasmid that produces T7 lysozyme, thereby reducing basal expression of target genes.
For the overproduction of a target protein, 50 ml of M9ZB-medium, containing 40 mg/l of ampicillin and 24 mg/l of chloramphenicol, were inoculated with a single colony and grown overnight at 37°C under agitation. One litre of M9ZB-medium was inoculated with 10 ml of this culture and grown at 37°C under agitation until the culture reached an absorbance value of 0.7 at 560 nm. The overproduction was induced with IPTG (final concentration 1 mM). After induction cellos were incubated for 3 hours at 37°C under agitation. Aliquots of 0.5 ml were collected before and after induction, centrifuged and the pellet was dissolved in 40 µl of urea 6 M and loaded onto a SDS-PAGE (see section 3.5.5) to check for target protein over-production.

3.5.2 Extraction of the overproduced protein from the cells

Three hours after induction the cells were harvested by centrifugation (30 min at 6000 rpm in a Sorvall GSA rotor). The pellet was suspended in 40 ml of lysis buffer per litre of culture and disrupted using a French Press at 600 bar. The lysate was centrifuged in an SS-34 rotor (Sorvall) at 15000 rpm, for 30 min to remove the cellular debris. The supernatant was then centrifuged in a Ti45 rotor (Beckmann) at 35000 rpm, for 16 hours to pellet the ribosomes and to isolate the supernatant (S100), which normally contains the protein of interest. The high salt concentration of the lysis buffer was necessary because at high salt concentration the target proteins remained in solution, while at low salt most the target proteins tend to aggregate.

Lysis buffer: 10 mM Tris-HCl pH 7,5
10 mM $MgCl_2$
100 mM NH_4Cl
800 mM NaCl
0.1 mM PMSF
0.1 mM Benzamidine
7 mM β-mercaptoethanol

3.5.3 Change of the protein buffer

When the buffer could not be changed by dialysis the Centriprep (Millipore) system was used. The protein solution was loaded on a centriprep, having the desired molecular

weight cut-off, and centrifuged at 3000 g until the solution was concentrated. An equal amount of buffer was then added and again centrifuged. This led to a buffer exchange of around 80%. By repeating twice this step a buffer exchange of 99% was reached.

3.5.4 Purification of protein

Purification of recombinant proteins is normally performed in as few purification steps as possible. The number and the nature of the steps depend on the protein and on the degree of purity that should be achieved.

3.5.4.1 Heat-denaturation of *E. coli* proteins

One advantage to purify thermophilic proteins is their heat stability. The cell lysate can be incubated at 60°C causing denaturation and precipitation of most of the *E. coli* proteins but not of *T. thermophilus* ones.
The S100 from the overproducing cells was dialysed over-night against buffer A and incubated for 30 minutes at 60°C and for other 30 minutes on ice. The denatured proteins were removed by centrifugation in an SS-34 rotor (Sorvall) at 10000 rpm, for 15 min, at 4°C, while the supernatant containing the over-produced protein, was kept.

Buffer A: 20 mM Tris-HCl pH 7.5
10 mM $MgCl_2$
50 mM NaCl
0.1 mM PMSF
0.1 mM Benzamidine
4 mM β-mercaptoethanol

3.5.4.2 Ion-exchange chromatography

Protein separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to an immobilised ion exchange group of opposite charge. Varying conditions such as ionic strength and pH can control these interactions.

Anion exchange. To eliminate DNA and RNA from the protein solution a DEAE-Sepharose CL-6B (Pharmacia) column 10 cm long and with diameter of 1.5 cm was used. The supernatant obtained after the separation of the heat-denatured proteins (see section 3.5.4.1) was loaded onto the column equilibrated with buffer A. The protein solution was eluted at a flow of 2 ml/min and the elution profile was monitored measuring the absorbance at 280 nm. The protein composition of each fraction was checked by SDS-PAGE (see section 3.5.5). At a salt concentration of 50 mM and pH 7.5, only nucleic acids and proteins with isoelectric point lower than 7.5 bind to the column and are eluted increasing the ionic strength of the buffer (i.e. using a linear gradient from 50 mM to 1.5 M NaCl). Proteins with isoelectric points higher than 7.5 do not bind to the column and are found in the flow through.

FPLC Cation/Anion exchange. The fractions eluted from the DEAE column, containing the protein of interest, were pooled, and concentrated with Amicon (see

section 3.5.6). The concentrated protein solution was loaded in a 6 ml Resource S or Resource Q column (depending on the isoelectric point of the protein to be isolated) in a FPLC (Fast Protein Liquid Chromatography, Pharmacia) system. The proteins were eluted using 40 ml of a linear gradient from 50 mM (buffer A) to 500 mM (buffer B) NaCl at a flow of 2 ml/min.

Buffer A:	20 mM Tris-HCl pH 7.5	Buffer B:	20 mM Tris-HCl pH 7.5
	10 mM $MgCl_2$		10 mM $MgCl_2$
	50 mM NaCl		**500 mM NaCl**
	0.1 mM PMSF		0.1 mM PMSF
	0.1 mM Benzamidine		0.1 mM Benzamidine
	4 mM β-mercaptoethanol		4 mM β-mercaptoethanol

3.5.4.3 Hydrophobic interaction chromatography

This technique is based upon interactions between solvent-accessible non-polar groups (hydrophobic patches) on the surface of biomolecules and the hydrophobic ligands (alkyl or aryl groups) covalently attached to a gel matrix.
Fractions containing the protein of interest were buffered in 1.5 M $(NH_4)_2SO_4$, 20 mM Tris-HCl buffer pH 7.5 and loaded in a Phenyl Superose HR5/5 column (FPLC, Pharmacia). The sample elution was performed using 60 ml of a linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ at a flow of 0.5 ml/min.

3.5.4.4 Gel filtration chromatography

In gel filtration chromatography the molecules are separated according to differences in their sizes. The protein sample, which has to be separated, is loaded at the top of the column bed. The sample zone moves down the bed as eluent is added to the top. The small molecules, which can diffuse into the pores of the gel beads, are delayed in their passage down the column in contrast to the large molecules, which cannot diffuse into the gel. The large molecules thus leave the column first, followed by the smaller in order of their sizes.
The protein of interest was loaded into the column and eluted isocratically with buffer C.

Buffer C:	20 mM Tris-HCl pH 7.5
	10 mM $MgCl_2$
	100 mM NaCl
	0.1 mM PMSF
	0.1 mM Benzamidine
	4 mM β-mercaptoethanol

3.5.5 SDS-Polyacrylamide-gel-electrophoresis

The qualitative analysis of fractions containing protein was performed using discontinuous SDS-Polyacrylamide-gel-electrophoresis (SDS-PAGE). To each samples 0.20 volumes of 5x sample loading buffer were added and after 3 minutes incubation at 95°C, were spinned down and loaded onto the gel. The electrophoresis was carried out at 250 volts for 3 hours. The gel was then stained for 1 hour in comassie solution and destained in EA-solution.

Stacking gel	6% Acrylamide
	0.16% Bis-acrylamide
	125 mM Tris-HCl pH 6.8
	0.1% SDS
	0.8% TEMED
	0.05% APS
Running gel	15% Acrylamide
	0.4% Bis-acrylamide
	375 mM Tris-HCl pH 8.8
	0.1% SDS
	0.025% TEMED
	0.05% APS
Running buffer	25 mM Tris-HCl pH 8.8
	200 mM glycin
	0.1% SDS
Sample loading buffer	125 mM Tris-HCl pH 6.8
	4% SDS
	20% Glycerol
	700 mM β-mercapthoethanol
	0.02% Bromophenol Blue
Stain solution	50% ethanol
	8% acetic acid
	0.25% Comassie Brilliant Blue
Destain solution	5% ethanol
	10% acetic acid

3.5.6 Concentration of protein solution

The protein solutions were concentrated using either Centripreps (see section 3.5.3) or by Amicon (Millipore) device. In this device a membrane, with molecular weight cut-off smaller than the protein in question, is placed at the bottom of a cell which is filled with

the protein solution. The concentration cell is closed and pressurised (4-5 bar) using nitrogen, while the contents of the cell is continuously stirred. In this way the flow through contains only low molecular weight components while the protein is concentrated inside the chamber.

3.5.7 Determination of the concentration of proteins

The determination of the protein concentration was performed according to the method described by Bradford (1976). This method is based on the shift of the maximum of absorbance of a protein bound to Comassie Brillant Blue G-250 from 465 to 595 nm. Few microlitres of the protein solution were incubated 10 min at room temperature with a comassie solution and the absorbance at 595 nm was measured. A calibration curve using increasing concentration of BSA was used as standard.

Comassie solution	0.01% (w/v) Comassie Brilliant Blue G-250
	4.7% (w/v) ethanol
	8.5% (w/v) phosphoric acid

Alternatively the concentration was determined using the extinction coefficient (ε) of the protein. The extinction coefficient can be determined using the amino acids composition of the protein and data coming from spectra of proteins and compounds measured in native and denaturing conditions (Gill and von Hippel, 1989).
The maximum of absorbance is normally measured at 280 nm and is coming from the tryptophan, tyrosine and cysteine.

$$\varepsilon = a\varepsilon_{Tyr} + b\varepsilon_{Trp} + \varepsilon_{Cys}$$

where ε_{Tyr}, ε_{Trp} and ε_{Cys} are the extinction coefficients for tyrosine, tryptophan and cysteine respectively, and a, b, c are the number of tyrosyne, triptophan and cysteine in the protein sequence.

The extinction coefficients used were the ones determined for the following standard compounds (Gill and von Hippel, 1989):

N-acetyl-tryptophanamide	$A_{280} = 5690$
Gly-L-Tyr-Gly	$A_{280} = 1280$
Cysteine	$A_{280} = 120$

From the absorbance at 280 nm, knowing the extinction coefficient, using the Lambert-Beer equation, the concentration can be determined:

$$A = \varepsilon \, l \, c$$

where: l = length of the cuvette in cm; c = molar concentration and ε = extinction coefficient.

3.5.8 Ribosome dissociation activity of IF3, IF3cys and IF3-C

One of the properties of IF3 is its ability to dissociate 70S ribosomes in 30S and 50S subunit. To check if the overproduced IF3 variants were active in 70S dissociation after purification, a 10-fold molar excess of IF3s was incubated with T70S for 30 min at 50°C in binding buffer (10 mM $MgCl_2$, 20 mM Hepes pH 7.5, 60 mM KCl, 10 mM NH_4Cl, 4 mM 2-mercaptoethanol). The mix was subsequently loaded on a sucrose gradient 10-30% and centrifuged for 16 h, at 19000 rpm and 4°C in a Beckman SW40 rotor. Fractions were collected, monitored at 260 nm and analysed by SDS-PAGE.

3.5.9 Preparation and isolation of the 30S·IF3 complex

3000 OD of crude 70S were incubated with 10-fold excess of IF3 for 30 min at 45°C in 10 mM Hepes pH 7.8, 60 mM NH_4Cl, 10 mM $MgCl_2$, 6mM β-mercaptoethanol. The 30S·IF3 complex was isolated by zonal centrifugation using a linear sucrose gradient from 0 to 40% in 10 mM Hepes pH 7.8, 60 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercapthoethanol. The fractions containing the 30S·IF3s complex were pelleted by ultra-centrifugation and the pellet suspended in 30S buffer (10 mM Hepes, 60 mM NH_4Cl, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol).
Alternatively the complex was formed by incubating one to ten-fold molar excess of IF3 with T30S for 30 minutes at 50°C in binding buffer. The sample was loaded on a sucrose cushion (37.5% w/v) and centrifuged in a Beckman TL-100 at 90000 rpm, for 3 hours, at 4°C to eliminate the excess of IF3. The pellet containing the 30S·IF3 complex was suspended in 30S buffer, dialysed over night against the same buffer and the concentration measured at 260 nm. Aliquots were frozen in liquid nitrogen and stores at -80°C. In both cases the presence of IF3 in the complex was checked by SDS-PAGE and western blot.

3.6 Immunologic methods

3.6.1 Production of anti-sera

Polyclonal antibodies against the different proteins used in these studies were raised in rabbits, injecting them with 300 µg of pure protein (SEQ-LAB, Goettingen).

3.6.2 Isolation of IgG from anti-sera

The isolation of pure IgG from the anti-sera was performed by affinity chromatography. For this purpose was used Protein A Sepharose CL-4B (Pharmacia), a column material to which immunoglobuline can bind. In this columns protein A from *Staphylococcus aureus* is immobilised onto a sepharose matrix. Protein A binds strongly to sites in the second and third constant regions of the Fc portion of the immunoglobuline heavy chain (Deisenhofer, 1981).
14 ml of anti-sera were loaded onto a 5 ml Protein A CL-4B column. The column was washed with 15 ml of TBS buffer, then with 10 ml of 2 M NaCl, 20 mM Tris-HCl pH 7.5 and again with 20 ml of TBS buffer, in order to remove all the non-specific bound proteins. The fraction containing the IgG was eluted using 0.2 M Glycine-HCl buffer pH 2.2 in a tube containing 200 µl of 1 M Tris-HCl pH 7.5 to a fraction of 1 ml final volume. In this way the pH was immediately risen after elution to prevent IgG degradation. The elution was followed at 280 nm. The eluate was treated with an equal volume of saturated $(NH_4)_2SO_4$ solution and let to precipitate completely for 30 min at 4°C. The precipitate was centrifuged for 20 min at 10000 rpm at 4°C (SS-34, Sorvall) and suspended in 3 ml of TBS buffer. The solution was then dialysed against TBS buffer and the concentration measured at 280 nm. A concentration of 1 mg/ml of IgG corresponds to an absorbance value of 1.4 at 280 nm. The average yield was of 8-12 mg of IgG per ml of anti-sera.

TBS Buffer	50 mM Tris-HCl pH 7.5
	150 mM NaCl

3.6.3 Western-Blot

Small amounts of proteins can be transferred onto a poly-vynylidene-di-fluoride (PVDF) membrane and detected using specific antibodies (Towbin et al., 1979). This method was used in this study to detect the presence of the C-terminal domain of IF3 in the 30S·IF3s complexes.
After electrophoretic separation of the proteins the gel was equilibrated in transfer buffer for 10 min at room temperature. The transfer took place from the polyacrylamide gel onto a PVDF-membrane (Millipore), in a tank filled with the transfer buffer for 16 hours at 4°C under constant current of 165 mA. The membrane was first washed with PBS-Tween and then incubated with 3% BSA for 1 hour, at room temperature. The primary antibody was then added in a dilution of 1:1000 to 1:10000, depending if sera or pure IgG was used, and incubated 1 hour, at room temperature. The membrane was then washed with PBS-Tween 3 times for 10 min and then incubated with anti-rabbit peroxidase-conjugate-secondary-antibody for another hour at room temperature. The membrane was then washed three times with TN buffer for 10 min each time at room temperature.
The detection can be done in two different ways:
using a colour reaction that takes place on the membrane with the detection buffer;
using a chemiluminescent substrate (Pierce) and auto-radiographing the blot.
Both methods are based on the reaction of the substrate used with the secondary antibody that is a peroxidase-conjugate. The second method is more sensitive, allowing the

detection of picogram quantities of antigen and was the one used in most of the western blots during this study.

Transfer Buffer	25 mM Tris-HCl pH 7.5
	0.5 mM DTE
	0.2% SDS
PBS-Tween pH 7.4	137 mM NaCl
	2.7 mM KCl
	4.3 mM Na_2HPO_4
	1.4 mM KH_2PO_4
TN Buffer	100 mM Tris-HCl pH 7.5
	150 mM NaCl
Detection Buffer	3 mM 4-Chloro-1-Naphtol
	17% Methanol
	100 mM Tris-HCl pH 7.5
	0.015% H_2O_2

3.7 Crystallographic methods

3.7.1 Crystallisation of the 30S subunit

There are many methods to crystallise biological macromolecules, all of which aim at bringing the solution of biological macromolecules to a supersaturated state. Among them, vapour diffusion technique is probably the most widely used and, since it was the method used in this study, it will be shortly described here.
The vapour diffusion method is very well suited for small volumes (down to 2 µl or less). A droplet containing the biological macromolecule to crystallise with buffer, crystallising agent and additives is equilibrated against a reservoir containing a solution of crystallising agent at a higher concentration than the droplet. Equilibration proceeds by diffusion of the volatile species (water or organic solvent) until vapour pressure in the droplet equals the one in the reservoir. If equilibration occurs by water exchange (from the drop to the reservoir), it leads to a droplet volume change. For species with a vapour pressure higher than the water, the exchange occurs from reservoir to drop. This method can be applied to three different systems: hanging drop, sitting drop and sandwich drop.

The crystallisation of the T30S subunit was performed using the hanging-drop vapour-diffusion method with 10 µl drops equilibrated against 1 ml of reservoir solution at 20°C as describe in Yonath et al., 1988. The typical 30S concentration was 21 nmol/ml (300 OD/ml). The 30S subunits were suspended in 30S buffer and incubated with spermidine

hydrochloride to a final concentration of 10 mM, then "heat activated" for 5 min at 65°C (Zamir et al., 1971). Methyl-pentan-diol (MPD) was used as precipitant agent. The final concentration of MPD in the drop was between 0 and 1.5% and in the reservoir was between 14 and 18%. The crystals of the T30S subunit obtained in this way belong to the space group $P4_12_12$ with unit cell axis a = b = 407 Å, c = 176 Å.

30S buffer	10 mM Hepes pH 7.8
	10 mM $MgCl_2$
	60 mM NH_4Cl
	4 mM β-mercaptoethanol

3.7.2 Soaking of the T30S crystals

The soaking experiments were performed incubating three crystals in 90 µl vessels containing the ligands to be diffused into the crystals for 12-24 hours at room temperature.
A special soaking technique was developed to diffuse into the 30S crystals oligonucleotides, IF3-C and antibiotics.
This technique consists in incubating with a 5-50 fold excess of ligand three crystals at 23°C and increasing the temperature to 40°C in 15 minutes. The temperature was kept at 40°C for 30 minutes and then decreased at 23°C in 25 minutes. In this way the ligand has time to diffuse into the crystals and in the meantime the increase in temperature allows a better binding of the ligand into the crystal. The crystals were then transferred to the stabilisation solution (MPD 30% v/v, in 30S buffer) and shock-frozen in liquid propane. All T30S crystals were also soaked, before or after the special soaking with the ligands, in 0.04 mM W18 for 12 hours. This step was performed because W18, which is a heteropolytungstate cluster ($[K_6(P_2W_{18})O_{62}]\cdot 14H_2O$), stabilised the 30S particles and improved the quality of the T30S crystals (Tocilj et al., 1999).

3.7.3 X-ray data collection

Single crystal X-ray and neutron crystallography are the only methods that offer direct structural information and have proven to be the most powerful technique for obtaining a reliable molecular model for large macromolecular complexes. Synchrotron radiation, which produces an intense, coherent and focused beam, generated in accelerators designed for high energy particle experiments, is being widely used in crystallographic studies of biological macromolecules. It turned out to be the only suitable X-ray source for crystallographic studies of the ribosomal crystals, because its high brightness and focusing are indispensable in order to record the measurable diffraction patterns from ribosomal crystals.
Data were collected with a bright synchrotron beam at cryo temperature (86-90 K) from flash frozen crystals as described by Tocilj et al., 1999. The data were recorded either on the 345 mm MAR or CCD detectors (QUANTUM4 or APS2) and processed by HKL2000 (Otwinowski and Minor, 1997). When collecting the 3-4 Å shell, decay was detected

within a period sufficient for 1-1.5° oscillations. To facilitate the collection of complete data sets, a beam with a smaller cross-section irradiated crystals. Translating the rod-like crystals (about 500x60x60 microns) once decay was observed allowed measurements from 2-6 separate regions from each crystal (Schlünzen et al., 1999).