

A New Heat Shock Protein That Binds Nucleic Acids*

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We describe the isolation of Hsp15, a new, very abundant heat shock protein that binds to DNA and RNA. Hsp15 is well conserved and related to a number of RNA-binding proteins, including ribosomal protein S4, RNA pseudouridine synthase, and tyrosyl-tRNA synthetase. The region shared between these proteins appears to represent a common, but previously unrecognized, RNA binding motif. Filter binding studies showed that Hsp15 binds to a 17-mer single-stranded RNA with a dissociation constant of 9 μM in 22.5 mM Hepes, pH 7.0, 5 mM MgCl_2 . A role of Hsp15 in binding nucleic acids puts this protein into a different functional category from that of many other heat shock proteins that act as molecular chaperones or proteases on protein substrates.

Temperature upshifts and a number of other stress conditions result in the rapid production of a number of proteins termed heat shock proteins (Hsps).¹ Although heat shock proteins were initially studied from a regulatory viewpoint, in recent years more and more attention has focused on determining the function of these proteins. All four major classes of heat shock proteins (Hsp90, Hsp70, GroE, and small heat shock proteins) are known to function as molecular chaperones, proteins that help other proteins adopt a biologically active conformation, without themselves becoming part of the final structure (1). In addition, a number of heat shock proteins are proteases, which hydrolyze irreversibly damaged proteins (2). Thus, heat shock proteins appear to be generally involved in the folding and degradation of proteins.

Sensitive RNA hybridization techniques and the availability of an ordered, sequenced library of *Escherichia coli* clones have made possible the discovery of 26 new heat shock proteins in *E. coli* (3, 4). This represents a largely untapped resource of novel heat shock proteins. In this paper, we report that one of these proteins, Hsp15, is an abundant, well conserved nucleic acid-binding protein, a function distinct from that of other previously described heat shock proteins.

EXPERIMENTAL PROCEDURES

Cloning of Hsp15—The *Bam*HI fragment encoding the Hsp15 gene, *yrfH* (*hslR*), was isolated from λ clone 621 (4) and ligated into pUC19 (New England Biolabs) giving the plasmid pTHZ13. The *hslR* gene was

isolated via PCR using pTHZ13 as the template, primer Hsp15-P1 (5'-TGAAGGAGACCATATGAAAGAGAAA-3'), primer Hsp15-P2 (5'-TCTTGCGAGGATCCAGTTATTCAC-3'), and *Taq* polymerase (Stratagene). The PCR product was purified with the QIAEX kit (QIAGEN, Valencia, CA), digested with *Bam*HI and *Nde*I, and ligated into the overexpression vector pET11a (Ref. 5; Novagen) to generate the plasmid pTHZ25. The sequence of this construct was confirmed by sequencing the entire insert region on both strands using an ABI automated sequencer (Perkin-Elmer).

Purification of Hsp15—The strain BL21(DE3) (Novagen) harboring the plasmid pTHZ25 was induced at an A_{578} of 0.8–1.0 with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 4 h and harvested by centrifugation (15 min, 5000 $\times g$, 4 °C). All further steps were performed at 4 °C. The cell pellet was resuspended in cold buffer A (30 mM sodium borate, 1 mM EDTA, pH 8.5) and lysis was performed by two passes through a French pressure cell (1 inch in diameter, American Instrument) at 18,000 p.s.i. The lysate was centrifuged (25 min, 39,100 $\times g$) and the supernatant immediately loaded onto a tandem ion-exchange chromatography system consisting of 50 ml of Q-Sepharose and subsequent 25 ml of SP-Sepharose (Amersham Pharmacia Biotech) both equilibrated with buffer A. After washing to base line at a flow rate of 1 ml/min and removing the Q-Sepharose column, the SP-Sepharose column was eluted with 10 column volumes (250 ml) of a linear KCl gradient (0.0–1.0 M KCl in buffer A). Hsp15-containing fractions as assayed by SDS-polyacrylamide gel electrophoresis were pooled and dialyzed over night against a >500-fold volume of buffer B (30 mM Hepes, 1 mM EDTA, pH 7.0). During this dialysis a precipitate formed that was removed by centrifugation (15 min, 34,800 $\times g$, 4 °C). The supernatant was brought to 1.5 M ammonium sulfate in buffer B and loaded onto a 25-ml Phenyl-Sepharose (Amersham Pharmacia Biotech) column equilibrated with the same buffer. After washing to base line, the protein was eluted with 250 ml of a linear gradient with decreasing ammonium sulfate concentration (1.5–0.0 M ammonium sulfate in buffer B). Fractions that contained Hsp15 only were pooled and concentrated by ultrafiltration (180-ml Amicon cell; 3-kDa exclusion volume membrane (Millipore)). To test for coelution of Hsp15 with nucleic acids, the Q-Sepharose column was eluted separately with a linear KCl gradient (500 ml, 0.0–1.0 M KCl in buffer A). Aliquots of the fractions were concentrated by ethanol precipitation (6) and assayed for nucleic acid content with agarose gels (6). Using the method of Edelhoch (7) and the variations of Pace (8) the molar extinction coefficient was determined as 15,524 $\text{M}^{-1} \text{cm}^{-1}$, which is in good agreement with the calculated value of 15,470 $\text{M}^{-1} \text{cm}^{-1}$ (8), and was used for determination of Hsp15 concentrations.

Heat Shock Induction at the RNA Level—*hslR* PCR product (0.1 pmol) was fixed to a Hybond (Amersham Pharmacia Biotech) nylon membrane and probed with labeled coding DNA as prepared by reverse transcription of 20 μg of total RNA (isolated with Trizol reagent; Life Technologies, Inc.) from log phase cultures grown at 37 °C or from the same culture heat-shocked for 10 min at 46 °C.

Determination of Cellular Abundance—Cultures of *E. coli* AB1157 or MC4100 were grown in LB (6) at 29 °C until an $A_{546} = 1.0$ (50 ml of culture in 250-ml Erlenmeyer flasks, 300 rpm air shaker, doubling time 40–50 min). Subsequent heat shock was achieved by transferring to a 42 °C shaking water bath, the new temperature in the culture being reached after <3 min. For the heat shock kinetic, aliquots were withdrawn, cooled in ice-water, and centrifuged (1 min, 7900 $\times g$, 4 °C), and the cells were washed in cold 150 mM NaCl and re-centrifuged. The cell pellet was lysed in 2.4% (w/v) SDS, 50 mM Tris-HCl, pH 8.0, 8.0% glycerol by heating to 95 °C for 10–30 min. The protein content of the lysate was determined by a modified Lowry assay using BSA as a standard (9). The amount of Hsp15 present was determined by Western immunoblotting analysis according to the protocol of the ECL system

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¶ This paper is dedicated to Tim Hering on the occasion of his 70th birthday and in honor of his encouraging example.

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¹ The abbreviations used are: Hsp, heat shock protein; PCR, polymerase chain reaction; BSA, bovine serum albumin; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

(Amersham Pharmacia Biotech). The films were scanned with an Elscrypt 400 AT densitometer (Hirschmann, Neuried, Germany) and analyzed using the PEAKFIT program (Jandel Scientific). Known quantities of purified Hsp15 were treated on the same blots for calibration. Both strains gave consistent results. Polyclonal rabbit antiserum against purified Hsp15 was a gift of Michael Ehrmann (Universität Konstanz, Konstanz, Germany).

Determination of Association State of Hsp15—The sedimentation analysis data were collected with a Beckman model E analytical ultracentrifuge in an AN-D rotor and double sector cells; one sector contained Hsp15 at 11.6 μM in buffer B, and the reference sector contained only buffer B. The molecular weight was determined by the meniscus depletion method (10), and the evaluation of the corresponding high speed (20,000 rpm) sedimentation equilibrium data ($\ln c$ versus r^2) was done by a computer program developed by Dr. G. Boehm, Regensburg, Germany. The specific volume of the protein was assumed to be 0.735 ml/g (11). The temperature at equilibrium was 19 °C. Analytical gel filtration was done on a Superdex 75 analytical grade HR10/30 (Amersham Pharmacia Biotech) in 10 mM Hepes, 150 mM NaCl, pH 7.0. The additional salt in the running buffer was necessary to prevent Hsp15 from sticking to the column material. 5–14 μg of Hsp15 were loaded at a concentration of 150 μM in buffer B.

Gel Retardation Assays—Single-stranded, circular M13mp18 DNA (7249 bases; U. S. Biochemical Corp.) was heated to 80 °C for 2 min prior to use. The plasmid pGEM-3Z (2743 base pairs; Invitrogen) was prepared with the kit Nucleobond AX 500 (Macherey-Nagel, Düren, Germany). The commercially available RNA preparation (yeast RNA type III; Sigma, catalog no. R7125) is reported to consist mainly of tRNA (12). Protein, nucleic acid, buffer, and salt were mixed in a final volume of 18 μl , and complex formation was allowed to proceed for at least 30 min at room temperature. To assure equilibrium, up to 1 week of incubation time was tested with no change in the apparent band patterns compared with the 30-min incubation time. Immediately prior to loading 2 μl of 10 \times loading buffer (50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol) was added. Electrophoresis was performed in 1% agarose (Biozyme) gels using TAE buffer (6) at 120 V for 3–4 h. Afterwards, the gels were stained with ethidium bromide (6).

Quantitative Zonal Affinity Chromatography—The quantitative zonal affinity chromatography was performed as described in Jenuwine and Shaner (13), except that instead of the high performance liquid chromatography we used a FPLC system with the HR 5/5 column (Amersham Pharmacia Biotech). The elution buffer was 1 mM sodium phosphate, pH 7.7, 0.1 mM EDTA, with NaCl added to the designated concentration (13). The protein concentrations and volumes loaded on the column were as follows: RNase A (Sigma), 6.6 mg/ml in H₂O, 10 μl loaded; Hsp15, 2.39 mg/ml in buffer B, 6 μl loaded; *cro* repressor (a gift from Katrin Ramm, Universität Regensburg, Regensburg, Germany), 1.6 mg/ml in 50 mM Tris-HCl, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 10 μl loaded; *lac* repressor (a gift from Sonya Melcher, University of Wisconsin, Madison, WI), 4.18 mg/ml in 10 mM Hepes, pH 7.55, 100 mM KCl, 0.1 mM 1,4-dithio-DL-threitol, 30% glycerol, 5 μl loaded. To calculate the affinities, the concentration of DNA in nucleotides was used. This was 1.26–2.73 mM accessible nucleotides depending on the DNA-cellulose preparation, determined as in Ref. 13. The data were consistent, independent of the batch of DNA-cellulose. The data were plotted and analyzed with the SigmaPlot program (Sigma).

Filter Binding Experiments—A DEAE filter (NA45 DEAE-cellulose, Schleicher & Schuell) was soaked for 10 min in 10 mM EDTA, pH 8.0, then 10 min in 0.5 M NaOH with shaking at room temperature, washed to neutrality with water, and incubated for >1 h in the wash buffer (22.5 mM Hepes, pH 7.0, 5 mM MgCl₂, 0.75 mM EDTA). The nitrocellulose-filter (Protran nitrocellulose, Schleicher & Schuell) was treated as the DEAE-cellulose but without the EDTA step. Immediately prior to application of the samples, the two filters were mounted in a 48-slot blotting device (Hoefer), the nitrocellulose filter directly on top of the DEAE filter. Only a very gentle vacuum was applied to prevent the filters from drying out. For the same reason, all samples were added as fast as possible, which usually did not take more than 5 min in total. The volume of the binding reaction was 10 μl ; the buffer was the same as the wash buffer plus additional 1.0 μM BSA (New England Biolabs), 8 mM 1,4-dithio-DL-threitol, and 3.2 units of RNasin (Promega). Binding reactions were incubated at room temperature (23–25 °C) for about 20 min. Addition of BSA prior to the addition of Hsp15 was important to prevent Hsp15-RNA-complexes from sticking to tube walls. The Hsp15 preparation was RNase-free, but the RNase inhibitor RNasin was always added when using BSA. When the liquid of the samples had completely passed the filters, the slot was immediately washed with 200 μl of wash buffer.

The sequences of the various RNA substrates were: GMP as monomer, CUG as trimer, CUCG as tetramer, CCCUC(dT) as hexamer, GGGAACGUC as nonamer, and GGGAACGUCGUCGUCGC as heptadecamer (17-mer). All substrates were 5'-³²P-labeled as described in Ref. 14. About 2000 cpm/slot were used. The dried filters were quantitated and analyzed with the PhosphorImager System (Molecular Dynamics). The curve fit in Fig. 7 was done with the program Kaleidagraph (Macintosh).

Enzyme Assays—The protease assay was done according to Twining (15); the chaperone assay was performed as described by Jakob *et al.* (16). Dialysis buffer was used as negative control. Positive controls were trypsin (Sigma) and Hsp90 (a gift from U. Jakob).

RESULTS

Characterization of New Heat Shock Proteins in *E. coli*

Twenty-six new heat shock genes were discovered in *E. coli* by global transcription analysis and mapped to specific λ clones in the ordered Kohara library (3, 4). Taking advantage of the estimated molecular weights of the corresponding heat shock proteins and the recently released sequence of the complete *E. coli* genome, we have been able to assign most of these "heat shock loci" (*hsl*) genes to sequenced open reading frames.² We consider these genes a largely unexplored resource of heat shock genes unrelated to previously studied heat shock genes. One of these, *yrfH* (*hslR*) encodes a 15,496-Da protein with a calculated isoelectric point (pI) of 9.94. Based on the molecular weight and the heat inducibility of the product of the *yrfH* (*hslR*) gene, we have assigned it the name Hsp15.

Hsp15 Is Highly Conserved in Prokaryotes

The Hsp15 sequence was used to search the nonredundant data base and the data base of unfinished microbial genomes maintained at National Center for Biotechnology Information (NCBI). Significant matches were found for sequences present in a wide variety of prokaryotic organisms including both Gram-negative and Gram-positive bacteria (Fig. 1). All of the homologues are very basic proteins, with pI values ranging from 9.9 to 10.3. We conclude that Hsp15 is a member of a previously undescribed family of well conserved, highly basic proteins. The molecular mass values of the Hsp15 homologues in Gram-negative organisms are in the range of 15.1–15.5 kDa. The proteins in the Gram-positive organisms *Streptococcus* and *Bacillus* are 5 kDa smaller in size due to a shortened C terminus. We decided to further investigate the function of this protein because its high degree of conservation implies that it plays an important role. We reasoned that, because it is not related to any known heat shock protein, this role may turn out to be new.

Hsp15 Shows Homology to RNA-binding Proteins

The search of the nonredundant data base with *E. coli* Hsp15 was iterated using the sensitive PSI-BLAST program, which includes profile construction (17). This search method automatically combines the significant alignments produced by BLAST into a matrix of position-specific scores. Searching the data base using this matrix in many cases allows the discovery of faint, but biologically relevant sequence similarities. Using this approach, a number of proteins that interact with RNA were found to contain a motif in common with each other and Hsp15 (Fig. 2). These include ribosomal protein S4, RNA pseudouridine synthase, and tyrosyl-tRNA synthetase. A 31-amino acid motif that usually begins with the sequence RLD and ends with the sequence NG is shared among Hsp15, these families of RNA-binding proteins, and HlyA. HlyA is homologous to FtsJ, a RNA methylase,³ suggesting that members of the HlyA family may bind RNA as well.

² W. B. M. Muse, T. Zander, and J. C. A. Bardwell, unpublished data.

³ H. Buegl, U. Jakob, and J. C. A. Bardwell, unpublished data.

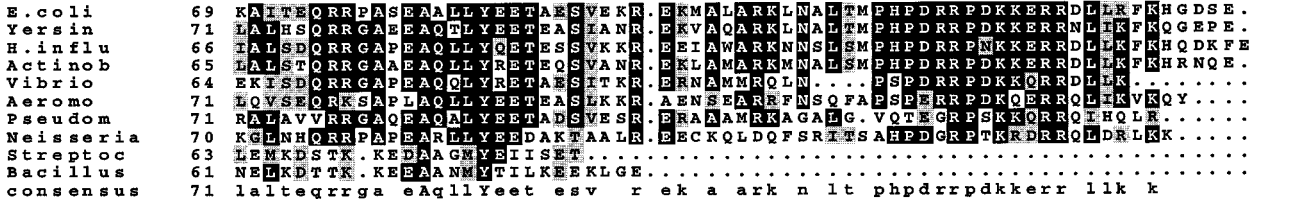
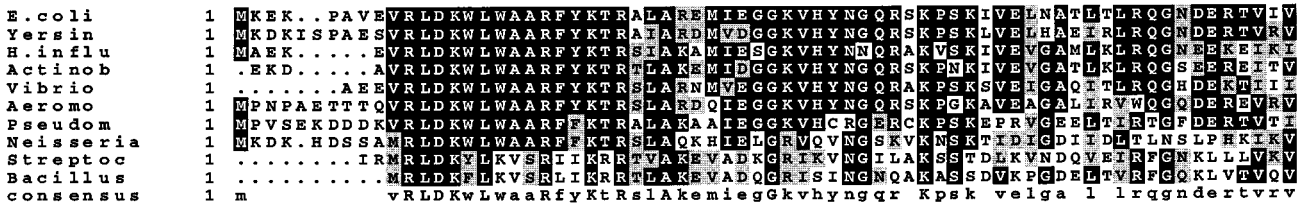
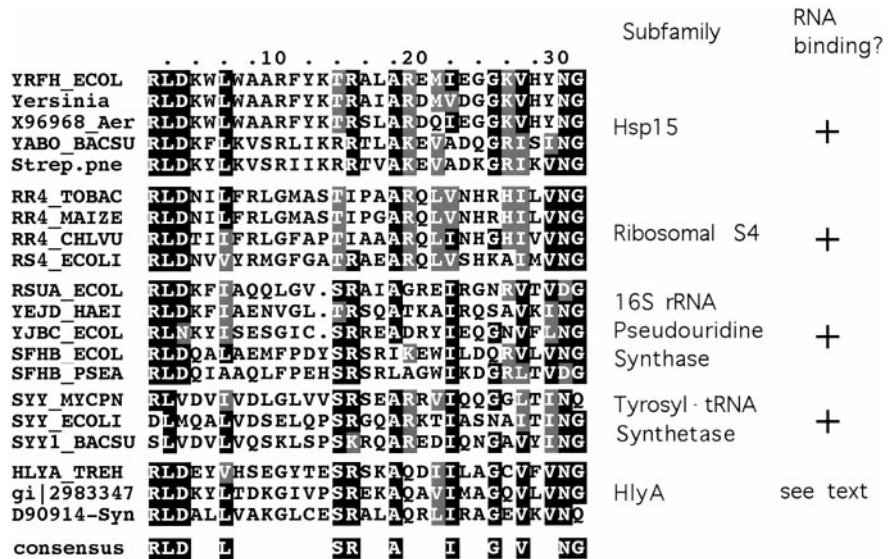


FIG. 1. Multiple alignment of the newly identified Hsp15 family. Sequence homology searches using GAPPED BLAST (17) against the GenBank nonredundant data base as well as searches in the incomplete microbial genome data base maintained at NCBI performed on May 22, 1998 revealed 14 members of the Hsp15 family. Sequence alignments were performed with CLUSTALW (18). Amino acid residues identical in 50 or more percent of the sequences are highlighted in black; related amino acids were shaded gray using the display program BOXSHADE. The source abbreviations and Swiss Protein or accession numbers, when available, are as follows: *E. coli*, *Escherichia coli*, YRFH_ECOLI; *Yersinia*, *Yersinia pestis* Sanger|Contig1280; *H.influ.*, *Hemophilus influenzae* YRFH_HAEIN; *Actinob.*, *Actinobacillus actinomycetemcomitans* OUACGT A-actin_Contig585; *Vibrio*, *Vibrio cholerae* TIGR|GVDCDC26R; *Aeromo.*, *Aeromonas salmonicida*, accession no. X96968; *Pseudom.*, *Pseudomonas aeruginosa* gnl|PAGP|Contig246; *Neisseria*, *Neisseria gonorrhoeae* OUACGT|Contig222; *Streptoc.*, *Streptococcus pneumoniae* TIGR stp_4125; *Bacillus*, *Bacillus subtilis* YABO_BACSU. Because the genomes of *Yersinia*, *Actinobacillus*, *Vibrio*, *Pseudomonas*, *Neisseria*, and *Streptococcus* are not yet complete, the sequences from these organisms should be regarded as preliminary.

FIG. 2. A new RNA binding motif. The Hsp15 sequence was used to search the nonredundant data base of the NCBI data base using the PSI BLAST program (17). Four protein families that contained a domain showing significant similarity to Hsp15 were found. Representative members of each family are shown. The Hsp15 family has 14 members, the S4 family has >200 sequenced members in the nonredundant data base, the 16 S pseudouridine synthase family has >50 members, >90 tyrosyl-tRNA synthetases are known, and the HlyA family has >10 members. Although HlyA has not yet been experimentally shown to bind RNA, it is homologous to the RNA-binding protein FtsJ, which functions as an RNA methylase (see Footnote 3).



Purification of Hsp15

A purification strategy based on the high isoelectric point of Hsp15 was devised. It involved a tandem ion-exchange chromatography system at pH 8.5 consisting of an anion exchange "pre-clearing" column, immediately followed by a subsequent cation exchange "trapping" column. Of the contaminating proteins co-eluting with Hsp15 from the cation exchange matrix, nearly all precipitated during the following dialysis, which therefore served as an effective purification step. A hydrophobic chromatography step separated Hsp15 from a small amount of degradation product. Hsp15 was assayed for purity by SDS-polyacrylamide gel electrophoresis and subsequent silver staining and was estimated by densitometry (data not shown) to be of greater than 99.7% purity. The identity of the purified protein was confirmed by N-terminal sequencing of the first nine amino acids; the initiation methionine was still present.

Although Hsp15 is expected to still be positively charged at pH 8.5, elution of the anion exchange column yielded some Hsp15-containing fractions. These fractions also showed the presence of nucleic acids in ethidium bromide-stained agarose

gels (data not shown). This co-elution suggested to us that Hsp15 bound to the anion exchange matrix via nucleic acids, suggesting a tight interaction between Hsp15 and nucleic acids.

Heat Shock Induction of Hsp15 and Steady State Expression Levels

To verify that Hsp15 is indeed a member of a new heat shock protein family, mRNA hybridization experiments were performed (Fig. 3, inset). A transient burst of heat shock RNA synthesis normally occurs in the 5–15 min following temperature shift. mRNA was prepared from log phase *E. coli* cultures grown at 37 °C or from the same culture after a 10-min heat shock treatment at 46 °C. As shown in the inset to Fig. 3 and characteristic of heat shock genes, Hsp15's mRNA is strongly up-regulated upon a temperature shift from 37 °C to 46 °C. This transient burst of heat shock mRNA synthesis has the effect of rapidly allowing the heat shock proteins to reach a new higher steady state level following temperature shift.

In order to quantitate the level of Hsp15 protein present

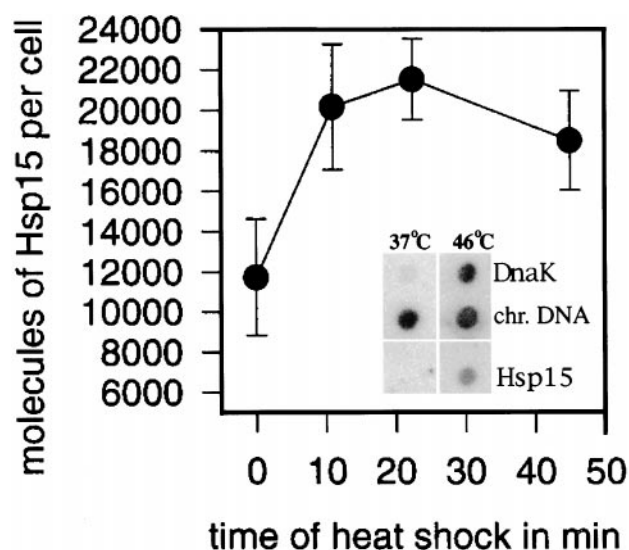


FIG. 3. The cellular abundance of Hsp15 was determined by quantitative Western immunoblotting. The determined percentage of Hsp15 mass per total cell protein mass was converted to the number of molecules per cell according to Ref. 19. The abundance corresponds to an intracellular concentration of 30–60 μM . Heat shock treatment was a shift of exponentially growing cultures from 29 °C to 42 °C. The factor of heat shock induction at the protein level was 1.8. The averages and standard deviations shown are the results from three independent experiments. *Inset*, heat shock induction monitored by RNA levels. Membrane-bound PCR product from Hsp15's gene (*hslR*) was hybridized with labeled coding DNA from cells grown at 37 °C or from the same cultures after 10-min heat shock at 46 °C. The DnaK gene (*dnaK*) PCR product was added as positive control and chromosomal DNA as internal normalization for comparison between different blots.

during steady state growth, we measured the intracellular abundance of Hsp15 in exponentially growing cultures at 29 °C and various times following a shift to 42 °C. Western immunoblot analysis showed that cultures at 29 °C contained 1.29% w/w Hsp15 per total cell protein. This fraction increased to 2.22%, 2.37%, and 2.04% after 11, 22.5, and 45 min of heat shock treatment (42 °C), respectively. Thus 12,000–22,000 molecules of Hsp15 are present per cell, as calculated using a conversion factor of 2.34×10^{-13} g of protein/cell (Ref. 19; Fig. 3). To calculate the intracellular concentration, the association state of Hsp15 needed to be determined. Hsp15 was found to be a monomer, in the absence of nucleic acid, by both analytical ultracentrifugation (14.7 ± 1.3 kDa) and gel permeation chromatography (15.8 ± 2.4 kDa) (Fig. 4). According to an average volume of an *E. coli* cell of $0.63 \mu\text{m}^3$ (Ref. 20; doubling time of 40 min), the intracellular concentration of Hsp15 is 30–60 μM . This makes Hsp15 a very abundant protein in the cell before and after heat shock, even more abundant than the GroEL 14-mer, which has been estimated (using slightly different conversion factors) to be present in 1580 copies or at a concentration of 2.6 μM at 37 °C (21). The observed ~2-fold increase in Hsp15 at the protein level is very similar to the degree of increase in protein level seen with the other characterized heat shock proteins in *E. coli*. The average increase in steady state level following shift from 30 °C and 42 °C for the 11 heat shock proteins that have been measured in *E. coli* is 1.97-fold (22). Many show similar *n*-fold increases such as GroEL (2.3-fold) or DnaK (2.0-fold); some, such as GrpE (1.3-fold) and lon (1.1-fold), show less of an increase; one other, HtpG, shows a substantially greater increase of 3.8-fold (22).

Nucleic Acid Binding

Gel Retardation Assays—To directly show that Hsp15 binds nucleic acids, we tested its ability to shift various kinds of

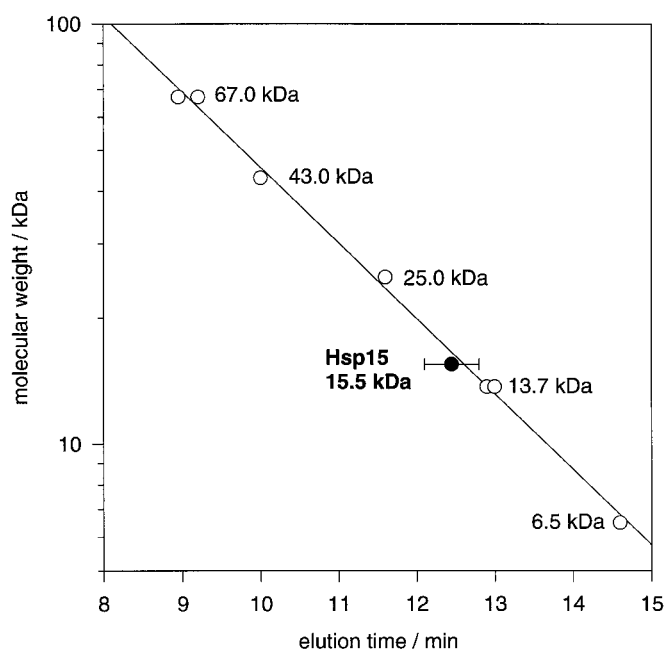


FIG. 4. Determination of molecular mass and association state of Hsp15 by gel permeation chromatography. Hsp15 elutes as expected for a monomer of its predicted molecular mass. The data point shown is the average of five experiments. Proteins used for calibration were: BSA (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen (25.0 kDa), RNase A (13.7 kDa), and aprotinin (6.5 kDa). The shown linear regression of the calibration accords to the equation $\log(\text{molecular mass/kDa}) = 3.45 - 0.18 t_{\text{elution}}/\text{min}$; $r^2 = 0.997$.

nucleic acids in gel retardation assays. Three different substrates were tested: the plasmid pGEM-3Z as an example of dsDNA, the single-stranded vector M13mp18 as an example of ssDNA, and a commercially available RNA preparation that consists mainly of tRNA. Under the same conditions and within the same gels, Hsp15 was compared with two well characterized nucleic acid-binding proteins: RNase A, as an example for a very weak binder (23), and the λ phage *cro* repressor, which exhibits high binding affinities (24). The binding reaction was performed in buffer B plus 150 mM KCl to mimic physiological ionic strength to a first approximation. The highest protein concentration was also tested in buffer B without additional KCl to give an impression of binding in low salt.

Fig. 5a shows the gel retardation assay using the dsDNA substrate (pGEM-3Z). The migration behavior of the naked DNA is seen in lanes 1 and 17. As is typical for plasmid preparations, there are four major bands corresponding to the supercoiled and nicked form of the plasmid monomers and dimers (25). RNase A shows hardly any effect at the RNase A concentrations tested, not even in low salt (Fig. 5a, lanes 2–6). This indicates that the assay in this range of protein concentrations is not sensitive enough to detect the low binding affinity of RNase A to dsDNA. In contrast, the *cro* repressor clearly shifts all four bands of the plasmid preparation (Fig. 5a, lanes 12–16). The retardation becomes more pronounced with rising protein concentration arguing for an additive binding mode. A very similar behavior is seen for Hsp15 (Fig. 5a, lanes 7–11). This is clear evidence that Hsp15 is binding to dsDNA.

The extent of band shift for both Hsp15 and the *cro* repressor was increased at the low salt conditions (Fig. 5a, lanes 11 and 16). This confirms that the binding interaction is sensitive to ionic strength.

The same type of experiment was repeated using a single-stranded DNA substrate (Fig. 5b). Here, RNase A shows a slight shift at high concentration reflecting its higher affinity for ssDNA than dsDNA (23). Hsp15 retards ssDNA to a greater

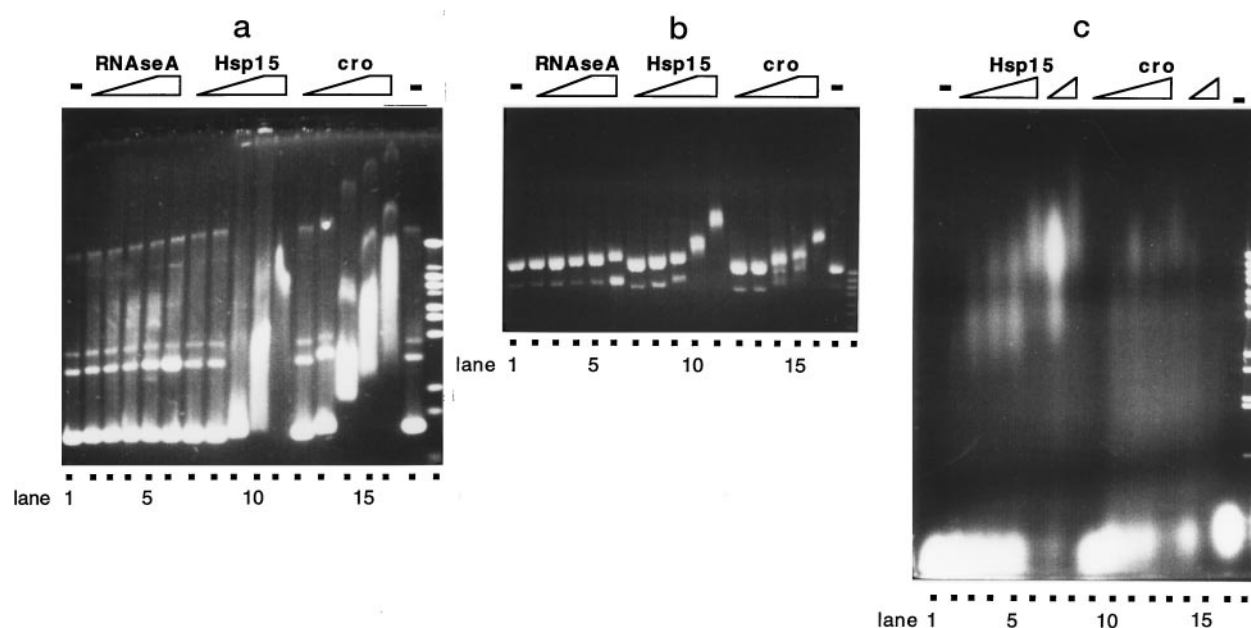


FIG. 5. Hsp15 binding to nucleic acids assayed in gel retardation experiments with three different substrates: dsDNA (a), ssDNA (b), and RNA (c). RNase A and the *cro* repressor were included for comparison. a, lanes 1–17 contain 440 ng of pGEM-3Z plasmid (2.74 kilobases) at a concentration of 14 nM (38 μ M in base pairs) in buffer B plus 150 mM KCl, with the exception of lanes 6, 11, and 16, which contain no added KCl. Lanes 1 and 17, no protein added. Lanes 2–6, RNase A at 0.8, 1.6, 3.2, 6.4, and 6.4 μ M. Lanes 7–11, Hsp15 at 0.8, 1.6, 3.2, 6.4, and 6.4 μ M. Lanes 12–16, *cro* repressor at 0.8, 1.6, 3.2, 6.4, and 6.4 μ M (dimer). Lane 18, 0.5 μ g of standard (λ phage DNA cut with *Eco*R91I; MBI Fermentas). b, lanes 1–17 contain 600 ng of M13mp18 DNA (7249 bases) at a concentration of 14 nM (100 μ M in bases) in buffer B plus 150 mM KCl, with the exception of lanes 6, 11, and 16, which contain no added KCl. Lanes 1 and 17, no protein added. Lanes 2–6, RNase A at 1.1, 2.2, 4.4, 8.8, and 8.8 μ M. Lanes 7–11, Hsp15 at 1.1, 2.2, 4.4, 8.8, and 8.8 μ M. Lanes 12–16, *cro* repressor at 1.1, 2.2, 4.4, 8.8, and 8.8 μ M (dimer). Lane 18, standard as in a. c, lanes 1–16 contain 9 μ g of yeast RNA (Sigma, type III; mostly tRNA) in buffer B plus 150 mM KCl, with the exception of lanes 7, 8, 14, and 15, which contain no added KCl. Lanes 1 and 16, no protein added. Lanes 2–8, Hsp15 at 16, 32, 48, 64, 97, 48, and 97 μ M. Lanes 9–15, *cro* repressor at 16, 32, 48, 64, 97, 48, and 97 μ M (dimer). Lane 17, standard as in a. The volume of all binding reactions was 18 μ l. Buffer B was 30 mM Hepes, pH 7.0, 1 mM EDTA.

extent at lower concentration than the *cro* repressor, perhaps suggesting a preference of Hsp15 for single-stranded over double-stranded substrates (see Fig. 5b, lanes 10 and 11 versus 15 and 16).

Fig. 5c shows that both Hsp15 and the *cro* repressor clearly retard the migration of a mainly tRNA substrate. The abundance of the shifted complex appears to be higher with Hsp15.

Zonal Quantitative Affinity Chromatography on DNA Cellulose—In order to assess the interaction of Hsp15 with dsDNA in a more quantitative way, we employed zonal quantitative affinity chromatography. This method provides a rapid and convenient means to quantitatively compare a protein of interest with standard proteins in its nucleic acid binding affinity as well as the salt dependence of this affinity (13).

Proteins with affinity for DNA will cyclically bind and unbind as they pass through a DNA cellulose column. The time it takes a protein to elute from such a column compared with the elution time under high salt conditions, where no binding occurs, is directly correlated to the affinity constant K_B , if chromatography is performed in the linear regime allowing rapid determinations of K_B values (13).

The range of K_B values for which the method is applicable is limited to the window of 10^2 to 10^4 M^{-1} . Higher affinities will generate prolonged elution times, diffusion of the protein peak, and subsequent detection problems. However, most nonspecific protein-DNA interaction equilibrium constants can be shifted into the 10^2 to 10^4 M^{-1} window by adjusting the salt concentration since the cation concentration is the major determinant for the affinity of a nonspecific protein-DNA interaction (26).

Using this method, Hsp15 was compared in its elution properties at varying salt concentrations to three proteins whose nonspecific binding affinities to DNA are well studied: the *lac* repressor, RNase A, and the *cro* repressor (Fig. 6, a–c).

The binding affinity of Hsp15 to the dsDNA matrix was found to be virtually the same as that of the *lac* repressor (Fig. 6a). Linear regressions for the data sets of Hsp15 and the *lac* repressor are in good agreement. Further, the measured values for the *lac* repressor meet very well previously published data for the nonspecific binding of the *lac* repressor to dsDNA (Fig. 6b; Refs. 27–30; corrected for pH according to Ref. 31). A linear regression for the combined sets of data from the literature and our measurements for the *lac* repressor ($\log(K_B/M^{-1}) = -2.92 - 9.61 \log([NaCl]/M)$; $r^2 = 0.941$) resembles very much the salt dependence of the affinity constant for the *lac* repressor as published by Jenuwine and Shaner (Ref. 13; $\log(K_B/M^{-1}) = -2.83 - 9.76 \log([NaCl]/M)$; $r^2 = 0.975$). For the other two tested proteins, RNase A and the *cro* repressor (Fig. 6c), measurements of binding constants at varying salt concentrations also agreed well with data from the literature (23, 24, 27). As already suggested by the gel retardation experiments, Hsp15 binds with much higher affinity to dsDNA than RNase A and with somewhat lower affinity than the *cro* repressor does. For each protein, the salt dependence of K_B appeared to be linear, as would be expected for the absence of significant specific ion binding effects associated with the proteins (26).

Filter Binding—The binding of Hsp15 to ssRNA was assayed by filter binding experiments. Labeled RNA substrate was incubated with increasing amounts of Hsp15 and analyzed by washing over a nitrocellulose and subsequent DEAE-cellulose filter. Protein-RNA complexes are retained on the first filter and free RNA on the second filter. The ratio of the amount of RNA on the nitrocellulose filter to the sum of the RNA on both filters gives a measurement of the fraction of protein-RNA complexes in the binding reaction. Because the RNA is present only in trace amounts, the free protein concentration can be assumed to be equal to the total protein concentration.

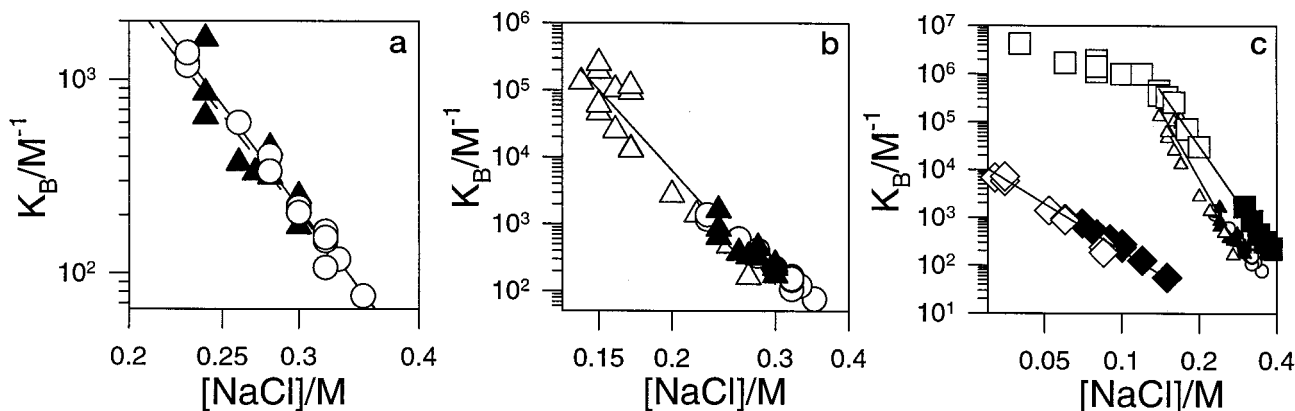


FIG. 6. Zonal quantitative affinity chromatography on a dsDNA (calf thymus, Sigma) column matrix. *a*, the dependence of the binding constant (K_B) for Hsp15 (\circ) and the *lac* repressor (\blacktriangle) on NaCl concentration was determined and is shown in a double logarithmic plot. The linear regression for Hsp15 (—: $\log(K_B/M^{-1}) = -1.15 - 6.66 \log([\text{NaCl}]/M)$; $r^2 = 0.987$) and for the *lac* repressor (---: $\log(K_B/M^{-1}) = -0.98 - 6.31 \log([\text{NaCl}]/M)$; $r^2 = 0.776$) were very similar, suggesting that Hsp15 resembles the *lac* repressor in its dsDNA binding properties. *b*, a comparison of literature data for the *lac* repressor (\triangle) and our measured values (\blacktriangle) shows good agreement for the *lac* repressor. The linear regression of all data sets for the *lac* repressor is $\log(K_B/M^{-1}) = -2.92 - 9.61 \log([\text{NaCl}]/M)$; $r^2 = 0.941$. The data for Hsp15 (\circ) are included for comparison. *c*, RNase A (\diamond) and the *cro* repressor (\blacksquare) were tested in the same column system to further confirm the reliability of the applied method in quantitative terms. Both proteins showed very good agreement of the measured data (closed symbols) to the literature data (open symbols). The lines are linear regressions to the combined data sets (RNase A: $\log(K_B/M^{-1}) = -0.98 - 3.27 \log([\text{NaCl}]/M)$; $r^2 = 0.989$; *cro* repressor: $\log(K_B/M^{-1}) = -0.92 - 7.75 \log([\text{NaCl}]/M)$; $r^2 = 0.997$). The data points and regression of *b* are included with reduced symbol size.

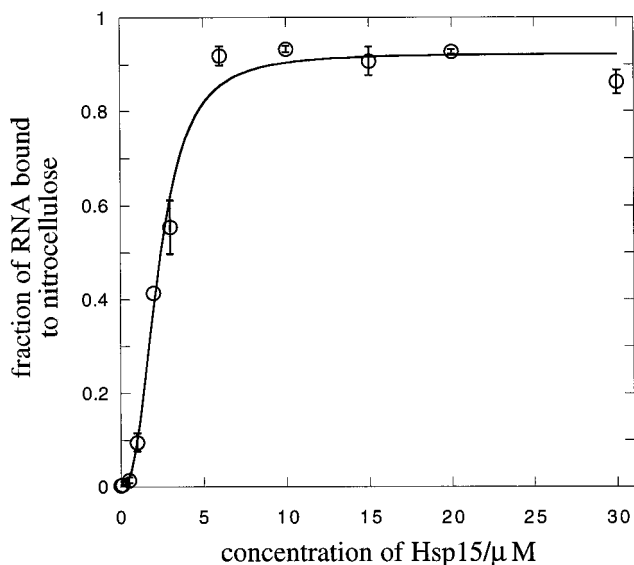


FIG. 7. A filter binding curve yields a dissociation constant of Hsp15 for ssRNA of 9 μM . Trace amounts of labeled 17-mer RNA were incubated with increasing amounts of Hsp15 and loaded onto a nitrocellulose filter with DEAE cellulose underneath. Protein-bound RNA trapped to the nitrocellulose in relation to the total RNA amount on both filters was plotted versus the Hsp15 concentration. The data were fitted according to the Hill equation $\Theta = \Theta_{\max} P_T^n / (P_T^n + K_D)$ (Θ , fraction of bound RNA; Θ_{\max} , maximal fraction of bound RNA; P_T , total protein concentration; K_D , dissociation constant; n , Hill constant reflecting the degree of cooperativity and the number of proteins bound per RNA substrate at infinite cooperativity) giving the parameters $K_D = 8.9 \pm 3.0 \mu\text{M}$, $\Theta_{\max} = 0.92$, and $n = 2.6 \pm 0.4$ with an error $r = 0.996$. The data points are the average of three experiments.

The binding curve in Fig. 7 shows a sigmoidal shape. A curve fit assuming a single site binding curve does not fit the data well (not shown). This speaks for more than one Hsp15 molecule bound to the 17-mer RNA substrate. A curve fit to a Hill-equation allowing the Hill constant to vary (see figure legend) agrees well with the experiment and gives a dissociation constant of $8.9 \pm 3.0 \mu\text{M}$ and a Hill constant of 2.6 ± 0.4 .

Substrate Length Effects—The filter binding curve suggested that 2–3 Hsp15 molecules could bind to a 17-mer RNA substrate, which would amount to a binding site size between 6

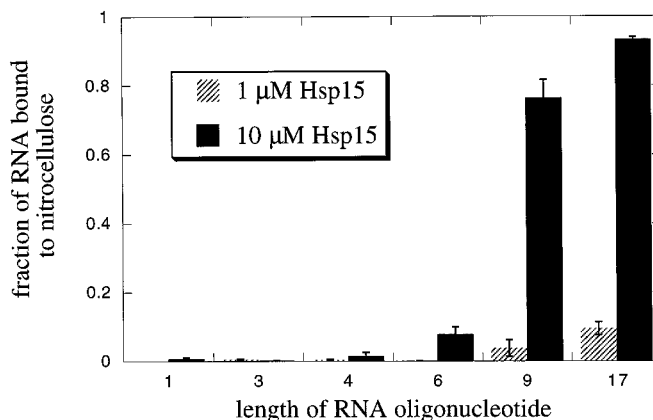


FIG. 8. Estimation of the binding site size. RNA substrates of various length were incubated with 1 or 10 μM Hsp15 and assayed for retention of RNA on nitrocellulose filters (see Fig. 7). The data for the 17-mer are taken from Fig. 7; all other data are the average of two experiments.

and 8 nucleotides. To further approximate the binding site size, RNA substrates of decreasing length were tested for binding to Hsp15 at two different concentrations (Fig. 8). Substrates with 1, 3, or 4 nucleotides were not significantly bound by Hsp15, as tested by filter binding. The onset of retention on the nitrocellulose was with the 6-mer RNA substrate, although only at the high concentration of Hsp15 and only to a small extent. The 9-mer was well bound by Hsp15 at both concentrations but still to a lesser extent than the 17-mer. Six nucleotides appeared to be the minimal required length for binding under these conditions consistent with the Hill constant of the binding curve for the 17-mer (Fig. 7), which suggested that 2–3 molecules of Hsp15 could be bound per 17-mer.

DISCUSSION

Our results define a newly recognized, well conserved, abundant heat shock protein that binds nucleic acids. Hsp15 was found to have a domain homologous to a number of RNA-binding proteins (Fig. 2) including ribosomal protein S4, RNA pseudouridine synthase, and tyrosyl-tRNA synthetase. The RNA binding domain on ribosomal protein S4 has been mapped to residues 48–177 (32), a region that includes the residues

97–127 that show homology to Hsp15. The region responsible for tRNA binding in the tyrosyl-tRNA synthetases is near the C terminus (33). The Arg-19 in the motif shown in Fig. 2 is at the homologous position to Arg-371 of the *Bacillus stearothermophilus* tyrosyl-tRNA synthetase. This arginine is directly implicated in tRNA binding (34). Since the only sequence shared by all these proteins is this motif and the only function they have in common is RNA binding, we propose that this RLD motif represents a previously unrecognized RNA binding motif.

This newly recognized potential RNA binding motif is surprisingly commonly found; eight proteins in *E. coli* contain this motif (Fig. 2 and data not shown).

The very basic properties of Hsp15 (pI of 9.94) and co-elution with nucleic acids from an anion exchange column during the purification further suggested a possible nucleic acid binding activity. The nonspecific nucleic acid binding properties were investigated by gel mobility shift assays, zonal quantitative affinity chromatography, and filter binding experiments.

In gel retardation experiments, Hsp15 was shown to bind to double-stranded plasmid DNA, to single-stranded phage m13 DNA, and to a yeast RNA preparation that mainly consisted of tRNA (Fig. 5, *a-c*). The more Hsp15 was added, the more retarded the nucleic acid band became, speaking for multiple binding sites on the substrates, which is typical for nonspecific protein-nucleic acid interactions (35). Also as expected was the sensitivity of the nucleic acid interaction to the salt concentration (26). This behavior was compared with the nonspecific nucleic acid binding properties of RNase A and the *cro* repressor in the same assay. Whereas RNase A showed hardly any binding in this system, the *cro* repressor clearly shifted the nucleic acid substrate, as expected for a protein with high nonspecific DNA binding activity (24). Hsp15 was shown to retard all three nucleic acid substrates. Compared under the same buffer conditions and protein concentrations to the *cro* repressor, the extent of the change in the migration position of the nucleic acid band in the gel was somewhat less for the dsDNA substrate, but similar if not greater with the ssDNA and the tRNA substrates. For both proteins the shifted bands had a smeared appearance, consistent with an intermediately cooperative binding mode (36).

The nonspecific binding affinity of Hsp15 to dsDNA was assessed via zonal quantitative affinity chromatography (13). Here, Hsp15 behaved virtually identically to the *lac* repressor on the DNA cellulose matrix in absolute terms as well as in the dependence of the affinity constant to the salt conditions (Fig. 6*a*). In order to confirm the applicability of the measurements with our column system, we compared our data for the *lac* repressor with the extensive data in the literature (Refs. 27–30; Fig. 6*b*). The measurements of the *lac* repressor on our column system met very well with the literature data. The published data stem from different methods, and there is a considerable amount of scatter among them. An agreement of the data within 0.2 log units is considered to be good (28). We also tested RNase A and the *cro* repressor (Fig. 6*c*), and both gave binding constants very similar to those in the literature, further corroborating the precision of this method (23, 24, 27). Data for the *cro* repressor are obtained at different conditions: pH 7.3 versus 7.7 and KCl versus NaCl, but the nonspecific DNA binding affinity of the *cro* repressor is insensitive to these changes.⁴

As Hsp15 behaved very similarly to the *lac* repressor, we used the literature data for the *lac* repressor to estimate Hsp15's affinity constant under conditions that, in first approximation, mimic physiological ionic strength (150 mM KCl). This

affinity constant cannot be measured directly with the affinity column because Hsp15 binds too tightly (see "Results" and Ref. 13). By correction for pH according to the relationship $\delta(\log(K_B/M^{-1}))/\delta\text{pH} = -2.1$ (31), we extract from Refs. 28 and 29 a range of binding affinities of the *lac* repressor at 150 mM NaCl, pH 7.7, of 25–4.7 $10^4 M^{-1}$. These correspond to dissociation constants of 4–20 μM . Thus, we conclude that Hsp15 binds to dsDNA in the micromolar range at conditions that mimic physiological salt concentration to a first approximation. The close resemblance of the salt dependence of Hsp15 K_B to that of the *lac* repressor in both the slope and y axis intercept also shows that this interaction for Hsp15 is like that of *lac* repressor mainly driven by the electrostatic contribution, *i.e.* counterion release (26).

The nonspecific binding of Hsp15 to ssRNA at 5 mM MgCl₂ was measured by filter binding experiments. A binding curve (Fig. 7) has a sigmoidal shape, suggesting, as seen with the gel retardation experiments, that more than one Hsp15 molecule bound to the nucleic acid substrate. Hsp15 is a monomer in the absence of nucleic acid at concentrations between 12 and 150 μM independent of the presence of 150 mM NaCl (analytical ultracentrifugation; see also Fig. 4). Both the gel retardation experiments and the binding curve with the 17-mer ssRNA show that Hsp15 seems to multimerize in a cooperative way on nucleic acid substrates that are long enough to accommodate several Hsp15 molecules. Whether the multimerization on the 17-mer ssRNA affected significantly the retention on the nitrocellulose and therefore enhanced the impression of cooperativity cannot be ruled out at this stage. The filter binding data are fitted well with the Hill equation that is based on a model of cooperative binding. The dissociation constant derived from the fit was $8.9 \pm 3.0 \mu\text{M}$. The Hill constant was fitted to be 2.6 ± 0.4 . It is a measure for the cooperativity of the interaction and gives also an estimate for the number of molecules bound, in this case speaking for 2–3 Hsp15 molecules being bound to a 17-mer ssRNA substrate. The binding site size therefore appears to cover between 6 and 8 nucleotides. This was confirmed by filter binding experiments with ssRNA substrates of decreasing length (Fig. 8). A 6-mer substrate is just on the verge of being significantly bound by Hsp15. We estimate the minimal length required for strong binding under the condition tested to be about 6 nucleotides.

The intracellular concentration of Hsp15 was determined to be 30 μM constitutively and 60 μM after heat shock (Fig. 3). In this concentration range of Hsp15, all the substrates in the gel retardation experiments (Fig. 6, *a-c*) and also the 17-mer ssRNA in the filter binding assay (Fig. 7) were tightly bound. The estimated K_D values for both DNA and RNA substrates are well below the determined intracellular concentration of Hsp15. Thus, it is very likely that the function of Hsp15 lies in binding of a nucleic acid substrate *in vivo*, too.

What is the *in vivo* substrate for Hsp15? The main clues so far are the high abundance of Hsp15 and its motif homology to four RNA-binding proteins. At 29 °C about 12,000 molecules of Hsp15 are present per cell and roughly 2-fold more (22,000) are expressed after heat shock. This abundance makes Hsp15 unlikely to recognize a specific DNA site. Nonetheless, a nonspecific DNA binding function is possible. The histone-like proteins HU, H-NS, and IHF are present in *E. coli* at about the same abundance (30,000, 18,900, and 15,000 molecules/cell, respectively, at 37 °C; Refs. 22 and 37).

The motif homology to RNA-binding proteins, however, strongly suggests that Hsp15 may recognize a RNA substrate *in vivo*, rather than a DNA substrate. If the *in vivo* function involves RNA binding, a specific binding site is certainly possible since tRNAs as well as rRNAs are present in high copy

⁴ Y. Takeda, personal communication.

numbers in the cell (7000–70,000 rRNA molecules per cell and about 200,000 tRNA molecules; Refs. 19 and 38).

Of the heat shock proteins in *E. coli* characterized so far, 13 of 21 exhibit either molecular chaperone or protease function (2). Hsp15 was tested for molecular chaperone and protease activity but gave negative results (data not shown). Besides the heat-inducible sigma factors (σ^{70} , σ^{32} , σ^E) that take part in the regulation of the heat shock response, only the lysyl-tRNA synthetase LysU is known so far as a nucleic acid-binding heat shock protein (2). Our isolation of the very abundant, nucleic acid-binding, heat shock protein Hsp15 suggests that the heat shock response may comprise not only functions acting on the protein but also on the nucleic acid level.

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