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Secretion of mammalian ribonucleases from *Escherichia coli* using the signal sequence of murine spleen ribonuclease

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A nucleotide sequence identical with that of the recently identified murine pancreatic ribonuclease (RNAase) was isolated from a murine spleen cDNA library. Active RNAase was expressed and secreted from *Escherichia coli lon⁻htpr⁻* transformed with a plasmid containing the *E. coli* trp promotor followed by the murine RNAase gene sequence, including the original eukaryotic 26-amino-acid signal sequence. Approx. 1 mg of properly matured RNAase protein/litre was secreted into the medium of a fermentor culture after the promotor was induced by tryptophan starvation. When the signal sequence was deleted from the plasmid, intracellular RNAase activity was very low and there was no significant supernatant RNAase activity. Even higher RNAase yields were obtained with a synthetic gene for bovine pancreatic ribonuclease cloned after the signal sequence of the murine gene. About 2 mg of correctly processed RNAase A/litre was isolated from the growth medium, and a further 8–10 mg of correctly processed RNAase/litre could be isolated from the soluble fraction of the cells. Thus this eukaryotic signal sequence is both recognized by the *E. coli* transport and processing apparatus and gives efficient *secretion*, as well as export, of active, mature mammalian RNAases.

INTRODUCTION

Most of the literature about ribonucleases (RNAases) deals with those isolated from bacterial sources (Deutscher, 1988) or with the bovine enzyme RNAase A (Benner & Allemann, 1989). Our goals are to (*a*) learn more about RNAases in mammalian cells that might be involved in cell growth and (*b*) determine the molecular basis of the specificity of pancreatic RNAases and to explain the differing activities of structurally related proteins like bovine seminal RNAase and angiogenin. In keeping with the first goal, we screened a murine spleen cDNA library with a probe to a highly conserved area in all known mammalian RNAases (Haugg *et al.*, 1990) to see what homologues of the pancreatic family were expressed in that tissue. We isolated a cDNA clone containing a gene sequence coding for an RNAase A homologue and found it to code for a protein identical with murine pancreatic RNAase (Schüller *et al.*, 1990). We then expressed the murine RNAase protein in *Escherichia coli* to compare its properties with those of its bovine counterparts. Like the genes for most secretory proteins in eukaryotic and prokaryotic cells, the murine RNAase gene contains a coding region for an *N*-terminal 'signal sequence' that is cleaved from the mature protein either during, or immediately after, transport through the cell membrane (Gierasch, 1989).

Although bacterial RNAases have been expressed to very high levels using *E. coli* secretion systems (Hartley, 1988; Heinemann & Hahn, 1989), mammalian RNAases were previously expressed in an insoluble form (Nambiar *et al.*, 1987; Schapiro *et al.*, 1988). We wanted to produce soluble murine RNAase protein in *E. coli* as we were not sure that all pancreatic RNAases could be refolded from inclusion bodies as easily as the bovine protein. Solubility in this case mandated a good secretion system, as RNAases are potentially toxic products for cells if produced in an active form intracellularly. As there had been previous reports that eukaryotic secretion signals would allow secretion to the periplasm of *E. coli* (Talmadge *et al.*, 1981; Gray *et al.*, 1985), we expressed the gene both with and without its own signal sequence. Although the mature protein, when produced intracellularly, appeared to be toxic, as constructions without the signal sequence either did not grow or produced little RNAase, expression of the protein with its own signal sequence allowed efficient secretion of active RNAase to the medium of *E. coli* cultures. The protein purified from the culture supernatant had been accurately cleaved to yield mature murine secretory RNAase.

We were also interested in expressing mutants of RNAase A (Benner & Allemann, 1989) in a soluble form. We therefore tested whether the murine signal sequence could also direct the secretion of bovine RNAase by inserting a synthetic gene for this protein into the original construct. Correctly processed RNAase A was secreted into the growth medium in even higher amounts than the murine RNAase. We have thus established a new secretion system for mammalian RNAases and mutants thereof.

MATERIALS AND METHODS

Bacterial strains and plasmids

The following *E. coli* strains were used: JM101 (for initial cloning after ligation of plasmids); *DS410* [mini-cell phenotype (Roozen *et al.*, 1971)]; and *lon⁻htpr⁻* (Baker *et al.*, 1984; Goff *et al.*, 1984). The preparation of murine spleen polyadenylated [poly(A)⁺]RNA and the λgt11 phage library was previously described (Hemmi *et al.*, 1989).

Abbreviations used: poly(A)⁺, polyadenylated; PBS, phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl, pH 7.2); PMSF, phenylmethanesulphonyl fluoride; IFN-γ, interferon-γ; VVM, volume of air/min per volume of liquid.

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RNA blotting

Total RNA was isolated from cells as previously described (Hemmi *et al.*, 1989; Chomczynski & Sacchi, 1987). For the isolation of RNA from adherent cells, the cells from the peritoneal fluid from two DBA mice were centrifuged, washed twice in phosphate-buffered saline (PBS) and then resuspended in Dulbecco's Modified Eagle's Medium plus 10% (v/v) fetal-calf serum in a Petri dish. After 5 h at 37 °C in a 5%-CO_2 incubator, settled macrophages could be seen on the bottom of the plate. The swimming cells were removed, the plates were washed twice with 5 ml of PBS and the cells removed from the plate by flooding the surface with 2.7 ml of solution D and the normal RNA-isolation procedure was followed (Chomczynski & Sacchi, 1987). The RNAs were separated on a 1%-agarose gel and transferred to a Genescreen filter. The blot was hybridized and washed as described for genomic blotting, but at differing stringencies (see the legend to Fig. 1); the formamide concentration during the hybridizations was increased to 50% for Figs. 1*d*, 1*c* and 1*e*. The whole murine gene was ^{32}P-labelled by using a 'random priming' kit from Pharmacia.

Construction of the expression plasmids

Plasmid pHR148, which contains the trp promotor and ribosome-binding site (Rink *et al.*, 1984), was cleaved with *Nco*I and *Bam*HI. The murine spleen RNAase cDNA was cleaved from a puc19-based plasmid with *Nco*I (there is a natural cleavage site for this enzyme at the start of the signal sequence) and then partially cleaved with *Bam*HI. The 4.7 kb vector fragment and the 716 bp fragment containing the whole coding sequence for the murine spleen RNAase were isolated from an agarose gel with Geneclean. Vector plus insert were ligated overnight with T4 DNA ligase (Biolabs) at 16 °C, to yield the plasmid designated pTrpmuRN + ss, and transformed into competent *E. coli* JM101 cells.

For the construction of pTrpmuRN, which codes for the sequence of the mature protein with one additional *N*-terminal methionine residue, the *Nco*I–*Bam*HI fragment of the RNAase gene, prepared as above, was cleaved with *Hin*fI and the resulting 630 bp fragment was isolated. This was combined with *Nco*I–*Bam*H1-cleaved pHR148 and two oligonucleotides (5′-CATGAGGG-3′ and 3′-TCCCTTA-5′, synthesized on an Applied Biosystems DNA synthesizer and purified by t.l.c. to fill in the 5′ *Nco*I site of the vector and the 3′ *Hin*fI site of the RNAase gene fragment. For construction of pTrpmuSSboRN, an *Nco*I/*Fok*I fragment containing most of the murine signal sequence and an *Nde*I/*Bam*HI synthetic gene fragment coding for most of the mature sequence of bovine pancreatic RNAase (Nambiar *et al.*, 1987) were joined in a four-way ligation to *Nco*I/*Bam*HI-cleaved pHR148 with a synthetic linker. All constructions were checked by sequencing (U.S. Biochemicals Sequenase kit) using the 31-mer screening oligonucleotide (see the Results section) as primer for the murine constructs and an internal segment of the bovine gene for the murine bovine hybrid.

Bacterial cultivation and expression of the RNAase

Initial testing of expression was done in shaker-flask culture. The trp promotor was induced by growth in Pipes/FM in shaker flasks as previously described (Schein & Noteborn, 1988). For production of RNAase in fermentor culture, 25 ml of an L-broth culture was used to inoculate 1.2 litre of medium containing (per litre): 5 g of casein enzymic hydrolysate (Sigma), 0.75 g of yeast extract (Difco), 4 g of K_2HPO_4, 1 g of KH_2PO_4, 1 g of NH_4Cl, 2.6 g of K_2SO_4, 0.01 g of $CaCl_2$, 20 g of glycerol (80%; Fluka), 5 ml of 200× trace-salt solution/ml, and about 500 μl of Dow–Corning 1510 silicon antifoam; $MgSO_4$ (to 2 mM) and sodium ampicillin (to 100 mg/l) were added after sterilization. Culture pH was controlled to 7.0 by addition of aq. 10% NH_3; aeration was about 0.9 VVM (dissolved oxygen was maintained to above 20% of saturation). Addition of 0.05% Tween 20 was found to significantly enhance the secretion of the bovine protein.

For RNAase assays, 1 ml samples were centrifuged in an Eppendorf Centrifuge at maximum speed for 5 min. This supernatant is referred to as 'culture supernatant' or simply 'culture medium'. The cell pellet was frozen at −20 °C and later taken up in 100 μl of water containing 0.02 mM-phenylmethanesulphonyl fluoride (PMSF) and 10 mM-EDTA. The resuspended cell pellets were frozen and thawed three times by cycling between liquid N_2 and cold water and centrifuged for 10 min in an Eppendorf centrifuge. This supernatant is the 'freeze–thaw supernatant' (FTS) and comprises the total soluble portion of the cells, including both the cytoplasmic and periplasmic compartments. To measure 'total intracellular RNAase activity' in the samples, the frozen pellet was taken up in 0.5 ml of 8 M-urea/20 mM-Tris/HCl(pH 8)/20 mM-NaCl/0.7% β-mercaptoethanol/0.35% sarcosine and freeze–thawed as described above, then transferred to 10000-M_r cut-off dialysis bags and dialysed overnight against 'renaturation buffer' [50 mM-Tris(pH 8)/100 mM-NaCl/2 mM-EDTA/0.02 mM-PMSF/GSH (3 g/l)/GSSG (0.6 g/l)]. Samples were centrifuged and the supernatant assayed.

Isolation of murine spleen RNAase and bovine RNAase A

The supernatant of a fermentor culture grown at 37 °C was used for the isolation of murine spleen RNAase. PMSF (0.02 mM) was added to all buffers just before use, as well as to the culture supernatant before starting the purification. Culture supernatant (40 ml) was combined with 8 ml of 5 M-NaCl and the sample was passed through a 1 ml column of phenyl-Sepharose (Pharmacia). The column was washed with 2–3 ml of 1 M-NaCl in 20 mM-Tris/HCl, pH 7.5. The flow-through and buffer wash were combined and dialysed against 2 × 1 litre of buffer (20 mM-Tris/HCl, pH 7.5). The dialysed sample was passed through a 1 ml column of DEAE-Sephacel (Pharmacia) equilibrated with the dialysis buffer; the flow-through and buffer washes were combined and applied directly to a 1 ml column of SP-Trisacryl (IBF Biotechnics), which was also equilibrated with the dialysis buffer. The SP-Trisacryl column was then washed with the dialysis buffer, followed by elution with 1.5 ml aliquots of the same buffer containing progressively more NaCl. RNAase activity eluted from the column between 120 and 180 mM-NaCl. The peak active fractions were combined and concentrated to about 0.3 ml in a Centricon 10 (Amicon) filter.

Bovine RNAase was isolated from 400 ml of a fermentor culture supernatant by a procedure modified for larger starting volumes. The culture supernatant was diluted 3-fold with 10 mM-Tris/HCl, pH 7.5, and applied to a 20 ml column of Matrex Blue Gel (Amicon) and the column eluted with a step gradient from 0.05 M- to 1 M-NaCl in 10 mM-Tris/HCl, pH 7.5. RNAase A was eluted at about 200 mM-NaCl. The eluates (less than 10% of the initial volume of culture) were dialysed, passed through a 5 ml DEAE column (as described above) and then purified and concentrated on a 2 ml column of CM-Sephadex G-25 using a step gradient elution like that described for Matrex Blue.

Intracellular soluble bovine RNAase was purified by lysing the frozen cell pellet from about 0.3 litre culture in 100 ml of 10 mM-Tris/HCl (pH 7.5)/10 mM-EDTA/0.02 mM-PMSF with a French press at 3450–6900 kPa (500–1000 lbf/in^2). The lysate was centrifuged for 30 min at 16000 *g* and the supernatant diluted to 400 ml and passed over a 70 ml column of DEAE-Sephacryl. The flow-through and buffer wash of this column were passed over a 2 ml column of CM-Sephadex G-25 (Pharmacia) and the

column was eluted with a step gradient from 50 mM- to 750 mM-NaCl in 20% (v/v) glycerol/10 mM-Tris/HCl, pH 7.5. The eluates were diluted and applied to a small Matrex Blue column and eluted as described above for the supernatant protein.

N-Terminal sequence determination

The murine protein was further concentrated to about 50 μl in the same Centricon, boiled in SDS sample buffer, and applied to a 1%-SDS/17%-(w/v)-polyacrylamide mini-gel. The appropriate band was cut out and eluted overnight into 25 mM-ammonium bicarbonate. The eluates were checked for protein using a silver-stained mini-gel and freeze-dried. Intracellular bovine RNAase was prepared analogously; that from the culture medium was sequenced directly from dialysed CM-Sephadex eluates.

Amino acid sequence determination was done on a protein sequencer model 810 from Dr. Herbert Knauer Wissenschaftliche Geräte (Bad Homburg, Germany), modified for isocratic reversed-phase h.p.l.c. determination of the amino acid phenylthiohydantoin derivatives (Frank, 1988).

Assays

Total protein was determined with the Bradford Coomassie Brilliant Blue assay (Bradford, 1976). As RNAase A reacts with the reagent in this assay with about 1/15th the intensity of BSA and is detected only slightly better with the Lowry protein reagent, final yields of both proteins were estimated from Coomassie Blue-stained mini-gels using Boehringer RNAase A as a standard.

RNAase activity in the supernatant was determined by the generation of acid-soluble radioactivity from a ^{32}P-labelled RNA substrate as previously described (Schein *et al.*, 1990). An RNAase A equivalent is the weight in mg (or μg) of RNAase A that would be needed to solubilize the same amount of RNA as the unknown sample (1 μl of a 0.1 μg/ml solution of RNAase A would degrade 60% of the labelled single-stranded RNA in the assay). The specific activity of the purified murine spleen RNAase in this assay was approximately 30% that of RNAase A (Table 1 below). The values given as μg of RNAase/ml in Fig. 2 (below) have been adjusted for this difference in specific activity.

RESULTS

Screening for RNAase A homologues expressed in murine spleen

The following 31-mer oligonucleotide probe, based on the sequence of the bovine gene for amino acids 40–50 of the RNAase A sequence [which are conserved in almost all the pancreatic protein sequences known, as well as that for the bovine seminal ribonuclease (Beintema *et al.*, 1988*a*)] was chosen:

40 50

-Cys-Lys-Pro-Val-Asn-Thr-Phe-Val-His-Glu

5′-ATGCAAGCCAGTGAACACCTTTGTGCACGAG-3′

3′-TACGTTCGGTCACTTGTGGAAACACGTGCTC-5′

The oligonucleotide (in bold type above) was used to screen Northern blots of total mouse spleen poly(A)+RNA as well as total RNA from the human cell lines Colo and Raji (Fig. 1). The probe detected a unique messenger of about 1 kb in murine spleen poly(A)$^+$ RNA. We therefore screened a λgt11 library made from the spleen RNA preparation. Nine clones isolated (from about 90000 plaques screened) contained an insert of about 750 bp; partial sequences of two and the complete sequence of another two of these isolates were identical with that obtained for the murine RNAase expressed in pancreas (Schüller *et al.*, 1990). We conclude that the same RNAase is expressed in murine spleen as in the pancreas, and protein heterogeneity must come from post-translational modifications (Beintema *et al.*,

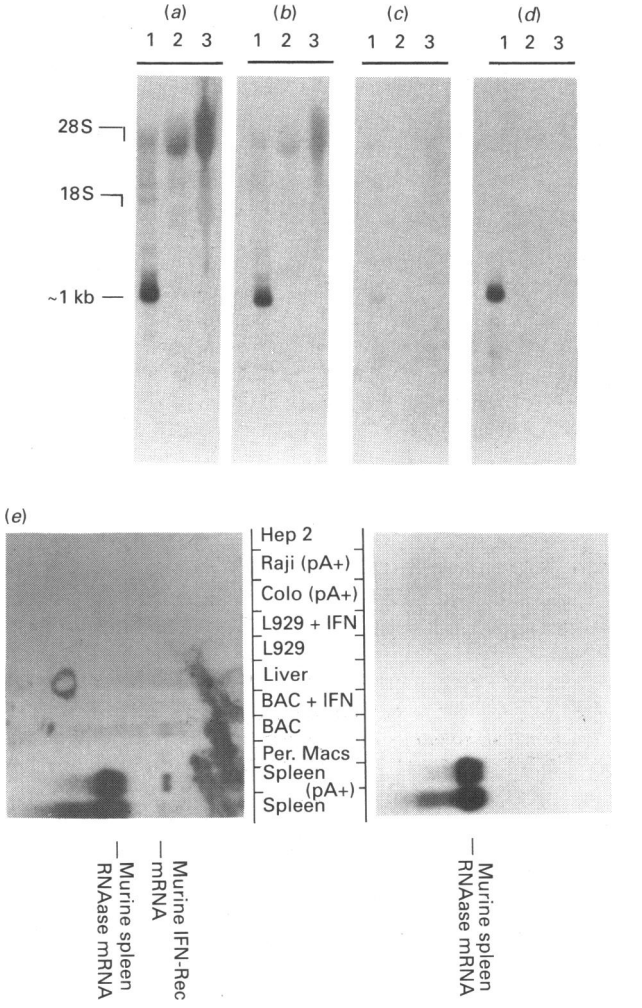

Fig. 1. Northern blotting of RNA from selected human and murine cell lines and from murine tissues using either the oligonucleotide described in the text or the whole murine spleen RNAase cDNA insert

(*a*)–(*d*) Northern blot of 10 μg of poly(A)$^+$ RNA from murine spleen cells (1), and 30 μg of total RNA from the human cell lines Colo205 (2) and Raji (3) hybridized with the 5′-^{32}P-labelled 31-mer oligonucleotide and washed at differing stringencies (A–C) or with random-primed-labelled whole murine spleen RNAase cDNA insert (*d*). The washing conditions were (*a*) 50 °C, (*b*) 55 °C, (*c*) and (*d*) 65 °C. The unique ~ 1 kb mRNA present only in the RNA isolated from murine spleen is indicated. (*e*) A blot of total or poly(A)$^+$ RNA from human (Hep2, Raji, Colo 205) or murine [L929, BAC (Schwarzbaum & Diamond, 1986)] cell lines and from murine liver, spleen, and the adherent cell populations from the peritoneum ('Per. Macs'), was first probed with random-primed-labelled whole murine spleen RNAase cDNA insert (top) and then rehybridized with the (constitutively expressed) gene for the receptor for murine IFN-γ receptor (bottom). The human and murine receptor genes do not cross-hybridize under these conditions. All samples are total RNA (10 μg for the peritoneal macrophage lane; somewhat less for the BAC+IFN-γ; 25 μg for the rest) except for lanes labelled (pA+), which contained 1 (spleen) or 5 μg of poly(A)$^+$ RNA. RNA was also isolated from both murine cell lines after treatment with murine IFN-γ (lanes labelled +IFN). The blots in (*c*), (*d*) and (*e*) were hybridized in 50% (v/v) formamide rather than 20% and washed at 65 °C. Abbreviations; Rec, receptor RNA; Per. Macs, peritoneal-wash macrophages.

1988*b*). The cell type responsible for mRNA production in spleen has not been determined.

The choice of spleen cDNA was fortunate, for RNA from murine liver or from the adherent macrophage population isolated from the peritoneal cavity of DBA mice showed no trace of messenger even after very long exposure of the blot (Fig. 1*e*, top). We also failed to detect a band hybridizing with the whole RNAase gene in total RNA isolated from two different murine cell lines (L929 and BAC). The total murine spleen RNA [before poly(A)$^+$ selection] contained a large amount of lower-M_r RNA, which is probably partially degraded mRNA that hybridized with the probe but did not bind to the poly(U) column. The blot of 1(*e*) was rehybridized with a probe for the murine interferon-γ (IFN-γ) receptor, a constitutively expressed protein (Fig. 1*e*, bottom). All of the murine RNA preparations except that from 'BAC+IFN' (see Fig. 1) showed a band hybridizing with this sequence, indicating that the RNA was intact.

Neither the 31-mer probe nor random-primed probe from the whole murine spleen RNAase gene (Fig. 1) detected any significant mRNA from any of the human cancer cell lines tested (Hep2, Colo205 or Raji).

Expression and secretion of active murine spleen RNAase

We attempted to express the murine RNAase gene in several different expression vectors for *E. coli*, but the highest production achieved was with the trp promotor construction described in the Materials and methods section. The pTrpmuRN+ss plasmid was transformed into several strains of *E. coli*, and the production of RNAase in the culture supernatant was compared in shaker-flask culture. The highest secreted activities were obtained with *DS410* and *lon*$^-$*htpr*$^-$; the latter was chosen as the host strain for fermentor cultivation. Table 1 compares RNAase activity in the supernatant and the supernatant fraction of cells from fermentor cultures of *E. coli lon*$^-$*htpr*$^-$ transformed with the murine spleen RNAase plasmids. When the construction containing the signal sequence of murine spleen RNAase (pTrpmuRN+ss) was used, RNAase activity was found in the culture medium. There was no significant activity secreted from the cells without plasmid nor from cultures transformed with the plasmid from which the signal sequence had been deleted (pTrpmuRN). RNAase activity in the freeze–thaw fraction of the cells for constructions with or without the signal sequence was higher than that of non-transformed *E. coli lon*$^-$*htpr*$^-$ control. Activity in the supernatant was also dependent on induction of the trp promotor; little activity was detected before nutrient depletion of the medium or in L-broth. If cultures were allowed to reach stationary phase, there was little difference in final RNAase activity in the medium, whether the culture was grown at 30 °C or 37 °C, although total RNAase production was higher at the higher temperature (Fig. 2). A significant proportion of the total RNAase activity was either insoluble or inhibited by a dialysable component (Table 1, last column), as the total activity after refolding and dialysis for all cultures was much higher than the soluble RNAase activity. There were, however, no obvious additional bands in Coomassie Blue-stained protein gels of the various cell fractions that would suggest the accumulation of substantial amounts of either precursor or mature protein (results not shown).

Table 1. Relative production and distribution of RNAase activity in fermentor cultures of *E. coli lon*$^-$*htpR*$^-$ transformed with expression plasmids for murine spleen RNAase based on the trp promotor

The samples were all taken after 24 h of growth. Samples were prepared for assay as described in the Materials and methods section; 'FTS' is the 'freeze–thaw supernatant' of the cells and is a measure of the soluble intracellular RNAase activity. RNAase A equivalents are the concentrations of RNAase A that would give approximately the same degree of substrate degradation as the sample after 10 min at 37 °C. ND, not detected.

Plasmid	Growth temp. (°C)	Culture A_{650}	RNAase A equivalents (mg/l) Medium	FTS	Total intra-cellular
None	37	6.3	ND	0.01*	0.5*
pTrpmuRN+ss	30	14.0	0.1	0.2	8
pTrpmuRN+ss	37	13.4	1	1	> 10
pTrpmuRN	37	7.8	ND	0.1	1

* This is the background RNAase activity due to bacterial nucleases.

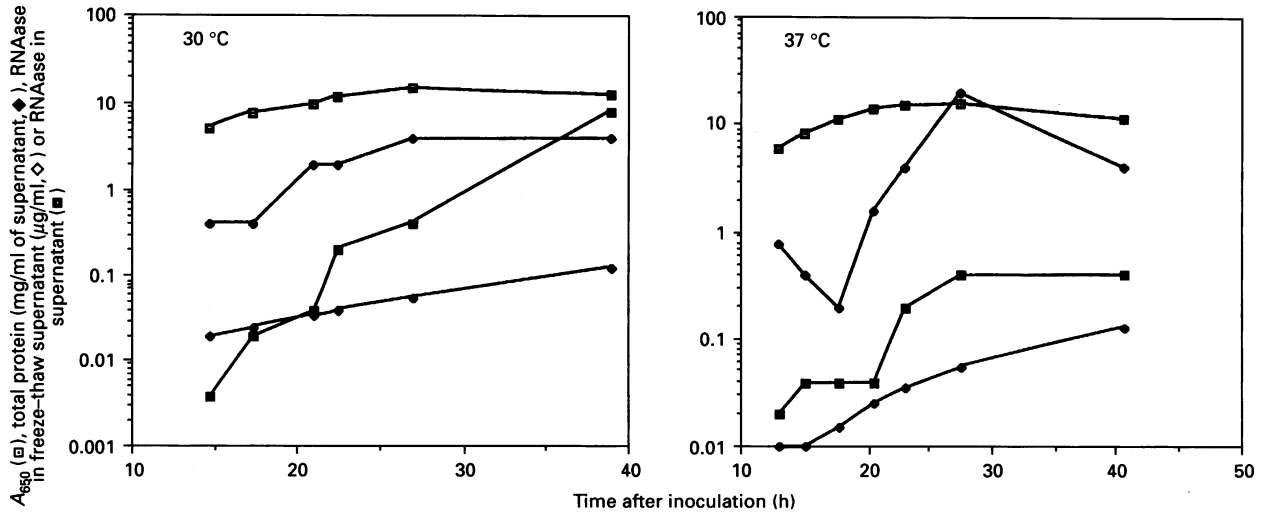

Fig. 2. RNAase activity in the supernatant and freeze–thaw soluble fraction of cells from fermentor cultures of *E. coli lon*$^-$*htpr*$^-$/pTrpMuRN+ss as a function of cell density (culture A_{650}) at two different growth temperatures

Total protein in the culture supernatant was measured with the Coomassie Brilliant Blue assay described by Bradford (1976). The RNAase assay and growth methods are described in the Materials and methods section.

Table 2. Purification of murine spleen RNAase from the supernatant of the 37 °C fermentor culture of pTrpmuRN+ss/*E. coli lon⁻htpR⁻* described in Table 1

'RNAase A equivalents' refers to the amount of RNAase A that would degrade as much RNA in the same amount of time as the sample.

Step	Total protein (mg)	RNAase A equivalents (μg)	Specific activity†
Supernatant	4	40–80	0.01–0.02
Phenyl-Sepharose flow-through	~1	40	0.04
DEAE flow-through	~0.5	40	0.08
SP-trisacryl eluate, after concentration	~0.01*	~3–6*	0.3–0.6

* Estimated from the gel; other concentrations were determined by the Coomassie Brilliant Blue assay described by Bradford (1976); only the peak fractions of the column were concentrated.

† μg of RNAase equivalents/μg of total protein.

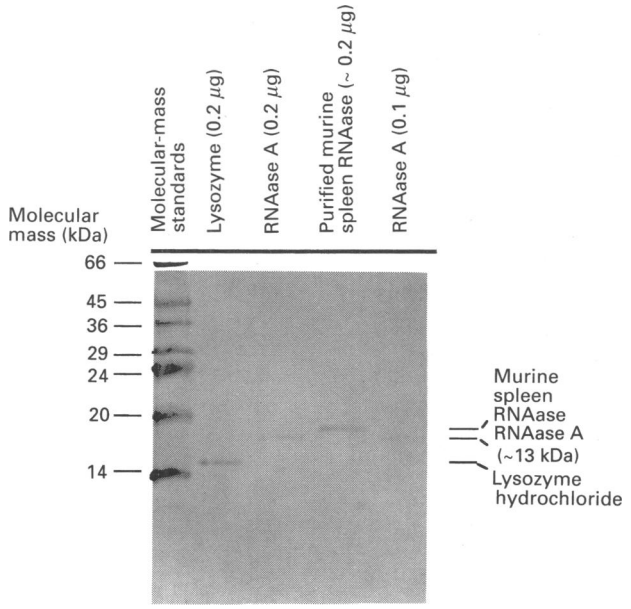

Fig. 3. Coomassie Blue-stained SDS/17% PAGE of the murine spleen RNAase purified as described in Table 1 and the Materials and methods section

In addition to normal molecular-mass markers (standards from Sigma; from top to bottom: BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor and α-lactalbumin), the indicated amounts of lysozyme hydrochloride (Sigma; molecular mass 14.3 kDa) and RNAase A (Boehringer-Mannheim, ~ 12.6 kDa) were used to quantify the protein content of the murine RNAase preparation; 1 or 2 μl of a 0.1 mg/ml solution of the standard proteins or 3 μl of the concentrated murine RNAase was combined with 1 μl of 5×-concentrated sample buffer [60% (v/v) glycerol, 5% (w/v) SDS and 10% (v/v) β-mercaptoethanol], heated 2 min in a boiling-water bath and applied to the gel. Note that the positively charged proteins have a relatively lower mobility on the gel than the markers of similar molecular mass.

Purification of mature murine spleen RNAase from the supernatant of *E. coli* cultures

Table 2 summarizes the purification of the RNAase from 40 ml of the culture supernatant of the 37 °C fermentor culture of *E. coli lon⁻htpr⁻* transformed with the pTrpmuRN+ss plasmid described in Table 1. A Coomassie Blue-stained gel of the purified protein is shown in Fig. 3. The *N*-terminus of the secreted RNAase had the sequence:

Determined: RESAAQKFQRQHMDPDGS(S)?N
Expected (if correctly cleaved): RESAAQKFQRQHMDPDGS S I N...

As acid cleavage between the aspartic acid and the proline residues lowered the yield, the sequencing was stopped six cycles later. The sequence was sufficient to establish that the isolated protein was properly processed murine spleen RNAase and that the eukaryotic signal sequence cleavage site was recognized by *E. coli* processing enzymes.

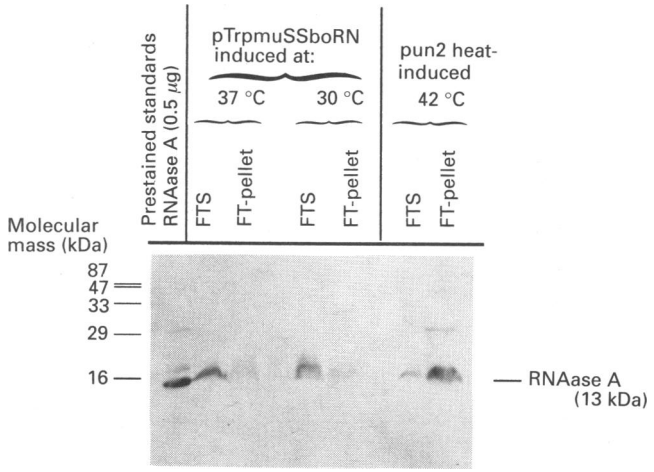

Fig. 4. Immunoblotting of bovine RNAase in the freeze–thaw supernatants (FTS) and pellets (FT-pellet) (prepared as described in the Materials and methods section) of cells from fermentor cultures of the secretion system (*E. coli lon⁻htpr⁻*/pTrpmuSSboRN) at two different growth temperatures as compared with *E. coli lon⁻hptr⁻*/pun2 produced from a cytoplasmic expression system in shaker-flask culture (pun2 heat-induced)

The fermentor cultures were grown in fermentor medium without added casein or yeast extract; the pun2 culture was induced in L-broth. Note that, even at the higher growth temperature, the RNAase produced in the secretion system remains in the soluble fraction of the cells, whereas in the heat-induced system the protein is mostly present in the pellet after freeze–thawing. Immunoblotting was done as previously described (Schein & Noteborn, 1988) using rabbit polyclonal antibodies to RNAase A and peroxidase-labelled goat anti-rabbit IgG. Each lane represents about 80 μl of initial culture {8 μl of either the FTS or the FT-pellet dissolved in FIF buffer [5 M-urea/1% SDS/50 mM-Hepes (pH 7.2)/1% β-mercaptoethanol/150 mM-NaCl (Schein & Noteborn, 1988)] were combined with 2 μl of 5× concentrated sample buffer (see Fig. 3), heated, and applied to the 17%-acrylamide mini-gel}; the RNAase A standard was from Boehringer-Mannheim.

Expression and purification of mature bovine RNAase A from *E. coli* cultures

We next cloned a synthetic gene for mature bovine RNAase A after the murine signal sequence as described in the Materials and methods section. We found that RNAase A was secreted in even greater amounts (~ 5 mg/l) to the culture medium than that observed for the murine protein. When the casein and yeast extract was removed from the fermentor medium, about 1 mg of RNAase A/litre was secreted into the growth medium of a 37 °C culture. There was also about 8 mg/litre in the freeze–thaw

Table 3. Purification of bovine pancreatic RNAase from 400 ml of the supernatant of a 37 °C fermentor culture of pTrpmuSSboRN/*E. coli lon⁻htpR⁻*

Step	Total protein (mg)	RNAase A equivalents (μg)	Specific activity†
Supernatant	40	2000–2500	0.06
Matrex Blue eluates	6.8	1500	0.2
DEAE flow-through	1.6	800–900	0.6
CM-Sephadex eluates	0.7*	600–800	0.9–1.1

* Estimated from the gel.
† μg of RNAase equivalents/μg of total protein.

supernatant of the cells (which was fully active and could be isolated) and less than 1 mg/litre was present in the insoluble fraction of the cells [as detected by immunoblotting with polyclonal anti-(RNAase A) rabbit antibodies and peroxidase-labelled goat anti-rabbit serum (Fig. 4)]. Only about 10% of the activity released by freezing and thawing could be extracted from the cells with low-salt buffers (results not shown); its location thus cannot be defined as strictly 'periplasmic', although it is probably cell-wall-associated. Fig. 4 shows that RNAase A expressed cytoplasmically after heat induction (pun2 lanes) is almost exclusively in inclusion bodies (in the insoluble fraction of the cells after freeze–thaw), whereas in the secretion system the RNAase is soluble, regardless of growth temperature.

The secreted bovine RNAase was purified from 400 ml of fermentor supernatant (Table 3; Fig. 5 left) and the *N*-terminal sequence determined:

Determined: KETAAAKFERQHMDSSTSA?
Expected (if correctly cleaved): KETAAAKFERQHMDSSTSAA...
(The sequencer was stopped after 20 cycles)

Again, the sequence was sufficient to establish that mature bovine pancreatic RNAase had been secreted into the growth medium of the culture.

To determine whether the immunoreactive active RNAase within the cells was also processed, about 10 g of frozen cell paste from the fermentor culture used for the supernatant isolation (equivalent to ~ 0.5 litre of culture) was lysed in a French press and purified in the same way as the activity from the supernatant. About 5 mg of RNAase was isolated that was identical with the secreted form in both its specific activity and its *N*-terminal sequence. Thus the overall yield for this production method is of the order of 12 mg of mature, active and soluble RNAase A/litre.

DISCUSSION

Expression of mRNA for 'pancreatic' RNAase in spleen

We have established that an mRNA identical with that for murine pancreatic RNAase (the sequence for which was unpublished when this work was done) is expressed in relative abundance in murine spleen. Although previous workers (Beintema *et al.*, 1988*a*,*b*) had found pancreatic-type RNAase protein in other tissues, this is the first evidence that the primary sequence of the proteins is also identical. Thus 'pancreatic RNAases', which are generally considered to be involved only in the digestion of foreign nucleic acids in the digestive system, may also degrade messengers in the spleen. This suggests that there may be extracellular RNA to be degraded in this organ.

A mammalian signal sequence directs secretion in *E. coli*

We have demonstrated that the murine spleen RNAase signal sequence can direct the secretion into the growth medium of two mature mammalian proteins. These results are consistent with previous reports (Talmadge *et al.*, 1981; Gray *et al.*, 1985) that at least some eukaryotic signal sequences can direct the processing and export of proteins to the periplasm. However, our protein yields are higher than that reported in either of these papers. In addition, the RNAases are to some extent secreted into the growth medium, greatly facilitating their purification. One additional advantage to secreting the protein is that we can directly

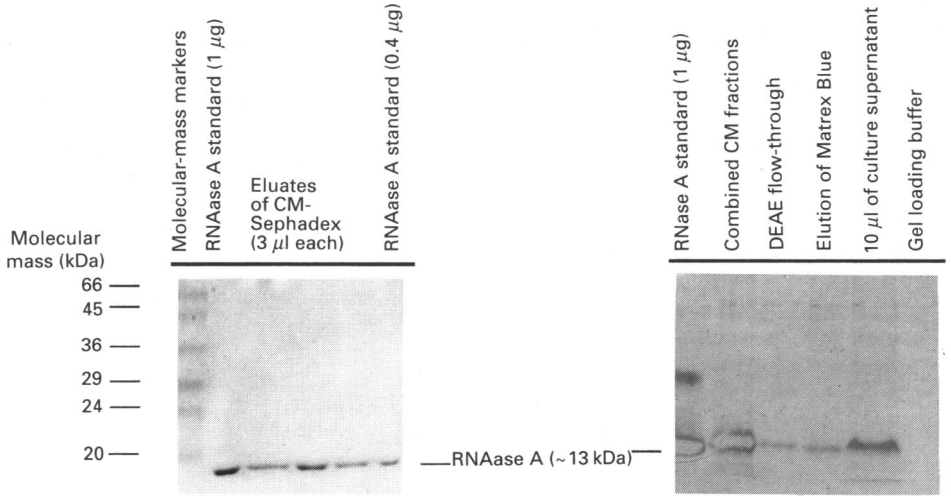

Fig. 5. Coomassie blue-stained (left) and silver-stained (right) SDS/17%-PAGE of samples from the indicated stages of the purification of bovine pancreatic RNAase from the supernatant of a 37 °C fermentor culture of pTrpmuSSboRN in *E. coli lon⁻htpr⁻*

The purification shown on the left was for the 400 ml of supernatant summarized in Table 3. The CM-Sephadex (CM) eluates on the left are each of 1 ml volume and were used to calculate the overall recovery in the purification. The gel to the right shows an initial small-scale purification (results not shown). Samples were prepared as described in Fig. 3.

select for mutant proteins with altered activities in plate assays without needing to lyse the cells.

It is known that many of the components involved in protein synthesis and secretion in *E. coli* resemble their mammalian counterparts (Randall & Hardy, 1989; Poritz *et al.*, 1990). It was previously established that although the amino acid sequences vary greatly, most *E. coli* and eukaryotic signal sequences [but not sequences from Gram-positive bacteria (Sarvas, 1986)] are similar in that they: (1) are 20–30 amino acids long; (2) contain a 'hydrophobic core' of about 12 apolar amino acids (predominantly Ile, Leu, Val, Phe, and Ala); (3) contain small amino acids (Ala, Gly, or Ser) at positions −1 to the start of the mature sequence, Ala, Gly, Ser, Val, or Leu at position −3, and a proline residue is often found between residues −4 and −6; and finally (4) their core region is typically preceded by one to three positively charged residues (Lys, Arg or, rarely, His) (Perlman & Halvorsen, 1983; von Heijne, 1983, 1985).

The signal sequence of the murine spleen RNAase conforms to most of these rules (the hydrophobic core region is underlined and charged residues are in bold face; * marks the cleavage site, and '+' and '−' are charged residues):

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-25                          -1 +1
MGLEKSLILFPLFFLLLGWVQPSLG*RESAAQ...
   -+                         +-
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The only exception is the glutamic acid (E) residue at position −22. The ability of the mammalian sequence to function in *E. coli* suggests that the overall requirements for secretion have been conserved throughout evolution. The last six amino acids of the signal sequence are conserved in all four of the 'pancreatic' RNAases for which the gene sequence is known (Table 4).

As *E. coli* generally does not secrete proteins [as opposed to 'export' of the protein to the perisplasmic space (Stader & Silhavy, 1990)], we have no real explanation for why so much of the protein, between 10 and 50 % of the total RNAase activity, was found in the culture medium. Our growth conditions may stimulate the release of proteins through the outer membrane; growth conditions alter the export and processing human growth hormone (Chang *et al.*, 1989) and the secretion of a HlyA–antibody fusion protein (Holland *et al.*, 1990). Possibly the murine RNAase primary sequence contains elements that facilitate its transport through the 'outer membrane'. This point is difficult to disprove, as there was little intracellular expression of active murine RNAase using the vector in which the signal sequence was deleted (pTrpmuRN). We do know that when large amounts of RNAase A are produced in the bacterial cytoplasm from a heat-shock-induced vector (pun2; Trautwein *et al.*, 1991; Fig. 4), there is no measurable activity in the growth medium (results not shown), although a small amount of active protein is found in the freeze–thaw supernatant of the cells. The intracellular RNAase A in the secretion system is presumably located within the 'periplasm' of the cells, as it is correctly processed and does not seem to be toxic for its host cells (the production phase of the culture continues for at least 15 h as opposed to the 2 h production phase with the cytoplasmic protein after heat induction).

Although it has been suggested that an arginine residue at the start of the mature protein prevents transport (Summers *et al.*, 1989), the murine protein was both correctly cleaved and secreted into the medium. One might suggest that, as the arginine residue is immediately followed by glutamic acid in murine spleen RNAase, the net positive charge is neutralized at the start of the coding sequence. We note that the mature sequence of the secreted pho A protein of *E. coli* also begins with an arginine residue that is followed after two residues by a glutamic acid residue (Gierasch, 1989). It is possible that the glutamic acid residue at position 4 of the signal sequence forms a salt bridge with this arginine residue; however, in the known sequences for pancreatic RNAase signal sequences, this glutamic acid is not conserved (Table 4). Further, this residue is also present in the rat pancreatic RNAase gene, which has three amino acids more at the start of the mature protein which are believed to come from

Table 4. Comparison of the coding and signal sequences of the pancreatic RNAase genes from bovine (BOVPRIBN), rat (RATPRNASE) and murine (MUPRNASE) sequences (the murine spleen and murine pancreatic genes have the same coding sequence) and the closely related bovine seminal RNAase (BOVSEMRNASE)

The alignment was done using the CLUSTAL program (Higgins & Sharp, 1988). Key to symbols: *, match across all sequences; ., conservative substitutions.

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Gene             Signal sequence                 Coding sequence

> BOVSEMRNASE    MAL-KSLVVLPLLVLVLLLVRVQPSLG---KESAAAKFERQHMDSGNSPSSSSNYCNLMM
> BOVPRIBN       MAL-KSLVLLSLLVLVLLLVRVQPSLG---KETAAAKFERQHMDSSTSAASSSNYCNQMM
> RATPRNASE      MGLEKSLFLFSLLV--LVLGWVQPSLGGESRESSADKFKRQHMDTEGPSKSSPTYCNQMM
> MUPRNASE       MGLEKSLILFPLFF--LLLGWVQPSLG---RESAAQKFQRQHMDPDGSSINSPTYCNQMM
                 *.* *** ...*.   *.* .******   .*..*.**.*****..... .*..*** **

> BOVSEMRNASE    CCRKMTQGKCKPVNTFVHESLADVKAVCSQKKVTCKNGQTNCYQSKSTMRITDCRETGSS
> BOVPRIBN       KSRNLTKDRCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSITDCRETGSS
> RATPRNASE      KRQGMTKGSCKPVNTFVHEPLEDVQAICSQGQVTCKNGRNNCHKSSSTLRITDCRLKGSS
> MUPRNASE       KRRDMTNGSCKPVNTFVHEPLADVQAVCSQENVTCKNRKSNCYKSSSALHITDCHLKGNS
                  .  .*...**********.*.**.*.*** .*.*** ..**..* *.. ****. .*.*

> BOVSEMRNASE    KYPNCAYKTTQVEKHIIVACGGKPSVPVHFDASV
> BOVPRIBN       KYPNCAYKTTQANKHIIVACEGNPYVPVHFDASV
> RATPRNASE      KYPNCDYTTTDSQKHIIIACDGNPYVPVHFDASV
> MUPRNASE       KYPNCDYKTTQYQKHIIVACEGNPYVPVHFDATV
                 *****.*.**. .****.**.*.* ******* *
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a duplication event (MacDonald *et al.*, 1982). We found that bovine RNAase A is better secreted than its murine counterpart; this may be due to its *N*-terminal sequence starting with a lysine rather than an arginine residue. However, there are many other reasons that could account for the difference in yield.

The amounts of secreted RNAase produced with the pTrpMuRN+ss or the pTrpmuSSboRN plasmid are higher than those reported for the secretion of bovine RNAase A from either *E. coli* (Presnell, 1988) or *Bacillus* (Vasantha & Filpula, 1989) vectors based on prokaryotic signal sequences. Significantly higher yields have been reported for secretion of mammalian–bacterial fusion proteins (Uhlén & Abrahmsén, 1989) from *E. coli*. However, the product must be treated after isolation to remove the bacterial portion.

Most secretion vectors for bacteria have coupled the gene for a mammalian protein to a signal sequence isolated from a prokaryotic protein. This approach has been only partially successful (Schein, 1989; Denèfle *et al.*, 1989), as the sequence of the mature protein also affects its ability to be transported through the bacterial cell wall (von Heijne, 1986; Schein *et al.*, 1986). The observed variability of signal sequences has led to the suggestion that signal sequences have co-evolved with the proteins transported to optimize the ability of the combined product to be properly transported and processed (Gierasch, 1989). Our results demonstrate that this eukaryotic leader can function very efficiently in *E. coli*. The original signal sequence of a mammalian protein should be tested for its ability to direct secretion, especially in cases where problems arise in the processing of fusions with bacterial signal sequences.

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REFERENCES

Baker, T. A., Grossman, A. D. & Gross, C. A. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 6779–6783
Beintema, J. J., Schüller, C., Irie, M. & Carsana, A. (1988*a*) Prog. Biophys. Mol. Biol. **51**, 165–192
Beintema, J. J., Blank, A., Schieven, G. L., Dekker, C. A., Sorrentino, S. & Libonati, M. (1988*b*) Biochem. J. **255**, 501–505
Benner, S. A. & Allemann, R. K. (1989) Trends Biochem. Sci **14**, 396–397
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Chang, J. Y.-H., Pai, R.-C., Bennett, W. F. & Bochner, B. R. (1989) Biochem. Soc. Trans. **17**, 335–337
Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
Denèfle, P., Kovarik, S., Ciora, T., Gosselet, N., Bénichou, J.-C., Latta, M., Guinet, F., Ryter, A. & Mayaux, J.-F. (1989) Gene **85**, 499–510
Deutscher, M. P. (1988) Trends Biol. Sci. **13**, 136–139
Frank, G. (1988) in Methods in Protein Sequence Analysis (Wittmann-Liebold, B., ed.), pp. 116–121, Springer Verlag, Berlin and New York
Gierasch, L. M. (1989) Biochemistry **28**, 923–930
Goff, S. A., Casson, L. P. & Goldberg, A. L. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 6647–6651
Gray, G. L., Baldridge, J. S., McKeown, K. S., Heyneker, H. L. & Chang, C. N. (1985) Gene **39**, 247–254
Hartley, R. W. (1988) J. Mol. Biol. **202**, 913–915
Haugg, M., Holliger, K. P., Hemmi, S. & Schein, C. (1990) (Abstr.) Experentia **46**, A15
Heinemann, U. & Hahn, U. (1989) Top. Mol. Struct. Biol. **10**, 111–141
Hemmi, S., Peghini, P., Metzler, M., Merlin, G., Dembic, Z. & Aguet, M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9901–9905
Higgins, D. G. & Sharp, P. M. (1988) Gene **73**, 237–244
Holland, I. B., Kenny, B., Steipe, B. & Plückthun, A. (1990) Methods Enzymol. **182**, 132–143
MacDonald, R. J., Stary, S. J. & Swift, G. H. (1982) J. Biol. Chem. **257**, 14582–14585
Nambiar, K. P., Stackhouse, J., Presnell, S. R. & Benner, S. A. (1987) Eur. J. Biochem. **163**, 67–71
Perlman, D. & Halvorsen, H. O. (1983) J. Mol. Biol. **167**, 391–409
Poritz, M. A., Bernstein, H. D., Strub, K., Zopf, D., Wilhelm, H. & Walter, P. (1990) Science **250**, 1111–1117
Presnell, S. R. (1988) Ph.D. Thesis, Harvard University
Randall, L. L. & Hardy, S. J. S. (1989) Science **243**, 1156–1159
Rink, H., Liersch, M., Sieber, P. & Meyer, F. (1984) Nucleic Acids Res. **12**, 6369–6387
Roozen, K. J., Fenwick, R. G. & Curtiss, R. (1971) J. Bacteriol. **107**, 21–33
Sarvas, M. (1986). Curr. Top. Microbiol. Immunol. **125**, 103–125
Schapiro, R., Harper, J. W., Fox, E. A., Jansen, H.-W., Hein, F. & Uhlmann, E. (1988) Anal. Biochem. **175**, 450–461
Schein, C. H. (1989) Bio/Technology **7**, 1141–1149
Schein, C. H. & Noteborn, M. H. M. (1988) Bio/Technology **6**, 291–294
Schein, C. H., Kashiwagi, K., Fujisawa, A. & Weissmann, C. (1986) Bio/Technology **4**, 719–725
Schein, C. H., Haugg, M. & Benner, S. A. (1990) FEBS Lett. **270**, 229–232
Schüller, C., Nijssen, H. M. J., Kok, R. & Beintema, J. J. (1990) Mol. Biol. Evol. **7**, 29–44
Schwarzbaum, S. & Diamond, B. (1985) J. Immunol. **135**, 141–146
Stader, J. A. & Silhavy, T. J. (1990) Methods Enzymol. **185**, 166–187
Summers, R. G., Harris, C. R. & Knowles, J. R. (1989) J. Biol. Chem. **264**, 20082–20088
Talmadge, K., Brosius, J. & Gilbert, W. (1981) Nature (London) **294**, 176–178
Trautwein, K., Holliger, K. P., Stackhouse, J. & Benner, S. A. (1991) FEBS Lett. **281**, 275–277
Uhlén, M. & Abrahmsén, L. (1989) Biochem. Soc. Trans. **17**, 340–341
Vasantha, N. & Filpula, D. (1989) Gene **76**, 53–60
von Heijne, G. (1983) Eur. J. Biochem. **133**, 17–21
von Heijne, G. (1985) J. Mol. Biol. **184**, 99–105
von Heijne, G. (1986) J. Mol. Biol. **192**, 287–290

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