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The 2′-5′ RNA Ligase of *Escherichia coli*

PURIFICATION, CLONING, AND GENOMIC DISRUPTION*

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An RNA ligase previously detected in extracts of *Escherichia coli* is capable of joining *Saccharomyces cerevisiae* tRNA splicing intermediates in the absence of ATP to form a 2′-5′ phosphodiester linkage (Greer, C., Javor, B., and Abelson, J. (1983) *Cell* 33, 899–906). This enzyme specifically ligates tRNA half-molecules containing nucleoside base modifications and shows a preference among different tRNA species. In order to investigate the function of this enzyme in RNA metabolism, the ligase was purified to homogeneity from *E. coli* lysate utilizing chromatographic techniques and separation of proteins by SDS-polyacrylamide gel electrophoresis. A single polypeptide of approximately 20 kilodaltons exhibited RNA ligase activity. The amino terminus of this protein was sequenced, and the open reading frame (ORF) encoding it was identified by a data base search. This ORF, which encodes a novel protein with a predicted molecular mass of 19.9 kDa, was amplified from *E. coli* genomic DNA and cloned. ORFs coding for highly similar proteins were detected in *Methanococcus jannaschii* and *Bacillus stearothermophilus*. The chromosomal gene encoding RNA ligase in *E. coli* was disrupted, abolishing ligase activity in cell lysates. Cells lacking ligase activity grew normally under laboratory conditions. However, moderate overexpression of the ligase protein led to slower growth rates and a temperature-sensitive phenotype in both wild-type and RNA ligase knockout strains. The RNA ligase reaction was studied *in vitro* using purified enzyme and was found to be reversible, indicating that this enzyme may perform cleavage or ligation *in vivo*.

RNA ligases have been detected in organisms throughout all major divisions of the phylogenetic spectrum. Specific metabolic functions, however, have been assigned to few of these enzymes. T4 RNA ligase, for example, repairs nicks introduced into the anticodon loops of tRNAs in phage-restrictive *Escherichia coli* strains upon T4 infection (1); the eukaryotic tRNA splicing RNA ligase joins tRNA half-molecules resulting from endonucleolytic removal of introns from eukaryotic tRNA precursors (2–4), and an RNA ligase is apparently responsible for the joining of guide RNA molecules to mRNA during RNA editing in kinetoplastids (5). At least two other species of RNA ligase, the metazoan-specific "animal pathway" RNA ligase (6) and the archaeal stable RNA splicing ligase (7), have been identified, but the precise *in vivo* substrate(s) and function(s) of these enzymes have yet to be determined.

The existence of RNA ligase in bacteria in the absence of bacteriophage infection was discovered in this laboratory in 1983 (8). An activity capable of performing the ligation step of eukaryotic tRNA splicing was detected in extracts of a wide range of bacteria including members of the Alpha and Gamma subdivisions of proteobacteria, green sulfur bacteria, and low G + C content Gram-positive bacteria (8). In extracts of *E. coli* the ligase activity had a substrate specificity restricted to 4 of the 10 *Saccharomyces cerevisiae* tRNA splicing intermediates: tRNA Tyr, Phe, Lys_2, and Trp half-molecules. The reaction mechanism of the *E. coli* RNA ligase apparently differed from that of known RNA ligases since it did not require a nucleoside triphosphate cofactor, and the product contained an unusual 2′-5′ phosphodiester bond at the ligated junction (8).

The discovery of RNA ligase in *E. coli* implies the existence of a novel form of bacterial RNA processing. No intervening sequences of the type found in eukaryotic nuclear or archaeal tRNA genes (which require enzymatic excision and religation) occur in known bacterial tRNA genes, although self-splicing introns are found in the tRNA genes of certain cyanobacteria (9) and some proteobacteria (10). In fact, no introns of any kind are found in the full genomic complement of tRNA genes in *E. coli, Mycoplasma capricolum,* and *Hemophilus influenzae* (11–13). To date no RNA processing event that would require the action of an RNA ligase enzyme has been observed to occur in any bacteria, indicating that elucidation of the substrate and function of the 2′-5′ RNA ligase should reveal a previously unknown step in bacterial RNA metabolism.

A genuine *in vivo* function for the *E. coli* RNA ligase activity observed *in vitro* is suggested by the occurrence of 2′-5′ linkages in native *E. coli* RNA. Several forms of 2′-5′-linked oligoadenylates have been detected in acid-soluble extracts of *E. coli* (14). The most abundant species of these oligoribonucleotides observed was 2′-5′-linked adenosine dinucleotide 3′-monophosphate, which was estimated to exist at an intracellular concentration over 100 nM. This dinucleotide could not be an intermediate in oligoadenylate synthesis, and it has been suggested that this species may be a degradation product of RNAs containing individual 2′-5′ bonds among standard 3′-5′ linkages (14). Since the formation of 2′-5′ linkages is not catalyzed by RNA polymerase, these bonds must be added in a post-transcriptional processing event by an enzyme such as the 2′-5′ RNA ligase.

In order to study the function and mechanism of the 2′-5′ RNA ligase, the enzyme was purified to homogeneity from *E. coli* extracts. A single polypeptide was found to contain RNA ligase activity. This protein was partially sequenced, and the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D26562 and Swissprot P37025.

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ORF[1] encoding it was identified. This ORF was amplified from *E. coli* genomic DNA and cloned. The chromosomal locus containing the ligase gene was disrupted, abolishing ligase activity in cellular extracts. Cells completely lacking ligase activity grow similarly to the parent strain. *E. coli* strains overexpressing recombinant 2′-5′ RNA ligase are temperature-sensitive for growth. The ligase reaction was also studied using purified enzyme and was found to be reversible *in vitro*.

EXPERIMENTAL PROCEDURES

Strains—E. coli strains utilized were *HB101*, XL1-Blue (Novagen), and *HSD947*, DH10B, RecA+.

Preparation of RNA Substrates for Ligation—RNA polymerase III transcription of pre-tRNA^{Tyr} in yeast extract was performed by the method of Evans and Engelke (15), using extracts prepared as described. The template for Pol III transcription of pre-tRNA^{Tyr} was supercoiled pYSUP6 (16). T7 RNA polymerase transcription was performed as described by Sampson and Saks (17). pre-tRNA^{Phe} was transcribed from the artificial pre-tRNA gene of Reyes cloned into pUC13 (18). The template for pre-tRNA^{Tyr} T7 transcription was created by PCR amplification from pYSUP6, adding a canonical T7 promoter at the 5′ end and a *Bst*NI restriction site at the 3′ end of the pre-tRNA, as well as altering the acceptor stem base pairs: $C^{1} \rightarrow G$, $T^{2} \rightarrow G$, $A^{87} \rightarrow C$, and $G^{88} \rightarrow C$ to improve transcription yields. This construct was cloned into the pBlueScript vector (Stratagene). tRNA substrates were modified by incubation at 24 °C for 45 min in the same extract and reaction conditions as were used for Pol III transcription. tRNA precursors were cleaved as described by Peebles *et al*. (2), using partially purified *S. cerevisiae* tRNA splicing endonuclease fractions from the hydroxyapatite step or later as described in the Rauhut protocol (2, 19).

All RNA transcripts were gel purified by polyacrylamide gel electrophoresis (PAGE) in 1 × TBE (Tris borate EDTA buffer), 7 M urea and visualized by autoradiography. RNAs were eluted from crushed gel slices in 0.6 M NH_4OAc, 2 mM EDTA, 0.005% Nonidet P-40 at room temperature with vortexing for 20 min. RNA eluates were extracted with phenol/chloroform (1:1, pH 4.5) and chloroform and then ethanol-precipitated in the presence of glycogen and resuspended in distilled water.

RNA Ligation Assay—tRNA half-molecules were annealed for ligation in 2 × ligation buffer (1 × = 40 mM HEPES, pH 7.8, 3 mM $MgCl_2$, 2 mM spermidine, 5% glycerol) by heating to 85 °C and slowly cooling to 30 °C over 20 min. Ligation reactions were typically performed in 4 μl of 1 × ligation buffer with 250 nM annealled tRNA half-molecules substrate. *E. coli* lysates or purification fractions (typically 1 μl) were added and incubated for 2–5 min at 30 °C. Reactions were stopped by the addition of 1.5 mg/ml proteinase K, 0.04% SDS, 5 mM EDTA, and 0.001 mg/ml *E. coli* total RNA for 20 min at 30 °C. An equal volume of 90% deionized formamide plus tracking dyes was added, and reactions were incubated at 65 °C for 5 min before cooling on ice and loading onto 8% acrylamide, 7 M urea, 1 × TBE gels for electrophoresis. RNA bands were visualized by autoradiography or by exposure to a Molecular Dynamics storage phosphor plate for quantification and analysis on a PhosphorImager using Imagequant software.

Large-scale Cell Growth—Cells were grown in media containing 16 g/liter Bacto-tryptone, 6 g/liter yeast extract, 50 mM phosphate buffer, pH 6.8, 4 g/liter $(NH_4)_2SO_4$, 0.5 g/liter $MgSO_4$ (anhydrous), and 20 ml/liter glycerol with aeration and constant feeding of 2 × media for 24 h. 5% methanol (which was found to induce ligase activity in extracts to approximately 1.5 × that found in extracts of uninduced cells) was added to media 2 h before harvest, and harvested cell paste was stored at −70 °C.

E. coli RNA Ligase Purification—All purification procedures were performed in extraction buffer (EB) containing 10% glycerol, 40 mM HEPES, pH 7.8, 2 mM EDTA, 1 mM Pefabloc (Boehringer Mannheim), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and stated concentrations of KCl. 200 g to 1 kg of HB101 cells frozen at −70 °C were thawed in a final volume of 1.6 ml/g cells of EB plus 125 mM KCl and dispersed with a hand blender. The cell suspension was sonicated on ice with a Branson Sonifier large tip at 80% power for 15 1-min periods with cooling on ice between. Cell lysate was centrifuged at 35,000 rpm in a Beckman 45Ti rotor at 3 °C for 1 h. The supernatant was mixed batchwise with 500 ml of DEAE-Sepharose CL-6B (equilibrated in EB + 125 mM KCl) for 30 min on ice. All subsequent steps were performed at 4 °C. The DEAE/extract slurry was poured into a column, and the DEAE effluent was loaded at approximately 1 ml/min onto a 200-ml bed of cellulose phosphate P11 (Whatman) equilibrated in EB + 125 mM KCl.

The phosphocellulose column was washed extensively with EB + 500 mM KCl and eluted with a linear KCl gradient from 0.5 to 1.5 M in EB. Peak ligase activity fractions were pooled, diluted to 150 mM KCl, and loaded onto 25 ml of heparin Hyper-D (Biosepra) column. Ligase activity was eluted from the heparin column with a 150–700 mM KCl gradient in EB, and peak fractions were pooled. The heparin pool was diluted to 150 mM KCl in EB and loaded onto a 15-ml bed column of *E. coli* tRNA linked to a Sepharose support. tRNA Sepharose (3 mg of RNA per ml of gel) was prepared as described by Rauhut *et al*. (19) but using *E. coli* tRNA (Sigma) instead of yeast tRNA. Ligase was eluted from this column using a gradient from 150 mM to 1 M KCl in EB, followed by a 2 M KCl wash. The ligase eluted in a broad peak beginning around 400 mM KCl, trailing into the 2 M wash.

Active fractions were pooled, diluted to 150 mM KCl, loaded onto a 2-ml column of heparin hyper-D, and eluted with EB + 600 mM KCl. This concentrated activity pool was then passed through a 115-ml bed of Superdex 75 gel filtration medium (Pharmacia Biotech Inc.) at a flow rate of 0.04 ml/min. Peak active fractions were diluted to 150 mM KCl in EB and loaded onto a column of *S. cerevisiae* tRNA linked to Sepharose (19). Ligase activity was eluted with a 150–800 mM KCl gradient. Note: For the 4-kg preparative purification mentioned under "Results," the purification procedure followed was the same as described above except that an *S. cerevisiae* tRNA affinity column was substituted for the initial *E. coli* tRNA step, and the tRNA pool was passed through Superdex 75 twice, sequentially.

SDS-PAGE, Elution, and Renaturation—Electrophoresis for preparative elution, as well as all analytical protein electrophoresis, was performed on 16% acrylamide/piperazine diacrylamide (2.67% cross-linking) gels under the conditions of Schagger *et al.* (20). Bands were visualized by staining with 0.3 M $CuCl_2$ for 15 min at room temperature, excised, and destained by successive washes in 0.25 M Tris-HCl, pH 8.8, 0.25 M EDTA, and distilled water. Excised bands were crushed and extracted overnight at 4 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, and 0.1 mg/ml acetylated bovine serum albumin (Life Technologies, Inc.). Eluted protein was precipitated with 4 volumes of cold acetone, incubated for 30 min at 0 °C, and pelleted by centrifugation at 15,000 rpm, 4 °C, for 20 min in an SS-34 rotor. Pellets were resuspended in EB containing 6 M guanidine HCl and microdialyzed *versus* EB plus 125 mM KCl at 4 °C. Silver staining of SDS-PAGE gels was performed according to the method of Wray *et al.* (21).

PCR—PCR was performed in 1 × PCR buffer with Mg^{2+} (Boehringer Mannheim), 0.4 mM dATP, dCTP, dGTP, and dTTP, 10 μM each oligonucleotide primer, and 0.7 units/μl *Taq* DNA polymerase. For reactions using degenerate primers, 400 μM total primers was used. For labeled probe production, 300 μM dCTP, dGTP, and dTTP were used in the presence 1 μM [α-^{32}P]dATP (Amersham Corp., 3000 Ci/mmol).

Mapping Blot Hybridization—Labeled PCR product was heated to 95 °C in 6 × SSC, 10 mM phosphate buffer, pH 6.8, 1 mM EDTA, 0.5% SDS, 100 μg/ml sonicated calf thymus DNA, and 0.1% dry milk, and then cooled on ice. The probe was hybridized to an *E. coli* Gene Mapping Membrane (PanVera) in the same buffer at 37 °C, overnight. The blot was washed with 6 × SSC, 0.1% SDS, and with 5 × SSC at room temperature and then exposed to film for autoradiography.

Genomic Knockout—The *E. coli* RNA ligase gene was cloned by PCR amplification from HB101 genomic DNA using primers corresponding to sequences located 213 bp upstream of the 5′ end and at the exact 3′ end of the predicted ORF, adding *Eco*RI and *Bam*HI restriction sites, respectively. Gene disruption was performed according to the method of Hamilton *et al.* (22). For genomic disruption, the Kan^{r} Genblock (Pharmacia) cassette was inserted into a *Pst*I site located at +144 bp in the ligase ORF clone. A ts plasmid bearing the interrupted ligase gene was created as follows. The origin-bearing *Pvu*II fragment of pFC20 (23) (bp 950-3838) was cloned into pMAK705 (22), replacing the *Pvu*II fragment spanning bp 5458–3722. The Kan cassette-interrupted ligase gene was then subcloned into the *Eco*RI-*Bam*HI sites of the pMAK polylinker in this construct to create pTSIL. pTSIL was transformed into a RecA^{+} strain of *E. coli* (HSD947) and plated at 43 °C on chloramphenicol to select for cointegration into the chromosome. Plasmid cointegrants were resolved by growth for several generations in liquid culture at 30 °C, at which temperature the plasmid replicon interferes with chromosomal replication. The chromosomal recA locus was then disrupted by P1 phage transduction to prevent further homologous recombination

[1] The abbreviations used are: ORF, open reading frame; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PNK, polynucleotide kinase; pre-tRNA, intron-containing tRNA precursor; bp, base pair(s); ts, temperature-sensitive; kbp, kilobase pair(s).

events. Cointegrant cultures were pelleted and resuspended in 0.1 M $MgSO_4$, 5 mM $CaCl_2$ and mixed with an equal volume of P1 lysate JC10240[2] for 20 min at 30 °C and plated on LB + tetracycline, chloramphenicol, and kanamycin to select for transductants. P1 transductants were screened for resolution of the ts plasmid by streaking separately on chloramphenicol, kanamycin, and nonselective LB plates at 43 °C.

RESULTS

E. coli 2′-5′ RNA Ligase Requires Base Modifications in tRNA Substrates—In previous studies of bacterial RNA ligase, activity had been assayed using substrates derived from tRNA precursors (pre-tRNAs) transcribed either *in vivo* in yeast or by endogenous RNA polymerase III activity in an *S. cerevisiae* extract (8). It was subsequently reported that pre-$tRNA^{Phe}$ transcribed from an artificial $tRNA^{Phe}$ gene by T7 RNA polymerase is an excellent substrate for the *S. cerevisiae* tRNA splicing enzymes (18), and this was also tested for ligation by *E. coli* RNA ligase. $tRNA^{Phe}$ half-molecules produced by digestion of T7-transcribed pre-$tRNA^{Phe}$ with *S. cerevisiae* tRNA splicing endonuclease were, however, poorly ligated in *E. coli* extracts (data not shown). Since $tRNA^{Phe}$ half-molecule substrates produced *in vivo* in yeast had previously been demonstrated to be ligated in *E. coli* extract (8), a requirement for some modification of substrate transcripts was implied.

In order to test the putative modification requirement, an *S. cerevisiae* $tRNA^{Tyr}$ gene was cloned under the control of a T7 RNA polymerase promoter. $tRNA^{Tyr}$ half-molecules derived from unmodified transcripts of this gene were poor substrates for the *E. coli* ligase, although they were utilized effectively by the *S. cerevisiae* tRNA splicing ligase. Fig. 1*A* shows that when pre-$tRNA^{Tyr}$ transcripts (*lane M*) were incubated in the same *S. cerevisiae* extracts utilized for RNA polymerase III transcription, $tRNA^{Tyr}$ half-molecules derived from them (*lane 1*) became substrates for the *E. coli* RNA ligase (*lane 4*). *E. coli* RNA ligase ligation of half-molecules derived from pre-$tRNA^{Tyr}$ modified *in vitro* produced a product of equivalent size to that produced by *S. cerevisiae* tRNA ligase or T4 RNA ligase and polynucleotide kinase (PNK) (*lanes 2–3*), although the bacterial enzyme is less efficient at joining these substrates. tRNA half-molecule substrates derived from modified T7 RNA polymerase transcripts were utilized by the *E. coli* RNA ligase as efficiently as yeast polymerase III transcripts, as quantified in a substrate titration experiment shown in Fig. 1*B*. This was presumably due to the formation of modified nucleosides in these transcripts, as observed by two-dimensional thin layer chromatography (TLC) of nuclease digests of substrate transcripts (data not shown). The identity and location of these modifications were not investigated further. The presence of a 2′-5′ linkage in ligated tRNA products was also observed by nuclease digestion and TLC (not shown), confirming that the reaction had been catalyzed by the previously described *E. coli* 2′-5′ ligase activity.

Pre-$tRNA^{Phe}$ T7 RNA polymerase transcripts modified by incubation in yeast extract were also used to produce substrates for ligation by the *E. coli* enzyme, although modified $tRNA^{Tyr}$ half-molecules were preferred as substrates by a factor of 2–3-fold over modified $tRNA^{Phe}$ half-molecules (Fig. 1*C*). Because $tRNA^{Tyr}$ half-molecules produced by endonuclease digestion of T7-transcribed, yeast extract-modified pre-$tRNA^{Tyr}$ were the most active substrates tested for ligation by the *E. coli* RNA ligase, they were chosen as substrates for quantitative assays of ligase activity during subsequent procedures.

Purification of the Ligase—A protocol for purification of the *E. coli* RNA ligase was developed and followed as detailed under "Experimental Procedures." A quantitative profile of RNA ligase activity throughout the purification procedure is given in Table I. The complexity of the protein population at each purification step was assayed by SDS-PAGE and silver staining and is shown in Fig. 2. Briefly, cells were disrupted by sonication, and the lysate (Fig. 2, *lane 1*) was subjected to

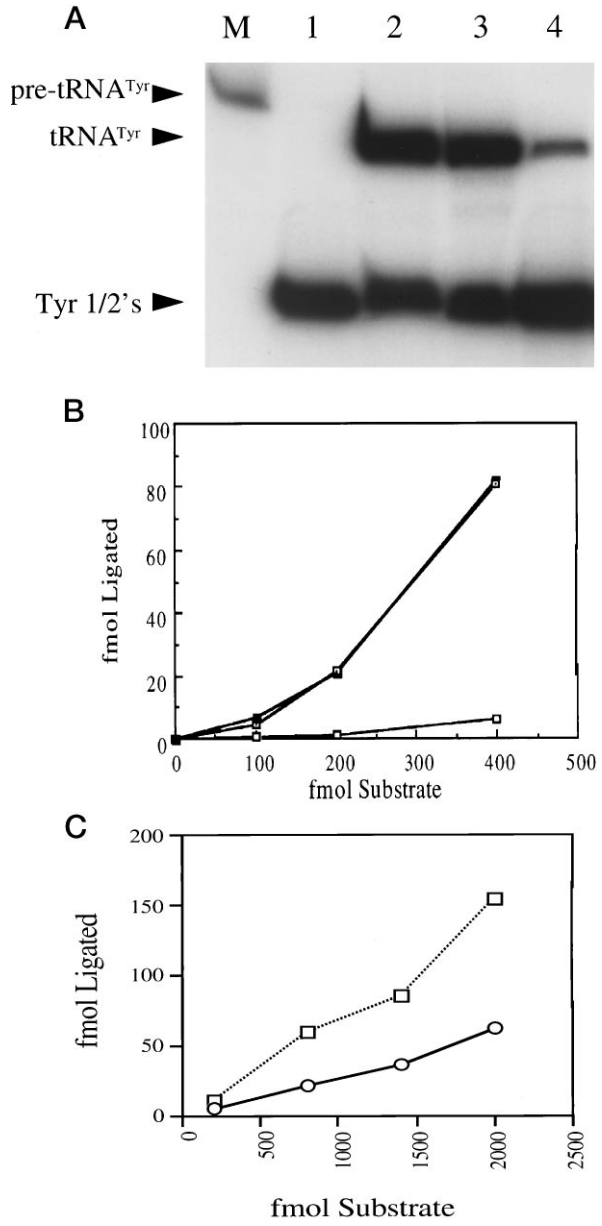

FIG. 1. **Ligation of tRNA half-molecule substrates.** *A*, ligation of $tRNA^{Tyr}$ half-molecules derived from T7-transcribed, yeast-modified $ptRNA^{Tyr}$ as described under "Experimental Procedures." Reactions including 0.35 pmol of half-molecules were incubated at 30 °C for 2 min and stopped to observe ligation rate. Reactions were supplemented with H_2O (*lane 1*), T4 RNA ligase, PNK, and ATP (*lane 2*), *S. cerevisiae* tRNA ligase and ATP (*lane 3*), and *E. coli* extract (*lane 4*). The location of pre-$tRNA^{Tyr}$ (*lane M*), Tyr halves, and ligated products are indicated. *B*, titration of $tRNA^{Tyr}$ half-molecule substrates for ligation in *E. coli* extract. The indicated molar amounts of $tRNA^{Tyr}$ half-molecules were incubated with 1 μl of *E. coli* lysate each at 30 °C for 15 min. Half-molecule substrates were derived from pre-$tRNA^{Tyr}$: transcribed by T7 RNA polymerase (⊡), transcribed by yeast RNA polymerase III in yeast extract (□), or transcribed by T7 polymerase and subsequently incubated in yeast extract (■). *C*, titration of $tRNA^{Tyr}$ and $tRNA^{Phe}$ half-molecule substrates for ligation. $tRNA^{Phe}$ half-molecules (○) and $tRNA^{Tyr}$ half-molecules (□) were derived from T7-transcribed, yeast-modified pre-tRNAs. The indicated molar amounts of substrate were incubated with 0.5 μl of partially purified *E. coli* RNA ligase (cellulose phosphate pool, see below) for 2 min at 30 °C.

[2] M. Saks, personal communication.

TABLE I
Purification profile for preparation of E. coli 2′–5′ RNA ligase

Analysis of the RNA ligase activity at each step of the purification process is shown. Yield was normalized to the S-100 supernatant, where the highest amount of overall activity was observed, and the comparative yield for crude extract is given in parentheses. ND, not determined because total protein amounts unavailable.

Fraction	Specific activity	Purification factor	Yield
	fmol ligated/mg protein/min		
Crude extract	17.5	1	(77%)
S-100 Supernatant	22.8	1.3	100%
DEAE effluent	246.5	14	ND
Cellulose phosphate	1517	87	60%
Heparin Hyper-D	2381	136	37%
E. coli tRNA-Sepharose	2780	159	23%
Heparin Hyper-D (II)	5826	333	ND
Superdex 75	8606	492	23%
S. cerevisiae tRNA-Sepharose	18930	1082	17%

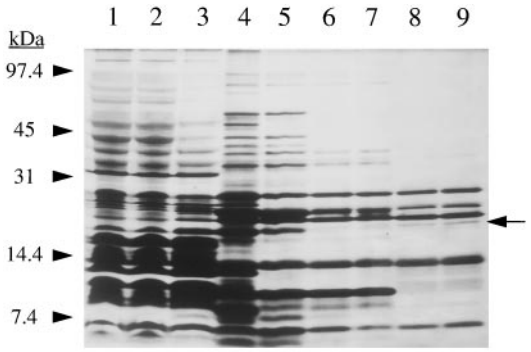

FIG. 2. **Purification of the *E. coli* RNA ligase enzyme.** Silver-stained 16% SDS-PAGE gel of aliquots from individual purification steps. Samples were normalized according to total fraction volume. *Lane 1*, crude extract; *lane 2*, S-100 supernatant; *lane 3*, DEAE effluent; *lane 4*, cellulose phosphate pool; *lane 5*, heparin hyper-D pool; *lane 6*, *E. coli* tRNA-Sepharose pool; *lane 7*, heparin II pool; *lane 8*, Superdex 75 pool; *lane 9*, *S. cerevisiae* tRNA-Sepharose pool. The 20-kDa ligase protein (identified as described under "Reconstitution of Ligase Activity") is indicated on the *right*.

centrifugation at 100,000 × *g*. The S-100 supernatant (*lane 2*) was mixed batchwise with a DEAE anion-exchange resin, and the unbound fraction (*lane 3*) was loaded onto a cellulose phosphate column. RNA ligase activity was eluted from the cellulose phosphate by an increasing gradient of KCl, and fractions containing peak levels of RNA ligase activity were pooled for further purification by sequential binding to, and salt gradient elution from, heparin and *E. coli* tRNA affinity matrices. Pooled active fractions were subjected to gel filtration through Superdex 75 media. Peak RNA ligase activity fractions after gel filtration contained a mixture of at least six polypeptides (Fig. 2, *lane 8*). Superdex 75 peak fractions were then pooled for binding to a column of *S. cerevisiae* tRNA-Sepharose. RNA ligase activity eluted from this matrix with a salt gradient still contained several polypeptide species (*lane 9*).

Reconstitution of Ligase Activity from a Single Polypeptide Following SDS-PAGE—Since the optimized purification procedure did not yield a single homogeneous polypeptide, separation by denaturing electrophoresis was also utilized. A large scale purification was undertaken using 4 kg of *E. coli* cells as starting material. Final peak ligase activity fractions were pooled and concentrated by dialysis, and an aliquot of the concentrate was subjected to SDS-PAGE. Successive regions of the SDS-PAGE gel lane were excised and individually extracted. Eluted protein from each gel slice was precipitated and resuspended in guanidine HCl. After dialysis, eluates were assayed for RNA ligase activity (Fig. 3*A*) and examined by

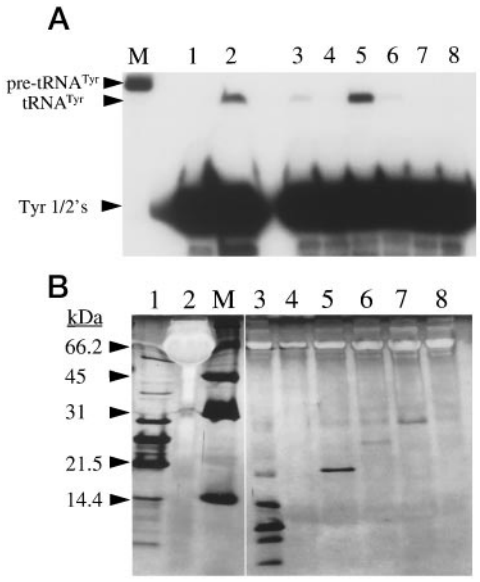

FIG. 3. **Identification of the RNA ligase polypeptide.** *A*, tRNA^{Tyr} half-molecule ligation assays containing 1 pmol of substrate were supplemented with 2 μl of dialyzed gel slice eluates and incubated at 30 °C for 30 min. Reactions supplemented with *lane 1*, buffer; *lane 2*, Superdex 75 activity pool; *lane 3*, eluate of ~3–12-kDa region of SDS-PAGE gel lane; *lane 4*, eluate of 16-kDa region; *lane 5*, eluate of 20-kDa region; *lane 6*, eluate of 27-kDa region; *lane 7*, eluate of 30-kDa region; *lane 8*, eluate of~ 35–100-kDa region of the gel lane. *B*, silver-stained 16% SDS-PAGE gel of renatured elution fractions. *M*, molecular mass standards; *lane 1*, Superdex 75 activity pool; *lane 2*, bovine serum albumin; *lanes 3–8*, same eluates as in *A*.

SDS-PAGE (Fig. 3*B*). Only a single eluate showed significant reconstituted activity (Fig. 3*A*, *lane 5*). The level of RNA ligase activity in this eluate was not affected by mixing with eluates of other gel slices (not shown). Fig. 3*B* shows that the active eluate contains only a single *E. coli* protein (of approximately 20 kDa) in addition to the bovine serum albumin carrier protein added during extraction (compare *lane 5* to *lane 2*). A small amount of a polypeptide of about 20 kDa is present in the low molecular weight eluate (Fig. 3*B, lane 3*) which may explain the trace ligase activity observed in this fraction (Fig. 3*A, lane 3*). Due to its ability to reconstitute RNA ligase activity, the 20-kDa protein alone was presumed to be the *E. coli* RNA ligase.

Ligase Protein Sequencing and Identification of the RNA Ligase Gene—A second aliquot of the concentrated RNA ligase activity pool was subjected to SDS-PAGE and transferred to a nylon membrane, from which individual protein bands were excised for sequencing. 15 residues of amino-terminal sequence of the 20-kDa RNA ligase protein were obtained and are given in Fig. 4*A*. This sequence did not match any known protein or predicted ORF in the then current Genbank/EMBL data bases. A set of degenerate oligonucleotide primers corresponding to possible coding sequences for an internal segment of the amino-terminal sequence (shown in Fig. 4*A*) was synthesized for use in PCR. Fig. 4*B* shows that these oligonucleotides successfully amplified a DNA fragment of approximately 55 bp from *E. coli* cells or genomic DNA (*lanes 1* and *2*) but produced no product using *S. cerevisiae* cells as a template, or in reactions lacking either primers or template (*lanes 3–5*). The amplified DNA was cloned and sequenced.

In order to facilitate the cloning of the *E. coli* RNA ligase gene, its chromosomal location was determined using a genomic mapping blot. A radiolabeled DNA probe was created by PCR using the degenerate primers described above and hybridized to a membrane containing the Kohara "mini-set" of ordered, overlapping *E. coli* genomic phage clones (24). Hybrid-

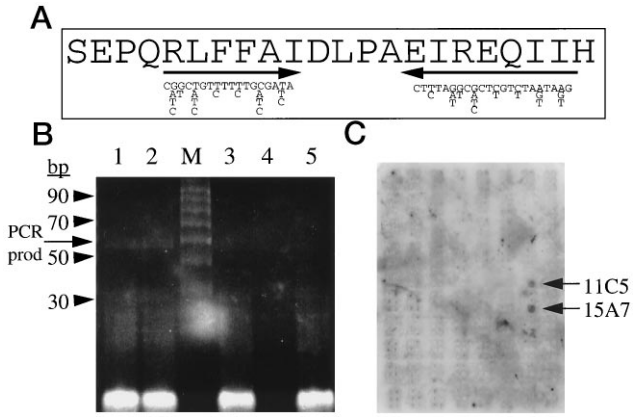

FIG. 4. **Detection of the RNA ligase gene.** *A*, determined amino-terminal amino acid sequence of 20-kDa ligase protein (*large letters*) and corresponding degenerate oligonucleotides (*small letters*) synthesized. *B*, PCR amplification of an *E. coli* genomic DNA fragment using the oligonucleotides indicated in *A* as primers. PCR reactions were separated on a 10% acrylamide gel and stained with ethidium bromide. *M*, DNA molecular mass standards; *lane 1*, *E. coli* genomic DNA template; *lane 2*, *E. coli* whole cells as template; *lane 3*, *S. cerevisiae* cells as template; *lane 4*, primers omitted; *lane 5*, template omitted. *C*, the *E. coli* genomic mapping blot hybridized with labeled PCR product created using the degenerate oligonucleotide primers in *A*. Positive clones are indicated by *arrows*.

ization to this blot (Fig. 4*C*) revealed two positive phage clones, 11C5 and 15A7, both mapping to the 3.0-min region of the *E. coli* chromosome and containing about 10 kbp of overlapping genomic DNA. Since sequencing of this genomic region had recently been completed at the Institute for Viral Research in Kyoto (25), the determined amino-terminal protein sequence and 14 bp of unambiguous genomic DNA sequence were submitted for matching against their sequence data base. Exact matches to both sequences were found in a theoretical ORF located at 2.8 min on the *E. coli* chromosome, and these researchers kindly provided the nucleotide sequence of a 3-kbp region surrounding this ORF.

Analysis of the 2′-5′ RNA Ligase Gene—Theoretical translation of the DNA sequence of the ligase ORF predicts a protein of 176 amino acids with a molecular mass of 19,934 Da (Fig. 5*A*). A methionine residue is encoded immediately prior to the first residue of the amino-terminal sequence obtained from the purified protein and is likely to be removed *in vivo*. A putative Shine-Delgarno ribosomal recognition site is located 14 bp upstream of the RNA ligase ORF (Fig. 5*A*). A possible match to the so-called "gearbox" transcriptional promoter consensus, which is typically found upstream of genes whose expression is inversely proportional to growth rate, can be found beginning at −119 upstream bp of the ORF (26). A possible σ^s −35 region (often found without a −10 consensus), which may be recognized by *E. coli* RNA polymerase bearing the stationary phase sigma factor, can be seen at −293 bp (27). The nearest potential σ^{70} housekeeping-type promoter consensus is found at −749 bp (not shown). Which promoter elements are utilized *in vivo* will need to be determined experimentally.

The RNA ligase ORF is closely flanked by two other ORFs. The previously characterized *sfs1* gene (28) lies immediately downstream of the ligase ORF with the same polarity and is transcriptionally regulated by control elements that overlap the ligase coding sequence (Fig. 5*A*). A theoretical ORF encoding a putative RNA helicase of the DEAH family is found immediately upstream of the RNA ligase ORF but with the opposite polarity (25).

Comparison of the predicted RNA ligase protein sequence to translations of the GenBank/EMBL data base using the BLAST algorithm (29) identified two highly similar protein sequences, predicted ORFs of unknown function from *Methanococcus jannaschii* (30) and *Bacillus stearothermophilus* (31). The *E. coli*, *M. jannaschii*, and *B. stearothermophilus* sequences are at least 23% identical and almost 50% similar over the entire length of the *E. coli* ligase protein (Fig. 5*B*). The *Bacillus* protein bears an extension of 129 amino acids at its carboxyl terminus, but this additional sequence does not have any significant matches in the available sequence data bases. Alignment of the three sequences reveals three highly conserved regions (*a*, *b*, and *c* in Fig. 5*B*) which may represent important functional domains of these proteins.

Genomic Disruption of the RNA Ligase Gene—A genomic disruption of the putative RNA ligase gene was performed in order to confirm that the protein identified by purification of RNA ligase activity was in fact the genuine ligase enzyme and to observe any phenotypes caused by a lack of RNA ligase function. The disruption was performed according to the method of Hamilton *et al.* (22) utilizing homologous recombination and subsequent resolution of a temperature-sensitive (ts) plasmid bearing an interrupted copy of the RNA ligase gene. First, the ligase ORF and 200 bp of upstream flanking sequence were amplified from genomic DNA using unique oligonucleotide primers and cloned into the pBlueScript vector to create the plasmid pBS-lig. A kanamycin resistance gene cassette was inserted into a unique restriction site at +45 bp in the ligase ORF, and the entire interrupted gene was subcloned into a plasmid containing a ts replicon and chloramphenicol resistance to create the plasmid pTSIL. After plasmid integration and resolution in a suitable $RecA^+$ *E. coli* parent strain (as described under "Experimental Procedures"), 64 candidate colonies were recovered. One of these was found to be both kanamycin-resistant and chloramphenicol-sensitive at 43 °C, indicating stable chromosomal insertion. Insertion of the kanamycin cassette into the correct chromosomal locus was confirmed by PCR amplification of genomic DNA from individual colonies of the knockout isolate (Fig. 6*A*). Amplification using primers hybridizing either 1 kbp upstream and at the 3′ end of the ORF (*set a*) or at the 5′ end of the ORF and 1.5 kbp downstream (*set b*) gave a product which in the disrupted isolates was increased by 1200 bp, precisely the size of this cassette. When whole cell extracts of isolates of this knockout strain were assayed for RNA ligase activity, none was detected (Fig. 6*B*, compare *lanes 3–5* with *6* and *7*).

Ligase Knockout Growth—Effects of the disruption of RNA ligase expression in *E. coli* on overall fitness were examined by assaying bacterial growth under a variety of conditions. RNA ligase knockouts were viable and showed wild-type growth rates at temperatures ranging from 23 to 43 °C (not shown). The growth curve of knockout isolates at 37 °C was essentially identical to that of the parent strain (Fig. 7*A*). The effects of moderate amounts of extra chromosomal expression of the 2′-5′ RNA ligase in knockout and wild-type strains were also tested. *E. coli* strains were transformed with the pBS-lig construct which fortuitously supported expression of RNA ligase activity at approximately 10 times wild-type levels (as assayed by measuring specific activity in whole cell extracts) but still at a level of protein undetectable in crude extracts by SDS-PAGE and silver staining (not shown). All RNA ligase overproducing strains (Fig. 7*B*, *B* + *D*), but not those transformed with vector alone (*A* + *C*), were temperature-sensitive, being viable at 37 °C but unable to grow at 43 °C. These overproducing strains also showed a slow growth rate and reduced carrying capacity at stationary phase at 37 °C, as shown in Fig. 7*A*. Thus, the overproduction of *E. coli* RNA ligase has a toxic effect.

Enzyme Equilibrium—The equilibrium of the RNA ligation

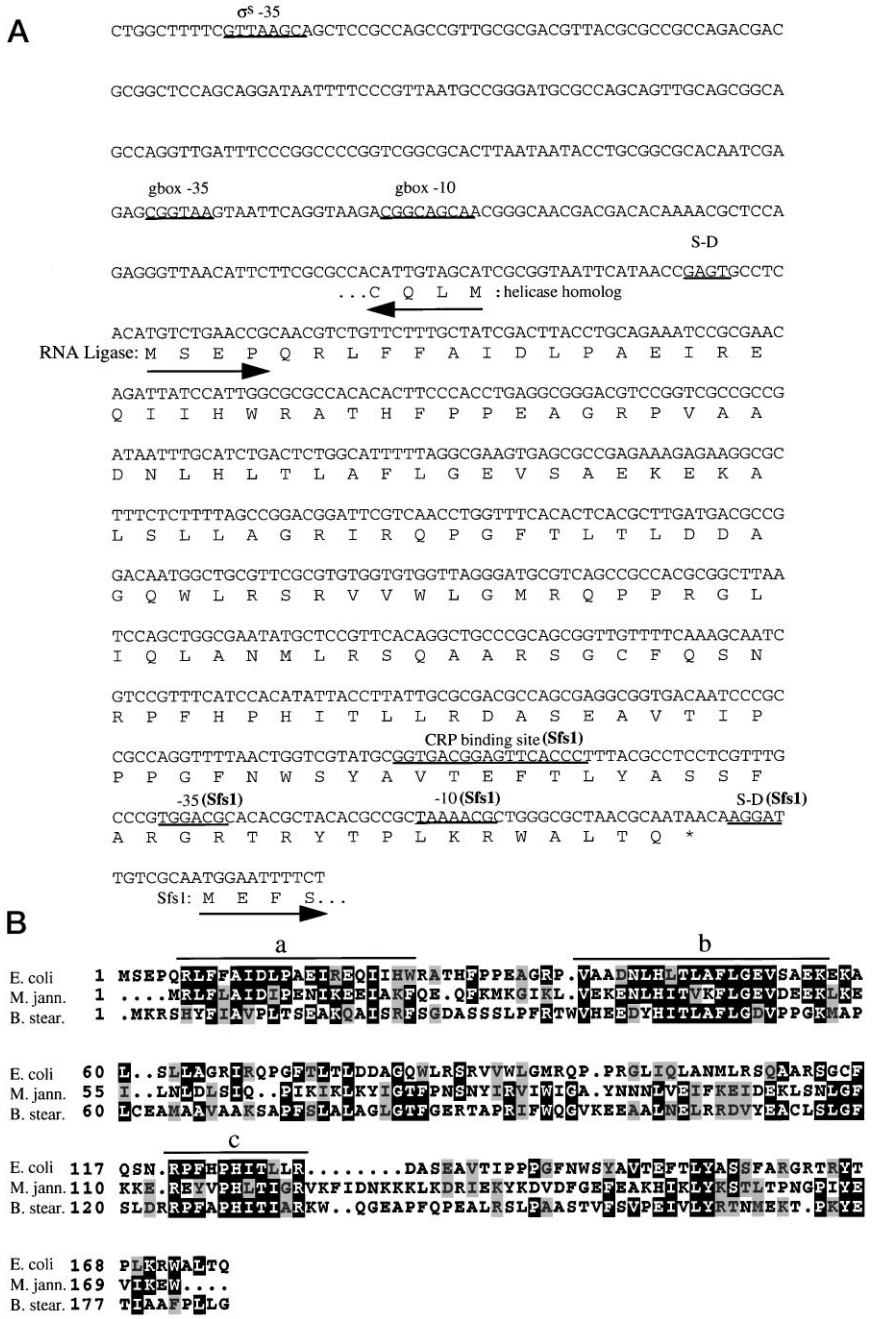

FIG. 5. **E. coli 2′-5′ RNA ligase gene.** *A*, DNA sequence of the *E. coli* genomic region including the RNA ligase gene, with translations. Starts of ORFs encoding a potential RNA helicase homolog and the *sfs1* gene are indicated. *S-D*, Shine-Delgarno ribosome binding site. −35 and −10 = consensus *E. coli* promoter sequences, *gbox −35* and *gbox −10*, potential gearbox promoter consensus sequences; σ^s *−35*, potential stress-induced promoter consensus sequence. *B*, alignment of *E. coli* RNA ligase protein sequence with potential homologs from *Methanococcus* and *Bacillus*. Identical residues are *boxed in black* and similar residues are *boxed in gray*. The species of origin of each ORF is indicated.

reaction was studied *in vitro* in order to gain insight into the *in vivo* function of this enzyme. The time course of action of purified *E. coli* RNA ligase on $tRNA^{Tyr}$ half-molecules and on $tRNA^{Tyr}$ produced by ligation of tRNA half-molecules with *E. coli* RNA ligase (creating a 2′-5′ linkage in the anticodon loop) was assayed. $tRNA^{Tyr}$ produced by ligation of half-molecules using T4 RNA ligase and PNK (to produce a 3′-5′ junction) was also tested as a control. Fig. 8*A* shows that purified *E. coli* RNA ligase specifically cleaved 2′-5′-linked substrates to fragments comigrating with authentic Tyr half-molecules (*lanes 0–5, E. coli lig*) with approximately the same kinetics as ligation of half-molecules by that enzyme (*lanes 0–5*, *Tyr 1/2's*). $tRNA^{Tyr}$ with a 3′-5′ linkage at the ligation junction was not cleaved (*lanes 0–5*, *T4 lig.*). The identity of the tRNA cleavage products was confirmed by the ability of purified *E. coli* RNA ligase to rejoin them, as demonstrated in Fig. 8*A* (*E. coli dig.*). Beginning with either pure half-molecules or ligated tRNA, at 5 min of incubation the molar ratio of substrates to products approached the same value (4–5:1, halves:full-length), as quantified in Fig. 8*C*.

DISCUSSION

E. coli RNA Ligase Substrates—The results shown in Fig. 1 demonstrate that the *E. coli* 2′-5′ RNA ligase requires modified nucleosides in artificial ligation substrates. This suggests that the *in vivo* substrate(s) of this enzyme is modified and therefore is likely to be a stable RNA as these modifications occur exclusively in stable RNAs in *E. coli* (32). Modified nucleosides may be recognized directly by the enzyme, as has been shown to occur in the interactions between some tRNAs and tRNA aminoacyl synthetases (33), or these base modifications may be required to stabilize the tRNA splicing substrates in a conformation that can be recognized by this enzyme. Modified nucleosides have been demonstrated to stabilize biologically active conformers of tRNAs in other systems (34, 35). The apparent requirement of *E. coli* 2′-5′ ligase for nucleoside modifications

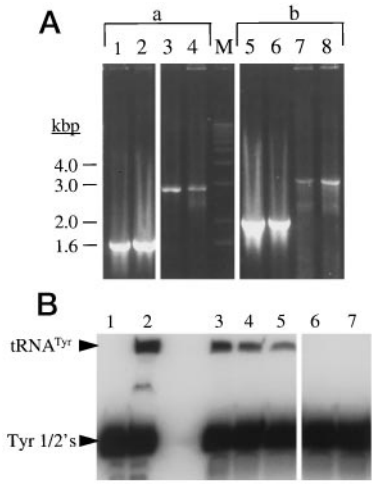

FIG. 6. **Genomic knockout of the RNA ligase gene.** *A*, PCR amplification of ligase gene from various genomic DNA preparations. Products were separated on a 1.5% agarose gel and stained with ethidium bromide. *M*, DNA molecular mass standards; *lanes 1* and *5*, HB101; *lanes 2* and *6*, HS947 (knockout parent strain); *lanes 3, 4, 7,* and *8*, disrupted isolates. *Set a,* primers located 1-kbp upstream and at the 3′ end of the ligase ORF; *set B,* primers located at the 5′ end of the ligase ORF and 1.5-kbp downstream. *B*, RNA ligation assay of lysates of indicated strains. Ligation reactions including 500 fmol were incubated at 30 °C for 10 min. Reactions supplemented with 1 μl each of the following: *lane 1*, buffer; *lane 2*, purified *E. coli* RNA ligase (Superdex 75 pool); *lanes 3–7*, crude lysates of HB101 (*lane 3*), HS947 (*lane 4*), a resolved cointegrate isolate not yet cured of the ts plasmid (*lane 5*), disrupted, resolved isolates cured of plasmid (*lanes 6* and *7*).

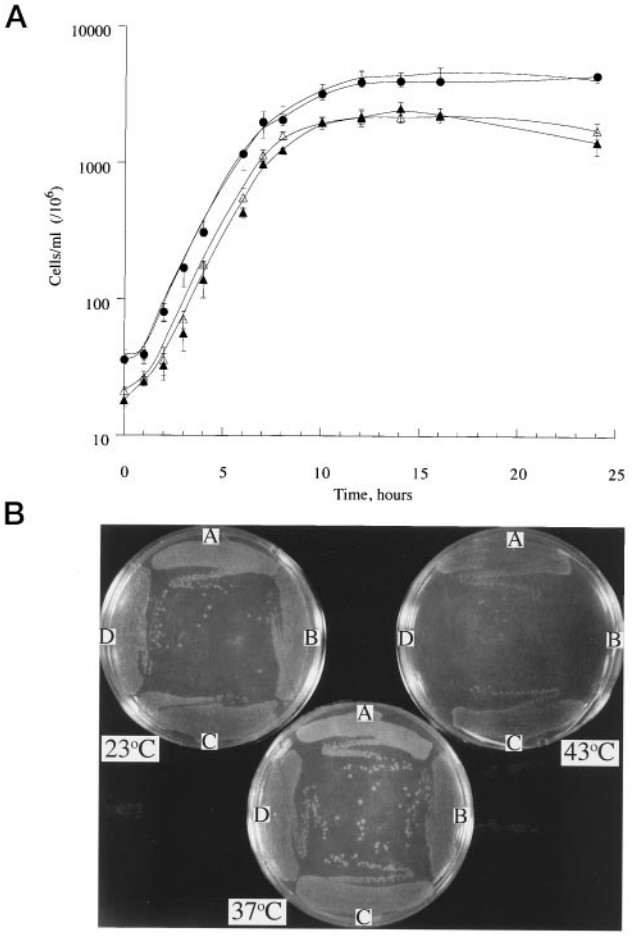

FIG. 7. **Growth phenotypes of RNA ligase knockout and overexpressing strains.** *A*, growth curves of *E. coli* strains at 37 °C derived by viable plating of three replicate cultures of each. Shown are the knockout parent strain HS947/RecA⁻ (○), HS947/RecA⁻ harboring the RNA ligase expression plasmid pBS-lig (△), an RNA ligase knockout isolate (●), and a knockout harboring pBS-lig. (▲). *Error bars* represent one standard deviation. *B*, growth of strains at indicated temperatures on LB agar. *A*, HS947/RecA⁻; *B*, HS947/RecA− + pBS-lig.; *C*, RNA ligase knockout; *D*, RNA ligase knockout + pBS-lig.

and the preference shown by this enzyme for a subset of *S. cerevisiae* tRNA splicing substrates suggest that the *E. coli* ligase is likely to act upon a tRNA or tRNA-like molecule *in vivo*. Comparison of the four *S. cerevisiae* tRNA species that are ligated by the *E. coli* RNA ligase to the six that are not (8) does not reveal any obvious consensus of sequence or base modifications that might be recognized. The preference of the *E. coli* RNA ligase for yeast $tRNA^{Tyr}$ half-molecules over $tRNA^{Phe}$ half-molecules (Fig. 1*C*) suggests that this enzyme has the ability to discriminate among individual tRNA species.

Ligase Purification—Purification of the *E. coli* 2′-5′ RNA ligase over 1000-fold from crude extracts provided highly purified protein fractions but not a single homogeneous polypeptide. Contaminating proteins remaining at the final stages of purification probably represent molecules with properties very similar to the 2′-5′ RNA ligase, but are not likely to be components of a macromolecular complex as their concentrations peak in different fractions during gel filtration (data not shown). The tight binding of *E. coli* RNA ligase to immobilized prokaryotic and eukaryotic tRNA provides additional evidence that the ligase recognizes a tRNA or tRNA-like substrate *in vivo*. The ability of the RNA ligase protein to refold and reconstitute enzymatic activity after SDS-PAGE suggests a stable, self-folding structure for this protein.

Ligase Gene Sequence—Theoretical translation of the nucleotide sequence of the RNA ligase gene predicts a polypeptide with size, charge, and other biochemical properties in excellent agreement with those observed for the ligase protein. The ligase does not appear to be expressed as part of a multicistronic operon as the nearest upstream ORF with the same polarity is located about 12 kbp away, and the adjacent downstream ORF (encoding the sfs1 protein) has its own promoter and regulatory elements (28). The cis-acting sequences controlling RNA ligase expression therefore remain to be determined.

The apparent conservation of the RNA ligase protein sequence between such distantly related bacterial species as *E. coli* and *B. stearothermophilus* and across kingdoms to the archaeote *M. jannaschii* suggests an ancient origin for this enzyme. This is in agreement with the observation of RNA ligase activity in extracts of a wide variety of bacterial species (8). The short blocks of high similarity between these predicted proteins as well as the lower dispersed similarity throughout suggest that the alignment is meaningful and is likely to represent a homologous origin and function for these proteins. If this alignment truly means that the 2′-5′ RNA ligase is highly conserved between proteobacteria and Archaea, an important metabolic function for this enzyme is implied.

The RNA ligase enzyme may have been lost from some evolutionary branches between the low G + C Gram-positive bacteria and the Gamma division proteobacteria however, since RNA ligase activity was not originally detected in *Desulfurovibrio*, *Paracoccus*, or *Rhodopseudomonas* species, although this may have been due to some artifact of extract preparation or ligation assay (8). No obvious homolog to the 2′-5′ RNA ligase protein can be found in the completed sequence of the *H. influenzae* chromosome which, although closely related to *E. coli* by rRNA sequence comparisons, has an extremely streamlined genome less than half the size of the chromosomes of other proteobacteria (13). *Haemophilus* may have discarded the ligase activity or transferred this function to another polypeptide due to the selection pressures that caused the

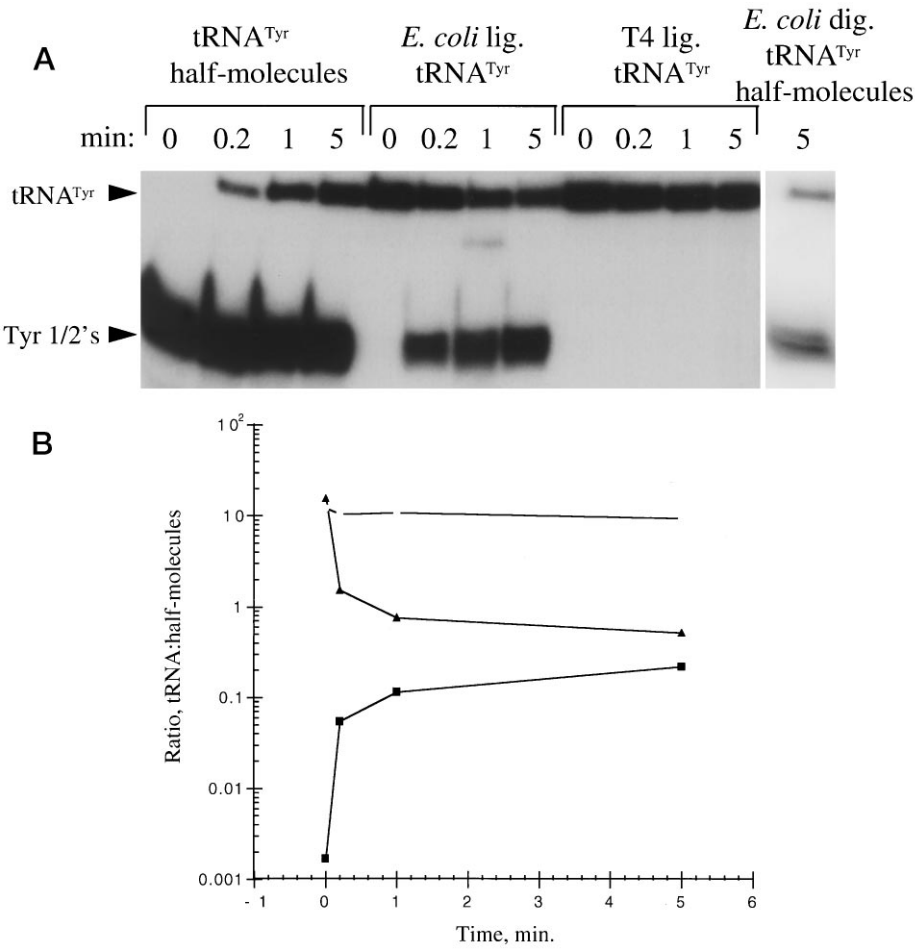

FIG. 8. **Equilibrium of the *E. coli* RNA ligase.** *A*, time course of forward and reverse reactions of highly purified *E. coli* RNA ligase. 4 μl of the Superdex 75 activity pool were added to 16 μl of the reactions containing the substrates indicated: *Tyr ½'s*, half-molecules derived from T7-transcribed, yeast-modified pre-tRNA^{Tyr} (4 pmol), *E. coli lig.tRNA*^{Tyr}, tRNA produced by ligation of tRNA^{Tyr} half-molecules with purified *E. coli* 2′-5′ RNA ligase (0.8 pmol); *T4 lig.tRNA*^{Tyr}, tRNA produced by ligation of tRNA^{Tyr} half-molecules with T4 RNA ligase and PNK in the presence of ATP (0.8 pmol). 4-μl aliquots were removed and stopped at the indicated times. *B*, quantification of the reactions in *A*, graphed according to the molar ratio of full-length tRNA to tRNA half-molecules at each time point. Shown are tRNA^{Tyr} half-molecules (■), *E. coli*-ligated tRNA^{Tyr} (▲), and T4-ligated tRNA^{Tyr} (○) substrates.

drastic decrease in size of its genome. This would imply (as did the lack of detectable activity in several bacterial species) that the RNA ligase may not perform a function absolutely necessary for bacterial survival but may be conditionally required under growth conditions encountered by a wide variety of species.

Genomic Disruption—Disuption of the genomic locus encoding the putative ligase protein confirmed that the correct polypeptide had been purified, since a complete loss of ligase activity in cell extracts ensued. This protein therefore appears to be the only enzyme in *E. coli* capable of ligating yeast tRNA half-molecules. The fact that genomic ligase knockout isolates were viable demonstrates that RNA ligase is not absolutely required for survival under laboratory growth conditions. Although the disrupted strains do not display a lethal phenotype, they can be examined for more subtle effects on growth and RNA metabolism. The availability of viable knockouts will also provide a useful null background for the expression of affinity-tagged or mutagenized ligase protein for use in further biochemical experiments. The toxic effects of moderate levels of ligase overexpression also imply some sort of interaction between the ligase enzyme and other cellular factors required for growth.

Examination of Enzyme Equilibrium—The reaction catalyzed by the *E. coli* ligase enzyme was shown to be fully reversible with an apparent equilibrium constant near unity, but favoring cleavage of 2′-5′ bonds. The tendency toward cleavage may perhaps be explained by the thermodynamics of phosphodiester bond cleavage and formation, which have been investigated thoroughly in the hammerhead ribozyme system. The favorable entropy of bond cleavage causes the internal equilibrium of the hammerhead ribozyme to favor cleavage of 3′-5′ phosphodiesters to 2′,3′-cyclic phosphate and 5′-hydroxyl termini despite the unfavorable enthalpy associated with cyclic phosphate formation (36). For the cleavage reaction catalyzed by the *E. coli* RNA ligase, an increase of entropy in what is essentially a unimolecular reaction (given the tight structural association of tRNA half-molecules) may be due to the additional degrees of freedom available to released termini and to the disruption of water ordered in and about the closed, structured anticodon loop.

This observed equilibrium of cleavage and ligation appears to explain the maximum extent of ligation observed in *in vitro* activity assays. However, it begs the question of whether the function of the enzyme is to catalyze ligation or cleavage *in vivo*. Despite the fact that the equilibrium observed *in vitro* favors cleavage, the direction of the equilibrium *in vivo* will depend on the effective concentrations of substrates available for each reaction. If the true *in vivo* substrate is tRNA, and ligated tRNA products are utilized for translation and thereby removed from the pool of substrates available to the ligase, then the ligation reaction will be favored. If, however, the cleavage products are removed by some process such as ribonucleolytic degradation, then the equilibrium will favor cleavage. To propose a cleavage function for the ligase enzyme *in vivo*, however, it is necessary to posit a source of substrates with 2′-5′ bonds, presumably in the context of a tRNA. No other *E. coli* enzyme is known or proposed that might form internal 2′-5′ linkages in a tRNA structure. A variety of endoribonuclease activities, however, could theoretically produce substrates for ligation by the *E. coli* RNA ligase, and in fact an activity

capable of doing so has been observed in *E. coli* extracts.[3] An enzyme capable of cyclizing free 3′-phosphates to 2′,3′-cyclic phosphates has also recently been discovered in *E. coli*.[4] Thus the available evidence, while circumstantial, favors a ligation function for this enzyme *in vivo*.

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