The Subnuclear Localization of tRNA Ligase in Yeast

Michael W. Clark and John Abelson

Division of Biology, California Institute of Technology, Pasadena, California 91125

Abstract. Yeast tRNA ligase is an enzyme required for tRNA splicing. A study by indirect immune fluorescence shows that this enzyme is localized in the cell nucleus. At higher resolution, studies using indirect immune electron microscopy show this nuclear location to be primarily at the inner membrane of the nuclear envelope, most likely at the nuclear pore. There is a more diffuse, secondary location of ligase in a region of the nucleoplasm within 300 nm of the nuclear en-velope. When the amount of ligase in the cell is increased, nuclear staining increases but staining of the nuclear envelope remains constant. This experiment indicates that there are a limited number of ligase sites at the nuclear envelope. Since the other tRNA splicing component, the endonuclease, has the characteristics of an integral membrane protein, we hypothesize that it constitutes the site for the interaction of ligase with the nuclear envelope.

I n eukaryotic cells, transfer RNA is transcribed in the nucleus as a precursor RNA. Before reaching its functional location in the cytoplasm, each tRNA molecule undergoes a distinct and complex set of processing and base modification reactions. Most of the studies on the localization of these reactions have been performed in the *Xenopus* oocyte system. They have shown that the RNA processing reactions, end maturation, CCA addition and splicing, take place in the nucleus (15). Some base modifications also take place in the nucleus (15). The partially matured tRNA molecule is then transported to the cytoplasm by a carrier-mediated translocation process (26). The remaining base modification reactions are performed by enzymes in the cytoplasm.

We have been studying one step in this pathway, the tRNA splicing reaction. In the yeast *Saccharomyces cerevisiae* ~20% of the tRNA genes contain a single intron (10, 16). The introns are small (14–60 b), and are located in the anticodon loop. The substrate for tRNA splicing is a precursor RNA which has been matured at its 5' and 3' ends and has been partially modified. Therefore, the tRNA splicing reaction could be the last step before its transport from the nucleus. If the spatial organization of the enzymes in the pathway is correlated with the temporal order of the reactions, the splicing enzymes might be expected to be localized at the nuclear membrane. Our previous studies on the biochemistry of tRNA splicing have suggested a membrane association of the splicing enzymes. These studies have also provided reagents to examine the subcellular site of the tRNA splicing process.

The tRNA splicing reaction is mediated by two enzymes: an endonuclease and a ligase (20). The endonuclease cleaves the splice sites and releases the intron (19). The ligase joins the 5' and 3' half molecules in an ATP-dependent reaction (9). We have purified the endonuclease to near homogeneity. This enzyme has the characteristics consistent with an integral membrane protein. It is particulate, even at high salt concentrations, but can be solubilized with nonionic detergents. Since the splicing reaction most likely takes place in the nucleus, we hypothesize that the endonuclease is a nuclear membrane protein. Although the endonuclease has been purified 200,000-fold (Green, P., and J. Abelson, unpublished results) we have not yet identified the polypeptide chain(s) nor have we obtained specific antisera. The tRNA ligase, however, has been purified to homogeneity (21). It is a soluble, 90,000-dalton protein. The gene has been cloned and sequenced. The gene can be expressed in *Escherichia coli*. We have used the pure ligase to raise specific antibodies in rabbits. It is known that the endonuclease and ligase reactions are concerted (8). It is therefore likely that the two proteins interact and are localized at the same site. The antibodies to tRNA ligase have been used to determine the location of ligase and presumably the location of the splicing reaction.

In this paper, we demonstrate by indirect immune fluorescence that the tRNA ligase protein is in a sector of the yeast nucleus. At higher resolution, studies using indirect immune electron microscopy on ultra-thin epon sections of yeast cells divides this site into two nuclear locations: a primary nuclear envelope site, most likely at the nuclear pore, and a less distinct, more diffuse secondary location in the nucleoplasm within 300 nm of the nuclear envelope. In a strain of yeast in which tRNA ligase can be overexpressed, we have observed a distinct nucleoplasmic tRNA ligase staining pattern which appears to indicate a nuclear substructure that determines the location of tRNA ligase.

Materials and Methods

Materials

chased from Seikagaka Kogyo Co. Japan. Colloidal gold goat anti–rabbit IgG conjugate was purchased from E. Y. Laboratories, Inc., San Mateo, CA. Propylene oxide, Epon 812, and 400 mesh nickel electron microscope grids were purchased from Polysciences, Inc., Warrington, PA. The immunoblot ELISA was done with the Anti-Rabbit IgG Vectastain ABC Kit from Vector Laboratories, Inc., Burlingame, CA.

Strains of Yeast

Strain 20B-12-1 (our nomenclature), a protease-deficient strain (α-pep4-3, prcl, prbl, his) obtained from Dr. E. Jones (Carnegie-Mellon University, Pittsburgh, PA), is the standard strain in this study. The yeast strains that overproduce tRNA ligase have been previously described (21). EMPy 47-1 is SS 328 (α-his3 δ 200 lys2-801° ade2-101° ura3-52 Gal suc2) containing the Cen IV plasmid pBM 150-RLGX with the tRNA ligase gene under GAL10 promoter control. Strains EMPy 268 and EMPy 284 have the genomic tRNA ligase gene disrupted by insertion of the HIS3 gene at a Bgl II restriction site in the coding region of the ligase gene (21). Cell viability in these strains is allowed by complementation with an 8-kb fragment of DNA containing the tRNA ligase gene. In the case of EMPy 268 the tRNA ligase gene is on a CEN IV-containing plasmid, pEP110, while EMPy 284 contains the tRNA ligase gene on a 2μ plasmid, pEP99. These two strains have the same phenotypic markers as in EMPy 47-1 except that the cells are made His+ by the gene disruption procedure. Strain 54-4-1 is an *E. coli* strain RZ510 that contains the ptac expression plasmid, pKK223-3 (5) with the tRNA ligase gene inserted.

Media

Wild type cells were grown in yeast extract/peptone/dextrose media. EMPy 47-1 was grown to the desired cell density in yeast extract/peptone media with 3% glycerol and 2% ethanol as a carbon source, then induced for tRNA ligase expression by the addition of galactose to a concentration of 2%. EMPy 268 and EMPy 284 were grown in YPD media at 30°C. *E. coli* strain 54-4-1 was grown in L broth.

Preparation of tRNA Ligase Antibodies

One antiserum (referred to as anti-tL3) was prepared against tRNA ligase protein purified from strain 20B-12-1 as described in reference 21 with one additional purification step by SDS-PAGE. A second antiserum (anti-tLE) was prepared against yeast tRNA ligase protein from *E. coli* strain 54-4-1 and purified as in reference 21. For both antisera, all innoculations were by subcutaneous injection in the back of a New Zealand White male rabbit.

Antibody Purification

Affinity-purified IgG was used for immune electron microscopy. For the anti-tL3 antibody we used the nitrocellulose filter antibody purification procedure (23). Because of the low titer of the anti-tL3 antiserum, we first isolated the IgG fraction of the serum by protein A-Sepharose 4B column chromatography (25), and subjected that IgG fraction to the affinity-purification procedure. The anti-tLE antiserum was affinity purified on a column of tRNA ligase protein attached to Affi-gel 10 (Bio-Rad Laboratories).

Immunoblot Procedure

Immunoblot analysis of the overproducing tRNA ligase strains was done by subjecting aliquots of yeast spheroplast extract to electrophoresis on a 9% polyacrylamide/SDS gel (13) followed by the electrotransfer of the protein to nitrocellulose (24). Antibody reactions of the filters were visualized as specified in the Vectastain Kit (Vector Laboratories) instructions, using an anti-tLE serum dilution of 1:2000. Densitometry was performed using a 2202 Ultroscan (LKB Instruments, Inc., Gaithersburg, MD).

Immune Fluorescence Microscopy

This procedure was modified from the techniques developed by Kilmartin for whole mount yeast cells (1, 12).

Postfixed Cells

200 ml of yeast culture were grown to an OD 600 less than 0.5. Cells were harvested by filtration through a 0.45 μm filtration unit (Nalgene Co., Rochester, NY). Cells were washed once with 10 ml of ice-cold distilled water and twice with 10 ml of 0.1 M K-phosphate pH 6.5. The cells were resuspended in 1 ml of 100 mM Tris-HCl pH 8, 25 mM dithiothreitol 5 mM

Na₂-EDTA, 1 M sorbitol, and incubated at 30°C for 10 min. Cells were pelleted by centrifugation and that pellet was washed in 5 ml of 0.1 M K/phosphate/citrate pH 5.8, 1.2 M sorbitol. The cell pellet was resuspended in 0.5 ml of the same buffer. 0.05 ml of β-glucuronidase (type-H2) and 0.05 ml of a 5 mg/ml solution of zymolyase were added. This cell suspension was incubated at 30°C for 1.5–2 h to thoroughly remove cell walls. The cells were centrifuged and the pellet washed twice with 0.1 M K/phosphate/citrate pH 6.5, 1.2 M sorbitol. The pellet was resuspended in the same buffer at an OD 600 of 0.5. 0.1 ml of the cell suspension was applied to a poly-lysine-coated microscope slide and permitted to stand for 5 min while the cells settled. The remaining supernatant was then removed. The cells were then fixed by the application of 5% formaldehyde in 0.1 M K/phosphate/citrate pH 6.5, 1.2 M sorbitol for 10 min. The supernatant was removed, the cells were washed once with the buffer alone, and then the slides were immersed in −20°C methanol for 6 min followed by two 30-s immersions in −20°C acetone. The slides were then air dried and stored at 6°C until needed.

Prefixed Cells

This procedure is the same as above except that after the cells were harvested by filtration and washed they were fixed by resuspension in 3.7% formaldehyde in 0.1 M K/phosphate pH 6.5 and incubated for 1.5 h at room temperature. After fixation, the cells were washed four times in the buffer alone to remove the formaldehyde. The cells were then treated as postfixed cells, except the fixation after mounting the cells on the slides was omitted.

Immune Fluorescence Staining

To the mounted cells, 0.1 ml of diluted anti–tRNA ligase antiserum in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mg/ml BSA (TBS/BSA) was applied and incubated for 16–18 h at 6°C. The dilutions for anti-tL3 and anti-tLE were 1:250 and 1:1,000, respectively. The slides were then placed at room temperature, the supernatant removed, and the cells incubated for 5 min with TBS/BSA. This treatment was repeated two times. 0.15 ml of a 1:100 dilution of FITC–IgG conjugate (Sigma Chemical Co.) in TBS/BSA was applied to the cells and incubated for 60 min at room temperature in a dark chamber. Cells were then washed with TBS/BSA four times for 5 min. After the washes, a drop of 90% glycerol buffered with 2 mM K/phosphate pH 9, 15 mM NaCl containing 1 mg/ml of ρ-phenylenediamine (17) was applied to the cells and a coverslip was sealed to the slide with clear fingernail polish. These slides were stored at 6°C in a dark chamber until they were viewed. Slides were viewed with a Leitz microscope, using brightfield and FITC excitation wavelengths or a Zeiss Standard microscope using Nomarsky Interference optics and FITC excitation wavelengths.

Immune Electron Microscopy

The fixation and embedding of the yeast cells was a modification of the procedure of Byers and Goetsch (3). In early experiments, wild-type cells were fixed only with 1% glutaraldehyde in 0.1 M Na/phosphate pH 7 and incubated in the cold overnight. In subsequent experiments, we used 4% paraformaldehyde/0.5% glutaraldehyde in the same buffer. Even though this last fixative did reduce the protein antigenicity slightly, the cell preservation was much improved so we continued to use the latter fixative. After fixation, the cells were washed with distilled water and the cell walls digested in the same manner as described above for the immune fluorescence cell preparation. With the cell walls removed, the fixed spheroplasts were washed in water, dehydrated through an ethanol series, embedded in Epon 812 (Polysciences), and heat hardened. Ultrathin sections of the cells were cut and taken up on 400 mesh nickel grids. These grids were then used for the "on-section" immune staining procedure.

On-Section Immune Staining of Ultrathin Sections

This antibody-staining procedure is a modification of the on-section immune electron microscopic methods described by DeMay (7). To help remove background staining, the antibody incubations for the on-section staining were performed in 20 mM Tris-HCl, pH 7.5, 0.1 mg/ml BSA with 500 mM NaCl (TBS0.5/BSA). All buffer solutions were filtered through a 0.45-μm filter unit (Nalgene Co.). Diluted antibody solutions were centrifuged for 10 min in a microfuge at 6°C. The 20 nm colloidal gold solutions were centrifuged at 2,100 rpm (700 *g*) in the rotor HB-4; Dupont Co., Sorvall Instruments Div., Newton, CT) for 15 min to remove large aggregates. The sections on the grids were treated for at least 10 min at room temperature with 20 mM Tris-HCl pH 7.5, 500 mM NaCl, and 8% BSA. After this

treatment, the grids were transferred directly to 0.1 ml of the prespun primary affinity-purified IgG in TBS0.5/BSA at an IgG concentration of ~0.25 μg/ml. The grids were incubated at 6°C for 16–18 h. The grids were removed from the primary antibody solution with teflon-coated forceps and rinsed by passing 1.5 ml of TBS0.5/BSA gently over the grids. These grids were placed in 0.1 ml of 0.4 μg/ml 20 nm colloidal gold goat anti-rabbit IgG conjugate and incubated for 1 h at room temperature. The next rinse was conducted by placing the grids in an E. M. multiple grid staining unit (No. 7332; Polysciences). The unit was immersed in 150 ml of TBS0.5/BSA and the solution was stirred for 3 min. After this rinse the grids were placed in 1% glutaraldehyde in TBS0.5 in the absence of BSA and fixed at room temperature for 10 min. These grids were rinsed with distilled water and then stained with 1% aqueous uranyl acetate for 5 min, rinsed with distilled water and then counterstained with 1% vanadatomolybdate (11) for 15 min. The grids were viewed in a Phillips 201-B transmission electron microscope.

Preadsorption Controls

When preadsorption control experiments were conducted, purified tRNA ligase protein was added to the affinity-purified IgG at 1–2 molar equivalents, incubated for 0.5 h on ice, spun in a microfuge for 10 min, and then treated like the other IgG.

Results

Localization of tRNA Ligase Immune Fluorescence

In initial experiments, using the fixation procedure that works well for abundant proteins such as tubulin (1, 12) (Fig. 1 A), we did not observe anti–tRNA ligase fluorescent stain-
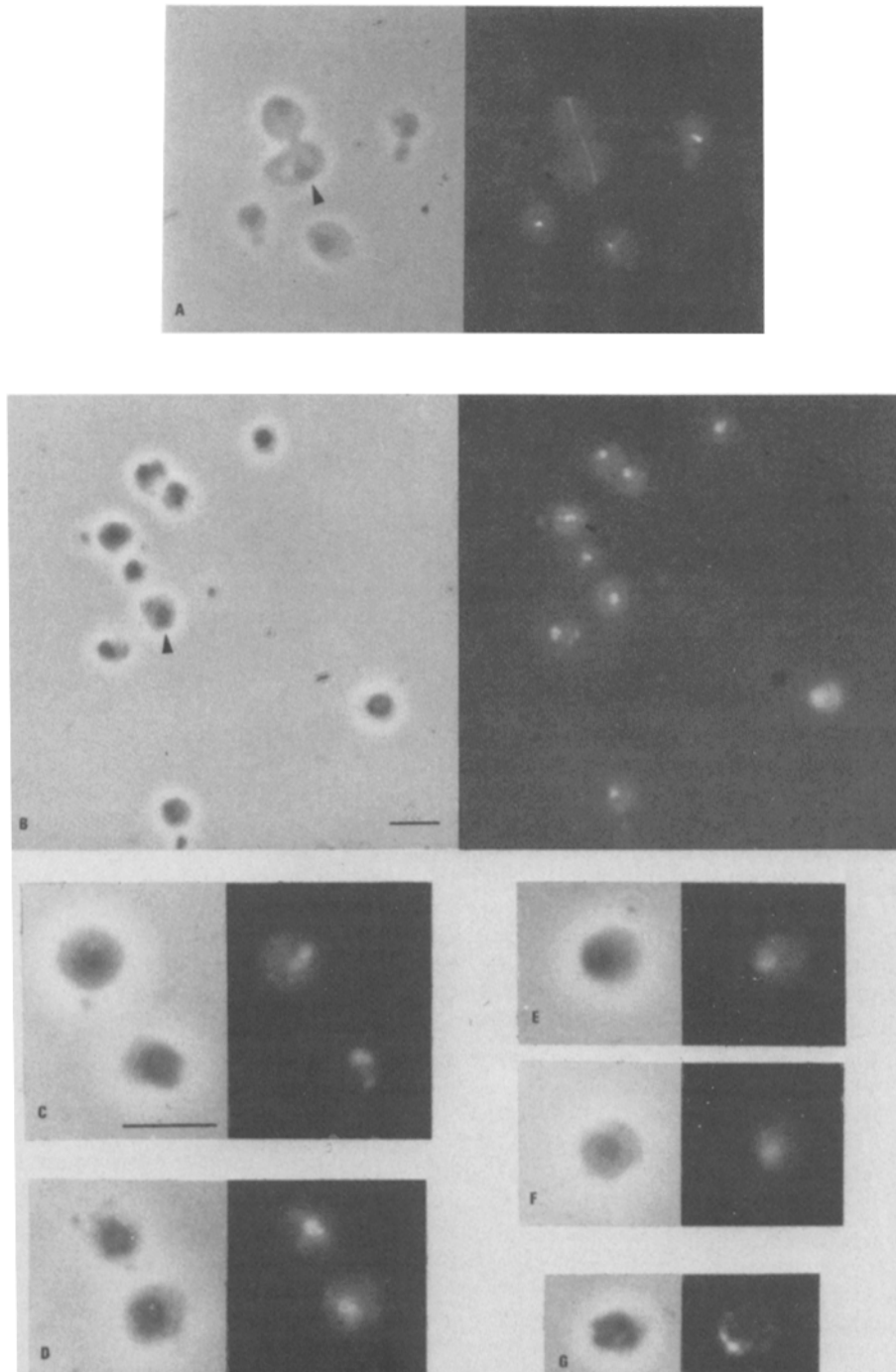

Figure 1. Immune fluorescence microscopy of wild-type yeast cells. (Left) Panels viewed under brightfield illumination where the cell nucleus appears as a dark spot (see arrow in A and B). This nuclear image has been verified by 4', 6 diamidino-2-phenylindole (DAPI) nuclear staining (data not shown). (Right) Panels viewed under FITC excitation wavelengths. (A) A view of prefixed cells (see Materials and Methods) incubated in the presence of 0.25 μg per ml of a rat monoclonal antibody against yeast β-tubulin, and visualized with a goat anti-rat IgG FITC conjugate. (B–F) Postfixed cells (see Materials and Methods) incubated in the presence of a 1:250 dilution of rabbit anti–tRNA ligase, (anti-tL3), and then visualized by a 1:100 dilution of goat anti–rabbit IgG conjugated to FITC. (G) An isolated yeast nucleus, slightly swollen by the isolation procedure, stained in the same manner as the cells in B–F. Bar, 5 μm.

ing of yeast. tRNA ligase is a rare protein in yeast (~500 copies per cell). There may be insufficient penetration of the IgG through the fixed cell matrix to observe such a weak signal. We therefore modified the procedure to remove the cell walls before fixation (see Postfixed Cells in Materials and Methods).

Using this procedure, it was found that the anti–tRNA ligase antibody stained the cell nucleus (Fig. 1, *B–F*). Note that the pattern of fluorescence revealed by the anti–tL3 antiserum is of a patched nature; that is, only a region of the nucleus (seen in the enlargements Fig. 1, *C–F*) is stained. Fig. 1 *G* demonstrates a similar staining pattern of an isolated yeast nucleus (22) swollen by the isolation procedure. In this case, fluorescence is observed only in the lower quadrant of the nucleus. No staining other than a faint overall background staining was observed using the preimmune serum (data not shown).

It is possible that the conditions for cell wall digestion might have influenced the tRNA ligase location in the cell. By engineering an increase in the number of tRNA ligase molecules in the cell, it was possible to localize the antigen in cells that were fixed before cell wall digestion. This experiment (described below) also allowed a correlation of the fluorescent signal with varying amounts of tRNA ligase.

Immune Fluorescence Staining of tRNA Ligase in a Strain Overproducing the Protein

In previous work, we constructed a strain of yeast in which expression of the tRNA ligase gene is under the control of the inducible GAL10 promoter (21). In the presence of galac-
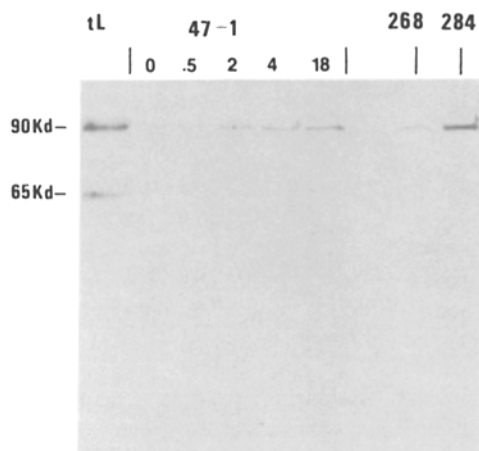

Figure 2. Immunoblot analysis of strains of yeast overproducing tRNA ligase. This immunoblot was stained with the anti–tRNA ligase (anti–tLE). Lane *1* (*tL*) contains 2 μg of purified yeast tRNA ligase. The prominent bands are the M_r 90,000 tRNA ligase and a M_r 65,000 proteolytic fragment of ligase. Aliquots of the various cell cultures were centrifuged, washed, and spheroplasts were prepared. 20 μg of total cell protein was loaded per gel lane. After electrophoresis, proteins were electroblotted to a nitrocellulose filter (see Materials and Methods). Lanes *2–7* (*47-1*) follow the induction of tRNA-ligase by addition of 2% galactose to strain 47-1, which contained a plasmid with the tRNA ligase gene under GAL10 promoter control. Lane *8* (*268*) is strain 268 that has the genomic tRNA ligase gene disrupted and cell viability complemented by a Cen IV plasmid containing the tRNA ligase gene. Lane *9* (*284*) is strain 284 that has the genomic tRNA ligase gene disrupted and contains a 2μ plasmid carrying the tRNA ligase gene.

tose the cellular concentration of tRNA ligase increases. Fig. 2, lanes *2–6*, (labeled *47-1*) shows the immunoblot of a time course for tRNA ligase induction in this strain, 47-1, as revealed by the anti–tRNA ligase antiserum, anti–tLE. The concentration of tRNA ligase continues to increase after 18 h in the presence of galactose. The tRNA ligase content is estimated to be ~15–20-fold higher after 18 h of induction on the densitometry of the immunoblot (see also Fig. 7 *A*). This finding is in agreement with the increase in ligase activity found in this strain by Phizicky and co-workers (21).

As seen in Fig. 3, the increase in tRNA ligase did have a dramatic effect on the fluorescent staining of the cell. Since the cells were fixed before the cell wall was removed, wild-type levels of tRNA ligase did not give a signal (Fig. 3 *A*). 4 h after the addition of galactose to the cell cultures, however, the fluorescence due to tRNA ligase staining was concentrated within a region of the cell nucleus (Fig. 3 *B*). This staining pattern is similar to the fluorescent pattern seen previously in wild-type cells. This induction experiment demonstrates that the fluorescence observed for wild-type cells was the result of the tRNA ligase protein. These experiments provide conclusive evidence that tRNA ligase is located in the yeast nucleus and perhaps in a compartment of the nucleus.

At 6 and 8 h after induction (Fig. 3, *C* and *D*, respectively), tRNA ligase staining was apparent in the cytoplasm as well. This change in staining was apparently the result of increased numbers of tRNA ligase molecules, which eventually fills the entire cell.

Immune Electron Microscopic Mapping of tRNA Ligase

It was necessary to determine the location of tRNA ligase at a higher resolution. To do this we used the technique of immune electron microscopy. The location of the primary antibody was visualized with a second IgG conjugated with a 20-nm colloidal gold particle (7). Because it destroys the antigenicity of most proteins (2), the conventional OsO_4 postfixation was not used in this procedure. An unforeseen advantage gained by the elimination of this step was the slight swelling of the lumen between the membranes of the nuclear envelope, which gave a better visualization of the inner and outer membranes of the yeast nuclear envelope than is obtained by the conventional procedure (3).

Fig. 4 *A* shows a representative image of the staining pattern observed when this procedure was conducted using wild-type yeast cells and the affinity-purified anti–tRNA ligase IgG, anti–tL3. Although there was an indication of a preference for the nucleus by the anti–tRNA ligase antibody (see *arrowheads* in Fig. 4 *A* indicating the 20-nm gold particles), the signal was weak. On these sections, colloidal gold particles could also be seen in the cytoplasm as well as in the nucleoplasm. Such a low signal could be expected given the low copy number of ligase and the finding that with Epon 812 sections, an antibody can only penetrate the section to a depth of 1 nm (4). Assuming a random distribution of 500 tRNA ligase molecules in the nucleus, we calculated there to be only 0.5–1 ligase molecule available for the anti–tRNA ligase antibody per section of nucleus. However, as we shall further demonstrate, the ligase molecules are not distributed randomly, but are concentrated in a region of the nucleus.

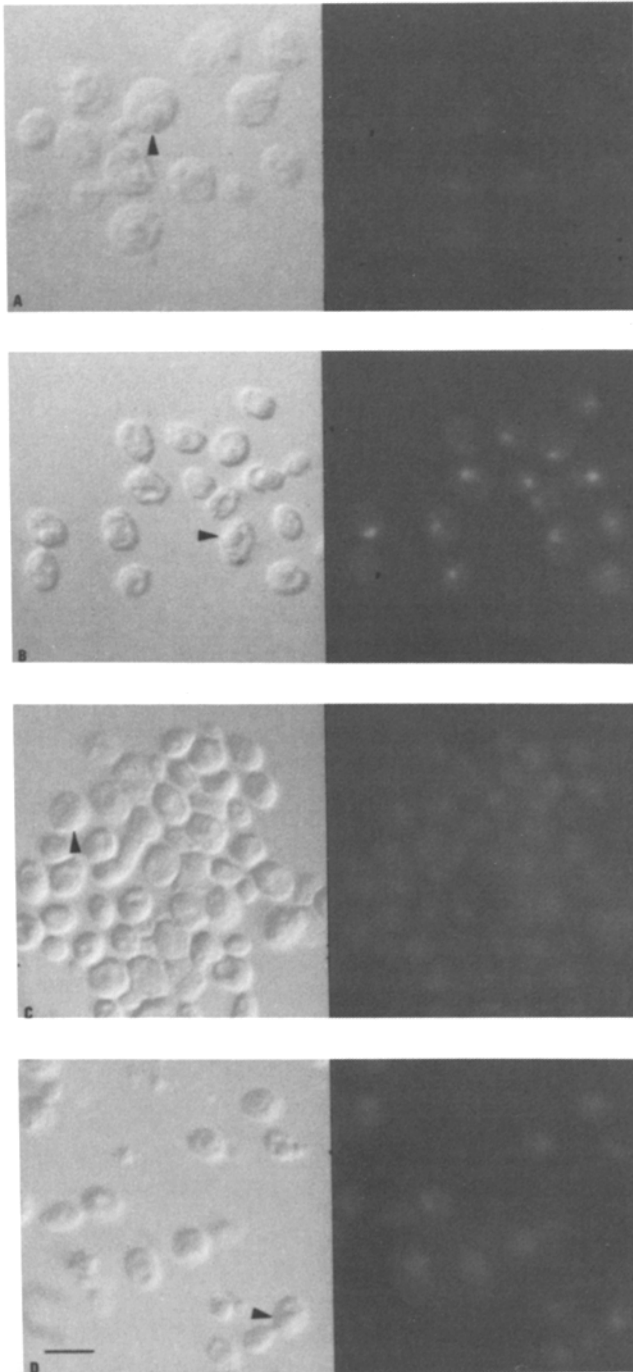To demonstrate a pattern of staining from this weak ligase

Figure 3. Immune fluorescence microscopy of cells induced for tRNA ligase expression. The cell strain 47-1 were prefixed (see Materials and Methods). (*Left*) Panels viewed by Nomarsky differential interference optics. (The nucleus is indicated by the *arrows.*) This image of the cell nucleus has been verified by DAPI nuclear staining (data not shown). (*Right*) panels viewed under FITC excitation wavelengths. The antibody-staining procedure was the same as in Fig. 1 for anti-tRNA ligase (anti-tL3). Aliquots of the cell culture were taken at the following time points after the addition of 2% galactose to the culture: *A*, 0; *B*, 4; *C*, 6; *D*, 8 h. Bar, 5 μm.

signal, data were compiled from many cells. The nuclear envelope was used as a reference point to plot the relative position of the colloidal gold particles in the cell. The shortest distance from a colloidal gold particle to the inner membrane of the nuclear envelope was measured. These distances were then plotted on a histogram. The error for these measurements is considered to be ±25 nm, which is the diameter of the colloidal gold particle plus a single IgG Fab.

The combined data from 320 cells are shown in Fig. 4 *B*. The plot reveals a preferred site of anti-tRNA ligase staining at the inner membrane of the nuclear envelope (see *arrowheads* in Fig. 4 *A*). Staining of the nuclear envelope was five to sixfold above the preimmune antibody staining (Fig. 4 *C*) and above the calculated random staining figures for the nuclear envelope of 3–4%. We also note that 40% of this staining is within 25 nm of a visible nuclear pore structure (see *lower arrowhead*, Fig. 4 *A*). Lesser additional staining was observed in the nucleoplasm within 300 nm of the inner membrane (see gold particles just behind *arrowheads* in Fig. 4 *A*) with its highest concentrations at 75–100 nm in from the nuclear envelope (Fig. 4 *B*). Some staining of the cytoplasm was also observed. The plot from sections stained with the preimmune IgG (Fig. 4 *C*) also shows staining of the cytoplasm in about the same quantities as with the immune IgG whereas the preimmune staining of the nucleoplasm, though, is slightly lower than the immune staining.

Based on the fluorescence results we expected to see a nucleoplasmic location for ligase. If tRNA ligase was only located on the nuclear envelope, it would generate a thin crescent-type fluorescent pattern not the "patch-type" staining seen. Although a nucleoplasmic location for tRNA ligase by immune electron microscopy is not as obvious as the nuclear envelope signal, there is a suggestion of a diffuse region of ligase staining in the nucleoplasm (compare Fig. 4 *B* and 4 *C* nucleoplasmic regions). The existence or not of this secondary site for tRNA ligase and the evidence that the primary nuclear envelope staining is truly a tRNA ligase location is further addressed in the control experiments described below.

Immune Electron Microscope Controls

To confirm the results described above three control experiments were performed. First, a second antiserum was produced against the yeast tRNA ligase. The protein used as the antigen for this antibody was purified from an *E. coli* strain that contained, in an expression vector, the gene for yeast tRNA ligase (21). From this strain 15 mg of purified tRNA ligase was obtained. A high titer, high affinity antiserum, called anti-tLE, was raised using this protein as the antigen. tRNA ligase produced in this manner contained no contaminating yeast proteins, so no yeast polypeptides other than tRNA ligase were stained by anti-tLE in an immunoblot of proteins from strains overproducing tRNA ligase (Fig. 2, lanes *2–8*, labeled *47-1*).

This antibody was used for immune electron microscopy. Sections of a yeast strain, 268, were used. This strain expresses tRNA ligase at ~1.5 times the wild-type levels (see Fig. 2, lane *7*, labeled *268*). The chromosomal tRNA ligase gene in this strain is disrupted and is complemented by a Cen IV plasmid that contains the tRNA ligase gene. The histogram of the combined data for 101 cells is given in Fig. 5 *A*. The staining pattern in this experiment is almost identical to
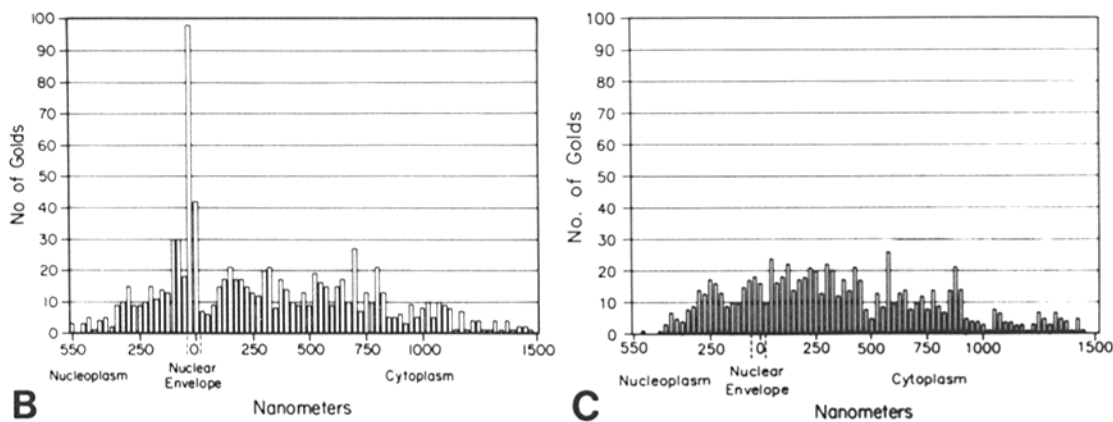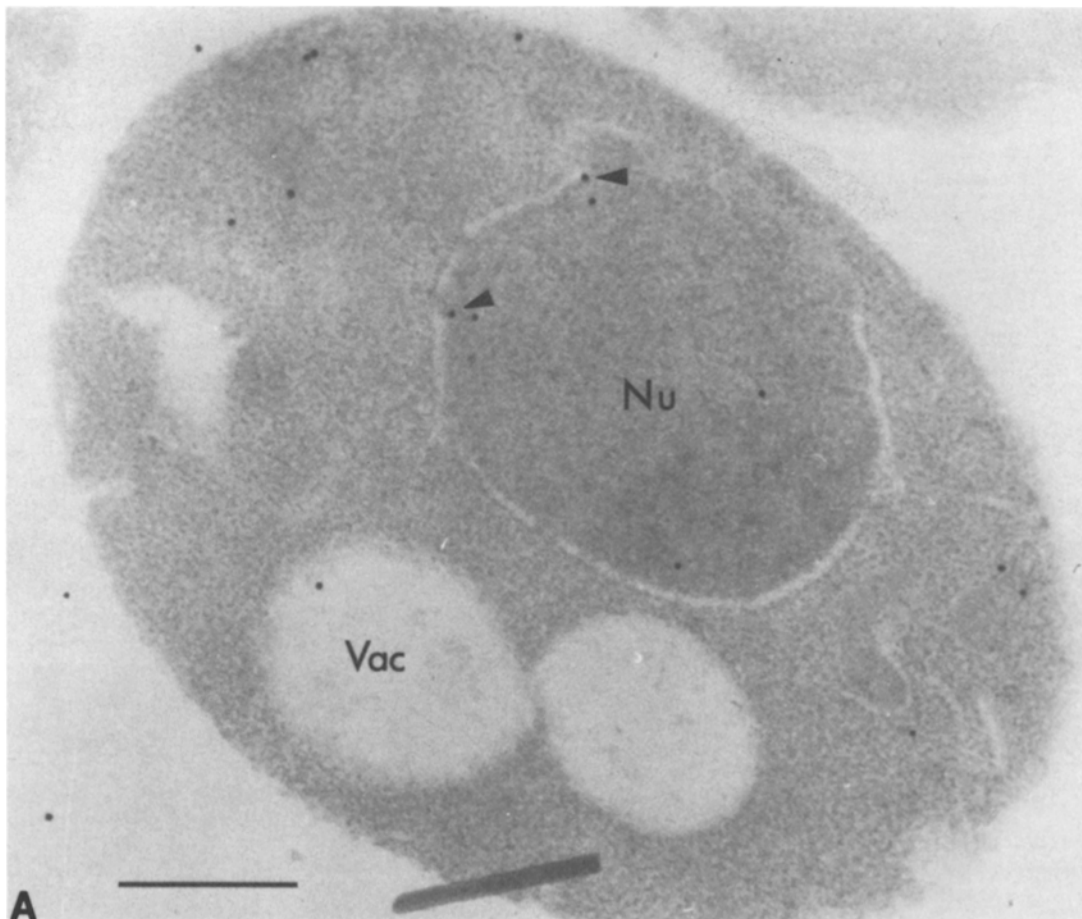
Figure 4. Immune electron microscopy staining for tRNA ligase at wild-type levels. An epon section of 1% glutaraldehyde fixed cells (strain 20B-12-1). The cells were stained with affinity-purified rabbit anti–tRNA ligase antibody (anti-tL3) and visualized with a goat anti–rabbit, 20-nm colloidal gold conjugate. (*Arrowheads*) Nuclear envelope-associated gold particles. The lower arrowhead points to a gold particle that is within 25 nm of a nuclear pore. *Nu,* nucleus; *Vac,* vacuole. Bar, 0.5 µm. (*B*) Histogram of the combined data from 320 yeast cell sections stained as in *A.* (*C*) Histogram of the combined data from 286 yeast cell sections stained as in *A* except preimmune IgG was used as the primary antibody. Measurements represent the shortest distance from the inner membrane of the nuclear envelope to a gold particle. The two bars of the nuclear envelope represent its inner and outer membrane. Each bar covers a distance of 25 nm.

the wild-type tRNA ligase staining (Fig. 4 *B*), with a predominant nuclear envelope staining, 42% of which was nuclear pore associated. Nucleoplasmic staining was present as before although a slightly higher cytoplasmic staining was observed.

To check the specificity of the immune reaction, a second type of control experiment was performed. In this experi-

ment anti-tRNA ligase was inactivated by preadsorption; adding 2 molar equivalents of pure tRNA ligase to the anti-tLE before use removed the tRNA ligase staining ability of the antibody in immunoblot analysis (data not shown). Fig. 5 *B* is the histogram of combined data from sections of strain 268 stained as in Fig. 5 *A* but using the preadsorbed antibody. The preadsorption regimen results in the total elimination of
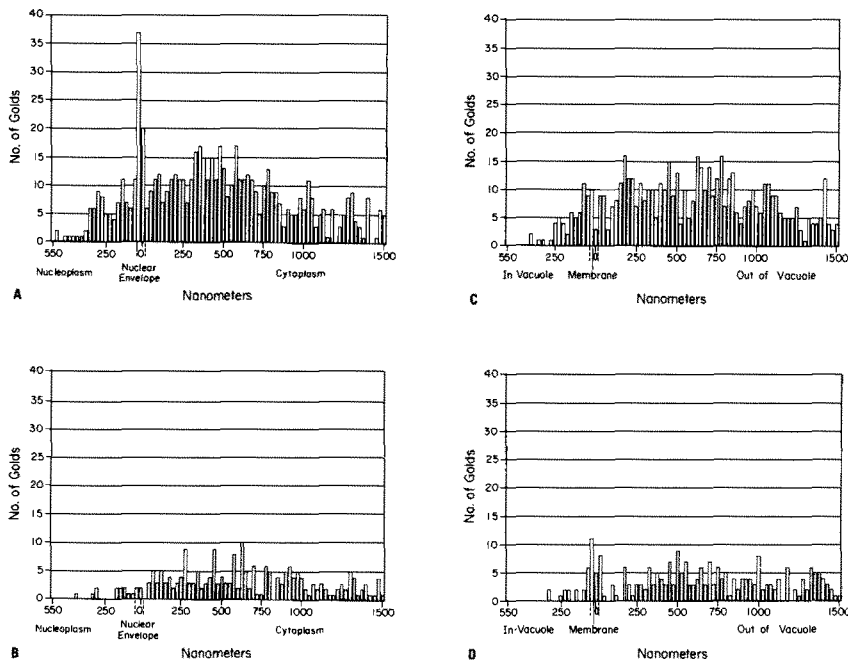
Figure 5. Histograms of the combined data from epon sections of yeast strain 268. This strain, which carries the tRNA ligase gene on a Cen IV plasmid, contains about 1.5 times the wild-type amounts of ligase. These sections were stained for immune electron microscopy with an affinity-purified anti-tRNA ligase antibody (anti-tLE) as the primary antibody. (*A*) Combined data from 101 cell sections stained as described for Fig. 4 *A*. (*B*) Combined data from 102 cell sections stained as in *A* except that 2 molar equivalents of pure tRNA ligase was added to the anti-tLE before use. (*C*) Combined data from 111 cell sections from the same electron micrographs used in *A* except that the reference point for data collection was the vacuole membrane not the nuclear envelope. (*D*) Combined data from 109 cell sections from the same electron micrographs used in *B* except that here the reference point for data collection was the vacuole membrane.

the nuclear envelope staining. We conclude from this that the nuclear envelope site is a primary location of tRNA ligase. The preadsorption of the anti-tRNA ligase antibody also removed most of the nucleoplasmic staining; it was decreased by 2.6-fold relative to total cellular staining (see Table I). In contrast, the preadsorption caused the proportion of cytoplasmic staining, relative to total percentage of cellular staining, to increase (see Table I). The decreased nucleoplasmic staining for ligase upon preadsorption of the antibody suggests a secondary, more diffuse nucleoplasmic location for tRNA ligase. This secondary location seems to be at the nuclear periphery within 300 nm of the nuclear envelope.

A bias could be created by using the annular nuclear envelope as a reference point for data presentation. Consequently, the positions of gold particles were measured relative to the membrane of the vacuole, an organelle that is approximately the same size as the nucleus. The histogram in Fig. 5, *C* and *D* are the results of measuring the same micrographs in Fig. 5, *A* and *B* from the vacuole membrane rather than from the nuclear membrane. No obvious staining pattern can be seen. Using an annular structure as a reference point for the measurement procedure does not introduce an obvious bias.

Steady-State Overproduction of tRNA Ligase Causes a Change in the Cellular Distribution of Protein

We were interested in determining the position of tRNA ligase in cells containing increased amounts of the enzyme. In this section, we will present the results of experiments in which tRNA ligase was overproduced by placing the ligase gene on a 2 μ, multicopy plasmid. This strain contains about 30–35 times the wild-type level of tRNA ligase (compare Fig. 2, lanes 2 [tL] and 8 [284]).

The histogram in Fig. 6 *A* summarizes the gold particle locations in this strain. The tRNA ligase staining pattern has been drastically changed. Here, the predominant staining is in the cytoplasm. The nucleoplasm staining has also increased 1.7-fold (see Table I), while the nuclear envelope staining remains about the same as in Cen IV plasmid and wild-type levels of tRNA ligase. The addition of the pure tRNA ligase to the anti-tLE removes most of the staining (Fig. 6 *B*). Thus, it appears that the nuclear "binding sites" for tRNA ligase become saturated when ligase is overexpressed and the residual ligase remains in the cytoplasm. The nuclear envelope site for tRNA ligase appears to be saturated at wild-type levels.

Table I. Quantitation of the Immune Electron Microscopic Data

Measured from the nuclear envelope	Wild-type	268	268+ ligase	284	284+ ligase	47-1* 0 time	47-1* 0 time+ ligase	47-1* 4 h	47-1* +4 h ligase
Golds in the nucleoplasm	216(23)‡	94(13)	12(5)	158(13)	16(7)	131(21)	50(13)	306(35)	90(23)
Golds on the nuclear envelope	140(15)	57(8)	4(2)	49(4)	5(2)	79(13)	16(4)	44(5)	9(2)
Golds in the cytoplasm	588(62)	553(79)	218(93)	972(83)	207(91)	425(66)	306(83)	531(60)	297(75)
Total golds counted	944	704	234	1179	228	635	372	881	396
Number of cells counted	320	101	102	107	113	123	116	113	109

* Galactose-induction experiment in which preimmune antibody staining has been subtracted.
‡ The number in parentheses is the percent of total cellular gold particles found within the indicated region of the cell.
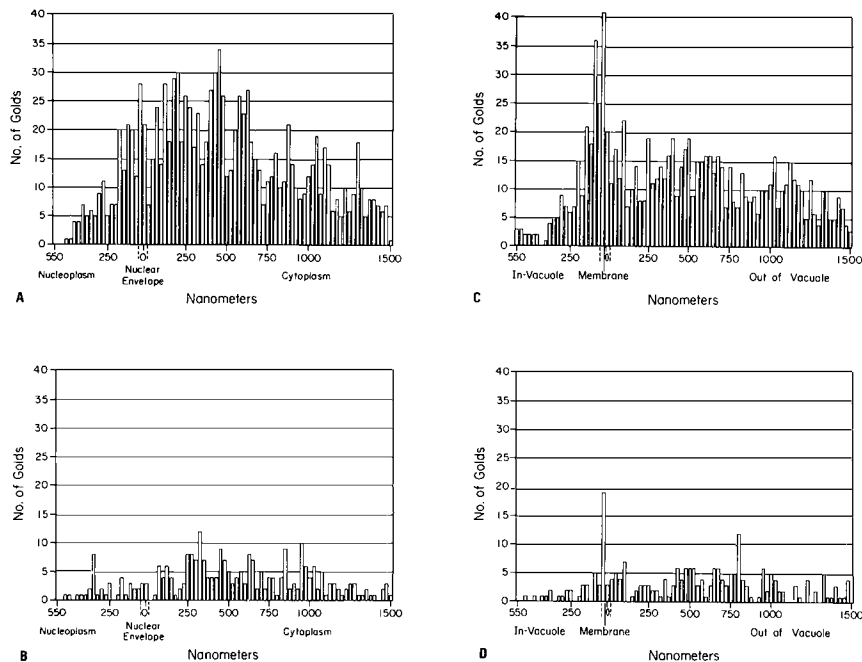
Figure 6. Histograms of combined data from epon sections of yeast strain 284. In this strain the ligase gene is carried on a 2µ plasmid and the level of ligase is 30–35 times wild-type. These sections were stained for immune electron microscopy with affinity-purified anti–tLE antibody. (*A*) Combined data from 107 cell sections stained as described in Fig. 4 *A*. (*B*) Combined data from 113 cell sections stained as in *A* except for the addition of 2 molar equivalents of pure tRNA ligase to the anti–tLE before use. (*C*) Combined data from 99 cell sections from the same electron micrographs used in *A* except the reference point for data collection was the vacuole membrane. (*D*) Combined data from 95 cell sections from the same electron micrographs used in *B* except that the reference point for the data collection was the vacuole membrane.

When the gold particle locations in this data set were plotted relative to the vacuole membrane an interesting result was seen. Staining was observed at the vacuole membrane and some staining within the vacuole (Fig. 6 *C*). These signals disappear when purified tRNA ligase was added to the antibody (Fig. 6 *D*), so the locations appear to be genuine. The cause of this vacuole staining is not known. It could be an artifact of ligase overproduction; for example, superfluous tRNA ligase protein could be "dumped" into the vacuole. It seems unlikely that these vacuole sites are functional ligase locations.

Limited Overproduction of tRNA Ligase Increases the Location of tRNA Ligase in the Cell Nucleus

When the tRNA ligase gene was expressed under control of the GAL10 promoter the fluorescent signal in the nucleus became saturated 4 h after induction. It was therefore of interest to monitor ligase location as a function of time after induction with the higher resolution of immune electron microscopy. Fig. 7 *A* shows a graphic comparison of immunoblot ligase staining quantitated by densitometry, to immune electron microsope/tRNA ligase staining in a galactose induction time course experiment. As can be seen, the number of gold particles per cell (Fig. 7 *A*, *solid squares*) increases with the time after galactose induction, but at a slower rate than is seen by the immunoblot analysis (Fig. 7 *A*, *solid triangles*). The lack of direct correspondence between the two techniques may be due to the relatively low resolution of the 20-nm gold particle. A single gold particle could cover many ligase molecules if they were clustered. Still, despite its lack of linearity, the immune electron microscopy staining technique reflects the increase in cellular tRNA ligase concentration.

In Fig. 7 *B* the immune electron microscopy data from Fig. 7 *A* are graphed according to cellular location: nucleus (*solid circles*) vs. cytoplasm (*crosses*). The result was similar to that seen in the fluorescent staining experiment (Fig. 3). Immunofluorescence and immune electron microscope techniques

revealed an increase of tRNA ligase in the nucleus until 4 h of induction. The nuclear concentration then leveled off and tRNA ligase in the cytoplasm began to increase.

Limited Overproduction of tRNA Ligase Reveals Distinct Accumulations of tRNA Ligase at the Nuclear Pore and in the Nucleoplasm

After 4 h of galactose induction, most of the tRNA ligase was concentrated in the nucleus. We have analyzed the tRNA ligase cellular location at 0 time and 4 h after galactose induction. Fig. 8, *A* and *C* are the histogram of ligase location in the induced strain at 0 and 4 h after galactose induction, respectively. Fig. 8, *B* and *D* are the control histogram in
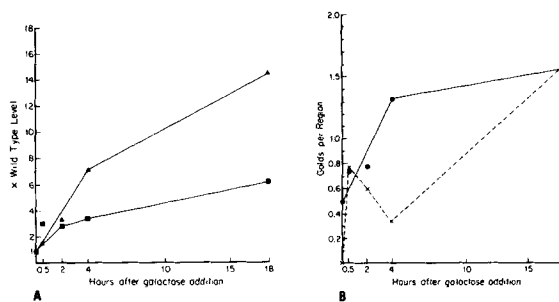

Figure 7. Immune electron microscopy of the galactose inducible strain, 47-1. (*A*) Graphic comparison of ligase levels as determined by immunoblot (Fig. 2, lanes 2–7) or immune electron microscopy. For each immune electron microscopic data point 70–90 sections were examined. (*Solid triangles*) Immunoblot band intensities as determined by densitometry. (*Solid squares*) Immune electron microscopy anti–tRNA ligase staining of whole cells. Each data point represents the average number of gold particles per cell divided by the average number of gold particles per cell at 0 time (with background subtracted). (*B*) Immune electron microscopy/anti–tRNA ligase data from *A* graphed as the number of gold particles per cell region (background subtracted). (*Solid circles*) Gold particles in nucleus. (*Crosses*) Gold particles in the cytoplasm.
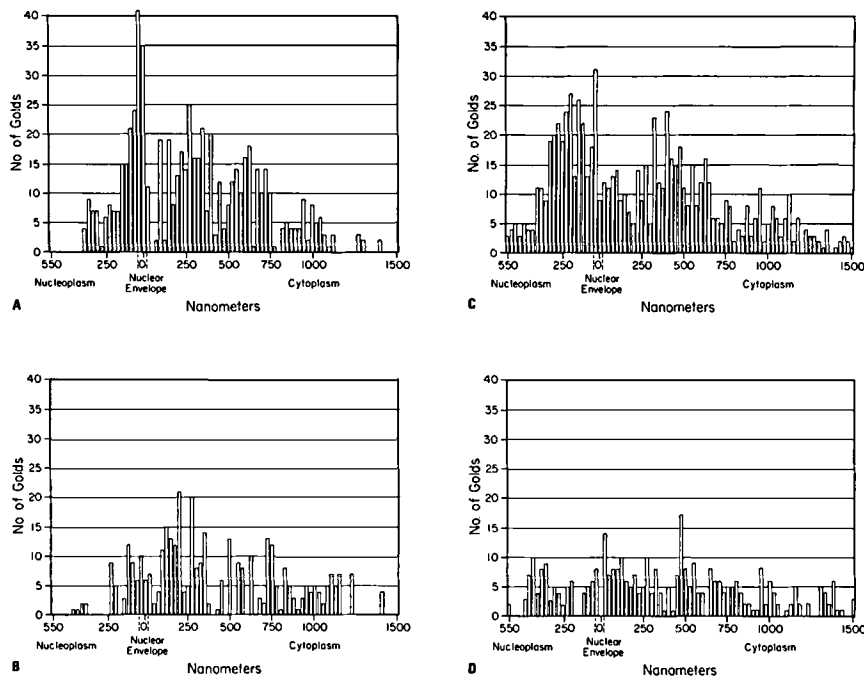
Figure 8. Immune electron microscopy/anti–tRNA ligase histograms of combined data from sections of the galactose inducible yeast strain (47-1) stained with anti-tL3. Because of the high background staining in this set of experiments, preimmune IgG staining was subtracted from every data set. (*A*) Combined data from 123 sections of cells at 0 time. These sections were stained as described for Fig. 4 *A*. (*B*) Combined data from 116 sections of cells at 0 time. These sections were stained as in *A* except that 1 molar equivalent of pure tRNA ligase was added to the anti-tL3 before use. (*C*) Combined data from 113 sections of cells at 4 h after galactose addition to the cell cultures. These sections were stained as in *A*. (*D*) Combined data from 109 sections of cells at 4 h after galactose addition to the cell culture. These sections were stained as in *B*.

which anti–tRNA ligase antibody was blocked by the addition of excess tRNA ligase.

At 0 time (Fig. 8 *A*) the distinct nuclear envelope staining was seen (cf. Fig. 4 *B*) where 35% of the particles were nuclear pore associated. The nucleoplasmic staining (cf. Fig. 4 *B*) was also observed. The addition of the purified tRNA ligase to the affinity-purified IgG (Fig. 8 *B*) removed all of the nuclear envelope staining and a majority of the nucleoplasmic staining.

After 4 h of galactose induction (Fig. 8 *C*), the nuclear envelope staining remained about the same as at 0 time, while the nucleoplasmic staining increased 2.3-fold over wild-type levels (see Table I). This increase was predominately at a region between 100–200 nm from the inner nuclear membrane. The staining of the nuclear envelope and nucleoplasm was removed by the addition of purified tRNA ligase to the affinity-purified antibody (Fig. 8 *D*). Apparently, the excess tRNA ligase in the nucleus accumulated at a distinct region 100–200 nm from the nuclear inner membrane. Again, it appears that the nuclear envelope sites are saturated by normal levels of tRNA ligase.

Close examination of the staining pattern after 4 h of galactose induction revealed some interesting aggregations of gold particles. Nuclear pore–associated accumulations were seen in 6% of the induced cells (Fig. 9). Arrows indicate pore structures with gold accumulated either in the nucleoplasm (Fig. 9, *A* and *B*) or just inside the pore in the nucleoplasm (Fig. 9 *D*). The accumulation of tRNA ligase molecules at the pore can be explained if tRNA ligase interacts with the pore structure or normally enters or leaves the nucleus through the pore. Also in 6% of the cells a series of gold particles could be seen that appeared to parallel the contour of the nuclear envelope within ~100–200 nm from nuclear envelope (*arrowheads*, Fig. 10). This nucleoplasmic pattern was never seen to completely circle the nucleus, but only occupied a sector of the nucleus. These data suggest a nucleoplasmic location for tRNA ligase in addition to the nuclear

envelope site. Also, the distinct nucleoplasmic pattern seen here suggests some nuclear substructure which could direct tRNA ligase to this region.

Discussion

We have demonstrated that yeast tRNA ligase has a primary location at the inner membrane of the nuclear envelope. 40% of the gold particles were located within 25 nm of a recognizable nuclear pore (i.e., an obvious connection between the inner and outer membrane that is 40–80 nm wide). Although we cannot conclude that all of the nuclear envelope staining is at pore sites, 40% association with the pore represents a fourfold increase over what would be expected if the gold particles were scattered at random along the nuclear envelope. The association with pores therefore appears to be significant.

The observation by Greer (8) of a functional association of ligase with endonuclease and the observation that the endonuclease is an integral membrane protein (19) strongly suggested a nuclear membrane location for tRNA splicing. Our localization of tRNA ligase supports such a conclusion. The small number of endonuclease molecules in a yeast cell (~150–200 copies per cell; Green, P., and J. Abelson, unpublished results) is of the same order as estimated for the tRNA ligase molecule. From the immune electron microscope studies, there appears to be limited number of sites for tRNA ligase on the nuclear membrane. A preferential association with the nuclear membrane was apparent in wild-type cells but when the level of tRNA ligase was increased further staining of the nuclear membrane was not observed. Thus the preferential ligase site in the nuclear membrane that we have observed here could be a membrane-bound endonuclease molecule.

Localization of tRNA splicing at a nuclear pore is very interesting and it suggests there is a connection of the nuclear pore with transport of tRNA to the cytoplasm. The precursor
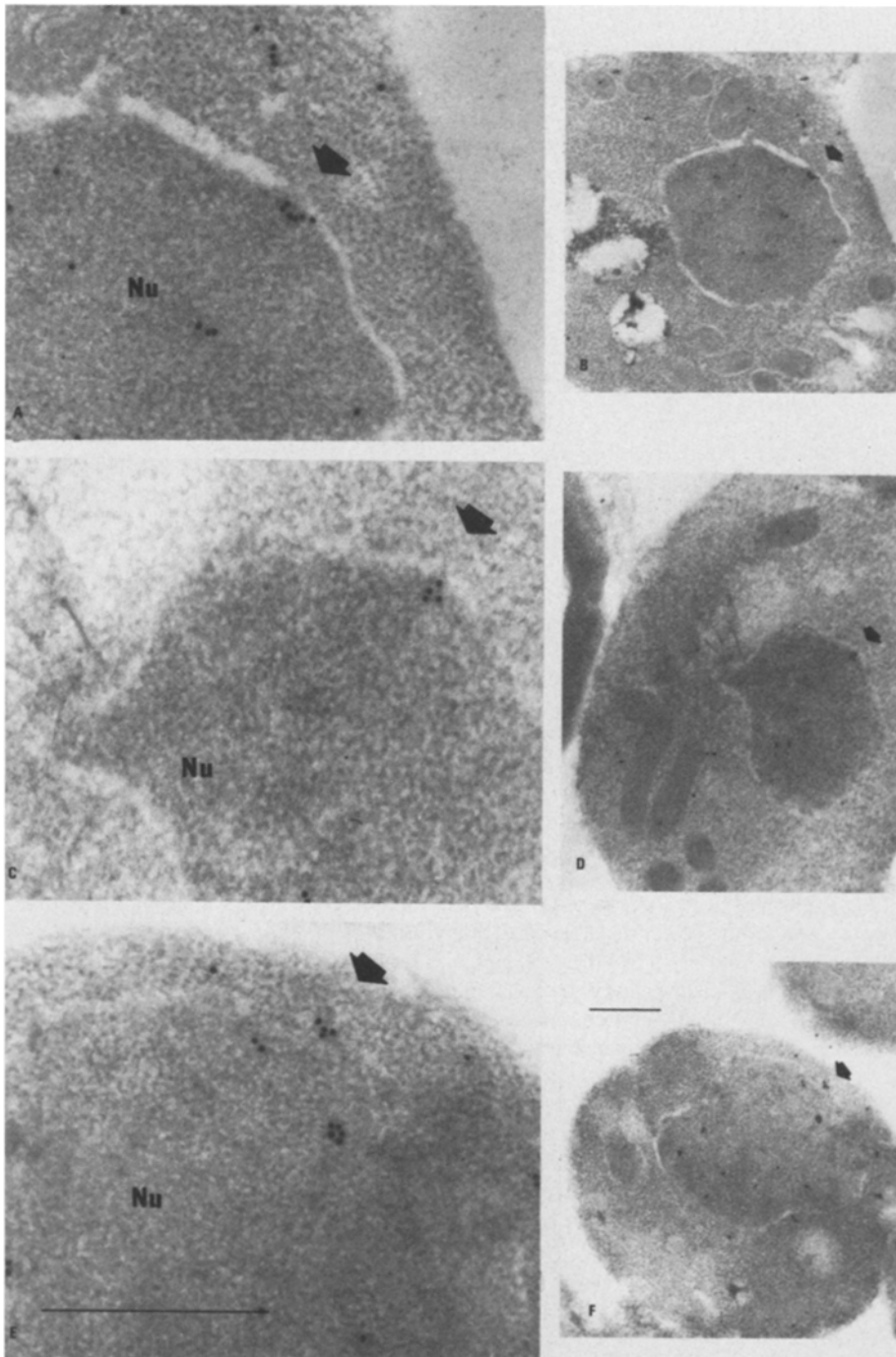
Figure 9. Gallery of electron micrographs of the galactose inducible strain (47-1), 4 h after galactose addition to the cell culture. The cells were stained for immune electron microscopy/anti-tL3. The data show nuclear pore–associated tRNA ligase staining. *A*, *C*, and *E* are enlargements of *B*, *D*, and *F*, respectively. The arrow indicates the nuclear pore with gold accumulation. *Nu*, nucleus. Bar, 0.5 μm.

substrates for splicing are end matured and partially modified. After the intron is removed they are ready for transport to the cytoplasm. Zasloff (26) has demonstrated that transport of tRNA from nucleus to the cytoplasm in *Xenopus* oocytes is not a simple diffusion process but rather is accomplished by a saturable, carrier-mediated, translocation process. An attractive hypothesis, suggested by our results, is that enzymes that mediate earlier steps in the progression of a tRNA molecule from transcript to fully functional tRNA are physically located at/or near the pore complex.

Both the immune fluorescence and electron micrographs suggested a second location for tRNA ligase in the nucleo-

plasm. In micrographs of cells that contained moderate increases in tRNA ligase, this location was revealed as an annulus parallel to the nuclear membrane, 100–200 nm into the nucleoplasm (Fig. 10). Recent experiments by H. Belford and C. Greer, (personal communication) have shown a stable complex formed between end-matured, intron-containing tRNA precursors with tRNA ligase. Their results suggest a model in which tRNA precursors containing introns are released from sites of transcription and/or end maturation and bind to tRNA ligase. This complex then moves to the membrane-bound endonuclease where the events of tRNA splicing occur. Following this model, the nucleoplasmic lo-
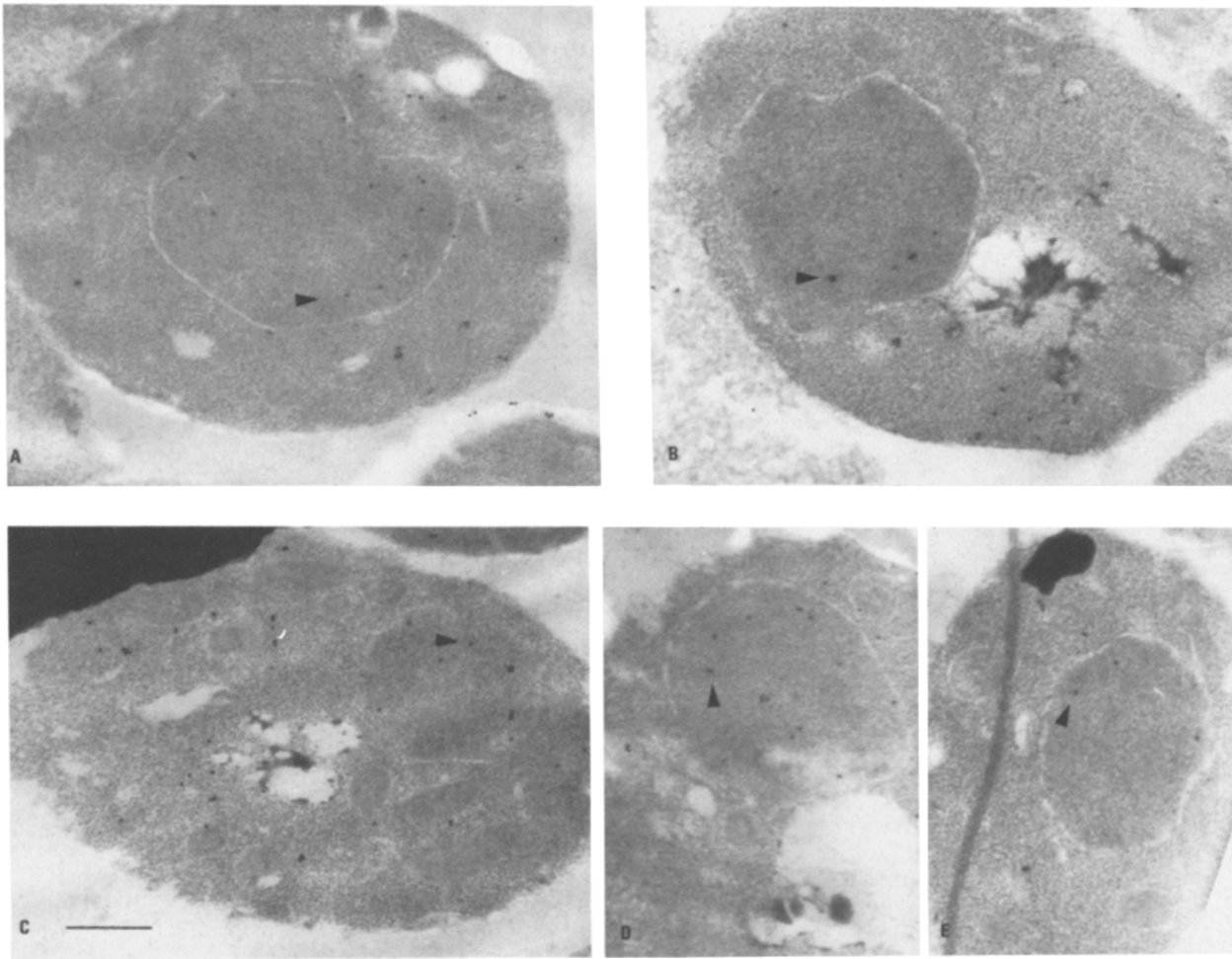
Figure 10. Gallery of electron micrographs of the galactose inducible strain (47-1), 4 h after galactose addition to the cell culture. The cells were stained for immune electron microscopy/anti-tL3. The data show the nucleoplasmic tRNA ligase staining pattern. The arrowheads indicate the start of a line of gold particles that are ~200 nm from the inner membrane of the nuclear envelope. Bar, 0.5 μm.

cation we observe for tRNA ligase could be the site of transcription and/or end maturation for the tRNAs. Such a spatially ordered model for tRNA maturation fits well with the temporally ordered sequence for these events reported for the *Xenopus* oocyte tRNA processing by Melton and co-workers (15). The annular array of tRNA ligase molecules in the nucleoplasm may also represent a distinct structure at that location, which reflects an element of nuclear architecture that has not yet been described. Further studies on the biochemistry and genetics of tRNA splicing in yeast may reveal elements of that structure.

Earlier studies on the localization of the tRNA splicing enzymes in the *Xenopus* oocyte by DeRobertis and co-workers (6) did not suggest a nuclear envelope localization. When germinal vesicles were manually dissected into a nuclear envelope and nucleoplasm fraction, splicing activity was only associated with the latter. tRNA splicing endonuclease from *Xenopus* oocytes purified by Tocchini-Valentini and his colleagues (18) does not appear to be an integral membrane protein. Thus the nuclear membrane localization that we have observed for tRNA splicing enzymes may not be a universal one. The two studies are operationally different, though, so it will be interesting to learn the results of a cyto-

logical study of the location of the *Xenopus* endonuclease when that experiment is possible.

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