Cytoplasmic Leucyl-tRNA Synthetase of Neurospora crassa Is Not Specified by the leu-5 Locus

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ABSTRACT

We generated a λgt11 Neurospora crassa cDNA library and screened the library for the cytoplasmic leucyl-tRNA synthetase (cyto LeuRS) clones using cyto LeuRS specific antibody. Two clones, λNCLRSC1 and λNCLRSC2, were obtained which have inserts of ~2 kbp and ~1.3 kbp, and which overlap at about 0.6 kbp. The following lines of evidence indicate that λNCLRSC1 and λNCLRSC2 encode parts of cyto LeuRS: (1) Antibodies affinity purified using either of the fusion proteins encoded by λNCLRSC1 or λNCLRSC2 inhibit cyto LeuRS activity. Thus, the fusion protein and cyto LeuRS share immunological determinants. (2) The same antibodies also react with an ~115-kDa protein, which comigrates with purified cyto LeuRS, in immunoblots of total N. crassa proteins. We used the cDNA clones to probe a N. crassa genomic DNA library and isolated two genomic DNA clones. Partial sequence analysis of cDNA and genomic DNA clones shows a methionine initiated open reading frame, which includes a stretch of amino acid residues that are highly conserved and that are at the ATP binding site in aminoacyl-tRNA synthetases. Using the cloned DNA as probe, we show that the cyto LeuRS mRNA is ~3900 nucleotides long. Finally, we have used restriction fragment length polymorphism mapping to show that the cyto LeuRS gene resides on the far right of linkage group II and not on linkage group V where the leu-5 mutation, which was previously reported to specify cyto LeuRS, is located.

PROTEINS which perform the same basic function can be localized in two or more cellular compartments in eukaryotes. These include proteins involved in the replication and expression of genetic material, and whether they are differentially regulated. Recent work in yeast on genes for aminoacyl-tRNA synthetases and tRNA modifying enzymes suggests that there are two different strategies for specifying proteins located in different cellular compartments. For histidyl-tRNA synthetase (Natsoulis, Hillger and Fink 1986), valyl-tRNA synthetase (Chatton et al. 1988) and three of the tRNA modifying enzymes (Hopper et al. 1982; Ellis et al. 1986; Dihanich et al. 1987), a single gene appears to code for both the cytoplasmic and mitochondrial forms of the enzyme. However, for threonyl-tRNA synthetases and tryptophanyl-tRNA synthetases (Myers and Tzagoloff 1985; Pape, Koerner and Tzagoloff 1985), different genes code for the cytoplasmic (cyto) form and for the mitochondrial (mito) form.

Little is known at the molecular level about the genes for the aminoacyl-tRNA synthetases or tRNA modifying enzymes in Neurospora except for an intriguing observation made by Gross and coworkers. These workers purified the cytoplasmic and mitochondrial leucyl-tRNA synthetases of N. crassa and showed that these enzymes have different tRNA substrate specificities and are immunologically distinct (Gross, McCoy and Gilmore 1968; Beauchamp, H orn and Gross 1977; Beauchamp 1975). However, it was noted that in the temperature-sensitive leucine auxotroph, leu-5, a single mutation affected both enzymes. In such a mutant the mitochondrial leucyl-

Abbreviations: cyto LeuRS, cytoplasmic leucyl-tRNA synthetase; mito LeuRS, mitochondrial leucyl-tRNA synthetase; PhRS, phenylalanyl-tRNA synthetase; kDa, kilodalton; IPTG, isopropyl thigalactoside.

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tRNA synthetase activity was much reduced and the cytoplasmic enzyme had an altered $K_m$ for leucine. In revertants which were prototrophic for leucine both of these phenotypes coreverted (Weeks and Gross 1971). These results suggested that the leu-5 genetic locus specified structural and/or regulatory information for both forms of LeuRS (Beauchamp, Horn and Gross 1977).

For our studies on analysis of genes for cytoplasmic and mitochondrial forms of aminoacyl-tRNA synthetases in N. crassa, and their regulation, we therefore selected leucyl-tRNA synthetase and as a particularly interesting system to study. Here, we describe the isolation and characterization of cDNA and genomic DNA clones for the cytoplasmic LeuRS of N. crassa and their use in chromosomal mapping of the cyto LeuRS gene using restriction fragment length polymorphism (RFLP) in N. crassa (Metzenberg et al. 1985). We show that the cyto LeuRS gene maps not on linkage group V where leu-5 maps but on linkage group II. This is in agreement with the recent reports of Kunugi et al. (1986) and Airas, Schischchoff and Cramer (1986) who purified cyto LeuRS from wild type and the leu-5 mutant strain of N. crassa and found no difference in $K_m$ for leucine between the cyto LeuRS from these strains. Thus, the leu-5 locus in N. crassa does not specify structural information for cyto LeuRS. In work currently underway, we have shown that the leu-5 locus does specify the mitochondrial enzyme. Therefore, the leucyl-tRNA synthetases of N. crassa are encoded by distinct genes.

MATERIALS AND METHODS

Materials: Staphylococcus aureus protein A and protein A-Sepharose were from Pharmacia, protein size markers were from either Bethesda Research Laboratories or Bio-Rad, and biotinylated anti-rabbit IgG and avidin horseradish peroxidase were from Vector Labs (Vectastain Kit). Yeast tRNA<sup>Ph</sup> and calf liver tRNA were from Boehringer Mannheim and yeast tRNA<sup>Ph</sup> was a gift from S. H. Chang. The rest of the materials were as described in Saches et al. (1986).

N. crassa CDNA and genomic DNA clones: Immunological screening of a Agt11 N. crassa CDNA library yielded two clones designated ANCLRS1, Neurospora cytoplasmic leucyl-tRNA synthetase cDNA clone, and ANCLRS2. The EcoRI inserts from these clones were excised and subcloned into the EcoRI site of pBR322 to yield pANCLRS1 and pANCLRS2, respectively. The same inserts were cloned into M13mp8 to yield mANCLRS1 and mANCLRS2.

N. crassa genomic DNA libraries containing partial Sau3A fragments, were obtained from M. J. Orbach, M. G. Schechtman and C. Yanofsky. Clone pNCLRSG11 (Neurospora cytoplasmic leucyl-tRNA synthetase genomic DNA clone, our designation) was isolated from a pBR3K-N. crassa genomic DNA library (Schechtman and Yanofsky 1983) using the cDNA insert in pNCLRSC1 as probe. Clone ANCLRS12 was isolated from a AJ1 N. crassa genomic DNA library (Orbach, Porro and Yanofsky 1986) using pNCLRSC1, pNCLRSC2 and pNCLRSG11 DNAs as probes. Each library was screened by hybridization to either colonies on filters (Hanahan and Meselson 1980) or by plaque lifts (Maniatis, Fritsch and Sambrook 1982).

pNCLRSG11 has ~1.5-kbp size insert whereas pNCLRSG12 has ~10-kbp size insert. A 4.4-kbp BamHI fragment derived from pNCLRSG12 was subcloned into the BamHI site of pBR322 to yield pNCLRSG13.

Enzymes: A crude enzyme fraction containing cyto LeuRS activity was obtained from N. crassa mycelia broken by bead beating (Cramer et al. 1983). Step elution of a S100 fraction following its adsorption on a DEAE-cellulose column (RajBhandary and Ghosh 1969) with a buffer containing 0.25 M potassium phosphate, pH 6.5, provided a protein peak depleted of nucleic acids. Material in the peak fractions was precipitated with 55% ammonium sulfate. The precipitated material was resuspended, dialyzed against buffer and made 50% in glycerol (RajBhandary and Ghosh 1969). This N. crassa extract (~5 mg protein/ml) was stored at -20°C and used for most of the experiments.

Purified cyto LeuRS (Kunugi et al. 1986) used as a master in some of these immunoblot experiments, was kindly provided by U. Englisch.

Antibodies: Rabbit anti-cyto LeuRS antiserum (Beauchamp, Horn and Gross 1977) was a kind gift of S. Gross. IgG fractions from this antiserum or nonimmune serum were obtained by affinity chromatography on protein A-Sepharose columns according to Goudswaard et al. (1978). IgG fractions were used for immunoblot analysis, immunoprecipitation of in vitro translation products or in experiments involving inhibition of N. crassa cyto LeuRS activity whereas total antiserum was used for immunological screening of the Agt11 N. crassa CDNA library and for affinity purification of the clones ANCLRS1 and ANCLRS2.

Immunoblot analysis of N. crassa proteins: Proteins in N. crassa extract were separated by electrophoresis on SDS-polyacrylamide gels (Laemmli 1970), electroblotted onto nitrocellulose filters (Burnette 1981) and were incubated overnight with antibodies at 4°C. Immune complexes were detected using either 125I-labeled protein A (40 μCi/μg) or biotinylated anti-rabbit IgG and avidin-conjugated horseradish peroxidase (Vectastain-ABC).

Aminoacylation assays and effects of antibodies on aminoacylation: These were essentially as described by Burnette, Von der Haar and Cramer (1972). The incubation mixture (120 μl) contained 150 mM Tris-HCl, pH 7.4, 150 mM KC1, 10 mM MgCl₂, 8 mM 2-mercaptoethanol, 0.2 mM CTP, 2 mM ATP, 20 μM 3H-Leu, yeast tRNA<sup>Ph</sup> (1 μg) or total calf liver tRNA (100 μg) and N. crassa extracts (~30 μg protein). Incubation was at 37°C. Assays for aminoacylation with phenylalanine were exactly the same except for substitution of 14C-Phe for 3H-Leu and yeast tRNA<sup>Ph</sup> (1 μg) for yeast tRNA<sup>Ph</sup>. Effects of antibodies on aminoacylation activity were assessed in one of two manners. N. crassa extract was preincubated with nonimmune or immune IgG for 1 hr at 4°C; immune complexes were cleared by the addition of protein A-Sepharose for 30 min at 4°C, followed by centrifugation. Alternatively, immunoadsorbants were incubated directly with the crude extract and then centrifuged. In both cases, activity remaining in the supernatant fraction was measured by 14C-Phe or 3H-Leu incorporation.

In vitro translation of N. crassa RNAs: Wheat germ extracts were used to translate N. crassa mRNA as described (Sachs 1986). Total translation products were incubated with anti-cyto LeuRS IgG or nonimmune IgG overnight followed by one hour with protein A-Sepharose. The immune complexes adsorbed to protein A-Sepharose were pelleted by centrifugation, washed extensively and used for electrophoresis on SDS-polyacrylamide gels. The 35S-labeled proteins were visualized by fluorography.
Construction of \( \lambda \)gt11 \( N. \) crassa cDNA library: RNA was isolated from \( N. \) crassa mycelia using the guanidinium isothiocyanate method (Chirgwin et al. 1979). Poly(A\(^{\ast}\)) RNA was prepared by oligo-dT-cellulose chromatography (Aviv and Leder 1972). cDNA synthesis was according to Huynh, Young and Davis (1985) with some modifications; cDNAs greater than 500 bp were pooled and used for ligation to EcoRI-treated \( \lambda \)gt11 DNA. The recombinant \( \lambda \)gt11 DNA was packaged in vitro (Maniatis, Fritsch and Sambrook 1982) and plated on Escherichia coli Y1090 in the presence of IPTG and X-gal. Recombinant phage, approximately 70% of the total phage, were identified as those yielding colorless plaques whereas the wild-type \( \lambda \)gt11 phage yielded blue plaques.

Immunological screening of the \( \lambda \)gt11 cDNA library and isolation of recombinant phage DNAs: The cDNA library was screened with anti-cyto LeuRS antiserum using published procedures (Young and Davis 1983). Approximately 150,000 recombinants were plated on two 150-mm plates. The plaques were transferred to nitrocellulose filters in duplicate, and screened with anti-cyto LeuRS antiserum diluted 1000-fold in the blocking agent, 1% hemoglobin in 50 mM Tris-HCl (pH 8.0) and 0.5% NaCl. \( ^{125}\)I-protein A prepared according to Dorval, Welsh and Wiszel (1975) was used for detection of immune complexes (~4 \( \times \) 10\(^5\) cpn 10 ml/plate). Five of the plaques gave positive signals on both sets of duplicate filters in the first round. Following three additional rounds of screening, two independent clones \( \lambda \)NCLRSC1 and \( \lambda \)NCLRSC2 were isolated. Plate stocks of the recombinant phages were made (Maniatis, Fritsch and Sambrook 1982) and used to lysogenize \( E. \) coli 1089. Lysogens induced by temperature shift were used for large scale isolation of \( \lambda \)NCLRSC1 and \( \lambda \)NCLRSC2 DNAs (Maniatis, Fritsch and Sambrook 1982).

Affinity selection of antibodies using \( \lambda \)NCLRSC1, \( \lambda \)NCLRSC2 and \( \lambda \)gt11 plaques: \( \lambda \)NCLRSC1, \( \lambda \)NCLRSC2 and \( \lambda \)gt11 phage were plated at ~5 \( \times \) 10\(^5\) plaques per 100-mm plate and the plaques were transferred to IPTG soaked nitrocellulose filters. The filters were then incubated with a 1:500 dilution of anti-cyto LeuRS antiserum, as described above for the immunological screening of recombinant phage. The filters were washed with TBS; antibodies bound to the filters were eluted (Weinberger et al. 1985), concentrated by ammonium sulfate precipitation and used for immunoblot analysis of \( N. \) crassa proteins. Alternatively, the eluted antibodies were adsorbed to protein A-Sepharose and used as an immunoadsorbant for cyto LeuRS activity in \( N. \) crassa extracts.

cDNA probes: Probes for screening of genomic libraries or for hybridizations to blots of DNA or RNA were prepared by nick translation (Rigby et al. 1977) of cDNA inserts in pNCLRSC1 or pNCLRSC2.

DNA sequencing: The genomic clone pNCLRSG11, was partially sequenced using restriction fragments and the MAXAM and GILBERT (1980) chemical degradation reactions. The cDNA clone, mNCLRSC1, was partially sequenced using the dioxy chain termination method (Sanger, Nicklen and Coulson 1977).

RNA blot hybridization: Poly(A\(^{\ast}\)) RNA (4 \( \mu \)g) was denatured by treatment with glyoxal (Mcmaster and Carmichael 1977), resolved on a 1 % agarose gel, transferred to nitrocellulose (Thomas 1980), and hybridized to \( ^{32}\)P-DNA (6 \( \mu \)Ci) labeled by nick translation of the pNCLRSC1 insert. \( ^{32}\)P-DNA size markers, made from \( \lambda \) DNA cut with HindIII and EcoRI, were labeled at their 3'-termini and also treated with glyoxal prior to electrophoresis.

Chromosomal mapping: The position of the cyto LeuRS structural gene with respect to the genetic map of \( N. \) crassa was determined using restriction fragment length polymorphism mapping (Metzenberg et al. 1984, 1985). Nick-translated pNCLRSC1 was used to probe Southern (1975) blots (provided by R. Metzenberg) of restricted genomic DNA from parental strains and from the progeny of the following crosses: Mauriceville-1c-A (FGSC #2225) \( \times \) al-2; nuc-2, arg-12; cot-1; ml-a (FGSC #4411); Mauriceville-1c-A \( \times \) multicent-2-a (FGSC #4488) and Mauriceville-1c-A \( \times \) pyr-4, arg-5, prep', trp-3-a. Of ten different restriction enzymes used to cut genomic DNA of the parental Mauriceville and Oakridge strains, restriction fragment length polymorphisms were observed with two, XhoI and EcoRV. Southern blots of genomic DNA cut with these enzymes were, therefore, used for chromosomal mapping of the cyto LeuRS gene.

RESULTS

Characterization of the anti-cyto LeuRS antibody: Prior to use in immunological screening of a \( N. \) crassa \( \lambda \)gt11 cDNA library, we established the specificity of the anti-cyto LeuRS antibody by its specific inhibition of cyto LeuRS activity and by immunodetection of \( N. \) crassa extracts.

Figure 1 shows the results of an experiment in which a \( N. \) crassa extract was incubated with anti-cyto LeuRS IgG fraction, immune complexes were removed by centrifugation and the supernatant was assayed for aminoacylation activity for leucine and for phenylalanine. Immune IgG inhibits the LeuRS activity in the \( N. \) crassa extract whereas the nonimmune IgG does not (Figure 1A). The inhibition is specific for LeuRS as there is no effect of immune IgG on the activity of \( N. \) crassa PheRS (Figure 1B). In addition, the anti-cyto LeuRS immune IgG has no effect on mito LeuRS activity (data not shown).
Immunoblotting experiments with the anti-cyto LeuRS Leucine Amino Transferase (L-A) antibody identified a ~115-kDa protein in N. crassa extracts (Figure 2, lanes 4 and 5). This band is absent in blots probed with nonimmune IgG (data not shown). The size of the major immunoreactive protein, ~110–115 kDa, is in close agreement to that of cyto LeuRS purified by two groups (KUNUGI et al. 1986; BEAUCHAMP, HORN and GROSS 1977).

Immunoprecipitation of in vitro translation products in a wheat germ cell free system directed by N. crassa poly(A)⁺ or total RNA followed by polyacrylamide gel electrophoresis shows a single band with a molecular weight of ~115 kDa (Figure 3, lanes 2 and 3) as expected for N. crassa cyto LeuRS. This protein is not immunoprecipitated by nonimmune IgG (lanes 4 and 5).

Preparation of a λgt11 N. crassa cDNA library and isolation of cyto LeuRS cDNA clones, λNCLRSC1 and λNCLRSC2: Besides providing evidence that the anti-cyto LeuRS antibody is specific, results in Figure 3 above also indicate that N. crassa poly(A)⁺ and total RNA preparations contain a full length functional mRNA for N. crassa cyto LeuRS. Therefore, a similar preparation of poly(A)⁺ mRNA was used for generation of the λgt11 cDNA library. Approximately 150,000 plaques on two 150-mm plates were transferred to nitrocellulose filters and the phage replicas were then probed with anti-cyto LeuRS antiserum. Of five positives, two (designated henceforth as λNCLRSC1 and λNCLRSC2) continued to show positive responses after three additional rounds of screening at progressively lower plating densities. In the last round of screening, all plaques were immunoreactive with anti-cyto LeuRS antibody. Analysis of the λNCLRSC1 and λNCLRSC2 DNAs showed that these have cDNA inserts of ~2 kbp and 1.3 kbp, respectively. The cDNA clones overlap by ~0.6 kbp and have been further characterized at both protein and DNA levels (see below).

The two cDNA clones direct the synthesis of significant amounts of β-galactosidase-cyto LeuRS fusion proteins upon thermal induction of the λNCLRSC1 and λNCLRSC2 prophages followed by IPTG induction of the lac promoter (Figure 4). The sizes of the fusion proteins are ~188 kDa and ~155 kDa corresponding closely to those expected on the basis of the size of cDNA inserts of ~2 kbp and 1.3 kbp in λNCLRSC1 and λNCLRSC2, respectively.

Identification of the cDNA clones: Because of the lack of amino acid sequence information on N. crassa cyto LeuRS, it was not possible to use DNA sequence of the cDNA clones alone to confirm their authenticity. However, the following lines of evidence, based on several immunological criteria, suggests strongly that the cDNA clones obtained are those for N. crassa cyto LeuRS. Antibodies affinity purified using the fusion proteins encoded in λNCLRSC1 and
Figure 4.—Analysis of β-galactosidase-cyto LeuRS fusion proteins. Lysates of E. coli Y1089 lysogenized by λNCLRSC1 (clone 1) and λNCLRSC2 (clone 2) or λgt11 were prepared after temperature induction of phage and incubation in the presence (+) or absence (−) of IPTG. The lysates were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and protein bands visualized by Coomassie blue staining. M, size markers in decreasing size order; myosin, phosphorylase b, bovine serum albumin and ovalbumin. Arrows on the left point to β-galactosidase (115 kDa) or the β-galactosidase-cyto LeuRS fusion proteins (155 kDa in clone 2 and 188 kDa in clone 1).

λNCLRSC2 inhibit cyto LeuRS activity. These same antibodies detect an ~115-kDa protein in immunoblots of total N. crassa proteins.

Fusion proteins present in induced λNCLRSC1 and λNCLRSC2 plaques were used to affinity purify the antiserum and the effect of the IgG fraction on cyto LeuRS activity was examined. Figure 5 shows that antibodies affinity purified using λNCLRSC1 encoded proteins inhibit LeuRS activity (compare curve 3 to curve 1, control). A similar result was obtained with antibodies affinity purified using λNCLRSC2 encoded protein (data not shown). In contrast, antibodies affinity selected using λgt11 encoded proteins show little effect on LeuRS activity (curve 2). The ability of cDNA encoded fusion proteins to react with cyto LeuRS antibodies was further demonstrated with isolated fusion proteins from λNCLRSC1. Preincubation of fusion proteins with antibodies decreased the activity of the latter in inhibition of cyto LeuRS activity from 80% to 33% (data not shown).

The affinity purified antibodies were also used in immunoblotting experiments on total N. crassa proteins separated on polyacrylamide gels. Figure 6 shows that antibodies affinity purified using either the λNCLRSC1 or the λNCLRSC2 encoded proteins react specifically with a protein in N. crassa extracts (lanes 2 and 3), which comigrates with a marker of purified cyto LeuRS (data not shown). In contrast, antibodies affinity selected using λgt11 encoded proteins do not react with this protein (Figure 6, lane 1). Thus, the λNCLRSC1 and λNCLRSC2 encoded fusion proteins share antigenic determinants with cyto LeuRS.

Isolation of genomic clones for N. crassa cyto LeuRS, derivation of a partial restriction map and DNA sequence: The cDNA inserts in λNCLRSC1 and λNCLRSC2 were isolated and subcloned into pBR322 and M13. The clones in pBR322 are henceforth designated as pNCLRSC1 and pNCLRSC2, respectively. The cDNAs were used as hybridization probes to isolate two overlapping genomic clones from partial Sae3A N. crassa genomic libraries (Schechter and Yanofsky 1983; Orbach, Porro and Yanofsky 1986). These genomic clones are designated as pNCLRSG11 and pNCLRSG13. The arrangement and partial restriction maps of the two genomic clones, and the two cDNA clones, pNCLRSC1 and pNCLRSC2, are shown in Figure 7. The genomic clones overlap each other by 0.2 kbp. pNCLRSG11 overlaps pNCLRSC1 but not pNCLRSC2. pNCLRSG13 hybridizes with both cDNA clones and extends beyond pNCLRSC2. The genomic and cDNA
Figure 6.—Immunoblot analysis of N. crassa proteins using antibodies affinity purified by adsorption to proteins present in Agt11 (control), λNCLRSC1 and λNCLRSC2 plaques. Proteins in N. crassa extracts (~10 μg) were separated by electrophoresis on an 8% SDS-polyacrylamide gel, electroblotted to nitrocellulose filter and incubated with antibodies affinity purified using Xgt1 plaques N. albumin. and ovalbumin. (lane 1). XNCLRSC1 plaques (lane 2) and XNCLRSC2 plaques (lane 3). Immune complexes were detected using Vectastain ABC. Numbers on the right indicate the positions of size markers, in decreasing size order, myo-in, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin.

Figure 7.—Arrangement and partial restriction map of cDNA and genomic DNA clones for cyto LeuRS. Restriction enzymes used for mapping were A, AvaI; B, BamHI; E, EcoRI; H, HindIII; K, KpnI; N, Nael; R, EcoRV; S, SacI; St, Stul, U, Sau3A and X, Xhol. No sites for PsI were found within the mapped region. Restriction sites generated during cloning which are not present in the original cDNA or genomic DNA are indicated within parentheses. Regions of overlap between the clones were confirmed by Southern analyses or by DNA sequencing. The wavy line shows the position of cyto LeuRS mRNA. Vertical arrows indicate mRNA 5'-ends as identified by S1 nuclease mapping and by primer extension (data not shown).

clones appear colinear within the limits of restriction mapping; however the presence of small introns, commonly found in N. crassa, is not excluded.

Figure 8 shows a partial DNA sequence based on analyses of pNCLRSG11 and pNCLRSC1. The DNA sequence contains a methionine initiated open reading frame that coincides with the β-galactosidase reading frame in ANCLRSC1. The codon usage in this frame agrees well with the codon preference in N. crassa genes (Sachs 1986). The deduced protein sequence from this reading frame includes a region of homology to other aminocyl-tRNA synthetases that has been identified previously (Freedman et al. 1985; Webster et al., 1984; Blow et al. 1983); the homology is shown in Figure 9. The histidine residues highlighted in Figure 9 are implicated in ATP binding by the positioning of substrate molecules on the known crystal structure of tyrRS from Bacillus stearothermophilus and by site specific mutagenesis (Leatherbarrow, Fersht and Winter 1985; Lowe, Fersht and Wilkinson 1985; Carter et al. 1984; Bhat, Blow and Brick 1982).

Size of mRNA: RNA gel transfer hybridization was used to identify the mRNA for N. crassa cyto LeuRS. Figure 10 shows that a single band, ~3.9 kb long present in N. crassa poly(A) RNA hybridized to the cDNA (lane 2). The size of the mRNA is sufficient to code for cyto LeuRS with a Mr of ~115 kDa.
Neurospora Leucyl-tRNA Synthetase

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<td><strong>tyr</strong> (<em>B. stearothermophilus</em>)</td>
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<td><strong>leu</strong> (<em>N. crassa</em>)</td>
<td>PYNRSIHAGN</td>
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**FIGURE 9.**—Amino acid sequence homology between the cyto LeuRS protein sequence as predicted from the pNCLRSC1 and pNCLRSC2 DNA sequences and other aminoacyl-tRNA synthetases. Numbers on the left refer to the position of the homology region relative to the N terminus of the protein.

**FIGURE 10.**—RNA gel transfer hybridization of *N. crassa* poly(A⁺) RNA using pNCLRSC1 DNA as probe. 32P-labeled marker DNAs (lane 1) and poly(A⁺) RNA (lane 2, 4 μg) were denatured by treatment with glyoxal before electrophoresis on a 1% agarose gel. Arrow indicates the mRNA band of ~3.9 kb which hybridized to the probe. Numbers on the left indicate size of 32P-DNA markers.

**Chromosomal mapping:** The cDNA clone pNCLRSC1 was used to determine the position of the cyto LeuRS structural gene with respect to the genetic map of *N. crassa* (PERKINS et al. 1982). RFLPs between Mauriceville-1c-A and marked strains in an Oakridge-type background were found with the enzymes *XhoI* and *EcoRV*. RFLP mapping using genomic DNA of progeny from standard crosses (FGSC #2225 × FGSC #4411 and FGSC #2225 × FGSC #4888; METZENBERG et al. 1984) (data not shown) indicated that the cyto LeuRS gene segregated most closely with the 5S RNA gene 17 on linkage group II (45/51) and not with the *inl* or *lys* markers on linkage group V (29/51) where *leu-5* maps. A finer mapping with a cross between Mauriceville-1c-A and an Oakridge strain carrying multiple markers for linkage group II (METZENBERG et al. 1985) was, therefore, carried out. The results (Figure 11) showed that cyto LeuRS co-segregated (39/41) with 5S RNA gene 17, the map order for the rightmost gene on linkage group II being *trp-*3, 5S gene 17, cyto LeuRS (Table 1).

**DISCUSSION**

As part of studies aimed at determining the structure, organization and linkage between the genes for the cyto and mito LeuRS in *N. crassa*, we have described the isolation and partial characterization of cDNA and genomic DNA clones for *N. crassa* cyto LeuRS. We generated a *Agt11* *N. crassa* cDNA library and used antibody raised against cyto LeuRS to screen for the cyto LeuRS clones. Two cDNA clones designated ANCLRSC1 and ANCLRSC2, which overlapped by about 0.6 kbp were obtained. The cDNA clones were used to screen a genomic DNA library and two genomic clones were isolated.
The lack of amino acid sequence information on *N. crassa* cyto LeuRS precludes a definitive assignment of the isolated clones by comparison of sequence data at the DNA level and at the protein level. However, the combined immunological and other evidence suggest that the cDNA clones λNCLRSC1 and λNCLRSC2 correspond to those for *N. crassa* cyto LeuRS: (1) The antibody used to screen the λgt11 cDNA library reacts specifically with *N. crassa* cyto LeuRS. It inhibits the LeuRS activity of *N. crassa* extracts (Figure 1A); it reacts with a single protein with a molecular weight of cyto LeuRS in immunoblots of total *N. crassa* proteins (Figure 2) and it immunoprecipitates a similar sized protein from a mixture of in vitro translation products directed by *N. crassa* poly(A') or total RNA (Figure 3). (2) Antibodies affinity purified using either of the fusion proteins produced by cDNA clones λNCLRSC1 or λNCLRSC2 inhibit cyto LeuRS activity (Figure 5). Isolated β-galactosidase fusion protein encoded by λNCLRSC1 blocks binding sites in the anti-cyto LeuRS antibodies that are needed for inhibition of cyto LeuRS in N. crassa extract (data not shown). (3) Both of the affinity purified antibodies react with a protein band having the size of cyto LeuRS in immunoblots of total *N. crassa* proteins (Figure 6).

The DNA sequence of the cDNA and genomic clones also provides additional evidence that λNCLRSC1 and λNCLRSC2 encode part of *N. crassa* cyto LeuRS. The DNA sequence contains a methionine-initiated open reading frame which not only uses codons highly preferred in *N. crassa* but the initiator codon is also preceded, as are most *N. crassa* mRNAs, by an A, G rich sequence. Most important, the protein sequence deduced from the DNA sequence contains a stretch of amino acids which is conserved in several ATP binding and amino acid activation (Figure 9).

The size of the mRNA detected in RNA blot hybridizations using λNCLRSC1 as probe is also consistent with λNCLRSC1 being an authentic clone for cyto LeuRS. The *N. crassa* cyto LeuRS is among the largest of *N. crassa* proteins (Figure 2A) with a *M*$_r$ of ~115 kDa. The observed size of cyto LeuRS mRNA (Figure 12), ~3.9 kb, is sufficient to code for cyto LeuRS.

KUNUGI et al. (1986) and AIRAS, SCHISCHICOFF and CRAMER (1986) have recently reported on the purification and analysis of kinetic parameters of purified *N. crassa* cyto LeuRS from wild type and the *leu-5* mutant. In contrast to the previous report of PRAINTZ and GROSS (1967), they have found that the cyto LeuRS from wild type and the *leu-5* mutant have virtually identical *K*$_m$ for leucine. This finding raises the possibility that the *leu-5* locus in *N. crassa* may not specify structural information for cyto LeuRS. Our
results on chromosomal mapping of the N. crassa cyto LeuRS gene provides direct evidence for this possibility. We have used the cyto LeuRS cDNA clones to map the gene in N. crassa chromosomes using restriction fragment length polymorphism (METZENBERG et al. 1985) (Figure 11) and have found that this gene maps not on linkage group V as reported for leu-5 (PRINTZ and GROSS 1967) but on the right arm of linkage group II (PERKINS et al. 1982) (Table 1). Finally, in work currently in progress (CHOW, METZENBERG and RAJBHANDARY, unpublished data) we have shown that the leu-5 locus in N. crassa specifies mitochondrial LeuRS. Thus, the cytoplasmic and mitochondrial forms of LeuRS are encoded by distinct genes.

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LITERATURE CITED


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