Theor Appl Genet (1985) 71:26–30

Species-specific proteins of the 50S subunit of the chloroplast ribosome in the genus *Nicotiana*

H. J. Smith [1,*] and D. P. Bourque [2]

[1] Department of Biochemistry and [2] Department of Nutrition and Food Science; 537 Biological Sciences West, University of Arizona, Tucson, AZ 85721, USA

Received January 25, 1985; Accepted March 25, 1985
Communicated by D. von Wettstein

Summary. The large subunits (50S) of chloroplast ribosomes were isolated from *Nicotiana tabacum,* a species of the Western Hemisphere, and from *N. excelsior* and *N. gossei,* Australian species. Their proteins were compared by two-dimensional gel electrophoresis. A pair of proteins (T_{12} and T_{15}) observed in *N. tabacum* has electrophoretic mobilities which differ from those of a similarly migrating, and probably homologous, pair of proteins observed in *N. excelsior* and *N. gossei.* The species-specific proteins in *N. tabacum* differ slightly in electrophoretic mobilities based on both charge and molecular weight from those in *N. excelsior* and *N. gossei.* Tryptic digests of radioiodinated proteins reveal that the peptide maps of all six proteins are similar. These results suggest that chemically altered forms of one or more proteins of the 50S chloroplast ribosome subunit may exist in vivo.

Key words: Chloroplast – Ribosomal proteins – Tobacco – Interspecific differences

Introduction

Genetic markers for eukaryotic ribosomal proteins have been detected in organelle ribosomes of *Chlamydomonas* (Mets and Bogorad 1972; Ohta et al. 1975), *Nicotiana* (Bourque and Wildman 1973), *Euglena* (Freyssinet 1977), and *Xenopus* (Leister and Dawid 1975) and in cytoplasmic ribosomes of *Drosophila* (Weber et al. 1976). However, for the ribosomes of a given organism, polymorphisms have been detected in only a few of the ribosomal proteins. If more markers existed, their mode of inheritance could be studied to determine the intracellular site of genes coding for these proteins. These studies would help to elucidate the mechanisms of control and integration of protein synthesis directed by nuclear and organelle genomes in eukaryotes.

Species of *Nicotiana* found in Australia and the Western Hemisphere have evolved in isolation since migrations between the moving land masses of South America and Australia were no longer possible (Raven and Axelrod 1975). There are distinct group-related differences between the electrophoretic properties of polypeptides of the chloroplast enzyme, ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) isolated from Australian and Western Hemispheric species of *Nicotiana* (Kung 1976). It is possible that ribosomal proteins of *Nicotiana* species might also exhibit such a pattern of evolutionary divergence.

Two-dimensional electropherograms of ribosomal proteins from Australian and Western Hemispheric species have been compared in an effort to detect differences in electrophoretic mobilities of proteins which might serve as genetic markers. Previous analysis (Bourque 1977) did not reveal characteristic differences in the 50S ribosomal proteins of *N. tabacum* and *N. excelsior.* However, in this paper we document our observation (Smith and Bourque 1977) that group-related differences are detected when the electrophoretic mobilities of proteins from different species are compared directly by the method of Leister and Dawid (1974). Our experiments employ co-electrophoresis of radioiodinated proteins of one species and unlabelled proteins of another species, followed by comparison of the stained gel and autoradiogram.

* Current address: Department of Biology, University of Chicago, Chicago, IL 60637, USA

Methods

Ribosome isolation

Leaves were homogenized (1.5 : 1, w/v) for 3 min in buffer (2.5 mM $MgCl_2$, 25 mM KCl, 50 mM Tris-HCl, pH 7.5, 60 mM β-mercaptoethanol). The brei was filtered through cheesecloth and Miracloth. After adjusting the $MgCl_2$ concentration to 25 mM, the filtrate was centrifuged for 60 min at 31,000 g. Ribosomes were then collected by centrifugation at 127,300 g for 8 h through a 1.3 M sucrose cushion containing 25 mM $MgCl_2$, 25 mM KCl, 25 mM Tris-HCl, pH 7.5, and 8 mM β-mercaptoethanol. The ribosomal pellet was resuspended in buffer containing 2.5 mM $MgCl_2$, 25 mM KCl, 25 mM Tris-HCl, pH 7.5 and 8 mM β-mercaptoethanol. The ribosomal suspension was dialyzed against this buffer for 36 h. As a result of the low Mg^{++} concentration during this dialysis the chloroplast (70S) ribosomes dissociate into large (50S) and small (30S) subunits, whereas cytoplasmic ribosomes (80S) remain undissociated (Bourque and Wildman 1973). Subunits and ribosomes were separated by zonal centrifugation on an isokinetic sucrose gradient. After gradient fractions were collected, the ribosome subunits were precipitated with ammonium sulfate (Bourque and Wildman 1973).

Extraction of ribosomal proteins

The ammonium sulfate precipitate was resuspended in 50 mM Tris-HCl, pH 7.0, to a protein concentration of approximately 3 mg/ml. The suspension was brought to a final concentration of 5 M urea and 2 M LiCl (Leboy et al. 1964) and incubated first for 30 min at room temperature, and then for 48 h at 4 °C. RNA was removed by centrifugation, and the pellets were re-extracted in urea – LiCl. Urea and LiCl were added to the protein-containing supernatant to final concentrations of 6.6 M and 2.66 MU, respectively. At this point, the ribosomal protein solution was divided into aliquots which were either radioiodinated or prepared directly for gel electrophoresis.

Salts from samples which were not iodinated and unbound iodine and salts from radioiodinated samples were removed by brief centrifugation on small (0.5 ml) columns of Sephadex G-25 (medium) equilibrated in 8 M urea and 1% β-mercaptoethanol (Neal and Florini 1973). Protein concentrations were estimated by a modification of the method of Schaffner and Weissmann (1973). Proteins complexed with SDS were precipitated with TCA, collected on nitrocellulose filters, and stained with a 1% solution of amido black (Eastman Organic Chemicals, Rochester, New York) for 4 min with constant stirring. The filters were washed and destained, the stained spots cut out and the stain eluted as previously described.

Radioiodination of ribosomal proteins

Proteins were radioiodinated by the method of Leister (1974). The reaction mixtures contained, in order of addition: 30 µl ribosomal proteins (1 µg/µl), 1 µl $Na^{125}I$ (10 µCi, carrier-free, Schwarz-Mann, Orangeburg, New York), 3 µl chloramine-T (10 mg/ml in 10 M urea, 50 mM Tris-HCl, pH 7.0). After 10 min at room temperature, the reaction was stopped by the addition of 1 µl 1 M β-mercaptoethanol. Any disulfides formed during the chloramine-T oxidation were reduced by adding 10 µl of a solution containing 7.5 M urea, 1 M Tris-HCl, 0.5 M EDTA, 0.25 M DTT, 1 M NaI, pH 8.9, and incubating the mixture overnight at room temperature. Reaction tubes were sealed with serum caps and individual components were added through the caps with a Hamilton syringe in order to prevent escape of volatile iodine. The caps remained in place throughout the reaction and subsequent incubation period in reducing solution. Reactions were performed in a well-ventilated hood using appropriate safety precautions.

Gel electrophoresis

Gel analysis of ribosomal proteins was carried out as soon as possible after the proteins were iodinated. Immediately prior to electrophoresis, desalted samples were concentrated by precipitation with an equal volume of 20% TCA (Howard et al. 1975). The precipitates were washed twice with ether, and resuspended in electrophoresis sample solution (8 M urea, 1% β-mercaptoethanol). Ribosomal proteins were analyzed by the two-dimensional polyacrylamide gel electrophoresis system of Mets and Bogorad (1974). The first dimension was run in 200 µl capillary tubes; the second, in 1.5 mm thick slab gels (13 × 16 cm).

The electrophoretic mobilities of chloroplast ribosomal proteins of any two species of *Nicotiana* were compared directly using the method of Leister and Dawid (1974, 1975). Stainable quantities (100–150 µg) of ribosomal proteins from one species were co-electrophoresed with small quantities (10 µg) of iodinated proteins from the second species. This amount of iodinated protein could not be detected in the second dimension gel by staining. After electrophoresis, the gels were stained, destained, and dried. Autoradiography was performed by exposing Kodak RP Royal X-Omat (R-5) Film to the gel for periods of from 24 h to 3 days. The stained pattern and autoradiogram, as well as tracings made from both, were compared.

Peptide mapping of iodinated proteins

Tryptic peptide analysis of iodinated peptides of the species-specific ribosomal proteins was performed by the method of Elder et al. (1977). Gel pieces containing the individual protein spots were excised and the stain removed. Gel pieces were then placed in reaction tubes, and a chloramine-T iodination performed. After dialysis to remove unbound iodine, the proteins were simultaneously digested and the peptides removed from the gel by incubation of the gel pieces in buffer containing trypsin. Iodinated peptides were separated on thin layer cellulose plates (EM Laboratories, Elmsford, New York) by high voltage electrophoresis in the first dimension and chromatography in the second dimension. No tracker dye was used in the electrophoresis. After the second dimension separation, the plates were dried, wrapped in Saran wrap, and autoradiography performed as described above for dried slab gels.

Results and discussion

It has been previously reported that no apparent differences in the electrophoretic mobilities were observed between 50S chloroplast ribosomal proteins of *N. tabacum*, a species of the Western Hemisphere and the Australian species, *N. excelsior* (Bourque 1977). However, when the 50S large subunit proteins of *N. tabacum* were co-electrophoresed with those of *N. excelsior*, using stainable amounts of non-iodinated proteins from one species, and trace quantities of radioiodinated proteins from the second, reproducible differences in a pair of proteins of molecular weight 16,000–20,000 were noted between the proteins of *N. tabacum* and

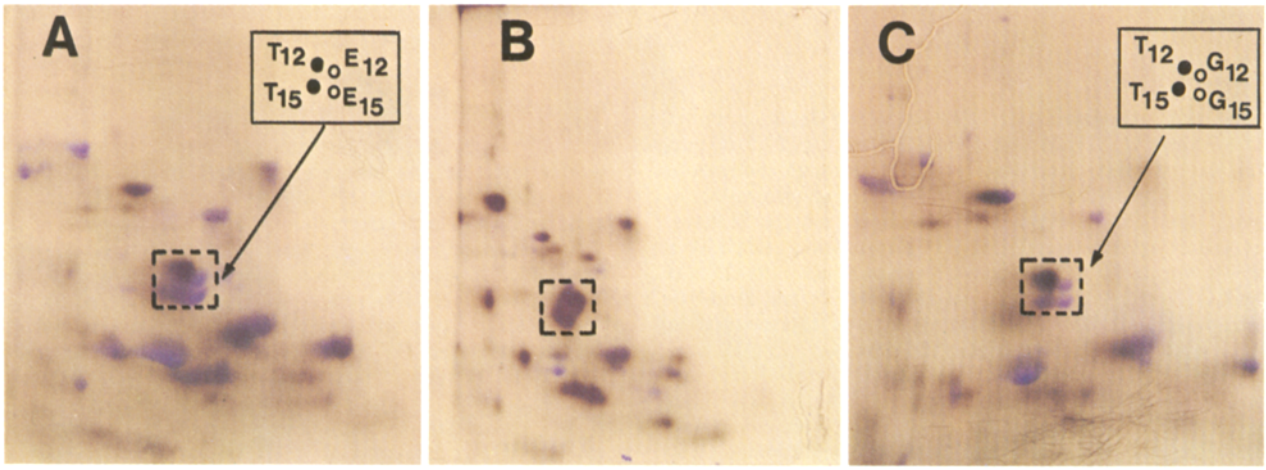

Fig. 1 A–C. Proteins of the large subunit (50S) of chloroplast ribosomes of *Nicotiana tabacum, N. excelsior,* and *N. gossei.* Radioactive and unlabelled protein samples from different species were mixed and then separated by two-dimensional gel electrophoresis. **A** ^{125}I-labelled *N. tabacum* (12 μg, 2×10^6 dpm) and 150 μg of *N. excelsior* ribosomal proteins; **B** ^{125}I-labelled *N. excelsior* (10 μg, 2×10^6 dpm) and 150 μg of *N. tabacum* 50S ribosomal proteins; **C** Proteins of *N. tabacum* (10 μg, 3×10^6 dpm of ^{125}I-labelled proteins) and *N. gossei* (160 μg). Included for clarity in *A* and *C* is a diagrammatic representation of the region of the gels in which the species-specific spots are located. The relative positions of these proteins are shown in the diagram as they appear in the figure, which is a photograph of the autoradiograph and the stained gel, superimposed on each other. The symbols T, E, and G represent the proteins which are specific to *N. tabacum, N. excelsior,* and *N. gossei,* respectively. Proteins T_{12} and T_{15} are present only in *N. tabacum.* Proteins E_{12} and E_{15} are identical to G_{12} and G_{15}, but both pairs differ from the homologous proteins (T_{12} and T_{15}) in *N. tabacum.* Proteins are numbered according to the convention of Capel and Bourque (1982)

those of *N. excelsior* (Fig. 1). In this region, *N. excelsior* has a pair of proteins, E_{12} and E_{15} (intense blue staining), which is slightly more basic than a pair of *N. tabacum* proteins, T_{12} and T_{15} (Fig. 1 A, B). The intensely blue staining spots of T_{12} and T_{15} (Fig. 1 A) are masked (Fig. 1 B) by the high density of spots E_{12} and E_{15} when *N. excelsior* is the sample which is iodinated and *N. tabacum* is nonradioactive. Control experiments (not shown) demonstrate that iodination causes no electrophoretic shifts in these proteins.

Three separate ribosomal preparations of *N. tabacum* and *N. excelsior* were run in both iodinated and non-iodinated form. Although the preparations varied with respect to relative amounts of individual proteins, the differences between the two species were always seen. Indirect evidence for the altered mobilities of T_{12} and T_{15} relative to E_{12} and E_{15} has also been noted in a related study using computer-mapping analysis of two-dimensional gels (Capel and Bourque 1977; Capel et al. 1979; Capel 1982).

The 50S ribosomal proteins of a second Australian species, *N. gossei,* were compared with those of *N. excelsior* and *N. tabacum* (Fig. 1 C). Using the *N. gossei* proteins as the non-iodinated proteins, G_{12} and G_{15} appear to have the same electrophoretic mobility as E_{12} and E_{15} of *N. excelsior* (Fig. 2). This pair, G_{12} and G_{15}, is also slightly more basic than T_{12} and T_{15}. Other differences between *N. tabacum, N. excelsior,* and *N. gossei* can be

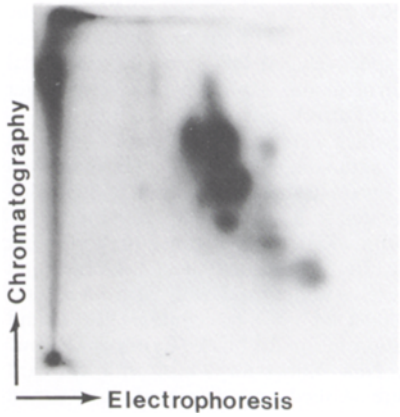

Fig. 2. Iodinated tryptic peptide map typical of the 50S ribosomal proteins T_{12}, T_{15}, E_{12}, E_{15}, G_{12}, and G_{15}

detected in the panels shown in Fig. 1. However, due to potential differences in relative iodination of different proteins, further studies are required to evaluate species-specific polymorphisms for additional protein pairs.

As a first step in examining the problem of whether the differences in electrophoretic mobilities observed reflect differences in structural genes for ribosomal proteins or in post-translational modification of the ribosomal proteins, fingerprints were made of the iodinated tryptic peptides of E_{12}, E_{15}, T_{12}, T_{15}, G_{12},

and G_{15}. Gel pieces containing the individual proteins in question were excised from the dried slabs and the pieces were treated by the method of Elder et al. (1977) to obtain radioiodinated tryptic peptides. A typical pattern of separated peptides is shown in Fig. 2. No differences were observed in the patterns between the proteins T_{12} and E_{12} (or G_{12}) or between T_{15} and E_{15} (or G_{15}) protein pairs which differ in charge. More strikingly, no major differences in peptide patterns were observed between proteins which were different in molecular weight and charge (e.g. E_{12} and T_{15}).

It is possible that the proteins of *N. excelsior* (or *N. gossei*) and *N. tabacum* which are distinguishable by electrophoretic mobility in two-dimensional gel electrophoresis differ with respect to tryptic peptides that lack amino acids which are iodinated, i.e. tyrosine, and histidine (Tsurugi et al. 1976). It may also be possible that minor differences in iodinated peptides were not detected by the methods of separation used here. The iodinated tryptic peptide patterns of the six proteins examined may, in fact, reflect fundamental similarities in these proteins which have different electrophoretic mobilities. Tsurugi et al. (1976) found that proteins of the 40S subunit of rat liver ribosomes which have distinct differences in electrophoretic mobility in polyacrylamide gel electrophoresis, nevertheless have amino acid compositions which are similar. In studies on the 50S ribosomal subunit proteins of *Escherichia coli*, Mora et al. (1971) observed that although each of the proteins had a distinct tryptic peptide map, there were similar peptides in the maps of different proteins. The iodinated tryptic peptide patterns that we observed suggest that those proteins which differ in charge (for example, T_{12} and E_{15}) not only contain similar amino acid compositions, but may also contain some identical peptides. However, the peptide mapping procedure may not be sensitive to certain single amino acid differences between homologous peptides.

Our results suggest that proteins T_{12} and T_{15} might differ from each other by only slight chemical modifications. Therefore, these proteins may represent multiple forms of a single polypeptide chain, as is the case fot the L7–L12 complex of *Escherichia coli* (Subramanian 1975) and the B-L13 protein of *Bacillus stearothermophilus* (Marquis and Fahnestock 1978). In both of these bacteria there are four copies of these proteins per ribosome. Since chloroplast ribosomes are similar in properties to procaryotic ribosomes, the homology proposed to interpret our results would not be unexpected. Additional studies are necessary to substantiate this possibility.

In conclusion, we have observed, as previously (Bourque 1977), similar overall patterns of electrophoretic mobility of 50S ribosomal proteins in the three species we have examined. Of the total number of iodinated 50S ribosomal proteins detected on gels, two pairs of proteins were detected which are species-specific in comparison of *N. excelsior* (or *N. gossei*) and *N. tabacum*. These proteins may also be group-specific to species of Australia relative to those of the Western Hemisphere. Peptide mapping analysis has shown that these species-specific proteins, however, appear to share similar peptides. The mode of inheritance of these proteins in interspecific hybrids is currently under investigation.

Nicotiana species of Australia and the Western Hemisphere have been separated for 36–41 million years (Raven and Axelrod 1975). *N. excelsior* (or *N. gossei*) and *N. tabacum* have different numbers of chromosomes (19, 18 and 24 pairs, respectively), and are sufficiently different in morphology to be classified in different subgenera (Goodspeed 1954). Such similarities in the complement of 50S ribosomal proteins of the two geographically isolated groups may be further evidence that the rate of evolution of ribosomal proteins is slow compared to those of other proteins (Hori et al. 1977). The two differences observed, however, may indicate that a number of other species-specific ribosomal proteins exist in the genus *Nicotiana*. In fact, at least four additional differences between the 50S chloroplast ribosomal proteins of *N. tabacum* and *N. excelsior* have been detected by computer-assisted analysis (Capel et al. 1979; Capel 1982).

There are 64 species of *Nicotiana*, 20 of which are found in Australia and 44 of which are natural to the Western Hemisphere (Goodspeed 1954). Among these species, it is probable that many amino acid sequence differences exist between homologous ribosomal proteins which could serve as genetic markers. Thus, the genus *Nicotiana* may yield additional evidence of chloroplast ribosomal protein polymorphisms.

Acknowledgements. This research was supported by Grant R01GM21242 from the National Institutes of Health. The technical assistance of Malcolm Capel and David Zaitlin is gratefully acknowledged. This is publication No. 3987 of the Arizona Agricultural Experiment Station.

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