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Distinctive Pattern and Translational Control of Mitochondrial Protein Synthesis in Rat Brain Synaptic Endings*

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Paola Loguercio Polosa and Giuseppe Attardi

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

Mitochondrial gene expression has been investigated in synaptic endings from rat cerebral cortex isolated at various stages during the postnatal development and maturation of the animal. The pattern of the mitochondrial translation products labeled *in vitro* in rat brain synaptosomes revealed some distinctive features when compared with the pattern observed in a rat fibroblast cell line, the most remarkable being the apparent absence of labeling of the ND5 product. This absence contrasted with the presence in synaptosomes of an amount of ND5 mRNA comparable with that found in the rat fibroblast cell line. The rate of mitochondrial protein synthesis per unit amount of mtDNA in brain synaptosomes showed a characteristic reproducible burst at 10–13 days after birth, thereafter declining sharply in the 3rd week to reach a level that remained constant over a 2-year period. The postnatal burst of mitochondrial protein synthesis coincided with a sharp increase in cytochrome *c* oxidase activity, pointing to a phase of rapid assembly of respiratory complexes. A comparison of the levels of mitochondrial mRNAs with the corresponding rates of protein synthesis during the animal development and maturation showed a lack of correlation. These observations, together with the apparent lack of translation of the ND5 mRNA, indicate that translational control plays a major role in the regulation of gene expression in rat brain synaptic mitochondria.

The expression of mitochondrial genes in mammalian cells has so far been studied, at the level of transcription or translation, mostly in cell culture systems (Attardi, 1985; Chomyn and Attardi, 1987; Attardi and Schatz, 1988). Very little is known about the expression of these genes in differentiated tissues. A quantitative analysis of mitochondrial RNA in rat hepatocytes has revealed that, in these cells, the levels of several specific mRNAs relative to that of the rRNAs are as much as an order of magnitude higher than observed in HeLa cells (Cantatore *et al.*, 1984). Similar results have been reported for mitochondrial RNA from rat cerebella (Renis *et al.*, 1989). Furthermore, in the case of rat hepatocytes, it appears that the higher relative levels of the mRNAs result from an increased rate of transcription of the whole heavy mtDNA strand transcription unit (Cantatore *et al.*, 1987a). On the contrary, no information is available concerning the expression of different mitochondrial genes at the level of translation in specialized cells. In the present work, we have chosen one differentiated cell type, brain nerve cells, to analyze the rates of synthesis of the various mtDNA-coded polypeptides and to compare them with the steady state levels of the corresponding mRNAs. The rat was used as an experimental system because of the facility of obtaining fresh material from this animal.

Nerve cell mitochondria are located in two compartments, the cell body and the nerve endings. In the present work, the rat brain mitochondria located in the nerve ending compartment were chosen for investigation for several reasons. First, it is possible to isolate a nerve ending (synaptosome) fraction not appreciably contaminated by glial mitochondria and thus to measure protein synthesis rates and mRNA levels in reasonably pure neuronal mitochondria. Furthermore, presynaptic ending mitochondria are of particular interest both from the point of view of biogenesis and from that of synaptic function. In fact, the segregation of these mitochondria in the nerve terminals creates the problem of a continuous supply of nuclear coded components to the peripheral organelles or possibly of a recycling of the organelles to the cell body, with intriguing implications from the point of view of regulation of gene expression. In another context, it can be expected that increased knowledge concerning the energetic metabolism that supports synaptic function will help in understanding the mechanism of this function.

The results obtained have indicated a distinctive pattern of mitochondrial protein synthesis in the rat brain synaptic endings, as compared with the pattern in a rat fibroblast cell line, the most remarkable difference being the apparent absence of synthesis of the ND5 subunit of NADH dehydrogenase. An analysis of the changes in mitochondrial gene expression during development and maturation has shown a burst of protein synthetic activity at the end of the 2nd week postbirth, which correlates with a sharp increase in cytochrome *c* oxidase activity. Furthermore, a comparison of the rates of mitochondrial protein synthesis with the steady state levels of the mRNAs has shown that translational control plays an important role in gene expression in neuronal mitochondria.

EXPERIMENTAL PROCEDURES

Animals—Sprague-Dawley male rats, from 3 days to 24 months, and Fisher 344 male rats, from 6 to 24 months, were used.

Isolation of Brain Synaptosomes—All the following preparative steps were carried out at 4 °C. The cerebral cortices were aseptically dissected out from one rat (or more animals, if less than 1 month old) and minced in 10 volumes of 10% (w/w) sucrose in 0.005 M Tris-HCl (pH 6.7, at 25 °C), 0.1 mM EDTA (Medium I). This suspension was gently homogenized (5 strokes) with a Dounce homogenizer. The crude homogenate was centrifuged at 1,300 × *g* for 5 min, and the low speed supernatant was then recentrifuged at 12,000 × *g* for 5 min to produce the 12,000 × *g* membrane fraction. The pellet was resuspended in 1 ml of Medium I/g of original wet tissue. Synaptosomes

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were isolated according to the procedure by Nagy and Delgado-Escueta (1984) with slight modifications. Briefly, the 12,000 × *g* membrane fraction was diluted with 4 volumes of 8.5% Percoll, 0.25 M sucrose medium (final concentration of Percoll, 6.8%), and the suspension (3 ml) was then layered onto a two-step Percoll density gradient (4 ml, 16%; 4 ml, 10%), and centrifuged at 15,000 × *g* for 20 min in a Beckman Ty65 fixed angle rotor. Synaptosomes, which formed a diffuse band at the 10–16% Percoll interphase, were collected and used without further purification.

Protein Labeling—One 1.5-ml sample of the membrane fractions recovered from the Percoll/sucrose gradient was mixed with 2 ml of methionine-free Dulbecco's modified Eagle's medium and incubated for different times, as specified below, in the presence of 1 mCi of [^{35}S]methionine (15 μCi/μl, ~1100 Ci/mmol; Amersham Corp.) and of 100 μg/ml cytoplasmic protein synthesis inhibitor, cycloheximide or emetine. Less than 1 h passed between killing the animal(s) and beginning the protein-labeling experiment. In some experiments, the effects of the mitochondrial protein synthesis inhibitor, chloramphenicol, added to a final concentration of 100 μg/ml, were investigated. Incorporation was terminated by the addition of 5 volumes of ice-cold incubation medium. Synaptosomes were collected by centrifugation at 12,000 × *g* for 10 min, washed two times with 0.25 M sucrose in 5 mM Tris, 0.1 mM EDTA, pH 7.0 (Medium II), and finally resuspended in a small volume (300 μl) of this medium in the presence of 5 mM phenylmethylsulfonyl fluoride. Protein concentration was determined by the Bradford method (Bradford, 1976).

Electrophoretic Analysis of in Vitro Translation Products—Just before gel electrophoresis, a sample of the synaptosome suspension (150 μg of protein) was treated with ethanol to a final concentration of 90% for 10 min on ice. After centrifugation of 12,000 × *g* for 10 min, the pellet was dried, dissolved in Laemmli buffer (Laemmli, 1970), and run on an SDS[1] 15–20% exponential polyacrylamide gradient gel (Chomyn and Lai, 1990). The gels were prepared for fluorography as described (Bonner and Laskey, 1974). The intensity of the bands in the autoradiograms was determined by densitometry (LKB laser-scanning densitometer). Exposure times were chosen to fall in the linear response range.

Extraction of Synaptosomal Nucleic Acids—Isolation of DNA and RNA from synaptosomes was performed by the proteinase K-SDS-phenol/chloroform method. Briefly, synaptosomes recovered from the gradient were washed three times in Medium II, resuspended in 5 ml of the same medium, and mixed with an equal volume of 240 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2.4% SDS (w/v), 200 μg/ml proteinase K. After incubation at 37 °C for 20 min, nucleic acids were extracted with phenol/chloroform/isoamyl alcohol and precipitated twice with ethanol. The concentration of nucleic acids (which were predominantly represented by RNA) was determined by measurement of absorbance at 260 nm, using a conversion factor of 40 μg/ml per absorbance unit.

RNA Transfer Hybridization Analysis—Polyadenylated RNA was isolated from each synaptosomal sample by a single passage of 30 μg of nucleic acids over an oligo(dT)-cellulose column (Type 3, Collaborative Research Inc.) (Amalric *et al.*, 1978). The bound fraction was collected by ethanol precipitation, electrophoresed on a 1.4% agarose/2.2 M formaldehyde gel in MOPS buffer (Lehrach *et al.*, 1977), and transferred onto a Zetaprobe nylon membrane (Bio-Rad) by electroblotting. The immobilized RNA was hybridized to rat liver mtDNA, isolated as previously described (England and Attardi, 1976), and labeled with ^{32}P by the random hexanucleotide priming method (Feinberg and Vogelstein, 1984). Prehybridization, hybridization, and washing the filters were performed as already reported (Cantatore *et al.*, 1987b). Before prehybridization, filters were washed at 58 °C for 1 h in 0.1 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% SDS. The intensity of the bands in the autoradiograms was determined by laser-scanning densitometry.

Analysis of Synaptosomal mtDNA—In order to normalize the protein synthesis data and mRNA levels from different experiments to a common internal marker, the mtDNA content of the synaptosomal fractions was determined. For this purpose, total nucleic acids extracted from a portion (0.7–0.8 mg of protein) of each synaptosome suspension used in the [^{35}S]methionine-labeling experiments or from synaptosome suspensions of rats of the same age as that used for mRNA analysis were digested for 3 h with *Eco*RI in the presence of RNase A (0.4 mg/ml). After incubation with 100 μg/ml proteinase K at 37 °C for 20 min, portions of the samples were directly electrophoresed on a 1% agarose gel in 40 mM Tris acetate (pH 7.8), 1 mM EDTA. The gel was stained with ethidium bromide, destained, and then photographed under UV light. mtDNA fragments were quantitated by densitometry using, as a standard, known amounts of CsCl-purified rat liver mtDNA cut with *Eco*RI. The relative labeling of the mitochondrial translation products and the relative amounts of mRNAs in different samples were determined by dividing the sum of the densitometric areas of the major bands (*COI*, *CYTb*, *COII*, *COIII*, and *A6* (Fig. 1)) in the protein-labeling profile and, respectively, of all the bands in the mRNA profile by the corresponding *absolute* amount of mtDNA in the sample.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.

RESULTS

Isolation of Synaptosomes and Characterization of in Vitro Protein Synthesis—A fractionation method utilizing an isoosmotic Percoll/sucrose gradient, introduced by Nagy and Delgado-Escueta (1984), has been used in the present work to isolate synaptosomes active in protein synthesis from rat cerebral cortices. This method allows the rapid isolation of reasonably pure, metabolically active synaptosomes. Three main membrane fractions are separated by centrifuging the 12,000 × *g* membrane fraction from a cerebral cortex homogenate in a Percoll/sucrose gradient under the conditions described by the authors. As characterized in the original paper, the two top bands (designated as bands A and B) consist predominantly of myelin sheath fragments and other membrane material; a diffuse band (designated as band C in the cited paper) at the 10–16% Percoll interphase is the fraction most enriched in intact synaptosomes, with little evidence of free mitochondria. A well defined pellet at the bottom of the gradient is represented mainly by free mitochondria, with only a small amount of synaptosomes.

Fig. 1*a* shows the protein-labeling pattern obtained after a 30-min *in vitro* incubation, in the presence of [^{35}S]methionine and 100 μg/ml cycloheximide, of a sample from the synaptosome band (band C) isolated by centrifugation in a Percoll/sucrose gradient of the 12,000 × *g* membrane fraction from a 21-day-old rat. For comparison, the 2-h *in vivo* emetine-resistant labeling pattern of the translation products from the mitochondrial fraction of R2 rat fibroblasts is also shown. The identification of the various rat mitochondrial translation products was made as previously reported (Attardi *et al.*, 1989), by a comparison of the R2 pattern with the HeLa cell pattern, and by immunoprecipitation experiments. These utilized antisera against peptides derived from the sequence of human mtDNA genes (Anderson *et al.*, 1981) that are completely or partially conserved in the rat (ND3, ND4L, and ATPase 6 (Gadaleta *et al.*, 1989)) or an antiserum against the intact bovine NADH dehydrogenase complex (Chomyn *et al.*, 1985, 1986) (Fig. 1), which cross-reacts with the rat enzyme (Cleeter and Ragan, 1985). Only the identification of ND6, a subunit of that enzyme, still remains uncertain.

The labeling profile observed for the synaptosome fraction in Fig. 1*a* (*−ETOH lane*) corresponded closely to the profile of the mitochondrial translation products from the R2 cell line (*−ETOH lane*), apart from the apparent absence of labeling of the ND5 product and a smeary appearance in the small size region of the electrophoretic pattern. This result confirmed the presence of synaptosomes in the fraction analyzed. In other experiments, it was shown that the *in vitro* labeling pattern of the upper two membrane fractions from a Percoll/sucrose gradient (bands A and B) had no resemblance to the pattern of the mitochondrial translation products, pointing to the absence of a significant amount of intact synaptosomes in these fractions (not shown). A pronounced labeled band moving somewhat more slowly than COI (marked with an *asterisk*) was observed in the protein-labeling patterns from both the synaptosomal fraction and the upper

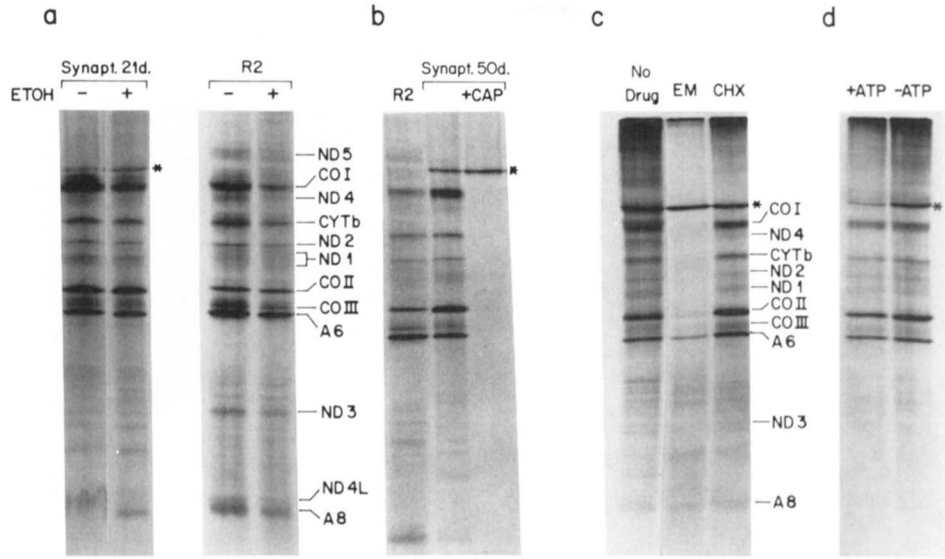

FIG. 1. **Characterization of *in vitro* protein synthesis in the synaptosomal fraction from Sprague-Dawley rat brain.** *a*, electrophoretic patterns of the *in vitro* translation products of the synaptosomal fraction from a 21-day-old rat labeled with [^{35}S]methionine for 30 min in the presence of 100 μg/ml cycloheximide or of the mitochondrial fraction from R2 cells labeled for 2 h in the presence of 100 μg/ml emetine. Equivalent samples of each fraction were electrophoresed directly, or after treatment with 90% ethanol, as described under "Experimental Procedures." *b*, effects of chloramphenicol (*CAP*) at 100 μg/ml on the protein-labeling pattern of the synaptosomal fraction from a 5-day-old rat incubated with [^{35}S]methionine for 20 min in the presence of 100 μg/ml cycloheximide. The 2-h emetine-resistant labeling pattern from R2 cells is shown for comparison. *c*, comparison of the *in vitro* protein-labeling patterns of the synaptosomal fraction from a 5-day-old rat incubated with [^{35}S]methionine for 20 min in the absence of inhibitors of cytoplasmic protein synthesis (*No Drug*), or in the presence of 100 μg/ml emetine (*EM*) or cycloheximide (*CHX*). *d*, effects of addition of ATP (1 mM) to the medium on the protein-labeling pattern of the synaptosomal fraction from a 5-day-old rat incubated with [^{35}S]methionine for 20 min in the presence of 100 μg/ml cycloheximide. In *b*, *c*, and *d*, the synaptosomal samples were treated with 90% ethanol prior to electrophoresis. *COI*, *COII*, and *COIII*, subunits I, II, and III of cytochrome *c* oxidase; *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*, subunits of the respiratory chain NADH dehydrogenase; *CYTb*, apocytochrome *b*; *A6* and *A8*, H^+-ATPase subunits 6 and 8.

two membrane fractions. Samples of nucleic acids from the synaptosomal fraction run on agarose gels revealed, after ethidium bromide staining, clear bands of the 12 S and 16 S mitochondrial rRNAs with relatively small amounts of 18 S and 28 S cytoplasmic rRNAs (not shown). By densitometry, the 18 S rRNA was estimated to be in 2–3-fold molar excess over the 16 S rRNA, as contrasted with the 100–200-fold molar excess found in HeLa cells (Attardi and Schatz, 1988). This suggests a low level of contamination of the synaptosomal fraction by cytoplasmic fragments from neuronal or glial cells.

It seemed likely that the smeary appearance in the small size region of the electrophoretic pattern of the synaptosomal fraction was due to the presence of lipids in these samples. Indeed, pretreatment of this fraction with ethanol removed the material producing this smeary appearance and revealed clearly the ND4L and ATPase 8 polypeptides (Fig. 1*a*, *+ETOH*). A similar treatment applied to the mitochondrial fraction from 2-h labeled R2 cells had no significant effect on the pattern of the mitochondrial translation products (Fig. 1*a*, *+ETOH*).

The nature of the unidentified labeled bands in the low molecular weight region of the synaptosomal pattern is unknown. A band migrating like the ND3 polypeptide in the R2 pattern is presumably the ND3 equivalent, although it is significantly less labeled than in R2 fibroblasts. It is not possible to say whether the two bands moving faster than ND3 in the synaptosomal pattern are related to ND3. However, the absence of abnormal bands in the high molecular weight region of the electrophoretic protein pattern (apart from the emetine-resistant band marked with an *asterisk* mentioned above) speaks against nonspecific phenomena of degradation or premature termination.

The labeling of all the bands in the synaptosome pattern, with the exclusion of the asterisked band, was found to be sensitive to 100 μg/ml chloramphenicol, as expected for mitochondrial translation products (Fig. 1*b*). The observation that the labeling of the asterisked band was resistant to both emetine and chloramphenicol suggests that its presence was not due to protein synthesis but rather reflected some form of end-labeling phenomenon. The nature of this band has not been investigated further. Interestingly, the pattern of mitochondrially synthesized polypeptides in the synaptosomal fraction was also clearly recognizable when the *in vitro* labeling was carried out in the absence of inhibitors of cytoplasmic translation, with relatively few extraneous bands appearing in the size range of the mitochondrial translation products (Fig. 1*c*). This observation indicates a low level of extramitochondrial protein synthesis in the synaptosomal fraction. Only in the high molecular weight region was an appreciable amount of emetine- and cycloheximide-sensitive translation products observed. Unexpectedly, in contrast to the situation observed in exponentially growing HeLa cells (Costantino and Attardi, 1977), it was found that emetine considerably inhibited the labeling of all mitochondrial translation products, whereas cycloheximide had no effect. Because of this observation, cycloheximide was used as a cytosolic protein synthesis inhibitor in all the subsequent experiments.

The kinetics of *in vitro* labeling of the synaptosomal proteins was investigated in experiments involving exposure of

synaptosomes from a 5-day-old and a 1-year-old rat to different [^{35}S]methionine pulses, from 3 to 45 min. In both cases, the labeling of mitochondrial translation products followed an approximately linear curve starting to level off after 20–30 min (not shown). The similarity in the time course of labeling observed for synaptosomes from a 5-day-old and a 1-year-old rat suggests that there was no substantial change in the size of the synaptosomal methionine pool during the first year of life. No appreciable differences were observed in the relative labeling of the various polypeptides after different labeling times up to 45 min. In particular, no evidence of labeling of the ND5 band was observed even after a 5-min pulse (not shown). Fig. 1*d* shows that addition of 1 mM ATP to the synaptosome incubation medium had no effect on the rate of labeling of the mitochondrial translation products, arguing against any significant contamination of the synaptosomes by free mitochondria (see "Discussion").

Age-related Changes in Synaptosome Protein Synthesis—A detailed analysis of the 20-min [^{35}S]methionine-labeling pattern of the synaptosome mitochondrial translation products from Sprague-Dawley rats of different ages, from 5 days up to 2 years, did not reveal any significant qualitative differences (Fig. 2, *a* and *b*). In particular, in the pattern from synaptosomes from animals of all ages examined, the ND5 product was not detectable, and the putative ND3 product was significantly less labeled than in mitochondria from R2 cells exposed to [^{35}S]methionine for 30 min. On a quantitative basis, the rate of labeling of the mitochondrial translation products per mg of protein measured 3 weeks after birth had declined by a factor of approximately 3, relative to the level observed in the 5- and 13-day-old rats (Fig. 2*a*). Thereafter, the rate of labeling did not decrease to any significant extent with age up to 10 months (Fig. 2a) with a moderate decrease in the 24-month-old rat (Fig. 2*b*). A similar analysis carried out on the synaptosomal fraction from four Fisher 344 rats, 1, 6, 12, and 24 months old, gave results comparable with those obtained with Sprague-Dawley rats (data not shown).

Synaptosome Mitochondrial mRNAs—In order to compare

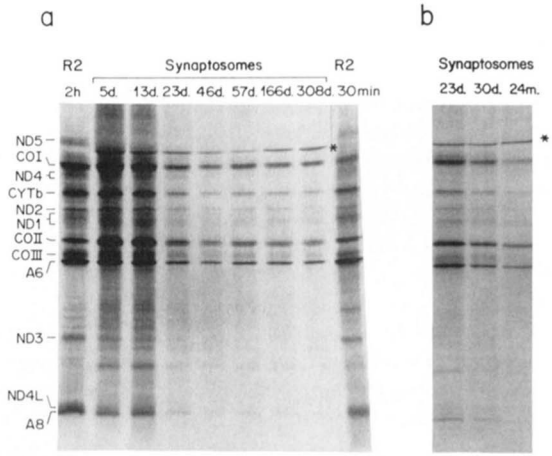

FIG. 2. **Display of newly synthesized mitochondrial translation products from brain synaptosomes of Sprague-Dawley rats of different ages.** Electrophoretic fractionation in an SDS-polyacrylamide gradient gel of proteins of the synaptosomal fraction from Sprague-Dawley rats of different ages labeled with [^{35}S]methionine for 20 min in the presence of 100 μg/ml cycloheximide. In different *lanes*, equal amounts of protein (120 μg) were subjected to electrophoresis after treatment with 90% ethanol. *Panels a* and *b* show independent electrophoretic runs of samples from rats of different ages, with the 23-day-old rat sample providing a common reference pattern. In panel *a*, the 30-min and 2-h emetine-resistant labeling patterns from the R2 cell line are shown for comparison. Explanation of the *symbols* is as in the *legend* of Fig. 1.

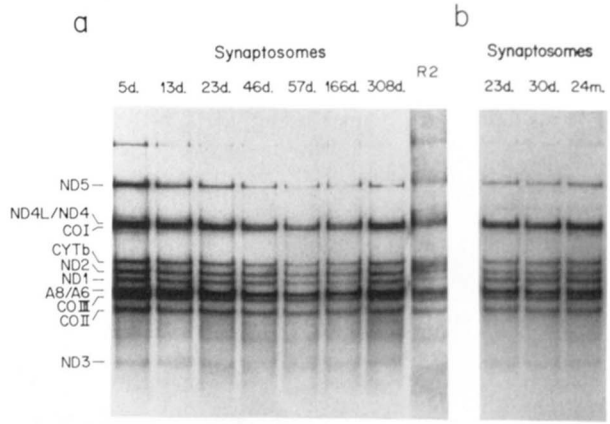

FIG. 3. **Mitochondrial mRNAs from brain synaptosomes of Sprague-Dawley rats of different ages.** The mtDNA-coded polyadenylated RNA components isolated from equivalent amounts (30 μg) of nucleic acids from synaptosomes and from the mitochondrial fraction of R2 cells were fractionated by electrophoresis in a 1.4% agarose-formaldehyde gel, electrotransferred onto a Zetaprobe membrane, and hybridized with a rat mtDNA probe ^{32}P-labeled by random priming. *Panels a* and *b* show independent electrophoretic runs and blots of samples from rats of different ages, with the 23-day-old rat sample providing a common reference pattern. Explanation of the *symbols* is as in the *legend* of Fig. 1.

the relative rates of synthesis of the various mtDNA-coded polypeptides in isolated synaptosomes with the steady state levels of the corresponding mRNAs, polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography from equal amounts of nucleic acids from synaptosomes of rats of different ages. A control experiment utilizing a rat COI gene-specific probe showed that no variation in yield of polyadenylated RNA between samples was introduced at this step. Equivalent RNA samples were fractionated by electrophoresis in a 1.4% agarose-formaldehyde gel in parallel with the polyadenylated RNA from an equivalent amount of nucleic acids from the R2 cell mitochondrial fraction. As shown in Fig. 3, *a* and *b*, the pattern of mitochondrial mRNAs from brain synaptosomes is substantially identical to that from R2 cells. The functional identification of the mitochondrial mRNAs in this pattern was made by comparison with the similar pattern previously described for HeLa cells (Attardi, 1986). In particular, one should notice the presence in the RNA from the two sources of approximately equivalent proportions of the ND5 and ND3 mRNAs. This contrasts with the strong underrepresentation or possible absence of ND5 and the reduced labeling of ND3 in the pattern of newly synthesized mitochondrial translation products from synaptosomes. As shown by an inspection of the autoradiograms and confirmed by densitometric measurements, the steady state amount of mtDNA-coded mRNAs per unit weight of synaptosome nucleic acids decreased progressively during the first 2 months and then remained constant over the next 3 months, with a tendency to increase in synaptosomes from a 10-month-old rat (Fig. 3*a*) and a 24-month-old rat (Fig. 3*b*). An analysis of the poly(A)$^-$ RNA components of the synaptosomal fraction revealed that the levels of the mitochondrial mRNAs are much higher, relative to those of the rRNAs, than in R2 fibroblast mitochondria. In particular, by a densitometric analysis of the autoradiograms, the molar ratio of 12 S rRNA to the best resolved mRNA species, COII mRNA, was found to be 3.1, 4.1, and 3.8 in the synaptosomal fraction of, respectively, a 23-, a 46-, and a 308-day-old rat, as contrasted with a value of ~15 previously determined for R2 fibroblast

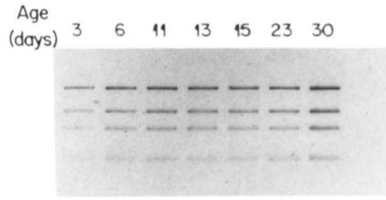

FIG. 4. **Quantitation of mitochondrial DNA from brain synaptosomes of Sprague-Dawley rats from 3 to 30 days of age.** Nucleic acids were extracted from synaptosomes as described under "Experimental Procedures," and equivalent amounts (2.5 μg) were digested with *Eco*RI and electrophoresed on a 1% agarose gel. After staining with ethidium bromide, the gel was photographed under UV light, and the intensities of the bands were determined by densitometry and compared with those of the bands produced by known amounts of *Eco*RI-digested rat liver mtDNA.

mitochondria[2]. These results are consistent with earlier observations made by comparing mitochondrial RNA from rat hepatocytes (Cantatore *et al.*, 1984) and rat cerebellum (Renis *et al.*, 1989) to mitochondrial RNA from HeLa cells.

Mitochondrial Gene Expression in Synaptosomes during Development and Maturation—In order to normalize the mitochondrial protein-labeling data and mRNA levels determined in isolated synaptosomes to a common internal marker, the mtDNA content was independently determined, as illustrated by the example shown in Fig. 4, in the synaptosomal fractions used for protein labeling and in those from rats of the same age used for mRNA analysis. The normalized values of protein labeling and mRNA amount for rats of different ages are shown in Fig. 5*a*. The mitochondrial mRNA level per unit amount of mtDNA shows a sharp decline in the first 3–4 weeks after birth and then remains fairly constant up to 6 months, with clear evidence of an increase in the older rats (10 and 24 months). The rate of protein labeling per unit amount of mtDNA appears to increase by a factor of about 2 from 5 days to 13 days after birth, and it then declines sharply in the 3rd week, and more slowly in the following weeks, reaching by 30–50 days a level that remains constant over the next 22 months. In order to verify the significance of the apparent burst of protein synthetic activity at the end of the 2nd week after birth, a more detailed analysis of protein labeling and mRNA level was carried out on brain synaptosomes from very young rats. For this purpose, two separate sets of rats from 3- or 5-day-old to 23- or 30-day-old (each set belonging to the same litter) were used. To minimize possible variations between synaptosome preparations, in one of these experiments the *same* preparation was used for both *in vitro* protein synthesis and mRNA level determinations, again with normalization of the data to the mtDNA content of the two synaptosomal portions analyzed. The results are shown in Fig. 5*b*. It is clear that the burst of protein synthetic activity around 13 days after birth is absolutely reproducible and does not correlate with any increase in mRNA levels.

Developmental Changes in Cytochrome c Oxidase Activity in Brain Synaptosomes of Young Rats—It seemed possible that the burst of mitochondrial protein synthetic activity observed in rat brain synaptic endings 2 weeks after birth corresponded to a phase of rapid assembly of respiratory complexes. In order to test this possibility, measurements of cytochrome *c* oxidase activity were carried out on synaptosomes isolated from Sprague-Dawley rats from 5- to 23-days-old. As shown in Fig. 5*b*, a rapid increase in cytochrome *c* oxidase activity per unit of mtDNA content was observed from 10 to 13 days after birth, corresponding precisely with the increase in rate

[2] A. Chomyn, personal communication.

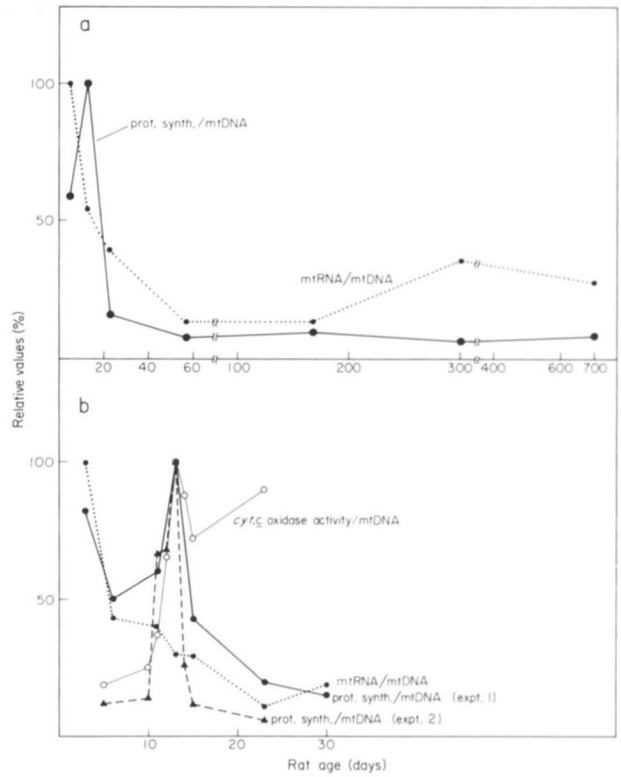

FIG. 5. **Mitochondrial gene expression in rat brain synaptosomes during development and maturation.** *a*, the rates of labeling of the mitochondrial translation products and the steady state amounts of the mtDNA-coded mRNAs in brain synaptosomes from Sprague-Dawley rats of different ages, which are shown in Figs. 3 and 6, have been normalized for the mtDNA content of the preparations used for *in vitro* protein synthesis analysis and, respectively, for isolation of the polyadenylated RNA components. *b*, normalized data for protein synthesis and mRNA levels from two additional experiments utilizing young rats are shown in parallel with the normalized values for cytochrome *c* oxidase activity measured in the synaptosomal fraction in one of the experiments. This activity was assayed as described by Mason *et al.* (1973) using treatment with 0.6% digitonin to disrupt the synaptosomal membrane and the outer mitochondrial membrane prior to the assay. See text for details.

of protein labeling. The cytochrome *c* oxidase activity remained high over the 10 days following the 13-day peak.

DISCUSSION

The purpose of this work has been to analyze the pattern of mitochondrial gene expression in rat brain synaptic endings during the postnatal development and maturation of the animal. The purity of the synaptosome preparations used in the present work was indicated by the presence of relatively small amounts of 18 S and 28 S rRNAs in the synaptosomal nucleic acids. These RNA species presumably derive from the postsynaptic contact regions, which contain cytoplasmic polyribosomes (Steward and Falk, 1986), as well as from contaminating microsomes; the latter were reported to be the main contaminant present in synaptosomes isolated by the Percoll/sucrose gradient fractionation method (Nagy and Delgado-Escueta, 1984). Also, the low level of extramitochondrial protein synthesis observed in the synaptosomal fraction in the absence of inhibitors of cytoplasmic protein synthesis argues in favor of a small contamination of this fraction by cytoplasmic fragments from neuronal or glial cells. The lack of any effect of added ATP on the rate of protein synthesis by the synaptosomal fraction indicates that this fraction was substantially uncontaminated by free mitochondria. In fact,

it has been shown that protein synthesis in isolated HeLa cell mitochondria is strictly depending upon the presence in the medium of ATP or, alternatively, of ADP, phosphate, and a respiratory substrate (Lederman and Attardi, 1970).

Under the conditions of rapid isolation and analysis of the synaptosomes used in the present work, the labeling of mtDNA-encoded polypeptides proceeded at a linear rate for at least 20 min. It is reasonable to think that the protein synthetic activity detected under these conditions reflected the *in vivo* processes and therefore could provide information on the changes in rate of mRNA translation occurring in the animal during development and maturation.

The pattern of the mitochondrial translation products synthesized *in vitro* in the rat brain synaptic endings revealed some distinctive features when compared with the labeling pattern of R2 fibroblasts, the most remarkable being the apparent absence of ND5 and the reduced labeling of ND3, two of the mtDNA-encoded subunits of NADH dehydrogenase. These differences were also observed when the two systems were subjected to short [^{35}S]methionine pulses (Fig. 2). The absence of labeling of ND5 did not appear to be due to a general tendency to artificial degradation of the mitochondrial translation products, which would have predominantly affected the high molecular weight components. In fact, other high molecular weight products, *i.e.* COI and ND4, were labeled in synaptosomes to a relative extent comparable with that observed in the rat R2 fibroblast line. On the contrary, the observation that no labeling of the ND5 polypeptide was observed even after a 5-min pulse suggests that ND5 is not synthesized or is synthesized only at a marginal rate in brain synaptic endings. However, one cannot absolutely exclude the alternative possibility that ND5 is synthesized at a normal rate but rapidly and specifically degraded. Also, in isolated rat quadriceps muscle, the ND5 product is the only polypeptide that is not labeled to any appreciable extent (Attardi *et al.*, 1989). By contrast, this polypeptide has been shown to be synthesized in all established human and rat cell lines, human fibroblast, and myoblast strains analyzed thus far.[2] An intriguing possibility is that the ND5 product is a growth-regulated component of NADH dehydrogenase. Nothing is known about the function of this subunit. It has, however, been suggested that this polypeptide may be an iron-sulfur protein and may participate in ubiquinone reduction (Chomyn *et al.*, 1988).

The coincidence observed between the postnatal burst of mitochondrial protein synthesis in rat brain synaptosomes and a rapid increase in cytochrome *c* oxidase activity per unit of mtDNA content suggests a correspondence of this burst with a rapid assembly of respiratory complexes. It is interesting that a rapid increase in the number and size of synapses and in the number of synaptic vesicles has been observed in the 2nd and 3rd postnatal week in the rat cerebral cortex (Bloom, 1972; Jones and Cullen, 1979; Blue and Parvanelas, 1983) and spinal cord (Weber and Stelzner, 1980). Also the oxidative metabolism of rat brain has been shown to increase considerably during postnatal development in correspondence with an increase in the number of mitochondrial profiles (Milstein *et al.*, 1968).

One interesting observation is that after the postnatal burst, the rate of mitochondrial protein synthesis per unit amount of mtDNA in isolated brain synaptic endings remained strikingly constant over a 2-year period. Since the amount of mtDNA/mg of synaptosomal protein declined only moderately during this period (~50%), the data strongly suggest that mitochondrial gene expression does not decrease substantially with age in brain cortex synaptic endings.

The sharp decline observed in the level of mitochondrial mRNAs per unit amount of mtDNA in brain synaptosomes in the first 3–4 weeks after birth is consistent with earlier observations (England and Attardi, 1976) indicating a strong decrease in the capacity for mitochondrial RNA synthesis of isolated synaptic endings from rat cerebral cortex as the age of the animal increased from 10 to 30 days. The significance of this decrease in transcription activity of mtDNA during the period of cortex synapse development is not obvious and requires further investigation.

Interesting insights into the regulation of mitochondrial gene expression in rat brain synaptic endings have been obtained by comparing the levels of mitochondrial mRNAs with the corresponding rates of mitochondrial protein synthesis during rat postnatal development and maturation. The main conclusion of this comparison is that translational control appears to play a major role in the regulation of gene expression in synaptic mitochondria. In particular, the apparent absence of ND5 and the reduction of ND3 among the newly synthesized mitochondrial translational products in brain synaptosomes is in striking contrast with the presence of relative amounts of ND5 and ND3 mRNAs comparable with those found in R2 fibroblasts. Furthermore, the postnatal burst of protein synthesis in brain synaptic endings occurs during a period of clear decline in the amount of mitochondrial mRNAs. The importance of translational control in mitochondrial gene expression in mammalian cells had previously been suggested by the observation that different mitochondrial mRNAs are translated in HeLa cells with efficiencies varying over almost an order of magnitude (Chomyn and Attardi, 1987). In yeast, there is good evidence indicating that specific nuclear coded factors play a role in the control of translation of individual mitochondrial mRNAs, in particular COIII mRNA, CYTb mRNA, and possibly COII mRNA (see review by Fox (1986)).

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