Comparison between the Time Course of Changes in Nerve Growth Factor Protein Levels and Those of Its Messenger RNA in the Cultured Rat Iris*

Rolf Heumann‡ and Hans Thoenen
From the Department of Neurochemistry, Max-Planck-Institute for Psychiatry, Am Klopferspitz 18a, D-8033 Martinsried/Munich, Federal Republic of Germany

In previous experiments, it has been demonstrated that, in rat irides in culture, a rapid increase in nerve growth factor (NGF) levels occurred (see Barth, E.-M., Korsching, S., and Thoenen, H. (1984) J. Cell Biol. 99, 839–843). We have now determined the levels of mRNA\textsuperscript{NGF} in rat irides as a function of time in culture as well. After an initial lag period of 2 h, mRNA\textsuperscript{NGF} levels were transiently increased, so that after 12 h, they had increased 35-fold with respect to zero time. In contrast, poly(A)\textsuperscript{+} RNA levels dropped to 55% of the zero time values within 5 h, recovered to 85% after 24 h, and remained constant until the end of the observation period. Total ribosomal RNA was found to remain constant, indicating that there was no nonspecific decline of overall metabolic function. Actinomycin D prevented the increase in mRNA\textsuperscript{NGF} without reducing the basic mRNA\textsuperscript{NGF} levels over a 5-h time period, indicating that the enhanced synthesis of NGF in the rat iris in culture is primarily mediated by an augmented production of mRNA\textsuperscript{NGF}. The increases of mRNA\textsuperscript{NGF}, cellular NGF, and NGF released into the medium were found to be strictly sequential. Monensin selectively abolished the increased production of mature NGF (see Barth et al.) but not of mRNA\textsuperscript{NGF}, suggesting that the processing of NGF precursor is prevented.

In previous experiments, it has been demonstrated that the density of sympathetic innervation was positively correlated to the density of sympathetic target organs (1, 2, 17). In contrast, the high NGF content of sympathetic ganglia was not accompanied by equivalent levels of mRNA\textsuperscript{NGF}, suggesting an NGF accumulation in sympathetic ganglia resulting from retrograde axonal transport rather than from local synthesis. This interpretation was further supported by the observation that blockage of retrograde axonal transport by 5-hydroxydopamine (selective destruction of sympathetic nerve terminals) and colchicine (disassembly of microtubules) resulted in a rapid NGF decrease in sympathetic ganglia (t0, 4–5 h) and a rapid concurrent NGF increase in the corresponding peripheral target organs (3). These observations raised the question whether the NGF increase in the target organs was exclusively due to an elimination of the efficient removal of NGF by retrograde axonal transport or whether there was also an enhancement of NGF synthesis.

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‡ To whom reprint requests should be addressed.

The abbreviation used is: NGF, nerve growth factor.

Preparation of Cultured Iris—Wistar rats (150–200 g, both sexes) were killed by cervical dislocation, and the irides were dissected and kept in culture as described previously (5). The time periods between killing and start of culture were always less than 5 min. The irides were cultured in Dulbecco's modified Eagle's H21 medium with 10% rat serum at 37°C. At the end of each incubation period, the irides were frozen and kept at −70°C until further use.

Isolation of RNA and Blotting Procedures—Total RNA was prepared according to Melera and Rusch (7) using bentonite (A1203) as a protein adsorbent and RNase inhibitor. The extracted RNA was treated with RNase-free DNase I (20 pg/ml) (Boehringer Mannheim) and proteinase K (200 μg/ml) (Boehringer Mannheim) essentially according to Tullis and Rubin (8). Electrophoresis of glyoxylated RNA was performed as described previously (9). Gels were run at 10 mA for 12 h. For the quantification of mRNA\textsuperscript{NGF}, the total RNA of four irides (19.2 μg) was applied to the wells, whereas for the quantification of 18S rRNA, only one-tenth of the total RNA from a single iris was loaded together with 1 μg of carrier r-RNA. Dot blots were prepared with the multifold apparatus (Schleicher & Schüll) using 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate) and 0.1% sodium dodecyl sulfate as sample buffer. The amount of total RNA applied in the serial 2-fold dilutions started with 10 μg.

Preparation of RNA Probes—The large PstI-cleaved fragment of a mouse cDNA\textsuperscript{NGF} insert (0.92 kilobase) obtained from J. Scott (Medical Research Center, Harrow, England) (10) and a mouse 18S rRNA-cDNA insert (1.85 kilobase) obtained from I. Grummt (University of Würzburg, Federal Republic of Germany) (11) were cloned into plasmids pSP6 in both orientations to the SP6 promoter of SP6 RNA polymerase (12). The recombinant plasmid templates were used for in vitro run-off RNA synthesis, and the polarities of the asymmetric transcripts were either complementary (RNA\textsuperscript{NGF+}, 18S rRNA−) or identical with (RNA\textsuperscript{NGF−}) the corresponding cellular RNA. Labeled RNA\textsuperscript{NGF+} and/or 18S rRNA− was used as a hybridization probe.
whereas unlabeled RNA NGF transcripts were used as standards (see "Results"). In the case of NGF transcripts, special care was taken to ensure purity. Removal of DNA by DNase I (20 μg/ml) treatment was found to be complete by addition and subsequent degradation to mononucleotides of a radioactively labeled DNA standard. In subsequent experiments, α-32P-labeled UTP was added after DNase treatment, and the removal of mononucleotides was monitored in the precipitations using 2 M ammonium acetate and 70% ethanol. The A260 (1 unit = 40 μg RNA) was subsequently read from the spectrum obtained by using a DU-8 spectrophotometer (Beckman Instruments). The in vitro synthesized RNA was glyoxylated, run on an agarose gel, and stained with acridine orange (30 μg/ml) (13). Single bands of the predicted size demonstrated the specificity of synthesis and the absence of template DNA. After addition of carrier tRNA (5 μg/ml), the in vitro synthesized RNA was stored in aliquots as ethanol precipitates at −70°C. Labeled RNA NGF+ and 18 S rRNA+ probes (specific activity 6 × 10^8 dpm/μg) were prepared as described previously (2).

Oligo(dT)12-18 (PL Biochemicals) was 5'-32P-labeled with T4 kinase (New England Nuclear) and separated from free [α-32P]ATP by Sephadex G-50 chromatography.

Hybridization and washing conditions for RNA and oligo(dT)12-18 probes were as described previously (2) using 50% formamide except that hybridization temperatures for RNA NGF+, 18 S rRNA+ and oligo(dT) were 65, 70, or 4°C, respectively. The differences in the optimal hybridization temperatures were mainly determined by the differences in the length and G-C content of the probes.

Densitometric Quantification of mRNA—Fold serial dilutions of standard RNA NGF+ (0.92 kilobase) were applied to the 1.5% agarose gels in parallel with the test samples. After Northern blot hybridization, the areas obtained from the densitometric scanning of the RNA NGF+ bands were plotted as standard curves. In most cases (depending on the intensity of the signal), the densitometrically obtained data were cross-checked with those from liquid scintillation counting of dissolved nitrocellulose bands cut from the appropriate region of the filter. All mRNA NGF values were corrected for the RNA recoveries.

The films (Fuji RX) were exposed to the Northern blots or dot blots for time periods that produced linear signals of the densitometric scanning at the given intensity of the bands.

RESULTS

Quantification Procedure for mRNA NGF—For the determination of the absolute quantities of mRNA NGF/iris, we used in vitro synthesized unlabeled RNA NGF as a calibration standard. The blotting and hybridization efficiencies were assumed to be identical for in vitro and in vivo synthesized RNA NGF because of their similar size (920 versus 1350 bases) and identical sequencing.

The SP6 system enabled us to synthesize an RNA fragment of suitable length which was used for the estimation of the recovery of RNA from individual preparations. The SP6 NGF+ template plasmid was cut with the restriction enzyme NcoI so that the template directed the synthesis of an RNA NGF− fragment of about 510 bases. A known amount of this short NGF− fragment (50 pg, if not otherwise stated) was added to the irides during thawing. The recovery of the RNA during the preparation was estimated from a calibration standard of this short fragment and was also included in the gels in separate lanes (data not shown). In Fig. 1, an example of the mRNA NGF+ quantification/iris is shown for zero time (in situ tissue) levels (0.65 ± 0.06 pg) and for 48 h (2.5 ± 0.2 pg) and 72 h (2.0 ± 0.19 pg) in culture. In view of the small variations of the recovery values (here, e.g. 93 ± 5%), the standard was only included in one of the triplicate preparations.

Time Course of Changes in mRNA NGF Levels—After a lag period of 2 h, the mRNA NGF− increased from a basal level of 0.63 ± 0.14 pg at zero time to a maximal value of 22 ± 0.6 pg/iris at 12 h (Fig. 2). Thereafter, the values declined to 7 ± 1 pg at 24 h, 1.6 ± 0.1 pg at 48 h, and 1.3 ± 0.1 at 72 h. Thus, the maximal increase was 35-fold. However, the total poly(A)+ RNA decreased to levels between 55 and 60% of the zero time values (see below); and therefore, the mRNA NGF increase calculated in terms of poly(A)+ RNA would be about 60-fold.

Time Course of 18 S rRNA and Total Poly(A)+ RNA—In contrast to the marked changes in the mRNA NGF levels, those of 18 S rRNA, which were determined as a measure for total cellular RNA, remained essentially unchanged over the whole time period investigated (Fig. 2). However, the poly(A)+ RNA content in total RNA decreased to 55% of the zero time values within the first 5 h (Table I) and remained at these low levels for up to 12 h. After 24 h, the poly(A)+ RNA increased to 80–90% of the zero time levels and remained constant up to 72 h, the latest time point investigated.

Comparison between the Time Courses of mRNA NGF, Cellular NGF, and NGF Released into the Culture Medium—As reported earlier, the NGF release into the culture medium was preceded by a corresponding increase in NGF tissue levels (5). As shown in Fig. 3, the increase in tissue NGF was preceded by an increase in mRNA NGF. The half-maximal level of mRNA NGF was reached after 4 h, that of tissue NGF after 9 h, and that of NGF released into the culture medium after 12 h (5).

Effect of Monensin and Actinomycin D on mRNA NGF—Previous experiments have demonstrated that the carboxylic monensin, which blocks the transfer of peptides through the Golgi apparatus (14), prevents both the
FIG. 2. Time courses of mRNA\textsuperscript{NGF} and 18 S rRNA (inset) in cultured iris. For each measurement of mRNA\textsuperscript{NGF} (Δ) four irides were pooled and the extracted total RNA was subjected to Northern blot analysis using \textsuperscript{32}P-labeled RNA\textsuperscript{NGF} as a probe. The values given represent the average of three independent preparations. Inset, for the determinations of 18 S rRNA (○), one-tenth of the total RNA extracted in triplicates from single irides was subjected to Northern blot analysis. In cases where no bars were drawn, the errors were within the size of the symbols.

TABLE I

Time course of the relative portions of the poly(A)+ RNA in the total RNA preparations

For each time point, the total RNA of four irides was extracted in triplicates and dotted onto nitrocellulose in serial 2-fold dilutions. After hybridization with 5' labeled oligo(dT)\textsubscript{12-18}, the values were obtained by liquid scintillation counting of the dissolved nitrocellulose spots.

| Time in culture (h) | Relative portions of poly(A)+ RNA (%) |
|---------------------|-------------------------------------|
| 0                   | 100 ± 3                             |
| 5                   | 55 ± 5                              |
| 12                  | 60 ± 5                              |
| 24                  | 85 ± 1                              |
| 48                  | 82 ± 10                             |
| 72                  | 86 ± 4                              |

FIG. 3. Time courses of mRNA\textsuperscript{NGF}, cellular NGF, and NGF released into the medium. The mRNA\textsuperscript{NGF} data (Δ) are drawn together with cellular NGF (○) and NGF released into the medium (●) as determined previously by a two-site immunoassay (5).

increase in tissue NGF and NGF released into the culture medium (5). We therefore investigated the effect of monensin on the levels of mRNA\textsuperscript{NGF}. In contrast to the production and release of NGF, the increase in mRNA\textsuperscript{NGF} was not affected by monensin using parallel cultures for the measurements of NGF and its mRNA. Actinomycin D, which has previously been shown to block the NGF increase of rat irides in culture (5), also prevented the increase in mRNA\textsuperscript{NGF} (Table II). It is noteworthy that actinomycin D prevented the rapid increase but did not reduce the basic levels of mRNA\textsuperscript{NGF}.

DISCUSSION

The results of this study show that in rat irides in culture, after a lag period of 2 h, the mRNA\textsuperscript{NGF} increased rapidly to a maximal 35-fold level at 12 h. This rapid increase was followed by a gradual decline to an 11-fold level at 24 h and thereafter remained constant at a 2–3-fold elevated level between 48 and 72 h. In contrast to the rapidly changing mRNA\textsuperscript{NGF} levels, the 18 S rRNA remained constant over the whole observation period of 72 h. The quantitative determination of 18 S rRNA was used as a measure for total tissue RNA (28 S and 18 S rRNA are derived from a common 45 S precursor RNA (15) and account for more than 90% of total cellular RNA). The fact that the ribosomal RNA remained constant over a 48-h cultivation period is a further indication that an impaired overall function of the iris in culture is not responsible for the observed changes in mRNA\textsuperscript{NGF}. This is in agreement with previous experiments in which no changes in the incorporation of [\textsuperscript{35}S]methionine into proteins over similar cultivation periods had been observed (5). The drop of poly(A)+ RNA to 55% of zero time values within the first 5 h therefore seems unlikely to reflect a general deterioration of the iris preparation, although the mechanism of such an apparent decrease of poly(A)+ RNA remains to be determined. Using denervated rat adrenals, a similar transient drop in total poly(A)+ RNA
has been previously observed (16). Thus, in cultured iris, the decrease in total poly(A)+ RNA serves to emphasize the specificity of the increase of mRNANGF. In terms of absolute values/iris, mRNANGF levels underwent a maximal increase of 35-fold after 12 h. Relative to poly(A)+ RNA, the increase is 60-fold after 12 h and approaches the 200-fold increase in NGF protein shown in previous experiments (5). Thus, a minor post-transcriptional contribution to NGF synthesis, e.g. by stabilization of the NGF protein, cannot be excluded. The fact that actinomycin D abrogated the rapid increase in the first 5 h, without reducing the mRNANGF level below the base line, indicates that the increase in mRNANGF levels predominantly results from an enhanced synthesis of mRNANGF rather than from a stabilization of pre-existing mRNANGF. Recently, Shelton and Reichardt (17) reported an initial experiment of changes in mRNANGF levels in rat irides in culture. Both the absolute values and the increases in culture are different from the present results in that the zero time mRNANGF values, are 13-fold lower and the maximal increase after 5–10 h in culture is only 7-fold. The lower absolute zero time values may be explained by differences in the RNA extraction procedure used. Since Shelton and Reichardt did not measure recovery, selective loss of mRNANGF is also possible. Their culture conditions were very similar to ours with the exception of the use of fetal calf serum in place of rat serum. In previous experiments, however, the differences in NGF tissue levels and NGF released into the culture medium were very few when cultures supplied with fetal calf serum were compared with those containing rat serum (5).

In previous studies (5), we have demonstrated that monensin prevented the increased production and release of NGF in rat irides in culture. In the present study, we have demonstrated that the changes in mRNANGF levels were not affected by monensin. This observation is in agreement with the previous interpretation that monensin blocks the transit of constituents stored in nerve terminals. These nerve terminals rapidly increasing levels of mRNA reaching a maximum after 12 h are suggestive of a mechanism resulting from the release of constituents stored in nerve terminals. These nerve terminals are disconnected from their cell bodies by the culture procedure and therefore degenerate. The degeneration process starts more rapidly the shorter the peripheral nerve stump (19) and therefore begins virtually immediately in the cultured iris system. At the present time, experimental efforts are in progress aimed at the identification of the substance(s) responsible for the rapid increase in mRNANGF. Interestingly, this increase is prevented by the addition of cycloheximide (5 µg/ml) to the culture medium, suggesting that a protein synthesis-dependent mechanism is involved.

The stably elevated mRNANGF levels between 48 and 72 h could suggest that under physiological conditions, the NGF production in the target cells is repressed by the presence of the innervating neurons. Such an assumption is in agreement with preliminary experiments in the sciatic nerve where we have observed an at least 10-fold increase within 6 h after nerve transection in segments distal to the cut, which contain the degenerating peripheral axons. Thus, the peripheral part of the sciatic could resemble the cultured iris, where all the innervating nerve terminals degenerate. Experiments are in progress aiming at the identification of the cells involved in the synthesis of NGF by in situ hybridization.

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