Serum and thyroid hormones T3 and T4 regulate nerve growth factor mRNA levels in mouse L cells

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Mouse L cells synthesize and secrete a neurotrophic factor related to the β subunit of the submaxillary gland nerve growth factor (NGF) of male mice. Use of a cDNA probe which encodes the β-NGF mRNA demonstrated that L cells produce a transcript identical in size to that of the submaxillary gland. Moreover, target sites of restriction enzymes *Eco*RI, *Pst*I and *Bam*HI were not significantly rearranged in the β-NGF gene locus of these cells. The abundance of the β-NGF transcript was found to depend on culture conditions. Removal of serum depressed the cellular content of polyadenylated RNA by a factor of 1.7, and decreased specifically the pool of β-NGF transcript by an additional factor of 4. The presence of 10^{-7} M testosterone in the serum-free medium did not modify the level of β-NGF mRNA, while addition of 10^{-7} M T3 (or T4) increased this level by a factor of 1.5. These data provide the first evidence that the β-NGF mRNA of L cells is subjected to regulation, but in a way apparently different from that described for the submaxillary gland.

Nerve growth factor *mRNA* *Serum* *T3* *Testosterone*

1. INTRODUCTION

NGF is a neurotrophic factor involved in the development of sympathetic and sensory neurons [1]. It is released by target organs which are innervated by NGF-sensitive nerve fibers [1–3], or by nerve-associated cells such as Schwann cells or astrocytes [4,5]. The production of NGF appears to be subject to regulatory events, since nerve injury, or transplantation of peripheral organs in vitro is accompanied by an enhancement of NGF production [6,7]. The factors controlling these events remain unknown. More information is available at the level of a unique organ, the male mouse submaxillary gland. This tissue contains exceptionally large amounts of NGF [8]. The neurotrophic molecule is a dimer, called β-NGF, which is associated with two other subunits, α and γ [9]. The biosynthesis of the submaxillary gland NGF is primarily controlled by testosterone, but is also stimulated by the thyroid hormone T4 [10–12].

Previous studies demonstrated that the mouse cell line L produces and secretes a neurotrophic activity which is immunologically and biochemically related to the submaxillary gland β-NGF [13,14], but which is not accompanied by the α and γ subunits [15,16]. Factor(s) influencing the rate of production of β-NGF in this cell line are at present unknown. Therefore, the ability of L cells to growth in defined conditions provides a model system for studying the expression of this gene. Here, we have used a cDNA probe encoding most of the mRNA of the submaxillary gland β-NGF [17] to characterize the β-NGF gene locus of L cells, and to examine the effect of serum, thyroid hormones and testosterone on the cellular levels of its mRNA.

2. MATERIALS AND METHODS

2.1. *Culture conditions*

The basal defined medium was a mixture of DMEM and F12 media (3:1, v/v) containing in-

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sulin (5 μg/ml), transferrin (100 μg/ml) and 2.5 × 10^{-8} M selenium [18]. This medium supports the proliferation of L-929 cells (here referred to as L cells). Growth was performed routinely in the presence of 5% horse serum. At the end of the growth phase, proliferation was irreversibly arrested with mitomycin C (40 μg/ml for 3 h) [14]. Cells were next detached with trypsin, washed in PBS and plated in serum-free medium (10^7 cells/15 cm diameter dish). They were incubated with or without thyroid hormone T3 or T4 or testosterone (10^{-7} M each). Alternatively, the medium was supplemented with 5% horse serum or with 10% foetal calf serum. Under such plating conditions, L cells were well separated from each other. Their number or density remained virtually constant, rendering the experimental system very reproducible and comparable, whatever the type of medium used. All media were renewed after 3 days. The RNA was extracted during the 4th day of culture.

2.2. *Preparation and blot analysis of RNA*

Total RNA from cells cultured in a single dish, or from a submaxillary gland, was prepared by the LiCl/urea method as described [19]. The relative content of poly(A)-rich RNA was determined by hybridization with [^{3}H]poly(U) (Amersham, M_r 12000–45000, 20–72 Ci/mmol) according to a procedure modified from [20]. Total RNA (0.5 μg) was diluted in 0.5 ml of 2 × SSC buffer (1 × SSC: 0.15 M NaCl and 0.015 M sodium citrate) and annealed with 0.2 μCi of radioactive poly(U) for 15 min at 45°C. Samples were chilled on ice, and supplemented with 2 ml of 2 × SSC buffer containing 20 μg RNase A. After 15 min at 0°C, 100 μg salmon sperm DNA and 150 μl of 100% trichloroacetic acid were added. The precipitate was collected on Whatman GF/B filters, dried and counted. The radioactivity increased linearly with the content of polyadenylated RNA, provided poly(U) remained in excess. A parallel treatment of poly(U) alone gave background values. For dot-blot analysis, samples of total RNA containing identical amounts of polyadenylated RNA were denatured with 1 M glyoxal and 50% dimethyl sulphoxide for 1 h at 55°C, and immobilized on Gene Screen membranes (New England Nuclear), as described [21]. After baking at 80°C, the membranes were subjected to prehybridization and hybridization following the procedure of Thomas [21] except that dextran sulphate was omitted. The ^{32}P-labeled probe (about 2 × 10^8 cpm/μg) was the nick-translated 917-base-pair *Pst*I/*Pst*I restriction fragment of the βNGF cDNA cloned by Scott et al. [17]. The nick translation reagent kit was from Bethesda Research Laboratories. Membranes were washed for 5 min in 2 × SSC, 0.5% SDS at room temperature, twice for 1 h in 0.1 × SSC, 0.5% SDS at 60°C, and radioautographed. Glyoxylated RNA was size fractionated on a 1.5% agarose gel, transferred to a membrane and hybridized as above.

2.3. *Preparation and blot analysis of DNA*

High-M_r DNA was isolated by the method of Blin and Stafford [22]. Restriction endonuclease digestion was performed according to the manufacturer's instructions (Amersham). Fragments were electrophoresed in 0.9% agarose gels, transferred to a membrane and hybridized as described [23].

3. RESULTS

3.1. *Characterization of the NGF transcript of L cells*

RNAs extracted from growing L cells and from the male mouse submaxillary gland were separated in agarose gels and transferred to hybridization membranes. Blots were hybridized with the denatured ^{32}P-labeled β-NGF cDNA, washed, and subjected to radioautography. The data in fig.1A show that L cells contained a single RNA species which hybridized with the β-NGF probe. Its size was identical to that of the 1.35 kilobase β-NGF transcript [17] found in the submaxillary gland. A similar pattern was obtained when the polyadenylated RNAs, enriched by 2 passages over oligo(dT)-cellulose, were fractionated and hybridized to the probe. The β-NGF mRNA represented about 0.02‰ of the poly(A)-containing RNA, instead of 1‰ in the submaxillary gland [17].

To determine whether the β-NGF genomic locus of L cells and of the submaxillary gland shared analogy, high-M_r DNA was extracted from both sources. It was cleaved with the restriction enzymes *Eco*RI, *Bam*HI or *Pst*I. The digestion products were fractionated on agarose gels, transferred to membranes and hybridized with the ^{32}P-labeled

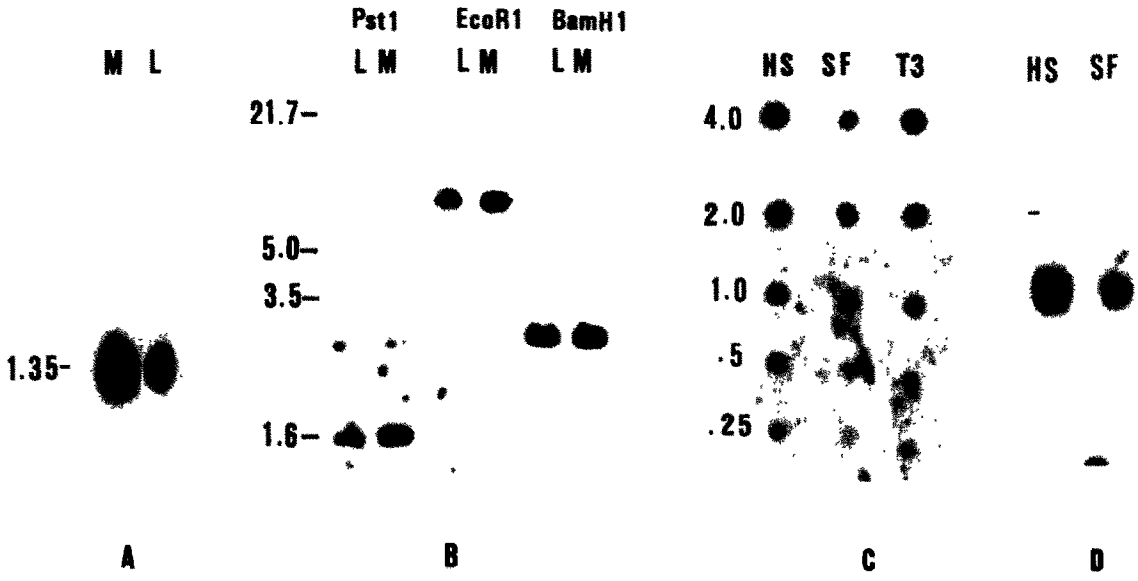

Fig.1. (A) Size determination of the β-NGF mRNA. Total RNA extracted from male mouse submaxillary gland (lane M, 4 μg) and L cells (lane L, 40 μg) was separated on the same agarose gel, transferred to a hybridization membrane, and hybridized with the ^{32}P-labeled β-NGF cDNA. Size is expressed in kilobases. Exposure times were: lane M, 12 h; lane L, 48 h. (B) Southern blot hybridization of the β-NGF cDNA to genomic DNA cleaved with *Pst*I, *Eco*RI or *Bam*HI. 20 μg digested DNA from submaxillary gland (M) or L cells (L) were subjected to electrophoresis, blotted and hybridized with the radioactive probe. Sizes are expressed in kilobases. (C) Dot-blot analysis of β-NGF mRNA. Total RNA was extracted from L cells cultured in 5% horse serum (HS), serum-free medium (SF) or the same medium supplemented with 10^{-7} M T3 (T3). Samples containing identical amounts of poly(A)-rich RNA were diluted serially, immobilized on a membrane, and hybridized with the radioactive β-NGF cDNA. Numbers represent the amounts of total RNA (μg) extracted from cells cultured with horse serum, and immobilized to the membrane. (D) Size fractionation of the L cell β-NGF mRNA. Total RNA was extracted from L cells culture in 5% horse serum (HS) or serum-free medium (SF). Samples containing identical amounts of poly(A)-rich RNA were fractionated, blotted and hybridized as in A.

cDNA probe. The radioautograms presented in fig.1B show that the various restriction fragments of L cell DNA and of submaxillary gland DNA that hybridized with the cDNA probe were very similar in size.

3.2. *Effect of serum on the NGF transcript in L cells*

RNA was extracted from mitomycin-treated L cells maintained for 4 days in 5% horse serum, or from similarly treated L cells cultured for the same period of time in serum-free medium. The relative amounts of poly(A)-containing RNA were first estimated in these samples by hybridization with [^{3}H]poly(U). The results from 4 independent experiments showed that the amount of total polyadenylated RNA per cell decreased by a factor of 1.7 when cells were cultured in the absence of serum. An additional, specific effect of the serum on the level of the β-NGF transcript was also observed. Identical amounts of polyadenylated RNA of either source were immobilized as dots on hybridization membranes, and were hybridized with the ^{32}P-labeled cDNA probe. The radioactivity associated with each spot in the radioautograms (see fig.1C) was estimated by densitometric scanning. The data summarized in table 1 indicate that the β-NGF transcripts were 4-times less abundant in cells cultured without serum. To verify further that the observed effect was associated with the β-NGF transcript, the RNAs from cells grown with or without serum were fractionated on agarose gels. Hybridizations showed that the β-NGF RNA was specifically diminished when cells were grown

Table 1

Levels of β-NGF transcript in L cells

Culture conditions	β-NGF RNA (relative units)
Serum-free	1
5% horse serum	4.22
10% foetal calf serum	3.8
Serum-free + 10^{-7} M T3	1.61
Serum-free + 10^{-7} M T4	1.5
Serum-free + 10^{-7} M testosterone	0.93

Dot blots performed as described (fig.1C) were subjected to densitometric analysis. A value of 1 was attributed to the relative level found in serum-free medium for each serial dilution point. Data are the mean of 6 experiments with horse serum (SD = ± 1.87), 4 with T3 (SD = ± 0.37), 3 with testosterone (SD = ± 0.17) and 1 determination for the others

in the absence of serum while the size of the transcript was unaffected (fig.1D).

3.3. *Effects of T3, T4 and testosterone*

RNA was extracted from L cells cultured for 4 days in serum-free medium supplemented with or without T3, T4 or testosterone (10^{-7} M each). Dot blots containing identical amounts of poly(A)-rich RNA were prepared and analysed as above. The results presented in table 1 (see also fig.1C) show that both T3 and T4 increased the relative amount of the β-NGF mRNA by a factor of 1.5–1.6, while testosterone had no effect.

4. DISCUSSION

The β-NGF mRNA produced by L cells has an M_r comparable to that synthesized in the male mouse submaxillary gland. Moreover, targets of restriction enzymes *Eco*RI, *Bam*HI and *Pst*I were not significantly rearranged in the genomic β-NGF locus. These 2 results provide strong evidence that the structure of the gene and of its transcript are very similar in either system. This would suggest that the precursor proteins translated from either RNA could be identical.

The results also showed that the cellular content of β-NGF RNA may be modulated by culture conditions. Withdrawal of serum had a double effect. First, it reduced the overall level of polyadenylated RNA by a factor of about 1.7. Furthermore, it reduced specifically the pool of the β-NGF transcripts by an additional factor of 4. It seems therefore that some factor(s) present in the serum increase the rate of transcription of the β-NGF gene or stabilize its mRNA.

We have investigated whether the presence of thyroid hormones and testosterone influenced the steady-state level of the β-NGF mRNA. T3 or T4 was added to the serum-free medium at 10^{-7} M, which is thought to keep the nuclear hormone receptors saturated [24]. Both compounds enhanced the concentration of the β-NGF transcript by a factor of 1.5. T4 was reported to increase the β-NGF content in the submaxillary gland and in the brain [12,25,26]. Our results with L cells suggest that thyroid hormones may exert a direct effect at some pretranslational level to regulate the β-NGF production in these organs.

In contrast, the pool of β-NGF mRNA of L cells was not affected by testosterone, in spite of the existence of functional androgen receptors in this strain [27]. Testosterone is known to stimulate the biosynthesis of the factor in the mouse submaxillary gland, and it is clearly established that the male gland contains more β-NGF mRNA than the female gland [3,17]. However, the concentrations of the β-NGF transcript found in some other peripheral organs, such as the heart atrium and ventricle, appear similar in animals of either sex [3]. The absence of any regulatory effect of testosterone on the β-NGF mRNA pool in L cells, which were initially derived from connective tissue [28], may reflect a general situation existing in several mouse tissues other than the submaxillary gland.

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