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DECREASED MICROCOCCAL NUCLEASE SENSITIVITY OF NUCLEI FROM NERVE GROWTH FACTOR-TREATED PC12 CELLS

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Summary: Nuclei from nerve growth factor-treated PC12 cells are more resistant to digestion with micrococcal nuclease than are nuclei from control cells. The production of oligosomal fragments is decreased, as is the generation of Mg^{2+}-soluble products. One interpretation of the data is that differentiation of these cells due to treatment with nerve growth factor involves a decrease in the total number of DNA sequences transcribed.

Nerve growth factor is a protein that is required for the survival and development of the sympathetic and sensory nervous systems (1, 2). A great deal of information has been obtained about its chemical nature, and about the specific actions it exerts on its target cells (3-5). Nevertheless, the exact mechanism(s) by which it works remains largely unknown.

It is clear that some of its actions require transcription. It is known, for example, that a number of enzymes are induced by nerve growth factor (6-9), and in many of these cases it has been shown formally that the synthesis of new RNA is required for the induction to occur. Indeed, even the most classic action of nerve growth factor, that of promoting neurite outgrowth, has been shown to require the synthesis of RNA (10).

Consistent with these observations of changes in nuclear function are the early findings in other laboratories that the DNA of nerve growth factor-treated neurons is more electron dense than that of controls (11), that nerve growth factor increases the size of the nucleoli (12), and that nerve growth factor antiserum induces cytotoxic changes in the nucleolus (13). More recent studies from this laboratory indicate that the phosphorylative metabolism of

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certain nuclear proteins in target cells is changed by nerve growth factor action (14), and changes in the activities of the RNA polymerases also occur (15). Shooter and his colleagues (16) have demonstrated that treatment of PC12 cells with nerve growth factor leads to a rapid and substantial increase in the cellular content of both RNA and protein. It seems reasonable then, that the nerve growth factor-induced changes in nuclear function might be accompanied by changes in the structure of the DNA.

Accordingly, we have done experiments using a nuclease probe to inquire into the state of the DNA in nerve growth factor-treated cells. We have used PC12 cells, a widely accepted model for the study of nerve growth factor action and for the investigation of neuronal differentiation (17). Our data indicate that nerve growth factor-induced differentiation in this cell system is accompanied by a overall decrease in the nuclease sensitivity of the DNA.

Materials and Methods: PC12 cells were maintained as monolayers in 150 cm^2 tissue culture flasks. They were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 7% horse serum, and 100 µg of streptomycin and 100 units of penicillin per ml. They were kept at 37°C in an atmosphere enriched in CO_2. Under these conditions the cells exhibited a generation time of between 48 and 72 hours. Accordingly, they were split in a 1-to-4 or 1-to-6 ratio each week and the medium changed once between splits. The cells were treated with nerve growth factor (18) (50 ng per ml) for 24 hours, usually on the day after the weekly change of medium.

Nuclei were prepared as previously described (14). Briefly, the medium was removed and the cells were detached by shaking with 0.32 M sucrose containing 3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium phosphate, pH 6.5 (Buffer A). The cells were collected by centrifugation at 7,000xg for 10 minutes. The cell pellets were rinsed thoroughly with Buffer A and then homogenized in an all-glass Potter-Elvehjem homogenizer. The homogenates were centrifuged at 750xg for 10 minutes and the pellets resuspended in Buffer A containing 0.2% Triton X-100. The centrifugation was repeated and the pellets suspended once again in Buffer A. The suspensions were layered on 3.9 ml discontinuous sucrose gradients composed of 1.3 ml portions of 0.8, 1.2, and 2.4 M sucrose, all brought to the ionic composition of Buffer A, and centrifuged at 58,400xg for 1 hour. The medium was then removed and the pellets frozen at -70°C until needed.

The nuclei were resuspended in water and incubated in 5 mM $MgSO_4$ and 25 mM Tris-HCl, pH 7.0, at 37°C in a final volume of 100 µl. Micrococcal nuclease (Worthington Biochemical Corp.) was added and the incubation continued for the times indicated in the Figures. Incubations for analysis on polyacrylamide gels were terminated by the addition of 10 µl of 0.2 M EDTA. Incubations for the determination of Mg^{2+}-soluble digestion products were terminated as described by Perry and Chalkley (19) by the addition of 1.1 ml of ice-cold 5 mM EGTA, pH 7.0. These latter samples were cooled to 0°C, and centrifuged at 5000xg for 10 minutes. The supernatant portions were read at 260 nm in a spectrophotometer.

Polyacrylamide gel electrophoresis was carried out on 5% gels in a buffer containing 0.1 M Tris, pH 8.0, 80 mM boric acid, and 1 mM EDTA. The samples were diluted 1:1 with a mixture of bromphenol blue in 25% glycerol and 75% sample buffer. The gels were run at 50 ma per gel. After electrophoresis the gels were stained for 15 minutes with ethidium bromide, 1 µg per ml, in water, rinsed with water, and photographed under ultraviolet light.

Results: Micrococcal nuclease digestion of nuclei from both control and nerve growth factor-treated cells resulted in the production of Mg^{2+}-soluble products. The nuclei from nerve growth factor-treated cells were digested less readily than the controls (Fig. 1). The decrease in digestion amounted to about 20 to 30% during the first one to two minutes.

This decreased digestion was also seen when the digested nuclei were analyzed on polyacrylamide gels (Fig. 2). The appearance of oligomers was greater in the control nuclei than in the nuclei from nerve growth factor-treated cells. In other experiments, not presented here, the digested nuclei

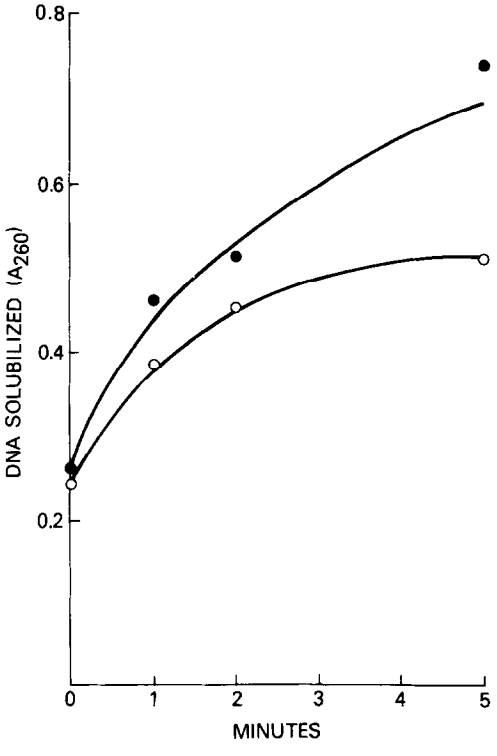

Figure 1. Micrococcal nuclease digestion of nuclei from control and nerve growth factor-treated cells to Mg^{2+}-soluble products. Nuclei from control (•) (1.5 A_{260} units) and from nerve growth factor-treated cells (o) (1.625 A_{260} units) were digested for various periods of time with 2 units of micrococcal nuclease. Incubations were stopped with 1.1 ml of ice-cold 5 mM EGTA, pH 7.0, and assayed as described in Materials and Methods.

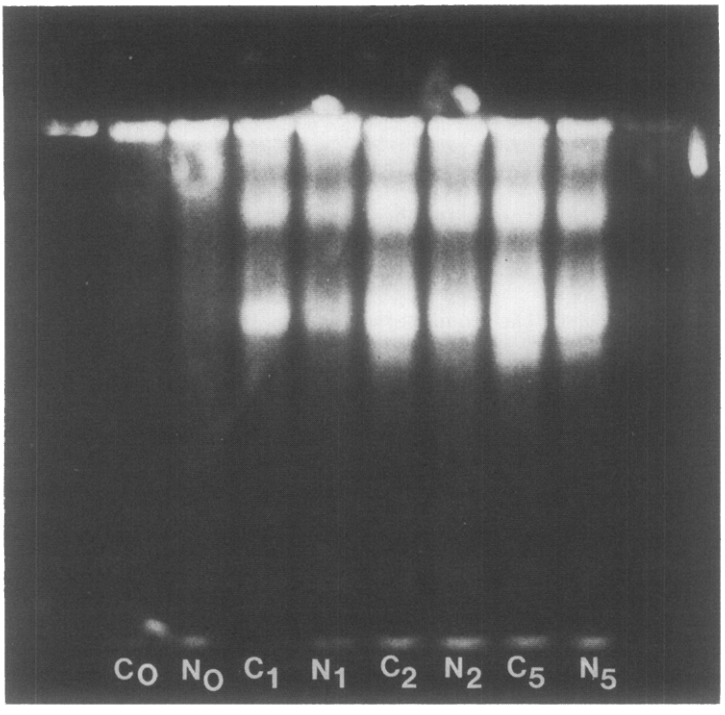

Figure 2. Micrococcal nuclease digestion of nuclei from control and nerve growth factor-treated cells analyzed by polyacrylamide gel electrophoresis. Nuclei from control (5.67 A_{260} units) and from nerve growth factor-treated cells (8.62 A_{260} units) were digested for various periods of time with 20 units of micrococcal nuclease. Incubations were stopped with 10 µl of 0.2 M EDTA and analyzed as described in Materials and Methods. (A) Polyacrylamide gel: Co, control-0 minutes; N_0, nerve growth factor-treated-0 minutes; C_1, control-1 minute; N_1, nerve growth factor-treated-1 minute; C_2, control-2 minutes; N_2, nerve growth factor-treated-2 minutes; C_5, control-5 minutes; N_5, nerve growth factor-treated-5 minutes.

were treated with proteinase K and the DNA precipitated with alcohol before analysis on the polyacrylamide gels. Under these conditions there was substantial trimming and the bands were not as sharp as those in Figure 2, but the gels show that the DNA is digested to mononucleosomes under these conditions and confirm that the digestion is indeed faster in nuclei from untreated cells.

Discussion: The structural basis for the decreased nuclease sensitivity of nuclei from nerve growth factor-treated cells is unknown. That structural alterations in the DNA occur is, however, not surprising. Early observations indicated (11) that changes in the DNA occurred in neurons treated with nerve growth factor. These changes led, within 4 hours, to a DNA structure with

greater electron density. Experiments not presented here have shown that the decreased nuclease sensitivity in PC12 cells is also apparent within 4 hours after nerve growth factor treatment. Our previous studies show that changes in the phosphorylation state of certain nuclear proteins occur within the same time frame (14). Such changes in phosphorylation could contribute to or even underlie the structural changes in the DNA. The structural changes could, in turn, be responsible for the long-term changes in gene transcription which follow nerve growth factor treatment of the cells (6-10).

Micrococcal nuclease preferentially attacks the internucleosome spacer regions of the DNA. The present data indicate, then, that nerve growth factor treatment causes an alteration in the structure of the nucleus or of the DNA itself such that these regions are less susceptible to nuclease attack. Some investigators have used micrococcal nuclease digestion to evaluate or even dissect out those sequences undergoing transcription (20, 21). Thus, one interpretation of the present data is that upon nerve growth factor treatment, which, in PC12 cells, leads to a differentiation closely resembling the changes seen in normal neurons, more sequences become silent than become active. Recently, however, the ability of micrococcal nuclease to distinguish between active and inactive chromatin has been reevaluated (22). In any case, our preliminary experiments suggest that, under the same conditions, DNase I, which clearly shows a preference for actively transcribed sequences (23-25), also digests control nuclei faster than nuclei from nerve growth factor-treated cells.

The PC12 cell system is an interesting one since there are many effectors, some of which, such as epidermal growth factor, have quite different actions on the cell than does nerve growth factor (26). The present observations will enable a detailed study of the actions of the several effectors on the structure of the DNA, and possibly to an interpretation of their actions on a structural basis.

Perhaps more significantly, the present observation provides a new window through which the transcriptionally-based actions of nerve growth factor can

be viewed. It may be that the exact structural basis of this alteration can be ascertained and that the reason for the alteration can be pinpointed, perhaps in the changes seen in nuclear proteins. For example, it might be possible to see if addition of specific classes of proteins from the nuclei of nerve growth factor-treated cells, caused the DNA of control cells to become nuclease-resistant. Alternatively, one could determine what proteins occur in the rapidly digested fractions of the DNA. Finally, using appropriate probes, it should be possible to determine if the structural alterations seen here change the nuclease sensitivity of specific genes.

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