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Transposition of DNase hypersensitive chromatin to the nuclear periphery coincides temporally with nerve growth factor-induced up-regulation of gene expression in PC12 cells

(DNase hypersensitive chromatin/snRNPs/nuclear topology)

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ABSTRACT **To test the hypothesis that the nonrandom organization of the contents of interphase nuclei represents a compartmentalization of function, we examined the relative, spatial relationship of small nuclear ribonucleoproteins (snRNPs) and of DNase I hypersensitive chromatin (DHC) in rat pheochromocytoma cells. In controls, DHC and snRNPs colocalized as pan-nuclear speckles. During nerve growth factor-induced differentiation, both snRNPs and DHC migrated to the nuclear periphery with the migration of DHC preceding that of snRNPs, resulting in their transient separation. The formation of DHC shells temporally coincided with an up-regulation of neurofilament light chain mRNA. This indicates that the expression of this sequence may be associated with its spatial transposition to the nuclear periphery.**

The molecular factors controlling RNA synthesis, processing, and transport occupy distinct, subnuclear regions in interphase nuclei. This has led to the concept that nuclei are topologically organized and that one level of control of gene expression is exerted by spatially coupling transcription and RNA processing (1–5). The scope of investigation of processes that control gene expression has thus expanded beyond molecular mechanisms to include transcription-associated changes in the supramolecular topology of nuclei. This concept of functional, nuclear compartmentalization is supported by the finding that factors involved in transcript processing, including small nuclear ribonucleoproteins (snRNPs) and the non-snRNP factor SC35, form discrete foci in nuclei, known as speckles (6–9). Also, specific chromosomes and specific domains thereof, including centromeric DNA, transcribed sequences as well as aggregates of RNA processing factors, are arranged in distinct, spatial relationships (1, 10–13). Although transcription occurs throughout nuclei in some cell types (14, 15), other evidence suggests that compartmentalization also applies to sites of transcription, represented by distinct, pan-nuclear foci of DNase I hypersensitive chromatin (DHC), a distribution that can be modulated by changes in cell function (16–22). Although DHC structurally represents all nucleosome-free DNA, regardless of transcriptional state, it does include transcriptionally active sequences (23, 24).

DHC (16, 18–22) and such nonexpressed sequences as centromeric DNA (25–28) and snRNP foci (8) change position under conditions that change gene expression. This indicates that changes in nuclear compartmentalization and gene expression may be functionally linked. We now report that differentiation of rat pheochromocytoma (PC12) cells is associated with a temporally out-of-phase transposition of DHC and snRNPs to the nuclear periphery, and that associated changes in gene expression temporally coincide with the transposition of DHC, but not with that of snRNPs.

MATERIALS AND METHODS

Cell Culture. PC12 cells were seeded on glass coverslips coated with rat tail collagen (type VII; Sigma), and maintained in a medium consisting of 85% MEM with Hanks' salt solution containing L-glutamine, 10% horse serum, 5% fetal bovine serum, 50 unit/ml penicillin and 50 μg/ml streptomycin (all from GIBCO). Three days after seeding, cultures to be differentiated were exposed to medium containing nerve growth factor (NGF, 7s, 100 ng/ml, Collaborative Research) for 2, 4, 8, and 24 h, and 5 days or 10 days, respectively. For each experimental condition, a duplicate culture was maintained without NGF to serve as a control. A minimum of three cultures were examined for each condition.

***In Situ* Nick Translation and Immunocytochemistry.** For double labeling of DHC and snRNPs, cultures exposed to NGF, and their respective controls, were fixed [1 h; 4% (wt/vol) paraformaldehyde in PBS] and permeabilized (0.5% Triton X-100, 45 min). Cultures were rinsed in nick translation buffer (50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/10 μg/ml BSA), then nick translated (40 μM dNTPs/40 μM bio-16-dUTP/10 unit/ml DNase I/10 unit/ml DNA polymerase I in nick translation buffer; 1 h). Following successive washes in 20 mM EDTA and PBS, cultures were incubated in 4% BSA to reduce nonspecific binding of antibodies. Nick translated DNA and snRNPs were labeled by anti-biotin antibody (Boehringer Mannheim, 1:100 dilution in 4% BSA; 1 h) and anti-Sm autoimmune serum (ANA reference serum no. 5, Center for Disease Control, Atlanta, 1:100 dilution in 4% BSA; 1 h), respectively, and visualized by fluorescein isothiocyanate-conjugated and tetramethylrhodamine B isothiocyanate-conjugated secondary antibodies, respectively. Signals were recorded by a Zeiss LSM410 laser scanning confocal microscope.

Quantitative Fluorescence *in Situ* Hybridization. To assess the changes in gene expression associated with NGF-induced differentiation, relative levels of neurofilament light chain (NF-L) transcripts were quantitatively examined by fluorescence *in situ* hybridization.

Biotinylated probes were prepared by nick translation (Boehringer Mannheim) of plasmids containing NF-L cDNA (29). Specificity of the labeled probes was confirmed by hybridization to dot blots of unlabeled NF-L cDNA (positive control), of lambda DNA and human Cot1 DNA (negative controls), on a single membrane. In all fluorescence *in situ* hybridization experiments probe from a single labeling reac-

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Abbreviations: DHC, DNase I hypersensitive chromatin; NGF, nerve growth factor; snRNP, small nuclear ribonucleoproteins; PC12 cells, rat pheochromocytoma cells; NF-L, neurofilament light chain.

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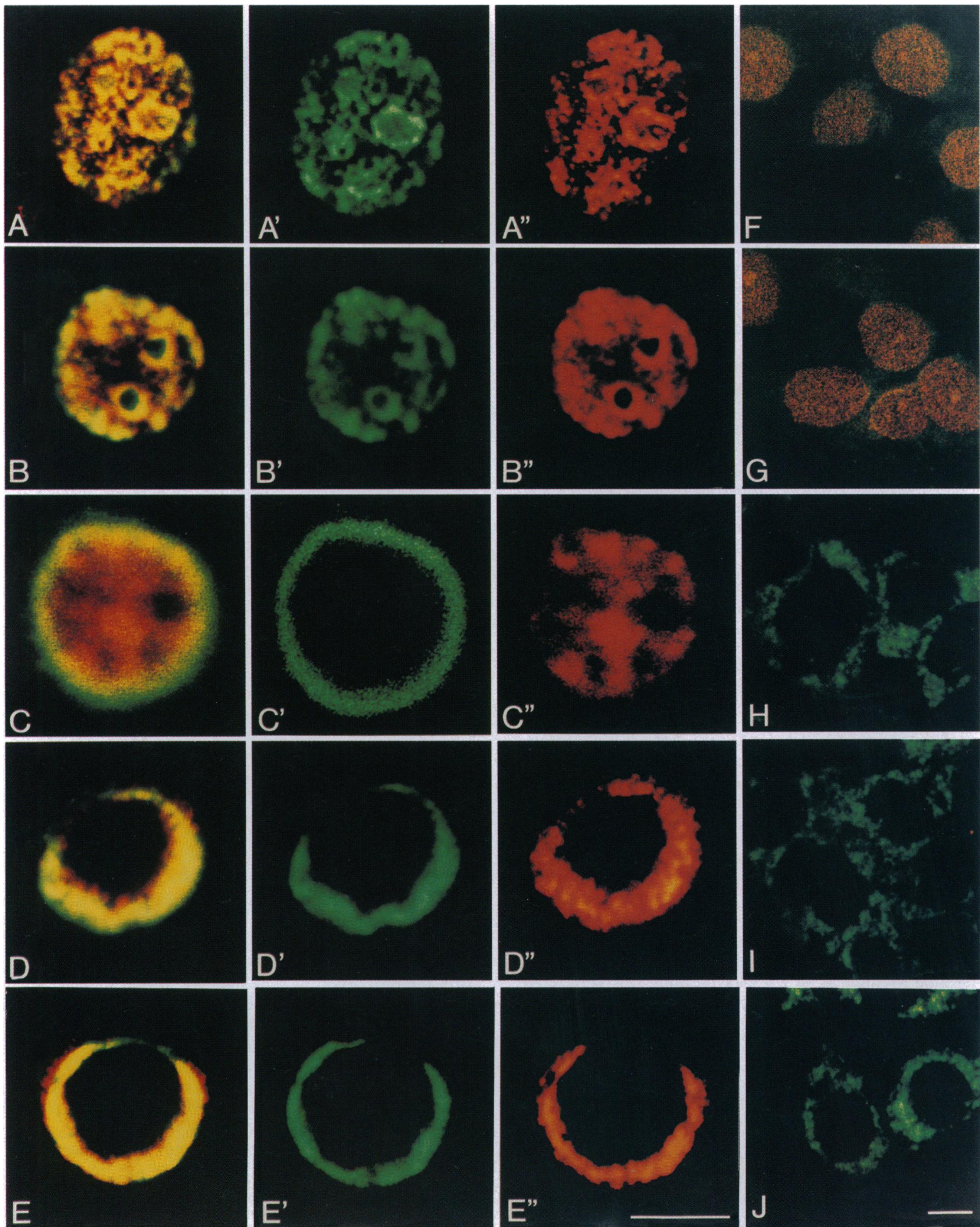

FIG. 1. Optical confocal sections showing NGF-induced changes in distribution of DHC and snRNPs in nuclei and changes in cytoplasmic levels of expression of NF-L. Merged images (*A–E*, yellow indicates colocalization) and corresponding unmerged images of nuclei labeled for DHC (green, *A′–E′*) and snRNPs (red, *A″–E″*), for controls (*A–A″*), and for 4, 8, 24, and 240 h post-NGF (*B–E″*), respectively. DHC forms peripheral shells at 8 h post-NGF (*C′*) that become progressively defined at later times (*D′* and *E′*). snRNPs disperse from speckles (*A″*) into a diffuse pan-nuclear pattern (*B″* and *C″*) and only later colocalize with DHC at the nuclear periphery (*D* and *E*). Fluorescence *in situ* hybridization of NF-L transcripts (green, *F–J*) at corresponding times shows low cytoplasmic levels in controls (*F*) and at 4 h post-NGF (*G*), up-regulation at 8 h post-NGF (*H*) and subsequent persistence (*I* and *J*). Nuclei in *F* and *G* are counterstained with ethidium bromide to show presence of cells. (Bar = 5 μm.)

tion was used. This ensured that hybridization occurred with probes of uniform nucleotide incorporation and concentration.

Cultures were fixed (as above), digested in HCl (0.1 M, 15 min) and permeabilized in Triton X-100 (as above). After washes in PBS and in protease buffer (50 mM Tris·HCl, pH 7.6/5 mM EDTA) cells were pretreated in proteinase K (10 μg/ml in protease buffer, 20 min) and acetylated to reduce nonspecific binding of probe. Two hundred nanograms of probe in 20 μl was applied to each culture and incubated (42°C, 16 h). Cultures were then washed (50% formamide, 2× SSC, 45°C, 3 × 10 min) and hybridization visualized by immunodetection of the biotinylated probe, as above. Fluorescence of NF-L transcripts was recorded by confocal laser microscopy, using identical microscope settings and laser intensity. Fluorescence was quantified by measuring an identical area in the cytoplasm of randomly chosen cells of each culture. Background fluorescence, measured in negative controls (probe omitted, 48 cells), was subtracted from each measurement and results expressed in arbitrary, relative units.

RESULTS

In PC12 cells, NGF induces a neuron-like phenotype and changes the expression of several early and late genes (30, 31). We have shown that NGF-induced differentiation is accompanied by a redistribution of snRNPs from a pan-nuclear distribution as speckles to a snRNP shell apposed to the inner nuclear membrane (8). To test whether this spatial reorganization results in altered spatial relationships between DHC and snRNP foci, their spatial distributions were detected by *in situ* nick translation and immunocytochemistry, respectively. In both naive and in fully differentiated cells, DHC and snRNPs colocalized extensively. In 97% of naive cells (n = 344), DHC and snRNPs colocalized as speckles throughout the nucleus (Fig. 1*A*). In contrast, in NGF-treated (240 h) cultures, 84% of differentiated cells (n = 249) displayed DHC and snRNP signals that colocalized at the nuclear periphery. In single optical sections, this was evident as an annulus, but represents a shell in three dimensions (8). No DHC remained detectable in the nuclear interior (Fig. 1*E*). In all cells, areas of labeled DHC were always smaller than associated snRNP areas. Morphometric analysis of 11 cells showed that 91% ± 3% (SEM) of the area occupied by DHC overlapped with snRNP-labeled areas. In contrast, a wide variability was observed in the extent of overlap of snRNPs with DHC. While the majority of differentiated cells showed near total overlap (Fig. 1 *D* and *E*), a subpopulation retained a diffuse distribution of snRNPs. This resulted in a distribution with a wide variability in that, on average, 67% ± 4% of the total snRNP positive areas were not overlapped by DHC. These proportions did not differ between control cells and NGF-treated cells.

To determine whether DHC and snRNPs remain associated during their transposition to the nuclear periphery, we temporally resolved their respective distributions at 2, 4, 8, and 24 h, post-NGF (Fig. 1 *A–E*). The fraction of cells exhibiting peripheral shells of DHC increased sigmoidally and rapidly within a narrow time window from 16.6% ± 10.1% (SEM) at 4 h to 79.3% ± 6.6% at 8 h post-NGF (Fig. 2). DHC was usually observed as clusters throughout the nucleus and prominently associated with nucleoli in naive cells and at 2 h and at 4 h post-NGF (Fig. 1). At 8 h post-NGF, the majority of cells exhibited a peripheral shell of DHC, indicating an apparent coalescence of DHC clusters (Fig. 1). In contrast to DHC, which persisted as clusters until their coalescence at the nuclear periphery, the snRNP speckles of naive cells dissociated into a pan-nuclear, diffuse distribution as early as 2 h post-NGF, most prominent at 8 h post-NGF (Figs. 1 and 3). This was associated with a concomitant, significant decrease in the mean number of snRNP foci from 30.5 ± 1.2 per nucleus

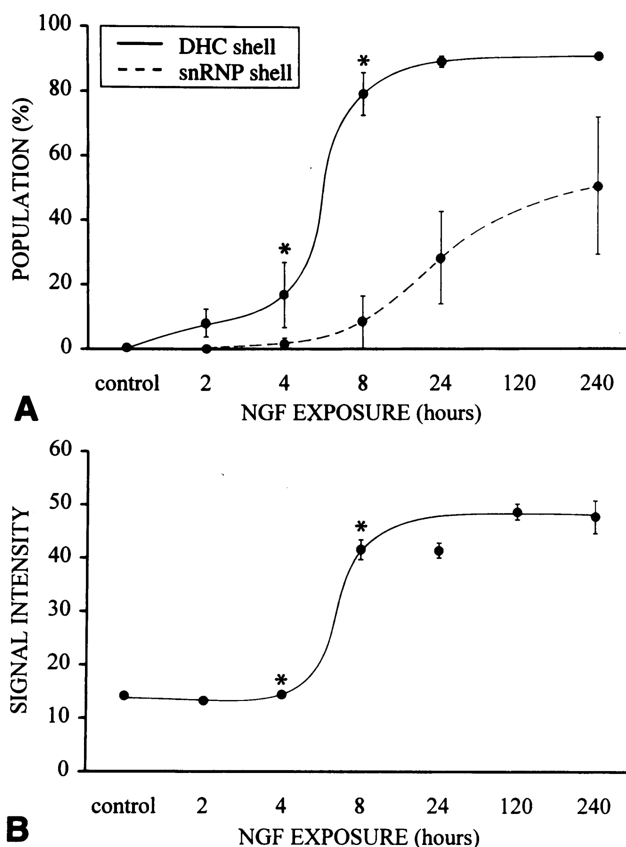

FIG. 2. NGF induces redistribution of DHC and snRNPs and expression of NF-L (mean ± SEM). (*A*) The fraction of cells that exhibit a peripheral shell of DHC and of snRNPs increases with time of exposure to NGF. Note sigmoidal, rapid increase of fraction that exhibits DHC shells (—). Redistribution of snRNPs to nuclear periphery occurs later and more gradually (- - -). (*B*) NF-L transcript levels as a function of time of NGF exposure. Sigmoidal increase temporally correlates with and mirrors that of redistribution of DHC, with closely similar inflection points at 6 h. Asterisks denote significantly different, successive pairs [$P < 0.05$, n = three experiments, 12 cultures, 3677 cells (*A*), 481 cells (*B*)].

in controls to 21.4 ± 1.9 and 15.5 ± 1.9 at 4 h and 24 h post-NGF respectively. Between 8 h and 24 h post-NGF, an increasing fraction of cells showed a shell of snRNPs at the nuclear periphery similar to that observed for DHC (Fig. 2). The migration of snRNPs occurred later than that of DHC, with the proportion of cells showing snRNP shells gradually increasing up to 50.7% ± 21.4% at 240 h post-NGF. This temporal lag between the transposition of snRNPs and of DHC resulted in their transient, spatial separation, most prominent between 4–8 h post-NGF.

Conflicting interpretations exists whether snRNP speckles represent storage pools or sites where splicing occurs (7, 9, 11, 32–35). To test if those sites that present as snRNP speckles represent functional compartments, and to determine whether it is the association of DHC with snRNP foci that modulates the expression of transcripts, NF-L mRNA was quantitatively assessed by fluorescence *in situ* hybridization. Cytoplasmic fluorescence intensity, specific for this transcript, in randomly chosen cells, increased in response to NGF, as previously shown (36). The temporal pattern of this up-regulation of NF-L (Fig. 1 *F–J*) was in phase with, and mirrored, the sigmoidal time course of formation of DHC shells, with inflection points occurring in both at 6 h post-NGF (Fig. 2). In contrast, no correlation was evident between NF-L up-regulation and formation of snRNP shells.

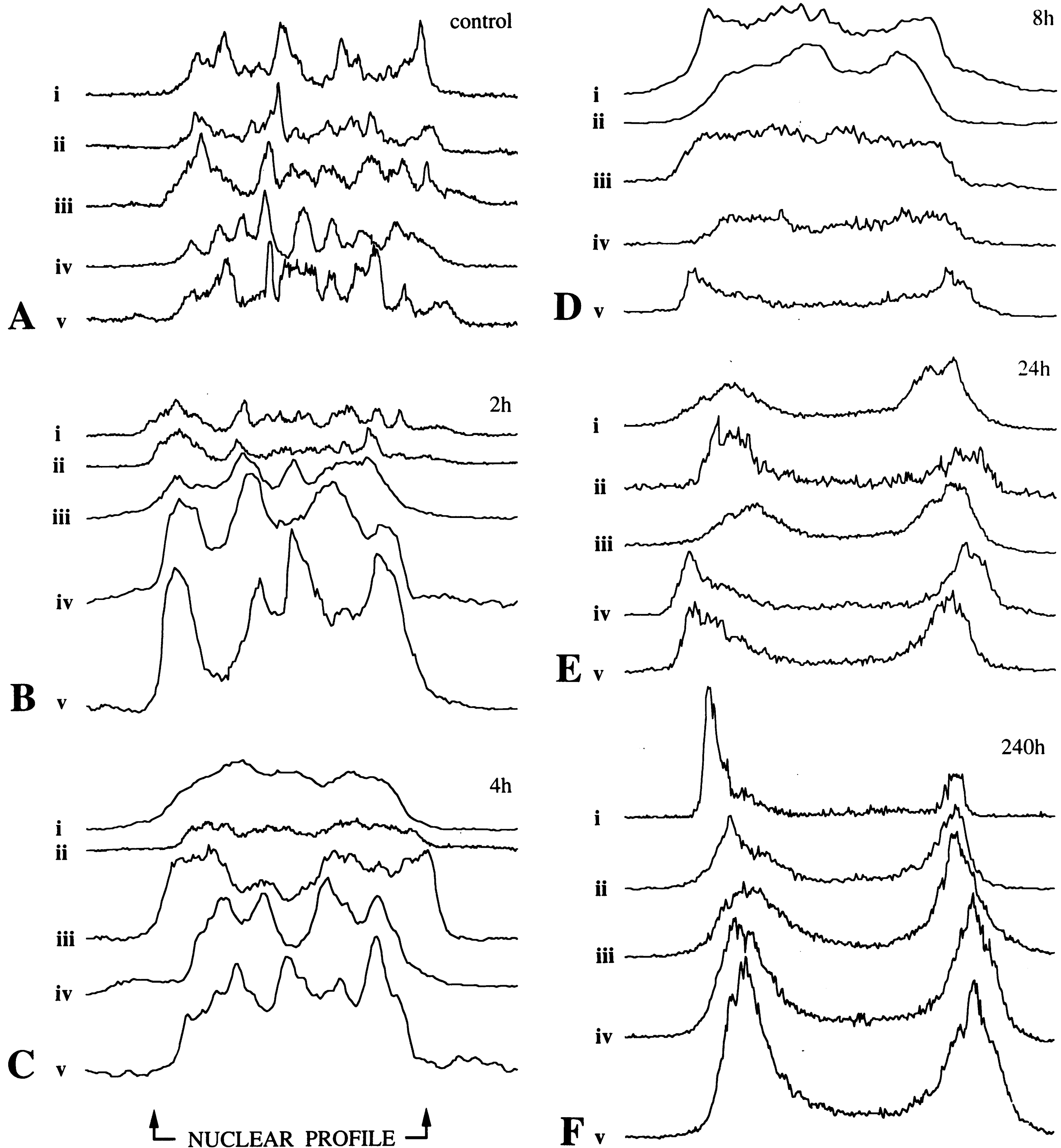

FIG. 3. Profiles of relative fluoresence intensity showing nuclear snRNP distribution as a function of time post-NGF. Each set shows nuclear profiles of five randomly chosen cells (i–v) for controls (*A*) and for 2, 4, 8, 24, and 240 h post-NGF (*B–F*, respectively). Recurrent fluctuations in intensity from near background level occur in controls (*A*), representing a speckled snRNP distribution. With increasing times post-NGF, peaks are less numerous and intensity remains above background (*B* and *C*). At 8 h post-NGF (*D*), snRNPs are diffusely distributed either in a pan-nuclear pattern (i–iv) or near the nuclear periphery (v), the latter increasingly evident at 24 h post-NGF (*E*) when the majority of cells exhibit an accumulation of snRNPs near the nuclear periphery. At 240 h post-NGF (*F*), snRNP fluorescence is intimately and exclusively associated with the nuclear periphery in all cells.

DISCUSSION

Nuclei of several cell types display a topological organization during interphase in which specific chromatin domains occupy distinct, nonrandom spatial positions. This organization is specific for a given cell type and may be associated with the transcriptional state of the cell (25–28). In fact, in nuclei of PC12 cells, a significant spatial repositioning of centromeres occurs in parallel with phenotypic changes during response to NGF (unpublished data) and in neurons regenerating processes *in vitro* (28). The observation that DHC and snRNPs also undergo spatial rearrangements with change in cell func-

tion indicates that the reorganization of the nucleus in response to changes in functional demand may be global and may include the entire genome. While it remains unclear whether spatial reorganization of chromatin is a consequence of or is causal in the activation of genes, the data presented here are consistent with the hypothesis that gene expression is in part regulated by spatially coupling chromatin sequences with functional subnuclear domains (25–28).

It is well documented that transcriptional activation of a gene sequence is mediated in part by its conformational change from a DNase resistant to a DNase-sensitive state (23, 24). In PC12 cells, NGF-induced differentiation is accompanied by an increase in susceptibility to DNase I of several genes, including the NF-L sequence (37). Although all actively transcribed sequences are susceptible to DNase I, correlations between transcriptional activity and sensitivity to DNase digestion, however, are not always observed (38). Given that only a small fraction of a typical eukaryotic genome is actively expressed in a given cell type, it is unlikely that the relatively large fraction of DHC represents transcriptionally active sites exclusively. It is more likely that the observed distribution of DHC represents a phenotype-specific set of exposed, transcriptionally competent sequences, the expression of which can be modulated upon demand, as previously suggested (20).

The work in the present study examined the distribution of total DHC, rather than specific sequences. It must be considered therefore, that the appearance of DHC at a different nuclear location, rather than representing their transposition to the nuclear periphery (S. Fakan, personal communication), may actually represent changes in the conformation of chromatin from DNase accessible to DNase inaccessible of one set of sequences and vice versa. NGF induces changes in the expression of several sequences in PC12 cells (36, 37). It is likely, therefore, that at least a part of the reorganization of DHC is due to activation and deactivation of NGF-responsive sequences. However, the observation that a spectrum exists in the extent of compaction of DHC from a diffuse to an increasingly compacted, narrow band at the nuclear periphery, and that this spectrum changes with increasing time of exposure to NGF (compare Fig. 1 *B′–E′*), indicates that a spatial migration of DHC does occur. Moreover, in contrast to reverse transformed cells that show persistence of DHC in the nuclear interior, including a region surrounding the nucleolus (21, 22, 39), no such persistence of DHC in the nuclear interior was detectable in the cell type employed in the present study. In fact, the interior of nuclei, including those regions surrounding nucleoli and prominent in naive cells (Fig. 1 *A′*), were totally devoid of DHC, following NGF treatment. Given that the NF-L sequence in PC12 cells is sensitive to DNase (37) and given that the majority of DHC is located at the nuclear periphery post-NGF, we suggest that the NF-L sequence is likely to be associated with one of the pan-nuclear DHC speckles in naive cells and is moved to the nuclear periphery during differentiation.

While an association exists between a change in the phenotype of a cell and its organization of DHC (16–22), the functional relevance and the mechanisms involved remains unclear. It is, however, probable that the topological organization of DHC is mediated by its attachment to the nuclear matrix (18). In addition, it has recently been shown that the eukaryotic genome also contains sequences that are distinct from scaffold attachment regions and that specifically mediate binding to nuclear envelope, possibly via lamin (40). It may be speculated, therefore, that interactions between different components of the nuclear matrix may be the basis for the nuclear reorganization observed in this study. The above, together with the observation that several DNA binding proteins are exclusively associated with the nuclear envelope (40–43), supports the hypothesis that interactions between chromatin and the nuclear envelope may function in chromatin condensation (44), gene regulation (16, 45), and nuclear organization (46).

While snRNPs have been shown to exist in speckles in several cell types, the functional implications of such a distribution remains controversial (7, 9, 11, 32–35). In the data presented here, the up-regulation of NF-L occurs at a time when snRNPS are diffusely distributed. RNA processing compartments are thus not limited to snRNP speckles and may also include unresolved sites. The migration of the majority of snRNPs to the nuclear periphery following NGF treatment may thus represent recruitment of snRNPs to sites of DHC. Such a recruitment has previously been documented in other cell types (47) and may involve the dissociation of snRNPs from speckles and their mobilization from a diffusible pool (48–50).

While transcription has been demonstrated to occur throughout the nucleus in some cell types (14, 15), these cells usually exhibit a nucleus with a very flat, prolate, ellipsoid geometry. In these nuclei, all sequences are therefore in a relatively close proximity to the nuclear envelope, a compartment previously assigned a functional role in the regulation of gene expression (45). In association with changes in gene expression, in PC12 cells, NGF also induces a change in nuclear geometry from a prolate ellipsoid to that approximating a sphere (data not shown). We therefore speculate that the formation of a DHC shell might function in the maintenance of a close proximity between actively transcribed sequences and the nuclear envelope. This is supported by the observation of a DHC shell in the spherical, vesicular nuclei of neurons *in vitro* (data not shown) and of a snRNP shell in spherical nuclei of NGF-differentiated PC12 cells (8).

In summary, we conclude that the up-regulation of the NF-L sequence is not associated with the extent of its colocalization with snRNP speckles, but rather, with its position at the nuclear periphery.

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