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Reassembly of C-myc and Relaxation of C-fos Nucleosomes During Differentiation of Human Leukemic (HL-60) Cells

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Human promyelocytic leukemic (HL-60) cells (1) have amplified c-myc protooncogene sequences which lead to an elevated level of c-myc gene expression (2,3). Induction of HL-60 cells by phorbol esters to undergo monocytic differentiation (4) results in the suppression of c-myc (5), but the activation of c-fos gene transcription (6). Chromatin structures of c-myc and c-fos were compared by measuring their sequences in nucleosome-associated DNA fragments. These nucleosomal particles were released from chromatin by micrococcal nuclease digestion and subsequently analyzed with two dimensional gel electrophoresis. C-myc related sequences were detected in nucleosomal DNA fragments of differentiated cells only, while the c-fos related sequences were found in nucleosomal DNAs of noninduced HL-60 cells. Since the enzyme preferentially digests relaxed DNAs, these results suggest that nucleosomal subunits of c-myc and c-fos chromatin are relaxed during the state of active transcription, and reassembled once their transcription is repressed.

In eukaryotes, the physical properties of chromatin structures have been associated with transcriptional activity of genetic elements. Electron microscopic and nuclease digestion studies of actively transcribed genes in eukaryotic chromatin have revealed that a more relaxed and extended configuration occurs in these regions relative to adjacent nontranscribed sequences (7-9). Extensive nucleosome-free regions are seen on active Xenopus ribosomal genes and their spacers (10). Reversible changes in nucleosome structure have been recently postulated in transcriptionally active and inactive states of

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ribosomal DNA chromatin (11). Relaxation of the nucleosome as a required mechanism for gene expression also occurs in the minichromosome structure of SV40 viruses (12,13). When HL-60 is induced to undergo either myeloid or monocytic differentiation, the elevated expression of the c-myc gene is dramatically reduced (5,14,15). In addition, rapid activation of the expression of the c-fos protooncogene is detected when HL-60 is induced by phorbol esters to differentiate into monocytes or macrophages (6). Changes of chromatin structures during differentiation of these cells have also been reported (16,17). Thus HL-60 provides a useful model to study the correlation between changes of the c-myc and c-fos chromatin structures and gene expression during the process of cell differentiation. First, if nucleosome relaxation indeed occurs at the sites of actively transcribed regions of chromatin, c-myc sequences in HL-60 and c-fos sequences in phorbol ester-induced HL-60 would be quickly digested by micrococcal nuclease, since the enzyme preferentially digests the linker DNA and those DNA regions which are free of nucleosome. However, inactive c-fos sequences in noninduced HL-60 would be protected from nuclease digestion. Second, if nucleosome relaxation is reversible after gene expression is suppressed, the c-myc sequences in differentiated cells should be protected from nuclease digestion by the reassembled nucleosome structures. Evidence is presented here to show that these two phenomena do occur in HL-60 cell differentiation.

Methods

Cell culture and phorbol ester-treatment: HL-60 cells were maintained in RPMI-1640 medium supplemented with 15.0% heat-inactivated fetal bovine serum. Cell morphology, density and viability were measured as described previously (24). Phorbol ester, 4-phorbol 12-myristate 13-acetate (PMA) induction was carried out by seeding 2 x 10^5 cells/ml in medium containing 10_8M PMA (Sigma, St. Louis, Mo.) for 6 days. Total of 1 x 10^9 cells each of HL-60 and PMA-induced cells were used for each experiment.

Nuclei preparation and nucleease digestion of chromatin: Noninduced or PMA-induced cells (1×10^9) were lysed in TMB buffer (10.0 mM Tris-HCl, pH 8.0 at 25°; 1.0 mM $MgCl_2$; 5.0 mM butyric acid) containing 1.0 mM phenylmethylsulfonylfluoride (PMSF) (Sigma) and 0.5% (v/v) Nonidet P-40 (Sigma), nuclei were isolated by centrifugation at 10,000 x g for 10 minutes at 4° and resuspended in a low ionic strength buffer (1.0 mM NaHEPES, pH7.5 at 25°). The nuclei preparation was then subjected to digestion with micrococcal nuclease (Sigma) at the concentration of approximately 125 units/600 ug DNA/ml at 37° for 10 minute. Under these conditions, about 10.0% of the total DNA became acid soluble (data not shown).

Separation of nuclease-released nucleosomal particles: The nuclease-released nucleosomal particles were isolated from undigested chromatin fragments by centrifugation at 25,000 x g for 5 minutes, and mono-, di- and oligo-nucleosomal particles were separated by electrophoresis on a 5.0% polyacrylamide tube gel using a low ionic strength buffer system (6.4 mM Tris-acetate, pH 8.0 at 25°; 3.2 mM Na-acetate; 0.32mM EDTA) as described (18).

Protamine-displacement of nucleosomal proteins: The nucleosomal proteins were subsequently displaced from nucleosomal particles in the tube gel by the in situ protamine displacement method and analyzed by a second dimension, 12.0% polyacrylamide gel electrophoresis (PAGE) in acid/urea buffer (5.0% acetic acid; 8.0 M urea) as previously described (18). The proteins were revealed by staining the gel with Coomassie brilliant blue.

Hybridization of nucleosomal DNAs with c-myc probes: The protein-stripped DNA fragments remaining in the tube gel were further analyzed on a second dimension, 9.0% PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) and stained with ethidium bromide as described previously (19). The DNA fragments were transferred to a Nytran filter (Schleicher and Schuell Inc., Keene, NH) electrophoretically as described earlier (25). The Nytran filters were subsequently hybridized (Southern hybridization)(21) with [^{32}P]-plasmid probes containing human c-myc inserts (pHSR-1) (22) and revealed by autoradiography on X-Omat AR films (Kodak, Rochester, NY).

Rehybridization of Nuclosomal DNAs with c-fos probes: Once the autoradiograms were obtained, the hybridized [^{32}P]-c-myc probes were dissociated by boiling the Nytran paper in water for 20 minutes before rehybridization with [^{32}P]-plasmid probes containing human c-fos inserts (pc-fos(human)-1)(23). The completeness of dissociation of original probes was monitored by autoradiography. Labelling of plasmids with [^{32}P] was done by the nick translation procedure (26). The results of rehybridization were again revealed by autoradiography.

Results

Induction of HL-60 cells with PMA for 6 days resulted at least 95% of cells differentiated into macrophages. Electrophoresis of the digested chromatin products in a 5.0% polyacrylamide tube gel resulted in the isolation of monomer,

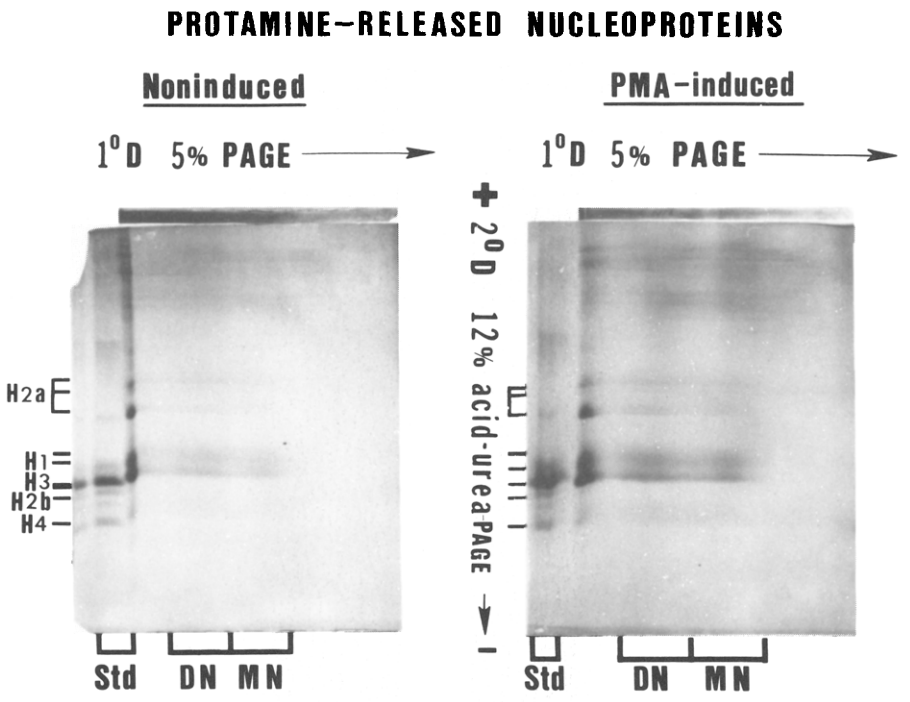

Figure 1. Two-dimensional electrophoresis of nucleosomal proteins isolated from noninduced HL-60 cells (left) and cells induced with PMA for 6 days (right). The first dimensional tube gels from a parallel experiment were layered over the second dimensional slab gels and photographed together. Both gels were stained with Coomassie brilliant blue. Histones were designated according to the migration pattern of a mixture of purified histones run on the left sides of the slab gels (Std). Designation of mononucleosomal (MN) and dinucleosomal (DN) regions was according to the sizes of correlating DNA fragments which were resolved in the second-dimensional DNA gels (see Figure 2).

dimer and oligomer nucleosomal particles as shown in the first dimensional gel pictures of Figure 1. The first dimensional tube gels from a parallel experiment were photographed horizontally on the top of the second dimensional gels. Analysis of the protein components of these nucleosomal particles which were protamine-displaced from their binding sites by electrophoresis in a second dimensional slab gel resulted in the identification of H4 histone, three subtypes of H2a, two subtypes of H1, H2b, H3 histones as well as numerous nonhistones with higher molecular weights as revealed in Figure 1. No significant difference was

found when these protein patterns were compared between noninduced HL-60 and PMA-induced HL-60 cells.

The protein-stripped nucleosomal DNA fragments remaining in the first dimensional tube gel were further characterized using electrophoresis in a second dimension, on a separate 9% SDS-PAGE as described in the "Methods". As expected for nucleosomal DNA composition (20), the major mononucleosomal DNA fragments migrated in the second dimensional gel at the regions of lengths between 140 base pairs (bp) and 200 bp, the dimer DNAs at the regions between 280 bp and 400 bp, and the oligonucleosomal DNAs retained at the top of the gel as revealed in the left panels of Figure 2. No significant difference in the distribution of DNA sizes between noninduced and induced cells was detected.

The two-dimensionally mapped nucleosomal DNA fragments were subsequently transfered to nitrocellulose (NT) paper by electrophoresis in order to detect c-myc and c-fos sequences by Southern hybridization. After hybridization with c-myc probes, the same NT paper was denatured and rehybridized with c-fos probes as described in the "Methods". The autoradiographic patterns of the nucleosomal DNAs hybridized with c-myc and c-fos radioactive probes respectively are shown in the middle and the right panels of Figure 2. Positive hybridization with c-myc sequences was detected in PMA-induced cells as dark exposure spots (middle lower panel) on X-ray film which coincide with nucleosomal DNA fragments revealed by ethidium bromide staining of the gels (left lower panel). No c-myc hybridization was detected in those of noninduced HL-60 cells (middle upper panel). However, positive hybridization with c-fos sequences was detected in the nucleosomal DNA fragments of noninduced cells (right upper panel) but not in those of PMA-induced cells (right lower panel).

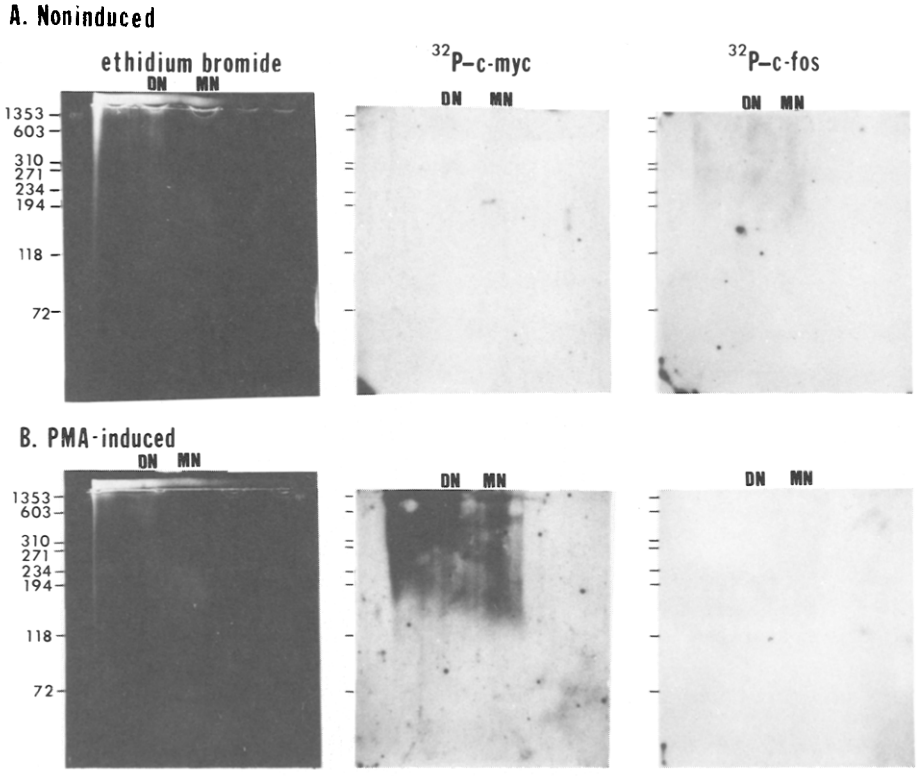

Figure 2. Southern hybridization analysis of nucleosomal DNA sequences resolved by two dimensional gel electrophoresis. The protein-stripped nucleosomal DNA fragments retained in the first dimension, tube gel were further analyzed by electrophoresis on a second dimension, 9% SDS-PAGE and stained with ethidium bromide as shown in the left panels. DNA fragments of mono-(MN) and di-nucleosomal (DN) particles were marked according to the sizes of DNA. The smeared patterns of larger DNAs above the MN and DN DNA regions were due to various amounts of proteins which were still bound to DNA fragments after protamine displacement. The DNA fragments were subsequently blotted to nitrocellulose paper, detected for c-myc and c-fos sequences by Southern hybridization and the radioactivities were detected by autoradiography as described in the "Methods". The middle panels are the hybridization patterns revealed by autoradiography using [^{32}P]-myc probes and the right panels are the patterns using [^{32}P]-fos probes. Sizes (bp) of Hind III-digested Phi-X 174 DNA fragments were also indicated. The top panels (Row A) are samples from noninduced HL-60 cells and the bottom panels (Row B) are from cells induced with PMA for 6 days.

Discussion

The hybridization signals could not be attributed to the random sequences of introns of the oncogenes or the host plasmid

sequences since no detectable hybridization was found either in noninduced HL-60 DNAs hybridized with c-myc probes or PMA induced HL-60 DNAs hybridized with c-fos probes. Although it is unlikely, we could not rule out the possibility that there may be unknown specific sequences in introns which are responsible for these results. The degree of hybridization of PMA-induced nucleosomal DNAs with c-myc probes is much greater than that of the noninduced DNAs with c-fos probes. Since the sizes of c-myc and c-fos inserts of plasmids are almost identical (9 kilo bp), this difference in the levels of hybridization are interpreted to indicate that c-myc sequences are amplified in HL-60 cells (2), whereas c-fos sequences are not (6). These data suggest that inactivation of c-myc gene expression results in the reassembly of nucleosomal particles at the c-myc gene loci, and that activation of c-fos gene expression results in the relaxation of the nucleosomal particles at the c-fos loci after HL-60 are induced with PMA to become macrophages. Whether a reversible mechanism to reassemble nucleosomes at the c-fos loci exists in differentiated cells is not known at this stage. The dynamic activity of the three dimensional chromatin structure at oncogene sites as reported here may facilitate explanation of some known regulatory mechanisms such as the termination of transcript elongation within exon 1 site which may be modulated by the relaxation of fourth DNase I hypersensitive site of c-myc sequence in differentiated HL-60 cells reported by Bentley and Groudine recently (27).

The fact that hybridization could be detected in mononucleosomal DNA fragments may also suggest that to detect nontranscribing gene sequences on nucleosomal DNA fragments may provide an advantage by increasing hybridization sensitivity.

The exons of each of human c-myc and c-fos protooncogenes are approximately 2.2 kilo bp (22,23) which theoretically span through 11 mononucleosomal particles in the inactive state; and more particles could be involved if introns are also considered. Assuming each nucleosomal DNA anneals to one [^{32}P]-gene probe, one can obtain at least an 11-fold increase in hybridization sensitivity when compared to using the purified DNA genome randomly sheared into large fragments in a standard hybridization assay.

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