Gene expression in mouse neuroblastoma cells: Properties of the genome

(Chromatin/differentiation/nonhistone chromosomal proteins/histones/cyclic AMP)

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ABSTRACT  Chromatin was prepared from isolated nuclei of proliferating and differentiated cultures of C1300 mouse neuroblastoma cells. Differentiation was induced by serum withdrawal or treatment with dibutyryl cyclic AMP. The ability to support DNA-dependent RNA synthesis when assayed in a cell-free system is three times greater for chromatin from proliferating cells. Histones isolated from proliferating and differentiated cells were fractionated electrophoretically. The relative amounts of proteins present in the five major histone fractions were similar. In contrast, there were significant differences in the nonhistone chromosomal proteins synthesized and associated with the genome of proliferating and differentiating neuroblastoma cells. Such differences are reflected by modifications in the electrophoretic banding patterns and in incorporation of [3H]tryptophan into various molecular weight classes of nonhistone chromosomal polypeptides. A functional relationship between changes in the nonhistone chromosomal proteins and variations in the transcriptional activity accompanying differentiation of neuroblastoma cells may exist.

For several years mouse neuroblastoma cells have provided an effective model system for studying biochemical and morphological parameters of neuronal differentiation and neoplasia. A variety of methods, including serum withdrawal and treatment with dibutyryl cyclic AMP (1), have been utilized to induce proliferating neuroblastoma cells to differentiate into cells that exhibit morphological (2–8), biochemical (4–7, 9–14), and electrical properties characteristic of neurons (15, 16).

The complex changes that accompany transition from the proliferating to the differentiated state may reflect alterations in the expression of information contained within the genome. One might anticipate modifications, therefore, in the macromolecules that comprise the genome and interact with DNA to regulate its function. Several lines of evidence suggest that the proteins associated with the genome play such a regulatory role in eukaryotic cells (17, 18). Histones have been shown to be involved in the repression of DNA-dependent RNA synthesis (19, 20), maintenance of chromosome structure (21), and cytodifferentiation (22). Nonhistone chromosomal proteins may recognize defined gene loci and thus mediate specificity of gene read-out (18, 23–29). The nonhistone proteins have been shown to direct the tissue specific transcription of globin genes (41–43) and the cell cycle stage-specific transcription of histone genes (44, 45). In the present study we have compared the histones and nonhistone chromosomal proteins associated with the genome in proliferating and differentiated mouse neuroblastoma C1300 cells. The possibility of a functional relationship between chromosomal proteins and differences observed in the transcriptional activity of chromatin from proliferating and differentiated mouse neuroblastoma cells is discussed.

MATERIALS AND METHODS

The neuro 2a line of mouse neuroblastoma C1300 cells was grown in monolayer culture in 1-liter glass Blake flasks in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum in a moist CO2 incubator. To induce differentiation, cells were plated in MEM with 20% fetal calf serum and incubated for 24 hr. The monolayers were then washed with MEM and subsequently maintained for 24 hr in either serum-free MEM or in MEM with 20% fetal calf serum and 1 mM dibutyryl cyclic AMP. Proliferating cultures were treated in a similar manner, but 20% fetal calf serum was present during both 24-hr incubation periods.

Nuclei and chromatin were prepared from mechanically harvested cells at 4°. Details of the procedures have been described (30, 31). Isolated nuclei were free of cytoplasmic contamination when examined by phase contrast microscopy. Little difference was observed in the patterns of proteins fractionated electrophoretically from either nuclei or isolated chromatin when comparisons were made with and without the inclusion of 50 µg/ml of L-1-tosylamide-2-phenyl ethyl chloromethyl-ketone (TPCK) in the isolation and washing media to inhibit proteolysis (32). The histone:nonhistone chromosomal protein ratios in chromatin from proliferating and differentiated cultures were 0.73 and 0.79, respectively.

The ability of chromatin from proliferating and differentiated cells to support RNA synthesis in vitro was determined using Escherichia coli RNA polymerase prepared according to the procedure of Burgess (33). Assay conditions were a modification of those described by Murphy et al. (34). The amount of DNA present in chromatin preparations was determined by Burton's modification of the diphenylamine reaction (35).

Histones were extracted from isolated nuclei with 0.25 M HCl at 4° as described (46) and fractionated electrophoretically on 15% polyacrylamide gels containing 2.5 M urea according to the method of Fanyim and Chalkley (36).

To study the distribution and synthesis of nonhistone chromosomal proteins in proliferating and differentiated mouse neuroblastoma cells, growth medium was removed from the monolayers and replaced with tryptophan-free MEM containing 5 µCi/ml of L-[3H]tryptophan (2.7 Ci/mol) for 60 min prior to harvesting. Chromatin was dissociated by homogenization in 1% sodium dodecyl sulfate, 1%...
2-mercaptoethanol, 0.01 M sodium phosphate, pH 7.0, dialyzed against 0.1% dodecyl sulfate, 0.1% 2-mercaptoethanol, 0.01 M sodium phosphate, and fractionated electrophoretically according to molecular weight on 7.5% polyacrylamide/0.1% dodecyl sulfate gels as described (46).

RESULTS

Twenty-four hours after serum was withdrawn from proliferating cultures, 71% of the neuroblastoma cells were differentiated. Twenty-four hours of treatment with 1 mM dibutyl cyclic AMP induced differentiation in 60% of the cells. In control cultures, where the medium was replaced with MEM containing 20% serum, only 18% of the cells were differentiated. Criteria for identifying cells as differentiated included the possession of one or more processes whose length was at least as great as the diameter of the cell body. When medium containing 20% serum was added to differentiated cultures induced by either serum withdrawal or cyclic AMP, the percentage of differentiated cells decreased to a level equivalent to that in cultures maintained continuously for similar periods of time in the presence of 20% serum, indicating that such differentiation was reversible.

Properties of the genome

Changes in neuroblastoma cells that accompany transition from the proliferating to the differentiated state may reflect modifications in the expression of information contained within the genome. To date, properties of the neuroblastoma genome in the two states have not been directly examined. In the present study the transcriptional activity of chromatin and the proteins associated with the genome in these cells are investigated.

The transcriptional activity of the genome in proliferating and differentiated neuroblastoma cells was determined by assaying the ability of isolated chromatin to serve as a template for DNA-dependent RNA synthesis in a cell-free system. The incorporation of [14C]ATP by E. coli RNA polymerase into acid-insoluble material was measured with the amount of DNA template being rate limiting. It has been previously reported that under such assay conditions chromatin transcription is a reflection of RNA synthesis that occurs in the nucleus of intact cells (38). A 3-fold increase in template activity was observed for chromatin from proliferating cells (645 cpm of ATP per mg of DNA) as compared to chromatin from cells induced to differentiate by serum withdrawal (245 cpm of ATP per mg of DNA). Although AMP residues may be added post-transcriptionally to RNA at the 3'-OH termini by ATP-polynucleotidyl exotransferases, such enzymes are inactive in the system used for chromatin transcription in these studies.

Although examination of the nuclei by phase contrast microscopy did not reveal the presence of visible cytoplasmic material, the possible contamination of chromatin by cytoplasmic proteins during the isolation procedure was excluded by mixing the cytoplasmic fraction prepared from 107 cells labeled for 30 min with [3H]leucine (5 μCi/ml) prior to harvesting with nuclei isolated from 107 unlabelled cells. The resulting chromatin preparations from proliferating and differentiated cells contained less than 0.6% of the counts in the added radioactive cytoplasmic fraction.

Histones were extracted with 0.25 M HCl from the nuclei of proliferating mouse neuroblastoma cells and of such cells induced to differentiate by serum withdrawal. These basic chromosomal polypeptides were then fractionated electrophoretically according to charge and molecular weight on polyacrylamide gels containing 0.9 M acetic acid and 2.5 M urea. The electrophoretic profiles are shown in Fig. 1. It is evident that the five principal histone fractions are represented in the nuclei of proliferating and differentiated neuroblastoma cells and that their electrophoretic mobilities do not differ significantly as a function of differentiation. The absence of variations in the histones of proliferating and differentiated neuroblastoma cells is further supported by the data in Table 1, which indicate that the relative amounts of protein present in each of the five histone fractions are similar.

In contrast to histones, variations in the composition and metabolism of nonhistone chromosomal proteins are evident.
Fig. 2. Dodecyl sulfate/polyacrylamide electrophoretic fractionation of chromosomal proteins from proliferating (a) and differentiated (b) neuroblastoma cells. Differentiation was induced by serum withdrawal. The incorporation of $[^{3}H]$tryptophan into each gel fraction was determined. Molecular weight estimations are based on the migration of proteins of known molecular weight which migrate linearly in proportion to the log molecular weight (cytochrome c, 12,400; horse myoglobin, 17,200; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase a, 94,000). Within the protein concentration ranges electrophoresed on these gels, the amount of dye bound is directly proportional to the concentration of protein, as determined by the method of Lowry et al. (37).

In proliferating and differentiated neuroblastoma cells, cells were labeled with L-$[^{3}H]$tryptophan, and the chromosomal proteins from isolated chromatin were fractionated electrophoretically according to molecular weight on dodecyl sulfate/polyacrylamide gels. Since histones do not contain tryptophan residues, incorporation of radioactivity reflects solely nonhistone chromosomal protein synthesis. Fig. 2 illustrates the electrophoretic profiles of total chromosomal polypeptides and the incorporation of radioactivity into various molecular weight fractions of nonhistone chromosomal proteins.
Table 1. Relative amounts of histone fractions in nuclei from proliferating and differentiated (grown without serum) mouse neuroblastoma cells

<table>
<thead>
<tr>
<th>Histone fractions</th>
<th>F1</th>
<th>F3</th>
<th>F2b</th>
<th>F2a2</th>
<th>F2a1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferating cells</td>
<td>14.6</td>
<td>29.2</td>
<td>21.0</td>
<td>16.2</td>
<td>15.8</td>
</tr>
<tr>
<td>Differentiated cells</td>
<td>14.0</td>
<td>26.6</td>
<td>19.4</td>
<td>15.3</td>
<td>18.1</td>
</tr>
</tbody>
</table>

Gels were stained with Amido Black, destained electrophoretically, and scanned at 600 nm. Relative amounts of protein in each histone fraction were determined by integrating areas under each peak of the gel scan and are expressed as percent of total protein.

for proliferating neuroblastoma cells (Fig. 2a) and for such cells induced to differentiate by serum withdrawal (Fig. 2b). Peaks P, Q, and R represent histone fractions F2b + F3, F2a2, and F2a1. The F1 histone fraction migrates anomalously in the 37,000 molecular weight region of the gels (39) (present in K). The relative amounts of protein in each region of the gels are similar for proliferating and differentiated cells (Table 2). However, a comparison of Fig. 2a and b indicates variations in specific fractions. Peak A, which consists of nonhistone chromosomal polypeptides that migrate in the 120,000 to 150,000 molecular weight region of these gels, is more pronounced in differentiated cells than in proliferating cells. Variations are also evident in the E complex, which contains nonhistone chromosomal polypeptides of molecular weight between 64,000 and 78,000. In the 120,000 to 145,000 molecular weight region of the gel, a three-fold increase in the incorporation of [3H]tryptophan is evident in the nonhistone chromosomal proteins which are synthesized and associated with the genome of proliferating cells (Fig. 2). A corresponding increase in the specific activity of these polypeptides is indicated in Table 2. Although there is an apparent 2-fold increase in the specific activity of peak K in proliferating cells, the presence of F1 histone in this peak makes it impossible to assign a specific activity to the nonhistone chromosomal protein component.

To eliminate the possibility that the modifications observed in nonhistone chromosomal protein metabolism are artifacts of serum withdrawal and to further establish that these modifications reflect changes in the genome that are associated with differentiation, proliferating neuroblastoma cells were induced to differentiate by treatment with 1 mM dibutyryl cAMP for 24 hr. Fig. 3 clearly indicates that, consistent with the decreased incorporation of [3H]tryptophan into the 120,000 to 140,000-dalton nonhistone chromosomal proteins which are synthesized and associated with the genome of mouse neuroblastoma cells when differentiation is induced by serum withdrawal, a similar decreased rate of synthesis of these proteins is apparent when differentiation is induced by dibutyryl cAMP.

DISCUSSION

The present studies demonstrate that modifications in the composition and transcriptional properties of the genome in mouse neuroblastoma C1300 cells are associated with the transition from the proliferating to the differentiated state. Chromatin from proliferating cells exhibited a 3-fold greater template activity for DNA-dependent RNA synthesis in a cell-free system than chromatin from differentiated cells. Numerous reports of alterations at the morphological and biochemical levels suggest that differentiation of neuroblastoma cells may involve specific changes in the expression of information contained within the genome (2, 4–6, 14). Al-

### Table 2. Relative amounts and incorporation of [3H]tryptophan in nonhistone chromosomal protein fractions of proliferating and differentiated (grown without serum) mouse neuroblastoma cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction and MW \times 10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-B</td>
</tr>
<tr>
<td>Proliferating cells</td>
<td>98.2–145.0</td>
</tr>
<tr>
<td>Relative amount</td>
<td>18.17</td>
</tr>
<tr>
<td>Specific activity</td>
<td>16.2</td>
</tr>
<tr>
<td>Differentiated cells</td>
<td>17.87</td>
</tr>
<tr>
<td>Relative amount</td>
<td>6.6</td>
</tr>
<tr>
<td>Specific activity</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Total chromosomal proteins from cells labeled with [3H]tryptophan were fractionated electrophoretically on dodecyl sulfate/polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue, destained electrophoretically, and scanned at 600 nm. Relative amounts of protein in each fraction were determined by integrating the area under the scan and are expressed as percent of total protein. Specific activities of each molecular weight (MW) region were calculated by dividing total [3H] cpm by the area under that region of the scan. Histones migrate in regions K and P-R, and therefore, specific activities for nonhistone proteins cannot be reliably assigned to these fractions.
though a variety of studies utilizing cell hybridization (40) and inhibitors of nucleic acid and protein synthesis have attempted to establish the site of control (1, 3, 8, 9), the results of these studies taken together are inconclusive. The present study represents the first direct evidence for differences at the level of the genome in proliferating and differentiated neuroblastoma cells. Transcriptional differences, however, do not rule out the possibility that regulation may also reside at post-transcriptional levels in the processing of mRNA precursors or in the utilization of mRNA as a template for protein synthesis.

Chromosomal proteins in proliferating and differentiated neuroblastoma cells were compared since these proteins have been implicated in the regulation of transcription. Differences in the five classes of histones were not observed. Variation, however, in post-translational modification of histone polypeptides (such as acetylation, methylation, and phosphorylation) may exist and are currently being investigated. In contrast, differences in the synthesis of nonhistone chromosomal proteins that are associated with the genome were observed. Such differences are particularly pronounced in the polypeptides that migrate in the 120,000 to 140,000 molecular weight region of dodecyl sulfate/polyacrylamide gels.

Several lines of evidence suggest that nonhistone chromosomal proteins play a key role in the regulation of the gene read-out (18, 23–29, 41–45). One can speculate, therefore, that a functional relationship may exist between the observed changes in the synthesis of these macromolecules and the variations in the transcriptional activity of the genome that accompany the transition of mouse neuroblastoma cells from the proliferating to the differentiated state.

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