Elevated expression of c-myc and N-myc produces distinct changes in nuclear fine structure and chromatin organization

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The proto-oncogenes c-myc and N-myc encode nuclear phosphoproteins with unknown function. Here, c-myc or N-myc, or hybrid constructs of the two, were transfected into fibroblastic cells (CV-1) using SV40-based high expression vectors. The cells were studied by indirect immunofluorescence microscopy and transmission electron microscopy to determine the localization of the two myc proteins within the nuclei and their influence on nuclear fine structure and chromatin organization. In c-myc transfected cells the overproduced protein product accumulated in large amorphous globules that displaced the normal chromatin and did not stain for DNA. In N-myc transfected cells condensed chromatin loops were formed. They were attached to the nuclear envelope and by traction in the latter they may have contributed to give the nucleus its irregular shape in these cells. During mitosis the chromatin loops persisted as clearly identifiable entities within the chromosomes, suggesting a rigid conformation that did not allow normal chromosome packaging. These findings suggest that the c-myc and N-myc proteins bind to different structures and may have different functions. Observations on cells transfected with hybrid constructs indicated that both the second and third exon of c-myc were required to yield a product that behaved like the c-myc protein. In contrast, domains encoded by the second exon of N-myc were sufficient to give rise to a product that morphologically behaved like the N-myc protein.

Introduction

The myc family of proto-oncogenes comprises five or more functional members (De Pinho et al., 1987). The best characterized of these, c-myc, is the cellular homologue of the v-myc oncogene carried by the MC29 virus. The latter is unique among the retroviral oncogenes in its ability to transform epithelial, fibroblastic, as well as hematopoietic cells (Cory, 1986). Also c-myc appears to be involved in the genesis and progression of a wide variety of tumors, usually after activation by retroviral insertion (Cory, 1986), chromosomal translocation (Klein & Klein, 1985), or gene amplification (Altalala & Schwab, 1986). The c-myc product is a nuclear phosphoprotein with a short half-life and with a high affinity for DNA and RNA (Donner et al., 1982; Persson et al., 1986; Spector et al., 1987). Although its precise function is unknown, there is strong evidence that it plays an important role in the regulation of cell proliferation.

For example, increased levels of c-myc mRNA were observed in lymphocytes and 3T3 cells in connection with the transition from a quiescent to a proliferating state (Kelly et al., 1983). Moreover, upon microinjection into 3T3 cells, the c-myc protein was found to cooperate with platelet-poor plasma in the induction of DNA synthesis, suggesting that it may act as a competence factor (Kaczmarek et al., 1985).

The second best characterized member of the family, the N-myc gene, was identified because of its partial homology to c-myc (De Pinho et al., 1987). Amplification of N-myc has been detected in human neuroblastomas, retinoblastomas, and small-cell lung carcinomas, often in a prognosis-related fashion (Schwab et al., 1983; Kohl et al., 1983, 1984; Brodeur et al., 1984; Nau et al., 1986). Like the c-myc product, the N-myc product is a nuclear phosphoprotein with a rapid turnover. Its expression is restricted to a limited range of tissues, and it was recently found that N-myc and c-myc are differentially regulated during the transition of murine embryonal carcinoma cells from a quiescent to a proliferating state (Sejersen et al., 1987). Both c-myc and N-myc are able to cooperate with activated ras genes in transforming primary rat embryo fibroblasts (Schwab et al., 1985; Yancopoulos et al., 1985).

Here, we have transfected c-myc, N-myc, or their hybrid constructs into CV-1 cells using SV40-based high expression vectors. The cells were studied by indirect immunofluorescence microscopy, staining with Hoechst dye, and transmission electron microscopy to determine the cellular localization of the c-myc and N-myc proteins and examine their influence on nuclear fine structure and chromatin organization. The findings indicate that these two closely related oncogene products are handled differently in the same cell system. Overexpression of c-myc led to the appearance in the nucleus of large globular structures which stained for the c-myc protein, but not for DNA. In contrast, the nuclei of N-myc transfected cells contained loop-like structures that were positive for both the N-myc protein and DNA. Hybrid constructs of the two genes gave rise to condensed chromatin regions of the same type as in N-myc transfected cells.

Results

Immunofluorescence microscopy

Following transfection with pSVEpR4P6, a plasmid construct that contained the two coding exons of the human c-myc gene, the CV-1 cells produced large amounts of the c-myc protein as demonstrated by metabolic labeling and immunoprecipitation in combination
with SDS-PAGE and fluorography (Classon et al., 1987). Indirect immunofluorescence microscopy indicated that the c-myc protein was concentrated in one or several large globules within the nucleus (Figure 1a–b).

As judged by the lack of staining with the Hoechst dye (Figure 1c), these structures did not contain appreciable amounts of DNA.

CV-1 cells transfected with pSVEpR4NHB, a plasmid construct that contained the two coding exons of the mouse N-myc gene, expressed the N-myc protein at a high level as demonstrated by immunoblotting (data not shown). Immunofluorescence staining with an antiserum against the N-myc protein revealed a complex pattern with thin strands of reactive material within the nucleus (Figure 2a–b). This pattern partly overlapped with that obtained for DNA using the Hoechst dye (Figure 2c).

The nucleoli did not show any c-myc or N-myc specific staining. This agrees with the lack of myc specific nucleolar staining in MC29 virus-transformed and non-transformed quail cells (Wingqvist et al., 1984). We found no myc specific staining in the cytoplasm of the transfected cells, in spite of the high nuclear expression of both proteins (Figures 1–2).

Nuclear fine structure and chromat in organization

CV-1 cells were transfected with c-myc, N-myc, or their hybrids and examined by transmission electron microscopy for changes in nuclear fine structure and chromat in organization. Cells from mock-transfected cultures and cultures transfected with the vector alone were structurally alike. They had a rounded nucleus surrounded by an envelope with numerous pore complexes. The nucleus contained one or more large nucleoli with a characteristic morphology, including pale-staining, fibrillar, and granular components. The rest of the nucleoplasm was composed of a smaller fraction of condensed chromatin (heterochromatin), mainly associated with the nuclear envelope, and a larger fraction of less condensed chromatin (euchromatin). Both types of chromatin had a diffuse, partly fibrillar, partly granular structure. In mitotic cells, all chromatin was packed into highly condensed metaphase chromosomes (Hancock & Boulkas, 1982). Except for the specific changes described below, cells transfected with c-myc, N-myc, or their hybrids retained a nuclear and metaphase chromosome structure as outlined above (Figures 3–6).

In the c-myc transfected cells large inclusion bodies appeared in the nucleus. They were distinct from the nucleoli, although often closely associated with them (Figure 3a–b), and displaced the normal chromatin towards the nuclear envelope (Figure 3c–e). They were formed by the aggregation of small spots of dense material with an amorphous or finely granular structure (Figure 3a–b). When fully developed, they were typically divided into three zones: a central zone of low density, without structural detail; an intermediate zone of higher density, composed of a complex network of strands with a globular substructure; and an outer zone of low density, containing dispersed small globules or spots of denser material (Figure 3c–e). Only few mitotic cells were found in these cultures and none of them were observed to contain any inclusion bodies of the type described above.

Transfection with N-myc produced nuclear changes of a different type (Figure 4). The overall rounded shape of the nucleus was retained but deep, tube-like or flattened invaginations were frequently observed on the surface. Spots or strings of condensed material appeared in the nucleoplasm. They were closely associated with the nucleoli and the nuclear envelope, and formed twisted fibers of varying width and large loop-like structures (Figure 4a–b). At higher magnification, these
entities could be resolved into 10-30nm diameter subunits intermixed with a fine, poorly defined material (Figure 4c). In mitotic cells, the chromatin was not normally packed and highly condensed material with a morphology similar to that described above remained associated with the metaphase chromosomes (Figure 4d–e). There was an increased number of dividing cells, suggesting either an increased mitotic rate or a prolongation of mitosis.

Transfection with the c-myc and N-myc hybrids gave different results depending on the order of the exons (see methods). pSVC/N induced nuclear changes only in few cells and there were no large inclusion bodies as in c-myc transfected cells (cf. Figure 3). Some cells contained smaller amounts of amorphous material of low or medium density (Figure 5a–b). In addition, highly condensed spots or strands of the same type as in the N-myc transfected cells (cf. Figure 4) were present both during interphase (Figure 5b–c) and mitosis (Figure 5d). More prominent effects were obtained with pSVN/C. The nuclear surface showed deep invaginations (Figure 6a) and long twisted fibers or loops of condensed material, closely associated with the nuclear envelope, were found in the nucleoplasm (Figure 6a–b), as in the N-myc transfected cells (cf. Figure 4). These structures were retained during mitosis and appeared as appendages on the metaphase chromosomes (Figure 6c).

Discussion

The contribution of illegitimately activated or amplified c-myc or N-myc genes to tumorigenesis and tumor progression is believed to be mediated by their respective protein products. Both genes code for nuclear phosphoproteins of similar molecular weight and with an amino acid sequence homology of 30–35% (Kohl et al. 1986; De Pinho et al., 1986; Stanton et al., 1986). We have recently found that human lymphoma cells with a low c-myc expression are nonpermissive for replication of SV40 DNA, but can be made permissive by cotransfection with a vector including c-myc (Classon et al., 1987). N-myc produces a similar effect in the same assay system (unpublished observations).

Here, we have studied the nuclear fine structure and chromatin organization of cells that were made to
express c-myc or N-myc after transfection with appropriate vectors. In c-myc transfected cells large amorphous globules accumulated within the nucleus. They were separate from the nucleoli, stained for the c-myc protein in indirect immunofluorescence microscopy, but did not stain for DNA with the Hoechst dye. It is suggested that these structures represent a storage compartment for the overproduced c-myc protein. Recent observations on the nuclear localization of v-myc and c-myc proteins in MC29-infected quail cells and human adenocarcinoma cells with an amplified c-myc gene revealed a staining pattern of a more finely speckled type (Spector et al., 1987). In this investigation, the v-myc and c-myc proteins were also reported to colocalize with small nuclear ribonucleoprotein particles (Spector et al., 1987).

Surprisingly, the N-myc transfected cells showed an entirely different picture from their c-myc transfected counterparts. Immunofluorescence for N-myc revealed a complex reticular pattern that overlapped with the staining for DNA. At the electron microscopic level, this corresponded to strands and loops of highly condensed chromatin that were attached to the nuclear envelope (or the underlying nuclear lamina). By pulling in the latter, they may have contributed to the irregular shape of the nucleus in these cells, with deep and narrow indentations. The condensed chromatin regions persisted in an essentially unaltered form during mitosis and appeared as interspersed strands or appendages on the mitotic chromosomes. This suggests a rigid conformation that opposed the packaging forces associated with normal chromosome condensation.

Computer analysis of the predicted amino acid sequences of c-myc and N-myc has revealed highly conserved clusters of amino acids, two in the 5' portion of the coding domain in exon 2 and two additional ones in the distal coding domain of exon 3 (Nau et al., 1985; De Pinho et al., 1986). The carboxyl terminus of both proteins contains an abundance of basic amino acids that could account for the affiliation of the protein with the chromatin (Persson & Leder, 1984). Our observations on cells transfected with chimeric constructs of c-myc and N-myc indicated that the protein segment encoded by the second exon of N-myc was sufficient to make the final product to associate with the chromatin in the same way as the N-myc protein. At least partly, also the
Figure 5. Nuclear fine structure in cells transfected with pSVC/N (interphase and mitosis). (a, b) Small nuclear inclusions (I) with amorphous structure, resembling the inclusions seen in c-myc transfected cells. (b, c) Nuclear spots and strings of highly condensed chromatin (S), similar in appearance to the structures seen in N-myc transfected cells. (d) Portion of a mitotic chromosome (C) with spots of highly condensed chromatin (S). Bars represent 0.5 μm. (a, b) 42,000 x, (c) 54,000 x, (d) 67,000 x.

Figure 6. Nuclear fine structure in cells transfected with pSVN/C (interphase and mitosis). (a, b) Nuclei with deep invaginations and strings of highly condensed chromatin (S), similar in appearance to the structures seen in N-myc transfected cells. This material is in many places closely associated with the nuclear envelope. Arrowheads mark nuclear pore complexes. (e) Portion of a mitotic chromosome (C) with attached strands of highly condensed chromatin (S). Bars represent 0.5 μm. (a) 33,000 x, (b) 79,000 x, (e) 25,000 x.
third exon of N-myc encoded sequences that made the protein product to behave in this way. In the case of c-myc, protein sequences encoded by both the second and third exon were evidently required to give a product that behaved as the c-myc protein.

In conclusion, this study demonstrates that overexpression of c-myc and N-myc give rise to distinctly different changes in nuclear fine structure and chromatin organization. In c-myc transfected cells the overproduced protein product accumulated in large amorphous globules that displaced the normal chromatin. In N-myc transfected cells condensed chromatin loops were formed. During interphase these structures were attached to the nuclear envelope. During mitosis they persisted as clearly identifiable entities within the chromosomes. These findings suggest that the c-myc and N-myc proteins bind to different structures and may therefore have different functions.

Materials and methods

Cell culture and transfections

Monkey CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Transfections were performed by a modification of the DEAE dextran method (McCUTCHEON & Pagano, 1968). Briefly, cells were detached by trypsinization and seeded in 90-mm plastic petri dishes at a density of 3 x 10^4 cells/cm^2. After 24 h, the cultures were washed once in TS buffer (140 mM NaCl, 25 mM Tris, 5 mM KCl, 0.5 mM NaHPO_4, 1 mM MgCl_2, 1 mM CaCl_2, pH 7.5) and once in PBS (Dulbecco’s phosphate-buffered saline; pH 7.3). The appropriate plasmid DNA was dissolved in TS buffer with 0.5 mg/ml -1 DEAE Dextran (5000 kD) at a concentration of 3.3 μg/ml and 1.5 μl was added to each petri dish. After 60 min at 37°C, the DNA-DEAE dextran mixture was withdrawn and 2 ml 20% glycerol in TS buffer was added for 2 min. The cells were then washed twice with TS buffer, exposed to 100 μg chloroquine for 5-7 h, and finally incubated in normal medium for 48 h at 37°C.

Plasmid constructions

The vector pSVEpR4 contains the SV40 early region that expresses both small and large T, the SV40 control region, and part of the pBR3 plasmid. It also has a XhoI site for insertion of foreign genes that will be expressed from the SV40 late promoter sequences (Hammarskjöld et al., 1986). The construct pSVEpR4P5 contains a 3.8 kb Smal-EcoRI fragment encoding the two coding exons from a human c-myc clone (CLASSON et al., 1987) and pSVEpR4NH a 4.5 kb HindIII-BamHl fragment from a mouse N-myc clone encoding the second and third exons of the gene (De Pinho et al., 1986). pSVc/N and pSVN/C are chimeric constructs between c-myc and N-myc; pSVc/N contains the second exon of the c-myc gene and the third exon of the N-myc gene and pSVN/C the second exon of the N-myc gene and the third exon of the c-myc gene, both constructs under control of the SV40 early promoter (Koskinen et al., manuscript in preparation).

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 3% formaldehyde in PBS, permeabilized with 0.2% NP-40, and rinsed with PBS. They were then exposed to rabbit antisera against the v-myc (Altitalo et al., 1983) or the N-myc protein (diluted 1:50 to 1:100 in PBS) for 30 min, rinsed with PBS, and exposed to fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (diluted 1:20 in PBS; Dakopatts) for 30 min (all at 20°C). After rinsing in PBS, the coverslips were mounted in 40% glycerol and examined in a Leitz microscope equipped with epifluorescent illumination. For staining of DNA, the Schiff reaction (0.5 μg/ml) was added for 5 min before the final rinsing and mounting.

Electron microscopy

The cultures were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) containing 0.05 M sucrose. After 2 h, the cells were scraped off the petri dishes and transferred to plastic tubes. They were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) containing 0.5% potassium ferrocyanate for 1 h at 4°C, dehydrated in ethanol, stained with 2% uranyl acetate in ethanol, and embedded in low-viscosity epoxy resin. Thin sections were cut on an LKB Ultratome IV, stained with lead citrate at alkaline pH, and examined in a Philips EM 300 electron microscope at 80 kV.

Acknowledgements

The authors thank Barbro Ehlin-Henriksson and Karin Blomgren for expert technical assistance. Antiserum against v-myc and N-myc were kindly provided by Kari Altitalo, University of Helsinki, Helsinki, Finland. Financial support was obtained from the Swedish Cancer Society, the Swedish Medical Research Council, NIH (5R01 CA14054-14), and Bristol-Myers. Janos Sümegi and Sigurdur Ingvarsson are recipients of fellowships from the Cancer Research Institute and from the Concern Foundation for Cancer Research.

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