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N-myc and c-src Genes Are Differentially Regulated in PCC7 Embryonal Carcinoma Cells Undergoing Neuronal Differentiation

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We examined the expression of N-myc, c-myc, and c-src in four embryonic carcinoma (EC) cell lines during different states of cell growth and following induction of in vitro differentiation. N-myc mRNA was detected in undifferentiated cells of four EC cell lines (PCC7, PCC3, PCC4, F9) neither of which showed N-myc gene amplification. No N-myc transcripts could be detected in mRNA prepared from a murine neuroblastoma cell line and from a murine fibroblast line. The level of N-myc mRNA decreased by 85% when PCC7 EC cells were induced by retinoic acid and cAMP treatment to form nerve-like cells. Six days after induction, the PCC7 cells changed into aggregates of neurofilament positive cells with massive neurite outgrowths. At this stage DNA replication had been reduced by more than 95%.The decreased N-myc expression in induced PCC7 cells was parallelled by 300-500% increase in c-src expression. Slowing of cell multiplication by serum starvation, on the other hand, did not affect the level of N-myc or c-src mRNA levels in PCC7 cells. C-myc was expressed in all EC lines except PCC7, which surprisingly did not express c-myc even at an exponential rate of proliferation. Chemical induction of F9 EC cells to form visceral endoderm or parietal endoderm resulted in markedly reduced (85%) levels of N-myc transcripts. A similar decline in c-myc expression was found in differentiated F9 cells. No c-src transcripts were detected in proliferating or differentiated F9 cells. These results suggest that N-myc may be expressed not only in neural development, but also in very early, undetermined embryonic cells. The activation of c-src expression when PCC7 EC cells differentiate into nerve-like cells shows that the pattern of proto-oncogene expression may change during a differentiation process, some proto-oncogenes increasing, others decreasing their representation in the mRNA pool.

Proto-oncogenes (c-onc), cellular sequences homologous to the transforming retroviral oncogenes (v-onc), may be of key importance in the control of cell proliferation and differentiation (Bishop, 1983). Recently a human gene denoted N-myc was identified and shown to be distantly related to the proto-oncogene c-myc (Schwab et al., 1984). N-myc is amplified and highly expressed in several human neuroblastomas and retinoblastomas (Kohl et al., 1983; Lee et al., 1984; Schwab et al., 1984; Kohl et al., 1984). A rapid decline in N-myc expression subsequent to retinoic acid (RA) induction of differentiation was recently reported in a human neuroblastoma cell line (Thiele et al., 1985). Based on these findings, it has been suggested that the expression of N-myc is limited to undifferentiated cells originating from neuroectoderm (Schwab et al., 1984). Another proto-oncogene, c-src, is also expressed in neural tissue. The $pp60^{c\text{-}src}$ product of the c-src gene has been found at high levels and in a stage- and cell type-specific fashion in chicken brain and retina (Cotton and Brugge, 1983; Sorge et al., 1984). The present study was undertaken in order to examine the developmental regulation of three proto-oncogenes implicated to be of importance for differentiation processes. To this end we examined the steady state levels of N-myc, c-myc and c-src mRNA in four embryonal carcinoma (EC) cell lines during different states of cell growth and following in vitro induced differentiation. The EC cell lines analysed were derived from the transplantable teratocarcinoma line OTT6050 (F9, PCC3, and PCC4) and from a spontaneous teratocarcinoma (PCC7). Teratocarcinomas are malignant tumors characterized by the presence of embryonal carcinoma (EC) cells as well as a variety of differentiated cell types originating from the EC cells (Kleinsmith and Pierce, 1964). The EC cells are the malignant stem cells of these tumors and they show remarkable similarities to undetermined cells of the early embryo (Martin, 1980). Some EC cell lines can form derivatives of all three germ layers as well as

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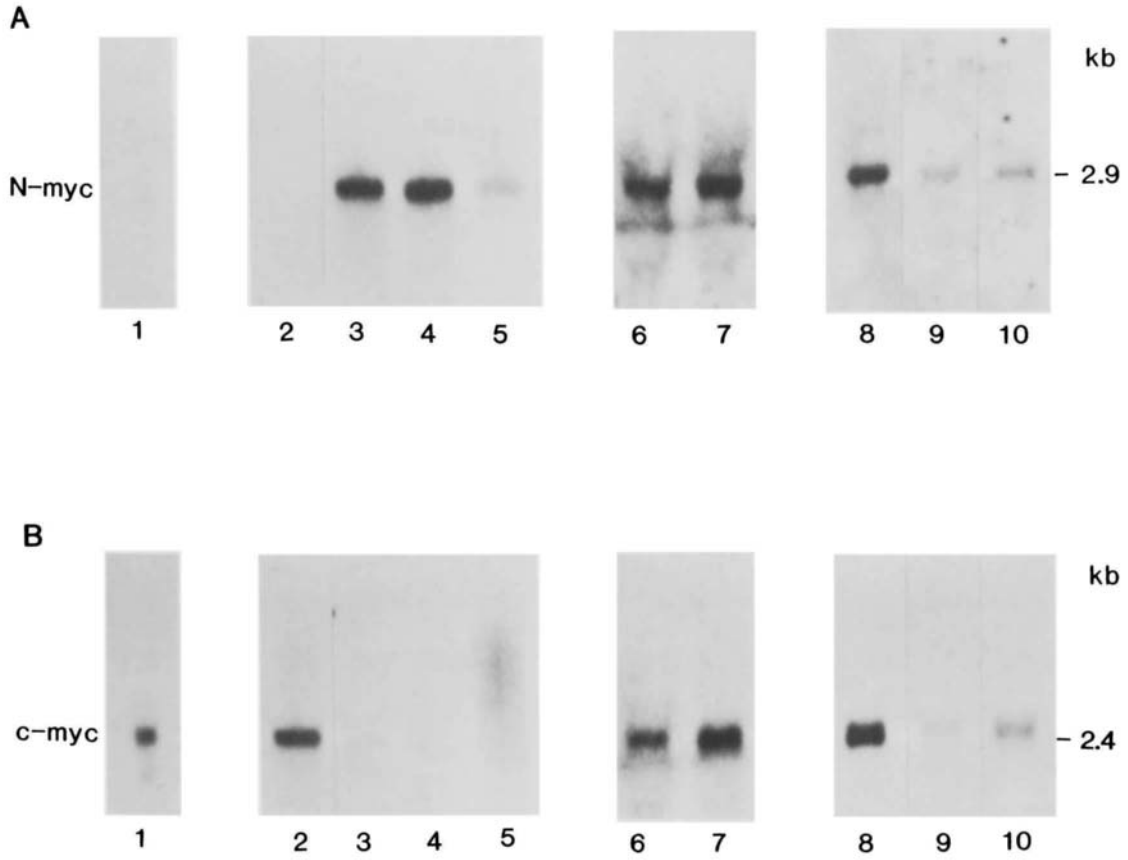

Fig. 1. A) Northern blots illustrating N-myc transcripts in murine embryonal carcinoma cell lines during different states of differentiation and proliferation. Lane 1: 15 μg of Balb/c 3T3 poly(A) + RNA; lanes 2–5: 10 μg of poly(A) + RNA from, respectively, NA, exponentially proliferating PCC7, serum-starved PCC7, and PCC7 nerve cells; lane 6: 40 μg of total PCC4 RNA; lane 7: 40 μg of total PCC3 RNA; lanes 8–10: 10 μg of poly(A) + RNA from, respectively, exponentially proliferating, F9 EC cells, F9 parietal endoderm, and F9 visceral endoderm. B) The same blots as in A were hybridized with a c-myc probe. Lanes 1–10 are the same as in A. C) Detection of transcripts homologous to v-src, actin and histone H2A in RNA from NA and PCC7 cells. The blot represented in A and B as lanes 2–5 was stripped from probe and rehybridized as detailed in Material and Methods. D) The blot represented in A and B as lanes 8–10 were stripped and rehybridized to detect expression of α-fetoprotein, histone H2A and actin in F9 stem cells (lane 8), F9 parietal endoderm (lane 9), and F9 visceral endoderm (lane 10). The weak hybridization signal in lane 8 is due to residual binding of the c-myc probe, and does not represent α-fetoprotein transcripts.

extraembryonic tissues. PCC3 and, to a lesser extent, PCC4 represent such "multipotent" EC lines. The two other EC cell lines studied here, PCC7 and F9, show more restricted and more homogeneous patterns of differentiation. PCC7 and F9 cells preferentially differentiate into cholinergic neurons and extraembryonic endoderm respectively (Pfeiffer et al., 1981; Strickland and Mahdavi, 1978).

Cell cultures

PCC3 A/1, PCC4 aza1 (Jakob et al., 1973), and PCC7 (Pfeiffer et al., 1981) embryonal carcinoma cell lines were generously provided by Drs. H. Jakob and J-F. Nicholas. F9 embryonal carcinoma cells (Bernstine et al., 1973) were obtained from Drs. S. Grandchamp and B. Ephrussi. NA, a hypoxanthine phosphoribosyl transferase deficient subline of mouse C1300 neuroblastoma (Augusti-Tocco and Sato, 1969), was obtained from Dr. P. Nelson.

EC-cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), non-essential amino acids, nucleosides, and sodium pyruvate. Balb/c 3T3 and NA cells were cultured in DMEM supplemented with 5% FCS. For induction of differentiation, PCC7 cells were treated for 3 days with 1×10^{-8}M retinoic acid (RA) dissolved in ethanol at a concentration of 1×10^{-5}M, and for 3 more days in DMEM with 2% FCS, 1×10^{-8}M RA and 1×10^{-3}M cAMP (Sigma). F9 cells were induced to form parietal endoderm by treating cultures with 1×10^{-7}M RA and 1×10^{-3}M cAMP for 10 days. Visceral endoderm (Hogan et al., 1981), was induced by culturing F9 cells for 10 days in cellophobic dishes in the presence of 5×10^{-8}M RA.

DNA synthesis

DNA synthesis was measured by determining the amount of radioactivity incorporated into an acid insoluble form after a 1-h pulse with 5 μCi/ml of ^{3}H-thymidine (^{3}H-TdR). The cells were then washed twice in trypsin-buffer and counted. After trichloroacetic acid (TCA) precipitation, the amount of ^{3}H-TdR incorporated was determined by scintillation counting.

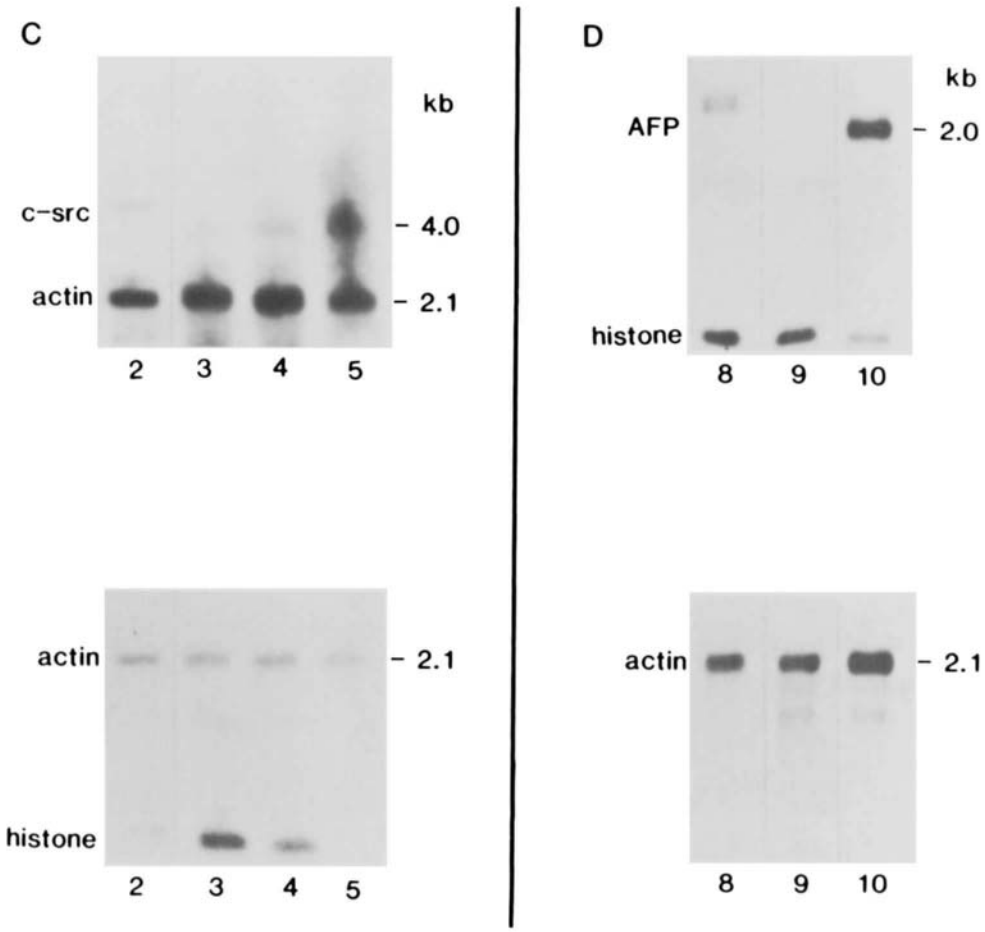

Figure 1

Plasminogen activator (PA) assay

Fibrinogen-agarose plates were used as a semi-quantitative measure of the amount of plasminogen activator (PA) secreted by cell cultures into the surrounding medium as described previously (Sejersen et al., 1985).

Isolation and blot analysis of RNA

One to five $\times 10^7$ cells were harvested at different states of differentiation and cell growth. RNA was isolated by hot phenol extraction (Edmonds and Caramela, 1969). Total RNA was selected for poly(A) + RNA by chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). Samples of total or poly(A) + RNA were analyzed for quantity and purity by A260/A280, denatured and fractionated through a formaldehyde-agarose (1.5%) gel (Lehrach et al., 1977), transferred to nitrocellulose (Thomas, 1980), and hybridized to DNA probes nick-translated in the presence of (α-^{32}P) dCTP (400 Ci/mmole) to specific activities of approximately 2×10^8 dpm/μg. The blots were hybridized at 42°C in 50% formamide and 10% dextran sulphate, washed at 42°C in 0.1 × SSC, 0.5% SDS and exposed to X-ray film (Fuji RX-L) for 7–48 h with enlightening screens at −70°C. For removal of probe and subsequent rehybridization, blots were washed at 65°C in 5 mM Tris-Cl, 0.2 mM EDTA, 0.05% pyrophosphate and 0.002% Denhard reagent. The sizes of the transcripts were determined relative to mouse 18S and 28S RNA markers. The rRNA's were visualized by methylene blue staining of the nitrocellulose filters. Relative intensities of the hybridization signals were quantified by scanning the RNA blot autoradiograms with a Shimadzu densitometer scanning apparatus. The following cloned DNA fragments were used as probes: N-myc, pNb1 (Schwab et al., 1983); c-myc, pM c-myc 54 (Stanton et al., 1983); v-src, PvuII E fragment (deLorbe et al., 1980); α-fetoprotein, AFP1 (Tilghman et al., 1979); actin, pAM91 (Minty et al., 1981); and histone H2A, pCH3, 3E (Harvey et al., 1981).

Southern blot analysis

High molecular weight DNA was cleaved with restriction enzyme Eco RI, and separated by agarose (0.8%) gel electrophoresis, transferred to nitrocellulose filter and hybridized with ^{32}P-labelled pNB1 DNA.

Immunofluorescence

Antiserum to neurofilament was prepared in rabbits. The preparation and characterization of the antiserum has been described previously (Dahl and Bignami, 1977; Raju et al., 1981). The immunohistochemical staining

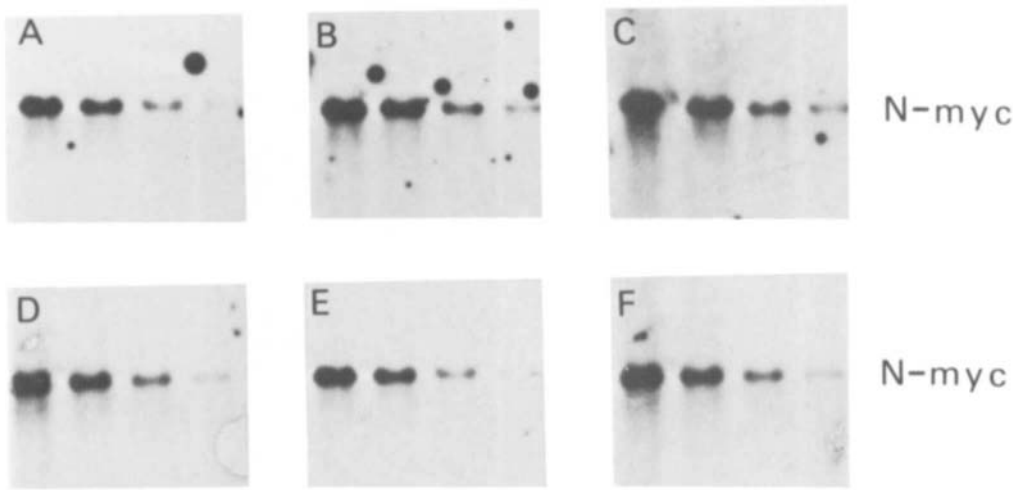

Fig. 2. Southern analysis of the N-myc locus in Balb/c mouse (A), NA mouse neuroblastoma (B), PCC3 (C), PCC7 (D), PCC4 (E), and F9 (F) DNA. Ten μg, 5 μg, 2.5 μg, and 1.25 μg of DNA was analyzed in each panel.

was carried out on air-dried cell cultures according to the indirect immunofluorescence technique of Coons (1958). In order to increase the fluorescence intensity, the slides were fixed in acetone (Seiger et al., 1984). Control slides were incubated in pre-immune serum followed by the FITC-labelled second antibodies.

RESULTS

N-myc and c-myc expression in EC stem cells

Total or poly-adenylated RNA extracted from exponentially proliferating EC stem cells was analyzed on Northern blots for N-myc expression by hybridization with the N-myc specific probe pNb1. All four EC cell lines (F9, PCC3, PCC4, PCC7) express a 2.9 kb N-myc transcript (Fig. 1A; lanes 3, 6, 7, 8). Expression of N-myc was not detected in Balb/c 3T3 cells or in NA murine neuroblastoma cells (Fig. 1A; lanes 1, 2). The N-myc gene was shown by Southern blot analysis not to be amplified in any of the four EC cell lines (Fig. 2). The c-myc gene was expressed in three of the EC cell lines (Fig. 1B), but not in PCC7 EC cells (Fig. 1B; lane 3).

Expression of N-myc and c-src during neuronal differentiation

Cells of the PCC7 EC line differentiate spontaneously into cholinergic neurons (Pfeiffer et al., 1981). The formation of nerve is, however, more pronounced and homogeneous in cultures induced by retinoic acid and cAMP (H. Jakob, personal communication). This differentiation process results in gross changes in cell morphology. In about 6 days, rounded EC cells growing as dispersed cells (Fig. 3A), change into aggregates of cells with massive neurite outgrowths (Fig. 3B–D). Immunofluorescence staining with antineurofilament antiserum showed no reaction with PCC7 stem cells but gave a strong fibrillar fluorescence with induced PCC7 nerve cells (Fig. 3E). Differentiation into nerve tissue resulted in 97% decrease in DNA synthesis (measured as incorporated ^{3}H-TdR after a 1-h pulse). Densitometry of Northern blot autoradiograms showed that the abundance of N-myc transcripts in mRNA preparations was 85% lower in PCC7 nerve cells than in PCC7 stem cells (Fig. 1A; lanes 3 and 5). Rehybridization with an actin probe showed that the RNA samples contained similar quantities of actin transcripts and that the N-myc differences were due to altered expression rather than technical errors. The change in N-myc expression occurred gradually over a 6-day period during which massive nerve differentiation took place (Fig. 4). The expression of histone H2A, a gene which is specifically transcribed during the S-phase of the cell cycle (Heintz et al., 1983), was reduced in parallel with N-myc (Fig. 4).

Formation of nerve from PCC7 cells resulted in altered expression also of the proto-oncogene c-src (Fig. 1c). C-src transcripts (4.0 Kb) were barely detectable in RNA from NA and PCC7 stem cells, analyzed by hybridization with a v-src probe. The level of c-src RNA in PCC7 nerve cells was 300–500% of that in PCC7 stem cells.

Expression of N-myc, c-myc and c-src during endodermal differentiation

In order to examine if the steady state levels of N-myc and c-src mRNA change in EC cells differentiating into tissues other than nerve, we examined two other pathways of EC cell differentiation. F9 EC cells can be induced to differentiate specifically into parietal and visceral endoderm (Hogan et al., 1981). Sublines of F9 have been reported also to be capable of nerve differentiation (Kuff and Fewell, 1980). The F9 cells examined in this study, however, did not form neuron-like cells. Parietal endoderm was formed from F9 cells cultured in the presence of retinoic acid and cAMP. Ten days after induction, close to 100% of the cells were of typical endoderm morphology. Cultures of F9 endoderm cells, but not F9 stem cells, were found on fibrinogen-agarose plates to secrete plasminogen activator (data not shown). The formation of parietal endoderm was accompanied by a reduction in the number of cells in S phase from 72% to 40% (percentage of cells incorporating ^{3}H-TdR during a 1-h pulse). Visceral endoderm was induced by

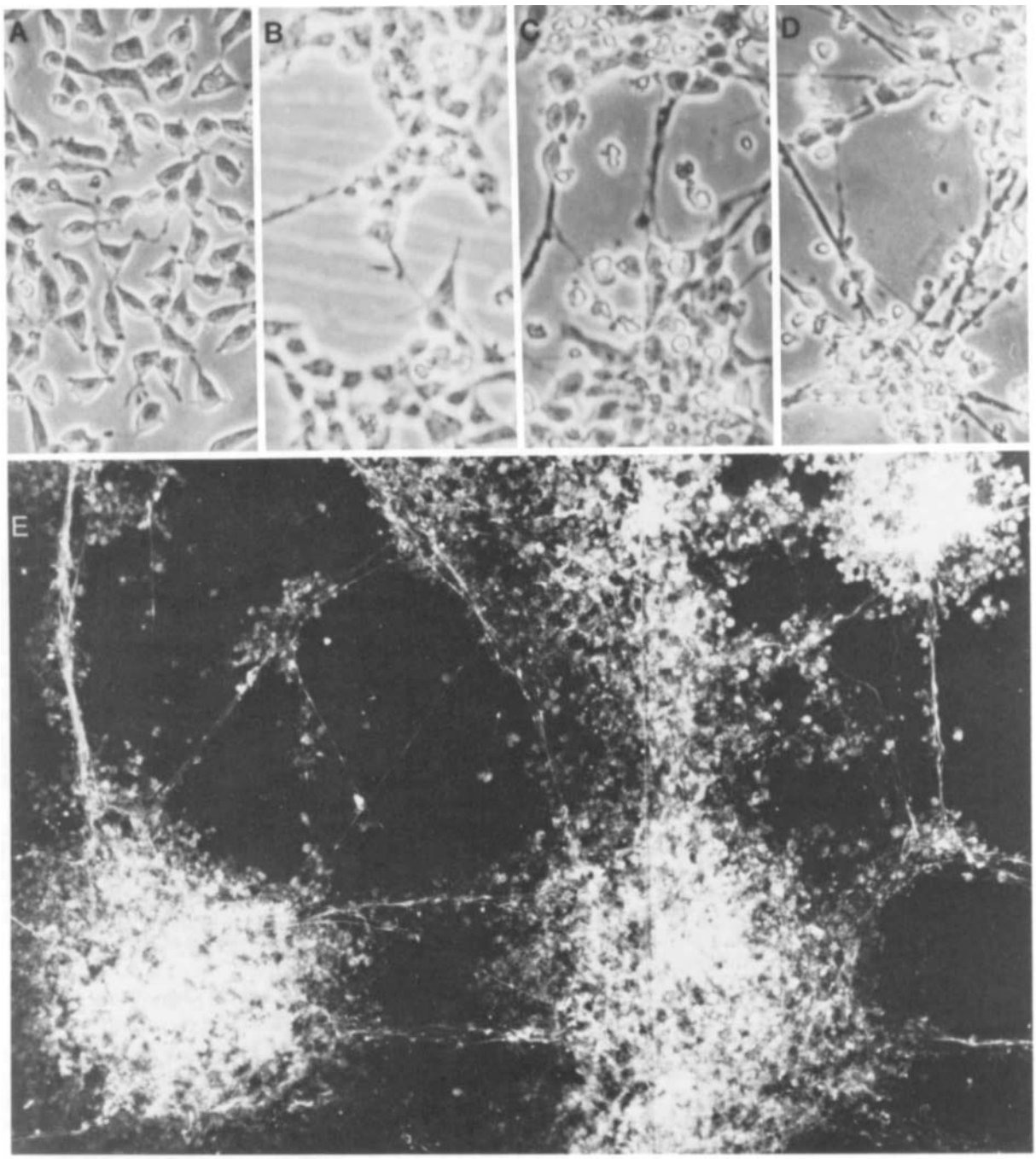

Fig. 3. PCC7 cells undergoing nerve differentiation. PCC7 stem cells cultured and induced as described in Figure 1 legend were photographed before treatment (A), and two (B), four (C), and six (D) days after induction. E) Expression of neurofilament immuno-reactivity in PCC7 cells 6 days after induction. Several large aggregates of brightly fluorescent cells are observed. These cell aggregates are interconnected by bundles of strongly neurofilament-positive fibers.

culturing the F9 cells for 10 days as embryoid bodies in the presence of retinoic acid.

α-Fetoprotein, a marker of visceral endoderm, was expressed in these cells, but not in F9 stem cells or F9 parietal endoderm (Fig. 1D; lanes 8–10). Incorporation of ^{3}H-TdR during a 1-h pulse in visceral endoderm cells was approximately 20% of that in exponentially proliferating F9 stem cells. By Northern hybridization we found that 10 days after induction of the F9 cells there was an 85% decrease in the level of N-myc expression, both in parietal and visceral endoderm (Fig. 1A; lanes 9 and 10). When the same blot was hybridized with a c-myc probe, we confirmed the previously reported decrease in the level of c-myc mRNA in F9 parietal endo-

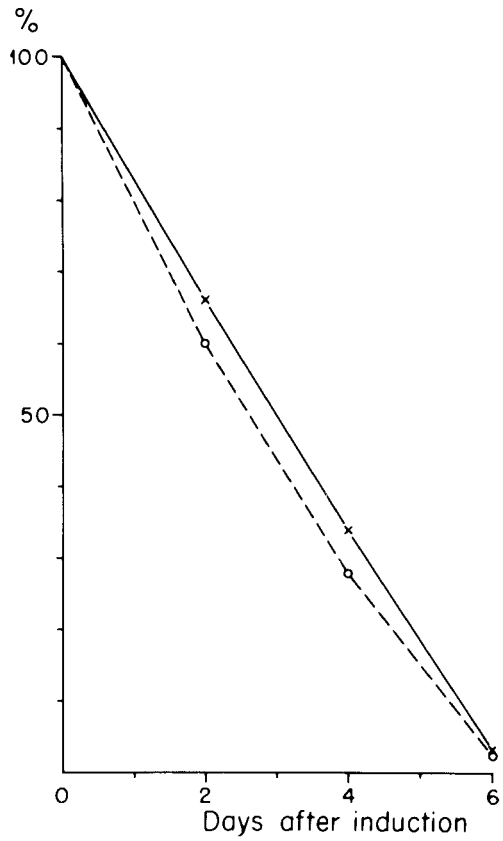

Fig. 4. Relative amounts of N-myc (—x—) and histone H2A (---o---) transcripts during differentiation of PCC7 cells, determined by densitometer scanning of Northern blot autoradiogram exposed in the linear range of the film.

derm (Campisi et al., 1984). We also found a similar decrease in F9 visceral endoderm (Fig. 1B, lane 10). No c-src transcripts were detected in proliferating or differentiated F9 cells (data not shown). Subsequently the blot was also hybridized with an actin probe and a histone H2A probe (Fig. 1D). These internal controls confirm that approximately equal amounts of RNA were analyzed (actin), and support the ^{3}H-TdR-incorporation results obtained from parallel cultures (histone H2A).

Serum starvation

Above we have reported how we examined N-myc and c-src expression in relation to differentiation and accompanying reduced rate of cell growth. Next we wished to know if the down-regulation of N-myc and up-regulation of c-src mRNA levels in differentiated PCC7 cells is related to the in vitro differentiation process or to the reduced rate of cell growth. It was of interest, therefore, to test whether N-myc and c-src expression changes when cell multiplication is arrested by serum starvation. For this purpose PCC7 cells were cultured in 1% FCS for 20 h. This treatment caused a 72% reduction in the amount of ^{3}H-TdR incorporated during a 1-h pulse, but did not affect the level of N-myc or c-src mRNA (Fig.1A,C; lane 3 and 4). The serum-starvation of PCC7 cells was not followed by any morphological signs of differentiation. PCC7 cells were very sensitive to serum-starvation and massive cell death resulted from prolonged culture (48 h) in 1% FCS.

DISCUSSION

N-myc is a proto-oncogene belonging to the myc family. It has no viral counterpart and its physiological role is unknown. Previous reports of amplification and elevated levels of N-myc transcripts in human neuroblastomas and retinoblastomas have implied that expression of this gene is confined to undifferentiated tumor cells of neuroectodermal origin (Kohl et al., 1983; Lee et al., 1984; Schwab et al., 1984; Kohl et al., 1984). Retinoic acid-induced differentiation of human neuroblastoma cells was recently reported to lead to an early decrease in N-myc expression (Thiele et al., 1985). Based on these results, it has been speculated that N-myc expression plays a role in the development of neuroectodermal tissue. Our finding of elevated levels of N-myc transcripts in four murine EC cell lines indicate that N-myc is highly expressed also in the malignant stem cells of teratocarcinoma. The EC stem cells of this germ cell tumor possess many properties of inner cell mass cells (embryoblasts) and primitive ectodermal cells (Martin, 1980). Developmentally, EC cells thus represent a stage preceding the formation of neuroectoderm. The EC cell lines used in this study vary in their capacity to differentiate. The high levels of N-myc mRNA in EC cells that preferentially differentiate into nerve tissue (PCC7) supports the suggestion that N-myc is expressed early in the development of neuroectodermal tissue. Furthermore, the finding of N-myc expression in two multipotent EC cell lines (PCC3, PCC4) and an EC cell line committed to differentiate into extra-embryonal endoderm (F9) suggests that N-myc is expressed already in undetermined cells present at the blastocyst stage.

The expression of N-myc was down-regulated during differentiation in both PCC7 and F9 EC cells. The 85% reduction of N-myc mRNA levels during differentiation into nerve tissue occurred gradually and in parallel with the slowing of cell proliferation. The reduction in N-myc levels in differentiating PCC7 cells appears to be a slower process than the decrease reported in a recent study of differentiating human neuroblastoma cells (Thiele et al., 1985). Formation of nerve from PCC7 cells also resulted in 300–500% increase in c-src expression at the mRNA level. The change in steady-state levels of N-myc and c-src mRNA in induced PCC7 cells appears to be related to the differentiation process and not to decreased cell multiplication since serum starvation did not affect the expression of N-myc or c-src. The increase in c-src expression corresponds well with previous reports by Cotton and Brugge (1983) and Sorge et al. (1984). Both groups found elevated levels of $pp60^{c\text{-}src}$ in neural tissue. Two recent reports have further elucidated the relationship between src expression and neural differentiation. Alemà et al. (1985) have presented data supporting an inductive effect of v-src expression on neuronal differentiation and Brügge et al. (1985) have found that a structurally modified form of the c-src protein is expressed at high levels in neurons. We are presently investigating a possible relationship between elevated c-src expression and neuronal differentiation of the PCC7 embryonal carcinoma line.

Differentiation of F9 EC cells into visceral as well as parietal endoderm was accompanied by 85% reduction in the level of N-myc transcripts. A similar reduction occurred in c-myc expression. Decreased c-myc expression in F9 cells differentiating into parietal endoderm has previously been found by Campisi et al. (1984). Since down-regulation of N-myc expression occurs both in PCC7 cells forming nerve-like cells and in F9 cells forming endoderm, it is clearly not a feature specific for neuronal differentiation.

To summarize, the present study shows that N-myc transcripts are abundant in undifferentiated embryonal carcinoma cells, i.e., a cell type which closely resembles the embryoblasts of early blastocysts. As these cells differentiate to visceral endoderm or parietal endoderm, the abundance of both N-myc and c-myc transcripts decreases by 85%. Differentiation of EC cells to nerve-like cells is accompanied by a similar decrease in N-myc expression, and an increase in the abundance of c-src transcripts.

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