Similarities and Differences in the Regulation of N-myc and c-myc Genes in Murine Embryonal Carcinoma Cells

THOMAS SEJERSEN,*† MAGNUS RAHM,* GABOR SZABO,† SIGURDUR INGVARSSON,† and JANOS SÜMEGI†

*Department of Medical Cell Genetics and †Department of Tumor Biology, Karolinska Institutet, S-10401 Stockholm, Sweden

c-myc and N-myc are closely related genes coding for putative DNA-binding proteins. The protein products of both genes have been implicated in the regulation of growth of normal and neoplastic cells. We compared the regulation of N-myc and c-myc expression under different growth conditions as well as in vitro differentiation of the murine EC lines F9 and PCC7. N-myc and c-myc expression was found to be regulated by distinct mechanisms, although similarities exist. Differences were found both at the transcriptional and at the post-transcriptional level. The two myc genes were regulated by mainly post-transcriptional mechanisms, but in PCC7 cells nuclear run-on assays indicated that c-myc was repressed at the level of transcription. N-myc and c-myc expression was negatively regulated at a post-transcriptional level in F9 and PCC7 cells during differentiation to visceral endoderm and nerve-like tissue, respectively. Serum stimulation of F9 cells for 4 h induced a sevenfold increase in c-myc transcripts but no significant elevation of N-myc transcripts. Mitogenic stimulation with insulin and transferrin also induced a marked elevation of c-myc but not of N-myc mRNA. In addition, the N-myc and c-myc genes differed in F9 cells with respect to (i) the kinetics of expression following induction of differentiation, c-myc undergoing quicker changes than N-myc; (ii) the response to cycloheximide inhibition of protein synthesis, indicating that c-myc but not N-myc is down-regulated by a short-lived protein; and (iii) the half-lives of the transcripts, estimated to be approximately 40 min for c-myc and 130 min for N-myc.

The myc family of proto-oncogenes contains three known members, namely c-myc, N-myc, and L-myc. These genes have been implicated in the initiation and progression of various animal tumors [1–5]. They may also be important during normal cell growth and development [4, 6–8]. Sequence comparisons between cloned c-myc and N-myc genes have revealed considerable homologies in two major domains of the coding exons [9]. The products of the c-myc and N-myc genes are nuclear proteins [10, 11] with similar transforming activity [12, 13]. Despite these similarities in structure and function, the two genes differ with respect to expression during normal mouse development [8]. c-myc appears to be expressed in all proliferating tissues, whereas N-myc expression is much more restricted. Developing mouse kidney, brain, and intestine are the only tissues with high levels of N-myc mRNA. Expression of c-myc, the best-studied member of the myc family, is known to be modulated in fibroblasts and lymphoid cells by serum [14], growth factors [15], and other modulators of cell proliferation [16–18]. Terminal in vitro differentiation of the monomyelocytic cell line HL60, erythro-leukemia cell lines, skeletal myoblasts, and the embryonal carcinoma (EC) cell

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line F9 are associated with decreased steady-state levels of c-myc mRNA [14, 19–22]. Similarly, down-regulation of the levels of N-myc transcripts has been observed in differentiating human neuroblastoma cell lines [23].

It has previously been reported that inhibition of protein synthesis increases c-myc expression in some, but not all, cell types [15, 24, 25]. Recent results indicate that regulation of c-myc expression following mitogenic stimulation, terminal differentiation, or inhibition of protein synthesis in several cell types is mediated by changes in the stability of c-myc mRNAs [24, 26, 27].

Certain murine embryonal carcinoma (EC) cell lines were recently shown to express both N-myc and c-myc [28, 29]. The present investigation was undertaken in order to compare the regulation of N-myc and c-myc in F9 and PCC7 EC cells. We found that c-myc and N-myc mRNA levels are both modulated by serum and differentiation in F9 EC cells. Nuclear run-on transcription assays indicated that the level of regulation in F9 cells is post-transcriptional for both genes. In differentiating F9 cells the reduction of c-myc mRNA levels appeared to be an event earlier than the down-regulation of N-myc expression. Furthermore, the expression of the two genes was differently affected in F9 cells growth-stimulated with serum or insulin/transferrin. The half-life of N-myc mRNA is longer than that of the c-myc transcript in F9 cells. In proliferating PCC7 cells the c-myc gene is transcriptionally silent as judged by RNA blot analysis and in vitro nuclear run-on transcription. The N-myc gene was, however, expressed in proliferating PCC7 cells and down-regulated post-transcriptionally in differentiated PCC7 cells.

MATERIALS AND METHODS

Cell cultures, (3H)thymidine labeling, and FACS analysis. F9 cells [30] and PCC7 EC cells [31] were cultured in DMEM supplemented with 10% fetal calf serum (FCS), nonessential amino acids, nucleosides, and sodium pyruvate. For induction of differentiation, PCC7 cells were treated for 3 days with 1×10^{-4} M retinoic acid (RA) and for 3 more days with 2% FCS, 1×10^{-4} M RA (Sigma), and 1×10^{-3} M dibutyryl cAMP (Sigma) in nutrient-supplemented DMEM. Neural differentiation was monitored by measuring neurite outgrowths. For each time point, the average neurite length was calculated on methanol-fixed Giemsa-stained PCC7 cells. Each data point represents the average of 30 randomly measured outgrowths longer than one cell body in length. F9 cells were induced to form visceral endoderm by culture for 10 days in cellobiose dishes in the presence of 5×10^{-5} M RA. For serum deprivation, F9 cultures were washed in serum-free medium. The cells were then kept for 48 h in nutrient-supplemented DMEM with 0.5 or 1% FCS as indicated in the text. Mitogenic stimulation of serum-deprived F9 cells was accomplished by the addition of fresh nutrient-supplemented DMEM containing 15% FCS or insulin (1 μg/mL, Sigma) and transferrin (5 μg/mL, Sigma). Transcription was inhibited by actinomycin D (5 μg/mL, Sigma) in serum-supplemented DMEM. Protein synthesis was inhibited by the addition of cycloheximide (Sigma) at 10 μg/mL to the culture medium. DNA synthesis was measured by determining the amount of (3H)thymidine incorporated into cells after a 1-h pulse as described [21] or by FACS analysis of the distribution of cells in the cell cycle.

Isolation of RNA and RNA blotting. RNA was isolated by hot phenol extraction [32]. Total RNA was selected for poly(A)^+ RNA by chromatography on oligo(dT)-cellulose [33]. Samples of total and poly(A)^+ RNA were analyzed for quantity and purity by measurements of UV light absorption (A_{260}/A_{230}). Total or poly(A)^+ RNA was denatured, fractionated through formaldehyde-agarose (1.1%) gels, transferred to nitrocellulose (Schleicher & Schuell), and hybridized to nick-translated DNA probes as described [29]. In some cases, electrophoretically separated RNA was blotted to Hybond-N nylon membranes (Amersham). N-myc and c-myc probes were in some experiments labeled with (32P)-dCTP by oligonucleotide primer extension [34]. For removal of probe and subsequent rehybridization, nitrocellulose filters were treated as previously described [29], and Hybond-N filters were
Fig. 1. Northern blot illustrating N-myc, c-myc, and actin transcripts in proliferating and serum-deprived (1% FCS) F9 cells. (P) Ten micrograms of poly(A)+ RNA from proliferating F9 cells; (S) serum-deprived F9 cells. Residual signal from previous c-myc hybridization is seen in lane P of the actin hybridization.

Washed for 2 h at 65°C in 0.005 M Tris–HCl, pH 8.0, 0.002 M Na2-EDTA, and 0.1× Denhardt’s solution. The following cloned DNA fragments were used as probes: N-myc, pNB1 [35], c-myc, pM c-myc 54 [36], α-fetoprotein, AFP1 [37], actin, pAM91 [38], and histone H2A, pCH3.3E [39]. Relative intensities of hybridization signals were obtained by densitometric scanning of autoradiographs exposed within the linear range of the film (Fuji RX-L).

In vitro nuclear run-on transcription. Nuclei were isolated and stored at −80°C as described by Dony et al. [27]. Nuclear run-on transcription was performed by the method of Schibler et al. [40] in the absence of creatine phosphate. The in vitro synthesized and 32P-labeled transcripts were hybridized to cloned DNA fragments bound to nitrocellulose filters [40].

RESULTS

N-myc and c-myc Regulation by Serum and Insulin/Transferrin

In order to investigate the relationship between expression of the protooncogenes c-myc and N-myc and cell proliferation, we analyzed their expression in serum-deprived and in serum-stimulated F9 EC cells. Exponentially growing F9 cells express both 2.9 kb N-myc and 2.4 kb c-myc mRNAs ([28, 29] and Fig. 1). The effect of growth inhibition on N-myc and c-myc expression in F9 cells was studied by exposing subconfluent cultures of F9 cells to 1% FCS for 48 h. The decrease in DNA synthesis following serum deprivation varied between 60 and 94% in different experiments. The amount of [3H]Tdr incorporated into DNA during a 1-h pulse was decreased by 94% following serum deprivation in the experiment illustrated in Fig. 1. The relative abundance of N-myc and c-myc mRNAs, measured by densitometer scanning of radiographs, was decreased by 88 and 92%, respectively, after 48 h of serum deprivation (Fig. 1). Actin expression was not affected by the serum starvation (Fig. 1). Differentiation-specific α-fetoprotein (AFP) mRNA was not synthesized after serum deprivation (data not shown).

In a separate experiment we investigated N-myc and c-myc mRNA accumulation in serum-deprived F9 cells following stimulation with serum or insulin and transferrin. F9 cells were treated with 15% FCS after 2 days of serum deprivation
Fig. 2. N-myc and c-myc expression in serum-deprived F9 cells (0.5% FCS) after stimulation with 15% FCS. The number of hours after readdition of serum is indicated. (A) Graphic illustration of the relative levels of N-myc mRNA (○), c-myc mRNA (×), and number of cells in the S/G2 phase of the cell cycle determined by FACS analysis (●). The relative levels of expression are compared with serum-deprived cells (100%). (B) RNA blot used for the graphic illustration. Eighteen micrograms of total RNA was analyzed in each lane. The blot was exposed for 6 days in order to allow comparisons with the residual expression at the 0 hour (serum-deprived) time point.

(0.5% FCS). As can be seen in Fig. 2, the abundance of c-myc mRNA underwent a transient 700% increase 4 h after serum addition. At the same time point, N-myc expression was elevated by only 23% (Fig. 2B), which was not significant with the limited accuracy imposed by the technique used.

Serum contains many growth factors, hormones, and other growth-supporting components. Therefore, growth stimulation of quiescent F9 cells by serum may conceal differences in the regulation of c-myc and N-myc in response to specific growth factors. In order to analyze further the relationship between growth induction and regulation of c-myc and N-myc expression, we added serum-free medium with insulin (1 µg/ml) and transferrin (5 µg/ml) to serum-deprived F9 cells. Insulin and transferrin have previously been reported to induce proliferation of F9 EC cells [41]. This was confirmed by [3H]TdR incorporation and FACS analysis (data not shown). The strong rise in c-myc expression induced by serum was reproduced by 4 h of insulin/transferrin treatment (Fig. 3). Expression of N-myc was not induced at the time points analyzed (Fig. 3). The transient accumulation of c-myc transcripts in serum-treated cells and in insulin/transferrin-treated cells can be due to a regulatory event(s) either at a transcriptional or at a posttranscriptional level. To distinguish between these alternatives, the density of
RNA polymerase B molecules engaged in transcription of the c-myc and N-myc genes, respectively, were measured by an in vitro nuclei transcription assay [40]. Nuclei were isolated from proliferating and from serum-starved F9 cells at various times after serum addition. The in vitro-synthesized nuclear run-on transcripts labeled with $^{35}$P]UTP were hydridized to c-myc- and N-myc-specific DNA probes immobilized on nitrocellulose filters. As shown in Fig. 4A, the number of RNA polymerases on both c-myc and N-myc genes remained unchanged during serum starvation and following serum stimulation.

**c-myc Regulation in PCC7 Cells**

We have previously shown that the poly(A) mRNA fraction from proliferating PCC7 cells lacks c-myc-specific transcripts [29], although the c-myc gene is
Fig. 4. Nuclear run-on transcription assay. (A) c-myc and N-myc transcriptional levels under various growth conditions of F9 cells. F9 cells were plated at $2 \times 10^6$ cells/cm$^2$ and cultured for one day in DMEM with 10% FCS. Nuclei were thereafter isolated from cells switched to 1% FCS for the indicated number of hours (a–c) or from cells deprived from serum (1% FCS) for 48 h and then switched to 15% FCS for the indicated time in hours (d–f): (a) 0, (b) 24, and (c) 48 h after changing to 1% FCS; (d) 0, (e) 1, (f) 4 h after adding 15% FCS. Nuclear isolation and nuclear run on transcription reactions were conducted as described under Materials and Methods. (B) c-myc and N-myc transcriptional levels during differentiation of F9 and PCC7 cells. F9 and PCC7 cells were induced to differentiate as described under Materials and Methods. At each time point (days) after induction of differentiation nuclei were isolated for nuclear run-on transcription: (a) 0, (b) 3, and (c) 10 days after RA/cAMP induction of F9 cells; and (d) 0, (e) 2, (f) 4, and (g) 6 days after the differentiation program was initiated with PCC7 cells. Equivalent counts ($5 \times 10^6$ cpm) of $^32$P-labeled nuclear run-on transcripts were hybridized to nitrocellulose filters containing 5 μg of c-myc, N-myc and c-src genomic fragments, as well as actin and α-fetoprotein cDNA fragments. Autoradiography was for 3 days using intensifying screens at −70°C.

present in the PCC7 genome (not shown). The absence of c-myc mRNA in these cells can be explained by transcriptional control or by increased turnover of the transcripts. Nuclei were isolated from proliferating and differentiated PCC7 cells in order to determine the transcriptional activity. $^32$P-labeled run-on transcripts were isolated and hybridized to immobilized DNA corresponding to the third exon of the murine c-myc gene. As shown in Fig. 4B, there was no RNA hybridizable to c-myc sequences. The N-myc DNA fragment, however, detected N-myc-specific mRNAs among the in vitro nuclear run-on transcripts (Fig. 4B). From the results of the nuclear run-on analysis we conclude that the expression of c-myc in PCC7 cells is repressed at the level of transcription.

Kinetics of N-myc and c-myc Expression during Neuronal and Endodermal in Vitro Differentiation

Previous studies have suggested a role for N-myc in neural differentiation of the murine EC cell line PCC7 [29] and of human neuroblastoma cell lines [23]. PCC7 cells differentiate into neural cells after induction with RA, cAMP, and low serum concentration (29). To investigate a possible involvement of N-myc in the induction of differentiation, the time course of N-myc down-regulation following
Fig. 5. Kinetics of N-myc expression in relation to neurite outgrowth and histone H2A expression in PCC7 cells after induction of neural differentiation. (A) Graphic representation of the relative levels of N-myc (○) and histone H2A (●) mRNAs compared to exponentially proliferating PCC7 cells. Average lengths of neurite outgrowths are also indicated. (B) RNA blot illustrating N-myc and actin expressions at the indicated number of hours after induction of differentiation. Ten micrograms of poly (A)+ RNA was analyzed in each lane.

RA/cAMP treatment was related to the appearance of a neuronal phenotype in the differentiating cells. Neural differentiation is characterized by the appearance of neurofilament-positive cells [29] and by gross changes in cell morphology, including neurite outgrowth ([29, 31] and Fig. 5). Analysis of RNA from RA/
Fig. 6. Kinetics of N-myc and c-myc expression in relation to α-fetoprotein transcription and histone H2A expression in F9 cells after induction of differentiation into visceral endoderm. (A) Graphic illustration of the relative levels of N-myc (O), c-myc (X), and histone H2A (■) mRNAs compared to exponentially proliferating F9 cells. Transcription of α-fetoprotein is also indicated (Δ). Relative levels of nuclear run-on transcription are compared with the maximal value obtained (100%). (B) Upper panel shows nondenaturing agarose/EtBr electrophoresis of small-scale preparations of samples analyzed by Northern blotting. Lower panels: RNA blot showing the N-myc and c-myc expression at the indicated number of hours after induction of differentiation. Forty micrograms of total RNA was analyzed in each lane.
cAMP-treated cells showed a down-regulation of N-myc expression (Fig. 5). The decrease in N-myc mRNA was detectable already 6 h after the induction of differentiation. By 12 h, there was a 70% decrease in the level of N-myc expression, compared to control cells. Thereafter, the down-regulation was not as drastic, but continued to reach the lowest value 6 days after induction. In the early phase of induction (Day 1), the decrease in histone H2A mRNA level was much less marked than the decrease in N-myc expression (Fig. 5). Histone H2A expression is known to correlate in time with the S phase of the cell cycle [42]. It can also be seen from Fig. 5 that after a lag period of about 2 days, the RA/cAMP-induced cells started to undergo a marked phenotypic conversion as assessed by the characteristic neurite outgrowth. Analysis of results obtained by in vitro nuclear run-on transcription shows, however, that N-myc is transcribed at a constant rate during the differentiation of PCC7 cells (Fig. 4B).

It has previously been reported that both c-myc and N-myc are down-regulated in F9 cells differentiating into visceral [29] and parietal endoderm [14, 27, 28, 43]. Here we present evidence that the transcription rate of both proto-oncogenes remains constant, while transcription of the gene for α-fetoprotein is induced, during formation of visceral endoderm from F9 cells (Fig. 4B). Northern blot analysis shows that the down-regulation of c-myc and N-myc associated with this differentiation followed different kinetics. The reduction of c-myc mRNA preceded the change in the level of N-myc transcripts in the cell (Fig. 6). c-myc down-regulation was dramatic during the first 24 h and was followed by a transient increase before returning to a low level of expression in differentiated F9 visceral endoderm (Fig. 6).
Stability of N-myc and c-myc mRNA in EC Cells

The c-myc mRNA has a rapid turnover and is degraded with a half-life of about 15–60 min in most of the cell types analyzed so far [24, 44, 45]. We have determined the intracellular turnover of N-myc and c-myc mRNA in proliferating F9 and PCC7 cells by following the change in the steady-state levels of both transcripts at various time points after the addition of actinomycin D, a potent inhibitor of RNA transcription. Figure 7A shows that the c-myc mRNA disappeared much more rapidly than the N-myc transcripts in actinomycin-treated F9 cells. A quantitative analysis of the turnover kinetics of steady-state levels of N-myc and c-myc RNA in F9 cells revealed half-lives of approximately 130 min for N-myc and 40 min for c-myc. The half-life of N-myc transcripts in PCC7 cells was approximately 60 min (Fig. 7B). Cytoskeletal actin transcripts served as a control and were not affected by 4 h of actinomycin D treatment, indicating a turnover rate comparable to that of bulk transcripts in the cells (not shown).

In view of the above findings, we wanted to test if the expression of N-myc in F9 cells, like c-myc, is regulated by a labile protein. We examined the effect of protein synthesis inhibition by cycloheximide on the steady-state levels of N-myc and c-myc mRNA before and after RA induction. Figure 8 shows that cycloheximide treatment for 3 h enhanced the abundance of c-myc mRNA approximately twofold in both undifferentiated and RA-induced F9 cells, while the levels of N-myc transcripts did not undergo an appreciable change during this time period.

DISCUSSION

The proto-oncogenes c-myc and N-myc show remarkable similarities and have both been implicated to be of importance in eucaryotic developmental processes [4, 6–8]. However, the two genes display different patterns of expression in developing mouse embryos, indicating that c-myc and N-myc are regulated independently [8].

The results presented here show that N-myc and c-myc are also regulated differently in two murine embryonal carcinoma cell lines, although similarities exist. Differences were found both at the transcriptional and at the post-transcriptional level.

N-myc and c-myc Regulation in PCC7 EC Cells

Post-transcriptional control dominates the regulation of both genes, but in PCC7 cells c-myc is repressed at the transcriptional level. N-myc, on the other hand, is transcriptionally active in PCC7 cells but undergoes post-transcriptional down-regulation during neuronal differentiation. The decrease in N-myc expression may be a prerequisite for neural differentiation of PCC7 cells, since N-myc expression in induced cells was down-regulated before DNA synthesis or neurite outgrowth was affected.

Absence of c-myc transcription in rapidly proliferating cells is rare, and it has been suggested that c-myc expression is required for cell cycle progression. Our results on the inactivity of c-myc in PCC7, and the finding that mitotically and
meiotically dividing germ cell have very few c-myc transcripts [46], suggest that c-myc expression is not a mandatory requirement for proliferation of certain cell types. We also speculate that a myc-like protein may be a requirement for initiation of DNA synthesis and that the N-myc product fulfills this function in PCC7 cells.

**N-myc and c-myc Regulation in F9 EC Cells**

In F9 cells, N-myc and c-myc are regulated differently in response to serum, growth factors, and induction of visceral endoderm differentiation. Expression of both N-myc and c-myc was drastically down-regulated at a post-transcriptional
level in F9 cells when these cells were serum starved. Other genes subject to
regulation by proliferation, such as thymidine kinase [47], dihydrofolate reduc-
tase [48], and the p53 cellular oncogene [27], are also post-transcriptionally
regulated. The serum dependence of N-\-myc expression in F9 cells contrasts with
the situation previously reported in PCC7 cells and neuroblastoma cell lines [23,
29]. N-\-myc expression was found not to be affected by serum starvation in the
latter cell types. The reason for this discrepancy is not known, but it is interesting
to note that serum contains factors capable of modulating the turnover rate of N-
myc transcripts, at least in F9 cells.

The marked elevation of c-\-myc expression in response to serum or to the
defined growth factors insulin/transferrin is not surprising in view of the large
number of reports on serum and growth factor-induced c-\-myc activity in a
number of different cell types [3, 14, 16, 25, 26, 49, 50]. The ability of insulin to
induce c-\-myc expression does, however, appear to differ between different cell
types. Insulin is a potent comitogen both for fibroblasts [16], L6 skeletal myo-
blasts [51], and F9 EC cells [41], whereas c-\-myc is induced by insulin in only F9
cells and L6 myoblasts [25], not in Balb/c 3T3 [15] or human diploid fibroblasts
[16].

N-\-myc and c-\-myc regulation in F9 cells differs not only in response to mitogen-
ic stimuli but also in following RA-induced differentiation into visceral endoderm.
The abundance of both N-\-myc and c-\-myc transcripts decreased due to post-
transcriptional events before DNA synthesis was inhibited and before overt
visceral endoderm differentiation could be observed. The kinetics of gene regulation
for the two oncogenes differs, however, since c-\-myc expression was down-
regulated before N-\-myc. It could be speculated that a decreased expression of
one or both of these myc genes is a prerequisite for visceral endoderm differenti-
ation of the F9 cells. The regulation of c-\-myc and N-\-myc expression during
visceral endoderm differentiation is similar to the post-transcriptional regulation
of c-\-myc recently reported in F9 cells differentiating into parietal endoderm [27,
52].

Further differences in the regulation of N-\-myc and c-\-myc in F9 cells are the
half-lives of the transcripts and the response to cycloheximide inhibition of
protein synthesis. The reason that a 3-h cycloheximide treatment increases the
level of c-\-myc but not N-\-myc mRNA may reflect that the turnover rate of c-\-myc
but not N-\-myc transcripts is down-regulated by a short-lived protein. It may,
however, at least partially also be explained by the shorter turnover rate of c-\-myc
compared to N-\-myc transcripts. The half-life of N-\-myc transcripts is approxi-
mately three times longer than that of c-\-myc mRNA. In both cases, the half-life is
sufficiently short to allow a quick and efficient post-transcriptional regulation,
but by distinct mechanisms.

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