Post-transcriptional control of c-myc proto-oncogene expression by glucocorticoid hormones in human T lymphoblastic leukemic cells

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ABSTRACT

We have studied the regulation of the human c-myc proto-oncogene by glucocorticoid hormones in T lymphoblastic leukemic cells. A significant decrease (50%) of the steady state levels of c-myc mRNA was observed as early as 3 h after dexamethasone treatment of CEM-1.3 human lymphoma cells, reaching less than 5% values, with respect to untreated cells, 24 h after hormone administration. Nuclear run-on experiments showed no modifications of the transcriptional rate from the first exon. However, a slight decrease (15%) of the transcript elongation from the first exon/first intron boundary was observed in the dexamethasone-treated cells. Using actinomycin D to block gene transcription, we have observed a significant increase in the rate of c-myc RNA specific decay after dexamethasone treatment. Furthermore, cycloheximide was able to overcome completely the dexamethasone-induced down-regulation of the c-myc gene expression. Our data suggest that dexamethasone is able to inhibit human c-myc gene expression primarily at the post-transcriptional level, through the synthesis of hormone-induced regulatory protein(s) controlling c-myc transcript stability.

INTRODUCTION

Several cellular proto-oncogenes (myc, fos, jun, myb, erbA and erbA-related steroid hormone receptors) encode nuclear proteins involved in various steps of the transcriptional control of gene expression (1–6). Increasing evidences suggest that gene transcription results from the cooperation of various transacting factors binding to cis-regulatory sequences (7,8). However, it is still rather poorly understood how each component of this transcriptional machinery coordinately modulates each other concerning the overall control of gene expression. After binding of the hormone, c-erbA oncogene-related steroid hormone receptors acquire transacting activity and participate in the control of gene expression (i) by binding to specific enhancer consensus sequences (6), (ii) by interacting with other transcription factors (9–12) and/or (iii) by modulating the intracellular levels of nuclear proteins subsequently responsible for gene transcription. For instance, estrogens enhance the expression of c-myc (13)

and c-fos (14) while glucocorticoids either inhibit or increase c-myc and c-myb (15) or c-erbA (16) expression, respectively. Because of the role played by c-myc in haematopoietic cell growth, the decreased expression of this oncogene by glucocorticoid hormones is suggested to represent a step in the cascade of events leading to the glucocorticoid-induced inhibition of lymphoid cell proliferation (17).

The expression of the c-myc proto-oncogene is controlled at the levels of both initiation and elongation of the transcript and of mRNA stability (18–23). This prompted us to study the mechanisms by which glucocorticoid hormones affect the transcriptional and post-transcriptional events leading to c-myc expression. In this paper we report that, while unaffected the transcriptional initiation of the gene, dexamethasone significantly increases the degradation of the c-myc transcript.

MATERIALS AND METHODS

Cell Culture

The human leukemic T cell line CEM-1.3 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (Flow Laboratories, Airshire, Scotland). Cells were treated with 1 μ M dexamethasone dissolved in ethanol (0.1% final concentration). Control cells were treated with the same concentration (0.1%) of solvent alone.

RNA isolation and Northern blot analysis

RNA was isolated by the guanidine isothiocyanate/cesium chloride technique (24). Twenty μ g of total RNA were fractionated by electrophoresis in 1.0% agarose gels containing formaldehyde, transferred to Hybond-N filters (Amersham International UK) and hybridized to human c-myc probe (plasmid pMC-41–3Rc, spanning the third exon, ref. 25) or human β -actin cDNA. Hybridization conditions were 50% formamide, 5 $\times$ SSC (1 $\times$ SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 0.05 M sodium phosphate, 1% SDS, 5 $\times$ Denhardt's solution (50 $\times$: 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 10% dextran sulfate, 0.1 mg/ml denatured salmon sperm DNA at 42°C for 24 h. For washing 2 $\times$ SSC, 0.5% SDS at room temperature and 0.1 $\times$ SSC, 0.5% SDS at 50°C were used.

Fig. 1. A–B). Northern blot analysis showing the time course of the effect of dexamethasone (DEX) on c-myc and β -actin expression in CEM cells. In B), amounts of c-myc transcripts were quantitated by densitometric scanning of autoradiographic bands (exposed for several times to allow comparisons within the linear range) and normalized to β -actin mRNA levels evaluated at each time in the same blot. Data represent the percent of control values (0 time, 100%) observed after hormone treatment. C). S1 mapping of c-myc RNA in untreated (C) and dexamethasone-treated cells (DEX). RNA was hybridized to the 354 nt-long single-stranded 5'-end labeled probe spanning the Sma I–Aha2 region of the first exon (–101 to +253 bp) shown at the bottom of the figure. P1 and P2 indicate protected RNA bands corresponding to transcripts starting from promoter 1 and 2 of the c-myc gene.

S1 mapping of RNA

The DNA probe was 5'-end labeled by T4 polynucleotide kinase (Amersham) and the single anti-strand was separated by polyacrylamide gel electrophoresis under denaturing conditions. Total RNA from untreated or dexamethasone-treated cells was coprecipitated with 50,000 cpm of the end-labeled single-stranded probe and dissolved in 10 μ l of 80% formamide, 0.5 M sodium chloride, 20 mM PIPES (pH 6.5), 1 mM EDTA, denatured at 85°C 15 min and hybridized at 37°C overnight. For S1 digestion, the hybridization mix was diluted with 300 μ l of 0.28 M sodium chloride, 30 mM sodium acetate (pH 4.5), 5 mM zinc acetate and 1,200 U/ml S1 nuclease (Amersham) and incubated 2.5 h at 14°C. Reactions were terminated by ethanol precipitation followed by electrophoresis on 8% acrylamide, 7 M urea gels.

Nuclear run-on transcription

Nuclei from untreated and dexamethasone-treated cells were obtained by lysis with 0.5% NP-40 in lysis buffer (Tris 10mM pH 7.4, 3 mM CaCl_2 , 2 mM MgCl_2) and centrifugation at 1500 rpm. The nuclear pellet was resuspended at a concentration of

Fig. 2. Nuclear run-on transcription of the human c-myc gene in CEM cells. Labeled nascent transcripts from untreated (Contr) and dexamethasone-treated cells (DEX) were hybridized to appropriate single-strand probes inserted into M13 in the orientation detecting sense (+) and antisense transcripts (–). The different c-myc probes (a, Sma I–Sma I; b, Xho I–Pvu II; c, Sac II–Sac II; d, Pst I–Pst I) used are indicated in the partial map of the c-myc gene represented at the bottom of the figure. P0, P1 and P2 represent the three described promoters of the c-myc gene. Hybridization to plasmid pUC8 and to β -actin (as an internal control) is also represented.

TABLE 1

DEXAMETHASONE/CONTROL RATIO		
exon 1	0.88 ^a	1.17 ^b
intron 1	0.64	0.85
exon 2	0.62	0.83
β -actin	0.75	1.0

Densitometric scanning of several different exposure times (to allow comparisons within the linear range) of the autoradiographs from the nuclear run-on transcription assay shown in Fig. 2. Ratios of the densitometric values of dexamethasone-treated versus untreated cells are shown.

^a actual densitometric ratio.

^b densitometric ratios normalized to β -actin transcription levels.

2.5×10^8 nuclei per milliliter in nuclear freezing buffer (40% glycerol, 50 mM Tris-HCl (pH 8.3), 5 mM MgCl_2 , 0.1 mM EDTA, 1.5 mM DTT, 200 U/ml RNasin) and 0.210 ml aliquots were immediately frozen in liquid nitrogen. Transcription assay was carried out as described by Linial et al. (26), using 5×10^7 nuclei and 0.5 mCi [^{32}P]UTP (3000 Ci/mmol, Amersham) and labeled RNA was isolated.

Hybond N filters (Amersham Int. UK) containing 1 μ g of the indicated purified inserts or plasmid DNA were prepared with Schleicher & Schuell Dot Blot apparatus under conditions suggested by the manufacturer except that wells were washed

Fig. 3. Increased decay of c-myc mRNA induced by dexamethasone. Cells were either untreated (control) or treated with dexamethasone (DEX) for 10 h. Actinomycin D (Act D) was then added, aliquots of the cultures were recovered and cytoplasmic RNA was isolated and analysed by Northern blotting. Autoradiographs corresponding to the DEX-treated cells are overexposed in the figure to allow a direct comparison to those of control cells. Autoradiographs of several different exposure times were subjected to densitometric scanning to allow comparisons within the linear range and values were normalized to β -actin mRNA levels evaluated at each time in the same blot using the same procedure. These densitometric data are represented in the right panel of the figure, which shows the percent of c-myc mRNA after the addition of actinomycin D (arrow) relative to the time 0 considered as 100%. The half-lives ($t_{1/2}$) of c-myc RNA decay were calculated from the line of best fits determined by the method of least squares after the initial lag in decay.

with $10\times$ SSC. Prehybridization and hybridization conditions were carried out as described by Linial et al.(26). All hybridization reactions contained the same nuclear equivalents of isolated RNA.

After hybridization, filters were washed with four changes of $2\times$ SSC/0.1% SDS at 65°C for 1 h and with two changes of $0.1\times$ SSC/0.1% SDS over 1 h at 60°C . Filters were then washed briefly with two changes of $2\times$ SSC at room temperature, dried and exposed to Kodak XAR films at -80°C with intensifying screens.

RESULTS AND DISCUSSION

Treatment of CEM-1.3 cells with $1\text{ }\mu\text{M}$ dexamethasone was followed by a significant decline of c-myc mRNA expression reaching barely detectable levels 24 h after hormone administration (Fig. 1A and B). The dexamethasone-induced decrease of the steady-state levels of c-myc mRNA was observed as early as 3 h after hormone treatment (Fig. 1A and B). The dexamethasone effect on c-myc mRNA was not due to a non specific cytotoxic effect of the hormone on CEM cells, since the hormone did not affect β -actin mRNA levels (Fig. 1A) or ribosomal RNA cell yield or cell viability during the period studied (not shown). Furthermore, the dexamethasone effect is rapid and preceded any detectable decrease of cell proliferation, evaluated by $[^3\text{H}]$ thymidine uptake, which only significantly decreased (by 38%) after 24 h of hormone treatment (not shown). The transcription of c-myc gene is mainly initiated from two promoters (P1 and P2) which are differentially used in various cell types (26–31) or conditions modulating the oncogene expression (32). Consequently we sought to investigate if dexamethasone was able to affect the levels of c-myc transcript from either P1 or P2 promoters. Fig. 1C shows that c-myc transcript uses preferentially P1 promoter in CEM cells.

Fig. 4. Effect of cycloheximide on the dexamethasone-induced decrease of c-myc gene expression. Cells were treated for 3 h with dexamethasone (DEX) and/or $5\text{ }\mu\text{g/ml}$ cycloheximide (CHX) as indicated in the figure and cytoplasmic RNA was isolated and analyzed by Northern blotting.

Dexamethasone significantly inhibited both P1 and P2 at the same extent, suggesting that the hormone does not regulate differentially the two c-myc promoters.

The transcription of the c-myc is regulated at the levels of both initiation and elongation of the transcript. A region near the exon 1/intron 1 junction has been described to be responsible for the block of the elongation of the c-myc transcript (18,22). To investigate the potential dexamethasone effects on c-myc transcription rates, we carried out nuclear run-on assays. Using single-strand DNA probes spanning the most upstream P0 promoter, the exon 1, the intron 1 and the exon 2, we wanted to determine if dexamethasone was able to decrease either or both

the initiation or the elongation of the c-myc transcript. As previously reported in other cell lines (18,22), the first exon was transcribed to a higher extent than the second one, suggesting a predominant attenuation of c-myc transcription in CEM cells (Fig. 2). A 18 h-dexamethasone treatment of CEM cells did not significantly decrease the transcription of exon 1. A slight decrease of the elongation of the transcript through exon 2 was observed (Fig. 2 and Table 1). However, after normalizing the data for transcription of the β -actin gene (Table 1), this hormone-induced inhibitory effect on c-myc transcript elongation only accounted for by 15%. Consequently, our results suggest that altered rate of RNA transcription does not significantly account for the dexamethasone-induced inhibition of c-myc transcript steady-state levels. Our data also suggest that the hormone-mediated control of c-myc mRNA levels might occur mainly at the post-transcriptional level.

In order to investigate if dexamethasone was able to influence the stability of c-myc mRNA, we treated CEM cells with actinomycin D to block gene transcription and followed the decay of c-myc mRNA levels in the presence or in the absence of dexamethasone. RNA synthesis was inhibited by about 90% at the concentration of actinomycin D used (5 μ g/ml). Fig. 3 shows the kinetics of disappearance of c-myc mRNA in actinomycin D-treated cells. In agreement to data previously reported (33), the half-life of c-myc mRNA was fast ($t_{1/2}$: 20 min). A 10 h-dexamethasone treatment was able to significantly increase the rate of c-myc mRNA specific disappearance ($t_{1/2}$: 10 min) (Fig. 3), while the stability of β -actin mRNA was not affected by the hormone (not shown). This suggests that dexamethasone controls c-myc gene expression primarily at the post-transcriptional level.

In order to study whether dexamethasone was able to control c-myc mRNA decay through the synthesis of a putative regulatory protein, we studied the effect of cycloheximide treatment of CEM cells on the dexamethasone-mediated decreased expression of c-myc gene. We observed that cycloheximide was able to overcome the dexamethasone-induced effect on c-myc mRNA, since the hormone treatment did not decrease c-myc mRNA levels compared to cycloheximide-treated, dexamethasone-untreated cells (Fig. 4). These data suggest that ongoing protein synthesis is necessary to elicit the hormonal effect.

The involvement of glucocorticoid receptor in the dexamethasone-induced inhibition of c-myc expression is suggested by the recently described failure of glucocorticoid hormone to decrease c-myc mRNA levels in glucocorticoid receptor-deficient CEM cells or cells bearing defective receptors (34). c-myc downregulation by dexamethasone was however observed after restoration of functional glucocorticoid receptor (34). Steroid receptor-mediated regulation of gene expression has been described to occur at the transcriptional and post-transcriptional levels (6,35). Our data provide the first evidence that the down-regulation of the human c-myc proto-oncogene by the hormone-driven glucocorticoid receptor occurs primarily at the post-transcriptional level. Although a slight decrease of the c-myc transcript elongation has been reported to be induced by dexamethasone, this does not seem to be a major regulatory pathway affected by the hormone in CEM cells. The overcoming of the dexamethasone-induced effect on c-myc transcript by protein synthesis blocking we have reported in this paper suggests the involvement of a hormone-induced regulatory protein in mediating the effect on c-myc mRNA stability.

Regulation of c-myc gene expression is mediated by a complex

array of transcriptional and post-transcriptional events. Agents which lead to growth arrest and cell differentiation decrease c-myc transcript elongation and mRNA stability (23). As a matter of fact, we have reported that dexamethasone, which is known to be a potent growth-inhibitory agent for lymphoid cells, is able to down-regulate c-myc primarily at a post-transcriptional level. Retinoic acid and estrogens have also been previously reported to regulate c-myc post-transcriptionally (13,23). Since all of these agents (retinoic acid, estrogens, dexamethasone) control cell functions by interacting with receptors belonging to the same erbA oncogene-related gene family, this suggests that they could share post-receptor molecular mechanisms regulating c-myc expression and eventually cell proliferation. However, Forsthoefel and Thompson have recently reported that dexamethasone inhibits both the initiation of transcription of c-myc and cell growth in murine lymphosarcoma P1798 cells (36). This discrepancy with our results could be a consequence of species-related differences in the pathways regulating c-myc gene. Cell-specific differences could also play a role, since retinoic acid has been shown to inhibit post-transcriptionally c-myc in murine F9 cells (23). The identical down-regulation of c-myc by single agents (i.e. dexamethasone) in spite of different molecular mechanisms (either transcriptional or post-transcriptional) further supports the heterogeneity and complexity of the regulatory pathways controlling the expression of this proto-oncogene and provide an experimental model for their dissection.

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