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Glucocorticoid mediated transcriptional repression of c-*myc* in apoptotic human leukemic CEM cells

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Abstract

Suppression of c-*myc* has been implicated as a critical event in some glucocorticoid-evoked apoptotic systems. It is therefore of interest to understand the mechanism of glucocorticoid-regulation of the c-*myc* gene. In the present study, a detailed analysis of dexamethasone (Dex)-evoked regulation of the human c-myc gene in human leukemic CEM-C7 cells has been performed. Dex suppresses c-*myc* mRNA and immunoreactive protein expression in clone CEM-C7 and subclone CEM-C7-14 cells. Nuclear run-on assays suggested that the regulation occurred at the level of transcription initiation. The half-life of c-*myc* mRNA was approximately 30 min and its stability was not affected by Dex treatment. In addition, Dex suppressed luciferase gene expression driven by −2052 to +34 bp c-*myc* promoter in transfected CEM-C7-14 cells. This result further supports that c-*myc* gene is suppressed by Dex at the transcriptional level in apoptotic human leukemic cells. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Glucocorticoids; c-*myc*; Apoptosis; Lymphoid; CEM cells

1. Introduction

The proto-oncogene c-*myc* is important for both cell proliferation and cell death [1]. Suppression of c-*myc* has been found to be part of various apoptotic induction pathways [2–8]. By using antisense and over-expressed c-*myc*, it has been demonstrated that rapid, strong suppression of c-*myc* is critical for glucocorticoid-induced apoptosis of sensitive clones of human leukemic lymphoblasts [2]. This occurs well before the cells start to become apoptotic. However, it is not clear how c-*myc* is regulated by glucocorticoids.

The product of c-*myc* gene is a 64–67 kDa nuclear transcription factor c-Myc, part of a complex family of proteins important for cell growth and viability [9]. The half-life of both c-*myc* mRNA and c-Myc protein is very short, varying between 30 and 60 min in different systems. The regulation of the c-*myc* gene and its products occurs by various mechanisms, including transcriptional, post-transcriptional, translational and combinations of these [1,10]. In vivo, the c-*myc* gene is transcribed from promoters P_0, P_1, P_2 and P_3, but in most cells, the P_1 and P_2 promoters within the untranslated exon I are the two major promoters, with the P_2 promoter predominating [10,11]. In addition to transcriptional initiation, the c-*myc* gene can be regulated by attenuation of its transcription. Sequences located at the 3′ end of exon I provide a signal leading to the failure of retention of RNA polymerase, thereby controlling mRNA elongation [12,13]. There are also known post-transcriptional mechanisms of control of c-*myc* mRNA, and c-Myc protein stability can be regulated independently.

Glucocorticoids exert a classic apoptotic effect on lymphoid cells, and several oncogenically transformed cell lines have been used to study it. Often glucocorticoids have been shown to suppress the expression of c-*myc* in such cell lines, including mouse S49 [14] and P1798 [15], and human CEM-C7 cells [16]. When active glucocorticoid receptor (GR) is provided by transfection, the expression of c-*myc* is also reduced by glucocorticoids in human Jurkat cells [4]. Studies of the mechanism of glucocorticoid down-regulation of c-*myc*

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have produced contrasting results. In P1798 cells, such regulation has been shown to be primarily at a transcriptional level [11,15]. In CEM 1.3 cells, however, Maroder et al. reported that the glucocorticoid dexamethasone (Dex) did not affect transcription, but mainly altered the stability of c-*myc* mRNA [17]. Because of the discrepancy in these results, we investigated further the control of c-*myc* by Dex, using CEM-C7 cells. In this paper it is demonstrated that in these cells c-*myc* gene expression is primarily regulated by Dex at the transcriptional level.

2. Materials and methods

2.1. Cell lines

The glucocorticoid-sensitive cell clone CEM-C7 [18] was derived from the CCRF-CEM cell line, grown from a female patient with acute lymphoblastic leukemia. CEM-C7-14 is a subclone of CEM-C7 cell clone. The subclone has the same phenotype as the original CEM-C7 cells, including diploid karyotype, growth, sensitivity to Dex, and GR content.

2.2. Protein extraction and Western blot

Cells were washed with isotonic phosphate-buffered saline (PBS) at 4°C and resuspended in lysis buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 20 μM leupeptin, and 400 μM 4-[2-aminoethyl] benzensulfonyl fluoride. The cellular lysates were centrifuged at 30 000 rpm for 30 min, at 4°C using Beckman TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatants were removed to fresh tubes. Protein concentration of the extract was then estimated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). In all samples, 2-mercaptoethanol was added to a final concentration of 5%. Whole cell extracts containing equal amounts (50 μg) of protein were electrophoresed in 10% polyacrylamide minigels (Bio-Rad) containing SDS and transferred to nylon membranes (Bio-Rad) using a semidry electroblotter (Integrated Separation Systems, Hyde Park, MA). After incubation in 10% dry milk powder in PBS, the membranes were incubated sequentially in PBS containing a solution of 5% powdered milk and the c-Myc monoclonal antibody and HRP conjugated secondary antibody. The monoclonal c-Myc antibody Myc1-9E-10.2, raised against a synthetic peptide in the COOH-terminal region of c-Myc, was generated as a culture supernatant of the hybridoma cell line CRL1729, purchased from American Type Culture Collection (Rockville, MD). After washing with PBS, the membranes were incubated for 5 min in the horseradish peroxide substrate (Pierce Chemical Company, Rockford, IL), and exposed to photographic film (Eastman Kodak Company, Rochester, NY) using various times of exposure to assure that evaluations for quantification of the signals were within the linear response range. The densitometric analysis of c-Myc protein was performed using an Image Analyser (Applied Imaging Corporation, Santa Clara, CA).

2.3. Nuclei isolation and nuclear run-on assay

Nuclei from CEM-C7 cells were isolated according to published methods (Current Protocols in Molecular Biology, Supplement 26, 1994). Briefly, cells were cultured as described above, and resuspended in an iso-osmotic buffer (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–HCl, pH 8.0, 1 mM DTT, 0.5% [v/v] Nonidet P-40). The nuclei were obtained by layering the lysed cell suspension on a sucrose cushion (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–HCI, pH 8.0, 1 mM DTT) and subjecting the samples to ultracentrifugation at 19 000 rpm at 4°C in a SW 50.1 rotor, for 47 min with slow acceleration and deceleration. Nuclei were immediately frozen and stored at −70°C until used in the assays. Nuclear run-on transcription assay was carried out according to Mahajan [19] with minor revisions. In a final volume of 400 μl, 200 μl of the nuclear suspension was mixed with the nuclear run-on transcription mixture (0.5 M Tris–HCl, pH 7.8, 0.25 M NaCl, 1.75 M ammonium sulfate, 10 mM EDTA), 0.2 M $MnCl_2$, 20 mg/ml heparin, a mixture of ATP, GTP, and CTP (20 mM stock), and labeled UTP (600 Ci/mmol, ICN Pharmaceuticals, Costa Mesa, CA). The reaction mixture was incubated at 30°C for 45 min followed by the addition of yeast tRNA (1 mg/ml), 20 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, and a solution of DNase I with proteinase K (v/v, 1:1). The samples were incubated for 30 min at 37°C, RNA was extracted, and to the aqueous phase, 20% TCA was added, incubated on ice, centrifuged for 15 min at $10\,000 \times g$, and rinsed twice with ice cold 5% TCA. After dissolving the pellet, RNA was precipitated overnight, dissolved in RNase-free water, and aliquots were added to filters and counted in a scintillation counter. The labeled RNA was used for hybridization as follows. The linearized double stranded c-*myc* and actin plasmids were denatured and applied onto nitrocellulose using a slot blot apparatus. The filter was baked for 2 h at 80°C, prehybridized in hybridization solution (0.01 M TES, pH 7.4, 0.2% SDS, 0.01 M EDTA, 0.3 M NaCl, 1 × Denhardt's, 100 μg/ml yeast tRNA, 100 μg/ml poly A) at 65°C for 2–6 h, and hybridized in the same hybridization solution but with added labeled RNA ($1-5 \times 10^7$ cpm/ml) for 30–40 h at 65°C. The filters were washed with 2 × SSC at 65°C for 1 h followed by a 30 min wash at 37°C with 2 × SSC and 10 μg/ml

boiled RNase A. The filters were then exposed to a phosphorimager screen and the relative amounts of c-*myc* and β-*actin* nuclear run-on transcripts were quantitated by using the MD ImageQuant software (version 3.3, Molecular Dynamics, Sunnyvale, CA). Normalization was carried out by using β-*actin* as the internal control.

2.4. RNA purification and RNase protection assays

Cells were collected, washed in PBS (4°C), quickly frozen and stored at −70°C until all samples were ready for analysis. Total RNA was isolated using the TRIZOL Reagent according to the method provided by the manufacturer (GIBCO BRL, Gaitherburg, MD). This RNA was used for RNase protection assay. A plasmid pTRI-c-*myc* containing 251 bp c-*myc* cDNA was constructed. ^{32}P-labeled antisense c-*myc* RNA probe was synthesized by T7 promoter dependent RNA polymerase according to standard in vitro transcription protocols, and purified by gel electrophoresis. RNase protection assay was done with a kit from Ambion (Austin, TX). Basically RNA samples were mixed with the labeled probe. One-tenth volume of 5 M NH_4OAc and 2.5 volumes of EtOH were added to the mixtures. The mixtures were then put in −20°C for 15 min and pelleted by centrifugation. The pellets were resuspended in hybridization buffer, incubated at 42–45°C overnight, digested by RNase, and precipitated. The pellets were resuspended in gel loading buffer, denatured and subjected to electrophoresis. The gel was dried and exposed to a phosphorimager screen (Molecular Dynamics).

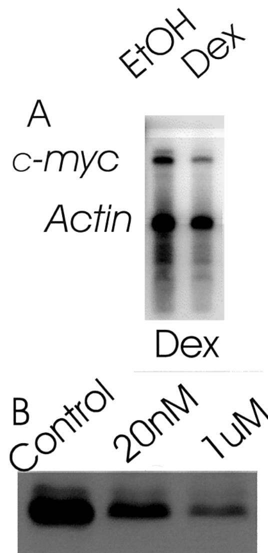

Fig. 1. Dexamethasone (Dex) suppresses both c-*myc* mRNA and protein in CEM-C7-14 cells. The CEM-C7-14 clone was subcloned from CEM-C7 cells. In panel A, CEM-C7-14 cells were treated with 20 nM of Dex or EtOH (control) for 24 h and then harvested. Total RNA samples were prepared and RNase protection assays performed. Protected c-*myc* and *actin* bands are shown. Panel B: CEM-C7-14 cells were incubated with 20 nM or 1 μM of Dex or ethanol (EtOH) for 24 h. Cells were collected and total protein extracted. Western blots were performed with monoclonal c-Myc antibody. The specific c-Myc band is shown. Data shown here are representative of multiple experiments.

2.5. Evaluation of the activity of human c-myc promoter

CEM-C7-14 cells were transfected with 15 μg of the human c-*myc* promoter-driven construct by electroporation [20]. The constructs, HBM-Luc and Mut-Luc, were kind gifts of Dr Linda Z. Penn (Department of Microbiology and Medical Biophysics at University of Toronto). HBM-Luc contains the sequence from −2052 to +34 of human c-*myc* promoter [21]. The negative control construct consists of mutant Mut-Luc sequence from −107 to +34 of the same promoter. After transfection, cells were selected with 400 μg/ml of G418. Recovered cells were incubated with EtOH or 1 μM of Dex for 24 h, then collected and lysed with 100 μl of lysis buffer provided in the luciferase assay kit from Promega (Madison, WI). After centrifugation at 13 000 × *g* for 20 min at 4°C, 20 μl of supernatant was analyzed for luciferase activity using the kit from Promega and a Packard LumiCount Luminometer (Downers Grove, IL). Results were normalized with protein content.

3. Results

3.1. Dex suppresses the expression of c-myc mRNA in CEM-C7 cells

It has previously been shown that both c-*myc* mRNA and protein are suppressed in the clonal CEM-C7 cells after treatment with Dex. Since then, to ensure purity of phenotype, CEM-C7 cells were recloned and the subclone CEM-C7-14 was used in the studies of the human c-*myc* promoter reported this paper. Clone CEM-C7-14 has virtually the same diploid karyotype, growth, sensitivity to Dex, and GR content as found in the original CEM-C7 clone (unpublished data). Here it is confirmed that in the subclone, as in the mother clone CEM-C7 c-*myc* mRNA is reduced by Dex, using the RNase protection method. It is then further shown that c-Myc protein is suppressed in a dose-dependent fashion, as shown by immuno-blot (Fig. 1A,B).

3.2. Dex suppresses c-myc at the transcriptional level

CEM-C7 cells were harvested and their nuclei isolated 0, 3, 6, 12, 24 h after treatment with 1 μM Dex. Using the full-length c-*myc* cDNA, nuclear run-on as-

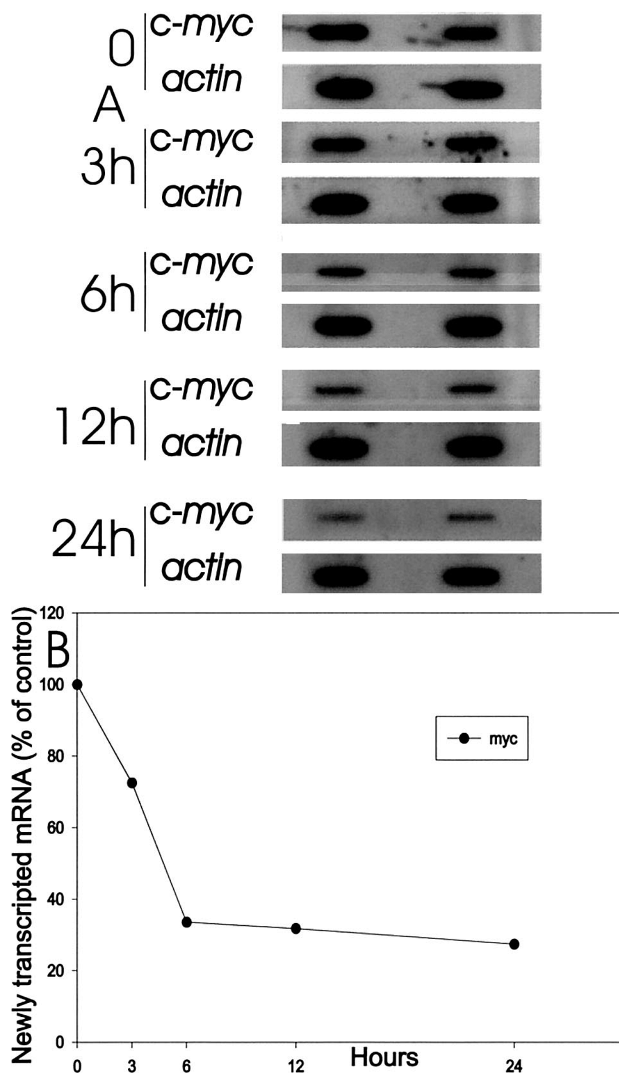

Fig. 2. Dexamethasone (Dex) suppresses c-*myc* gene transcription. CEM-C7 cells were grown in 1 μM Dex and collected at 0, 3, 6, 12 and 24 h after the addition. Nuclei were isolated and nuclear run-on assays performed. Plasmids containing full-length c-*myc* cDNA or β-*actin* (control) were used as probes. Panel A: A representative slot blot of one of three independent experiments is shown (duplicate samples). Panel B: The specific bands for c-*myc* from Panel A were quantified and normalized with corresponding *actin* bands.

says were performed. At all the time points tested, the newly synthesized c-*myc* mRNA levels were decreased in nuclei from the Dex-treated cells (Fig. 2). A region near the exon 1/intron 1 junction has been reported as the region responsible for the *elongation attenuation* of c-*myc* mRNA transcription. Since the probe which was used was the full-length c-*myc* cDNA, it was possible that the decrease of c-*myc* mRNA transcripts was due to attenuation of c-*myc* mRNA transcription. In order to rule out this possibility, a plasmid containing the 5′ flanking region and half of the first exon of the c-*myc* gene was employed. This plasmid will only bind to c-*myc* mRNA corresponding to the region prior to the attenuation point in the c-*myc* gene. With it, one could determine whether Dex affected the initiation of c-*myc* transcription, and not down-stream effects on elongation or attenuation. At the time points indicated, nuclei were isolated and nuclear run-on experiments performed. Newly synthesized c-*myc* mRNA was suppressed to about the same extent as found with the full length cDNA probe of c-*myc* (Fig. 3), suggesting that transcriptional initiation was primarily affected by Dex treatment.

3.3. Dex does not stablize c-myc mRNA

In order to determine the effect of Dex on the stability of c-*myc* mRNA, RNase protection assays were employed to determine the half-life of c-*myc* in

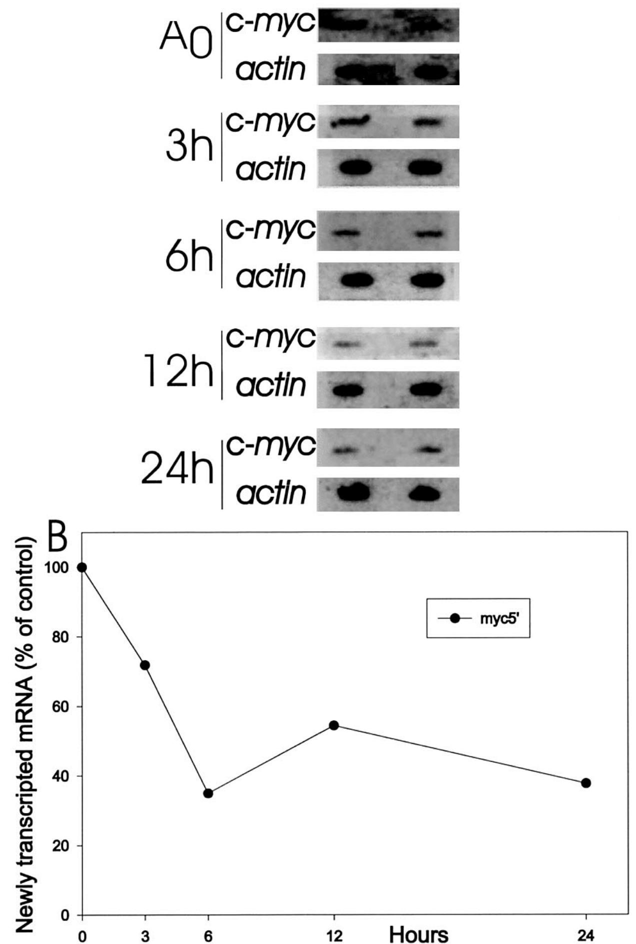

Fig. 3. Dexamethasone (Dex) suppresses transcription initiation of the c-*myc* gene. CEM-C7 cells were treated with 1 μM Dex for 0, 3, 6, 12 and 24 h and cells collected. Nuclei were isolated and nuclear ran-on assays performed. A plasmid containing 148 bp of 5′ flanking region and 339 bp of the first exon of c-*myc* or β-*actin* (control) were used as probes. Panel A: A slot blot with duplicate samples is shown. Panel B: The bands from panel A were quantified, normalized with corresponding *actin* bands, and data were plotted as a line graph. A representative of two independent experiments is shown.

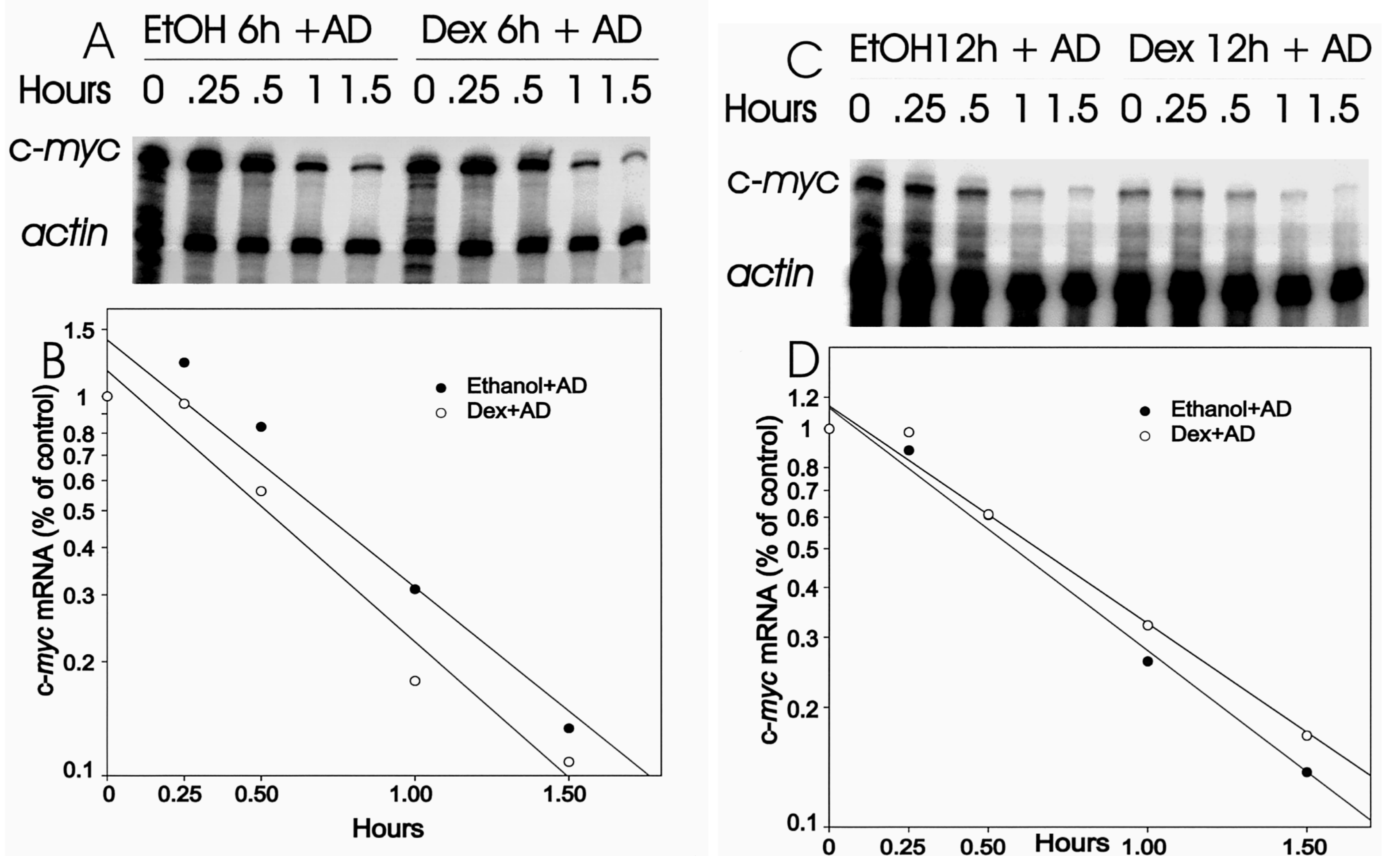

Fig. 4. Dexamethasone (Dex) does not alter the stability of c-*myc* mRNA. CEM-C7 cells were treated with EtOH or 1 μM Dex for 6 (A, B) or 12 h (C, D). Cells were then treated with actinomycin D (3 μg/ml) and collected 0, 0.25, 0.5, 1, 1.5 h later. Total RNA was isolated and RNase protection assays performed with full length human c-*myc* and β-*actin* (control) probes. In panel A and C, the specific *myc* and *actin* protected bands are indicated. In Panel B and D, the specific *myc* bands were quantified and normalized with the corresponding *actin* bands. Data are plotted in a semi-log scale. The half-life of cmyc was determined for Dex- or EtOH-treated cells, in each case.

cells grown with or without Dex. After incubation with 1 μM Dex or EtOH for 6 or 12 h, cells were treated with actinomycin D to block further RNA synthesis. Cells were collected at 0, 0.25, 0.5, 1, and 1.5 h after treatment with actinomycin D and RNA was prepared. The level of c-*myc* mRNA was determined by RNase protection assays. Fig. 4 shows that the half-life of c-*myc* mRNA is equal in the cells treated with Dex or EtOH vehicle. The half-life was 30 min at both time points which were tested.

3.4. Dex suppressed the activity of a luciferase reporter gene driven by human c-myc promoter

Further proof that Dex regulates c-*myc* gene primarily at the transcriptional level comes from studies of the c-*myc* promoter-reporter gene. A plasmid carrying the sequence spanning -2052–$+34$ (relative to P_2) of the human c-myc promoter driving a luciferase reporter gene (Fig. 5A) was transfected into CEM-C7-14 cells along with a neomycin resistance conferring construct, PMTneo. After selection with 400 μg/ml of G418, the mass cell culture was treated with 1 μM Dex or EtOH vehicle for 24 h. Cells then were collected and lysed. Luciferase activities were determined for each sample and normalized with its protein concentration. In Fig. 5B, the data show clearly that Dex suppressed the c-*myc* promoter-driven luciferase activity by 50%. This further confirmed that the c-*myc* gene is primarily regulated by Dex at the transcriptional level, and provides initial evidence that a significant part of the regulatory region resides within the promoter fragment used. Dex showed minimal repression of luciferase activity from a shorter, mutant *myc* promoter construct, employed as a related non-specific promoter. It was concluded that the observed Dex repression requires sequences found in the non-mutated, longer c-*myc* promoter.

4. Discussion

In contrast to growth factor-dependent epithelioid and fibroblastic cells, in most lymphoid cell systems

tested, apoptosis triggered by a variety of agents is preceded by a severe down-regulation of c-*myc* message and protein expression. Examples include glucocorticoid-evoked apoptosis of mouse S49 cells and human CEM and Jurkat cells [4,14,16]. Though the precise pathway by which c-*myc* suppression causes apoptosis has not been deciphered, the possibilities include loss of the Myc repression of death genes and/or down-regulation of vital genes that require Myc for activation.

Suppression of c-*myc* has been shown to be critical in the glucocorticoid-induced apoptotic pathway in human leukemic lymphoblasts of the CEM line. Both mRNA and protein of c-*myc* have been shown to be rapidly and deeply down-regulated by glucocorticoids in the receptor-positive, glucocorticoid-lysed CEM-C7 cells, but not in CEM-C1 cells, which have intact glucocorticoid receptors but resist glucocorticoid-induced apoptosis [22] or in ICR-27 cells, which lack functional receptors [23]. In CEM-C7 cells an antisense c-*myc* oligomer has been shown to block the expression of c-*myc* and induce cell death. Moreover, over-expression of c-*myc* driven by heterologous promoters all rescued Dex-induced cell death in CEM-C7 cells [2]. These observations strongly suggest that suppression of c-*myc* is crucial to glucocorticoid-induced apoptosis in this system. To be able to understand fully how c-*myc* is involved in this apoptotic pathway and especially what the upstream events of suppression of c-*myc* are, one has to know how c-*myc* gene is regulated.

Studies of various systems have shown that the expression of c-*myc* can be regulated at several levels, including transcription, post-transcription, and translation. In mouse lymphoid P1798 cells, Dex did not affect the stability of *myc* mRNA, but instead showed suppression at transcriptional initiation (shown by nuclear run-on assay) [15]. However, a study of CEM cells designated CEM 1.3 yielded data that Dex had no effect on transcription initiation. In that paper, assays

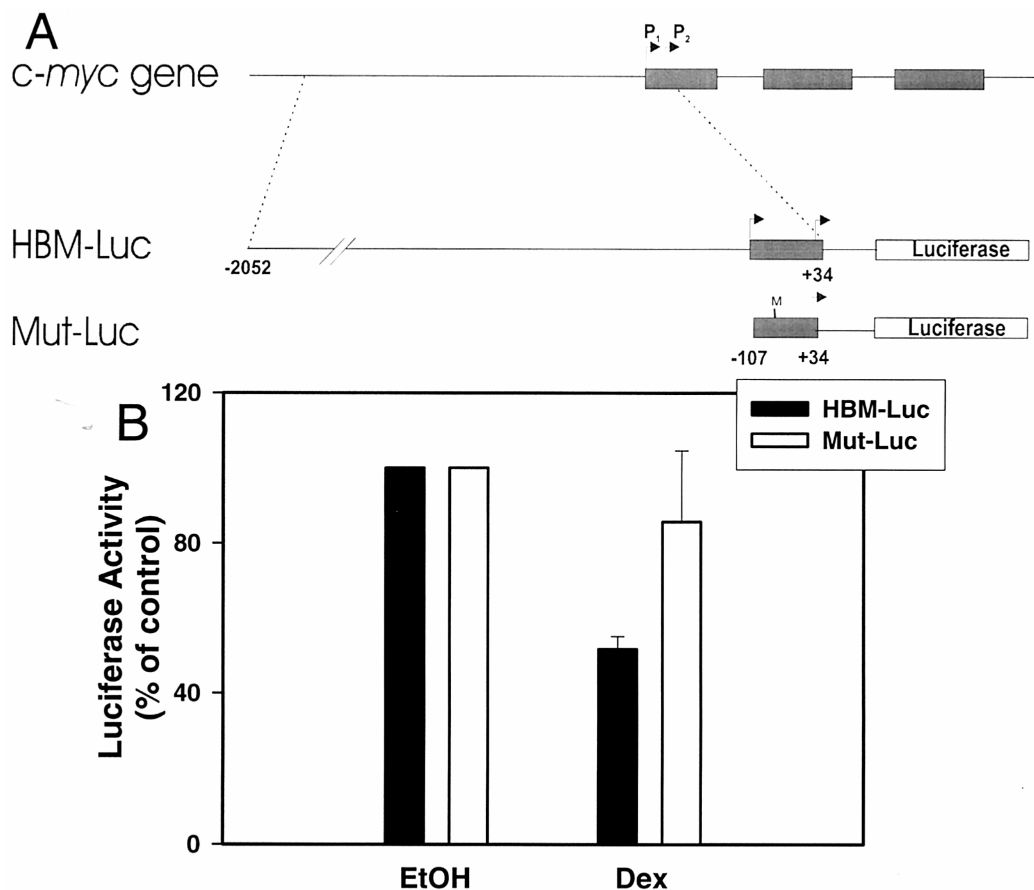

Fig. 5. Dexamethasone (Dex) suppresses human c-*myc* promoter-driven luciferase activity. CEM-C7-14 cells were cotransfected with pMTneo and HBM-Luc or Mut-Luc. Transfected cells were selected with 400 μg/ml of G418. Mass cultures were then treated with EtOH or Dex for 24 h and analyzed for luciferase activity, using the luciferase assay kit from Promega. Panel A: Schematic illustration of human c-myc genes with three exons (Gray boxes) and the two major start sites (P_1 and P_2) shown. The construct HBM-Luc contains the sequence corresponding to −2052 to +34 of the c-*myc* promoter relative to the P_2 Start site, driving a luciferase reporter gene. Mut-Luc contains a mutant sequence corresponding to −107 to +34 of the same promoter driving luciferase. Panel B: Luciferase activities were measured in cells transfected with either HBM-Luc or Mut-Luc and treated for 24 h with ethanol or 1 μM Dex. Data are average ± S.D. from three independent experiments.

were reported on nuclear run-on and mRNA half-life, each at a single time point after Dex administration [17]. More thorough analyses have been done in CEM-C7 and subclone CEM-C7-14 cells. Nuclear run-on assays were carried out at multiple time points up to 24 h after Dex treatment. Using a full length c-*myc* cDNA probe we show that transcription of c-*myc* is clearly suppressed by Dex to a minimum 30% of the control level at 6 h and remains at that level up to 24 h. To confirm that this suppression is at the level of transcription initiation, a plasmid containing some 5′ region and half of exon 1 of c-*myc* was employed, as a probe designed so as to measure transcripts preceding the major known control point for elongation of c-*myc* transcripts. Newly synthesized c-*myc* mRNA is reduced by Dex treatment using this probe, to about the same extent as that observed using the full-length cDNA probe of c-*myc*. This clearly demonstrates that in the cells studied the c-*myc* gene is suppressed at the transcription initiation level. In addition, the half-life of c-*myc* in cells treated with or without Dex for 6 and 12 h was followed. At both time points, the half-life of c-*myc* is about 30 min, identical in EtOH (vehicle) or Dex treated CEM-C7 cells. Moreover, a luciferase plasmid under the control of the region from -2052 to $+34$ of the c-*myc* promoter was transfected into CEM-C7 cells. The luciferase activity was suppressed by Dex to about 50% of the control. All these data are consistent and suggest that Dex regulates c-*myc* gene at the transcriptional, not the post-transcriptional, level in CEM-C7 cells. One cannot explain the discrepancy from the earlier report, save that CEM cells used in the prior report was designated CEM-1.3. No source and history of those CEM cells are given; therefore the relationship between CEM-1.3 and CEM-C7 cells is not clear. Hence, the discrepancy in results might be explained by the difference between cells, or by phenotypic drift and genotypic diversity, if CEM-1.3 cells share the origin of CEM-C7 cells. It does seem significant that in both rodent P1798 cells and human CEM-C7 cells, the negative regulation of c-*myc* by Dex was transcriptional. It was suspected that this is the general case.

Acknowledgements

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