PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 75 (2000) 91–99

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

The delayed induction of c-*jun* in apoptotic human leukemic lymphoblasts is primarily transcriptional

Feng Zhou [1], Rheem D. Medh, Weiping Zhang, Naseem H. Ansari, E. Brad Thompson *

Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX 77555-0645, USA

Received 9 May 2000; accepted 5 October 2000

Abstract

Because of their ability to induce lymphoid cell apoptosis, glucocorticoids have been used for decades to treat certain human leukemias and lymphomas. Studies presented in this paper complement our previous work demonstrating that sustained induction of the proto-oncogene c-*jun* plays a crucial role in the glucocorticoid-induced apoptotic pathway in CEM cells, human leukemic lymphoblasts. Results from measurements of c-*jun* mRNA half-life with RNase protection assays and of transcription by nuclear run-on assays indicate that, in the dexamethasone-sensitive cloned CEM-C7 cells, c-*jun* is induced at the transcriptional level. Consideration of time-course, however, suggested that this might be a secondary or possibly a delayed primary response. Use of cycloheximide to block protein synthesis strongly induced c-*jun* mRNA, suggesting that there had been relief from a labile protein repressor of transcription. Comparing the level of induction by cycloheximide with that of dexamethasone indicated that the two did not induce by an identical mechanism. The high induction by cycloheximide obscured simple interpretation of elevated c-*jun* mRNA levels after concomitant administration of cycloheximide and dexamethasone. This was resolved by nuclear run-on experiments, which showed that the dexamethasone induction of c-*jun* mRNA in this system does require protein synthesis.

Keywords: Glucocorticoids; c-*jun* Gene regulation; Apoptosis; Lymphoid; CEM cells

1. Introduction

The sustained induction of c-*jun* has been implicated as a causative signal in various apoptotic pathways [1–5]. We previously reported data suggesting that c-Jun, the protein coded by the c-*jun* proto-oncogene, is critical to the glucocorticoid-evoked apoptosis of a human leukemia lymphoblast line [6]. The evidence showed that: (1) c-*jun* mRNA and c-Jun protein, but not c-Fos, JunB or JunD, are induced by dexamethasone (Dex) in CEM lines that are sensitive to glucocorticoid-dependent apoptosis; (2) c-*jun* induction occurs before overt apoptosis; (3) among CEM clones, a correlation exists between high-level expression of c-*jun* and glucocorticoid-evoked apoptosis; and (4) antisense c-*jun* blocks both Dex induction of c-*jun* mRNA and apoptosis.

The proto-oncogene c-*jun* is an immediate early response gene. Thus, when resting cells are stimulated to grow, a transient burst of c-*jun* transcription occurs shortly after addition of the stimulus. In cycling cells, c-*jun* is expressed briefly during a part of the G_1 phase of cell cycle. The effects of c-*jun* transcription are manifested through its coded protein c-Jun, part of the AP-1 transcription complex family of proteins [7]. Inappropriate, often prolonged expression of c-Jun has been found correlated with apoptosis and, in several cases, blocking this expression has been protective [3,6].

When glucocorticoids are added to cultures of cloned CEM-C7 cells, several early molecular events occur. These include rapid downregulation of c-*myc* [8,9], followed in a few hours by induction of c-*jun* mRNA and protein [6]. This continues to increase to a maxi-

* Corresponding author. Tel.: +1-409-7722271; fax: +1-409-7725159.

E-mail address: bthompso@utmb.edu (E.B. Thompson).

[1] Present address: National Flow Cytometry Resource, Life Science Division, LS-5, M-888, Los Alamos, NM 87545, USA.

0960-0760/01/$ - see front matter
PII: S0960-0760(00)00161-8

mum, plateauing at around 24 h. About that time, cells begin to show several biochemical and gross morphologic manifestations of apoptosis [10], which increase thereafter, until most cells are dead. As long as agonist steroid is present and cells are viable, the c-Jun levels remain high. The pattern of delayed induction displayed by c-*jun* is atypical for a 'primary' induced gene. It is known that genes simply and directly induced by glucocorticoids show an increase in transcription in a short time after addition of steroid (see Ref. [11] and references cited therein). Activation of the glucocorticoid receptor (GR) and its nuclear translocation take only minutes [12]. The delay of ≥ 6 h before accumulation of mRNA over basal levels, as is seen with c-*jun* induction [6], suggests either a very large initial pool of mRNA (so it is a while before total c-*jun* mRNA can be seen to increase), very weak induction, delayed post-transcriptional regulation, or 'secondary' induction. In secondary induction, either a necessary factor must be induced or a repressor destroyed before the mRNA measured can increase. The present studies were designed to distinguish between these general mechanisms, specifically to test whether induction of c-*jun* was by transcriptional or post-transcriptional mechanisms and, if transcriptional, whether by the primary or secondary mode.

2. Materials and methods

2.1. Cell lines

The glucocorticoid-sensitive cell clone CEM-C7 was derived from CCRF-CEM, obtained from a female patient with acute lymphoblastic leukemia [13]. ICR-27 cells are a clonal derivative of CEM-C7 cells, obtained after mutagenesis and selection for resistance to high-dose Dex and have a defective GR [14,15].

2.2. Protein extraction and Western blot

Cells were washed with phosphate-buffered saline (PBS) (4°C) and resuspended in a protease inhibitor cocktail (50 mM Tris (pH 7.4), 0.6 M NaCl, 0.4 mM AEBSF, 80 μM leupeptin). Cells were lysed by performing three cycles of freezing for 5 min in an alcohol/dry ice mixture followed by thawing for 30 min in a 4°C ice water bath. The cellular lysates were centrifuged at 30,000 r.p.m. for 30 min, at 4°C using a 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 Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Whole cell extracts containing 50 μg protein were electrophoresed in 12% polyacrylamide minigels (Bio-Rad) containing sodium dodecyl sulfate (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 at 4°C in PBS containing a solution of 5% powdered milk with the c-Jun polyclonal antibody source. After washing with PBS, the membranes were incubated for 5 min in the horeradish peroxide substrate (Pierce Chemical Company, Rockford, IL), and developed on film 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-Jun protein was performed using a Lynx densitometer (Applied Imaging, Santa Cruz, 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 already described and resuspended in an iso-osmotic buffer (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 10 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol (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–HCl (pH 8.0), 1 mM DTT) and subjecting the samples to ultracentrifugation at 19,000 r.p.m. 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 use. Nuclear run-on transcription assays were carried out according to Mahajan and Thompson [16]. Briefly, in a final volume of 400 μl, 200 μl 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 adenosine triphosphate, guanosine triphosphate, and cytidine triphosphate (20 mM stock, 1 mM final concentration), and labeled uridine triphosphate (600 Ci/mmol, 150 μCi/reaction). 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. After incubating on ice, the samples were centrifuged for 15 min at $10{,}000 \times \boldsymbol{g}$, 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-*jun* and

actin plasmids vector or a single-strand plasmid were denatured and applied onto nitrocellulose using a slot blot apparatus. The filter was baked for 2 h at 80°C, prehybridized in the 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-*jun* 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 using β-*actin* as the internal control.

2.4. Determination of caspase activity

Caspase activity was measured as described by Sarin et al. [17] with slight modifications. CEM-C7 cells treated with or without 100 nM Dex were harvested and 2.5×10^5 cells were lysed in 50 μl buffer containing 50 mM HEPES (pH 7.5), 10% sucrose, 0.1% Triton X-100 and 10 μM DTT. Substrates for measurement of caspase activity were obtained from Enzyme Systems Products (Livermore, CA). Caspase 1, 2, 3 and 8 were measured using substrates Z-YVAD-AFC, Z-VDVAD-AFC, Z-DEVD-AFC and Z-IETD-AFC, respectively. The cell lysate (100 μl) was mixed with 50 μM appropriate fluorogenic substrate, incubated at room temperature for 1 h, diluted to 1 ml with PBS and the fluorescence of the AFC [7-amido-4-trifluoromethylcoumarin] released was measured using the F-4500 fluorescence spectrophotometer (Hitachi Instruments, San Jose, CA).

2.5. RNA purification and RNase protection assays

Cells were collected, quickly frozen and stored at −70°C until all samples were ready for analysis. Total RNA was isolated using the TRIzol reagent (Gibco/BRL Life Technologies, Grand Island, NY) according to the method provided by the manufacturer. A plasmid pTRI-c-*jun* containing 340 base pairs (bp) c-*jun* cDNA was constructed. ^{32}P-labeled antisense c-*jun* RNA probe was synthesized by T7 promoter-dependent RNA polymerase according to standard in vitro transcription protocols (Ambion, Austin, TX), and purified by gel electrophoresis. RNase protection assay was carried out with a kit from Ambion. Basically, RNA samples were mixed with the labeled probe. The mixtures were added with 5 M NH_4OAc and 2.5 volumes of EtOH and kept at −20°C for 15 min, then 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. Image bands were quantified by the MD IMAGEQUANT software.

3. Results

3.1. Induction of c-jun is receptor dependent

Since glucocorticoid activation of its receptor and subsequent nuclear translocation are rapid processes, delay in the induction of c-*jun* could be due to some non-traditional, receptor-independent mechanism. Previously, we showed that *c-jun* induction occurred at Dex concentrations consistent with those required to occupy the GR. In this paper, we have tested whether these receptors are required for the induction. ICR-27 cells, a receptor-defective subclone of CEM-C7, were employed because their failure to respond is due simply to lack of wild-type receptor. Their Dex resistance is due to the deletion of the normal GR allele [15]. Restoring GR to these cells by complementation or transfecting it via an expression plasmid restores their apoptotic response to Dex [11,18]. ICR-27 cells were therefore treated with 1 μM Dex or ethanol vehicle for up to 48 h, during which time they grew continuously. At intervals, samples were taken for immunochemical analysis of c-Jun. No induction was seen at any time. An example of one experiment is shown in Fig. 1. Clearly, Jun levels were not increased in ICR-27 cells; if

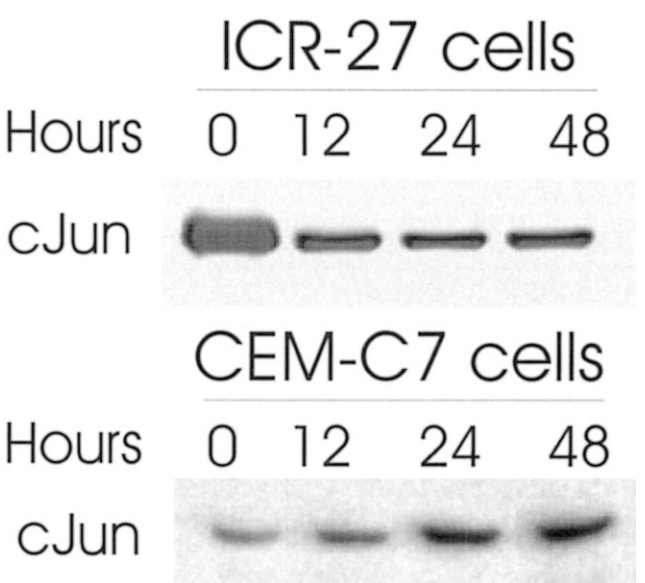

Fig. 1. Dex did not induce c-Jun in cells lacking functional GR, but did in cells with functional GR. ICR-27 (upper panel) or CEM-C7 (lower panel) cells were treated with 1 μM Dex. At 0, 12, 24 and 48 h, cell samples were extracted for immunoblot analysis of c-Jun levels.

anything, they were slightly reduced and were constant at all time points following Dex treatment. Control CEM-C7 cells treated in parallel showed the typical induction.

3.2. The expression of c-jun is regulated by Dex at the transcriptional level

To determine whether c-*jun* induction occurs at a transcriptional or post-transcriptional level, cell nuclei were isolated from CEM-C7 cells at 0, 6, 12, 24 h after addition of 1 μM Dex to the cultures. Nuclear run-on experiments were performed to test for induction of transcription. The results indicate that c-*jun* transcription increased beginning at 6 h and continued to rise thereafter, reaching an average maximum of 2.6-fold at 24 h (Fig. 2). In no experiment was evidence of transcription induction seen earlier than 6 h.

3.3. Capase activity increases after c-jun induction

Our previous work [6] showed that c-*jun* induction begins 6–12 h after Dex is added to CEM-C7 cells. In this paper, we confirm this result and show that this induction is transcriptional (Fig. 2). Overt apoptosis begins later, after about 24 h. To determine the chronological relationship between c-*jun* induction and the known predominant effector of apoptosis, the caspases, we assayed for changes in activity of caspases 1, 2, 3 and 8. The results showed (Fig. 3) that, whereas there was no change in activity of caspase 1 or 8, caspases 2 and 3 increased strongly between 24 and 36 h. These increases in activity were GR dependent, since they were not observed in the GR-deficient ICR-27 cells (data not shown).

3.4. Dex does not affect the stability of c-jun mRNA

Measurements of c-*jun* mRNA pools were performed to determine the half-life of the message after the addition of 1 μM Dex or ethanol (EtOH, control) for 6, 12, or 24 h. Fig. 4A–D shows the results from an experiment at the 12 and 24 h time points. The half-life of c-*jun* was identical in control and Dex-treated cells, although of course the absolute level of c-*jun* mRNA was much higher in the Dex-treated cells. The half-life of c-*jun* also showed no change after treatment of the cells with Dex 1 μM for 6 h (data not shown). In all cases, c-*jun* mRNA had a half-life of approximately 0.7 h.

3.5. Levels of c-jun mRNA were increased by cycloheximide

A classic test for secondary induction is to block protein synthesis while inducer is added; then to follow

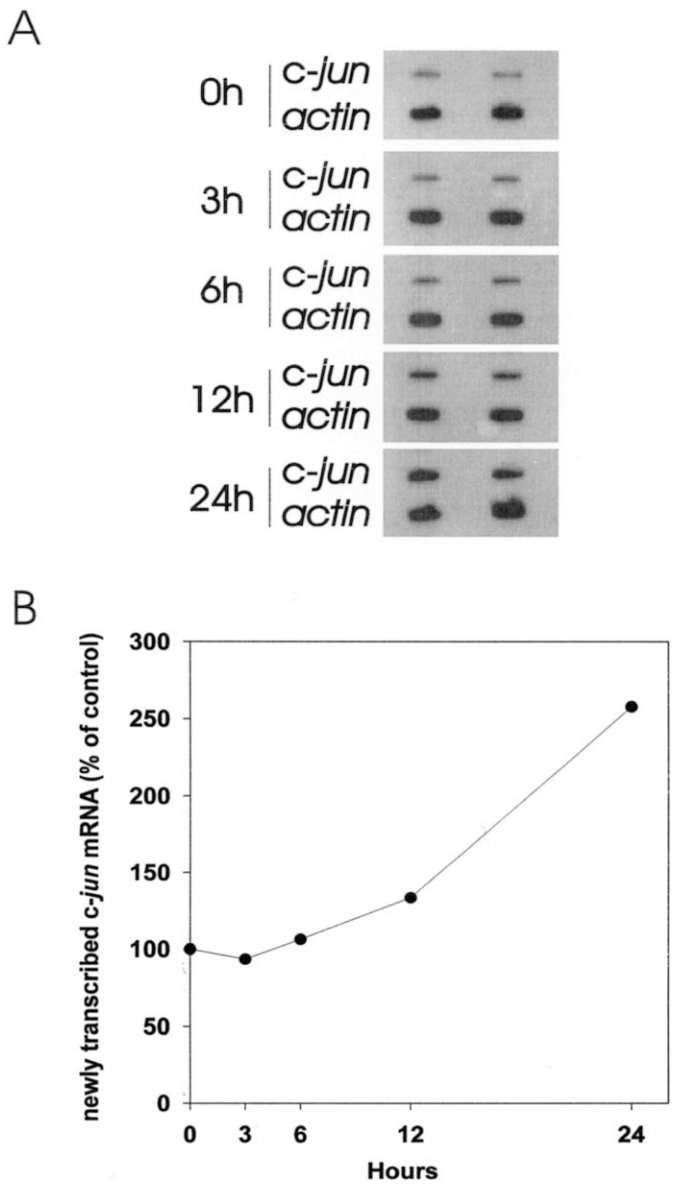

Fig. 2. Dex induced c-*jun* mRNA at the transcriptional level. CEM-C7 cells were treated with Dex for 0, 3, 6, 12, or 24 h. Nuclei isolated from cells sampled at each time point were used to assay for c-*jun* transcription, as described in Section 2. Data from one of three such experiments are shown. (A) Slot blots of the duplicate samples; (B) line graph of the data quantified by image analysis. The c-*jun* bands from (A) at each time point were quantified, averaged, and normalized to the β-*actin* bands for the respective time points. The experiment was repeated three times (except the 12 h time point, which was tested in four experiments) with similar results.

the relevant mRNA [19]. Hence, to try to determine whether de novo synthesis of protein was required for the Dex induction of c-*jun*, 10 μg/ml cycloheximide (CHX) was added to the cultures to inhibit protein synthesis. However, CHX itself induced c-*jun* mRNA levels strongly. Continuous incubation with CHX alone for 6 or 12 h resulted in an eight- to tenfold increase in

c-*jun* mRNA over the ethanol control-treated cells (Fig. 5A). This suggested that the CHX treatment had removed a repressor of c-*jun*. Perhaps the Dex induction occurred by removal of the same repressor. Consideration of the timing and quantity of c-*jun* mRNA pool induction suggested that Dex and CHX were not operating on the same repressor, at least not with equal efficiency. Fig. 5B presents data comparing the pool size change of c-*jun* mRNA after treatment with CHX alone (the same data as in Fig. 5A), Dex alone, or both together for 12 h. Obviously, the increase in c-*jun* mRNA was much greater after CHX treatment, and this large induction obscured any effect that blocking protein synthesis might have on the Dex induction process.

As quickly as CHX induces, so does its removal allow the c-*jun* mRNA to return to basal levels. Exposing cells to CHX for 6 or 12 h, followed by washes to remove the inhibitor, and reincubation for an equal time caused the mRNA to fall substantially towards basal levels (Fig. 5C,D). However, if CHX was present during the first 6 of 12 or 12 of 24 h while Dex was continuously present for the entire period, the final c-*jun* mRNA levels were as high as if Dex alone had been present for the entire 12 or 24 h (compare Fig. 5C,D). Two possibilities suggest themselves: either protein synthesis was not required for the Dex induction, and the superimposed CHX effect came and went unrelated to Dex induction; or the treatment with CHX together with Dex did in fact block induction by Dex but left the c-*jun* gene in a state that subsequently allowed Dex to induce it to a greater extent than would have been reached in the length of time available without blocking protein synthesis. Use of nuclear run-on assays permitted a more direct test of the need for prior protein synthesis for the Dex induction to occur.

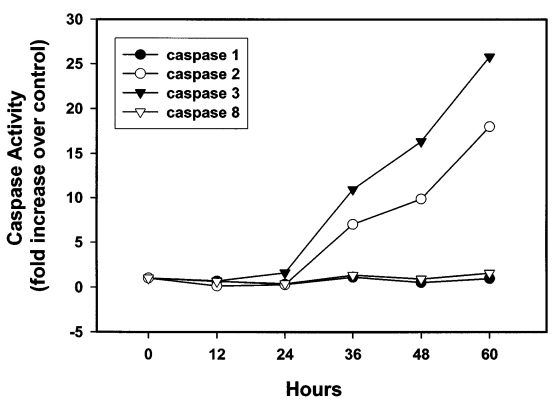

Fig. 3. Time course of Dex-evoked caspase activation in CEM-C7 cells. CEM-C7 cells were treated with 100 nM Dex for the indicated times; after which cells were harvested and lysed as described in the Section 2. The activities of caspases 1, 2, 3 and 8 were determined fluorometrically, using specific AFC substrates for each. The data shown are averages of duplicate determinations from a representative experiment. In five experiments, a similar time course of caspase 2 and 3 activation was observed.

3.6. Dex induction of c-jun transcription does require newly synthesized protein

Since CHX elevated c-*jun* mRNA to higher levels than that induced by Dex, making interpretation of its effects on the Dex-dependent induction by assays of mRNA pools difficult, nuclear run-on experiments were employed to address whether de novo protein synthesis was required. CEM-C7 cells were treated with EtOH, Dex, Dex + CHX, or CHX for 16 h, and their nuclei prepared for nuclear run-on assay. Fig. 6 shows that the level of new c-*jun* transcripts initiated after treatment with Dex for 16 h in the presence of CHX was significantly less than the level induced by Dex alone for the same period of time. This implied that CHX alters the effect of Dex on the expression of c-*jun*, and that de novo protein synthesis was required for the transcriptional induction of c-*jun* by Dex. We noted that Dex + CHX slightly increased the synthesis of c-*jun* mRNA. However, that increase was significantly less than that induced by Dex alone and might be due to the effect of CHX itself, which elevated c-*jun* synthesis 2.2-fold. Thus, a significant part of the Dex-dependent transcription induction of c-*jun* was blocked by CHX.

4. Discussion

Products of the nuclear proto-oncogenes *jun* and *fos* play a major role during cell growth, differentiation and development [20]. Induction of c-Jun has been implicated as a mediator of lymphoid cell apoptosis evoked by various stimuli, including ceramide, stress, bufalin and corticosteroids [21–23]. Antisense c-*jun* or circumin, an inhibitor of AP-1 activity, was able to inhibit various instances of apoptosis [6,21]. It has been suggested that glucocorticoid-evoked apoptosis of Jurkat cells requires a repressive function of GR, involving interactions with c-Jun [24]. In CEM-C7 cells, a strong correlation between GR and c-Jun transcription has been reported to facilitate functional cross-talk between the two pathways [25].

With accumulating evidence suggesting a significant role for c-*jun* in apoptotic pathways, there is increasing interest in understanding how c-*jun* is regulated. In general, the regulation of c-*jun* is highly complex and varied in different cell types, as well as with different stimuli. The regulation can be purely transcriptional, post-transcriptional, or both [26–30]. Glucocorticoid induces c-*jun* expression in some cells, but suppresses it in others [25,31–33]. In both AtT-20 pituitary tumor cells and U-937 monocytic leukemia cells, Dex represses the transcription of c-*jun* [25,34]. Interestingly, both cell lines resist Dex-dependent apoptosis. In CEM-C7 cells, on the other hand, the results from our previous work

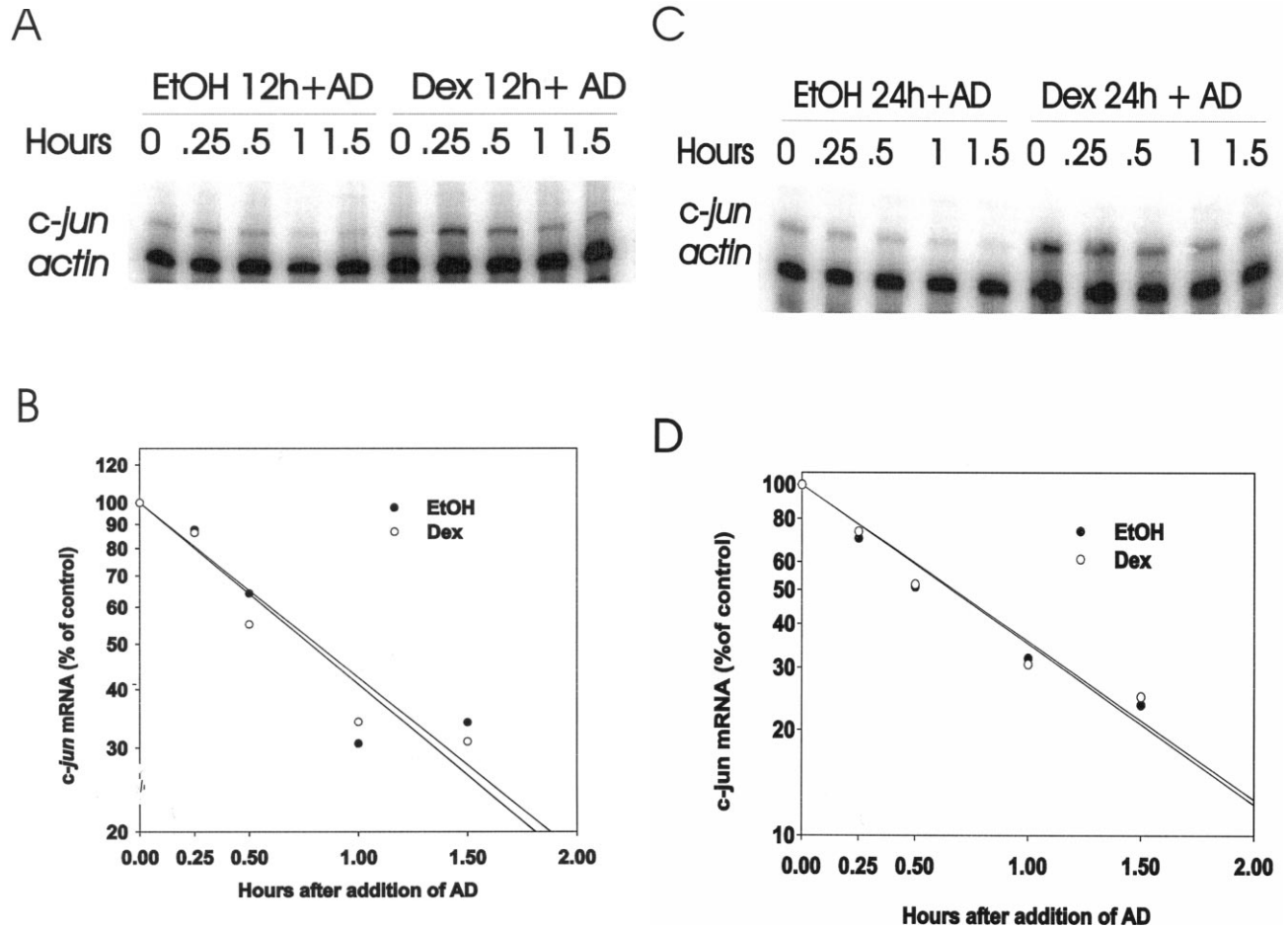

Fig. 4. Dex treatment did not stabilize c-*jun* mRNA in CEM-C7 cells. CEM-C7 cells were incubated with EtOH or Dex for 12 h (A, B), or 24 h (C, D). Actinomycin D (AD) was added to cells to a final concentration of 3 μM. Cells were collected at time intervals shown thereafter. The mRNA for c-*jun* in extracts containing total cellular RNA was estimated by RNase protection assay, performed as described in Section 2. The specific protected bands of c-*jun* from (A) and (C) were quantified and normalized to the corresponding β-*actin* bands. Data were plotted on a semi-log scale in (B) and (D). The same results were obtained in two independent experiments.

and this paper demonstrate that the expression of c-*jun* is induced by Dex in a time-, dose- and receptor-dependent manner. These cells are killed apoptotically by Dex. The receptor-deficient ICR-27 subclone shows no cell death, growth inhibition, or induction of c-*jun*. Nuclear run-on assays showed that the induction was transcriptional, and estimate of mRNA half-life showed no effect of Dex treatment.

Jun induction clearly precedes the onset of apoptosis in CEM cells, and we postulate that this sustained induction is relevant to the ultimate cell death [6]. When resting cells are stimulated to grow, a well-documented brief increase in c-*jun* expression occurs, but prolonged induction of c-*jun* may be harmful. In CEM-C7 cells, Jun is clearly elevated by 12 h and beyond, after adding Dex to the cells. To determine the role of caspases in their ultimate apoptosis, we have carried out detailed studies of their activity and relationships to the final outcome. This complete set of data is too extensive to present fully in this paper, but we do include (Fig. 3) data germane to our hypothesis, showing the chronology of altered activity of several caspases. We found that caspase 1, known to respond to activation of Fas or TNF-like receptors, was not activated by Dex in these cells. Caspases 2 and 3, 'effector' caspases, were activated, however, after the induction of Jun, and just as overt apoptosis begins. This timing is consistent with our hypothesis that the elevation of Jun is important for the eventual recruitment of the caspase cascade, which we have documented elsewhere [35].

The time course of c-*jun* mRNA expression and the data from nuclear run-on experiments have shown that c-*jun* induction only began at ≥ 6 h after addition of Dex to the culture. It is well known that only a few minutes are required for activated GR to enter the nucleus and bind to a simple promoter regulating gene expression [12,19]. This implies that the regulation of c-*jun* expression by Dex most likely is not by the mechanism of directly and solely binding of GR to the

c-*jun* promoter. Furthermore, there are no classic palindromic glucocorticoid response elements (GRE) in the known portions of the c-*jun* gene's regulatory region. Although a putative GRE site has been mentioned to exist at the 5′ end of the −1.6 kbp region of c-*jun* promoter [36], no species and sequence information are available, nor have any functional tests of it been reported. In short, Dex induction of c-*jun* is more likely to be a secondary rather than a primary response.

Since concomitant protein synthesis is typically required for secondary response mechanisms [19], the protein synthesis inhibitor CHX was employed. CHX itself, however, was found to induce c-*jun* mRNA levels higher than did Dex alone in an equivalent time. This created obvious difficulty in interpreting whether CHX alters the effect of Dex on the expression of c-*jun*. Washing out CHX after a period of exposure led to the rapid reversal of its inductive effect. Keeping Dex present continually during the time of exposure to CHX and the subsequent period following CHX washout resulted in induced c-*jun* mRNA levels equivalent to those reached by continuous exposure to Dex without CHX. This could occur either because the Dex induction was insensitive to CHX and proceeded unabated through the +CHX period, or because the CHX did indeed block Dex induction, but left the cells in a state such that after CHX removal, induction by Dex was more rapid. We therefore tested the effect of CHX on the Dex induction of c-*jun* mRNA transcription more directly, by nuclear run-on assays.

In the presence of CHX, Dex could only slightly induce new synthesis of c-*jun* mRNA, to a level that

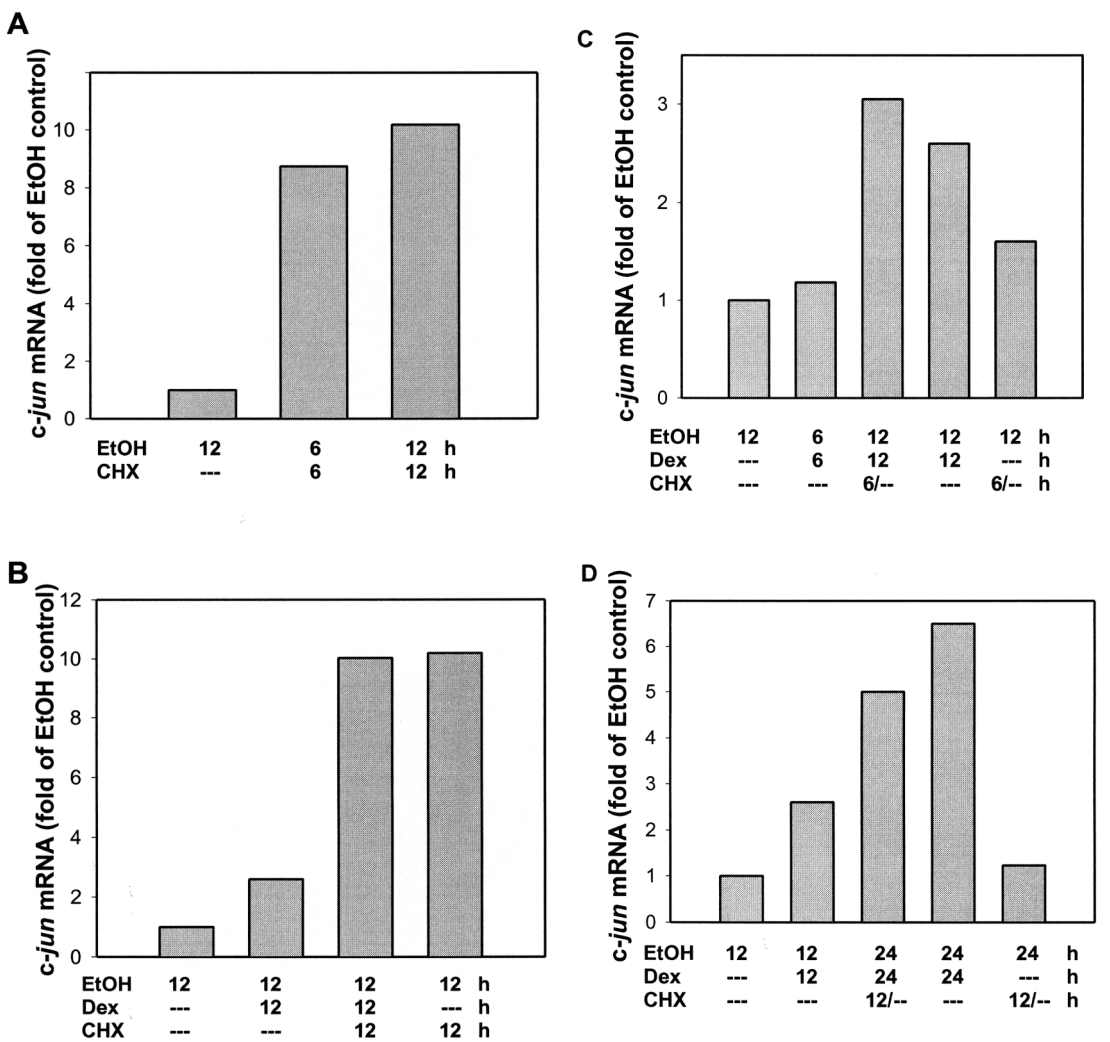

Fig. 5. CHX increased c-*jun* mRNA to a higher level than did Dex. Total RNA was extracted from CEM-C7 cells, and the RNase protection assay was used to determine the c-*jun* mRNA levels. (A) Cells were treated with EtOH or CHX for 12 h, or CHX for 6 h; (B) cells were treated with EtOH, Dex, Dex + CHX and CHX for 12 h; (C) cells were incubated with ethanol for 12 h, Dex for 6 h, Dex for 12 h with CHX present for the initial 6 h (Dex12 + CHX6a), Dex for 12 h, and EtOH for 12 h with CHX for initial 6 h (CHX6a); (D) cells were incubated with EtOH for 12 h, Dex for 12 h, Dex for 24 h with CHX present for the initial 12 h (Dex24 + CHX12a), Dex for 24 h, and CHX for initial 12 h (CHX12a). All experiments were repeated with essentially the same results.

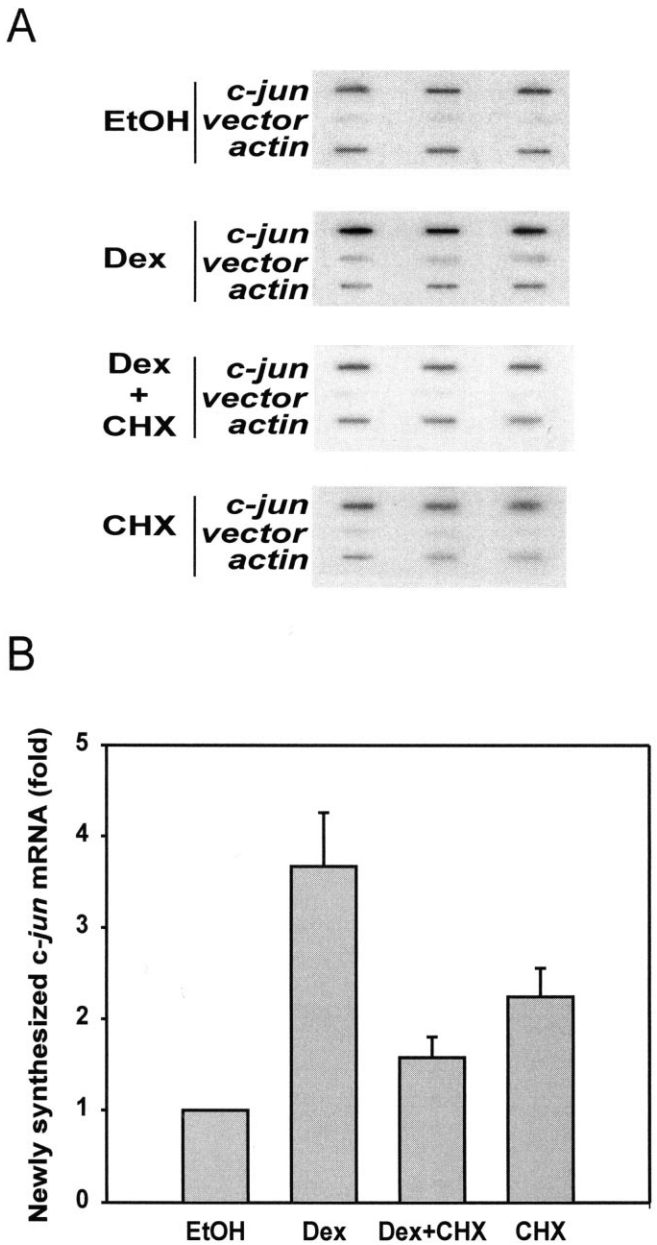

Fig. 6. De novo protein synthesis was required for the induction of c-*jun* with Dex in CEM-C7 cells. Nuclei were isolated and the nuclear run-on assay was used to determine the newly synthesized c-*jun* mRNA levels. Single-strand DNA of a plasmid with c-*jun* in an antisense orientation was used to determine c-*jun* expression. A plasmid-containing *actin* was used as internal control and a vector without a c-*jun* insert was used for a negative control. (A) CEM-C7 cells were treated with EtOH, Dex, Dex + CHX or CHX alone for 16 h. Three lanes show triplicates. (B) The bands in (A) were quantified. Data from triplicates were averaged and subtracted by data from vector, and then normalized by data from *actin*. The data for EtOH treated were set as one-fold. The bars represent the range between two independent experiments.

was significantly less than that caused by Dex alone. This clearly indicated that CHX prevented much of the inductive effect of Dex on c-*jun* transcription. It seems that de novo protein synthesis was required for induction of c-*jun* gene by Dex in these cells, a classic 'secondary response.' Since ongoing protein synthesis was necessary, relatively labile proteins must be required for Dex-regulation of the c-*jun* gene. This was not an effect relevant to general protein synthesis machinery, because the rate of transcription of the normalizer housekeeping gene measured was untouched. A search for the relevant primary gene(s) seems warranted.

Our results do not distinguish a pure secondary response from a recently proposed delayed primary response mechanism [19]. A classic secondary response consists of inducer 1 inducing an independent inducer 2 that is solely responsible for the induction of the measured gene product. In a delayed primary response, there is still induction of inducer 2, but both inducers then participate in the induction of the measured mRNA. Since GRs frequently act in conjunction with other transcription factors, a delayed primary response is possible. This possibility is the more likely since our preliminary data (not shown) indicate that the continued presence of Dex was necessary for maintaining the transcription regulation of the c-*jun* gene. Further supporting such a mechanism is the coordinate regulation of the GR and c-*jun*, which has been suggested by Vig et al. [25]. Both the mRNA and protein of GR are increased by glucocorticoids in CEM cells. Nuclear run-on experiments have also demonstrated that the *gr* gene is regulated at the transcriptional level (data not shown). Newly synthesized c-Jun, GR or both proteins might function as activators for transcription regulation of both c-*jun* and *gr* genes. All these data suggest that there is a coordinate regulation between the *gr* and c-*jun* genes after addition of Dex to CEM cells.

Acknowledgements

This research was conducted in conjunction with the Walls Medical Research Foundation. The authors thank Betty H. Johnson for help in preparing the manuscript.

References

[1] F. Colotta, N. Polentarutti, M. Sironi, A. Mantovani, Expression and involvement of *c-fos* and *c-jun* protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines, J. Biol. Chem. 267 (1992) 18278–18283.
[2] S. Estus, W.J. Zaks, R.S. Freeman, M. Gruda, R. Bravo, E.M. Johnson, Jr, Altered gene expression in neurons during programmed cell death: identification of *c-jun* as necessary for neuronal apoptosis, J. Cell Biol. 127 (1994) 1717–1727.
[3] E. Bossy-Wetzel, L. Bakiri, M. Yaniv, Induction of apoptosis by the transcription factor *c-Jun*, EMBO J. 16 (1997) 1695–1709.
[4] Y. Ishikawa, T. Yokoo, M. Kitamura, *c-Jun*/AP-1, but not NF-kappa B, is a mediator for oxidant-initiated apoptosis in

glomerular mesangial cells, Biochem. Biophys. Res. Commun. 240 (1997) 496–501.
[5] M. Qian, J. Kralova, W. Yu, H.R. Bose, Jr, M. Dvorak, B.G. Sanders, K. Kline, *c-Jun* involvement in vitamin E succinate induced apoptosis of reticuloendotheliosis virus transformed avian lymphoid cells, Oncogene 15 (2) (1997) 223–230.
[6] F. Zhou, E.B. Thompson, Role of *c-jun* induction in the glucocorticoid-evoked apoptotic pathway in human leukemic lymphoblasts, Mol. Endocrinol. 10 (1996) 306–316.
[7] P. Angel, M. Karin, The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation, Biochim. Biophys. Acta 1072 (1991) 129–157.
[8] Y.S. Yuh, E.B. Thompson, Glucocorticoid effect on oncogene/growth gene expression in human T lymphoblastic leukemic cell line CCRF-CEM. Specific *c-myc* mRNA suppression by dexamethasone, J. Biol. Chem. 264 (1989) 10904–10910.
[9] R. Thulasi, D.V. Harbour, E.B. Thompson, Suppression of *c-myc* is a critical step in glucocorticoid-induced human leukemic cell lysis, J. Biol. Chem. 268 (1993) 18306–18312.
[10] B.H. Johnson, S. Ayala-Torres, L.N. Chan, M. El-Naghy, E.B. Thompson, Glucocorticoid/oxysterol-induced DNA lysis in human leukemic cells, J. Steroid Biochem. Mol. Biol. 61 (1997) 35–45.
[11] Y.S. Yuh, E.B. Thompson, Complementation between glucocorticoid receptor and lymphocytolysis in somatic cell hybrids of two glucocorticoid-resistant human leukemic clonal cell lines, Somat. Cell Mol. Genet. 13 (1987) 33–45.
[12] H. Htun, J. Barsony, I. Renyi, D.L. Gould, G.L. Hager, Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera, Proc. Natl. Acad. Sci. USA 93 (1996) 4845–4850.
[13] M.R. Norman, E.B. Thompson, Characterization of a glucocorticoid-sensitive human lymphoid cell line, Cancer Res. 37 (1977) 3785–3791.
[14] J.M. Harmon, E.B. Thompson, Isolation and characterization of dexamethasone-resistant mutants from human lymphoid cell line CEM-C7, Mol. Cell. Biol. 1 (1981) 512–521.
[15] J. Ashraf, E.B. Thompson, Identification of the activation-labile gene: a single point mutation in the human glucocorticoid receptor presents as two distinct receptor phenotypes, Mol. Endocrinol. 7 (1993) 631–642.
[16] P.B. Mahajan, E.A. Thompson, Jr, Cyclosporin A inhibits rDNA transcription in lymphosarcoma P1798 cells, J. Biol. Chem. 262 (33) (1987) 16150–16156.
[17] A. Sarin, M.L. Wu, P.A. Henkart, Different interleukin-1 beta converting enzyme (ICE) family protease requirements for the apoptotic death of T lymphocytes triggered by diverse stimuli, J. Exp. Med. 184 (6) (1996) 2445–2450.
[18] D.V. Harbour, P. Chambon, E.B. Thompson, Steroid mediated lysis of lymphoblasts requires the DNA binding region of the steroid hormone receptor, J. Steroid Biochem. Mol. Biol. 35 (1990) 1–9.
[19] D.M. Dean, M.M. Sanders, Ten years after: reclassification of steroid-responsive genes, Mol. Endocrinol. 10 (1996) 1489–1495.
[20] L.J. Ransone, I.M. Verma, Nuclear proto-oncogenes fos and jun, Ann. Rev. Cell Biol. 6 (1990) 539–557.
[21] H. Sawai, R. Okazaki, H. Yamamoto, H. Okano, Y. Takeda, M. Tashima, H. Sawada, M. Okuma, H. Ishikura, H. Umehara, N. Domae, Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells, J. Biol. Chem. 270 (45) (1995) 27326–27331.
[22] M. Faris, N. Kokot, K. Latinis, S. Kasibhatla, D.R. Green, G.A. Koretzky, A. Nel, The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression, J. Immunol. 160 (1) (1998) 134–144.
[23] M. Watabe, K. Ito, Y. Masuda, S. Nakajo, K. Kadaya, Activation of AP-1 is required for bufalin-induced apoptosis in human leukemia U937 cells, Oncogene 16 (6) (1998) 779–787.
[24] M. Maroder, A.R. Farina, V. Vacca, M.P. Felli, D. Meco, I. Screpanti, L. Frati, A. Gulino, Cell-specific bifunctional role of Jun oncogene family members on glucocorticoid receptor-dependent transcription, Mol. Endocrinol. 7 (4) (1993) 570–584.
[25] E. Vig, T.J. Barrett, W.V. Vedeckis, Coordinate regulation of glucocorticoid receptor and *c-jun* mRNA levels: evidence for cross-talk between two signaling pathways' at the transcriptional level, Mol Endocrinol. 8 (1994) 1336–1346.
[26] P. Angel, K. Hattori, T. Smeal, M. Karin, The *jun* proto-oncogene is positively autoregulated by its product, Jun/AP-1, Cell 55 (1988) 875–885.
[27] D. Chauhan, S.M. Kharbanda, E. Rubin, B.A. Barut, A. Mohrbacher, D.W. Kufe, K.C. Anderson, Regulation of *c-jun* gene expression in human T lymphocytes, Blood 81 (1993) 1540–1548.
[28] G. Kick, G. Messer, G. Plewig, P. Kind, A.E. Goetz, Strong and prolonged induction of *c-jun* and *c-fos* proto-oncogenes by photodynamic therapy, Br. J. Cancer 74 (1996) 30–36.
[29] A.R. Patel, J.Y. Wang, Polyamines modulate transcription but not posttranscription of *c-myc* and *c-jun* in IEC-6 cells, Am. J. Physiol. 273 (1997) C1020–C1029.
[30] S.E. Adunyah, R. Chander, V.K. Barner, R.S. Cooper, Regulation of c-jun mRNA expression by hydroxyurea in human K562 cells during erythroid differentiation, Biochim. Biophys. Acta 1263 (1995) 123–132.
[31] T.J. Barrett, E. Vig, W.V. Vedeckis, Coordinate regulation of glucocorticoid receptor and *c-jun* gene expression is cell type-specific and exhibits differential hormonal sensitivity for down- and up-regulation, Biochem. 35 (1996) 9746–9753.
[32] M. Subramaniam, D. Colvard, P.E. Keeting, K. Rasmussen, B.L. Riggs, T.C. Spelsberg, Glucocorticoid regulation of alkaline phosphatase, osteocalcin, and proto-oncogenes in normal human osteoblast-like cells, J. Cell Biochem. 50 (1992) 411–424.
[33] H. Lee, Y.T. Shaw, S.T. Chiou, W.C. Chang, M.D. Lai, The effects of glucocorticoid hormone on the expression of *c-jun*, FEBS Lett. 280 (1991) 134–136.
[34] R. Hass, M. Brach, S. Kharbanda, G. Giese, P. Traub, D. Kufe, Inhibition of phorbol ester-induced monocytic differentiation by dexamethasone is associated with down-regulation of *c-fos* and *c-jun* (AP-1), J. Cell Physiol. 149 (1991) 125–131.
[35] E.B. Thompson, R.D. Medh, F. Zhou, S. Ayala-Torres, N. Ansari, W. Zhang, B.H. Johnson, Glucocorticoids, oxysterols, and cAMP with glucocorticoids each cause apoptosis of CEM cells and suppress *c-myc*, J. Steroid Biochem. Mol. Biol. 69 (1999) 453–461.
[36] C. Jonat, H.J. Rahmsdorf, K.K. Park, A.C. Cato, S. Gebel, H. Ponta, P. Herrlich, Antitumor promotion and antiinflammation: down-modulaiton of AP1 (Fos/Jun) activity by glucocorticoid hormone, Cell 62 (1990) 1189–1204.