Transcriptional down-regulation of c-myc in human prostate carcinoma cells by the synthetic androgen mibolerone

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Summary The mechanism of down-regulation of c-myc RNA associated with androgen-induced suppression of the transformed phenotype in the human prostate carcinoma cell line LNCaP is investigated. The synthetic androgen mibolerone (17α-17β-Dimethyl-19-nortestosterone) reversibly inhibits the proliferation of LNCaP cells and, from 12-72 h after hormone addition reduces the level of c-myc transcripts to a few per cent of controls. P1, P2, and P6 c-myc transcripts decline at the same rate, whereas P3 transcripts are much less hormone sensitive. Nuclear run-on analysis revealed that c-myc is down-regulated at the level of transcription initiation in LNCaP cells. The level of c-myc transcripts prevailing in untreated control cells can be restored in androgen-induced cells by excess antiandrogen, indicating the involvement of the androgen receptor in c-myc down-regulation.

The cellular proto-oncogene c-myc is known to be involved in the regulation of cell growth and differentiation (for review see Cole, 1986). The level of c-myc RNA is invariably higher in proliferating than in quiescent cells, and remains roughly constant throughout the cell cycle (Thompson et al., 1985). C-myc RNA is markedly induced upon stimulation of resting cells by mitogens to pass from G0 to G1 (Kelly et al., 1983; Campisi et al., 1984). In complementary experiments, c-myc RNA levels fall dramatically when cells withdraw from the cell cycle into G0 or undergo terminal differentiation (for review see Spencer & Groudine, 1990). Steady state levels of c-myc RNA are subject to distinct control mechanisms: exceptionally short half-life of c-myc RNA (Dani et al., 1984; Pichacezy et al., 1987), impaired maturation of the primary transcript (Eick, 1990), and a block to RNA-elongation at the first exon-intron boundary (Bentley & Groudine, 1986a; Eick & Bornkamm, 1986) as rapid means of c-myc regulation, and modulation of the rate of initiation as a late acting mechanism (Siebenlist et al., 1988).

The structure of the gene with two major, P1 and P2 (Batley et al., 1983), and two minor, P3 and P6 (Bentley & Groudine, 1986a; ar-Rushdi et al., 1983; Hayday et al., 1984; Bentley & Groudine, 1986b), sites of transcription initiation has been well characterised. Additionally, positive and negative regulatory elements have been found within or flanking the human and mouse c-myc gene (Yang et al., 1986; Chung et al., 1986; Remmers et al., 1986; Lipp et al., 1987; Kakkis & Calame, 1987; Hay et al., 1987; Iguchi-Ariga et al., 1988; Weissinger et al., 1988; Asselin et al., 1989; Hall 1990) which may modulate initiation (Thalmeier et al., 1989; Pieterpol et al., 1990; Hall, 1990; Saecc & Cochran, 1990) and elongation of transcription (Bentley & Groudine, 1988; Miller et al., 1989). Several transcription factors have been described which bind to sequences upstream of c-myc including NF1 (Siebenlist et al., 1984), AP2 (Imagawa et al., 1987), AP1, and octamer binding factors (Takimoto et al., 1989; Hay et al., 1989), NFkB (Duyao et al., 1990), and a mouse plasmacytoma specific repressor protein (Kakkis et al., 1989).

Stimuli from steroid hormones are generally considered to have a key-role in regulating cell proliferation and tissue development. Despite the increasing molecular data on steroid hormone-receptor complex action on individual response elements (for review see Beato, 1989), until now, the intricate cell-biological processes leading from hormonal signals to the modulation of cell proliferation remain poorly defined. Cell cycle arrest associated with c-myc down-regulation by steroid hormones has hitherto been described in lymphocytes and promyelocytes. Glucocorticoids block lymphocytes and lymphoma cells at the G1 phase of the cell cycle. Among a panel of known growth-related genes, only c-myc expression was reduced by dexamethasone (Yuh & Thompson, 1989). In a T lymphoblastic leukaemic cell line, immediate post-transcriptional down-regulation of c-myc has been demonstrated in response to glucocorticoids (Maroder et al., 1990). In the promyelocytic cell line HL60, 1,25-Dihydroxyvitamin D3 (1,25-(OH)2D3) induced differentiation along the monocyte lineage is preceded by a decrease in the steady state level of c-myc RNA (Reitsma et al., 1983). The 1,25-(OH)2D3 effect on c-myc mRNA was shown to occur at the transcriptional level (Simpson et al., 1987). Androgen analogues containing a 17α-methyl-testosterone backbone inhibit the proliferation (Sonnenschein et al., 1989) and suppress the transformed phenotype (Wolf et al., 1991) in the androgen responsive (Horoszewicz et al., 1983; Schulz et al., 1985; Berns et al., 1986) human prostate carcinoma cell line LNCaP (Horoszewicz et al., 1980). The androgen receptor of LNCaP cells carries a point mutation in the steroid binding domain but activates transcription in an androgen-dependent manner (Veldscholte et al., 1990). LNCaP cells are considered to be the best-suited in vitro model of prostate cancer available (Thompson, 1990). Recently we could show that the synthetic androgen mibolerone represses anchorage-independent growth and concomitantly reduces the level of c-myc RNA in LNCaP cells (Wolf et al., 1991). Here we have studied the details of hormonal c-myc repression. We demonstrate late transcriptional repression of the P1, P2, and P6 promoters of c-myc.

Materials and methods

Cell culture and hormones

The prostate carcinoma cell line LNCaP (Horoszewicz et al., 1980) was obtained from the Human Tumor Cell Laboratory, Sloan Kettering Institute for Cancer Research, Rye, NY. LNCaP cells between passages 75 and 90 were used for the experiments described. Cells were maintained in RPMI medium as monolayers in the presence of 10% FCS and phenol red. For the preparation of seed stocks, cells were
grown to 50 to 75% confluency before use. Hormones were added 48 h after seeding as ethanol solutions to give a final concentration as indicated in the figures. Synthetic androgens: 7a-17a-Dimethyl-19-nortestosterone (mibolerone; Upjohn). Antiandrogens: 6-chloro-6-dehydro-17a-acetoxyl-12a-methylene-progesterone (ciproterone acetate; CA; Schering).

**RNA extraction and Northern blot analysis**

Standard protocols were followed as described elsewhere (Wolf et al., 1991).

**S1 mapping**

Single-stranded uniformly labelled DNA probes were prepared by primer extension of M13 clones, double-stranded probes by end-labelling with T4-poly nucleotide kinase. Hybridisation of labelled DNA fragments to total RNA was carried out using a modification of the method of Berk and Sharp (1977). Hybridisation mixtures of 20 μl containing 10 c.p.m. of the labelled probe (specific activity 10 c.p.m μg⁻¹), 40 μg RNA, 90% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.5. 1 mM EDTA were denatured at 90°C for 5 min and immediately transferred to 58°C. After 15 h the hybridisation was terminated by addition of 180 μl ice-cold buffer containing 250 mM NaCl, 30 mM Na-acetate, pH 4.5, 2 mM Zn-acetate, 5% glycerol, and 400 units of nuclease S1 (Boehringer, Mannheim). The samples were incubated at 25°C for 1 h, extracted twice with phenol-chloroformisoamylalcohol (25:24:1, v:v:v), and precipitated with ethanol. Protected DNA fragments were separated on 5% polyacrylamide gels containing 7 M urea.

**Nuclear run-on analysis**

Preparation of nuclear extracts and the hybridisation procedure were performed as described (Eick & Bornkamm, 1986) with slight modifications. 2 x 10⁶ cells were scraped from culture dishes and washed twice in PBS. Cells were resuspended in 10 mM Tris- HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% (v: v) NP40 and incubated on ice for 5 min. The nuclear pellets were spun down at 500 g and washed by resuspension in 10 ml of the same buffer. The pelleted nuclei were resuspended in storage buffer (50 mM Tris- HCl, pH 8.3, 40% (v:v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA) and frozen in liquid nitrogen in portions of 100 μl corresponding to 2 x 10⁶ nuclei. The nuclei were mixed with 100 μl reaction buffer (10 mM Tris- HCl, pH 8.0, 5 mM MgCl₂, 300 mM KCl, 0.5 mM of each ATP, CTP, GTP and 100 μCi of (α-²P) UTP (800 Ci mmol, Amersham)) and incubated for 20 min at 28°C. DNaseI was added to a final concentration of 10 μg ml⁻¹ and the incubation was continued for 5 min at 28°C. After addition of 200 μl STE buffer (100 mM Tris- HCl, pH 7.5, 50 mM EDTA, 0.5% SDS) and 20 μl proteinase K (10 mg ml⁻¹), preincubated at 37°C for 1 h) the samples were incubated for 1 h at 40°C. Nuclear transcripts were separated from unincorporated nucleotides on a Sephadex G-50 column equilibrated with 10 mM Tris- HCl, pH 7.5, 1 mM EDTA, 1% SDS. The labelled RNA was boiled for 10 min, chilled on ice and hybridised to LA immobilised on nylon filters (PALL) in Church-buffer (0.5 mM sodium phosphate, pH 7.1, 7% SDS, 0.1 mM EDTA) after preincubation of the filter in the same buffer. After hybridisation the filters were washed twice at 50°C in 0.1 x SSC, 1% SDS, twice in 2 x SSC containing 10 μg ml⁻¹ RNAase A at 25°C and finally once again in 0.1 x SSC, 1% SDS at 50°C. The filters were exposed to Kodak XAR-5 films using a Dupont Cronex Lightning Plus intensifying screen.

**Results**

**Down-regulation of c-myc RNA in response to synthetic androgen is slow**

The effect of the synthetic androgen mibolerone (7a-17a-Dimethyl-19-nortestosterone) on c-myc expression in LNCaP cells was studied. Cells were incubated for 0-120 h in the presence or absence of 3.3 x 10⁻⁴ M mibolerone followed by RNA extraction. Steady state c-myc RNA levels were analysed by Northern blotting using an exon 3-specific probe. The structure of the c-myc gene and all probes used in this work are depicted in Figure 1. When LNCaP cells are maintained in the presence of mibolerone a decline in c-myc RNA levels becomes clearly visible after 12-48 h (Figure 2a). After 72 h, the amount of c-myc RNA decreases to about 10% of control cells, but is still detectable in Northern blots. As shown in Figure 2b, the decrease of c-myc RNA is monophasic. In LNCaP control cells c-myc RNA levels begin to decline slightly after 24 h, and reach about 60% of the starting value after 120 h. RNA levels of the housekeeping enzyme glyceraldehyde-phosphate-dehydrogenase (GAPDH) did not change significantly during the experiment. Thus, the substantial reduction of c-myc RNA levels by androgen is a late effect. Androgen does not trigger any early down-regulation of c-myc. The effect of mibolerone is reversible: 48 h after withdrawal of the hormone c-myc steady-state RNA reached pretreatment levels (data not shown).

**Excess antiandrogen represses c-myc down-regulation**

If c-myc down-regulation is mediated by the androgen receptor, the effect of mibolerone must be suppressed by the simultaneous addition of antihormone which competes with androgen for specific receptor binding. In previous work (Wolf et al., 1991) we found that a large excess of the antiandrogens cyproterone acetate (CA) or hydroxyflutamide is required to antagonise growth related effects of synthetic androgens in LNCaP cells. CA has a much lower affinity for the androgen receptor than synthetic androgens with a 17a-methyl-testosterone backbone (Wakeling et al., 1981). The suppression of c-myc RNA levels is counteracted by the antiandrogen CA (Figure 2c) at the same concentration ratio (1:750 w:w) at which the growth inhibiting effect of androgens on LNCaP cells is reversed (Wolf et al., 1991). This finding indicates that the androgen receptor is involved in the signal transduction chain leading to c-myc down-regulation and to inhibition of proliferation by synthetic androgen.

The levels of P₄, P₃, and P₃ transcripts decline, whereas P₃ transcripts remain almost constant

In order to resolve the contribution of the four known c-myc promoters to the steady-state c-myc signal seen in Northern blots, we performed nuclearase S1 protection assays with probes derived from the promoter region of the human c-myc gene. Southern analysis of the c-myc locus of LNCaP cells revealed no rearrangement or amplification (data not shown). Therefore, the expected sizes of S1 protected fragments were calculated on the basis of the germline configuration of the human c-myc gene. As shown in Figure 3a, the ratio of P₄: P₃: P₃: P₄, P₃ transcripts in control cells is about 80:20:3. This ratio is not changed in the presence of mibolerone. P₄, P₃ and
$P_0$ transcripts are reduced to the same extent (Figure 3a). In contrast, $P_3$ transcripts which comprise only 3–5% of the total $c$-myc transcripts in LNCaP cells, remain at a level almost equal to controls in the presence of mibolerone (Figure 3b). Regulation of the $P_1$-promoter independently of the $P_0$, $P_1$, and $P_2$-promoter has recently been reported for the normal $c$-myc allele in the Burkitt's lymphoma cell line Raji (Eick et al., 1990).

![Northern blot analysis of total RNA of LNCaP cells after treatment with the synthetic androgen mibolerone and the anti-androgen cyproterone acetate. LNCaP cells were cultivated in the presence (+) or absence (−) of $3.3 \times 10^{-9}$ M mibolerone (MIB) for various periods from 0–120 h. RNA was extracted and analysed on Northern blots (20 μg per lane) hybridised with a $c$-myc third exon specific probe labelled by random priming (Clal-EcoRI, 1.4 kb. probe c in Figure 1). Subsequently, the probe was washed off and the filter was rehybridised with a glyceraldehyde-phosphate-dehydrogenase (GAPDH)-specific probe (Allen et al., 1987) a. The autoradiograms were scanned densitometrically and $c$-myc RNA levels normalised to GAPDH RNA levels are shown schematically in a block diagram b. The effect of mibolerone is antagonised by cyproterone acetate (CA) $\left(1.8 \times 10^{-6} \text{M}\right)$. LNCaP cells were incubated in the presence of MIB and CA as indicated and RNA was extracted after 48 h c.]

**Figure 2** Northern blot analysis of total RNA of LNCaP cells after treatment with the synthetic androgen mibolerone and the anti-androgen cyproterone acetate. LNCaP cells were cultivated in the presence (+) or absence (−) of $3.3 \times 10^{-9}$ M mibolerone (MIB) for various periods from 0–120 h. RNA was extracted and analysed on Northern blots (20 μg per lane) hybridised with a $c$-myc third exon specific probe labelled by random priming (Clal-EcoRI, 1.4 kb. probe c in Figure 1). Subsequently, the probe was washed off and the filter was rehybridised with a glyceraldehyde-phosphate-dehydrogenase (GAPDH)-specific probe (Allen et al., 1987) a. The autoradiograms were scanned densitometrically and $c$-myc RNA levels normalised to GAPDH RNA levels are shown schematically in a block diagram b. The effect of mibolerone is antagonised by cyproterone acetate (CA) $\left(1.8 \times 10^{-6} \text{M}\right)$. LNCaP cells were incubated in the presence of MIB and CA as indicated and RNA was extracted after 48 h c.
C-myc is down-regulated at the level of transcription initiation

C-myc RNA is subject to an unusually rapid turnover (Dani et al., 1984). In several cellular systems, c-myc RNA stability is the primary control mechanism to modulate steady-state levels (for review see Piechaczyk et al., 1987). We have determined the contribution of posttranscriptional mechanisms to c-myc RNA repression in LNCaP cells and measured the stability of c-myc RNA after addition of actinomycin D. In the absence and presence (24 h) of mibolerone the half life of c-myc RNA was measured to be 15 to 20 min (data not shown). Therefore, a considerable contribution of a post-transcriptional mechanism is unlikely, and we have studied transcriptional control of c-myc RNA levels in nuclear run-on experiments. This method measures the density of actively transcribing RNA-polymerase II molecules on individual segments of the c-myc gene and allows to estimate the rate of c-myc RNA initiation and elongation.

In LNCaP control cells similar signals were obtained for the transcription rate of c-myc exon 1, 2, and 3 (Figure 4). However, the probe used for exon 1 has a size of 446 bases and is more than 3-fold smaller than the exon 2 and 3 probes with 1533 and 1405 bases, respectively. The relatively high transcription rate of c-myc exon 1 compared to exon 2 indicates that a fraction of RNA polymerases becomes blocked on the way to exon 2. A block to RNA-elongation in the c-myc gene has been described at the boundary of exon 1 to intron 1 in many different cellular systems (for review see Spencer & Groudine, 1990).
In the presence of mibolerone, the density and distribution of RNA polymerases on the c-myc gene remained unchanged for the first 3 h. Subsequently, transcriptional activity declined over a period of 2 days to about 10% of controls. The transcription of exon 1 and exon 2 slowed down with a similar rate. However, the data presented in Figure 4 do not rule out a contribution of the RNA-elongation block for c-myc down-regulation between 6 and 12 h. In the presence of mibolerone and antihormone, transcription of c-myc was unaffected over a period of 2 days (data not shown).

The transcription rate for the PSA (prostate-specific antigen [Schulz et al., 1988]) gene was increased 3 h after addition of mibolerone and subsequently declined towards pretreatment levels after 4 days (Figure 4). Thus, the decrease in c-myc transcription is not due to a general decrease in RNA synthesis concomitantly to inhibition of cell proliferation.

**Discussion**

The synthetic androgen mibolerone is capable of triggering a set of fundamental changes in the growth behaviour of LNCaP cells: inhibition of proliferation, abrogation of anchorage-independent growth, morphological change, and reduction of c-myc RNA levels (Wolf et al., 1991). The signal transduction pathway from androgen binding to the ultimate cellular responses remains to be elucidated. In this report, the level of c-myc down-regulation by mibolerone in LNCaP cells has been analysed.

Mibolerone induces a late transcriptional repression of c-myc in LNCaP cells. The transcription rate for c-myc exon 1 and 2 decreased with a similar rate 3 to 6 h after addition of hormone. Thus, a reduced rate of RNA-elongation which has been described as fast control mechanism in c-myc down-regulation (Eick & Bornkamm, 1986) does not significantly contribute to c-myc repression in LNCaP cells. However, mibolerone represses c-myc at the level of transcription initiation. The lag phase between hormone addition and the decline of c-myc transcription (>3 h) indicates that androgen receptor-mediated repression of c-myc involves additional regulatory steps.

Androgen regulation of c-myc has also been studied in vivo. C-myc RNA in the ventral prostate epithelial cells of rats increases nearly 4-fold within 1 day and 6- to 7-fold within 2 days after castration. The castration induces atrophy of prostatic epithelial cells while androgen treatment causes an increase in cell size and number. Administration of testosterone at the time of castration prevents the atrophy and the increases in c-myc RNA levels (Quarmby et al., 1987). Similar observations were made studying the repression of androgen-dependent Shionogi mouse mammary carcinoma cells in castrated syngeneic animals. 3–6 days after castration, the tumour mass began to regress accompanied by a continuous increase of c-myc RNA (Rennie et al., 1988).

Repression of c-myc has also been reported for other steroid hormones. Glucocorticoids induce growth inhibition and c-myc repression in the human T lymphoblastic leukemic cell line CCRF-CEM (Yuh & Thompson, 1989), in mouse lymphoma cells (Eastman-Reks & Vedeckis, 1986), in oestrogen-treated oviducts of immature chickens (Kories et al., 1989), and in the murine lymphosacoma cell line P1798 (Forsthoefel & Thompson, 1987). A direct transcriptional repression of the murine c-myc gene by binding of the glucocorticoid receptor complex to a response element upstream of exon 1 has been discussed as a possible mechanism of c-myc shutoff (Forsthoefel & Thompson, 1987).

The response elements for the glucocorticoid and androgen receptor share the imperfect palindromic GTTACANN-NTGTTCT (Beato, 1989). In the androgen-regulated C3 gene of rat prostate androgen response is conferred by an element in the first intron (Claesens et al., 1989). This element AGTACGTGATGTTCT differs in only two positions of the left hand part to the consensus sequence. The first intron of the c-myc gene harbours also a potential glucocorticoid androgen receptor binding site GGTAGCAGCTGTTCT which diverges in two positions. All elements have the TGT-TCT motif in common which has been shown to be functionally important for glucocorticoid and androgen response. Whether glucocorticoid and androgen receptors can bind to the described element in the first intron of c-myc has yet to be proven.

Alternatively, steroid-receptor complexes may exert their negative effect on c-myc expression without direct binding to a response element. The glucocorticoid receptor (GR) and the transcription factor API can reciprocally repress one another's potency to activate transcription. In this particular antagonistic relationship, the negative factor (GR) does not displace the positive transcription factor (API) from its response element, but appears to interact directly with API (Jonat et al., 1990, Yang-Yen et al., 1990; Schüle et al., 1990). Thus, glucocorticoids may repress c-myc by direct binding to API I (20 located at a binding site which has been described close 330 bp upstream of the P-promoter. This region has been identified as negative element for c-myc transcription (Hay et al., 1987; Takimoto et al., 1989; Hay et al., 1989). Whether the androgen receptor can reduce transcription initiation of c-myc via API binding is not yet known.
In vivo androgen has a keyrole in the maintenance of prostate cells. The hormone preserves the differentiated state of the cells and represses c-myc. High expression of c-myc accomplished by a retroviral vector results in benign hyperplasia in mouse androgen-reconstituted prostate model (Thompson et al., 1989). Thus, regulation of c-myc in prostate cells by androgen is evident. However, the precise role of c-myc in growth control of normal and neoplastic prostate cells remains to be elucidated.

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