Cyclic AMP and c-myc gene expression in PY815 mouse mastocytoma cells

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The possibility was examined that inhibition of growth of PY815 mouse mastocytoma cells by N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (DB cyclic AMP) results from inhibition of c-myc gene expression. Temporary increases in c-myc RNA which occurred soon after DB cyclic AMP treatment and upon removal of the drug were not consistent with direct inhibition of c-myc gene expression by DB cyclic AMP. The increases in c-myc RNA coincided with the passage through, or accumulation of cells in late G_1 – early S phase. It is proposed that cyclic AMP may stimulate c-myc gene expression which normally occurs only in late G_1 – early S phase in PY815 cells and that cyclic AMP prevents c-myc expression in cells at other phases of the cell cycle by inhibiting their progression past a cyclic AMP-sensitive restriction point in early G_1 phase.

Cyclic AMP *c-myc gene* *Gene expression* *PY815 mouse mastocytoma cell*

1. INTRODUCTION

Adenosine 3',5'-cyclic monophosphate (cyclic AMP) has been implicated as a regulator of mammalian cell growth [1–3]. With PY815 mouse mastocytoma cells we have shown that the cyclic AMP analogue N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (DB cyclic AMP) increases the intracellular concentration of cyclic AMP and inhibits growth by slowing progression past a cyclic AMP-sensitive point in the G_1 phase of the cell cycle [4–6]. After removing DB cyclic AMP the growth-arrested PY815 cells require 2–3 h to become insensitive to readdition of DB cyclic AMP and progress into their normal 9–10 h cell cycle [5]. The initiation of growth of G_1 phase-arrested cells has recently been shown to involve the expression of growth-related genes or oncogenes [7–9]. Therefore it was possible that cyclic AMP might reduce the expression of specific oncogenes, to regulate PY815 cell growth. To test this hypothesis we examined the effect of inhibiting PY815 cell growth with DB cyclic AMP on the availability of c-myc RNA in PY815 cells. Our results indicate that DB cyclic AMP does not inhibit PY815 cell growth by decreasing c-myc RNA. Instead, c-myc RNA increased in a cell cycle-dependent manner which may be affected by cyclic AMP according to the position of cells within the cell cycle.

2. MATERIALS AND METHODS

PY815 mouse mastocytoma cells were grown as described [5]. The v-myc gene was provided by Dr A. Reeve (Biochemistry Department, Otago University, New Zealand) as a 1.5 kilobase DNA fragment cloned into the *PstI* site of pBR322 [10].

Total cellular RNA was prepared from 7×10^7 PY815 cells washed once with phosphate-buffered saline and recovered by centrifugation. The cells were resuspended at 2°C in lysis mixture (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.6), 0.5% Nonidet NP40, 10 mM vanadyl-ribonucleoside complexes) vortex-mixed for 10 s then underlayered with an equal volume of lysis mixture containing 24% sucrose and 1% NP40. After 5 min the lysate was centrifuged at $10000 \times g$ for 2 min at 4°C in a swinging bucket rotor and the

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upper layer of cytoplasmic material was recovered and mixed with an equal volume of 0.3 M NaCl, 2% SDS, 25 mM Na_2EDTA, 0.2 M Tris-HCl (pH 7.5). Proteinase K (200 μg/ml) was then added and the cytoplasmic extracts were incubated for 30 min at 37°C. Finally, the mixture was extracted several times with phenol:chloroform (1:1, v/v) and after centrifugation RNA in the aqueous phase was recovered by precipitation with 2 vols ethanol at −20°C. The RNA precipitates were recovered by centrifugation, washed once with 75% ethanol, 0.1 M Na acetate (pH 5.2), redissolved in 0.1 M Na acetate (pH 6), then phenol extracted at 60°C to ensure total protein removal [11].

To clone the v-myc gene into the phage M13mp8 DNA vector the 1.5 kilobase-pair v-myc DNA fragment was excised from the pBR322 v-myc hybrid with the restriction enzyme *PstI* and recovered by agarose gel electrophoresis followed by electroelution [12]. The v-myc DNA (100 ng) was then ligated into the *PstI* site of phage M13mp8 DNA (20 ng) in a final volume of 10 μl containing 1 mM ATP, 10 mM $MgCl_2$, 10 mM dithiothreitol, 66 mM Tris-HCl (pH 7.6) and 0.1 units of T4 DNA ligase at 14°C overnight. The ligated DNA was transformed into competent *E. coli* JM101 [13]. Transformed bacteria containing recombinant phage M13mp8 DNA were selected by plating aliquots of transformed cells in soft agar containing 0.2 ml of exponential phase *E. coli* JM101, 40 μl of 100 mM isopropyl thiogalactoside, 40 μl of 2% X-gal in dimethylformamide onto YT agar plates. The plates were incubated at 37°C for 12 h when recombinant phage replication produced clear plaques due to the v-myc DNA inserts placing the β-galactosidase gene out of phase with its promoter.

Single-stranded phage DNA was prepared from recombinant M13mp8 phage as described [14] and checked for v-myc DNA inserts by agarose gel electrophoresis (0.7% agarose in borate buffer). Recombinant M13mp8 phage DNA containing v-myc DNA showed reduced mobility on agarose gels. To identify clones with complementary v-myc DNA strands and ensure the transcribed strand of v-myc DNA was cloned, template DNAs containing inserts were screened by hybridising all M13mp8 DNAs (0.5 μg) to a single recombinant M13mp8 DNA in 10 μl of 10 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM Tris-HCl (pH 7.5). The DNA was denatured at 95°C and annealed by slow cooling to 20°C. Complementary phage template DNAs were distinguished from non-complementary phage template DNA by their reduced mobility in 0.7% agarose-borate buffer gels. To identify which clones carried the transcribed (+) strand of v-myc DNA the first 60 nucleotides of two complementary phage template DNAs were sequenced as described by Sanger et al. [15]. The recombinant M13mp8 DNA containing the (+) strand of v-myc was used to prepare a v-myc DNA hybridisation probe.

A high specific activity v-myc DNA probe (1×10^8 cpm ^{32}P per μg DNA) was synthesized by reverse priming the v-myc (+) template DNA using the method of Hu and Messing [16]. The New England Nuclear 13-nucleotide oligomer 1201 was used as primer. The primer was annealed and extended as described by Meinkoth and Wahl [17].

To detect c-myc RNA transcripts samples of total cellular RNA isolated from 1×10^6 cells at various times during and after drug treatment were resuspended in 10 μl of 0.75 M NaCl, 0.075 M Na_3^+ citrate, 7% formamide and denatured at 60°C for 15 min. The cellular RNA was then dotted onto nitrocellulose filters previously washed with 3 M NaCl, 0.3 M Na_3^+ citrate. The filters were baked, prehybridized and hybridized with the v-myc(+) probe as described [17]. Finally, the filters were washed 3 times with 15 mM NaCl, 1.5 mM Na_3^+ citrate, 0.1% SDS at 20°C for 5 min and with 1.5 mM NaCl, 0.15 mM Na_3^+ citrate, 0.1% SDS at 50°C until all background radioactivity was removed. The hybridized 'dots' were then cut out and associated Cerenkov radiation was measured in a liquid scintillation counter.

Cell cycle distributions were measured using an Ortho ICP-22A microfluorimeter after staining DNA with diamphinophenylindole.

3. RESULTS AND DISCUSSION

Initial attempts to detect c-myc gene expression in PY815 cells using a 'nick translated' v-myc DNA probe (spec. act. 5×10^6 cpm $^{32}P/\mu$g DNA) prepared from the 1.5 kilobase-pair v-myc gene according to the method of Rigby et al. [18] were unsuccessful. These v-myc probes were probably unable to detect a single messenger RNA species within a mixture of total cellular RNA due to their

low specific activity. Therefore, a probe with much higher specific radioactivity was prepared by cloning a 1.5 kilobase-pair fragment of DNA containing the v-myc gene into the phage gene vector M13mp8.

Recombinant M13mp8 DNAs were screened initially to detect clones containing complementary sequences by hybridizing a single clone to all other clones. Then the first 60 nucleotides of two complementary recombinant M13mp8 clones were sequenced to identify the clone containing the (+) sense transcribed strand of v-myc (cf. [19]). The M13mp8 v-myc clone containing the (+) strand of v-myc was used to make a radioactive probe by primer extension in the presence of [^{32}P]dCTP. Probes synthesised by this technique (spec. act. 1 $\times 10^8$ cpm per μg DNA) were used to detect c-myc gene expression by Northern dot-blot hybridisation. Probes were hybridised to total cellular RNA prepared from untreated PY815 cells, cells grown for 3, 6, 9, 12 and 15 h with 0.1 mM DB cyclic AMP + 1 mM theophylline and cells treated with drug for 15 h then transferred to drug-free medium for 3, 6, 9 and 12 h.

Fig.1A shows that 3 h after treating PY815 cells with DB cyclic AMP and theophylline the level of c-myc gene expression had increased 1.8-fold. However, this increase was not sustained despite the continued presence of DB cyclic AMP and theophylline for up to 15 h and between 6 and 15 h c-myc expression was reduced to half that in untreated cells. Three hours after removing the drugs c-myc expression again increased briefly to 1.5-fold that in untreated cells before decreasing again to low levels. These results showed no correlation between the availability of DB cyclic AMP and theophylline and c-myc RNA.

Other researchers have suggested that c-myc gene expression is associated with the G_1 phase of the cell cycle [7,8,20]. Therefore, we analysed the distribution of PY815 cells within the cell cycle at various times during and following treatment with DB cyclic AMP and theophylline, to determine whether the level of c-myc RNA was cell cycle related under the conditions of our experiments. Fig.1B shows that the peaks of c-myc RNA that occurred during DB cyclic AMP treatment, or when DB cyclic AMP was removed, corresponded to times when the majority of cells were present in late G_1 or early S phase of the cell cycle, rather

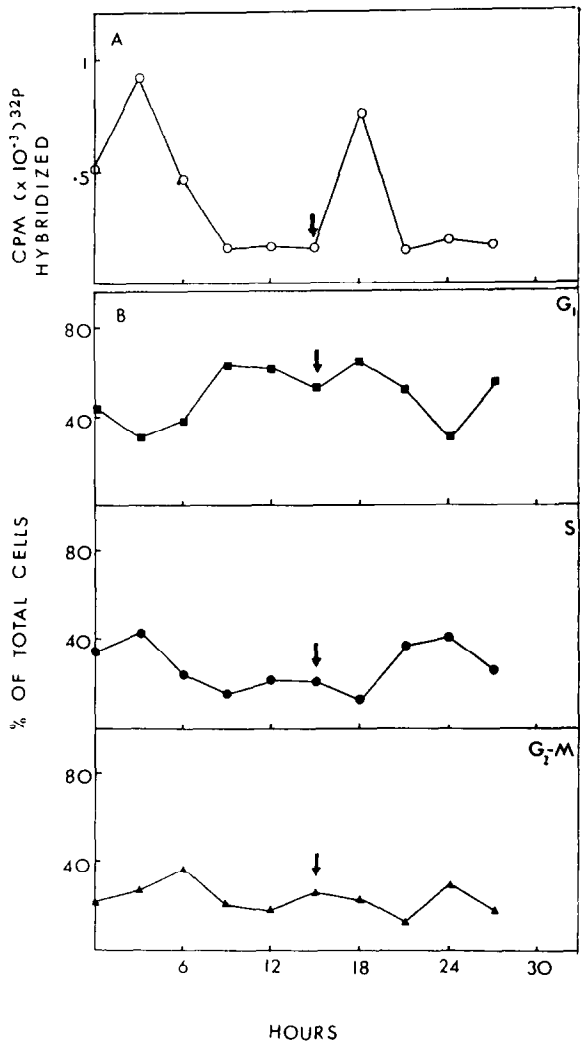

Fig.1. (A) Detection of c-myc RNA in total PY815 cell RNA by Northern dot-blot analysis. Hybridised ^{32}P-labelled v-myc DNA was measured at various times after adding DB cyclic AMP + theophylline to cultures and after transferring drug treated cells to drug-free medium after 15 h (arrowed). (B) Cell cycle distribution at various times after adding DB cyclic AMP + theophylline to cultures and after transferring drug-treated cells to drug-free medium at 15 h (arrowed).

than to the availability of DB cyclic AMP and theophylline.

The preceding results do not support the idea that inhibition of PY815 cell growth by cyclic AMP is a consequence of inhibition of c-myc gene expression. In fact, c-myc RNA increased, when cells were treated with DB cyclic AMP and

theophylline and again soon after the drugs were removed. These increases in c-myc RNA occurred when the majority of cells resided in late G_1 or early S phase, beyond the cyclic AMP-sensitive restriction point in early G_1 phase of the PY815 cell cycle [5,6]. Thus the increases in c-myc RNA appeared to be associated with the movement of cells out of G_1 into S phase. c-myc RNA eventually decreased below that in untreated log phase cells when cells accumulated at the cyclic AMP-sensitive point in early G_1 phase (fig.1A and B).

These observations would be consistent with the proposal that DB cyclic AMP stimulates the normal increase in c-myc RNA that occurs in PY815 cells beyond the cyclic AMP-sensitive restriction point in G_1 phase. In addition cyclic AMP would prevent other cells from passing the cyclic AMP-sensitive restriction point and increasing c-myc RNA production in late G_1–early S phase. This would occur if c-myc expression is normally confined to a period in late G_1–early S phase [8,20] and stimulated by cyclic AMP.

While our research was in progress Huang and Cho-Chung [21] reported that hormone withdrawal or DB cyclic AMP suppresses the c-ras^H oncogene in rat mammary carcinomas.

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