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EFFICIENT RETROVIRAL TRANSFER OF A MOUSE c-myc CONSTRUCT INTO HL60

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SUMMARY We introduced an LTR-driven mouse c-myc second and third exon, Tn5Neo gene construct into the inducible human leukemia line HL60 using an amphotropic retroviral vector system. Over 90 % of the cells became neo-resistant and the transfected myc gene was transcribed in several neomycin resistant clones. Making use of the simultaneous presence of the different myc genes in the same cell, we compared expression of the corresponding mRNAs after differentiation and their decay mechanisms.

The MC29 virus-related proto-oncogene c-myc has been implicated in the initiation and/or maintenance of cellular proliferation in a wide variety of biological systems (1-7). myc mRNA expression is usually elevated in proliferating normal and transformed cells (1,8-10). Constitutive expression of transfected myc genes prevented dimethyl sulfoxide (DMSO) induced terminal differentiation of murine erythroleukemia (MEL) cells (11-13), suggesting that myc down-regulation regularly accompanying cellular differentiation (6,14) is required for the progression of differentiation after acquisition of the committed state (11).

DMSO induces granulocytic (15), 12-0-tetradecanoylphorbol-13-acetate (TPA) induces monocytic differentiation (16) in the human promyelocytic leukemia cell line HL60 (17). myc, which is amplified and overexpressed (6,18) in HL60, is rapidly down-regulated after differentiation induction. Formation of transcription elongation block at the first exon, first intron boundary seems to be the primary mechanism of myc down-regulation upon granulocytic differentiation (19).

We transfected HL60 with an Mo-MuLV long terminal repeat (LTR) driven myc construct that also contained the neomycin selection marker pSV2-neo (20) via retroviral infection (21). Expression of the transfected (designated +myc) and resident (c-myc) genes was demonstrated by RNase protection analysis. This sys-

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tem allowed us to compare some features of the regulation of the (resident) c-myc and (transfected) +myc genes operating in the same cell.

METHODS

HL60 cells were cultured in RPMI 1640 + 10 % fetal calf serum (FCS) and induced to differentiate by cultivation for several days in the presence of dimethyl sulfoxide (DMSO, Sigma Chemical Co., used at 1.25 %) or 12-0-tetradecanoylphorbol-13-acetate (TPA, Sigma Chemical Co., used at 10 nM or as noted).

The retroviral vector expressing the mouse c-myc gene was constructed (see Fig. 1) by inserting the XhoI-XhoI fragment containing the coding exons of the mouse myc gene from the cDNA clone pMC-myc 54 (a gift from Dr. K. Marcu) into the Sal I site of the retroviral vector pLJ (A. Korman and R. Mulligan, unpublished). This vector is a MoMuLV based vector with the Tn5 Neo gene placed under the control of the SV40 early promoter. The proximal splice donor of the MoMuLV has been removed by a point mutation. The myc gene is transcribed from the LTR-promoter. The PLJ-myc construct (see Fig. 1) was transfected into $\psi 2$ cells (22) by the calcium-phosphate technique (23), and Neo-resistant colonies were selected. Virus was transferred into the amphotropic PA-12 (24) cells by infection with the $\psi 2$ culture supernatant filtered through 0.22 µm membranes. Infection by PA-12 supernatants was done in the presence of 4 µg/ml of Polybrene for 24 hrs. Selection on 1 mg/ml of G418 was commenced 48 hrs after infection and continued for 2 weeks. Clones of G418-resistant HL60 were isolated by limiting dilution. Efficiency of gene transfer, as estimated from the percentage of G418 survivors, was >90%.

To demonstrate (mouse) +myc mRNA expression in the presence of high levels of (human) c-myc mRNA in HL60, RNase protection analysis (25) was performed. [^{32}P] -labeled RNA probes were synthesized by T3 or T7 polymerases from a mouse pMC-myc 54 (a gift from Dr. K. Marcu) exon 3 pvu II - xho I fragment cloned into Bluescribe M13+ (Vector Cloning Systems) vector (or by Sp6 polymerase in the Amprobe system for the human abl gene), after appropriate linearization. RNA, prepared (26) from clones of transfected HL60 that survived G418 selection was hybridized with the RNA mouse c-myc exon 3 probe.

RESULTS AND DISCUSSION

+myc mRNA expression

A 370 b RNA fragment can be protected from RNase from the 410 b probe that contains 40 bases from the Bluescribe vector. Fig. 2A shows that a 370 b frag-

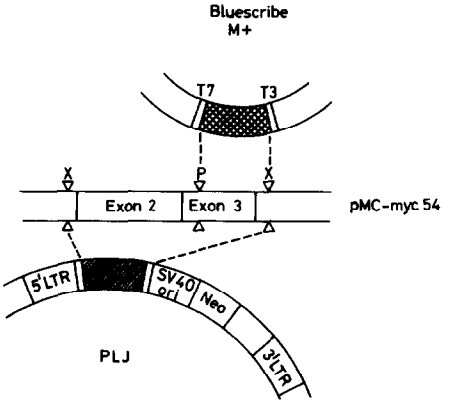

Fig. 1. Schematic structure of the PLJ-myc construct and mouse exon 3 myc probe. P: PvuII, X:XhoI restriction sites.

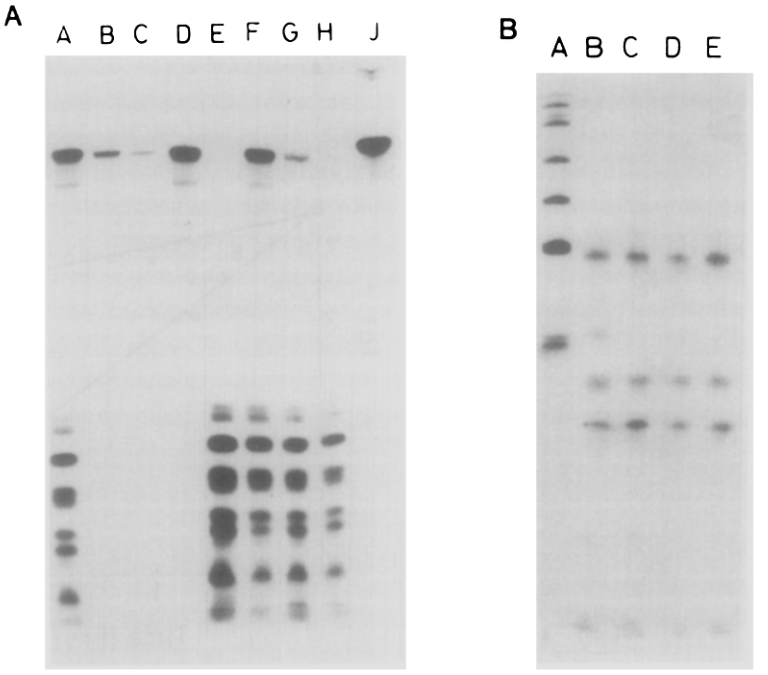

Fig. 2A. RNase protection analysis. The antisense exon 3 mouse myc probe (see Fig. 1) was hybridized with RNA prepared from: transfected HL60 clone 20 before (lane A), after 3 days of DMSO (B), 2 days of TPA (C) induction; non-transfected HL60 (E); transfected cl.2, 3, 4 (lanes F,G.H, respectively) and A9 plasmocytoma cells (D). Lane J: 5000 cpm of the 410 b-long probe.

2B. RNase protection analysis. RNA prepared from transfected HL60 before (lane B), after 3 (C) and 5 (D) days of DMSO induction, or after 2 days of induction with TPA (E), were hybridized with a human c-abl probe. Lane A: pBR322 HgaI fragments.

ment was protected in clones 20, 2, 3 (also in clone 1, see Fig. 3) but not in clone 4. The levels of +myc expression varied 10-fold between clones 20 and 3. There was no variation in c-myc expression, as judged by the band intensities of the degraded human myc fragments. Nontransfected cells did not have +myc while mouse plasmocytoma cells (A9) expressed only +myc (mouse) specific mRNA. Correspondingly, single copy myc band on the Southern was detected only in case of the transfected cells (data not shown).

Effect of differentiation induction

Fig. 2A also demonstrates that +myc was present after 3 days of DMSO, and 2 days of TPA induction, although in quantities diminished (to varying extent in different experiments) as compared to the preinduction level. Fig. 2B shows that in contrast with c-myc, c-abl expression did not change during differentiation, in agreement with earlier data (6).

In view of the early down-regulation of c-myc upon differentiation we wanted to know if there is an early period when c-myc is already down-regulated while +myc is still unchanged. Fig. 3 (lanes A, C, E) shows that 18 hrs after addition of DMSO c-myc is barely visible while +myc is just as highly expressed as before induction.

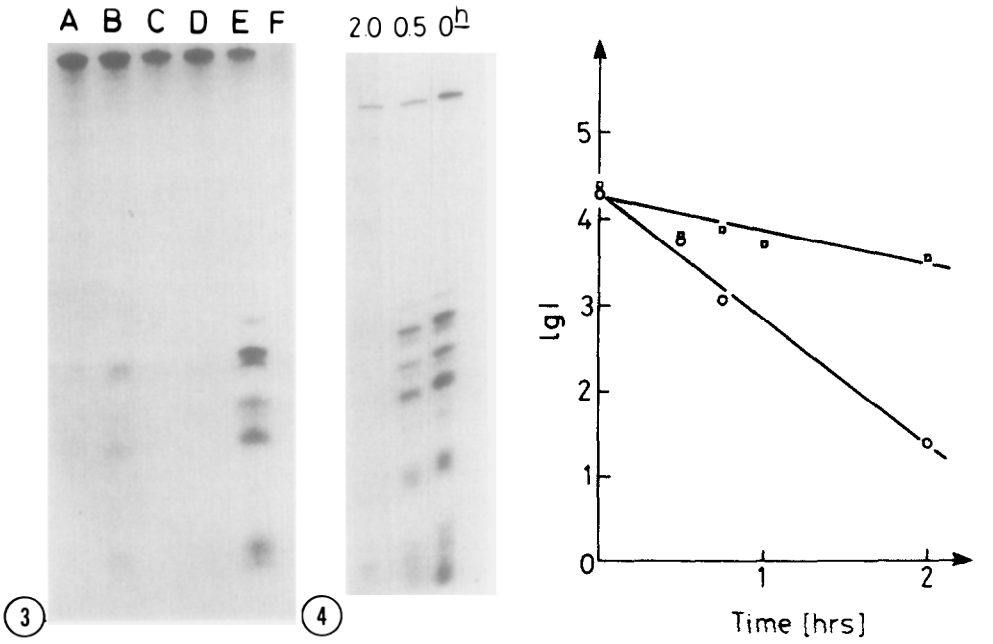

Fig. 3. RNase protection analysis. RNA from transfected HL60 (cl.1) was hybridized with the antisense exon 3 probe (A-E). A: 24 hrs of DMSO induction; B: 24 hrs of DMSO induction, 1 hr cycloheximide (10 µg/ml) treatment; C: 24 hrs of TPA induction; D: 24 hrs of TPA induction, 1 hr cycloheximide; E: non-induced cells, no treatment; F: RNA hybridized with the sense (T7-transcribed) myc probe.

Fig. 4. Decay kinetics of c-myc and +myc. Transfected HL60 (cl.20) was treated with 0.5 µg/ml actinomycin D for 0-2 hrs. RNA was prepared from aliquots of $2x10^7$ cells for RNase protection analysis using the mouse exon 3 probe. Logarithms of densitometric readings (LKB Ultroscan) of the +myc (square symbols) and (one of the) c-myc (circle symbols) bands are plotted on the Y axis (lg I).

Comparison of turnover of the c-myc and +myc transcripts

Decay of the two myc mRNAs after blocking transcription with actinomycin D followed different kinetics (see Fig. 4). c-myc had a half-life of about 30 mins, while +myc about 120 mins. In agreement with these $t_{1/2}$ values, cycloheximide, an inducer of gene expression (by preventing mRNA degradation; see below), induced a ∿3-fold increment in 1 hr for c-myc and in ∿3 hrs for +myc (Fig. 5). After 3,5 hrs, however, (or later, data not shown) c-myc mRNA levels were greatly reduced in the same cells, suggesting that cycloheximide only transiently prevented c-myc degradation, while +myc stayed invulnerable to this degradation mechanism.

Cycloheximide did not restore c-myc to its pre-differentiation level (Fig. 3), in agreement with the notion that c-myc down-regulation upon differentiation occurs primarily at the transcriptional level (19).

The prolonged decay of +myc mRNA may be accounted for by the lack of destabilizing 5' and 3' sequences (27-29). The half-life values measured in our studies are close to those measured in Colo 320 cells by Rabbitts et al. (30), in

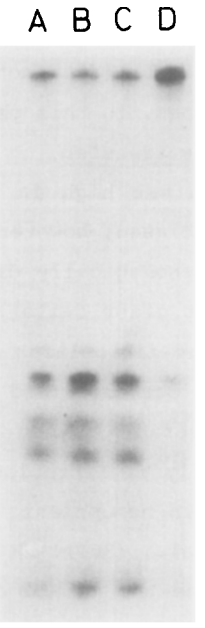

Fig. 5. Effect of cycloheximide on +myc and c-myc expression. RNA was prepared from transfected HL60 (cl.20) before (A), after 1 hr (B), 1.5 hr and 3.5 hrs of cycloheximide treatment (10 µg/ml). RNase protection analysis was performed using the mouse exon 3 probe.

Burkitt's lymphoma lines (28), and murine plasmocytomas (29) carrying truncated myc genes. Earlier data on myc mRNA degradation in HL60 gave shorter value (15 m. (31)). The degradation process may be directly coupled to completion of translation as suggested by Linial et al. (5) or to the activity of a specific labile RNase as suggested by Kelly et al. (4).

Inhibition of protein synthesis would prevent mRNA degradation in either case. +myc seems to follow this pattern. The (transient) c-myc mRNA increment early after cycloheximide treatment is also in agreement with a protein synthesis dependent mRNA degradation mechanism. The selective reduction of c-myc mRNA after 3 hrs cycloheximide treatment, however, suggests that c-myc mRNA is subject to a degradation mechanism not affecting +myc. This mechanism is activated long after protein synthesis has been inhibited. We speculate that there is a protein synthesis dependent mRNA stabilizing mechanism that is inactivated late upon cycloheximide treatment. This mechanism should be specific for c-myc (as opposed to +myc). Alternatively, the mRNA degradation process is not fully inactivated in the presence of cycloheximide and within 2-3 hrs it will overcome the rate of transcription that is also declining in the absence of protein synthesis. In the latter case we must suppose that labile positively acting transcription factors, not involved in +myc (LTR-driven) transcription, are required for c-myc transcription.

c-myc expression decreased after prolonged (2 hrs) cycloheximide treatment in non transfected HL60 cells as well (data not shown), ruling out the role of an interaction between the myc genes, in this phenomenon.

Efficiency of retroviral gene transfer

The level of myc expression was high in 4/6 of the neo-resistant clones investigated. This figure may be biased, however, since those clones were chosen for RNA analysis that seemed morphologically different from the parental cells (by the presence of a few percent giant cells). Shiroki et al. (32) achieved a level of LTR-myc expression in rat fibroblasts as high as that in ours in HL60. Expression of an LTR-construct in our case (and in that of Collins, see ref. 33) is in variance with the less favorable experience of Magli et al. (34). Thus, LTR appears to function efficiently in fibroblasts (see also Rasmussen and Gilboa (35)), and at least in certain hemopoietic cells, in line with earlier experience (reviewed by Stewart et al., (36)).

Retrovirus vectors may become the method of choice for gene transfer into hematopoietic cells, should the problems of variable efficiency be resolved. Collins (33,37) recently reported the efficient use of an amphotropic retrovirus vector in the transfer of a Moloney-LTR-driven neo marker into K562 cells, while only a few percent of HL60 or KG-1 cells became infected. HL60 exhibits a remarkable phenotypic heterogeneity (38) that could explain subline differences in amphotropic virus receptor density. However, the subline used in our experiments was relatively resistant to infection by the same amphotropic pseudotype carrying a different construct (our unpublished results), demonstrating that infectivity of the retrovirus depends partly on the construct used. The main difference between the construct used by Collins (33,37) and us seems to be that his was shorter and gave rise to spliced (therefore not packaged) RNA, as well as full-length RNA molecules.

The high efficiency of gene transfer in our system will hopefully enable us to study the vectorial factors influencing the efficiency and the sequence determinants for the differential +myc mRNA turnover.

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