Eur J Cancer Clin Oncol, Vol. 24, No. 11, pp. 1727–1735, 1988.
Printed in Great Britain

0277-5379/88$3.00 + 0.00

The Same External Signal Differentially Induced the c-myc Expression in Burkitt Lymphoma and B-Lymphoblastoid Cell Lines

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Abstract—*An extracellular signal, such as phorbol-12-myristate-13-acetate (PMA), was found to reduce the c-myc expression in Burkitt lymphoma (BL) cells but augment the expression of the same gene in a B-lymphoblastoid cell line (B-LCL). Studies with Epstein–Barr virus (EBV)-converted BL cells demonstrated that the differential effect of PMA on c-myc expression was not due to alterations in the structure of the translocated c-myc gene, but to the difference in the intracellular milieu of the B cells. Experiments on the degradation rate in c-myc RNA suggested that this phenomenon in c-myc expression was exerted, at least in part, at the transcriptional level.*

INTRODUCTION

C-MYC EXPRESSION IN lymphocytes and fibroblasts has been found to be under the direct control of growth signal and responsible for the progression of resting cells from G_0 into G_1 phase [1]. Therefore, perturbation of myc expression may play an important role in the malignant transformation of B cells [2, 3].

Burkitt lymphoma (BL) cells carry a reciprocal translocation between chromosome 8 involving the c-myc gene and either chromosome 14, 2 or 22 [4–7]. The consequence of such a translocation may induce the deregulation of c-myc expression. Several studies have shown not only quantitative but also qualitative changes in c-myc expression in BL cells [8–11]. In general, this is reflected by an increase in c-myc expression. However, in some BL cell lines, c-myc expression was less than that in some B lymphoblastoid cell lines (B-LCLs) [12], suggesting the level of expression may not be of critical importance between BL and B-LCL cells. As a qualitative change, it has been reported that the more distal promoter, P_1, is preferentially activated in BL cells, whenever the dual promoter region of the c-myc gene is preserved during translocation [12]. However, the preferential usage of the P_1 promoter does not always apply to all BL cell lines. Recent reports indicate that the activation of the translocated c-myc gene depends on the stage of B cell maturation [13]. But, a precise analysis of this mechanism has not yet been reported. The present study shows that PMA stimulation which augments c-myc expression in B-LCL cells paradoxically reduces a similar expression in BL cells. In experiments using EBV-converted BL (E95-C) cells that reveal the phenotypic characteristics specific for B-LCL cells, it is suggested that the same external signal may provide a reciprocal effect on c-myc expression depending on differences in the intracellular milieu.

Accepted 22 June 1988.

MATERIALS AND METHODS

Cells

All cell lines used are shown in Table 1 and were maintained at a cell density of 1–5 × 10^5 cells/ml in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 5 × 10^{-5} M 2-ME. All BL cell lines were kindly provided by G. Klein (Karolinska Inst., Sweden). E95-C cells, which are more B-LCL like than E95C-BL-41 cells [15], were cloned from E95C-BL-41 cells in our laboratory.

Reagents

PMA (phorbol-12-myristate-13-acetate) and actinomycin D were obtained from Sigma Co. Ltd. (St. Louis, MO). Monoclonal antibodies against B-LCL associated antigens (LB-1 and BB-1) were kindly provided by E.A. Clark (Washington Univ., U.S.A.).

Table 1. Characteristics of BL and B-LCLs used in this study

Cell line	Growth pattern	EBV	Monoclonal antibody binding* LB-1†	BB-1†
BL				
Jijoye	Single cells	+	−	−
BL-29	Single cells	+	−	−
BL-36	Single cells	+	−	−
Ramos	Single cells	−	−	−
BL-41	Single cells	−	−	−
EBV-converted EL-41 (E95-C)	Small clumps	+	++	+
B-LCL				
RPMI8866	Large clumps	+	++	++
RPMI1788	Large clumps	+	++	++
CL-4	Large clumps	+	++	++

*The fluorescence intensity was evaluated as negative (−), weak (+) and strong (++).
†B-LCL associated antigen [14].

RNA extraction

Total RNA was isolated from cells by the procedure of Auffray and Rougeon [16], utilizing urea and LiCl. The RNA pellet was washed twice in cold ethanol and quantitated by absorbance at 260 nm.

Northern blotting assay

For hybridization analysis, 10 μg of total RNA preparations were denatured by formaldehyde, fractionated on 1% agarose gel and transferred to a nitrocellulose filter [17]. DNA probes were labelled by nick-translation and hybridization was conducted at 42°C in 5 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.2% Ficoll, 0.2% bovine serum albumin (BSA), 0.2% polyvinylpyrolidone (PVP), 0.1% SDS and 100 μg/ml heat denatured salmon sperm DNA. After 16 h, the filter was washed at 42°C in 0.1 × SSC and 0.1% SDS to remove unhybridized probes prior to autoradiography. A 1.4 kb ClaI-EcoRI fragment of the c-myc gene including the third exon was used as a probe for the analysis of c-myc expression. The fragment was prepared from PSV2.26 provided by S. Tonegawa (M.I.T., MA) [18]. A 2 kb BamHI–PVuII fragment of the pseudo-human β actin gene provided by T. Kakunaga (Osaka University) was utilized as a probe for measurement of β-actin expression.

Dot hybridization

Dot hybridization was carried out as described by White and Bancroff [19]. Seventy-five microliters of RNA solution containing 10 μg of total RNA was transferred to a 1.5-ml tube containing 45 μl of 20 × SSC plus 30 μl of 37% (w/w) formaldehyde. The mixture was then incubated at 60°C for 15 min. For analysis, 50 μl of each sample were serially diluted with 100 μl of 15 × SSC, and 100 μl of each dilution were applied on a nitrocellulose filter supported with a 96-hole minifold apparatus (Schleicher and Schuell). The nitrocellulose filter was then baked at 80°C under vacuum and hybridization was carried out as in the Northern blotting assay.

s_1-Nuclease protection assay

This assay was followed as described by Weaver and Weissmann [20], but slightly modified. A single stranded 860 bp PvuII–PvuII fragment DNA of the first c-myc exon cloned in M13 and labelled with [α-^{32}P]dCTP by primer extension was hybridized with 20 μg of total cellular RNA. Hybridization was carried out in 80% (v/v) formamide, 400 mM NaCl, 40 mM PIPES (ph 6.4) and 1 mM EDTA at 55°C for 12 h followed by 100 units s_1-nuclease digestion for 40 min at 30°C and separated on 5% urea-denatured polyacrylamide gel.

Fluorescence-activated cell sorting (FACS)

FACS was performed as described in [21].

RESULTS

c-myc expression in PMA-stimulated BL and B-LCL cells

In order to demonstrate the differential effect of PMA on c-myc expression in Burkitt lymphoma (BL) and B-lymphoblastoid cell line (B-LCL) cells, exponentially growing BL (Jijoye) and B-LCL (RPMI8866) cells were stimulated with 100 ng/ml of PMA and the total cellular RNA was extracted at various time periods. c-myc expression was analyzed by a Northern blotting assay utilizing a ClaI–EcoRI fragment including the c-myc third exon as a probe. A representative result shown in Fig. 1 demonstrates that PMA induced about a 3–5-fold decrease in the c-myc expression in BL cells, whereas about a 2–5-fold increase was

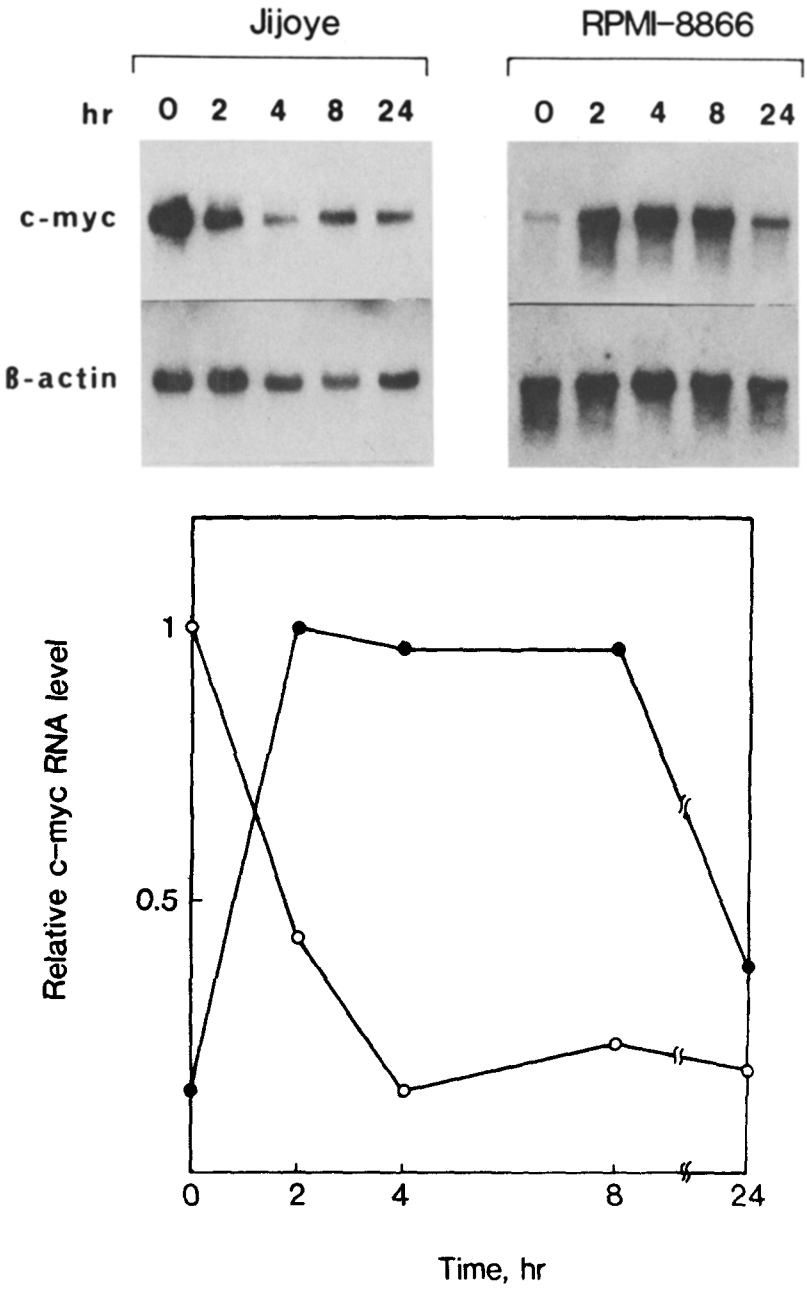


Fig. 1. Effect of PMA on c-myc expression in BL (Jijoye) and B-LCL (RPMI8866) cells. (Upper) RNA blot analysis. Ten micrograms of total RNA from each sample were analyzed on a 1% agarose gel. As an internal control, the same filter was rehybridized with a β-actin probe. (Lower) Densitometric analysis. The c-myc RNA levels were expressed as the ratio of c-myc/β-actin signals. Open circle, Jijoye cells. Closed circle, RPMI-8866 cells.

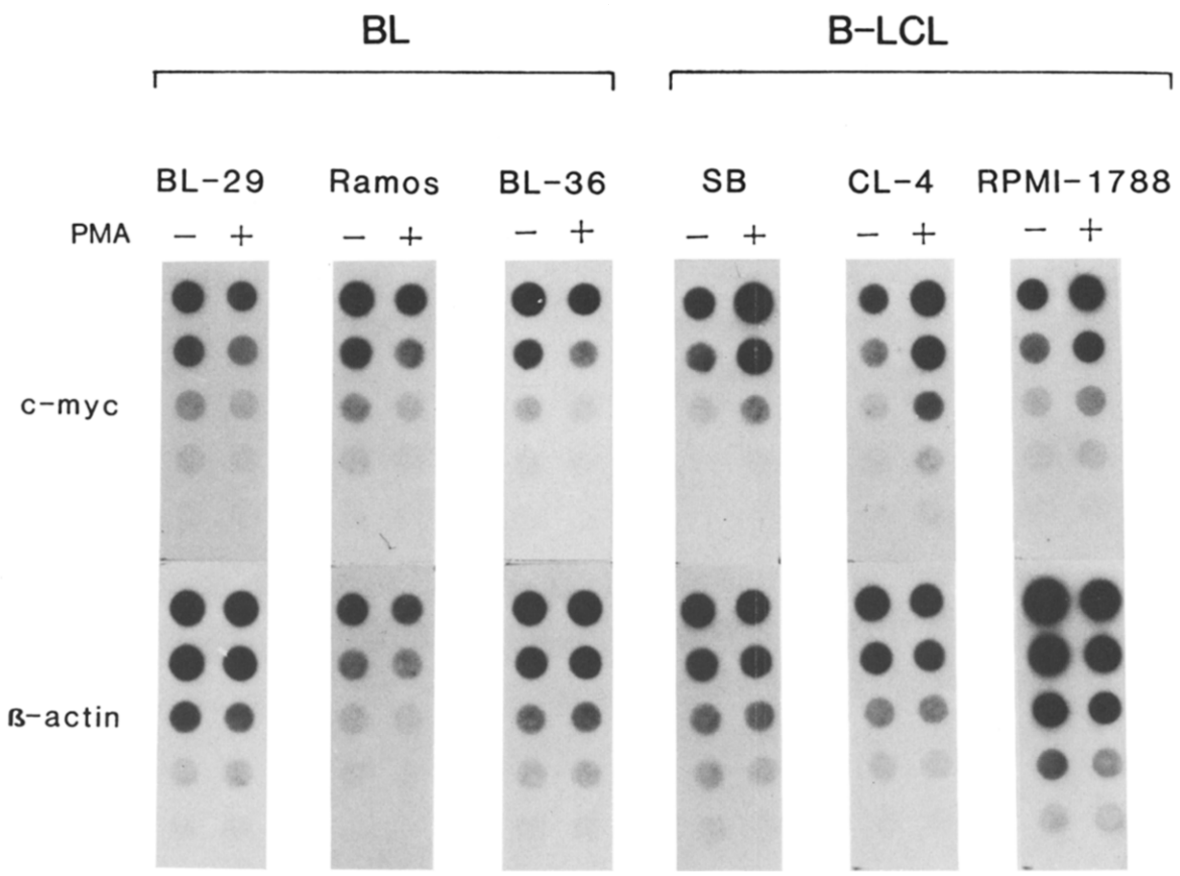


Fig. 2. Effect of PMA on c-myc expression in BL and B-LCLs. Ten micrograms of the RNA and four successive 1:3 *dilutions were spotted on the blot. All other experimental details are the same as described in the legend to Fig.* 1.

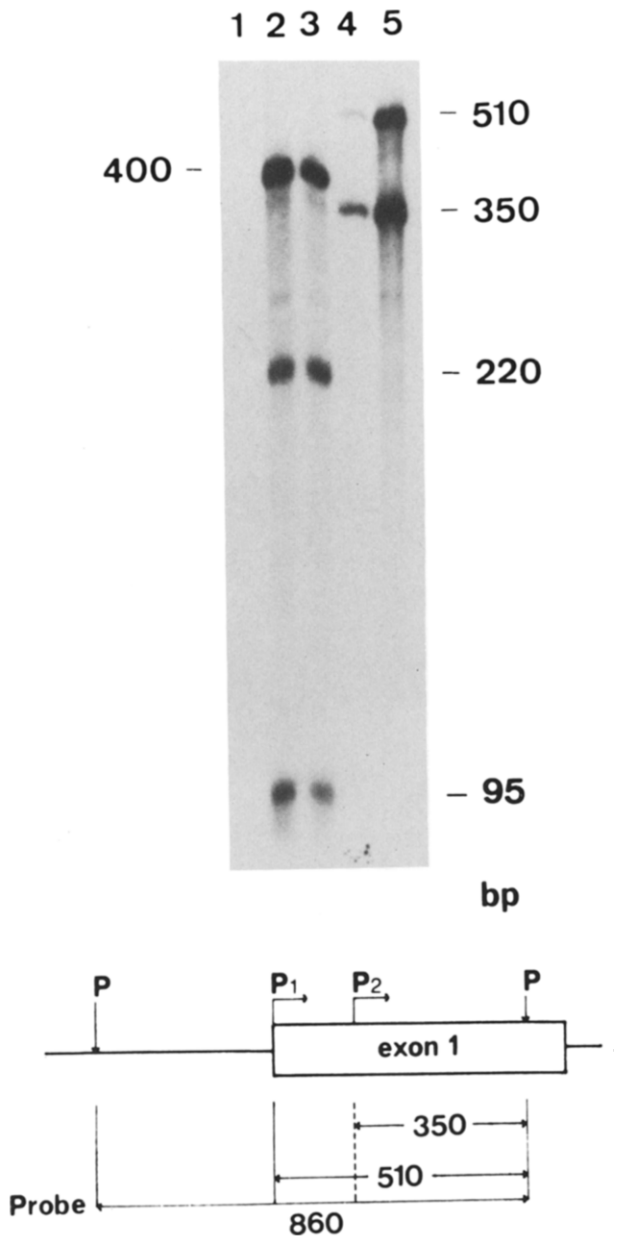
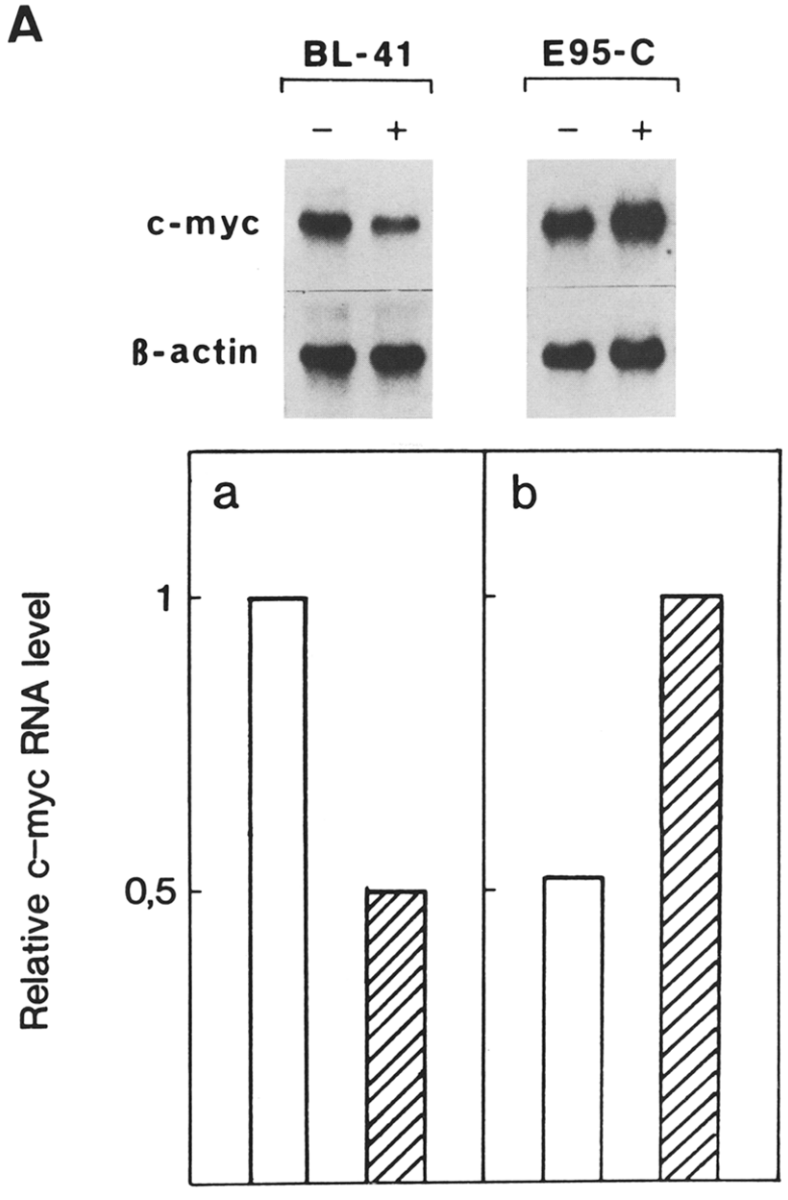


Fig. 3. s_1-Nuclease protection assay in BL (Jijoye) and B-LCL (RPMI-8866 cells). A 860 bp PvuII-PvuII fragment containing c-myc first exon, labelled with [α-^{32}P]dCTP by primer extension was used as a probe for hybridization with 20 μg of total RNA in Jijoye or RPMI-8866 cells. Lane 1 shows tRNA. Lanes 2 and 4 show non-treated Jijoye and RPMI8866 cells, respectively. Lanes 3 and 5 show PMA (100 ng/ml)-treated Jijoye and RPMI8866 cells, respectively. The bands of 510 and 350 bp represent the normal c-myc transcripts initiating at P_1 and P_2, respectively. P = PvuII.



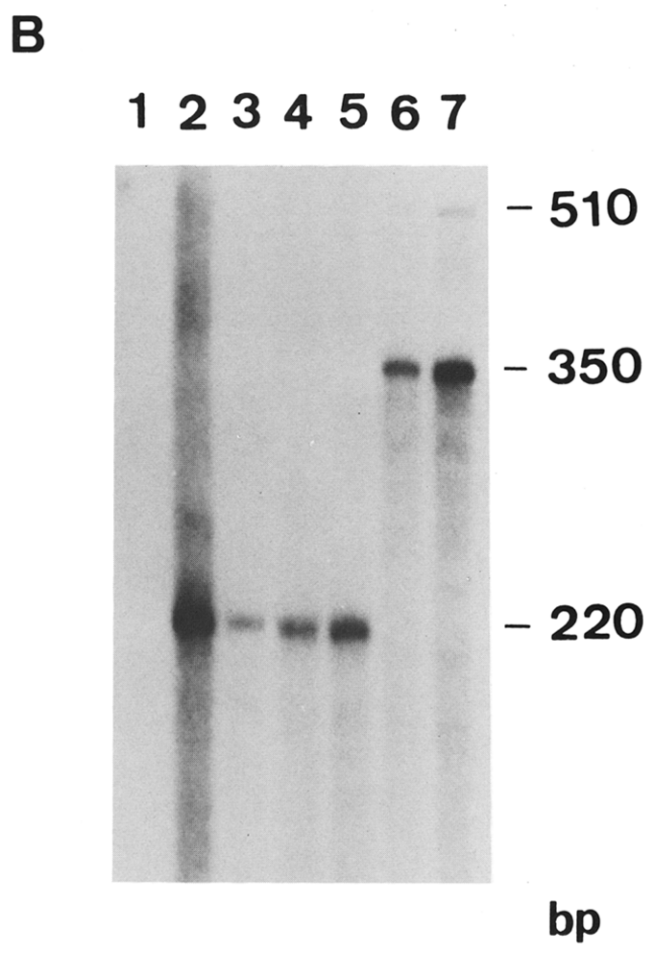


Fig. 4. (A) Effect of PMA stimulation on the c-myc expression in BL-41 and EBV-converted BL-41 (E95-C) cells. Cells were stimulated with 100 ng/ml of PMA for 2 h and total RNA were subjected to Northern blotting analysis. All other experimental details are the same as described in the legend to Fig. 1. (Upper) RNA blot analysis. (Lower) Densitometric analysis. (a) BL-41 cells. (b) E95-C cells. Hatched bars, PMA-treated cells. Open bars, non-treated cells. (B) s_1-Nuclease protection assay in BL-41 and E95-C cells. Lane 1 shows tRNA. Lanes 2 and 4 show non-treated BL-41 and E95-C cells, respectively. Lanes 3 and 5 show PMA-treated BL-41 and E95-C cells, respectively. Lanes 6 and 7 show PMA-treated and non-treated RPMI8866 cells, respectively. All other experimental details are the same as described in the legend to Fig. 3.

observed in B-LCL cells. The differential effect of PMA on c-myc expression already appeared at 2 h following stimulation. The expression of the β-actin gene was measured as a control for the relative amount of RNA in each sample, and the result indicated that β-actin expression remained nearly constant throughout the course of PMA stimulation. We interpret small variations in the amount of β-actin RNA as resulting from experimental error.

In order to exclude the possibility that a reciprocal effect of PMA on the c-myc expression is the case only for these two cell lines, Jijoye and RPMI-8866, the c-myc expression in the other BL cell lines (BL-29, Ramos and BL-36) and B-LCLs (SB, CL-4 and RPMI1788) was examined. PMA (100 ng/ml) stimulation for 2 h induced a decrease in the c-myc expression in all BL cell lines and an increase in all B-LCLs tested, although the degree of the suppression and the augmentation was variable. In contrast, the expression of the β-actin gene was nearly constant (Fig. 2). Therefore, these results confirmed that PMA induced a reciprocal effect on c-myc expression in BL and B-LCL cells.

The expression of the translocated c-myc gene in BL cells

In order to examine whether the c-myc expression in 2 h PMA-treated or non-treated BL cells comes from only the translocated c-myc gene, we studied the s_1-nuclease protection assay using the c-myc first exon as a probe. As shown in Fig. 3, we could detect the c-myc expression in Jijoye cells (lanes 2 and 3) at three bands (400, 220 and 95 bp). On the other hand, c-myc expression in RPMI8866 cells (lanes 4 and 5) used as a normal control of the c-myc gene was clearly detected at two bands (510 and 350 bp). Furthermore, PMA stimulation could not induce a new initiation site of the c-myc transcript in Jijoye and RPMI8866 cells (lanes 3 and 5). In other BL cell lines used in this experiment (BL-29, Ramos and BL-36), no normal transcripts (510 and 350 bp) were detected (data not shown). These results indicate that almost all c-myc expression in PMA-treated or non-treated BL cells result from the translocated c-myc gene.

Effect of the EBV on the regulation in the c-myc expression

It is said that some EBV-converted BL cell lines reveal the phenotypic characteristics specific for B-LCL [22]. Table 1 shows that EBV can induce the B-LCL phenotype in EBV-negative BL cells. In order to elucidate an effect of the B-LCL phenotype on c-myc expression, we compared c-myc expression of EBV-negative BL (BL-41) cells and that of EBV-converted BL-41 (E95-C) cells, which were subcloned as described in Materials and Methods. As shown in Fig. 4(A), stimulation of PMA reduced c-myc expression in BL-41 cells as in the case of the other BL cell lines. On the other hand, PMA induced an increase in c-myc expression in E95-C cells, although the degree of augmentation was less than that in B-LCL cells.

To investigate whether the c-myc expression in E95-C cells did not come from the normal c-myc gene, the s_1-nuclease protection assay was carried out using the first c-myc exon as a probe. As shown in Fig. 4(B), c-myc expression in PMA-treated (lane 5) and non-treated E95-C cells (lane 4) occurred from the translocated c-myc gene and the initiation site was the same as that in BL-41 cells (lanes 2 and 3). These results indicate that the EBV did not induce expression in the normal c-myc gene, but induced the reciprocal change of the expression in the translocated c-myc gene.

Effect of PMA on c-myc RNA degradation

Cells stimulated with PMA for 2 h were exposed to an inhibitory dose of actinomycin D to block all transcriptional activity. The fate of the c-myc RNA was followed by densitometric scanning of bands on autoradiograms at different time intervals. A representative result is shown in Fig. 5. Under these conditions, no significant difference in the degradation rate of the c-myc RNA was observed between PMA-treated and non-treated cells, and this was the case both for Jijoye and RPMI8866 cells. Two other independent experiments showed nearly the same results (data not shown).

DISCUSSION

This study demonstrates the reciprocal effect of PMA on c-myc expression in BL and B-LCL cells: PMA reduces c-myc expression in BL cells, while augmenting it in B-LCL cells. These observations were not simply due to a difference in the level of c-myc expression in BL and B-LCL cells. In RPMI-8866 cells, c-myc expression was higher than that in certain BL cells.

In order to elucidate whether the reciprocal effect of PMA on c-myc expression is due to a difference in the time course of c-myc expression or in the optimum concentration of PMA required, more detailed studies on the kinetics of c-myc expression and dose–response of PMA were carried out. The RNA samples extracted from the stimulated cells at 0, 10, 30, 60 and 120 min were examined for c-myc expression. A decrease in c-myc expression could be detected at 10 min after stimulation in BL cells without any temporary increase in the expression in that length of time, compared to 0 min (data not shown). Whereas in B-LCL cells the c-myc expression started increasing also at 10 min following stimulation and no decrease was observed in that interval, compared to 0 min (data not shown). Furthermore, both cell lines were stimulated with various concentrations of PMA and c-myc expression at 2 h following stimulation was

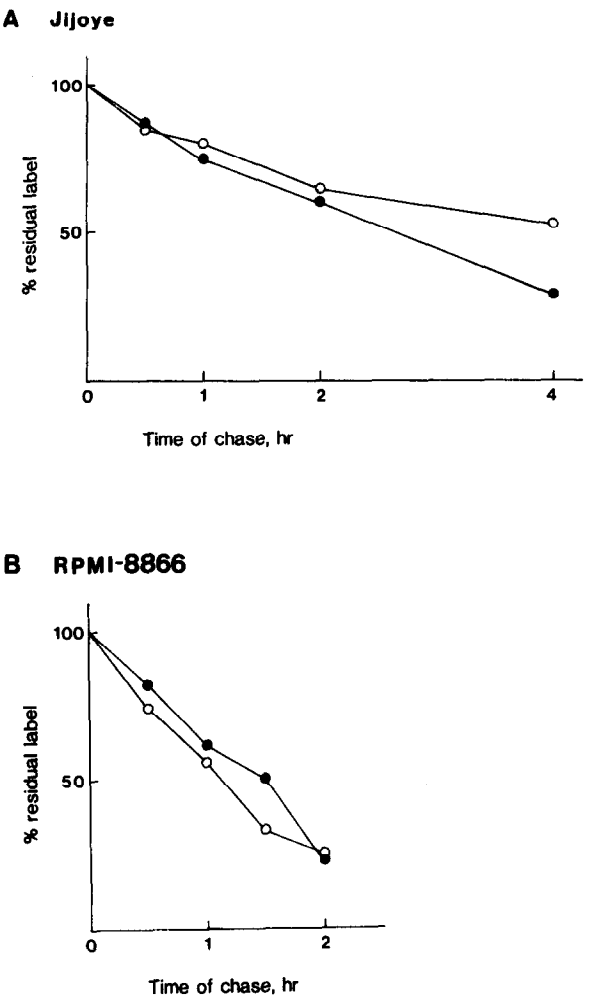


Fig. 5. The degradation rate of the c-myc RNA in PMA-treated and non-treated cells. Cells were pre-treated for 2 h *with* 100 ng/ml *of PMA and incubated further with* 5 μg/ml *of actinomycin D for the indicated time periods. Ten microliter aliquots of total RNA prepared at each time point were analyzed for the c-myc expression by Northern blotting assay. The c-myc RNA levels were expressed as described in the legend to Fig.* 1. *Closed circle, PMA-treated cells. Open circle, non-treated cells.*

examined. PMA induced a dose-dependent decrease in c-myc expression in BL cells, while a dose-dependent increase in B-LCL cells was observed (data not shown).

Anti-immunoglobulin (anti-Ig) and 1-oleoyl-2-acetyl-glycerol (OAG) also showed the same effect on c-myc expression in BL and B-LCL cells (data not shown). Anti-Ig can activate protein kinase C through the activation of phosphatidyl-inositol (PI) turnover and an increase in the intracellular Ca^{2+} [23, 24], whereas both PMA and OAG are direct activators of protein kinase C [25]. These observations, therefore, suggest that activation of protein kinase C induced a different effect on c-myc expression in BL and B-LCL cells.

As shown in Table 1, an EBV-carrying BL cell line does not always represent the phenotypic characteristics specific for B-LCL. It is, therefore, conceivable that EBV infection *per se* is not responsible for the reciprocal effect of PMA on the c-myc expression. Furthermore, c-myc expression in E95-C cells came from only the translocated allele regardless of PMA stimulation [Fig. 4(A), (B)]. One possible explanation for these observations is that the reciprocal effect of PMA on c-myc expression was not due to alterations in the structure of the translocated c-myc gene, but to a difference in the transcriptional mechanism or signal transduction, i.e. a difference in intracellular milieu. However, in order to generalize this possibility, it may be necessary to elucidate c-myc expression in other cell lines.

Several reports still debate whether the regulation of c-myc expression occurs at the transcriptional or the post-transcriptional level [26–30]. Furthermore, an elongation block of c-myc transcription in down-regulation of c-myc expression was recently reported [31]. In the present study, it is suggested that the reciprocal effect of PMA was due to regulation, at least in part, at the transcriptional level. Nevertheless, it is not clear whether PMA could induce a block of the transcript elongation in BL cells. The half-life of the c-myc RNA in BL cells was found to be slightly longer, in other words, the RNA was more stable than that in B-LCL cells, in agreement with the results reported by other investigators [32, 33]. However, we cannot understand what this result means.

Acknowledgements—The author wishes to thank G. Klein for providing all BL cell lines and E.A. Clark for providing monoclonal antibodies against B-LCL associated antigens. I also thank E. Barsumian for his critical reading of the manuscript and M. Kawata for her secretarial assistance. This study was supported by a special Grant in Aid from the Ministry of Education, Science and Culture, Japan.

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