GTPγS inhibits early c-*myc* protein accumulation but not DNA synthesis in Swiss 3T3 fibroblasts

Stephen R. Pennington*, John P. Moore**, Gerard I. Evan***, T. Robin Hesketh and James C. Metcalfe

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, UK

Received 10 August 1990

Quiescent Swiss 3T3 fibroblasts stimulated with epidermal growth factor and insulin showed large transient increases in c-*myc* mRNA and c-*myc* protein accumulation which were maximal at about 2 h after addition of the co-mitogens. When the cells were loaded with 0.1 mM of guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) by transient permeabilisation immediately before mitogenic stimulation, the increase in c-*myc* mRNA was similar to that observed in unloaded cells but the corresponding c-*myc* protein peak was reduced by at least 95%. The GTPγS completely blocked incorporation of [^{35}S]methionine into cell proteins for 3–4 h after addition of the mitogens, but not thereafter, and caused a delay in the subsequent onset of DNA synthesis by the same period. The data show that less than 5% of the early increase in c-*myc* protein normally observed after mitogenic stimulation is required for its obligatory role in the progression of cells to S phase implied by other evidence.

GTPγS; c-*myc* Protein; DNA synthesis; 3T3 fibroblast; Transient permeabilisation

1. INTRODUCTION

It has recently been shown that when GTPγS (a non-hydrolysable analogue of GTP) is loaded into Swiss 3T3 fibroblasts by transient permeabilisation, it causes substantial accumulation of inositol phosphates and cyclic AMP [1] consistent with activation of the GTP-dependent proteins coupled to phosphatidylinositol 4,5-bisphosphate hydrolysis (G_p) [2,3] and adenylate cyclase (G_s) [4–6]. These G proteins are activated by a variety of mitogens that stimulate quiescent fibroblasts to progress to DNA synthesis in S phase [7–10] and this raised the question of whether GTPγS also caused mitogenic activation.

A variety of studies have implied that activation of the c-*myc* proto-oncogene, one of a family of related genes which encodes a nuclear DNA binding protein, is necessary but not sufficient for the progression of normal cells from the quiescent G_0 state to S phase [11–22]. For example, microinjection of bacterially expressed c-*myc* protein into Swiss 3T3 fibroblasts enabled the cells to grow in platelet-poor plasma lacking platelet-derived growth factor [13] and antisense c-*myc* oligodeoxynucleotides have been shown to cause sequence-specific inhibition of mitogenic stimulation of both fibroblasts and lymphocytes [11,12]. However, the role of the c-*myc* protein in the transition to S phase is not clearly resolved. Some studies have suggested that it may be directly or indirectly involved in DNA replication [23] although this was discounted by subsequent work [24]. Others have implied a role for c-*myc* protein in trans-activation or trans-repression of other genes [25–27] or in the regulation of mRNA turnover [28].

In examining whether GTPγS activated all of the early responses to normal mitogens in fibroblasts, we compared the expression of the c-*myc* gene in response to GTPγS with the response to stimulation by the co-mitogens epidermal growth factor (EGF) and insulin. It was found that GTPγS caused a complete but temporary block on protein synthesis for 3–4 h and as a result of this block the normal accumulation of c-*myc* protein after mitogen addition was almost abolished. The GTPγS delayed the subsequent entry of the cells into S phase by the same period as the inhibition of protein synthesis but did not inhibit the extent of DNA synthesis. The implications of these observations for the role of c-*myc* protein in the G_0 to S phase transition are discussed.

Correspondence address: J.C. Metcalfe, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, UK

**Present address:* Dept of Human Anatomy and Cell Biology, University of Liverpool, PO Box 147, Liverpool, L69 3BX, UK

***Present address:* Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London, SW3 6BY, UK

****Present address:* Imperial Cancer Research Fund Laboratories, Dominion House, Bartholemew's Close, London, EC1A 7BE, UK

Abbreviations: GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2. EXPERIMENTAL

2.1. *Cell culture and transient permeabilisation*

Swiss 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Gibco), 44 mM $NaHCO_3$, 25 mM glucose, penicillin G (35 U/ml) and streptomycin (80 mU/ml) in a humidified atmosphere of air/CO_2 (90:10), pH 7.4, at 37°C. Cells were seeded onto 90 mm plastic Petrid dishes (3×10^5 cells) and grown to confluence (5–7 days) before use. DNA synthesis was measured by the incorporation of [^{3}H]thymidine (0.5 μCi/ml) into acid precipitable material [1] in non-permeabilised cells and in transiently permeabilised cells re-seeded to 30 mm Petri dishes which had been coated with freshly prepared rat tail collagen [29]. Transient permeabilisation of the cells was achieved by a modification of the scraping procedure of McNeil et al. [30,31] as described previously [1].

2.2. c-myc *mRNA and* c-myc *protein assays*

The quantitation of c-*myc* mRNA from non-permeabilised or transiently permeabilised cells was performed as described previously [1,32]. The amounts of c-*myc* protein in cell lysates prepared from non-permeabilised and transiently permeabilised Swiss 3T3 fibroblasts were measured by the enzyme-linked immunosorbent assay described by Moore et al. [33]. To prepare the lysates, cell monolayers were washed twice with 5.0 ml of ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, pH 7.2), 5.0 ml of ice-cold PBS was added and the cells were harvested by gentle scraping with a rubber policeman. The cell suspension was centrifuged (120 × *g*, 6 min) and the pellets resuspended in 50 μl of lysis buffer (1% sodium dodecyl sulfate (SDS), 1% aprotinin, 0.5 mM phenylmethylsulfonylfluoride and 50 mM dithiothreitol in PBS). Cellular DNA was sheared by repeated passage of the cell lysates through a 26-gauge needle and the samples were boiled for 5 min. After incubation with 100 mM iodoacetamide for 30 min on ice the samples were again passed through 26-gauge needles, diluted 10-fold in PBS and stored at −80°C before assaying.

2.3. *[^{35}S]Methionine incorporation*

Transiently permeabilised cells were re-seeded to 30 mM Petri dishes coated with freshly prepared rat tail collagen and incubated in DMEM containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid and 20 μCi/ml [^{35}S]methionine (Amersham Int.). Incubations were terminated by washing the cells twice with ice-cold PBS (1.0 ml) and the cells lysed by the addition of 1% SDS (0.5 ml). The SDS solution was transferred to 1.5 ml of ice-cold trichloroacetic acid (TCA) and incubated on ice for 30 min. The samples were filtered under suction through Whatman GF/C filters, washed 4 × with ice-cold TCA, air-dried and their radioactivity determined by liquid scintillation counting.

3. RESULTS AND DISCUSSION

The time courses of c-*myc* mRNA and c-*myc* protein accumulation in response to EGF with insulin in Swiss 3T3 fibroblasts are shown in Fig. 1a,b. Both the mRNA and protein showed large transient increases that were maximal at about 2 h after the addition of the mitogens with a smaller and variable maximum at about 8 h. The amounts of both c-*myc* mRNA and protein were only slightly increased compared with unstimulated cells after 12 h when the onset of DNA synthesis occurs.

Fibroblasts can be transiently permeabilised to a wide range of molecules by the scrape-loading technique first described by McNeil and co-workers [30,31]. When Swiss 3T3 fibroblasts were permeabilised in the absence of GTPγS, there was a substantial increase in c-*myc* mRNA and c-*myc* protein (Fig. 1c,d). There were small additional increases in the amounts of both mRNA and protein in response to stimulation by EGF and insulin (Fig. 1c,d). It was shown previously that 98% of the surviving cells re-seal within 2 min of permeabilisation as judged by the exclusion of Lucifer yellow and fluorescein-labelled dextrans [1]. The similarity of the time course of the initial transient in c-*myc* protein in the re-sealed cells and those which had not been permeabilised implies that functional recovery to synthesise macromolecules in response to mitogens occurs without significant delay in the re-sealed cells.

When the cells were loaded with GTPγS by transient permeabilisation, the large increase in the amount of c-*myc* mRNA (Fig. 1e) caused by permeabilisation was unaffected but the corresponding early burst of c-*myc* protein at 2–3 h was abolished and only a small accumulation was observed at 8 h (cf. Fig. 1d and 1f). This small and delayed increase in c-*myc* protein in cells transiently permeabilised in the presence of GTPγS was further reduced by the action of EGF and insulin (Fig. 1f) to less than 5% of the transient peak in normally stimulated cells (Fig. 1b).

The data for c-*myc* protein accumulation (Fig. 1f) strongly suggested that GTPγS might inhibit protein synthesis, at least for a few hours after loading the cells. The time course of incorporation of [^{35}S]methionine into cells is shown in Fig. 2. GTPγS completely inhibited the incorporation of [^{35}S]methionine into cells for 3–4 h either with or without the addition of EGF and insulin. Subsequently the rates of [^{35}S]methionine incorporation were similar for cells stimulated with EGF and insulin irrespective of prior loading with GTPγS, implying that the inhibitory effect of GTPγS on protein synthesis was fully relieved. Recovery from GTPγS inhibition was also observed for unstimulated cells which incorporated [^{35}S]methionine more slowly (Fig. 2). Control experiments demonstrated that extracellular GTPγS did not affect [^{35}S]methionine incorporation in non-permeabilised cells whereas cycloheximide (100 μM) substantially reduced uptake in these cells and blocked the response to EGF and insulin throughout G_1 (not shown). The inhibitory effect of GTPγS on protein synthesis is consistent with previous reports demonstrating that non-hydrolysable analogues of GTP inhibit cell-free protein biosynthesis (see, for example, [34]). From the data here it is clear that GTPγS inhibits protein synthesis for 3–4 h in intact cells and that this accounts for the abolition of the early peak of c-*myc* protein accumulation.

The delay in onset of protein synthesis induced by GTPγS implied that it might have an effect on the time course of DNA synthesis. This was compared for cells transiently permeabilised in the presence or absence of GTPγS and then stimulated by EGF and insulin (Fig. 3a). There was a delay in the onset of [^{3}H]thymidine in-

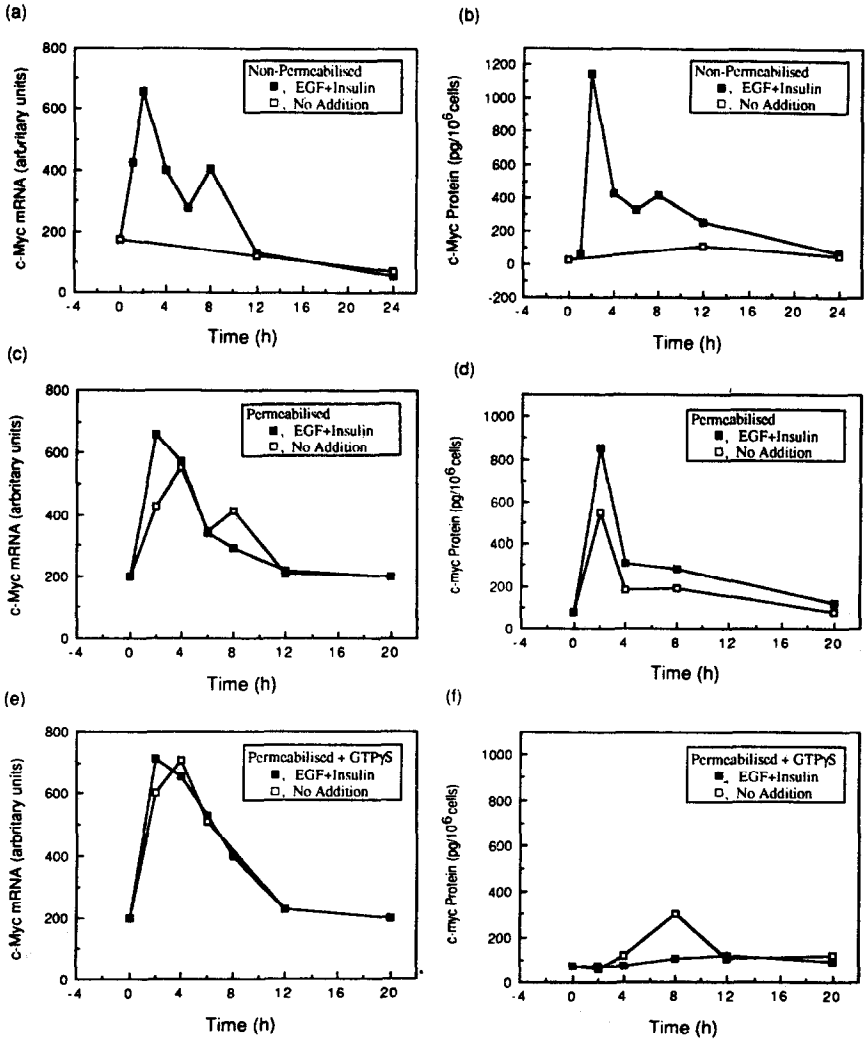

Fig. 1. Time courses of c-*myc* mRNA and c-*myc* protein accumulation in fibroblasts. Cells were either non-permeabilised (a,b) or transiently permeabilised in the absence (c,d) or presence (e,f) of 0.9 mM GTPγS. In each experiment cells were treated either without (□) or with (■) 10 ng/ml EGF and 1 μg/ml insulin. Data for c-*myc* mRNA and c-*myc* protein are the means of duplicate determinations from a representative experiment of three. Errors were less than ± 10% of the mean values.

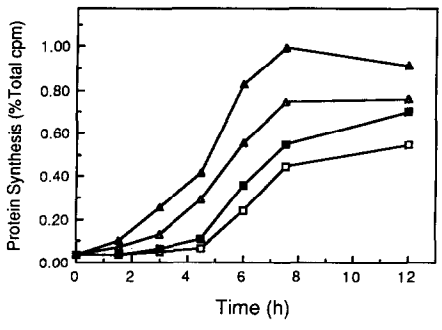

Fig. 2. Time courses of [^{35}S]methionine incorporation into fibroblasts. Cells were transiently permeabilised in the presence (□,■) or absence (△, ▲) of 0.9 mM GTPγS and were treated with (▲,■) or without (△,□) EGF (10 ng/ml) and insulin (1 μg/ml).

corporation of 3–4 h, similar to the delay in [^{35}S]methionine incorporation, but no effect on the final extent of [^{3}H]thymidine incorporation. Control experiments showed that transiently permeabilising the cells was sufficient to stimulate a small but consistent increase (about 5%) in the uptake of [^{3}H]thymidine (Fig. 3b). However, GTPγS alone had no additional effect on DNA synthesis in permeabilised cells and any effects of GTPγS on DNA synthesis in response to either EGF and insulin or foetal calf serum were small (<15%) (Fig. 3b). Taken together, the data demonstrate that cells transiently permeabilised in the presence of GTPγS and stimulated with EGF and insulin show only a small increase in c-*myc* protein which is less than 5% of the amount accumulated in non-permeabilised cells

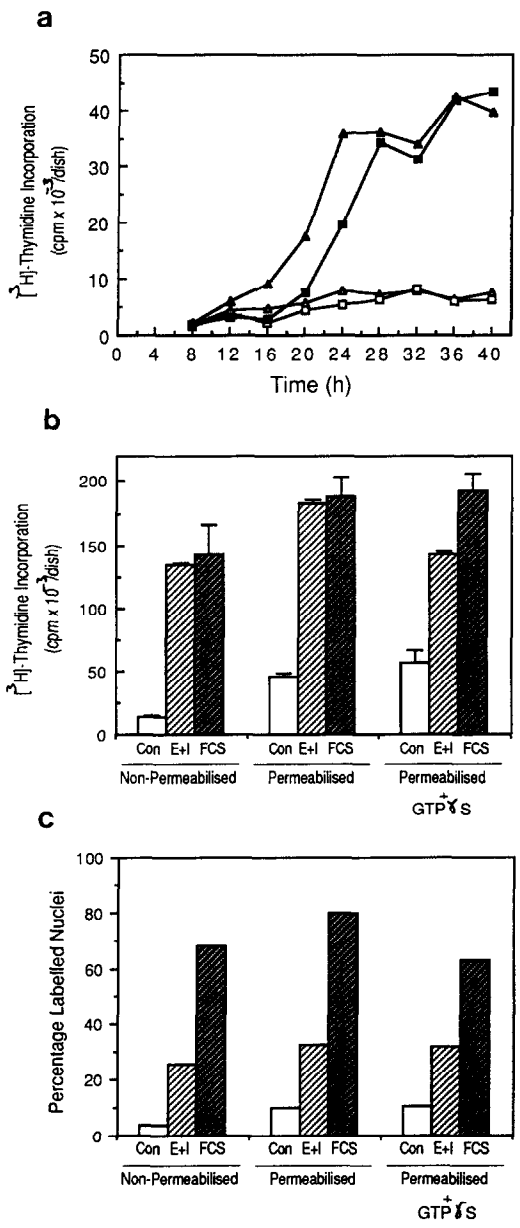

Fig. 3. DNA synthesis in fibroblasts. (a) Time courses of [^{3}H]thymidine incorporation into acid-precipitable material in cells transiently permeabilised in the presence (□,■) or absence (△, ▲) of 0.9 mM GTPγS and treated with (▲,■) or without (△,□) 10 ng/ml EGF and 1 μg/ml insulin. (b) [^{3}H]thymidine incorporation at 30 h after stimulation. Cells received no addition (Con), 10 ng/ml EGF and 1 μg/ml insulin (E+I) or 10% FCS (FCS).

stimulated with the mitogens, but will nevertheless progress to DNA synthesis in S phase.

The overall effects of GTPγS can be rationalised according to the scheme in Fig. 4 in which GTPγS activates early c-*myc* gene transcription via second messengers from G_p and/or G_s but inactivates the GTP-dependent proteins required for subsequent translation (see [35]). The full relief of inhibition after 3–4 h, as evidenced by the subsequent rates of [^{35}S]methionine incorporation, must be due to metabolic inactivation of

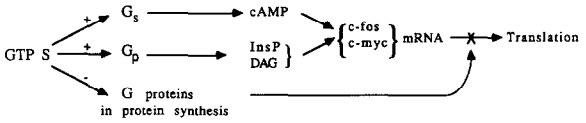

Fig. 4. Early effects of GTPγS on fibroblasts.

GTPγS since the alternative possibility of overproduction of the GTP-dependent proteins required for protein synthesis cannot occut in the presence of GTPγS. The activation of G_p and G_s (and any other unidentified G proteins coupled to the mitogenic pathway) is presumably reversed when the GTPγS is inactivated, so that in the absence of normal mitogenic stimulation the cells fall back into the quiescent state. It is clear that the failure of GTPγS to act as a mitogen cannot be attributed to its inhibitory effect on early c-*myc* protein synthesis, since the amount of this protein at 8 h after permeabilisation in the presence of GTPγS was much larger than when the GTPγS-loaded cells were stimulated by the co-mitogens and the cells subsequently progressed to S phase. At the time that the GTPγS-induced inhibition of protein synthesis was relieved after 3–4 h, substantial amounts of c-*myc* mRNA were still present in the cells. The inhibition of the residual c-*myc* protein response by the EGF and insulin was therefore unexpected but may result from activation of the normal mechanisms that cause the net amounts of c-*myc* protein to decline later in G_1 in non-permeabilised cells.

The delay in onset of DNA synthesis in cells that had been located with GTPγS by a period equivalent to the block in early protein synthesis strongly suggests that the early activation of protein synthesis is a critical determinant of the duration of G_1. Delays in entry into S phase can also be induced by the addition of a conventional inhibitor of protein synthesis, cycloheximide, with the co-mitogens, and the length of the delay is determined by the duration of the exposure to the inhibitor (data not shown).

Assuming, as the evidence summarised earlier very strongly implies, that there is an obligatory requirement for c-*myc* protein in G_1 for progression to S phase, the amount that is required soon after mitogen addition is less than 5% of the maximum c-*myc* protein concentration normally observed in the cells. From current evidence it is at least plausible that an essential role for c-*myc* protein is to regulate the expression of other genes required for progression through G_1, possibly through a nuclear post-transcriptional mechanism [28] and that these genes are activated by a relatively low concentration of c-*myc* protein. It is also possible that the much higher concentrations of c-*myc* protein normally observed when the cells are stimulated by mitogens are involved in the regulation of genes controlling differentiated functions of the cells that are not directly involved in proliferation. The use of GTPγS to

restrict c-*myc* protein accumulation without affecting DNA synthesis may therefore facilitate the identification of those genes activated via c-*myc* that are required for proliferation.

Acknowledgements: This work was supported by a grant from the Cancer Research Campaign (to J.C.M. and J.P.M.). S.R.P. was the recipient of an Elmore Medical Research Studentship.

REFERENCES

[1] Pennington, S.R., Hesketh, T.R. and Metcalfe, J.C. (1988) FEBS Lett. 227, 203–208.
[2] Cockroft, S. and Gomperts, B.D. (1985) Nature (Lond.) 314, 534–536.
[3] Cockroft, S. (1987) Trends Biochem. Sci. 12, 75–78.
[4] Pfeuffer, T. and Helmreich, E.J.M. (1975) J. Biol. Chem. 250, 867–876.
[5] Smigel, M.D., Katada, T., Northup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17, 1–18.
[6] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[7] Paris, S. and Pouyssegur, J. (1987) J. Biol. Chem. 262, 1970–1976.
[8] Paris, S., Chambard, J.-C. and Pouyssegur, J. (1987) J. Biol. Chem. 262, 1977–1983.
[9] Rozengurt, E., Legg, A., Strang, G. and Courtenay-Luck, N. (1981) Proc. Natl. Acad. Sci. USA 78, 4392–4396.
[10] Rozengurt, E. (1982) Exp. Cell Res. 139, 71–78.
[11] Heikkila, R., Schwab, G., Wickstrom, E., Loke, S.L., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987) Nature (Lond.) 328, 445–449.
[12] Wickstrom, E.L., Bacon, T.A., Gonzalez, A., Freeman, D.L., Lyman, G.H. and Wickstrom, E. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 1028–1032.
[13] Kaczmarek, K., Hyland, J.K., Watt, R., Rosenbert, M. and Baserga, R. (1985) Science 228, 1313–1315.
[14] Armelin, H.A., Armelin, M.C.S., Kelly, K., Stewart, T., Leder, P. and Stiles, C.D. (1984) Nature (Lond.) 310, 655–660.
[15] Mougneau, E., Lemieux, L., Rassoulzadegan, M. and Cuzin, F. (1984) Proc. Natl. Acad. Sci. USA 81, 5758–5762.
[16] Prochownik, E.V., Kudowsda, J. and Rogers, C. (1988) Mol. Cell. Biol. 8, 3683–3695.
[17] Cavalieri, F. and Goldfarb, S. (1987) Mol. Cell. Biol. 7, 3554–3560.
[18] Cory, S., Bernard, O., Bowtell, D., Schrader, S. and Schrader, J.W. (1987) Oncogene Res. 1, 61–76.
[19] Stern, D.F., Roberts, A.B., Roche, N.S., Sporne, M.B. and Weinberg, R.A. (1986) Mol. Cell. Biol. 6, 870–877.
[20] Sorrentino, V., Drozdoff, V., McKinney, M.D., Zeitz, L. and Fleissner, E. (1986) Proc. Natl. Acad. Sci. USA 83, 8167–8171.
[21] Kelekar, A. and Cole, M.D. (1986) Mol. Cell. Biol. 6, 7–14.
[22] Holt, J.T., Redner, R.L. and Neinhuis, A.W. (1988) Mol. Cell. Biol. 8, 963–973.
[23] Iguchi-Ariga, S.M., Itani, T., Kiji, Y. and Ariga, H. (1987) EMBO J. 6, 2365–2371.
[24] Gutierrez, C., Guo, Z-S., Farrell-Towt, J., Ju, G. and DePamphilis, M.L. (1987) Mol. Cell. Biol. 7, 4594–4598.
[25] Kingston, R.E., Baldwin, A.S. and Sharp, P.A. (1984) Nature (Lond.) 312, 280–282.
[26] Kaddurah-Daouk, R., Greene, J.M., Baldwin, A.S. and Kingston, R.E. (1987) Genes Deriv. 1, 347–357.
[27] Schweinfest, C.W., Fujiwara, S., Lau, L.F. and Papas, T.S. (1988) Mol. Cell. Biol. 8, 3080–3087.
[28] Prendergast, G.C. and Cole, M.C. (1989) Mol. Cell. Biol. 9, 124–134.
[29] Bornstein, M.B. (1958) Lab. Invest. 7, 134–137.
[30] McNeil, P.L., Murphy, R.F., Lanni, F. and Taylor, D.L. (1984) J. Cell. Biol. 98, 1556–1564.
[31] McNeil, P.L. and Taylor, D.L. (1985) Cell Calcium 6, 83–93.
[32] Moore, J.P., Todd, J.A., Hesketh, T.R. and Metcalfe, J.C. (1986) J. Biol. Chem. 261, 8158–8162.
[33] Moore, J.P., Hancock, D.C., Littlewood, T.D. and Evan, G.I. (1987) Oncogene Res. 2, 65–80.
[34] Hershey, J.W.B. and Monro, R.E. (1966) J. Mol. Biol. 18, 68–76.
[35] Proud, C.G. (1986) Trends Biochem. Sci. 11, 73–77.