(Promega, Biotec) and oligomers were added when indicated to the reaction mixture.

### *Artificial chain termination reaction using 3′-O-methyl guanosine triphosphate*

Chain termination of nascent RNA transcripts by incorporation of 3′-*O*-methyl guanosine triphosphate was utilized to generate RNA chains of known length and sequence from SP6 promoter. RNA synthesis reactions with modified chain-terminating substrate was performed as in the standard *in vitro* synthesis assays except for the addition of 0.1 μM 3′-*O*-methyl guanosine (Pharmacia).

### *Cell lines and proliferation assays*

Human urinary bladder cancer cell line T24, rat NIH 3T3 and human HBL 100 cell lines were obtained from the American cell culture collection (Rockville, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Flow) supplemented with 7% heat inactivated (30 min at 65°C) foetal calf serum, antibiotics (50 U/ml of penicillin and 50 U/ml streptomycine) and 4 mM glutamine. Cells were seeded on microtitre plates at a density of $2 \times 10^3$/well. Oligomers were added at various concentrations, each well contained 200 μl medium. After 3 days the microcultures were fed by replacing 200 μl medium with fresh medium containing oligomers until the fifth day. Cell numbers were determined daily using a counting chamber for quantification of cells or colourimetric assay using (3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyltetrazolium bromide) as described (Denisot and Lang, 1986). Treated and untreated cells showed 98–100% viability after 5 days of growth. In 5 days the number of untreated cells was increased 30-fold on the average.

### *Transport and stability of oligonucleotides in T24 cells*

For each time point $5 \times 10^6$ c.p.m. of 5′ $^{32}$P-labelled oligodeoxynucleotide (2 pmole of labelled oligonucleotide and 1 μM carrier unlabelled oligonucleotide) was added to $5 \times 10^5$ T24 cells in 2 ml of cell culture medium. Following incubation for the specified period, the cells were collected and oligomer extracted from cytoplasm and nuclei as previously described (Teichman-Weinberg *et al.*, 1988). Oligonucleotide extracted from 50 μl culture medium, cytoplasm and nucleus was subjected to electrophoresis on 20% polyacrylamide gels containing 7 M urea.

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# N-*myc* disrupts protein kinase C-mediated signal transduction in neuroblastoma

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**In neuroblastoma, amplification of the N-*myc* gene is closely correlated with increased metastatic ability. The mechanism by which N-*myc* acts to increase neuroblastoma malignancy is poorly understood as yet. It is shown here that transfection of N-*myc* in a neuroblastoma cell line causes suppression of one isoform of protein kinase C, named δ, and induction of an unusual type of protein kinase C, named ζ. N-*myc*-transfected neuroblastoma cells were found to be blocked in the activation of both c-*fos* mRNA and the NF-κB transcription factor by phorbol ester. Introduction of a protein kinase C expression vector in N-*myc* transfected neuroblastoma cells restored inducibility of both c-*fos* and NF-κB by phorbol ester. These observations indicate that changes in protein kinase C gene expression significantly alter the response of N-*myc*-amplified neuroblastomas to a variety of external signals.**

*Key words:* N-*myc*/neuroblastoma/protein kinase/signal transduction

## Introduction

Amplification of N-*myc* is associated with increased metastatic ability and poor prognosis of human neuroblastomas (Brodeur *et al.*, 1984; Seeger *et al.*, 1985). We have developed a model system to study the effects of N-*myc* on neuroblastoma tumor progression by transfecting the rat neuroblastoma cell line B104 with the N-*myc* oncogene. We found that N-*myc* elicits a series of responses in these cells that are reminiscent of advanced grade neuroblastoma, i.e. increased growth rate and ability to form metastases (Bernards *et al.*, 1986). These data indicated that N-*myc* amplification is not a mere fortuitous marker of advanced stage disease, but rather that N-*myc* plays a causal role in neuroblastoma tumor progression.

N-*myc* is a member of a family of transcription factors that share a common structural motif: a stretch of basic amino acids followed by a domain having a helix–loop–helix structure (Murre *et al.*, 1989). This motif is thought to be responsible for both DNA binding and dimerization of these proteins (Jones, 1990). It is therefore likely that N-*myc* functions by altering the expression of certain key cellular genes, the altered expression of which is ultimately responsible for the increased malignancy of N-*myc*-amplified neuroblastomas.

We have shown previously that N-*myc* suppresses the expression of MHC class I antigens and neural cell adhesion molecule (NCAM) in neuroblastoma (Bernards *et al.*, 1986; Akeson and Bernards, 1990). In each case it was shown that suppression of gene expression required the continuous presence of high levels of N-*myc*, indicating that N-*myc* was responsible for the observed changes in cellular gene expression.

The identification of two genes that are altered in expression by N-*myc* provided an entry into examining the possible function of N-*myc* in gene regulation. Based on the structural relationship of N-*myc* with other transcription factors, it was expected that N-*myc* would interact directly with the promoters of the genes that it regulated. However, I show here an entirely different and unexpected mechanism which reveals surprising regulatory pathways in the cell.

## Results

### *N-myc blocks activation of NF-κB*

We have previously shown that N-*myc* suppresses expression of MHC class I genes by reducing the binding of a nuclear factor, named H2TF1, to the MHC class I gene enhancer (Lenardo *et al.*, 1989). The mechanism by which N-*myc* interferes with the binding of H2TF1 to DNA is unclear as yet. One clue to how N-*myc* might operate stems from the observation that several transcription factors, such as NF-κB and CREB, are regulated by phosphorylation events (Sen and Baltimore, 1986; Yamamoto *et al.*, 1988; Ghosh and Baltimore, 1990). I therefore explored the possibility that N-*myc* acted to prevent phosphorylation of H2TF1, thereby causing it to lose DNA binding activity.

To investigate this, the following experiment was designed: both B104 cells, which have active H2TF1 transcription factor, and B104 N-*myc*-transfected cells, which have greatly reduced levels of H2TF1, were treated with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) to activate protein kinase C (PKC). If N-*myc*-transfected neuroblastoma cells lacked H2TF1 binding activity because of lack of proper transcription factor phosphorylation, then stimulation of protein kinases, such as PKC, could potentially increase DNA binding of H2TF1 in the N-*myc*-transfected neuroblastoma cells. The abundance of active H2TF1 transcription factor was monitored by preparing nuclear protein extracts from phorbol ester-treated cells and using these extracts in an electrophoresis mobility shift assay (EMSA) with oligonucleotides corresponding to both the H2TF1 and NF-κB recognition sites as probes. The H2TF1 binding motif has previously been shown to bind both the H2TF1 and the NF-κB transcription factors with high affinity (Baldwin and Sharp, 1988). Since NF-κB can be activated by phorbol ester in all non-lymphoid cells tested thus far, activation of NF-κB was thought to serve as an internal control in this experiment (Sen and Baltimore, 1986).

The results of this experiment, shown in Figure 1, indicate that TPA, as expected, did activate NF-κB in untransfected B104 neuroblastoma cells. A small, but reproducible, increase in the amount of H2TF1 transcription factor was

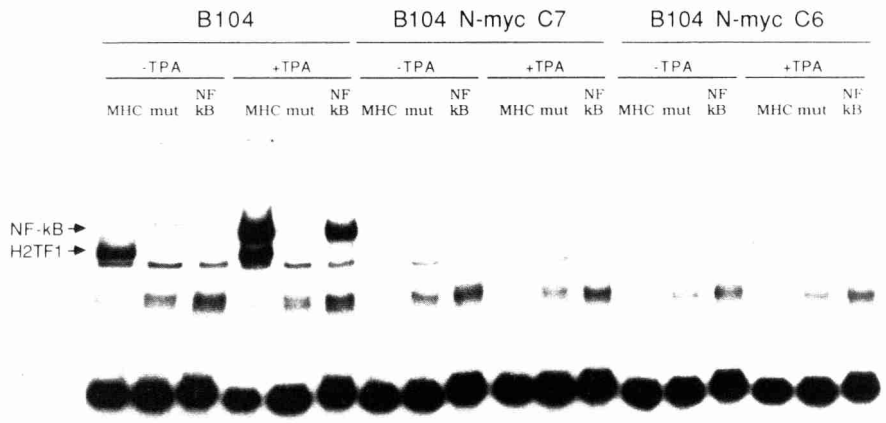


**Fig. 1.** EMSA assay of neuroblastoma cells before and after TPA treatment. B104 neuroblastoma cells and two N-*myc* transfected derivatives were treated with 100 ng/ml of phorbol ester for 1 h at 37°C. After this, nuclear protein extracts were prepared and used in an EMSA. As a control, extracts were made from untreated cells. The following oligonucleotide probes were used: H2TF1 oligonucleotide (lanes labeled MHC), NF-κB oligonucleotide (lanes labeled NF-κB) and an oligonucleotide containing a mutated H2TF1 and NF-κB recognition motif (lanes labeled mut). The positions of the H2TF1 and NF-κB complexes are indicated by arrowheads.

also found in B104 cells after phorbol ester treatment (Figure 1). This latter result suggests that H2TF1 DNA binding activity can, at least to some extent, be regulated by protein kinase C. Phorbol ester treatment of two lines of B104 N-*myc*-transfected cells did not result in the appearance of any H2TF1 transcription factor (Figure 1). Unexpectedly, however, NF-κB, monitored here just as control, was also not activated by TPA in the N-*myc*-transfected neuroblastoma cells. This latter observation was very puzzling: how could a nuclear oncogene interfere with cytoplasmic events in which a (ostensibly ubiquitously present) kinase phosphorylates one of its substrates? Since the regulation of NF-κB has been elucidated in much greater detail than that of H2TF1, I focused in subsequent experiments on the suppression of the phorbol ester-mediated activation of NF-κB by N-*myc*.

### *Cytosolic NF-κB in neuroblastoma cells*

It has previously been shown that in non-lymphoid cells, NF-κB is found in the cytosol, bound to an inhibitor named I-κB (Baeuerle and Baltimore, 1988a). *In vitro*, NF-κB can be liberated from the inactive NF-κB/I-κB complex by treatment with the detergents deoxycholate and NP40 (Baeuerle and Baltimore, 1988b). Detergent treatment thus provides a means of assaying cytoplasmic levels of the NF-κB/I-κB complex.

One way in which N-*myc* could interfere with the activation of NF-κB in neuroblastoma is by reducing the amount of cytosolic NF-κB/I-κB complex in these cells. To investigate this possibility, cytosolic protein extracts were prepared from B104 cells and two N-*myc*-transfected derivatives of this cell line. These extracts were then treated with the detergents deoxycholate and NP40 and used in an EMSA.

The results of this experiment, shown in Figure 2, indicate that B104 N-*myc*-transfected cells have only slightly less detergent-activatable NF-κB in the cytosol than the untransfected neuroblastoma cells. The small decrease in inactive NF-κB/I-κB complex in N-*myc*-transfected cells is hardly enough, however, to explain the virtually complete

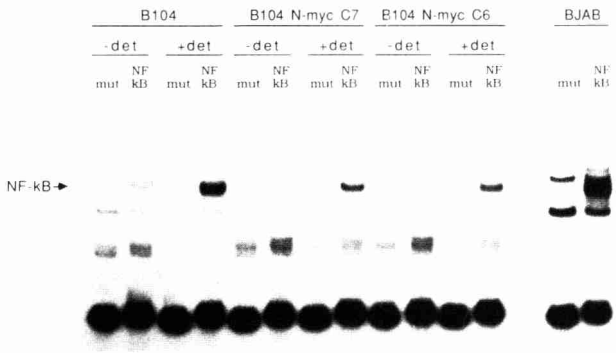


**Fig. 2.** EMSA assay of cytosolic protein extracts. Cytosolic protein extracts were prepared from untransfected B104 neuroblastoma cells (lanes labeled B104) and two N-*myc* transfected derivatives (lanes labeled B104 N-*myc* C6 and C7). These extracts were used in an EMSA, either in the absence of detergent (lanes labeled −det) or in the presence of 0.2% deoxycholate and 0.2% NP40 (lanes labeled +det). As a control, a nuclear extract was used of human B cell lymphoma BJAB, which express constitutive NF-κB. The following oligonucleotides were used: NF-κB (lanes labeled NF-κB) and an oligonucleotide specifying a mutant NF-κB motif (lanes labeled mut). The position of the NF-κB retarded complex is indicated by an arrowhead.

block in the TPA-mediated activation of NF-κB in these cells. Moreover, I found that several independently derived B104 N-*myc*-transfected cells also had only two- to three-fold less NF-κ-B mRNA than the parental neuroblastoma cells (data not shown). These results indicate that N-*myc* does not cause major changes in the amount of cytosolic NF-κB/I-κB complex in neuroblastoma cells and suggest that N-*myc* primarily interferes with the activation of NF-κB from cytosolic NF-κB/I-κB complex.

The finding that the detergent-activatable transcription factor in the B104 cells co-migrated with NF-κB from a B cell lymphoma (BJAB, Figure 2), suggests that the factor binding to the NF-κB recognition motif in the neuroblastoma cells is NF-κB and not one of the NF-κB-like factors described recently (Ballard *et al.*, 1990).

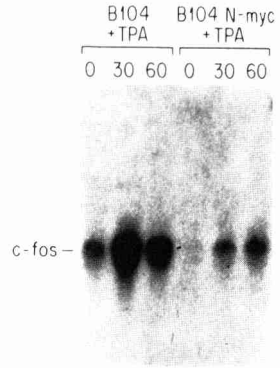


**Fig. 3.** Northern blot analysis of c-*fos* expression. B104 neuroblastoma cells and B104 N-*myc* C6 cells were treated for 0, 30 and 60 min with 100 ng/ml of phorbol ester. After this, total cellular RNA was extracted and 20 μg of RNA from each time point was electrophoresed through a 1% agarose formaldehyde gel. After transfer to nitrocellulose, the filter was probed with a c-*fos* cDNA. Conditions for hybridization and Northern blot analysis have previously been described (Bernards *et al.*, 1986).

### *N-myc suppresses TPA induction of c-fos*

It was recently shown that purified PKC can release active NF-κB from inactive NF-κB/I-κB complex *in vitro*, suggesting that PKC directly phosphorylates one of the proteins in the complex to activate the NF-κB transcription factor (Ghosh and Baltimore, 1990). The finding that N-*myc* almost completely abolished activation of cytosolic NF-κB/Iκ B complex therefore raised the possibility that N-*myc* interferes with PKC function. To find further support for this hypothesis, I monitored another response of activation of PKC: the rapid and transient induction of c-*fos* mRNA (Prywes and Roeder, 1986; Fish *et al.*, 1987).

To test the effect of N-*myc* on the TPA induction of c-*fos* mRNA, both B104 cells and B104 *N*-myc-transfected cells were treated with phorbol ester and RNA was extracted at t = 0, 30 and 60 min after TPA stimulation. The induction of c-*fos* mRNA was examined by Northern blot analysis.

In Figure 3 it can be seen that stimulation of B104 cells with TPA resulted in a rapid and transient induction of c-*fos* mRNA. In the N-*myc*-transfected derivatives, however, the induction of c-*fos* was greatly suppressed. These data support the notion that N-*myc* interferes with PKC function in neuroblastoma cells.

### *N-myc causes changes in protein kinase C expression*

The data shown above indicate that N-*myc* suppresses at least two different responses to phorbol ester in neuroblastoma cells. Although alternative interpretations could be entertained, this began to suggest that PKC itself, rather than its network of target proteins, was defective. To investigate this, PKC enzyme activity was measured on partially purified extracts from B104 cells and B104 N-*myc*-transfected cells in a histone phosphorylation assay (Housey *et al.*, 1988). The results of these experiments indicated that extracts from B104 cells reproducibly contained 2- to 4-fold more PKC enzyme activity than B104 N-*myc*-transfected cells (data not shown).

Since N-*myc* is a nuclear protein (Ramsay *et al.*, 1986), it was unlikely that N-*myc* caused these alterations in PKC activity by interfering directly with PKC function in the cytoplasm. Moreover, N-*myc* is widely believed to be a transcription factor. I therefore examined the expression of PKC in the neuroblastoma cells.

Thus far, six related genes have been isolated that encode different PKC isoforms, named α, β, γ, δ, ε and ζ (Nishizuka, 1988; Ono *et al.*, 1988). To measure expression levels of these six genes in the rat neuroblastoma cells, six replicate Northern blots were prepared, each containing equivalent amounts of RNA from parental B104 cells, B104 cells containing the neomycin resistance gene only, and two N-*myc*-transfected derivatives. These filters were then hybridized with specific cDNA probes for the six rat PKC genes (probes kindly provided by Dr John Knopf, Genetics Institute, Cambridge, MA).

The results of this, shown in Figure 4, indicate that B104 cells, and B104 cells transfected with the neomycin resistance marker alone, express PKC type δ. However, in two N-*myc*-transfectants of the B104 cell line, a significant reduction in the expression of PKC δ was found (Figure 4, left panel). Surprisingly, the N-*myc*-transfected derivatives were found to express a novel type of PKC, not found in the parental neuroblastoma cells, named ζ (Figure 4, center panel). No significant expression of the other four isoforms of PKC was found in these cells (data not shown). I conclude that N-*myc* causes a major alteration in the pattern of PKC gene expression in neuroblastoma cells.

### *PKC transfection restores TPA responsiveness*

The finding that the N-*myc*-transfected neuroblastoma cells did not induce c-*fos* and NF-κB in response to phorbol ester stimulation was unexpected, since the N-*myc*-transfected cells still expressed one isoform of PKC, namely ζ. However, PKC ζ has recently been shown to be unable to bind phorbol ester or its natural ligand diacylglycerol (DAG, Ono *et al.*, 1989). The TPA non-responsiveness of the N-*myc*-transfected neuroblastoma cells could therefore very well be caused by the suppression of PKC δ expression in these cells.

To investigate this, I used an expression vector that directs the synthesis of a TPA-responsive PKC gene under the control of a promoter that is not sensitive to N-*myc* down regulation. Because the PKC δ cDNA was not made available to us, I used a rat PKC γ cDNA for transfection, since PKC γ, like δ, binds to and is activated by TPA and DAG. This vector was used for transfection into B104 N-*myc*-C7 neuroblastoma cells. Transfectants were screened by Northern blot analysis for the expression of PKC γ.

As can be seen in Figure 5, several stably transfected lines were obtained that expressed PKC γ. Reprobing the filter shown in Figure 5 with N-*myc* indicated that all PKC γ transfectants had retained very high levels of N-*myc* expression (not shown). This ruled out the possibility that the PKC γ transfectants had altered properties as a result of a loss of N-*myc* expression.

The ability of the PKC γ-transfected neuroblastoma cells to respond to TPA was tested in two independent assays. In the first assay I measured the induction of c-*fos* mRNA in response to phorbol ester stimulation of the PKC γ-transfected neuroblastoma cells. To do this, B104 cells, B104 N-*myc* C7 cells and two independently derived PKC γ transfectants of B104 N-*myc* C7, were treated with TPA. RNA was isolated at t = 0 and 30 min after stimulation and analyzed by Northern blot hybridization for the expression of c-*fos*.

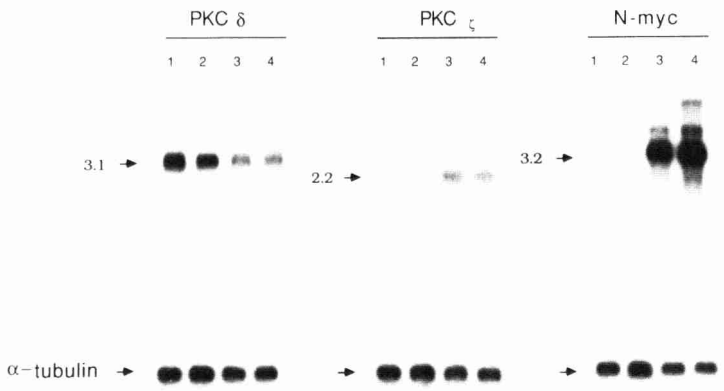


**Fig. 4.** Northern blot analysis of PKC expression. Twenty micrograms of total RNA isolated from untransfected B104 neuroblastoma cells (lanes labeled 1), B104 neuroblastoma cells transfected with the neomycin resistance marker only (lanes labeled 2) and two N-*myc*-transfected B104 cell lines (lanes labeled 3 and 4) was electrophoresed through a 1% agarose–formaldehyde gel, transferred to nitrocellulose and probed with a rat PKC δ-specific probe (left panel), a rat PKC ζ-specific probe (center panel) and with a N-*myc* probe (right panel). As a control, all filters were reprobed with a rat α-tubulin cDNA to check RNA integrity and for quantification (bottom panels).

In Figure 6, it is shown that both PKC γ transfectants had a dramatic increase in the amount of c-*fos* mRNA in response to TPA stimulation, whereas the B104 N-*myc* C7 cells, from which they were derived, showed virtually no c-*fos* induction. The level of induction of c-*fos* in the PKC γ transfectants was comparable with that of the parental B104 neuroblastoma cells, indicating that PKC γ expression could completely override the N-*myc*-mediated suppression of c-*fos* induction.

Next, I tested the ability of the PKC γ transfectants to activate the NF-κB transcription factor in response to TPA stimulation. To do this, I treated B104 N-*myc* C7 cells and two PKC γ-transfected derivatives of this cell line with phorbol ester and made whole cell protein extracts from these cells 1 h after stimulation. These extracts were then used in an EMSA using both the mutant and wild-type H2TF1 oligonucleotides as probes. For comparison, the protein extracts of the TPA-stimulated cells were also treated with detergents NP40 and deoxycholate to liberate all NF-κB present in the extracts.

The results of this experiment, shown in Figure 7, indicate that in the two PKC γ transfectants almost all of the NF-κB transcription factor present in the cytosol can be activated following TPA stimulation, whereas control B104 N-*myc* C7 cells again failed to activate NF-κB following TPA stimulation. The combined results of these two experiments indicate that suppression of phorbol ester of c-*fos* and NF-κB by N-*myc* is almost certainly due to a lack of sufficient TPA activatable PKC in these cells. Furthermore, these data indicate that cells can grow very well in the apparent absence of the DAG-activated signaling pathway.

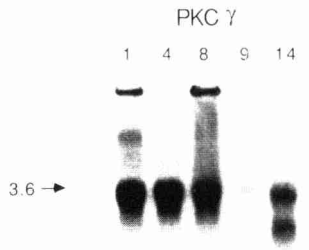


**Fig. 5.** Northern blot analysis of PKC γ-transfected cells. B104 N-*myc* C7 cells were transfected with PJ4PKCγ and, as a selectable marker, pSV2gpt. Colonies of mycophenolic acid-resistant cells were isolated, expanded and analyzed for expression of PKC γ by electrophoresis of 20 μg total RNA through a 1% formaldehyde–agarose gel. The probe used was the rat PKC γ cDNA. Shown are five independently derived transfectants labeled B104 N-*myc* PKC γ1, 4, 8, 9 and 14.

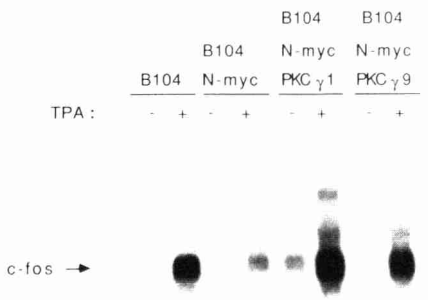


**Fig. 6.** c-*fos* induction in PKC γ-transfected cells. B104 cells, B104 N-*myc* C7 cells and two transfectants of B104 N-*myc* C7 that expressed greatly different levels of transfected PKC γ (Figure 5), were incubated for 30 min with 100 ng/ml of TPA. After this, RNA was extracted. RNA was also prepared from untreated cells as a control. Twenty micrograms of total RNA from either untreated cells (lanes −TPA) or TPA-treated cells (lanes +TPA) was electrophoresed through a 1% agarose gel, transferred to nitrocellulose and probed with a c-*fos* probe.

### *Restoration of gene induction*

The data shown above suggest that N-*myc* can interfere with the expression of a number of genes whose transcription regulatory factors depend on phosphorylation by PKC δ for activity. Among the genes that are expected to be affected by the N-*myc*-induced changes in PKC gene expression are those which are induced by NF-κB (Lenardo and Baltimore, 1989). To test whether this is indeed the case, I used a plasmid in which an enhancerless promoter, fused to two tandem copies of the NF-κB recognition motif, is linked to

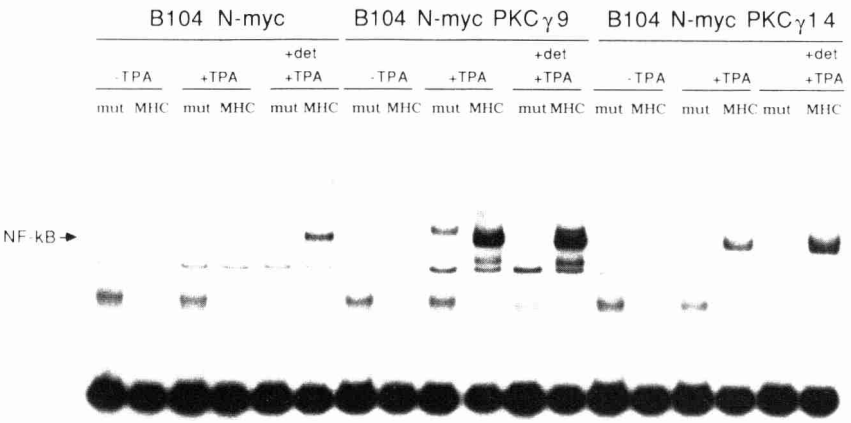


**Fig. 7.** EMSA assay of PKC γ-transfected neuroblastoma cells. Whole cell protein extracts were prepared from B104 N-*myc* cells and two PKC γ-transfected derivatives of this cell line (γ9 and γ14) that expressed moderate levels of exogenous PKC γ (Figure 5). Extracts were made both before (lanes labeled −TPA) and after treatment with 1000 ng/ml of TPA for 1 h (lanes labeled +TPA). As a control, the extracts of the TPA-treated cells were also incubated with DNA probes in the presence of deoxycholate and NP40 (0.2% each) to liberate all NF-κB present in the extracts (lanes labeled +det +TPA). This allowed an estimation of the percentage of NF-κB that can be activated by phorbol ester treatment in these cells. The probes used were the H2TF1 oligonucleotide, which binds both H2TF1 and NF-κB (lanes labeled MHC), and a control mutant oligonucleotide (lanes labeled mut).

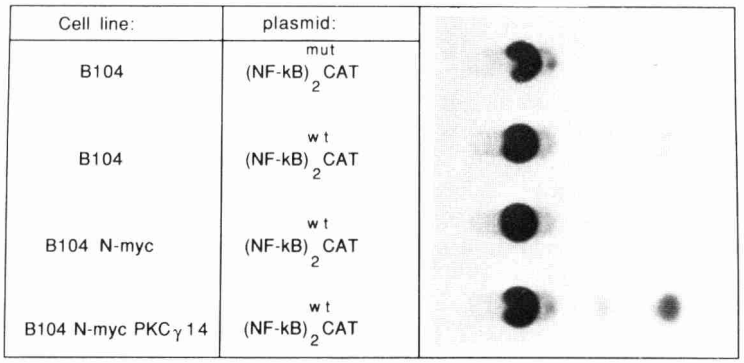


**Fig. 8.** Transient transfection of NF-κB-CAT plasmid in neuroblastoma cells. B104 cells, B104 N-*myc* C7 cells and B104 N-*myc* PKC γ14 cells were transiently transfected with a plasmid in which an enhancerless c-*fos* promoter, linked to two copies of the NF-κB recognition motif is fused to the chloramphenicol acetyltransferase (CAT) gene. The c-*fos* promoter fragment used in this construct contains only 71 bp of the *fos* promoter (Pierce *et al.*, 1988) and lacks all regulatory elements including the phorbol ester and serum responsive elements (Fisch *et al.*, 1987). As a control a plasmid was used in which two copies of a mutant NF-κB is fused to the same promoter−CAT construct: $(NF\text{-}\kappa B)_2^{mut}CAT$ (Pierce *et al.*, 1988). The CAT reporter constructs were co-transfected with pRSVβ-gal, a β-galactosidase expression plasmid, to control for differences in transfection efficiency. After correction for β-galactosidase activity, the B104 cells and B104 N-*myc* PKC γ14 cells were found to have approximately equal levels of CAT activity, whereas the B104 N-*myc* C7 cells completely lacked any detectable CAT activity (data not shown).

the chloramphenicol acetyltransferase (CAT) reporter gene. It has previously been shown that this reporter gene construct is only expressed in cells that harbor active NF-κB (Pierce *et al.*, 1988). The NF-κB-CAT plasmid is therefore a convenient tool to monitor levels of active NF-κB in cells. As a control, a plasmid was used that contained two mutant copies of the NF-κB recognition motif linked to the same promoter−CAT construct. Both plasmids were used in a transient transfection in B104 neuroblastoma cells, the B104 N-*myc* C7 cells and the B104 N-*myc* PKCγ14 cells. Forty-eight hours after transfection, TPA was added for another 3 h to activate PKC, after which the cells were harvested and CAT activity was measured.

As can be seen in Figure 8, B104 cells transfected with the wild type, but not the mutant, NF-κB-CAT plasmid displayed CAT activity. As expected, when the same construct was transfected in the B104 N-*myc*-transfected cells, no CAT activity was observed. The PKCγ-transfected derivative of this cell line, however, had significant CAT activity following TPA stimulation. These results confirm that N-*myc*, by altering PKC gene expression, perturbs the PKC-mediated signal transduction from the plasma membrane to the nuclear transcription machinery.

## Discussion

In the present study it is shown that in neuroblastoma, N-*myc* prevents the induction of both c-*fos* and NF-κB by phorbol ester. Furthermore, it was found that N-*myc* suppresses the expression of PKC type δ in neuroblastoma, and activates an unusual isoform of PKC, named ζ. The relationship between these two sets of observations was demonstrated by showing that introduction of a PKC expression vector into N-*myc*-transfected neuroblastoma cells restored phorbol ester-induction of both c-*fos* and NF-κB. In a preliminary survey of four human neuroblastomas, TPA

did activate NF-κB in two non-N-*myc*-amplified cell lines, but not in the two N-*myc* amplified neuroblastomas (R.Bernards, unpublished data). These data suggest that N-*myc* also acts in human neuroblastoma to perturb PKC-mediated signal transduction.

PKC is a gene family consisting of at least six related members, all of which show evolutionary conservation and are expressed in distinct tissues (Nishizuka, 1988; Ono *et al.*, 1988). This suggests that the members of the PKC gene family have distinct functions, probably because they phosphorylate different substrates. The substitution by N-*myc* of PKC ζ for PKC δ in neuroblastoma is particularly significant, because PKC ζ is the only isoform of PKC that is not activated by TPA or DAG (Ono *et al.*, 1989). It is therefore likely that the N-*myc*-induced alterations in PKC gene expression will dramatically alter the ability of N-*myc*-amplified neuroblastomas to respond to a variety of extracellular signals.

The effects of N-*myc* on PKC gene expression were not readily detectable in a PKC enzyme assay. This is probably due to the fact that the decrease in PKC δ in the N-*myc*-transfected neuroblastoma cells is balanced by an increase in PKC ζ. Thus, our data indicate that small differences in PKC enzyme activity as measured in a histone phosphorylation assay can nevertheless be associated with dramatic alterations in cellular responses to PKC-activating signals. Recently, alterations in PKC isoform expression were also found in fibroblasts transfected with a mutant Ha-*ras* oncogene. The *ras*-induced changes in PKC isoform expression were also not detectable in a PKC enzyme activity assay (Borner *et al.*, 1990). Alterations in PKC isoform expression may thus be a more common event in transformation than generally believed, as alterations in PKC isoform expression may not be readily detectable.

We have previously shown that N-*myc* suppresses the binding of a nuclear factor, named H2TF1, to the MHC class I gene enhancer (Lenardo *et al.*, 1989). In the present studies, I found that transfection of PKC γ restored NF-κB inducibility in N-*myc*-expressing neuroblastoma cells, but did not restore binding of H2TF1 to the MHC class I gene enhancer (Figure 7). These results could indicate that alterations in PKC expression are not responsible for the suppression of H2TF1 binding by N-*myc* in neuroblastoma. However, in the present experiments, a PKC γ expression vector was introduced into the B104 N-*myc* cells, whereas the predominant isoform of PKC in the untransfected B104 cells was δ. The failure to restore H2TF1 binding in B104 N-*myc* cells by PKC γ could therefore also be caused by differences in substrate specificity between the PKC γ and δ isoforms. A further study of the effects of transfection of the different PKC isoforms in N-*myc*-expressing neuroblastoma cells may reveal important differences in substrate specificity of the different PKC isoforms, the nature of which has remained obscure thusfar.

An interesting parallel exists between neuroblastoma cells and pre-B lymphocytes. Pre-B lymphocytes characteristically express extremely high levels of N-*myc*, and have no active NF-κB transcription factor in the nucleus (Zimmerman *et al.*, 1986; Baeuerle and Baltimore, 1988b). During the transition to mature B cells, N-*myc* expression is turned off and NF-κB becomes constitutively active. The present data suggest that in pre-B lymphocytes, N-*myc*, by acting on PKC, can suppress signals that result in activation of NF-κB. In agreement with this, I found that TPA did activate NF-κB in a late pre-B lymphoid line that does not express N-*myc* (70Z/3, Zimmerman *et al.*, 1986), but not in two mouse pre-B cell lines that expressed high levels of N-*myc* (R.Bernards, unpublished data).

An important question is whether the N-*myc*-induced alterations in PKC gene expression contribute to the increased malignancy of N-*myc*-amplified neuroblastomas. In a recent study, transfection of PKC β in a colon cancer cell line was shown to cause growth inhibition and suppression of tumorigenicity (Choi *et al.*, 1990). However, expression of the same isoform of PKC in a fibroblasts cell line enhanced tumorigenicity (Housey *et al.*, 1988). These apparently conflicting data probably indicate that alterations in PKC expression can either enhance or suppress tumorigenicity, depending on the cell type studied. Consistent with this notion is the fact that TPA stimulates proliferation in some cell types but inhibits growth and induces differentiation in others. For instance, treatment of the human promyelocytic leukemia cell line HL60 with phorbol ester leads to macrophage differentiation (Rovera *et al.*, 1979), and induction of differentiation by phorbol ester has also been found in a non-N-*myc*-amplified human neuroblastoma cell line (Pahlman *et al.*, 1981). These data could indicate that N-*myc* enhances tumorigenicity by preventing differentiation-inducing signals from reaching the nucleus.

PKC can phosphorylate a number of growth factor receptors, including the insuline receptor, the EGF receptor, $pp60^{src}$, and the c-*met*-encoded growth factor receptor (Hunter *et al.*, 1984; Gould *et al.*, 1985; Bollag *et al.*, 1986; Lin *et al.*, 1986; Gandino *et al.*, 1990). In each case, it was shown that phosphorylation by PKC leads to down-modulation of receptor activity. N-*myc* could therefore significantly alter the repertoire of cell surface receptors involved in mediating growth and differentiation in neuroblastoma. It will therefore be of interest to evaluate how transfection of PKC expression vectors in N-*myc*-amplified neuroblastomas alters their *in vivo* growth potential.

## Materials and methods

### *Cell culture and transfections*

Culture conditions of B104 cells and the generation of N-*myc*-transfected derivatives have been described (Bernards *et al.*, 1986). Transient transfections were performed essentially as described by Van der Eb and Graham (1980). For transient transfection assays, 100 mm plates of neuroblastoma cells were transfected with 20 μg of CAT construct and 10 μg of β-galactosidase control plasmid. Cells were harvested 51 h post transfection for analysis of enzyme activity. To generate stable transfectants, 30 μg of PKC expression vector and 2 μg of pSV2gpt were transfected into a 100 mm plate of neuroblastoma cells. After 4 h precipitate was removed. Twenty-four hours later, cells were split 1:10 into medium containing mycophenolic acid and were subsequently fed every third day. Colonies of mycophenolic acid-resistant cells were isolated 14 days post transfection, expanded, and analyzed for expression of PKC.

### *CAT assays*

Forty-eight hours post transfection, TPA (100 ng/ml) was added to the transiently transfected cells. After an additional 3 h of incubation, cells were washed with phosphate-buffered saline (PBS) and incubated for 2 min at 37°C with 2 ml of a solution containing 10 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA. Cells were scraped off the plates, collected by centrifugation and resuspended in 100 μl of 0.25 M Tris pH 8.0. To obtain a cytoplasmic protein fraction, cells were freeze–thawed three times, cellular debris was removed by centrifugation and supernatants were used for enzyme assays. Chloramphenicol acetyl transferase (CAT) activity was determined as described by Gorman *et al.* (1982). β-galactosidase activity was determined as described by Hall *et al.* (1983). To measure β-galactosidase activity

a plasmid was used in which the Rous sarcoma virus LTR is fused to the β-galactosidase coding region.

### *PKC enzyme assays*

Extracts from B104 cells and B104 N-*myc*-transfected cells were partially purified over a DEAE–Sephacel column as described by Housey *et al.* (1988). Total PKC enzyme activity was measured using a modified mixed micelle assay system (Amersham, UK).

### *Gel electrophoresis DNA-binding assays*

Nuclear extracts were prepared from various cell lines using essentially the procedure of Dignam *et al.* (1983) except that the protease inhibitors leupeptin, chymostatin and antipain were added. Cytosolic extracts were simultaneously prepared as described previously (Baeuerle and Baltimore, 1988a). Whole cell protein extracts were prepared by the following protocol. $3 \times 10^7$ cells were harvested, rinsed in PBS and resuspended in 100 μl buffer C (Dignam *et al.*, 1983). After this, cells were disrupted by freezing and thawing once. Nuclear proteins were extracted by incubating on ice for 20 min, after which cellular debris was removed by centrifugation at 75 000 *g* for 20 min. The protein concentration of the supernatant was determined, after which extracts were quick-frozen in an ethanol–dry ice bath. Extracts were stored at −70°C until used.

EMSA assays were carried out for 20 min at room temperature using 0.1 ng (6000 c.p.m.) of radiolabeled probe, 3 μg of poly(dI-dC)·poly-(dI-dC), and 10 μg of protein extract. Binding buffer contained 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 5% glycerol, 3 mM GTP, 5 mM $MgCl_2$, 1 mM DTT and 1 mM EDTA. Electrophoresis was carried out for 2 h at 100 V on 4% polyacrylamide gels (30:1 crosslinking) containing 1 × TGE (1 × TGE = 50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.7). The oligonucleotides used were: H2TF1: TGGGGATTCCCCA, NF-κB oligonucleotide: AGGGGACTTTCCG, mutant oligonucleotide: TGCGGATTCCCGA. All three oligonucleotides were cloned into the polylinker cloning site of the pUC13 plasmid. For labeling of EMSA probes, 3 μg of plasmid was digested with *Eco*RI and *Hin*dIII, and labeled with [$^{32}$P]dCTP and Klenow enzyme. The ~75 bp probes were separated from the plasmid vector on a non-denaturing 6% polyacrylamide gel and recovered from the gel by electroelution.

### *PKC expression vector and probes*

A 2.6 kb cDNA containing the entire coding sequence of the rat PKC γ gene was cloned into the mammalian expression vector pJ4Ω. This plasmid contains the Moloney leukemia virus LTR as a promoter, followed by a polylinker cloning site. A splice signal and polyadenylation site are present downstream of the insertion site in the vector. The resulting plasmid, PJ4PKCγ, was used for transfection in B104 N-*myc* C7 cells.

Short (150–180 bp) probes corresponding to the most divergent regions of the six rat PKC genes, subcloned in the vector pGEM4 were used to detect isoform-specific transcripts. For Northern blot analysis, inserts were removed from the vectors and isolated and a 1.5% agarose gel.

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