STUDIES ON BEHAVIOR OF PROTEIN KINASE C IN TRANSFORMED NIH3T3 CELLS

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https://doi.org/10.11501/3086539

出版情報：九州大学, 1991, 博士（理学）, 課程博士
バージョン：
権利関係：

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1991

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SUMMARY

To make clear the mechanism of signal transduction leading to cellular transformation, I investigated the behavior of protein kinase C in the normal and oncogene-transformed NIH3T3 cells. By looking at phosphorylation of the 80K protein, a specific substrate for protein kinase C, by means of two dimensional (2D) gel analysis, the response of protein kinase C to 12-o-tetradecanoylphorbol-13-acetate (TPA) was compared between normal and the transformed NIH3T3 cells. The results are summarized as follows; (1) Normal NIH3T3 cells maintained a certain degree of phosphorylation in the 80K protein in Dulbecco modified Eagle's medium supplemented with 0.1%, 0.3%, 1%, 3% or 10% fetal calf serum, and the phosphorylation of the 80K protein was stimulated by TPA; (2) NIH3T3 cells transformed with c-*raf* or H-*ras* maintained a decreased level of phosphorylation of the 80K protein and the phosphorylation could not be stimulated by TPA in all the serum concentrations tested; (3) Similar results were obtained in the analysis of N-*ras*, K-*ras*, v-*src*, v-*mos*, polyoma middle T antigen-transformed cells; (4) These different responses of protein kinase C to TPA between normal and transformed NIH3T3 cells were confirmed by analysis of hormone-inducible H-*ras* transformants I constructed. (5) Furthermore, *in vitro* assay of the activity of protein kinase C showed that the activity in the membrane fraction is elevated more than that in the cytosol in the transformants in contrast with those of NIH3T3 cells. (6)By utilizing the transformation-inducible cells described above, transcription of c-*fos* gene and activation of nuclear factors were observed in the course of transformation. (7)A protein kinase C inhibitor, H-7, inhibited the anchorage-independent growth of the

transformation-inducible cells. These results suggest that oncogene products may induce the change of protein kinase C activity and bring about translocation from cytosol to membrane, which lead to cellular transformation.

LIST OF ABBREVIATIONS

k b	1000 base pairs or 1000 bases
DMEM	Dulbecco modified Eagle's medium
FCS	fetal calf serum
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol bis (β-aminoethyl ether)-N, N, N', N'- tetraacetic acid
MMTV	mouse mammary tumor virus
LTR	long terminal repeat
SDS	sodium laurylsulfate
MOPS	morpholino propanesulfonic acid
DTT	dithiothreitol
PMSF	phenylmethylsulfonyl fluoride
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

INTRODUCTION

Oncogene

It has been shown that certain genes are involved in malignant transformation of cells when a point mutation or chromosome translocation occurs. These genes have been termed as oncogenes.

Activated oncogene products are specifically localized in the cell membrane, cytoplasm or nucleus, and may be classified into some groups in respect to the specified activity and characteristics; (1) growth factor family such as *sis* (Doolittle *et al.*, 1983, Waterfield *et al.*, 1983), *int*-2 (Peters *et al.*, 1983) and *hst*-1 (Sakamoto *et al.*, 1986), (2) receptor and non-receptor type tyrosine kinase family such as *erb*-B (Downward *et al.*,1984) and *src* (Collett *et al.*, 1978), (3) serine/threonine kinase family such as *raf* (Jansen *et al.*, 1984) and *mos* (Maxwell and Arlinghaus ,1985), (4) G-protein family such as *ras* (Perucho *et al.*, 1981), (5) nuclear oncogene such as *myc* (Bister and Duesberg ,1982), *fos* (Cohen and Curran, 1989) and *jun* (Maki *et al.*,1987). The normal type genes of these oncogenes are termed as proto-oncogenes, and it is believed that proto-oncogenes are normally involved in controlling proliferation, for instance, as mediators of signal transduction pathways. Of these genes, details of two oncogene families, *raf* and *ras* , are described below.

i) raf

The *raf* oncogene was originally found as a murine gene involved in murine sarcoma virus, 3611 MSV (Rapp *et al.*, 1983), and was found to be identical with the *mil* oncogene derived from chicken (Jansen *et al.*, 1984). In addition to the cellular counterpart c-*raf*-1, related genes A-*raf*-1 and B-*raf* have been isolated as

members of the *raf* gene family which contains also other pseudogenes c-*raf*-2 and A-*raf*-2 (Ikawa *et al.*, 1988). The c-*raf*-1 gene consists of 17 exons and encodes a 73k daltons cytoplasmic protein which contains 648-amino acid residues. Within the carboxy-terminal half, serine/threonine-specific protein kinase domain is conserved among c-raf-1, A-*raf*-1 and B-*raf* gene products (Rapp *et al.*, 1988). The retroviral *gag-raf* fused protein was demonstrated to have serine/threonine protein kinase activity by using *in vitro* autophosphorylation and phosphorylation assays with exogeneous substrates (Moelling *et al.*, 1984). Incorporation of amino-terminally truncated versions of c- or A-*raf*-1 gene into a retrovirus renders these viruses transforming, and the amino-terminal half of most activated versions of c- or B-*raf* genes have been truncated and the carboxy-terminal half kinase domains have fused non-specifically to other proteins by gene rearrangement. Therefore, it is thought that the amino-terminal truncation is necessary for activation of its kinase activity (Rapp *et al.*, 1988). In the amino-terminal halves of *raf* kinase family, the homology was reduced, but some conserved regions, a cysteine-rich domain and a serine/threonine-rich domain, were noted (Ishikawa *et al.*, 1987). Indeed, it was reported that a serine/threonine-rich domain within the amino-terminal half functions as regulatory region of kinase activity (Ishikawa *et al.*,1988 , Rapp *et al.*, 1988b) and that the cysteine-rich regions in *raf* gene products are highly homologous with the cysteine-rich regions found in protein kinase C (Ishikawa *et al.*, 1986). While the c-*raf* gene product was suggested to be a mediator in the intracellular signal transduction system, mechanism of cellular transformation by the gene product remains to be clarified.

ii) *ras*

H-*ras* and K-*ras* genes have been detected in the acutely transforming Harvey and Kirsten murine sarcoma viruses, respectively. Furthermore, in a large number of human tumors, activated forms of H-*ras* and K-*ras* as well as of the related N-*ras* gene have been identified by DNA-transfection assay (Shimizu *et al.*, 1983) and by polymerase chain reaction (Bos, 1989). These eukaryotic *ras* genes encode proteins of 21k daltons that appear to be important in control of cell growth. The *ras* proteins are membrane proteins that bind GDP and GTP, and hydrolyze GTP. Most of the activated forms of the *ras* proteins contain a mutation at position 12 or 61 and have the reduced GTPase activity. The region around the position 12 glycine is thought as a catalytic domain of the GTPase. Alteration in the GTPase activity and GDP/GTP exchange are responsible for their transforming activity; GTP-bound forms are active, and GDP-bound forms are inactive (Barbacid, 1987).

The *ras* proteins share structural and biochemical properties with signal transducing G proteins, which are known to depend on GDP/GTP exchange for activation. In the resting state, they exist as stable, inactive GDP-bound complexes, and are converted to active GTP-bound forms in response to an upstream signal. Conversion to the GDP-bound form is achieved through rapid hydrolysis of bound GTP. G proteins are involved in the coupling of cell-surface receptor proteins to intracellular signalling systems, for instance, adenylate cyclase and phosphodiesterases (Gilman, 1987).

Some reports have presented evidence that *ras* proteins may be involved in the control of phospholipase C which is a phosphodiesterase responsible for phosphatydilinositol breakdown

(Wakelam *et al.*, 1986, Chiarugi *et al.*, 1986). The breakdown products act as intracellular regulators and activate protein kinase C in growth factor stimulation of cell proliferation, so the relation of the protein kinase C and *ras* gene products are desired to be clarified.

GTPase activating protein (GAP) was recently found and shown to accelerate conversion of GTP-bound forms to GDP-bound forms of *ras* proteins (Gibbs *et al.*, 1988). This reaction, when accelerated by GAP, proceeds at rates more than 100 times higher than intrinsic rates, but the GAP has no effect on mutated *ras* proteins. Most *ras* mutaions in the effector binding site ,which destroy the biological activity of oncogenic *ras* proteins, also make them insusceptible to GAP to stimulate the GTPase, suggesting that GAP is a candidate for a *ras* effector protein (McCormick, 1989).

Rapp and his co-workers have reported that a *raf*-specific antibody inhibited DNA synthesis in H-*ras*-transformed NIH3T3 cells but that *ras*-specific antibody did not inhibit DNA synthesis in *raf*-transformed NIH3T3 cells (Rapp *et al.*, 1987). It is therefore thought that *raf* is located downstream to *ras* in the signal transduction pathway utilized by growth factors. Studies of the protein kinase C in the *ras* and *raf* transformed cells may be important to clarify the role of the oncogene products in the signal transduction pathway.

Protein Kinase C

Stimulation by extracellular signals such as growth factors and hormones is transduced into nucleus through the receptors. One of the well known pathways is that through protein kinase C. Protein kinase C is a Ca^{2+}-activated, phospholipid-dependent serine/threonine-specific protein kinase. This enzyme is known to

be activated by diacylglycerol, a product of phosphatidylinositol breakdown which is stimulated in response to growth factors and hormones, and both stimulation of transcription by transcriptional activators and cellular proliferation occur through phosphorylation by this enzyme (Nishizuka, 1988). Furthermore, protein kinase C is known to be a receptor for phorbol esters such as 12-o-tetradecanoylphorbol-13-acetate (TPA) (Kikkawa *et al.*, 1983). Since TPA, a constituent of cloton oil, is the strongest tumor promoter and acts in promotion of chemical carcinogenesis, the protein kinase C which directly binds TPA is thought to be important in inducing the cellular transformation. Thus, the protein phosphorylation system through the protein kinase C appears to be involved in widespread functions.

Upon stimulation with hormones, serum and purified growth factors, phospholipase C-mediated hydrolysis of phosphatidylinositol and of its phosphorylated derivatives, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate, is stimulated and produces diacylglycerol and inositol-1,4,5-triphosphate. The former activates protein kinase C and the latter stimulates Ca^{2+} release from intracellular vesicles into the cytosol (Nishizuka, 1984). Both the activated protein kinase C and the stimulated Ca^{2+} release activate a plasma membrane Na^{+}-H^{+} exchanger, which elevates cytoplasmic Na^{+} and pH. Treatment with TPA causes the translocation of protein kinase C from cytosol to membrane, increases the activity of membrane-associated protein kinase C transiently, and maintains the prolonged lack of the total protein kinase C activity in the cells (Kraft and Anderson, 1983). The activated protein kinase C is also known to down-regulate the epidermal growth factor receptor by

phosphorylation of its intracellular domain (Schlessinger, 1986). Alteration in the phosphatidylinositol turn over and the following metabolic events may be related with cellular transformation. It is reported that the breakdown products of phosphatidylinositol was elevated in *ras*-transformed cells compared with nontransformed cells (Fleishman *et al.*, 1986, Wolfman and Macara, 1987). Analysis of relationship between the oncogene products and protein kinase C is of interest to gain insight into the mechanism in the cellular transformation.

It is known that agents which stimulate the protein kinase C, such as serum, growth factors and TPA, induce the transcription of some genes including c-*fos*, c-*jun* and c-*myc* (Rabin *et al.*, 1986). In the promoter regions of these genes, a common sequence has been detected. The sequence, termed as serum responsive element (SRE), is necessary for the induction of transcription in response to the stimulation (Prywes and Roeder, 1986), and it is thought that the transcription is activated through the activation of protein kinase C (Cohen and Curran, 1989). Thus, the activation of immediate early genes might be one of the controlling mechanisms through protein kinase C.

AP-1 is one of known transcriptional factors , which is composed of c-*fos* and c-*jun* gene-products. AP-1 binds to a specific sequence, termed as AP-1 binding site or TPA responsive element, located upstream of some genes which are induced by TPA or growth factors (Angel *et al.*, 1987). Since TPA directly activates protein kinase C, it is likely that phosphorylation through protein kinase C is a crucial modification for AP-1 regulation.

80K Protein

Treatment with TPA, growth factors or an exogenous phospholipase C, activates the protein kinase C in intact cells, and rapidly increases the phosphorylation of an 80K daltons protein which migrates at the acidic end of an isoelectric focusing gel (Rozengurt *et al.*, 1983, Rodriguez-Pena and Rozengurt, 1985, Bishop *et al.*, 1985, Blackshear *et al.*, 1985, 1986). As a variety of agents that cause large increases in the level of c-AMP, including prostaglandin E_1, adenosine agonists and cholera toxin, did not affect the phosphorylation, it has been proposed that the phosphorylation of this protein, termed 80K protein, is mediated by protein kinase C (Rozengurt *et al.*, 1983). Furthermore, it has been reported that the 80K protein is phosphorylated by purified protein kinase C *in vitro* (Moolenaar *et al.*, 1984, Rodriguez-pena and Rozengurt,1986). Therefore, an analysis of the 80K phosphoproteins by 2D gel electrophoresis is a useful assay for the activity of protein kinase C *in vivo* (Rodriguez-Pena and Rozengurt, 1986).

Here, I investigated the action of protein kinase C, by the assay of the 80K protein phosphorylation, in the transformants, especially *raf* and H-*ras* transformants, to address the effect of activated oncogene products on protein kinase C in these transformants.

MATERIALS AND METHODS

I. Materials

Cell Lines

SCT1025 is an NIH3T3 cell line transformed with the activated c-*raf*-1 gene derived from human stomach cancer DNA (Shimizu *et al.*, 1985). TFN-75 and Lu65 are NIH3T3 cell lines transformed with human N-*ras* and K-*ras* gene, respectively. TFH-2 and 45-3-A are NIH3T3 transformants with H-*ras* proteins, which contain valine substituted for glycine at position 12 and contain leucine substituted for glutamine at position 61, respectively. Lu65 and 45-3-A are kindly provided by Dr Y. Taya and Dr Y. Yuasa, respectively. Biologically active v-*src* and v-*mos* clones were kindly gifted from N. Tsuchida and introduced to NIH3T3 cells to give TF-v-*src* and TF-v-*mos* in our laboratory. MT-3 is a NIH3T3 cell line transformed with polyoma virus middle T antigen, kindly provided by Dr Y. Itoh.

Chemicals

TPA (12-O-tetradecanoylphorbol-13-acetate), dexamethasone (9α–fluoro-16α-methylprednisolene) and H-7 (1-(5-isoquinolinyl sulfonyl) -2-methylpiperazine) were purchased from Sigma Chemical Company. Bombesin was purchased from Funakoshi Chemical Inc. Nitrocellulose membrane filters were obtained from Schleicher & Schuell Company. Radioactive nucleotides were purchased from Amersham.

II.Methods

Metabolic Labeling

NIH3T3 and the transformants were precultured for two days in DMEM supplemented with FCS at indicated concentrations described in figure legends. The cells were fed with phosphate-free

DMEM containing dialyzed FCS and incubated with ^{32}P-orthophosphate (200μCi/ml) for 24 h to label the endogenous ATP pool. If stimulation was desired, 200nM or 50nM TPA, 10nM Bombesin or 20% FCS was added for last 5 min. For protein synthesis analysis, the cells were precultured in methionine-free DMEM supplemented with 10% FCS for one hour prior to labeling with ^{35}S-methionine(100μCi/ml) for 4 hours. These labeled cells were washed with cold phosphate buffered saline (PBS), treated with 10% trichloro acetic acid (TCA) for 20 minutes. The cells were washed with cold PBS, and suspended in the nuclease reaction mixture [10mM Tris-HCl (pH7.5) / 5mM $MgCl_2$ / 0.5% NP40 / 200μg/ml DNase / 100μg/ml RNase A] and incubated in ice for 10 min. The pellet precipitated with 4 volume of cold ethanol was dissolved in SDS-urea solution [0.5% SDS / 9.5M urea / 5% β-mercaptoethanol / 1% ampholine (pH3.5-10)] and mixed with an equal volume of lysis buffer [9.5M urea / 4% NP40 / 2% ampholine (pH3.5-10) / 5% β-mercaptoethanol] described as Yamashita *et al.* (Yamashita *et al.*, 1984). An aliquot of ^{32}P-labeled proteins incorporating 1 x 10^6 cpm was applied upon 2D gel electrophoresis.

Two Dimensional Gel Electrophoresis

Nonequilibrated polyacrylamide gel electrophoresis was performed as described by O' Farrell (O' Farrell, 1975). The first dimension was carried out at 500V for 4 h at 10°C by using pH 3.5-10 ampholine (LKB Bromma, Sweden). For second dimension, the proteins were analyzed on a 12.5% polyacrylamide slab gel at 20mA per gel at 4°C. Following electrophoresis, the gels were fixed, dried and exposed to X-ray film (Kodak XAR-5).

Analysis of Phosphoamino Acids on 80K Protein

The labeled proteins of NIH3T3 cells treated with TPA were applied upon 2D gel electrophoresis. The gels were fixed with 10% acetic acid / 10% methanol and incubated in 1M KOH at 55°C for 2 hours. The gel was, then, washed with 10% acetic acid / 10% methanol, dried and exposed to X-ray film.

Preparation of Cell Lysates for Protein Kinase C Assay

Subconfluent cells were washed with cold PBS, harvested and collected by centrifugation at 2000 rpm for 5 min. The cells were suspended in buffer A [50mM Tris-HCl (pH 8.0) / 2mM EDTA / 10mM EGTA / 0.25M sucrose / 10% glycerol / 0.1mM leupeptin], sonicated and centrifuged at 88,000g for 45 min at 4°C. Cytosolic supernatant was retained and the membrane pellet was suspended in buffer B [20mM Tris-HCl (pH 8.0) / 2mM EDTA / 2mM EGTA / 50mM 2-mercaptoethanol / 10% glycerol / 0.1mM leupeptin] containing 1% Triton X-100, rotated at 4°C for 30 min and the detergent-solublized membrane preparation was obtained after centrifugation. The cytosol and membrane preparations were applied on a column of DE-52 cellulose (Whatman) and fractions eluted with buffer B containing 0.15M NaCl were assayed for protein kinase C activity.

Assay of Protein Kinase C Activity

Protein kinase C activity was assayed by measuring the incorporation of ^{32}P into histone from [γ-^{32}P] ATP. The standard reaction mixture contained an aliquot of the cell fraction, 20mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 10μg histone Hl, 1μg phosphatidylserine and 10μM [γ-^{32}P]ATP (750cpm/pmol) with or without 100μg/ml TPA and 750μM $CaCl_2$ in 50μl final volume. After incubation at 30°C for 3 min, the reaction was terminated by

addition of 50μl of cold 50% TCA. The precipitate collected by filtration was counted for radioactivity.

Construction of Recombinant DNA

Recombinant plasmids (pMDSS) containing the activated H-*ras* coding region under the control of the MMTV-LTR were constructed as follows. The H-*ras* coding region, contained within a 2.9kb *Sac* I fragment derived from T24 human bladder carcinoma DNA, was subcloned into pUC19. The MMTV-LTR sequence, contained within 1.45kb *Hind* III fragment of pMDSG (kindly gifted by Dr. T, Yamamoto), was introduced into this plasmid. Recombinant plasmids (pMDN) containing the activated human N-*ras* coding region under MMTV-LTR were constructed as follows. The 1.45kb *Hind*III fragment containing MMTV-LTR promoter was subcloned into pUC19. Into this plasmid, a 7kb *Eco*RI fragment containing N-*ras* exon 3 and 4 from pGNS7.0 was introduced following ligation of a 4kb *Bgl*II-*Eco*RI fragment containing N-*ras* exon 1 and 2 from pGNS9.2.

Establishment of Transformation-inducible Cell Lines

After NIH3T3 cells were transformed with pMDSS or pMDN plasmid DNA by calcium-phosphate co-precipitation method (DNA-transfection), some colonies in the presence of 2μM dexamethasone were picked into duplicate wells in a 24-well plate: one well with 2μM dexamethasone and one well without dexamethasone. One clone, which was morphologically untransformed without dexamethasone and transformed with dexamethasone, was picked and tested for the ability of anchorage-independent growth in 0.3% soft agar with but not without dexamethasone. Hormone-inducible mRNA specific for human H-ras gene was detected by northern analysis.

Preparation of Cellular DNA

Cells were harvested, suspended in 10mM Tris-HCl (pH 7.4) and 10mM EDTA, and lyzed by adding SDS to 0.5% and proteinase K to 100μg/ml. The lyzed cells were heated at 65°C for 15 min, then more proteinase K was added to 200μg/ml and the mixture was incubated overnight at 37°C. After the lysate was treated with phenol, high molecular weight DNA was isolated by ethanol-precipitation.

Southern Blot Analysis

Ten μg of DNA was digested with restriction endonucleases and subjected to electophoresis through 1% agarose gel at 1V/cm, and then transferred to a nitrocellulose filter by the method of Southern (Southern, 1975). Then the filter was baked at 80°C for 2 h in a vacuum oven and blocked in a solution containing 6 x SSC [1 x SSC is 0.15M NaCl / 0.015 M sodium citrate], 1 x Denhardt's solution [0.02% Ficol400 / 0.02% polyvinylpyrrolidone / 0.02% bovine serum albumin], 10μg/ml denatured salmon sperm DNA at 70°C for 5 h, followed by hybridization with nick-translated ^{32}P-labeled DNA probes. Hybridization was performed in 5 x SSC, 40% formamide, 3 x Denhardt's solution and 10μg/ml denatured salmon sperm DNA at 42°C for 20 h. The filter was washed several times for 20 min each at 70°C with 2 x SSC buffer [0.3M NaCl / 0.03M sodium phosphate / 0.06% sodium pyrophosphate / 0.05% SDS] and sequentially with 1 x and 0.5 x SSC buffer, and exposed to X-ray film (Kodak XAR-5) at -70°C with an intensifying screen.

Northern Blot Analysis

Cells were homogenized in 4M guanidium thiocyanate, and total RNA was extracted by centrifugation through a CsCl cushion as described by Chirgwin *et al* (Chirgwin, 1979). Twenty μg of total

RNA was ethanol-precipitated, washed with 70% ethanol and dissolved in 10μl of 62.5% formamide / 1 x MOPS buffer [20mM MOPS / 5mM sodium acetate / 1mM EDTA]. The RNA was heated at 65°C for 5 min and 2.5 μl of 11M formaldehyde was added, followed by heating at 65°C for 5 min. After mixing with tracking dye, the RNA sample was electrophoresed in 1.5% agarose in 1 x MOPS buffer / 2.2M formaldehyde at 15mA for 16 h. The RNA was transferred to a nitrocellulose filter, and the filter was baked at 80°C for 2 h in a vacuum oven and blocked in a solution of 50% formamide / 5 x SSC / 1 x Denhardt's solution / 100μg/ml denatured salmon sperm DNA / 50mM sodium phosphate at 43°C for 5 h. Hybridization was done at 43°C for 20 h in the same buffer after adding nick-translated ^{32}P-labeled DNA probe. The filter was washed once with 2 x SSC buffer at 43°C, sequentially with 2 x, 1 x and 0.5 x SSC buffer at 70°C, for 20 min each. The filter was exposed to X-ray film (Kodak XAR-5) at -70°C with an intensifying screen.

Soft Agar Assay

Upon 1.5% bottom agar, trypsinized cells were split into 0.3% agar in DMEM containing 3% FCS with or without chemicals (200nM TPA or 50μM H-7). Dexamethasone was added to the cells two days after, and cells were cultured for 10 days. The size of colonies was compared.

For assay of the effect of the chemicals upon the cell growth, 1 x 10^6 cells were pretreated for 24 hours with or without the chemicals (200nM TPA or 50μM H-7) in DMEM supplemented with 3% FCS, treated with or without 2μM dexamethasone for additional 24 hours, and counted.

Preparation of Nuclear Extracts

Nuclear extracts were prepared by the method of Dignam *et al* (Dignam, 1983). Cells grown to subconfluency in 100 mm dishes were scraped and washed with PBS. The cells were suspended in buffer A [20mM Hepes (pH7.9) / 5mM KCl / 0.5mM $MgCl_2$ / 0.5mM DTT / 0.5mM PMSF], and allowed to stand in ice for 10 min. After the cells were lyzed by 20 strokes of Dounce homogenizer (B type pestle), nuclear fraction obtained by centrifugation was extracted with buffer B [20mM Hepes (pH 7.9) / 25% glycerol / 0.5M NaCl / 1.5mM $MgCl_2$ / 0.2mM EDTA / 0.5mM DTT / 0.5mM PMSF / 0.5μg/ml pepstatin A] by gently rotating at 4°C for 60 min. Extract clarified by centrifugation at 15,000 rpm for 1 h was dialyzed against 50 volumes of binding buffer [10mM Tris-HCl (pH 7.5) / 50mM NaCl / 1mM EDTA / 10% glycerol / 0.5mM DTT / 0.5mM PMSF / 0.5μg/ml pepstatin A] at 4°C for 5 h, and kept frozen at -80°C.

Mobility Shift Assay

To prepare probes for mobility shift assay, plasmid pYT25-11 containing polyomavirus enhancer sequence (kindly gifted by Dr. Satake) was digested with *Eco*RI and *Hind*III (see Fig. 14A), treated with bacterial alkaline phosphatase followed by phenol treatment. The DNA fragments were labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase, and electrophoresed upon 20% polyacrylamide gel. After the gel was exposed to X-ray film, the region in the gel containing a ^{32}P-labeled *Eco*RI-*Hind*III 73 bp fragment of A element of polyomavirus enhancer was cut off by referring to the profile of autoradiograms. After the gel fragment was crushed in the deionized water, the supernatant after centrifugation was used as ^{32}P-labeled probe.

Binding reaction was performed in a total volume of 25 μl contained the following components: 0.1ng of ^{32}P-labeled DNA fragment (10,000 cpm), 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM EDTA, 10% glycerol, 0.5mM DTT, 0.5mM PMSF, 0.5μg/ml pepstatin A and 4μg of poly(dI-dC) (Pharmacia). Nuclear extract (10μg protein as standerd reaction) was added to the above mixture and incubated at 25°C for 30 min. The mixture was mixed with 1μl of dye solution and loaded on a 4% polyacrylamide gel in a buffer containing 40mM Tris-acetate and 1 mM EDTA. Gels were dried and exposed to X-ray film (Kodak XAR-5) with an intensifying screen at -70°C.

RESULTS

Identification of the 80K Protein.

The 80K protein, which is a specific substrate for protein kinase C as described by Rodriguez-Pena and Rozengurt (1986), was identified by 2D gel electrophoresis in this study (Fig. 1, (1), indicated by the arrow). This identification was supported by several lines of evidence. (1) A rapid increase of the phosphorylation in this 80K daltons protein (Isacke *et al.*, 1986) was induced upon addition of 50nM TPA (Fig. 1, (2)), 10nM Bombesin (Fig. 1, (3)), and fetal calf serum to 20% (Fig. 1, (4)). (2) The phosphorylation of this protein disappeared on treatment with 1M KOH (Fig. 2) as described by Isacke (Isacke *et al.*, 1986), indicating that the majority of phosphoamino acids in this protein are phosphoserine and phosphothreonine. (3) The tailing of phosphorylation in this molecule to the acidic end of the 2D gel is observed as previously reported (Isacke *et al.*, 1986). (4) No other proteins with similar molecular weight and the pI range 4-5 were not detected.

Reduction of the Phosphorylation of 80K Protein in Transformants but not in Normal NIH3T3.

To investigate the response of protein kinase C to TPA in the transformants, I must consider the effect of serum concentrations on the protein kinase C activity. First of all, NIH3T3 cells were labeled with ^{32}P-orthophosphate in DMEM supplemented with 0.1%, 0.3%, 1%, 3% or 10% FCS for 24 h, and analyzed by 2D gel electrophoresis. In Fig. 3A a-e, the basal level phosphorylation of the 80K protein was detected in the presence of various concentrations of serum.

To investigate the response of protein kinase C under these conditions, TPA-inducible phosphorylation of this protein was analyzed by the addition of 200nM TPA (Fig. 3A, f-j). The increase of the phosphorylation of 80K protein was detected in the presence of 0.1%-3% FCS (Fig. 3A, f-i). In the presence of 10% FCS, TPA-treatment induced no remarkable increase of the phosphorylation of the 80K protein (Fig. 3A, j). As a phosphoprotein, which is indicated by red arrow in Fig. 3A (a), was constantly detected in the presence of various concentrations of serum, this phosphoprotein was used as internal control. The radioactivity in the spots of the 80K protein and of the internal control protein, which were cut off from the dried gels, was measured in a liquid scintillation counter, and the amounts of radioactivity were compared. The result showed that TPA-inducible phosphorylation of the 80K protein was much increased in the presence of lower concentrations of serum.

SCT1025, an NIH3T3 cell line transformed with activated human c-*raf*-1, was grown in DMEM supplemented with 0.1%, 0.3%, 1%, 3% or 10% FCS and labeled as the case of NIH3T3 cells, and the phosphorylated proteins were analyzed by 2D gel electrophoresis. The basal level phosphorylation of the 80K protein was undetectable at all (Fig. 3B, a-e). Similarly, the phosphorylation of the 80K protein in TFH-2, NIH3T3 cells transformed with H-*ras* gene containing valine substituted for glycine at position 12, was also undetectable in all the serum concentrations (Fig. 3C, a-e).

Labeled protein samples were also prepared after treating these transformants with TPA, and analyzed by 2D gel electrophoresis. Neither the SCT1025 nor TFH-2 exhibited TPA-inducible phosphorylation of 80K protein (Fig. 3B, f-j and 3C, f-j). The internal control protein described above was equally labeled in

these cell lines. To investigate the effect of the activated H-ras gene with another point mutation, which contains leucine substituted for glutamine at position 61, 45-3-A cells were used. But the effect of this distinct mutation was not detected (Fig.4, (1)). As it was reported that *raf* oncogene may function in the pathway of transformation by H-*ras* oncogene (Rapp, 1987), I addressed whether these phenomena are observed only in H-*ras* and *raf* transformants. The results indicated that the phosphorylation of the 80K protein was not commonly detected in N-*ras*, K-*ras*, v-*src*, v-*mos* or polyoma middle T antigen-transformed NIH3T3 cells (Fig. 4).

To examine a possibility that the apparent reduction of the phosphorylation of the 80K protein is due to the decreased synthesis of the protein itself, I analyzed the synthesis of this protein in normal and transformed cells (Fig. 5). The level of the synthesis in the transformants by *raf*, *src* or *mos* were similar to that in NIH3T3 cells. An increase and decrease were observed in H-*ras* and N-*ras* transformants, respectively. To examine a possible effect of phosphatase during the sample preparation, NIH3T3 and SCT1025 (*raf* transformant) were mixed, processed and the proteins were applied on 2D gel. This mixed preparation exhibited the phosphorylated 80K protein as NIH3T3 preparation did (Fig. 6), suggesting that the disappearance of the phosphorylated 80K protein in SCT1025 cells is not due to the action of a common phosphatase during sample preparation. These results described above suggest that protein kinase C activity responsible for the phosphorylation of the 80K protein is not detected in the transformants in contrast with normal NIH3T3 cells.

Establishment of Hormone-Inducible H-*ras* Transformants.

I demonstrated that the reduction of phosphorylation of 80K protein commonly occurs in transformants with *raf*, H-*ras*, N-*ras*, K-*ras*, v-*src*, v-*mos* or polyoma middle T antigen, as compared with NIH3T3 cells. To confirm the correlation between cellular transformation and the reduction of phosphorylation of 80K protein, I established hormone inducible H-*ras* transformants. A transcriptional promoter inducible by steroid hormone, mouse mammary tumor virus-long terminal repeat (MMTV-LTR), was used in this experiment.

Construction of recombinant plasmids containing the activated human H-*ras* coding region under the MMTV-LTR is illustrated in Fig. 7. The plasmids containing one and two fragments of MMTV-LTR are termed pMDSS-I and pMDSS-II, respectively. After NIH3T3 cells were transfected with each pMDSS plasmid DNA, the cells were cultured in the presence of 2μM dexamethasone, one of steroid hormones. Colonies which exhibit morphological change and anchorage-independent growth in soft agar only in the presence of dexamethasone were isolated (MATERIALS AND METHODS).

DNA of TF-MDSS3-2-2 cells, a transformant of the pMDSS-II, was analyzed by southern blot (Fig. 8). By using a 2.9kb *Sac*I fragment containing human H-*ras* coding region as probe, a single band of about 9.2kb was shown in the transformant but not in normal NIH3T3 cells. As only one *Eco*RI site exists in pMDSS-II plasmid, an *Eco*RI site in the host cell DNA might be utilized for the appearance of the 9.2kb band. This result suggests that one copy of pMDSS plasmid is integrated in a cellular chromosome of the transformants. Two faint signals, which were commonly observed in lane 1-4, might be contamination with plasmid DNA. As shown in Fig. 9, morphological change of TF-MDSS3-2-2 cells become visible

at 12 h after addition of dexamethasone and exhibits completely transformed phenotype after 48 h. As TF-MDSS3-2-2 cell line exhibits remarkably morphological change between in the absence and in the presence of dexamethasone, this cell line was used in following experiments. In the same way, two recombinant plasmids containing activated human N-*ras* gene were constructed (Fig. 10, and MATERIALS AND METHODS), but no transformed NIH3T3 cell line containing the plasmid was detected.

Reduction of the Phosphorylation of 80K Protein in Inducible H-*ras* Transformant.

To confirm the correlation between the cellular transformation and the reduction of phosphorylation of 80K protein, phosphorylation of the 80K protein in the inducible transformant TF-MDSS3-2-2 was analyzed by a 2D gel. In the absence of dexamethasone, phosphorylation of the 80K protein was enhanced with TPA in this cell line (Fig 11. (1) and (2)), suggesting that this cell line under the condition behaves as normal NIH3T3 cells. This cell line was also analyzed by 2D gel after two days of H-*ras* gene expression induced by dexamethasone. Phosphorylation of the 80K protein of this cell line was not enhanced with TPA in the presence of dexamethasone (Fig. 11, (4) and (5)), as is the case of stable transformed cell lines with H-*ras* gene products. These results strongly suggest that reduced activity of protein kinase C in the transformants may be related to cellular transformation events rather than to divergence of the cell lines during long-term culture.

Different Distribution of Protein Kinase C Activity between Normal and Transformed NIH3T3 Cells.

As the reduction of phosphorylation of 80K protein in the transformants suggests decreased activity of protein kinase C *in*

vivo, *in vitro* activity of this enzyme was measured. Cytosol and membrane fractions were prepared from normal, H-*ras* or *raf* transformed NIH3T3 cells as described in MATERIALS AND METHODS, and the protein kinase C activity in these fractions was assayed by measuring the incorporation of ^{32}P into histone from [γ-^{32}P] ATP. Protein kinase activity of each fraction in the presence or absence of both calcium and TPA was shown in Fig. 12A. Protein kinase C activity was calculated as the difference between the activities in the presence and absence of TPA plus calcium (Fig. 12B). By using 1μg protein of each fraction where linear dependence of reaction on the protein amount was observed, protein kinase C activity was re-assayed for normal NIH3T3, H-*ras* and *raf* transformants (Table 1). In NIH3T3 cells, protein kinase C activity in the cytosol fraction was higher than that in the membrane fraction, while the activity in the membrane fraction was higher than that in the cytosol fraction in the *raf* or H-*ras* transformants.

Analysis of mRNA in Early Stage of Cellular Transformation.

To demonstrate the change of protein kinase C activity in early stage of the transformation induced by activated H-*ras* gene products, the transcription of c-*fos* gene was analyzed by northern blot. As the transcription of c-*fos* gene is known to be induced by TPA or certain growth factors which activate protein kinase C , the transcript of c-*fos* gene is expected to be induced if protein kinase C is activated through a signal transduction pathway involving H-*ras* gene products. Total RNA was isolated from TF-MDSS3-2-2 cells at 0 h, 1 h, 2 h, and 3 h after exposure with dexamethasone, and 20μg of the RNAs were electrophoresed in duplicate and transferred to

nitrocellulose filter. One filter was hybridized with ^{32}P-labeled human H-*ras* cDNA probe and a major band of about 5.8kb was detected (Fig.13). The other filter was hybridized with ^{32}P-labeled v-*fos* probe and a band of about 2.2 kb was observed. As the former filter was washed out and re-hybridized with human ferritin probe as internal marker, a single band of about 1.1kb was detected. The transcript of human H-*ras* gene under the control of MMTV-LTR was increased at 1 h after exposure with dexamethasone but not 0 h. Similarly, the increased transcript of endogeneous mouse c-*fos* gene was observed at 1 h, suggesting that change of protein kinase C activity occurs in relation to the expression of the activated H-*ras* gene products.

Effects of Inhibition of Protein Kinase C on Cell Growth and on Anchorage-independent Growth

TPA is known to cause the down-regulation of protein kinase C in a long-term treatment. So, by using the TPA and an inhibitor of protein kinase C, H-7, I examined the effect of inhibition of protein kinase C on the cell growth in early stage of cellular transformation. The TF-MDSS3-2-2 cells were pretreated with or without these chemicals for 24 h in order to inhibit or down-regulate the protein kinase C, then cultured with or without dexamethasone for another 24 h and counted (Table 2, left column). As compared with non-transformed cells which were pretreated without the chemicals (None - Dex), the transformed cells induced by dexamethasone (None + Dex) showed faster growth. But the growth of transformed cells pretreated with H-7 (H7 + Dex) were slower than that of the transformant without H-7 (None + Dex), suggesting that the inhibition of protein kinase C may give growth inhibition to the cells which are in the early stage of cellular transformation.

However, H-7 treatment seems to have stimulated the growth of non-transformed cells ((H7 - Dex) and (None - Dex)). Long term treatment with TPA (TPA - Dex) gave the cells a growth-stimulating effect as in the transformed cells induced by dexamethasone (None + Dex), and seems to have given somewhat synergistic effects with dexamethasone (TPA + Dex), which will be discussed below. Furthermore, the effects of H-7 and TPA were also assayed in the soft agar to distinguish from cell growth to tumorigenicity. The inducible transformants were pretreated with or without these chemicals in 0.3% soft agar for 2 days, and the size of colonies was measured 10 days after induction of transformation by dexamethasone (Table 2, right column). Presence of the inhibitor H-7 (H7 + Dex) caused a decrease of anchorage-independent growth as compared with the transformant without H-7 (None + Dex), suggesting that the activation of protein kinase C is important in the early stage of transformation. The treatment with H-7 alone formed no colony (H7 - Dex), suggesting that H-7 stimulates the cell growth, but not anchorage-independent growth. The finding that pretreatment with TPA decreased the anchorage-independent growth (TPA + Dex) is different from the synergistic effects on the cell growth. The TPA treatment (TPA - Dex), unlike H-7 treatment (H7 - Dex), stimulated anchorage-independent growth, while both exhibited similar effect on the cell growth. There are other possibilities to explain the results; that H-7 may inhibit other kinases and that TPA or H-7 may indirectly regulate MMTV promoter.

Mobility Shift Assay.

As certain nuclear factors, such as AP-1, are thought to be activated by protein kinase C , the activity of these factors may

change in the signal transduction pathway induced by H-*ras* gene products if protein kinase C is activated in the pathway. Some enhancer binding proteins are known to show qualitatively different change between normal and oncogene-transformed cells (Wasylik *et al.*,1988, 1989, Piette *et al.*,1988). Therefore, I attempted mobility shift assay with polyoma virus enhancer DNA by using the nuclear extracts from TF-MDSS3-2-2 cells exposed with dexamethasone. The enhancer region of polyomavirus is a *cis*-acting regulatory element required not only for transcriptional activation but also for viral DNA replication. An enhancer A element used in this study is 24bp between nucleotide 5107 and 5130 in the virus genome, and contains a binding site of transcriptional factor AP-1 which is activated by protein kinase C (Satake *et al.*, 1988).

First of all, to determine the proper amounts of poly (dI-dC), as carrier for blocking non-specific binding, and of a nuclear extract, ^{32}P-labeled *Eco*RI-*Hind*III fragment containing polyomavirus enhancer A element (Fig. 14A) was incubated with a nuclear extract of NIH3T3 cells and poly (dI-dC) described in the figure legend, and electrophoresed through 4% polyacrylamide gel. Three major bands were observed and named I, II and III as indicated in Fig. 14B. Formation of these bands were prevented when excess amounts of unlabeled *Eco*RI-*Hind*III fragment was included in the reaction mixture (Fig. 15, lanes 3 and 4), while formation of these bands was not affected when similar size fragments of plasmid pUC19 DNA were used as a competitor (Fig. 15, lanes 5 and 6). These results suggest that these three bands represent complexes resulted from specific interaction of proteins with the DNA.

Some nuclear extracts of oncogene-transformed NIH3T3 cells were used in this assay (Fig. 16). N-*ras*, H-*ras* or *raf* transformed cell extracts exhibit more intense signals rather than NIH3T3. In the assay with N-*ras* transformed cell extract, new two bands were observed (Fig. 16, lane 2). The top band may result from an aggregation.

To investigate the change of enhancer binding proteins in the process of transformation, nuclear extracts were prepared from TF-MDSS3-2-2 cells at 0 h, 6 h, 2 days and 4 days after exposure with dexamethasone and assayed (Fig. 17). When the nuclear extract from the cells exposed with dexamethasone for a longer time was used, band III appeared to be converted to an upper band (Fig. 17, lanes 2-5). These results suggest that a new binding protein is produced or that affinity or amount of binding protein may increase in the process of cellular transformation caused by activated H-*ras* gene products.

TF-MDSS3-2-2 cells without dexamethasone (at 0 h), however, may not be identical to normal NIH3T3 cells, as the assay with the cell extract at 0 h showed a distinct profile from that with NIH3T3 extract (Fig. 17, lane 1 and 2). The activated H-*ras* gene under the MMTV promoter may be expressed slightly in the cells even in the absence of dexamethasone.

DISCUSSION

Reduction of Phosphorylation of 80K Protein in Transformants.

The significance of participation of protein kinase C in transformation is supported by several lines of evidence. (1) TPA, which binds directly to protein kinase C, acts as a strong tumor promoter and stimulates chemical carcinogenesis in mice (Nishizuka, 1988). (2) Application of TPA to mouse skin causes a rapid and sustained loss of protein kinase C (Fournier, 1987). (3) Oncogene-induced transformation in the rodent fibroblasts is enhanced by tumor promoters such as TPA (Hsiao *et al.*, 1984, 1986, Dotto, 1985). (4) A mutant protein kinase C can transform fibroblasts (Megidish and Mazurek, 1989). These results suggest that change of protein kinase C activity may be prerequisite for the cellular transformation.

To investigate the action of protein kinase C in the transformants, an assay for the activity of protein kinase C *in vivo* was used since there are some difficulties in measuring protein kinase C activity *in vitro*, such as a large background of calcium- and phospholipid-independent activities. The assay was a measurement of phosphorylation of an endogenous substrate for protein kinase C, 80K protein, in response to activation of this enzyme by hormones or extracellular agents, for instance, TPA.

Although the purified protein kinase C can phosphorylate many proteins *in vitro* including histone, protamin, myosin light chain and fibrinogen, only a few proteins which are phosphorylated by protein kinase C *in vivo* have been reported: epidermal growth factor receptor (Cochet *et al.*, 1984, Iwashita and Fox, 1984), insulin

receptor (Jacobs *et al.*,1983), c-*src* gene product pp60$^{c\text{-}src}$ (Gould *et al.*, 1985), transferrin receptor (May *et al.*, 1984), vinculin (Werth and Pastan, 1984) and 80K protein (Rozengurt *et al.*, 1983, Rodriguez-Pena and Rozengurt, 1986). Out of them, the protein which can be detected most easily by 2D gel electrophoresis is the 80K protein.

This 80K protein is an 80,000 mol. wt. cellular protein, originally found as a protein phosphorylated within seconds of protein kinase C activation by such stimuli as growth factors, phorbol ester and cell-permeable diacylglycerols. Stimulation of phospholipid breakdown leading to generation of diacylglycerol by exogenous phospholipase C also causes a rapid enhancement of 80K protein phosphorylation, while the agents that increase the level of c-AMP does not affect the phosphorylation. Prolonged pretreatment of the cells with phorbol ester, which leads to a marked decrease in the number of specific phorbol ester binding sites and to disappearance of protein kinase C activity measured *in vitro*, prevents the increase in 80K protein phosphorylation elicited by phorbol esters and growth factors. All these findings suggest that 80K phosphorylation reflects the activation of protein kinase C in intact cells. As the 80K phosphoprotein migrates at the acidic end of 2D gels, within the pH range 4.4-4.9, it is useful as a specific marker of protein kinase C activity in 2D gel analysis (Rodriguez-Pena and Rozengurt, 1986).

In this study, I demonstrated that the phosphorylation of the 80K protein was not stimulated in *raf* and H-*ras* transformed NIH3T3 cells by TPA addition even under the low serum concentration (Fig. 3). These results suggest that activity of protein kinase C responsible for phosphorylation of 80K protein is reduced

in the transformants. The same results were observed, furthermore, in N-*ras*, K-*ras*, v-*src*, v-*mos* or polyomavirus middle T antigen transformants (Fig. 4). There was no difference of phosphorylation level in 80K protein between the distinct H-*ras* transformed cell lines which have different point mutation sites, TFH-2 and 45-3-A cells (Fig. 3C and Fig. 4 (1)). No remarkable change was detected in the level of the synthesis in the 80K protein itself in normal and transformed cells (Fig. 5). The reduction of phosphorylation in the 80K protein was not due to the phosphatase action during sample preparation (Fig. 6).

The reduction of phosphorylation in 80K protein observed commonly in c-*raf*, H-*ras*, N-*ras*, K-*ras*, v-*src*, v-*mos* and polyomavirus middle T antigen transformed cells can be either due to one signal in the cascade of signal transduction following to cellular transformation or to secondary consequence of divergence of the cell lines resulting from long-term culture. To make clear this problem, I established hormone-inducible H-*ras* transformants. Two dimensional gel analysis of the transformed cells two days after treatment with dexamethasone confirmed reduction of phosphorylation of 80K protein (Fig. 11), strongly suggesting that the reduction of protein kinase C activity responsible for phosphorylation of 80K protein may be required for cellular transformation.

By assay of morphological change and DNA synthesis using microinjected anti-*ras* monoclonal antibody, Stacey *et al.* have reported that transformation by *src*, *fms*, or *fes* gene products depended on c-*ras* protein while the transformation by *mos* or *raf* gene products did not (Mulcahy *et al.*,1985). Rapp *et al.* have reported that the DNA synthesis of H-*ras* transformed cells was

inhibited by injection of anti-*raf* antibody (Rapp *et al.*,1987), and that expression of v-*fms*, v-*src*, v-*sis*, H-*ras* or polyoma middle T antigen increased the apparent molecular weight and phosphorylation state of the c-*raf* protein (Morrison *et al.*, 1988). Noda *et al.* have reported that *raf* or *mos* gene products can transform variant cell lines which were resistant to transformation by *ras* gene products (Noda *et al.*, 1983). These findings suggest that the position of *raf* and *mos* protein kinase action is downstream to that of *ras* in the signal transduction by *src* tyrosine kinase. These views are supported by my results that the phosphorylation of 80K protein is commonly reduced in the discussed transformants .

NIH3T3 fibroblasts transformed by the H-*ras*, K-*ras*, v-*src*, v-*fms* or v-*sis* oncogene products possessed elevated levels of diacylglycerol, the endogenous activator of protein kinase C, as compared to the nontransformed parental cells (Wolfman and Macara, 1987, Fleischman *et al.*, 1986, Preiss *et al.*, 1986). Wolfman suggested that constitutively elevated diacylglycerol in NIH3T3 cells transformed by *ras*, *src* or *fms* gene products might cause down-regulation of protein kinase C (Wolfman and Macara, 1987), which is not inconsistent with my results. Decreased response of 80K protein phosphorylation to TPA in the transformants in this study may reflect the "down-regulation" called by Wolfman *et al.*

Kamata *et al.* reported that in a K-*ras*-transformed NIH3T3 cell line the phosphorylation of 80K protein is stimulated by TPA treatment (Kamata *et al.*, 1987), which does not accord with my finding. This discrepancy may be explained in either one of the following ways. (1) As the DT cells which Kamata *et al.* used were transformed by Kirsten murine sarcoma virus and my

transformants were derived from human cellular oncogenes, the transformants might show different responses to TPA. (2) The cell culture lineage might contribute to the divergence of tranformants which might, for example, express a subspecies of protein kinase C responding differently to TPA. (3) Different experimental conditions might be involved in the discrepancy. A protein kinase C subspecies which can respond to TPA might be maintained in the transformants starved of phosphate for 4 h of pre-labeling with [^{32}P] orthophosphate under their conditions, but might not survive in the transformants after 24 h of pre-labeling under my conditions. Fu *et al.* (1991) have recently reported that the level of 80K phosphorylation in TPA-stimulated DT cells was lower than that in unstimulated NIH3T3 cells , which almost coincide with my results. More detailed analysis is necessary.

Effect of mixing NIH3T3 and SCT1025 cells suggests that the reduction of the phosphorylated 80K protein in SCT1025 cells is not due to the action of phosphatases during sample preparation (Fig. 6). However, it can not rule out the possibility that the reduction of 80K phosphorylation may be affected by phosphatase action *in vivo*; the activity of phosphatases may be higher in the transformants than in the normal NIH3T3 cells. Furthermore, the different action of the phosphatases may explain the discrepancy in the K-*ras* transformants discussed above. An experiment using inhibitors of serine/threonine-protein phosphatase, such as okadaic acid, may be desired.

Recently, cDNA coding the 80K protein has been cloned and analyzed (Stumpo *et al.*, 1989). One striking finding is that the open reading frame of 1005 bp predicts a protein of 335 amino acid residues (mol. wt. 31,949). Despite this predicted size, the protein

migrated on SDS/polyacrylamide gel with an apparent molecular weight of 80,000-87,000 after expression of the cDNA in cells normally lacking the protein. TPA-response of the phosphoprotein was also confirmed. It is highly enriched in alanine (28.4 mol %), contains an amino-terminal myristoylation consensus sequence, and includes a 25-residue basic domain containing the known protein kinase C phosphorylation site. A pI value is calculated as 5.46 for the entire protein. The discrepancy between its predicted and apparent molecular masses on SDS gels is thought to stem from factors such as its acidic pI and general hydrophilicity, allowing for incomplete binding of SDS, as well as its apparent rod-like helical structure. The mRNA and the immunoreactive protein are densely distributed in brain, spinal cord, spleen and lung. Stumpo *et al.* proposed the name myristoylated alanine-rich C kinase substrate (MARCKS) for this protein. The determination of the physiological role of this protein is desired.

***In vitro* Activity of Protein Kinase C.**

Protein kinase C is a large family of proteins with multiple subspecies, and the general mechanism of activation of these enzymes depend on the presence of calcium, diacylglycerol and phospholipid. Diacylglycerol, a hydrolyzed product of inositol phospholipids which is increased in response to growth factors and hormones, activates protein kinase C, which in turn phosphorylates a range of cellular proteins, for example, proteins controlling gene expression. In addition to the positive action, protein kinase C appears to provide negative feedback control over various steps of signal transduction, such as phosphorylation of receptors by this enzyme resulting in a rapid decrease of binding affinity for the ligands. The negative feedback control is, however, relieved by

cleavage of protein kinase C by calpain I. A target of calpain I is the activated form of protein kinase C because the calpain is activated in the presence of phosphatidylserine, diacylglycerol and calcium.

In this study, protein kinase C activity *in vitro* was lower in the cytosol fraction and higher in the membrane fraction of *raf* and H-*ras* transformants as compared to those of normal NIH3T3 cells (Fig. 12 and Table 1). The similarity of the distribution in *raf* and H-*ras* transformants in this study suggests that both gene products may act in the same signalling pathway involving protein kinase C. Similar subcellular distribution was reported for the protein kinase C activity in H-*ras*-transformed rat fibroblasts (Huang *et al.*, 1988) and in K-*ras*-transformed NIH3T3 cells (Kamata *et al.*, 1987). Possible reasons for reversed protein kinase C activities between cytosol and membrane fractions of the transformants are; (1) translocation of protein kinase C from cytosol to membrane, (2) decrease of calpain activity in the membrane and (3) function of distinct subspecies of protein kinase C with the individual enzymological characteristics. Nishizuka has reported that several subspecies of protein kinase C are co-expressed in a single cell type and that they disappear at different rates upon treatment with TPA (Nishizuka, 1988). The enhancement of protein kinase C activity associated with the membrane, which is caused by oncogene products, may induce the uncontrolled cell proliferation, while the decrease of protein kinase C activity in the cytosol may bring about the failure of phosphorylation of 80K protein in response to TPA. When compared to NIH3T3 cells, *raf* transformants have a slight lower level of total protein kinase C activity (Table 1). While TFH-2 possesses an increased level of the total activity, it has been

reported that total protein kinase C activity in H-*ras*-transformed rat fibroblasts was 40% less than that of untransformed cells (Huang *et al.*, 1988). The biased distribution of protein kinase C activity in membrane and cytosol fraction rather than the total activity may be significant for transformation .

As the difficulty of measurement of *in vitro* protein kinase C activity has been pointed out (Ohta and Hidaka, 1987), first of all, the response of protein kinase C to TPA was analyzed *in vivo*. When the *in vitro* activity was assayed, calcium- and phospholipid-independent protein kinase activity was apt to disturb the assay. It might be caused by insufficient purification of a kinase fraction or by heterogeneous phosphatidylserine emulsion or the resolution of the emulsion by carried-over detergents in the eluted fraction from DEAE cellulose column. Therefore, some indirect analyses of the protein kinase C were attempted as follows.

Change of Protein Kinase C in Early Stage of Transformation

To investigate the behavior of protein kinase C in the course of conversion from normal cells to the transformed cells, the expression of c-*fos* gene, which is thought to be induced through protein kinase C activation, was analyzed. Induction of c-*fos* mRNA was detected in this course of conversion in parallel with H-*ras* gene expression (Fig. 13). Furthermore, by mobility shift assay using a DNA fragment containing AP-1 binding site, it was shown that some enhancer binding proteins, probably AP-1, were activated in the course of the cellular transformation caused by activated H-*ras* gene products (Fig. 17). The protein kinase C inhibitor H-7 has repressed the anchorage-independent growth of the inducible transformed cells Table 2). These results suggest that

protein kinase C is activated , at least once, in the course of cellular transformation and that the activation is necessary for the transformation. It is known that as soon as normal cells are stimulated by TPA, protein kinase C translocates transiently from cytosol to membrane and is down-regulated for several hours (Kraft and Anderson, 1983, Farrar and Anderson, 1985, Hirota *et al.*, 1985, Fearon and Tashjian, 1987). Considering this evidence, my results described above may suggest that protein kinase C is activated and translocated from cytosol to membrane in the course of cellular transformation induced by activated H-*ras* gene products. In fact, the reversed subcellular distribution of this enzyme activity has been observed in the transformants (Table 1). The sustained activation of oncogene products in the transformants may bring a constitutive effect, such as the activation of transcriptional factors, and may give the cells perpetual growth capability. The disappearance of TPA-response of 80K protein phosphorylation in the transformants (Fig. 3) may reflect the constitutive activation of the protein kinase C.

In the analysis of mRNA in the inducible transformed cells, a major band of about 5.8kb was detected by human H-*ras* cDNA probe. As the reason for having given rise to the size, there are some possible explanations; (1) Recombinant plasmid pMDSS-II, which contains two copies of 1.45kb MMTV-LTR fragment upstream of 2.9kb H-*ras* gene, is integrated in the ~~inducible~~ transformed cells. The transcription might be initiated from the distal promoter. (2) The transcription might be terminated at AATAAA sequence in the pUC vector or host genomic DNA, although an intrinsic polyadenylation signal motif, AGTAAA, exists in the 2.9kb H-*ras* gene fragment. (3) The 5.8kb signal may show

an unspliced precursor RNA, as some smaller signals were observed under long-exposure. As regards c-*fos* and ferritin RNA, the reasonable size was detected (Greenberg and Ziff, 1984, Beaumont *et al.*, 1987)

Satake *et al.* have reported that some transcriptional factors including AP-1 are activated in H-ras transformed cells (Satake *et al.*, 1988). In their report, polyomavirus enhancer A element forms 3 major bands with nuclear factors containing AP-1 upon their mobility shift assay using mouse 3T6 nuclear extract. Their results coincide with my results using nuclear extracts of mouse NIH3T3 and the transformed cells. But the band complexed with AP-1 described by them could not be identified in my conditions, possibly because the conditions of electrophoresis with Tris/acetate/EDTA buffer in this study was different from their conditions with Tris/borate/EDTA. Anyway, my results show that the nuclear factors from the inducible transformant after exposure with dexamethasone exhibited both the disappearance of band I, II and III and the conversion to another upper band, suggesting that DNA element containing AP-1 binding site is activated in the early stage of transformation induced by H-*ras* proteins. The assay with nuclear extract at 4 days of dexamethasone exposure showed a distinct profile from that with the established H-*ras* transformant TFH-2 (Fig, 17, lane 5 and 6). This difference may suggest either that the transformational change is not enough in the inducible transformant exposed with dexamethasone for 4 days or that secondary changes have occurred in TFH-2 cells during the long-term culture.

Effect of protein kinase C inhibition in the transformation is shown in TABLE 2. (1) H-7, a protein kinase C inhibitor, inhibited

the cell growth and anchorage-independent growth of dexamethasone-treated transformed cells [(None + Dex) and (H7 + Dex)]. It suggests that activation of protein kinase C is necessary for cell growth and anchorage-independent growth of the transformed cells. (2) Without dexamethasone, H-7 stimulated the cell growth [(None - Dex) and (H7 - Dex)], suggesting that inhibition of protein kinase C may stimulate the mitogenesis of this cell line. (3) The cell growth of transformants treated with TPA (TPA + Dex) seems to be more than that by dexamethasone treatment alone (None + Dex) or TPA treatment alone (TPA - Dex). But, the effect of TPA upon the transformed cells, that is, (TPA + Dex)/(None + Dex) is lower than the effect of TPA upon the non-transformed cells (TPA - Dex)/(None - Dex), suggesting that the true effect of TPA pretreatment, namely down-regulation of protein kinase C, may be suppressive for cell growth and anchorage-independent growth. As H-7 may not have a high specificity for protein kinase C, it may also have inhibited other kinases, such as c-AMP dependent protein kinase. More detailed investigation is desired by using novel, selective inhibitors of protein kinase C which prevent cell growth in response to mitogens (Davis *et al.*, 1989).

Cellular Transformation and Behavior of Protein Kinase C

In this study, my results revealed that; (1) the phosphorylation of the 80K protein, a specific substrate for protein kinase C, was maintained at decreased levels and could not be stimulated by TPA in H-*ras*, N-*ras*, K-*ras*, c-*raf*, v-*src*, v-*mos* or polyoma middle T antigen-transformed NIH3T3 cells, but stimulated in NIH3T3 cells; (2) These different responses of protein kinase C to TPA between normal and transformed cells were confirmed by analysis of hormone-inducible H-*ras* transformant;

(3) The activity of protein kinase C of the membrane fraction was elevated more than that of the cytosol in the transformants when compared with those of NIH3T3 cells; (4) The transcription of c-*fos* gene, one of immediate early genes, and activation of enhancer element containing an AP-1 binding site were observed in the early stage of transformation; (5) A protein kinase C inhibitor H-7 inhibited the anchorage-independent growth of the transformation-inducible cells. These results suggest that *ras* and *raf* gene products induce the change of protein kinase C activity. At least one way of signalling pathways mediated by *ras* or *raf* gene products might lead activation of protein kinase C.

In regard to the relationship between oncogenic signal transduction pathways and phosphatidylinositol turnover, there are recently two views.

(I) By using anti-oncoprotein antibodies, some reports suggested that *src*-tyrosine kinase, *ras* family, and *raf*- or *mos*-serine/threonine kinase may act in the same pathway described above. Furthermore, some reports have presented evidence that *ras* gene products may be involved in the direct control of phospholipase C, the phosphodiesterase responsible for phosphatidylinositol turnover (Wakelam *et al.*, 1986, Chiarugi *et al.*, 1986). It has been described that cells stably transformed by various oncogenes gain constitutively enhanced level of diacylglycerol (Fleischman *et al.*, 1986, Preiss *et al.*, 1986, Wolfman and Macara, 1987) and of inositol phospholipid metabolism (Kaplan *et al.*, 1986, Huang *et al.*, 1988), in spite of a report of decreased level of diacylglycerol in human colon tumors (Phan *et al.*, 1991). The increase of diacylglycerol seems, however, to be produced by

activation of other phosphodiesterase-mediated degradation of phosphatidylcholine, but not of phosphatidylinositol (Lacal *et al.*, 1987, Moscat *et al.*, 1989, Price *et al.*, 1989, Lopez-Barahona *et al.*, 1990). Downward *et al.* (1988) have reported that the marked enhancement of phosphatidylinositol turnover in response to a peptide mitogen is due to changes in the receptor number rather than to direct coupling by *ras* products of the receptor and phospholipase C. Biochemical measurement showed that activated protein kinase C translocates from cytosol to membrane (Kraft and Anderson, 1983, Farrar and Anderson, 1985, Hirota *et al.*, 1985, Fearon and Tashjian, 1987). Moscat *et al.* have recently reported that, by using immunocytochemical detection of protein kinase C, activation of *ras* or *src* gene products induces permanent translocation of protein kinase C to the cytoplasmic membrane and that down-regulation (loss) of the enzyme is not observed in the transformants (Diaz-Laviada *et al.*, 1990). My results are confirmed by their report; the constitutively elevated activity in membrane fraction of H-*ras* transformants might be just caused by both permanent translocation of protein kinase C and the lack of destruction of the activated enzyme. Furthermore, the lack of down-regulation observed in the transformants is mimicked by chronic treatment with exogenous phosphatidylcholine-hydrolysing phospholipase C, but not with exogenous phosphatidylinositol-hydrolysing phospholipase C or TPA, suggesting that diacylglycerol derived from phosphatidylcholine, but not from phosphatidylinositol, is responsible for the regulation of protein kinase C in the transformed cells (Diaz-Laviada *et al.*, 1990). The sustained activity of protein kinase C is thought to affect the transcriptional factors. Wasylik *et al.* (1989) have reported that

PEA-1 motif, which is recognized by transcriptional factor AP1, was activated by transformation with *raf* gene, as well as *src* , *ras, mos, fos* and polyoma middle T antigen genes. In the H-*ras* transformant, enhancer binding proteins containing AP-1 are modulated by H-*ras* gene product (Satake *et al.*, 1988), and H-*ras* gene product augments c-*jun* activity and stimulates phosphorylation of its activation domain (Binetruy *et al.*, 1991). My study has shown that H-*ras* gene product led to a new complex with the AP-1 binding site during the process of transformation and that inhibition of protein kinase C suppressed the anchorage-independent growth. As the change of behavior of protein kinase C was observed in the transformants with N-*ras*, K-*ras, raf, src, mos* and polyoma middle T antigen-transformed cells as well as H-*ras*, it is suggested that constitutive tranlocation of protein kinase C may be required in the process of transformation induced by the oncogene products on the same signalling pathway.

In addition to the evidence indicating that the nuclear transcriptional factors are regulated by protein kinase C, H-*ras* transformed cells exhibits the translocation of protein kinase C to nucleus, leading to increase of nuclear-associated protein kinase C activity (Chiarugi *et al.*, 1990). For example, c-jun gene product which is a component of AP-1 is activated by phosphorylation through activation of H-ras gene product (Binetruy *et al.*, 1991), while DNA binding activity of c-jun gene product is activated by dephosphorylation through activation of protein kinase C (Boyle *et al.*, 1991). As it suggests that AP-1 is regulated by some protein phosphatases through activation of protein kinase C, analysis for the localization of such phosphatases may give a hint to understand

the mechanism of the translocation of protein kinase C.

Introduction of *ras* genes into mouse teratocarcinoma F9 cell and rat chromaphin PC12 cell induces the differentiation. Activation of AP1 and the following gene expression are observed in the course of the differentiation (Imler *et al.*, 1988, Noda *et al.*, 1985, Bar-Sagi and Feramisco, 1985). It is of interest to see whether the same signalling pathway is utilized in the cell differentiation, cell growth and transformation.

(II) Recently, receptor type tyrosine kinases, such as PDGF receptor, colony stimulating factor-1 receptor, epidermal growth factor receptor and insulin receptor, and non-receptor type tyrosine kinases, such as *src, abl, fms, ros* and *fyn* oncogene products, were reported to associate with phospholipase C-γ , phosphatidylinositol-3 kinase (PI-3 kinase), GAP and c-*raf*-1 gene product (Kaplan *et al.*, 1990). A complex of polyoma middle T antigene and *src* gene product binds also the PI-3 kinase (Whitman *et al.*, 1985). These proteins except for *raf*-1 protein were shown to have SH-2 domain which is found as a specific amino acid sequence conserved in *src* family gene products, and the SH-2 domain was shown to bind to phosphotyrosine-containing proteins some of which are autophosphorylated upon its ligand binding (Matsuda *et al.*, 1990). As the GAP binds to and activates the *ras* proteins, it is likely that receptor-tyrosine kinases can activate multiple pathways, such as *ras, raf* and phosphatidylinositol turnover, at the same time. The findings suggest that these signal transduction pathways may play roles independently .

Mechanisms of signal transduction must be very complicated. The signals through the mediators may be twined again; in a

signalling pathway from extracellular factors through the receptors, an activated intracellular factor may transfer a part of the signal to the membrane receptors negatively in the normal cells, but positively in the transformed cells. Activation of T-cell receptor causes a rapid hyperphosphorylation of c-*raf* and an increase in the kinase activity through activation of protein kinase C (Siegel *et al.*, 1990). This activation of c-*raf* through protein kinase C presents an example of the distinct mechanism from that reported for growth factor receptor tyrosine kinases. Protein kinase C appears to provide positive and/or negative regulation over various steps of signal transduction. My results show obviously that the change of protein kinase C activity occurs in the transformation induced by oncogene products. If the 80K protein is a factor mediating a negative feedback signal of protein kinase C, reduced phosphorylation of the protein in transformants may bring about a blockade of such a negative control, leading to transformed phenotype. The recent isolation of the gene encoding the 80K protein should be helpful to gain insight into signalling mechanisms through protein kinase C.

ACKNOWLEDGEMENTS

I greatly acknowledge Dr. Kenji Shimizu for his kind and excellent guidance, stimulating discussion and generous encouragement through this work. I wish to express my deep gratitude to Professor Yasumi Ohshima for his encouragement through this study and for critical reading of the thesis. I also thank Professor Mutsuo Sekiguchi for helpful discussions. I am also thankful for much help from members of the Department of Biology and of Biochemistry, Kyushu University.

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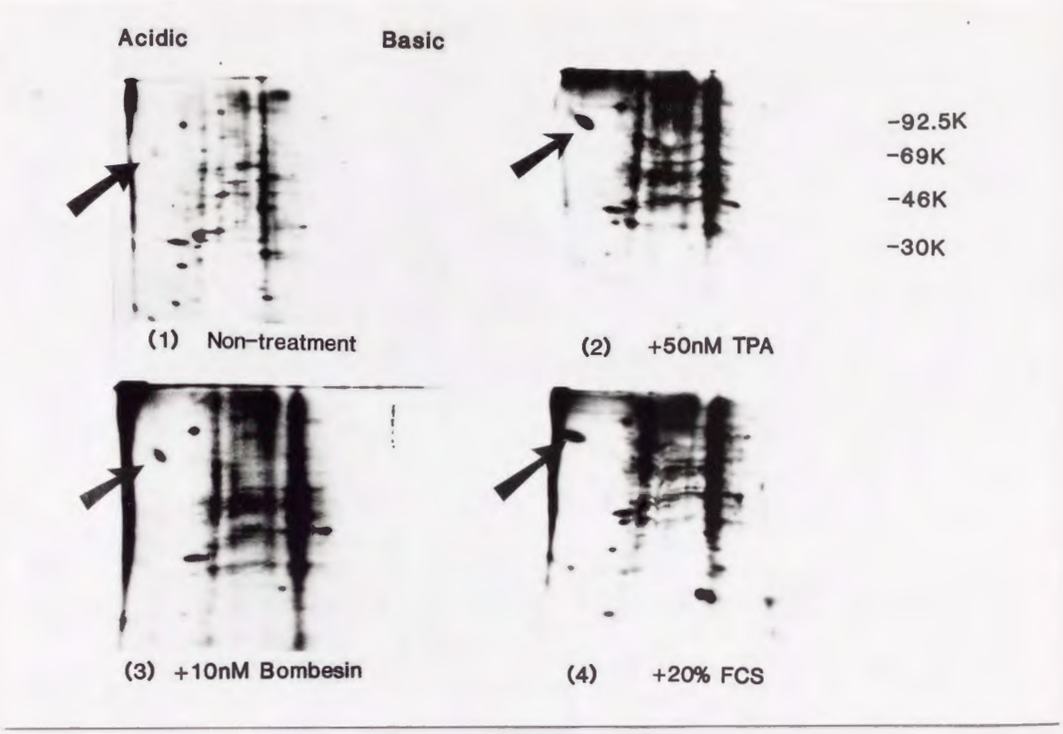
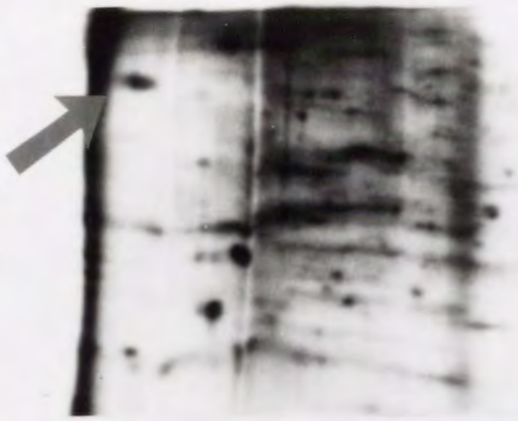


Fig. 1. Identification of the phosphorylated 80K protein. Semiconfluent NIH3T3 cells were pre-labeled in DMEM supplemented with 10% FCS (except that 0.5% FCS was used for +20% FCS sample) containing [^{32}P]orthophosphate (see "MATERIALS AND METHODS"), processed 5 min after addition of activators of protein kinase C as follows: (1)no-addition control, (2)50nM TPA, (3)10nM bombesin, (4)20% FCS. These labeled cell preparations were subjected to 2D gel electrophoresis, using 12.5% polyacrylamide gel.

(1) Non-treatment



(2) Treatment with 1M KOH

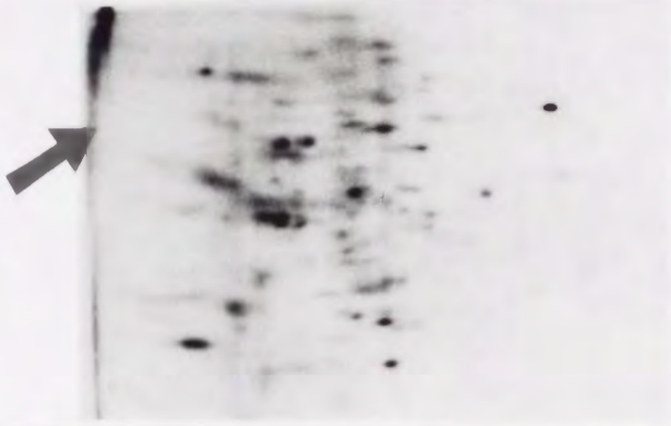
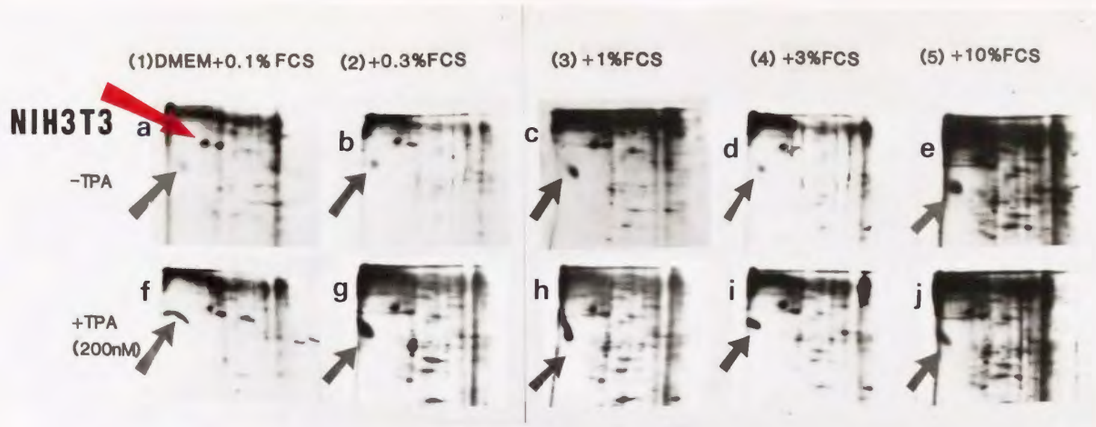
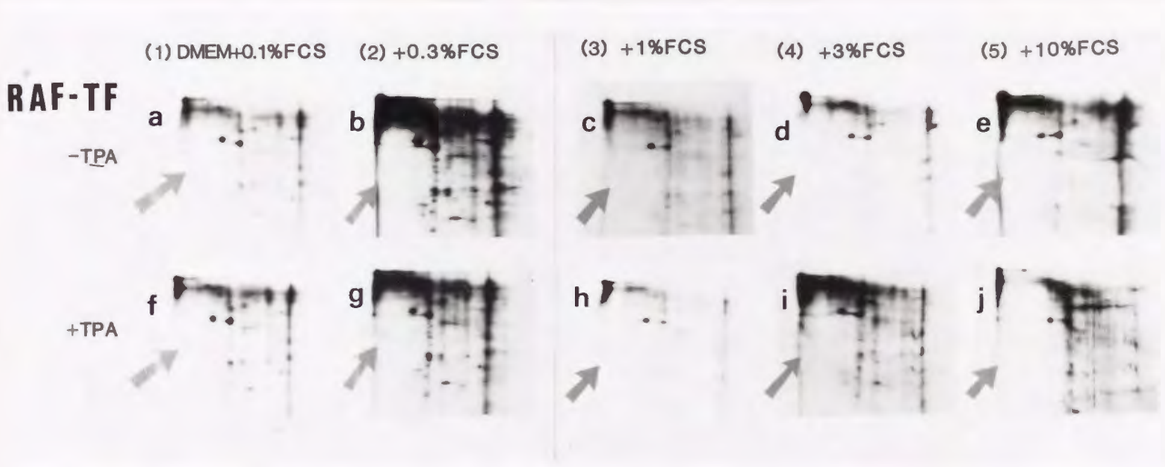


Fig. 2. Analysis of phosphoamino acids of the 80K protein. The [^{32}P] labeled proteins of NIH3T3 cells treated with 50nM TPA were applied on 2D gel electrophoresis. The gels were fixed with 10% acetic acid / 10% methanol and treated without (1) or with (2) 1M KOH at 55°C for 2 hours to hydrolyse the serine-bound and threonine-bound phosphates. Then, the gels were washed with 10% acetic acid / 10% methanol, dried and exposed to X-ray film.

A


(1)DMEM+0.1% FCS
(2)+0.3%FCS
(3)+1%FCS
(4)+3%FCS
(5)+10%FCS
NIH3T3
a
b
c
d
e
-TPA
f
g
h
i
j
+TPA
(200nM)

B


(1) DMEM+0.1%FCS
(2) +0.3%FCS
(3) +1%FCS
(4) +3%FCS
(5) +10%FCS
RAF-TF
a
b
c
d
e
-TPA
f
g
h
i
j
+TPA

C

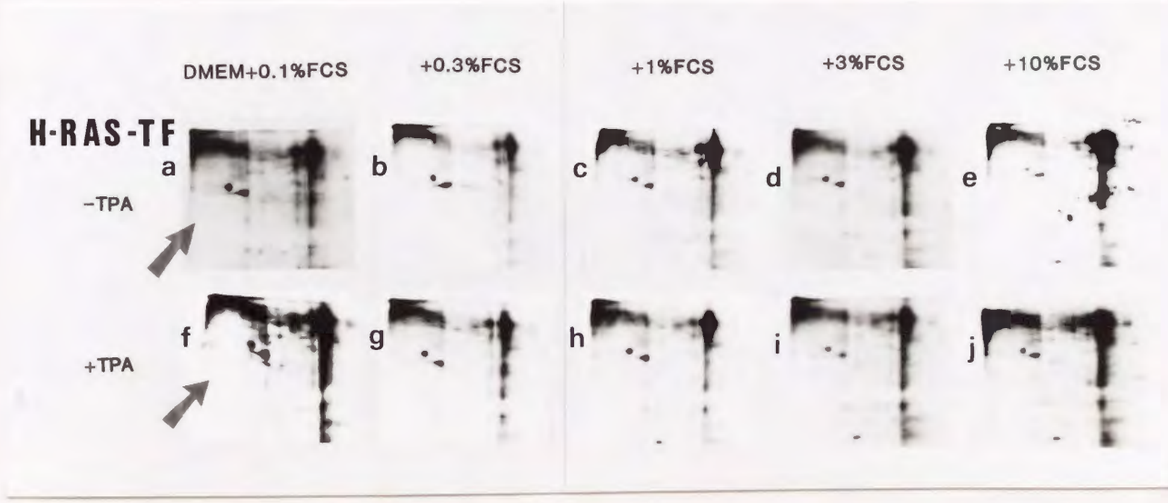

DMEM+0.1%FCS
+0.3%FCS
+1%FCS
+3%FCS
+10%FCS
H-RAS-TF
a
b
c
d
e
-TPA
f
g
h
i
j
+TPA

Fig. 3. Effect of TPA on phosphorylation of the 80K protein in normal and transformed NIH3T3 cells. NIH3T3 cells (A), SCT1025 transformed with c-*raf* (B) and TFH-2 transformed with H-*ras* (C) were grown in DMEM supplemented with 0.1% FCS (a and f), 0.3% FCS (b and g), 1% FCS (c and h), 3% FCS (d and i) and 10% FCS (e and j) for 24 hours, and pre-labeled with [^{32}P]orthophosphate under the same conditions for 24 hours, and then samples were prepared 5 min after treatment with 200 nM TPA (f - j) or without treatment (a - e). The acidic end of the 2D gels is shown.

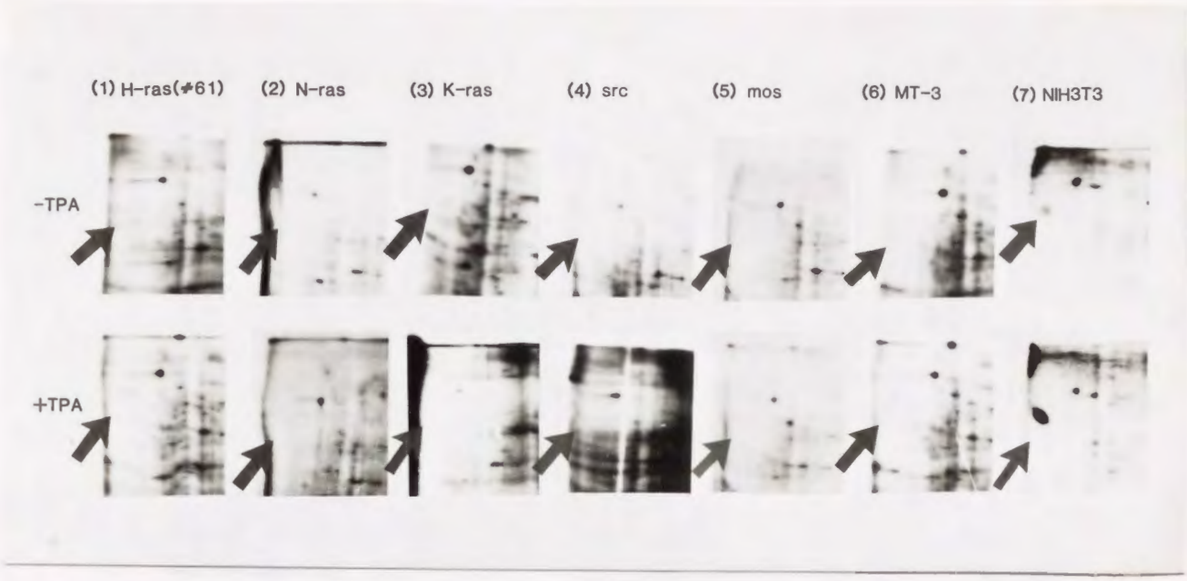


Fig. 4. Effect of TPA on phosphorylation of the 80K protein in other transformed NIH3T3 cells. 45-3-A (1), TFN-75 (2), Lu65 (3), TF-v-src (4), TF-v-mos (5), MT-3 (6) and NIH3T3 cells (7) were pre-labeled in DMEM supplemented with 3% FCS containing [^{32}P] orthophosphate, and processed 5 min after addition of 200nM TPA (lower) or without addition (upper). The acidic end of the 2D gels is shown.

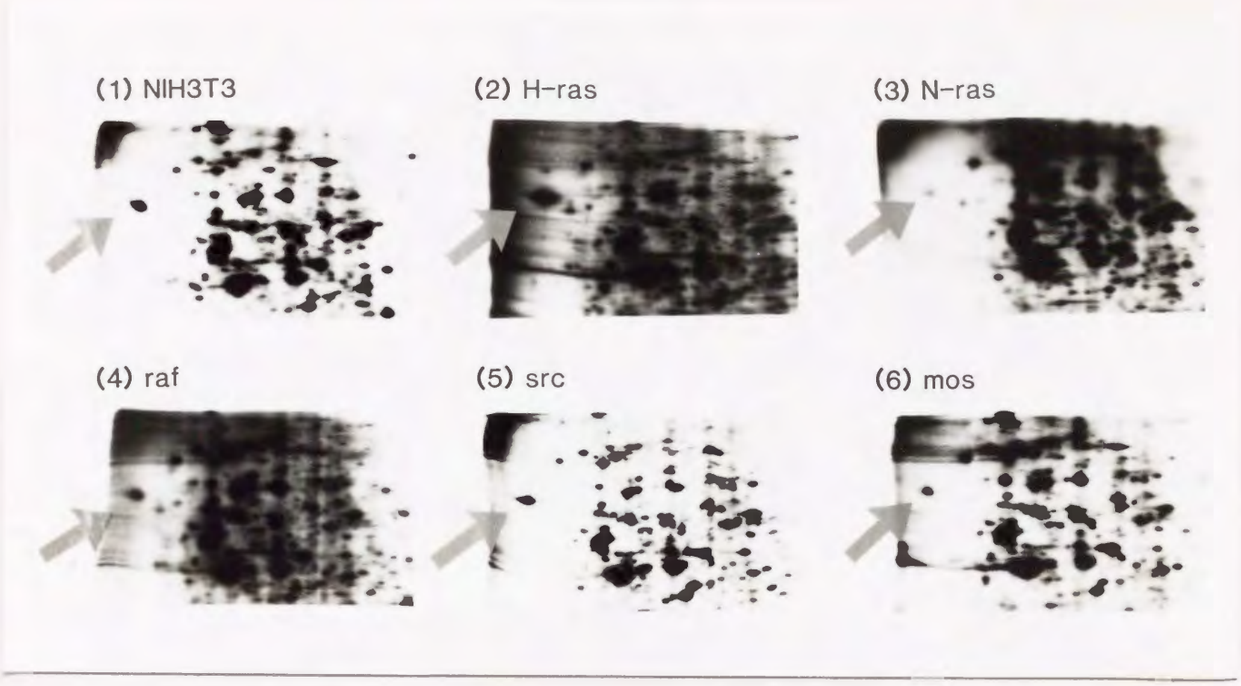


Fig. 5. Synthesis of the 80K protein in normal and transformed NIH3T3 cells. NIH3T3 (1), TFH-2 (2), TFN-75 (3), SCT1025 (4), TF-v-src (5) and TF-v-mos (6) were precultured in methionine-free DMEM supplemented with 10% dialyzed FCS for 1 hour prior to labeling with [^{35}S]methionine for 4 hours. Cell preparation and 2D gel electrophoresis were performed as described in "MATERIALS AND METHODS".

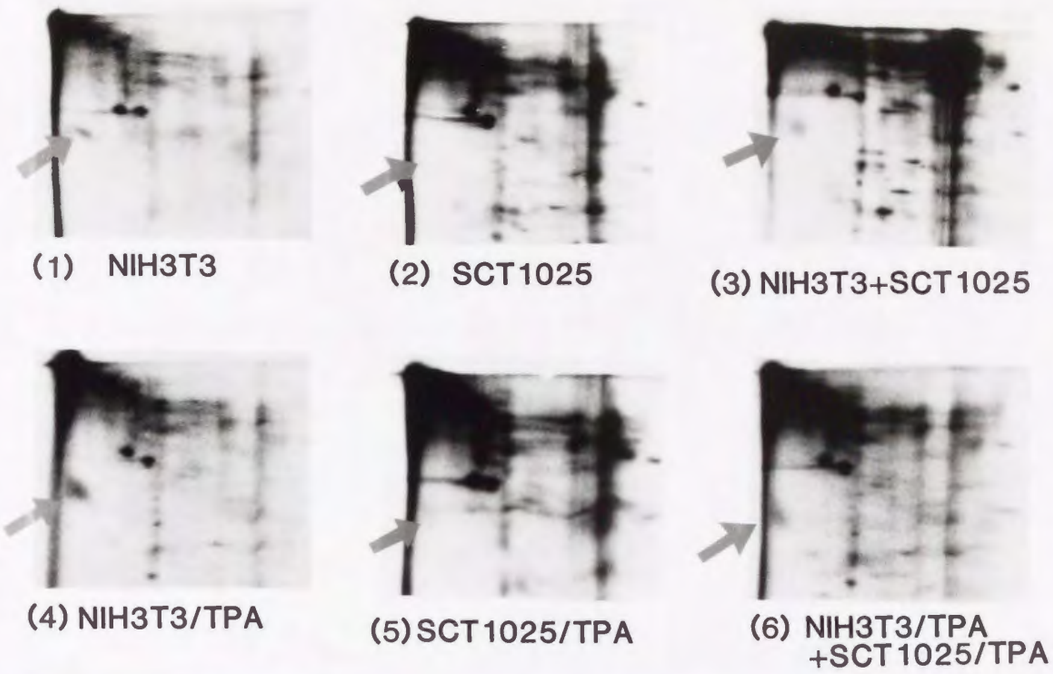


Fig. 6. Effect of mixing cells on phosphorylation of the 80K protein during the sample preparation. NIH3T3 treated with(4) or without(1) 200nM TPA and SCT1025 treated with (5) or without (2) 200nM TPA were washed with cold PBS, treated with 10% TCA for 20 min. The cells were, mixed (3 and 6) or separately (1, 2, 4 and 5), processed as described in MATERIALS AND METHODS.

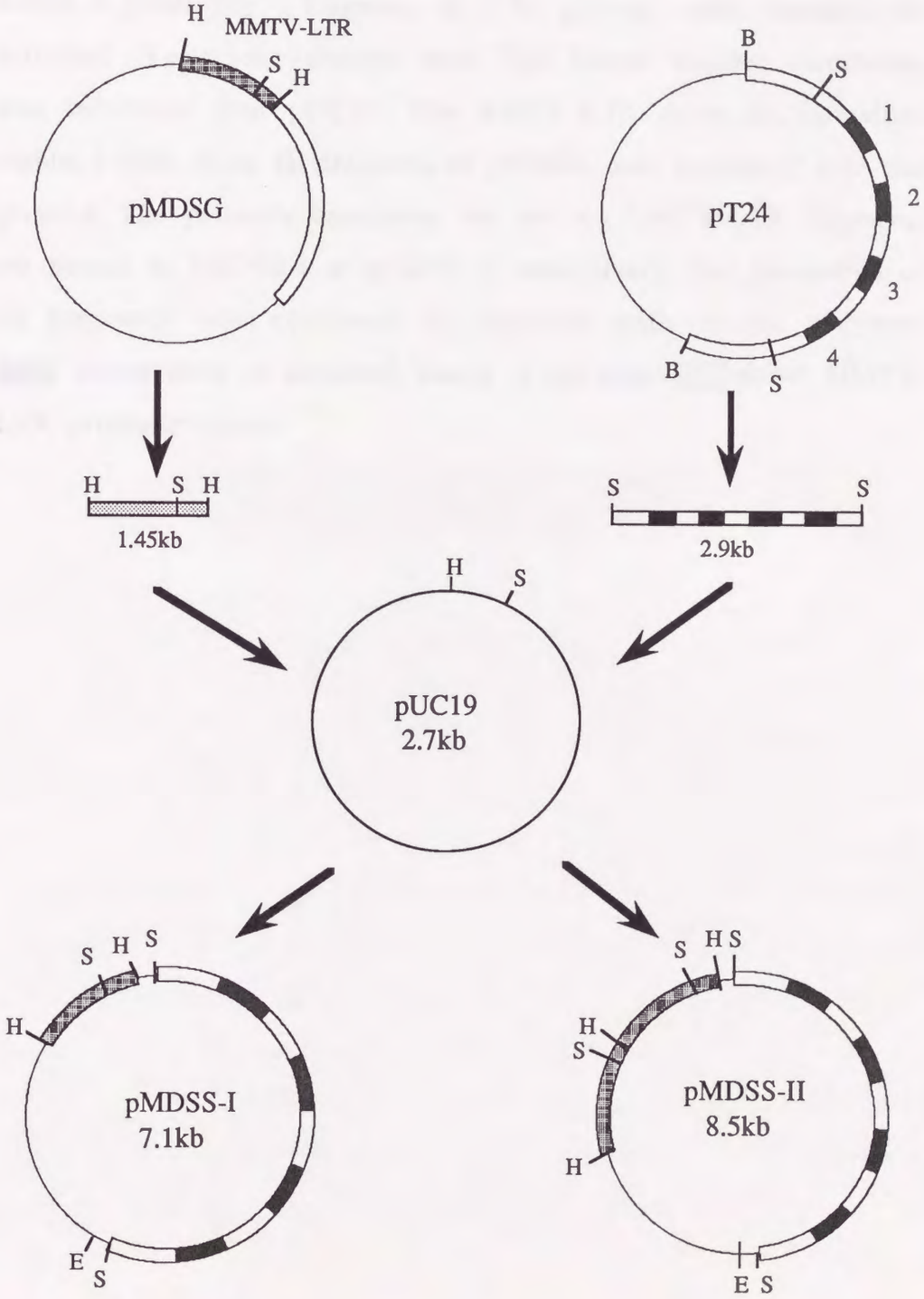

H
MMTV-LTR
S
H
pMDSG
B
S
1
2
pT24
3
4
B
S
H
S
H
1.45kb
S
S
2.9kb
H
S
pUC19
2.7kb
S
H
S
H
pMDSS-I
7.1kb
E
S
S
H
S
H
S
pMDSS-II
8.5kb
H
E
S

Fig. 7. Schematic representation of construction of inducible plasmids containing an activated human H-*ras* coding region under the MMTV-LTR promoter. The H-*ras* coding region, contained within a 2.9kb *Sac* I fragment of pT24 plasmid which contains the activated H-*ras* gene derived from T24 human bladder carcinoma, was subcloned into pUC19. The MMTV-LTR promoter, contained within 1.45kb *Hind* III fragment of pMDSG, was introduced into this plasmid. The plasmids containing one or two MMTV-LTR fragments are named as PMDSS-I or pMDSS-II, respectively. The orientation of all fragments was confirmed by digestion with several enzymes. ■ shows exon of activated human H-*ras* gene. ▒ shows MMTV-LTR promoter region.

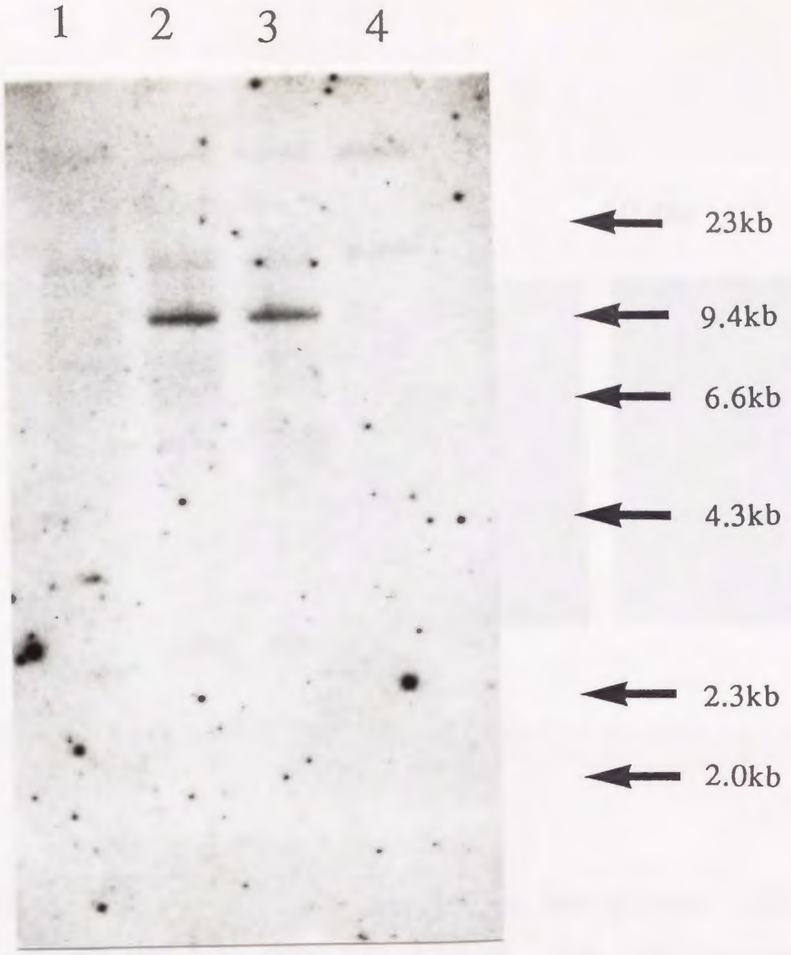


Fig. 8. Analysis of the integrated plasmid DNA in the hormone-inducible transformants. DNA prepared from NIH3T3 cells (lane 1) and TF-MDSS3-2-2 cells treated with (lane 2) or without (lane 3) 0.5μM dexamethasone were digested with *Eco*RI, electrophoresed, transferred to nitrocellulose filter and baked at 80°C for 2 h. The filter was hybridized with ^{32}P-labeled 2.9kb *Sac*I fragment from pT24 containing human H-*ras* gene described in Fig.7, washed and exposed to X-ray film. *Hind*III-digested λphage DNA marker was applied in lane 4.

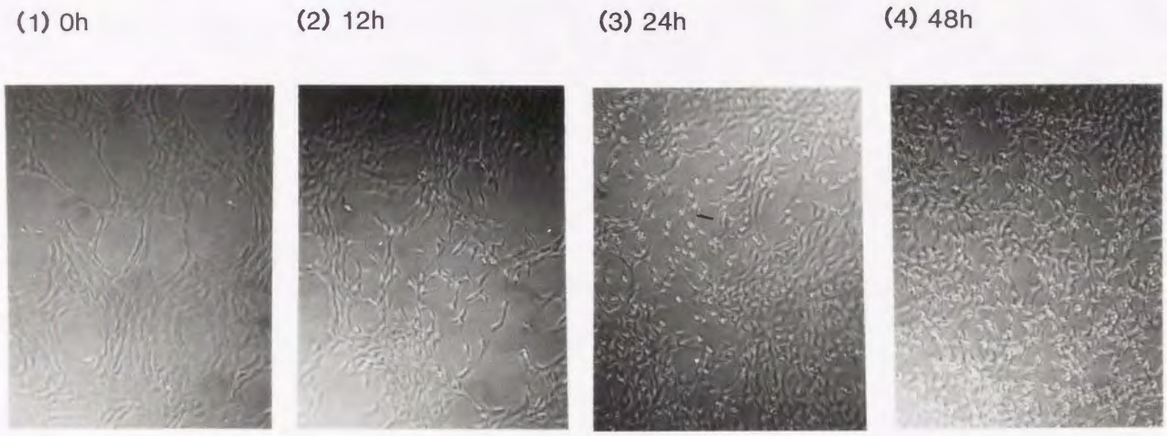


Fig. 9. Morphological change of the inducible transformed cells. Morphology of TF-MDSS3-2-2 was shown at 0h, 12h, 24h and 48h after treatment with 0.5μM dexamethasone. This cell line exhibits flatten shape in the absence of dexamethasone, but exhibits completely transformed phenotype at 48h after addition of dexamethasone. This cell line exhibits anchorage-independent growth in soft agar only in the presence of dexamethasone.

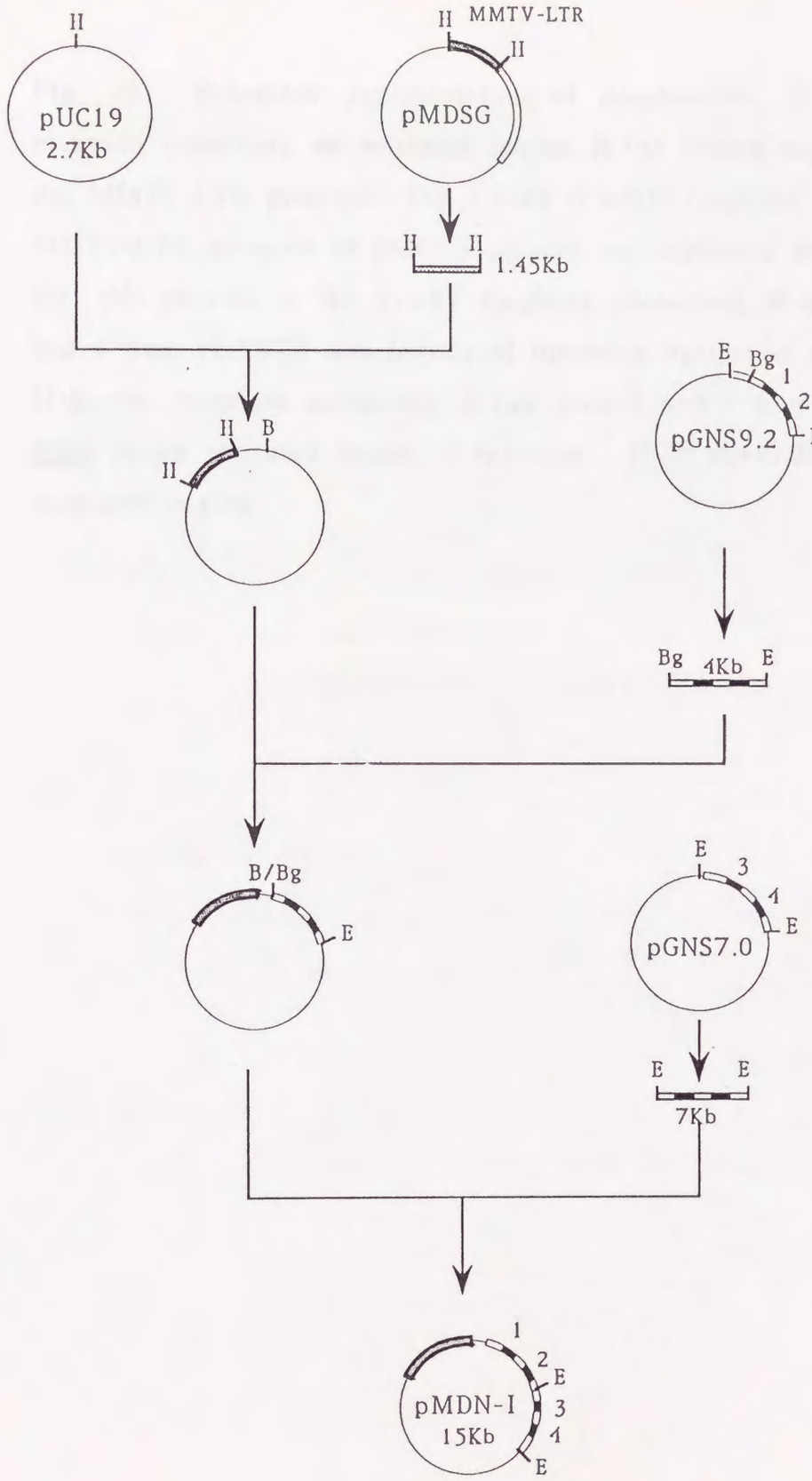

H
pUC19
2.7Kb
H MMTV-LTR
H
pMDSG
H H
1.45Kb
H B
H
E Bg 1
2
pGNS9.2
E
Bg 4Kb E
B/Bg
E
E
3
4
pGNS7.0
E
E E
7Kb
1
2
E
pMDN-I
15Kb
3
4
E

Fig. 10. Schematic representation of construction of inducible plasmids containing an activated human N-*ras* coding region under the MMTV-LTR promoter. The 1.45kb *Hind*III fragment containing MMTV-LTR promoter of pMDSG plasmid was subcloned into pUC19. Into this plasmid, a 7kb *Eco*RI fragment containing N-*ras* exon 3 and 4 from pGNS7.0 was introduced following ligation of a 4kb *Bgl* II-*Eco*RI fragment containing N-*ras* exon 1 and 2 from pGNS9.2. ■ shows activated human N-*ras* exon. ▭ shows MMTV-LTR promoter region.

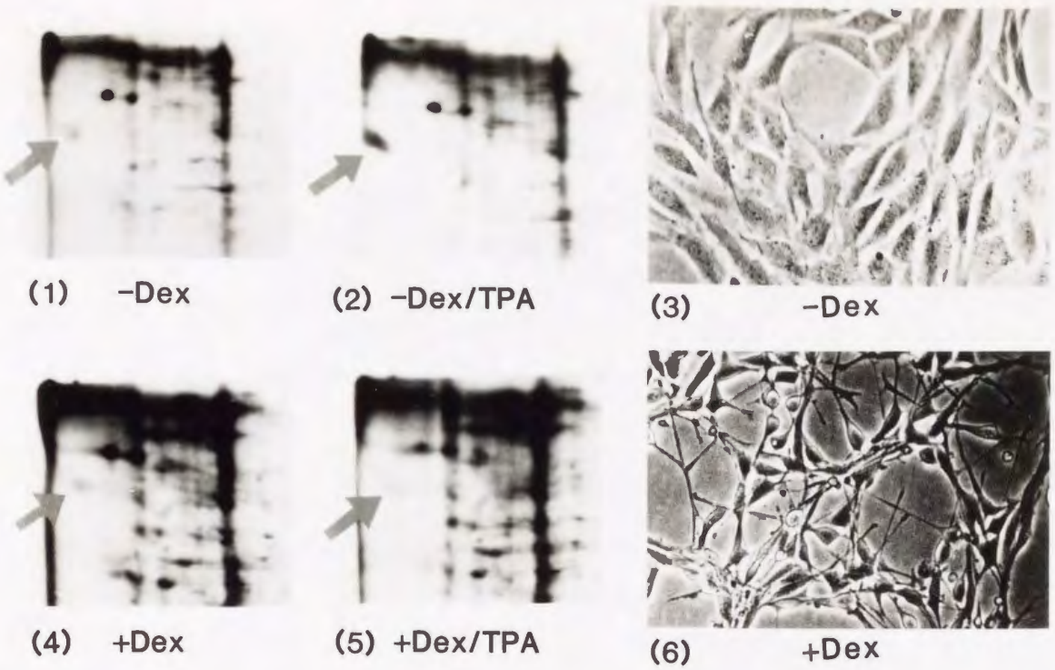
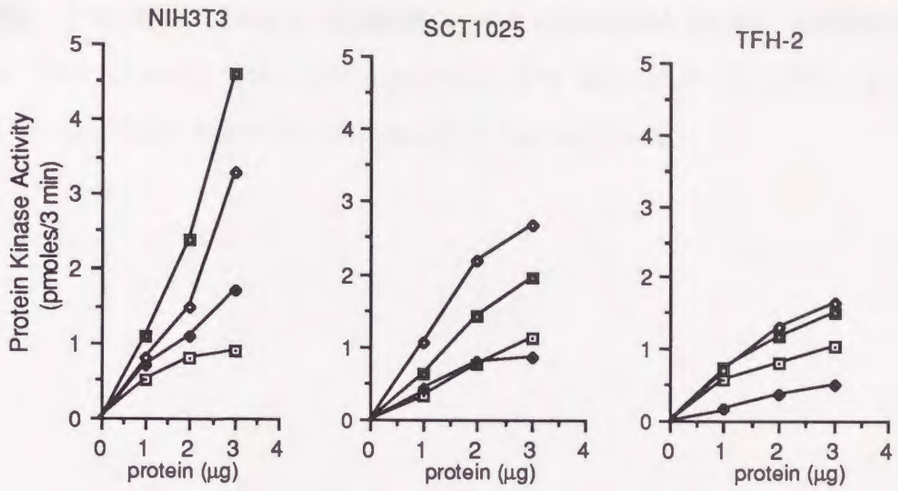


Fig. 11. Effect of TPA on the phosphorylation of the 80K protein in an inducible transformant. The TF-MDSS3-2-2 cell line was grown in DMEM containing 3% FCS, without (1-3) or with (4-6) 2μM dexamethasone (Dex) for 2 days, and processed 5 min after adding 200nM TPA (2 and 5), or with no addition. Labeling of cells and 2D gel electrophoresis were performed as described in "MATERIALS AND METHODS". The morphology of this cell line without and with 2μM dexamethasone is shown in (3) and (6), respectively.

A



B

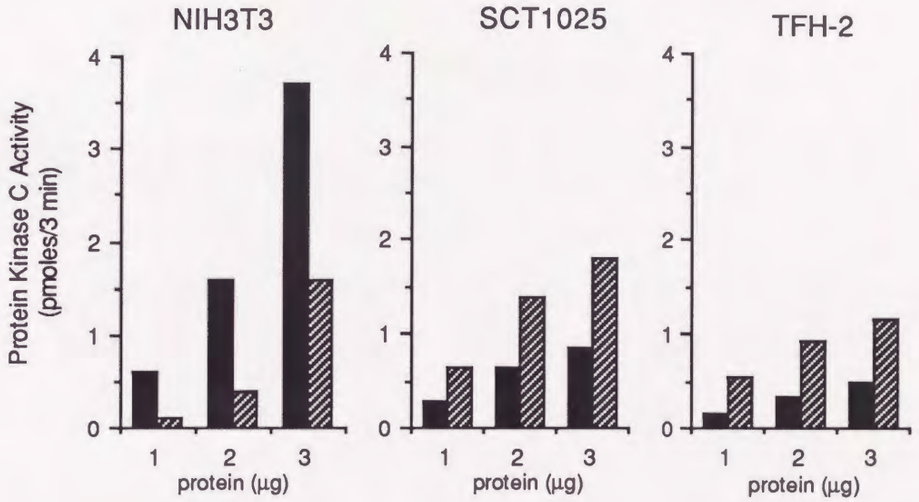


Fig. 12. Protein kinase activity (A) and protein kinase C activity (B) in normal and transformed NIH3T3 cells. Cytosol and membrane fractions were prepared from normal, H-ras or raf transformed NIH3T3 cells, and the protein kinase activity was assayed by measuring the incorporation of ^{32}P into histone from [γ-^{32}P]ATP as described in MATERIALS AND METHODS. The protein kinase activity of cytosol (square) and membrane (diamond) fraction in

the presence (■ and ◇) or absence (⊡ and ◆)of both calcium and TPA was shown in A. Protein kinase C activity of cytosol (▬) or membrane (▨) was calculated as the difference between the activity in the presence and absence of TPA plus calcium in B. Each fraction was assayed in duplicate.

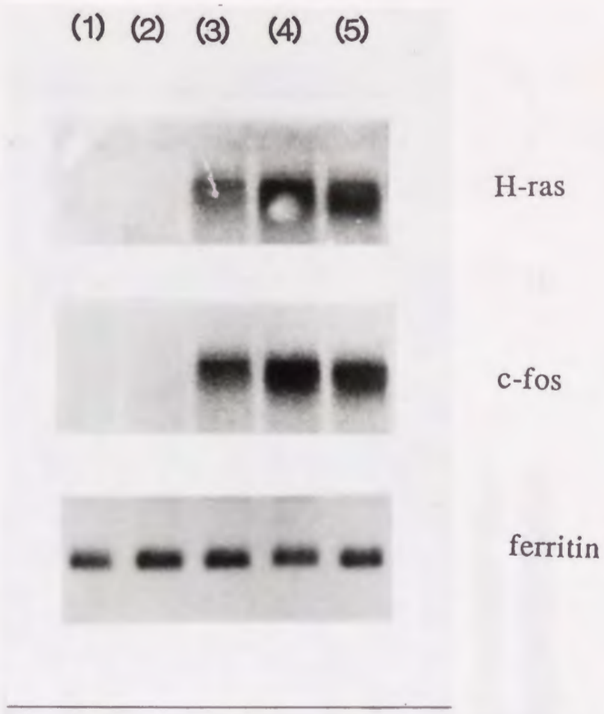


Fig. 13. Analysis of mRNA in early stages of cellular transformation. Twenty μg of total RNA isolated from TF-MDSS3-2-2 cells at 0h (lane 2), 1h (lane 3), 2h (lane 4) and 3h (lane 5) after exposure with 0.5μM dexamethasone and 20μg of total RNA from normal NIH3T3 exposed with dexamethasone for 3h (lane 1) were duplicatively electrophoresed, transferred to nitrocellulose filter and baked at 80°C for 2h. One filter was hybridized with 32P-labeled human H-ras cDNA probe, RC-1, and the other filter was hybridized with 32P-labeled v-*fos* probe, p-v-*fos*. The former filter was washed with 50% formamide / 10mM phosphate buffer(pH7.4) at 70°C for 1 h, and re-hybridized with human ferritin as an internal marker. H-*ras*, *fos* and ferritin probes have hybridized with transcripts of about 5.8kb, 2.2kb and 1.1kb, respectively.

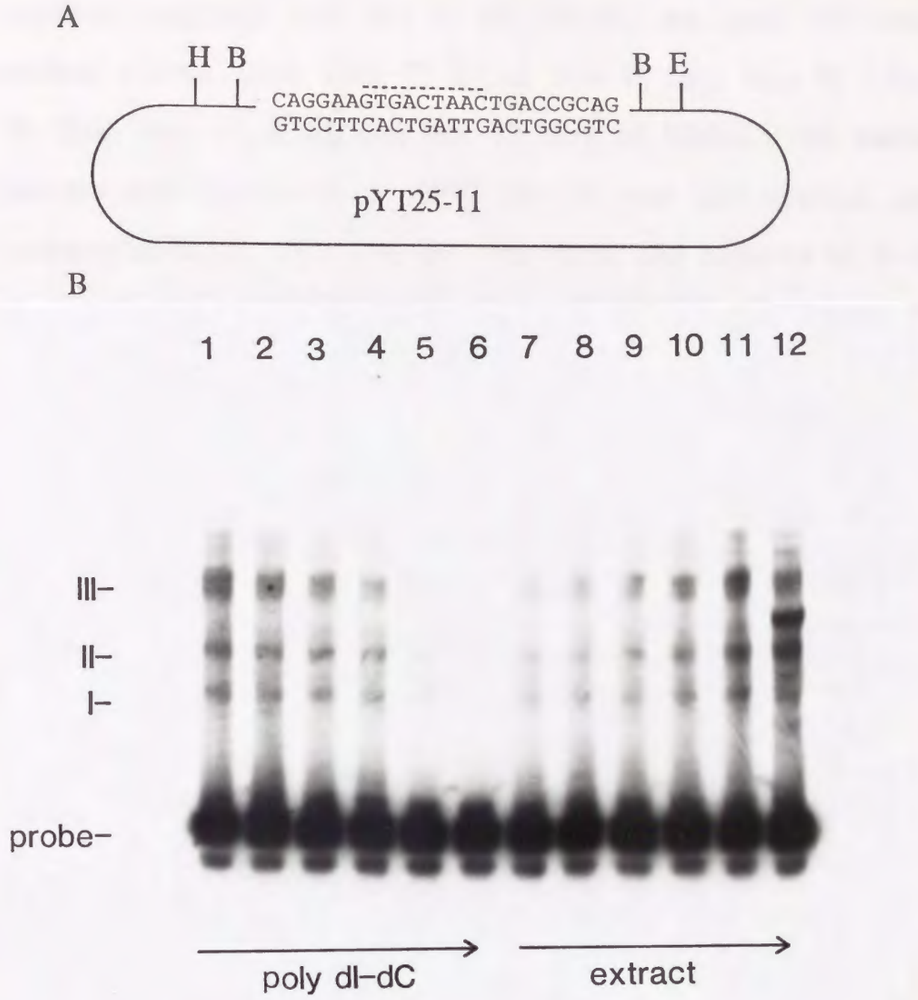


Fig. 14. Determination of conditions in mobility shift assay.

A. polyomavirus enhancer A element used as probe. -----; AP1 binding site. E; *Eco*RI, B; *Bam*HI, H; *Hind*III. The 73 bp *Eco*RI-*Hind*III fragment containing this enhancer A element was labeled with ^{32}P, purified by 20% polyacrylamide gel and used for mobility shift assay. B. Determination of the proper amounts of poly(dI-dC) and nuclear extract. In lanes 1-6, 55pg of enhancer fragment (10,000cpm) and 2μg of NIH3T3 cell nuclear extract are used. The amount of poly(dI-dC) used; lane 1; 0μg, lane 2;1μg, lane 3; 2μg, lane 4; 3μg, lane 5; 4μg and lane 6; 5μg. In lanes 7-12, 55pg of

enhancer fragment and 2μg of poly(dI-dC) are used. The amount of nuclear extract used; lane 7; 0.5μg, lane 8; 1μg, lane 9; 1.5μg, lane 10; 2μg, lane 11; 2.5μg and lane 12; 3μg of NIH3T3 cell extract. The mixture was incubated at 25°C for 30 min and applied upon 4% polyacrylamide at 4°C. The gel was dried and exposed to X-ray film as described in MATERIALS AND METHODS. Three major bands were named I, II and III.

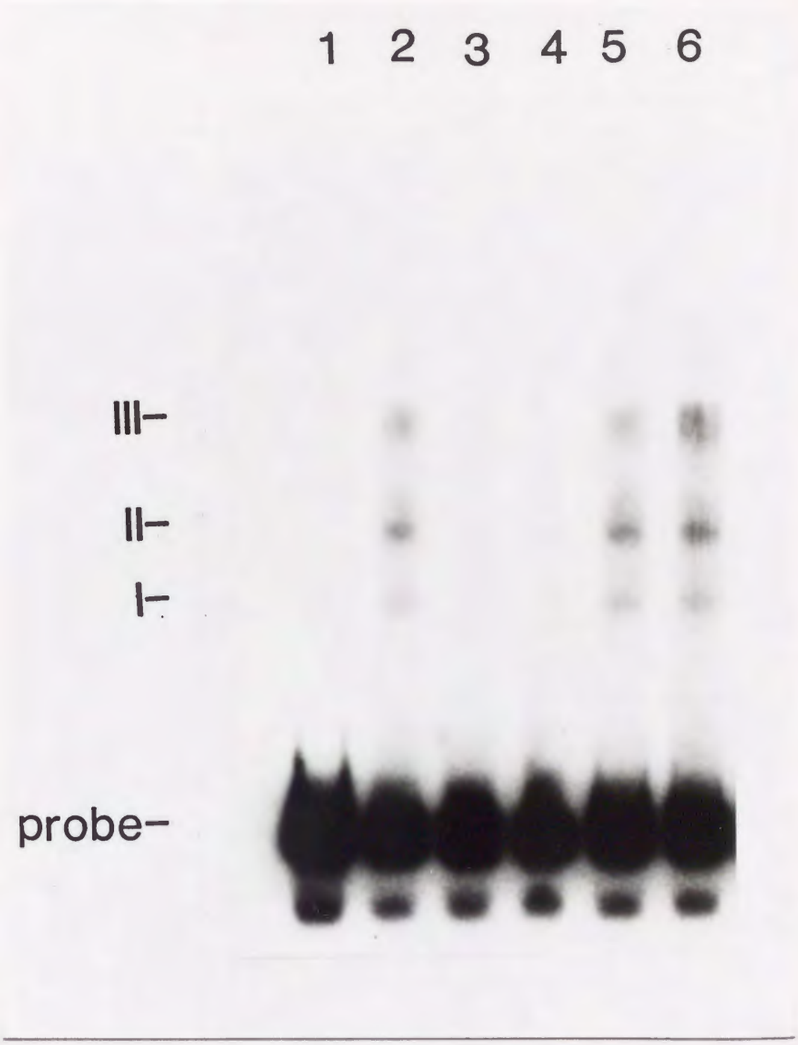


Fig. 15. Competition assay by non-labeled enhancer A element and non-specific fragments. For all lanes, the reactions contain 55pg of ^{32}P-labeled enhancer A element, 3μg of NIH3T3 nuclear extract and 4μg of poly(dI-dC) except that no extract was added for lane 1. Lane 2; no competitor, lane 3; 6ng of non-labeled enhancer A element, lane 4;12ng of non-labeled enhancer A element, lane 5; 6ng of *Alu*I-digested pUC18 fragments, lane 6; 12ng of *Alu*I-digested pUC18 fragments.

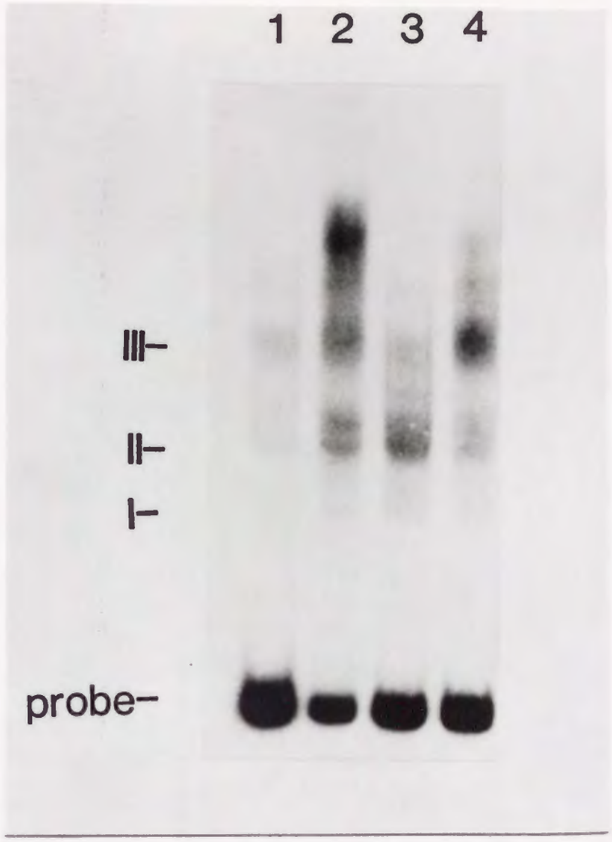


Fig. 16. Analysis of enhancer binding proteins in oncogene-transformed NIH3T3 cells. For all lanes, the reactions contain 55pg of ^{32}p-labeled enhancer A element, 4μg of poly(dI-dC) and 10μg of nuclear extract. Lane 1; NIH3T3 nuclear extract, lane 2; TFN-75 (N-*ras*) nuclear extract, lane 3; TFH-2 (H-*ras*) nuclear extract, lane 4; SCT1025 (*raf*) nuclear extract.

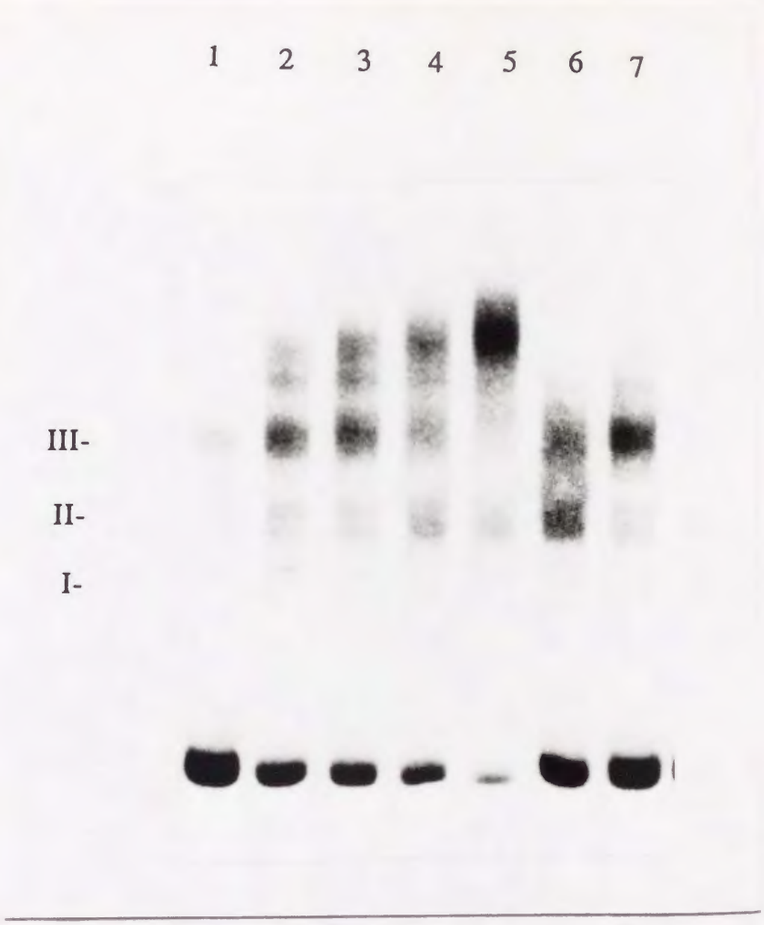


Fig. 17. Changes of enhancer binding proteins in the process of transformation. Nuclear extracts were prepared from TF-MDSS3-2-2 cells at 0h, 6h, 2 days and 4 days after exposure with 0.5μM dexamethasone and assayed. Lane 1; NIH3T3 nuclear extract, lane 2; TF-MDSS (0 h) nuclear extract, lane 3; TF-MDSS (6 h) nuclear extract, lane 4; TF-MDSS (2 days) nuclear extract, lane 5; TF-MDSS (4 days) nuclear extract, lane 6; TFH-2 nuclear extract, lane 7; SCT-1025 nuclear extract. The other components are the same as described in Fig. 16.

Table1. Protein kinase C activity in normal and transformed NIH3T3 cells

Cell line	Cytosol (C)			Membrane (M)			Total (C)+(M)
	PKC activity (pmoles/μg /3min)	Total protein (μg)	Total activity (pmoles/10^8cells /3min)	PKC activity (pmoles/μg /3min)	Total protein (μg)	Total activity (pmoles/10^8cells /3min)	Total activity (pmoles /10^8cells/3min)
NIH3T3	0.62±0.09	475	295	0.12±0.05	672	81	376
SCT1025	0.20±0.07	323	65	0.71±0.02	422	300	365
TFH-2	0.22±0.06	1577	347	0.60±0.09	1574	944	1291

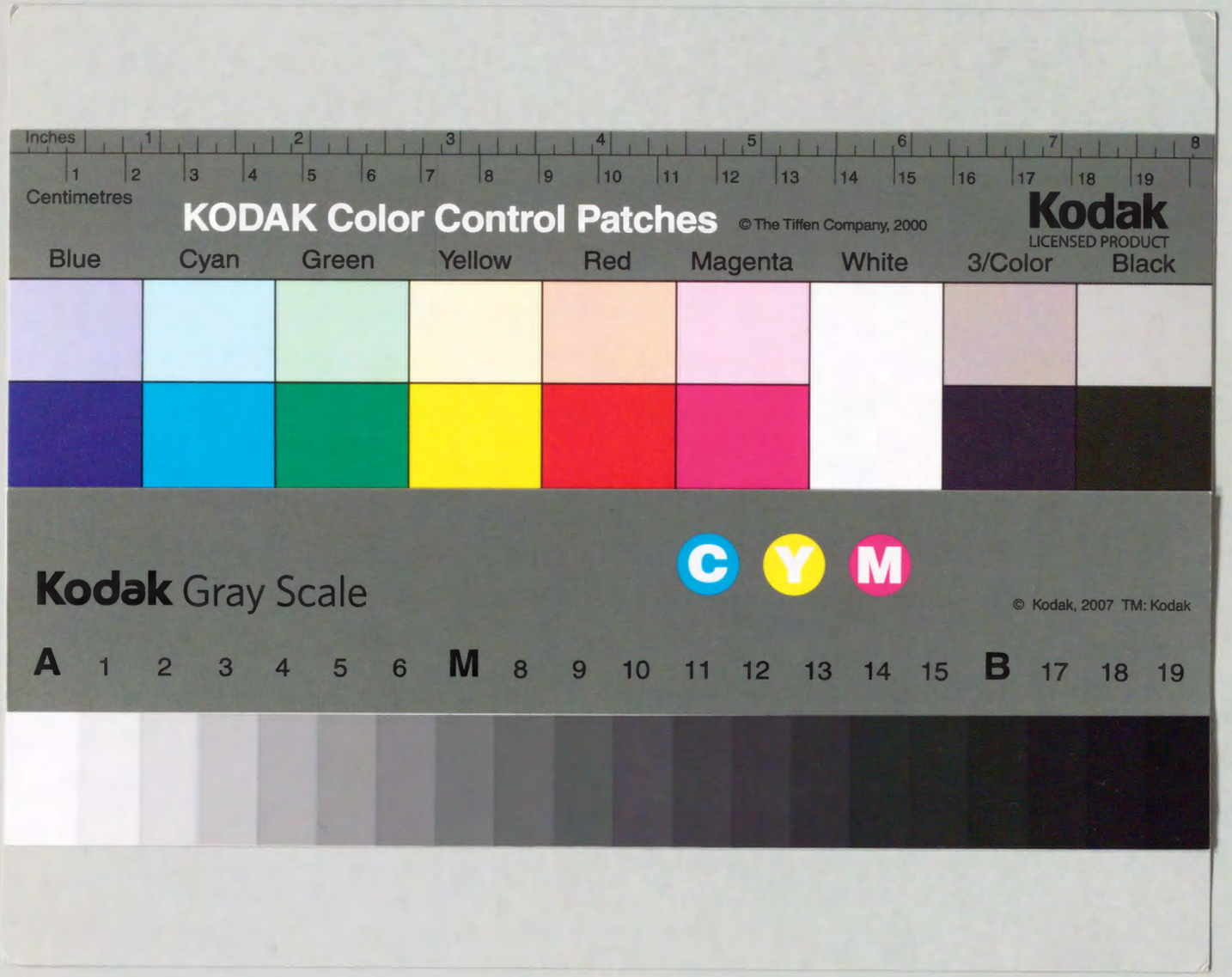
Protein kinase C activity was re-assayed using one μg of cytosol or membrane prepared from normal, H-*ras* and *raf* transformed NIH3T3 cells as described in Fig. 12 and MATERIALS AND METHODS. Each fraction was assayed in duplicate. PKC; protein kinase C.

Table 2. Effects of inhibition of protein kinase C on cell growth and on anchorage-independent growth.

Treated cells	Cell growth (x 10^6 cells)	Anchorage-independent growth
None - Dex	1.46	-
None + Dex	2.63	+++
H7 - Dex	2.00	-
H7 + Dex	2.18	++
TPA - Dex	2.67	+ *
TPA + Dex	3.04	++

For cell growth assay, 1 X 10^6 cells of TF-MDSS3-2-2 were pretreated with or without chemicals (50μM H-7 or 200nM TPA) in DMEM supplemented with 3% FCS for 24 h, and cultured with or without 2μM dexamethasone for another 24 h and the cell numbers were counted. For anchorage-independent growth assay, 3 X 10^4 cells were pretreated with or without the chemicals (200nM H-7 or 50μM TPA) in 0.3% soft agar containing DMEM supplemented with 3% FCS for 2 days, and the colony size was measured 10 days after addition of 2μM dexamethasone. "None - Dex" or "None + Dex"; none-pretreated cells treated without or with dexamethasone. "H7 - Dex" or "H7 + Dex"; H-7 pretreated cells treated without or with dexamethasone. "TPA - Dex" or "TPA + Dex"; TPA pretreated cells treated without or with dexamethasone. Cell growth is shown as the cell number. Colony size is shown by the number of "+" in proportion to the magnitude as the largest colony is defined as

"+++". "-"; no colony. *; colony had stopped the growth at 20-30 cells. Each assay was done in duplicate. The same results were obtained in 25μM H-7 and 100nM TPA in place of 50μM H-7 and 200nM TPA, respectively.


Inches
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© The Tiffen Company, 2000
Kodak
LICENSED PRODUCT
Blue
Cyan
Green
Yellow
Red
Magenta
White
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Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19