Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 283-291

PROTEIN KINASE C mRNA LEVELS AND ACTIVITY IN RECONSTITUTED NORMAL HUMAN EPIDERMIS: RELATIONSHIPS TO CELL DIFFERENTIATION

Roberto Gherzi[1*], Bianca Sparatore[2], Mauro Patrone[2], Andrea Sciutto[3], and Paola Briata[3]

[1]Cellife Biotecnologie s.r.l., [2]Istituto di Chimica Biologica, Università di Genova, [3]Istituto Nazionale Ricerca sul Cancro, IST; Genova, Italy

Received February 27, 1992

Although keratinocytes are a major target of phorbol ester actions, the activity and the expression of the eight cloned protein kinase C (PKC) isoenzymes have not been studied in detail in human epidermis. Starting from normal human keratinocytes, we reconstituted in culture a mutilayered epithelial tissue which presents many hystological, biochemical, and molecular features of the authentic epidermis and we used it as a model to investigate the PKC activity and mRNA levels. We found that *i)* PKC activity is higher in differentiated than in non-differentiated cells; *ii)* the mRNA levels of PKCδ and -η/L, while are differently affected by spontaneous keratinocyte differentiation, are down-regulated during phorbol esters-induced cell differentiation. Our findings could represent a basis to investigate the involvement of PKC isoforms in the keratinocyte differentiation process.

The protein kinase C (PKC) is a phospholipid-dependent protein serine/threonine kinase actively involved in cell proliferation and differentiation which binds phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (1-3 for reviews). Molecular cloning analyses have revealed that PKC molecules consist of two major groups of eight enzymes having closely related structures (2, 3). Conventional PKC comprises four isoforms, PKCα, -βI, -βII, and -γ, which are characterized by the presence of the C2 calcium binding domain and by their requirement for Ca^{2+}, in addition to phospholipids and diacylglycerol, for activation (4,5). Recently cloned PKCδ, -ε, -ζ, and η/L isoforms (so called novel PKC) seem to be independent of Ca^{2+} for activation and lack the C2 domain (6-9). Within the two major groups, each enzyme presents peculiarities in term of either tissue distribution and requirement of activators and cofactors (2, 3).

Keratinocytes (KC), the prevalent cell type in epidermis, are a well known target for the cellular effects of phorbol esters (10). *In vivo*, phorbol esters induce simultaneous changes characteristic of both the differentiative and proliferative programs of mouse epidermis (11,12). In primary cultures of murine KC, it has been reported that phorbol esters induce a subpopulation of epidermal basal cells to undergo terminal differentiation as well as accelerate the proliferation of another cellular subset (13, 14). Several attempts to correlate PKC activity to keratinocyte differentiation did not give any consistent result (13-15). Moreover, some reports indicate that, in primary cultures of mouse KC, PKC levels and activity are discordant (16). The discovery of multiple PKC isoforms led to the idea that different

*To whom correspondence should be addressed at Istituto Nazionale Ricerca sul Cancro (IST), Viale Benedetto XV, 10, 16132 Genova, Italy.

0006-291X/92 $1.50

isoenzymes serve distinct functions through differences in activator and/or substrate specificity (3). The co-expression of multiple PKC isoenzymes in epidermis could also help to explain the different effects of phorbol esters on epidermis.

Normal human KC can be cultured to reconstitute a multilayered epithelium which displays many hystological, biochemical, and molecular aspects of the authentic epidermis (17-19) and which maintain virtually the same differentiation features and gene expression pattern of its authentic counterpart (18,19). Recently, we studied in detail the differentiation program of human epidermis reconstituted in culture and characterized the expression of the GLUT1 glucose transporter as a marker of cell differentiation (20).

Aims of the present study were to investigate the PKC activity and the mRNA levels of the eight different isoenzymes during keratinocyte differentiation using the reconstituted human epidermis as a model.

EXPERIMENTAL PROCEDURES

Reagents, plasmids and oligonucleotides. T4 polynucleotide kinase was from Boehringer Mannheim. A 1.9 kb EcoRI fragment of the rat brain GLUT1 cDNA (pGTH-14) and a 1.2 Kb PstI fragment of a β actin cDNA (pBAC) were prepared and used as described (20). PMA was from Fluka. All other reagents, of molecular biology grade, were obtained from sources listed in (20, 21). To derive isoform-specific oligonucleotides, the sequences of the eight PKC clones (from the EMBL data base) were analyzed utilizing μVAX Digital and Macintosh PowerBook 140 (with DNA Strider 1.1 software) computers. The sequences of oligonucleotides used are presented in Table I. Oligonucleotide probes were labeled with [γ-^{32}P]ATP to an activity of 1-2 x 10^9 cpm/μg with T4 polynucleotide kinase. Temperatures for hybridizations were calculated with the formula: [4(G + C) + 2(A+T)] x 20/number of nucleotides in the probe (23), and hybridizations were carried out as described (24). pGTH-14 and pBAC were labeled by random priming with hexanucleotide fragments using [α-^{32}P]-dCTP (25).

Cell cultures. Normal human KC, obtained from healthy volounteers, were cultured to form non-confluent colonies (NCC) or to reconstitute a multilayered tissue (multilayered epidermis: ME) according to the technique of Rehinwald and Green (17) essentially as previously described (20).

Flow Cytometric Analysis. Flow cytometric analysis of normal human keratinociyes, obtained from either NCC or from ME was performed exactly as previously described (20, 22).

RNA preparation and Northern blot analysis. Total RNA was prepared from NCC and ME by the guanidine/cesium chloride method (26). Polyadenylated RNA (poly(A)$^+$RNA) vas prepared according to Jakobsen et al. (27) using Dynabeads Oligo(dT)$_{25}$ (Dynal). Polyadenylated RNA (10 μg per lane) was denatured, electrophoresed and blotted to Hybond-N nylon membranes as reported (21). Filters were hybridized using radiolabeled pGTH-14, pBAC or oligonucleotide probes and washed as previously described (21,24). Blots were exposed to Hyperfilms at -70° C.

Table I. Oligonucleotides utilized as isoform-specific PKC probes

isoform	sequence	position	Ref.
PKCα	5'-TATGTTAGCGATGACCAGTTG-3'	[1924-1944]	(4)
PKCβI	5'-CAAGTTCATGATGAAGAGCTT-3'	[1936-1956]	(4)
PKCβII	5'-ATATTCCTGATGACTTCCTG-3'	[1933-1953]	(4)
PKCγ	5'-GGGCACGGGCACGGGGCTGCT-3'	[2065-2085]	(5)
PKCδ	5'-GTCCATAGAGTCGATGAGGTTCTT-3'	[2292-2315]	(6)
PKCε	5'-AGGGGTTTCCTGTTTCTTTAGCCC-3'	[848-871]	(6)
PKCζ	5'-TTGGGAAGGCATGACAGAATCCAT-3'	[250-273]	(6)
PKCη/L	5'-CACTTTGTTAATATTGTTTTGGCA-3'	[672-696]	(8)

The sequences (antiparallel to the cDNA sequences reported into square brakets) of the isoform -specific PKC oligonucleotides used to hybridize Northern blots are presented. In brakets are listed the references from which sequences were derived.

Identification of protein kinase C activity in KC. To remove all the residual feeder layer from the cultures without affecting keratinocyte colonies, NCC were treated with 0.05% Trypsin and 0.02% EDTA in Special Salt Solution (ICN) for 5 min at 37° C and the supernatants were discarted. This procedure was not neccessary for ME since confluent cultures are not contaminated by residual feeder layer. Then, cultures were washed twice in PBS and KC scraped from the plates, collected by centrifugation, and counted. 10^8 cells were suspended in 2 ml of 10 mM Hepes buffer, pH 7.4, containing 0.25 M sucrose, 10 mM 2-mercaptoethanol (2-ME), 1 mM EDTA, 100 μg/ml leupeptin, and 3 mM phenylmethylsulfonil-fluoride. The cells were lysed by sonication with 6 strokes for 10 sec each at 0° C, then centrifuged at 100,000 x g for 10 min. The protein kinase C activity was purified from the supernatant (referred to as the cytosolic extract) and from the pellet (particulate cell fraction) by chromatography onto a DEAE (DE-52) column as described (28). PKC activity was assayed using 100 μl of the eluted fractions as described (28). One unit of PKC activity was defined as the amount of the enzyme that causes the incorporation of 1 pmol of ^{32}P/min into Histone S-III under the assay conditions.

RESULTS AND DISCUSSION

Although epidermis is a major target of phorbol ester tumor promotion, the relatively few studies published on epidermal PKC have been performed exclusively in mouse skin bioptic specimens or in primary cultures of mouse KC (11-15). Recently, we reported that it is possible to obtain epidermal keratinocyte cultures at different stages of confluence and stratification, enriched in small, basal (stem-like) cells or in large and differentiated cells, respectively, without modifying the concentration of Ca^{2+} in the culture media (20). We also described the high expression level of the glucose transporter GLUT1 gene as a marker of non differentiated, basal, KC (20). In this study, we utilized non confluent secondary cultures (NCC) of normal human KC and multilayered reconstituted epidermis (ME) (obtained from the same cellular strain) as sources of non differentiated and differentiated cells, respectively. For some experiments, we treated NCC with PMA (10 and 100 ng/ml) for 16 h. As reported, PMA predominantly induces cell differentiation in our culture system (20). The average dimensional distribution and GLUT1 expression levels of cell cultures used for the experiments, assumed as a keratinocyte differentiation marker, are presented in Table II (see also ref. 20).

Table II. Dimensional distribution and GLUT1 protein expression of normal human keratinocytes in non-confluent colonies and in multilayered epithelia

	ME	NCC	NCC+PMA
size	percent of total keratinocytes		
small	27.5	67.3	42.0
medium	22.7	20.1	21.5
large	49.8	12.6	36.5
	αCT reactivity (% of NCC keratinocytes reactivity)		
	18.1	100	22.5

Keratinocytes obtained from non confluent colonies (NCC) or from multilayered epithelia (ME) were analyzed by flow cytometry as described under Experimental Procedures. Cells were arbitrarily grouped in small, medium, and large according to their dimension and side scatters as described in ref. 20. The reactivity of KC to the anti Glut-1 polyclonal antipeptide antibody αCT antibody was assayed as reported in ref 20 and it is indicated as the percentage of the αCT reactivity of KC obtained from NCC.

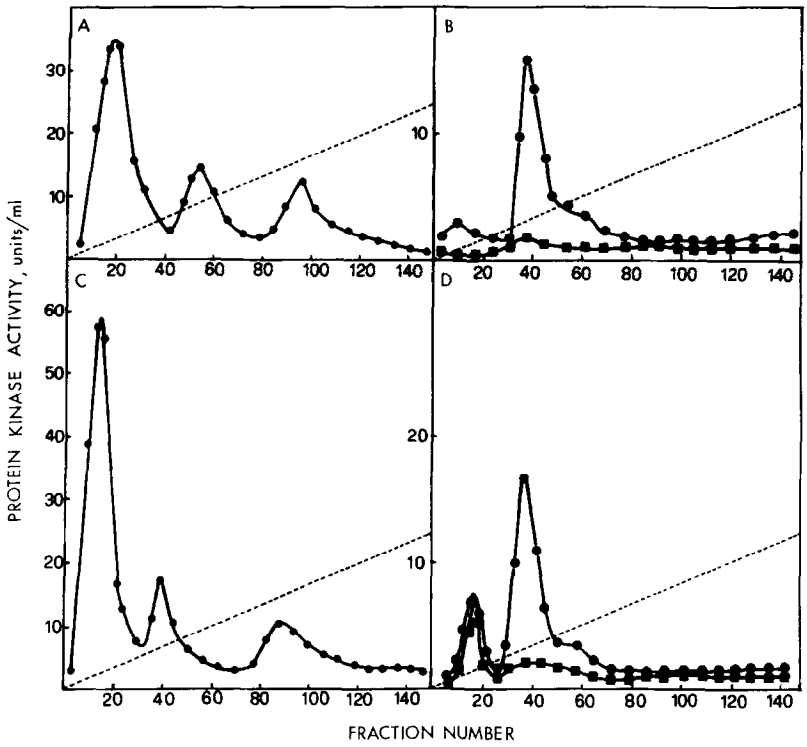

Figure 1. Separation of protein kinase activities by DEAE-chromatography.
Keratinocytes (10^8 cells) were collected from NCC (panels A and B) or ME (panels C and D) and the cytosolic (A and C) and particulate (B and D) cellular fractions were prepared as described under Experimental Procedures. Each sample was submitted to DEAE-chromatography and assayed for protein kinase C activity using 100 µl of the eluted fractions. The assay mixture contained all PKC effectors (i.e. 0.5 mM Ca^{2+}, 10 µg phosphatidylserine, 0.2 µg dioleoylglycerol,(●) or 0.5 mM Ca^{2+} alone (■). The dotted line represents the NaCl elution gradient from 0 to 0.6 M.

Table III. Protein kinase C activity in cytosolic and in particulate fractions of cultured normal human keratinocytes obtained from non-confluent colonies or multilayered epithelia

Culture conditions	PEAK	PROTEIN KINASE ACTIVITY (total units)			
		Ca^{2+}+lipids	EDTA	Ca^{2+}	EDTA+lipids
NCC	I	980	320	312	688
	II	400	400	343	421
	III	344	398	384	364
ME	I	1300	200	216	690
	II	325	258	269	356
	III	448	436	400	370

Each peak of protein kinase activity, obtained from DEAE chromatographies presented in Fig.1, was collected, concentrated to 2 ml by ultrafiltration and assayed (100 µl) in a standard assay mixture (see the legend to figure 1) or, alternatively, the PKC effectors (Ca^{2+} and lipids) were substituted with 1 mM EDTA, 0.5 mM Ca^{2+} alone, or 1 mM EDTA plus lipids.

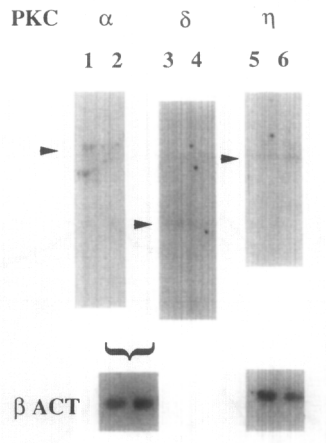

Figure 2. Northern blot analysis of poly(A)+ RNA from either non confluent colonies of keratinocytes or from multilayered epithelia.

Poly(A)+ RNA was prepared from either non confluent colonies of keratinocytes or from multilayered epithelia according to the procedures detailed under Experimental Procedures. 10 μg of poly(A)+ RNA were electrophoresed and transferred to Hybond-N membranes. Filters were hybridized with radiolabeled PKCα, -δ, and -η/L specific oligonucleotides, or with β actin radiolabeled cDNA probe (as indicated), washed and autoradiographed. Figure shows representative autoradiograms (exposed 6 days at -80°C for PKC oligonucleotides or 16 h at -80°C for β actin) of three performed. A single blot was hybridized with PKCα, PKCδ, and β actin probes while another one was hybridized with PKCη/L and β actin probes.

First, we measured PKC activity in cytosolic and particulate fractions of cultures enriched in non differentiated KC. We found that in the cytosolic fraction of normal human KC are present both a Ca^{2+}-dependent and a Ca^{2+}-independent PKC activity (Fig. 1A and Table III). The kinase activity, eluted in fractions 15-35 of chromatogram shown in Fig. 1A, is ~75% inhibited by 0.01 μM staurosporine, as previously reported for PKC activity purified from other sources (28). As shown in Fig. 1B, the PKC activity associated to the particulate fraction of keratinocytes (fractions 30-45 in Fig. 1B) accounts for the 17.2% of the total cellular activity. A comparable percentage of PKC activity has been found associated to the particulate fraction of other cell types (29,30).

Although the implication of PKC in the regulation of many cellular processes including growth, differentiation, and gene expression regulation has been proven (1, 3), the role of each PKC isoform in the regulation of different cellular functions is unknown. We investigated the mRNA levels of the different kinase isoenzymes in cultured KC by Northern blot analysis of poly(A)+ RNA using specific oligonucleotides deduced from the sequences of the eight PKC cDNA clones (Table I). We found that KC express three PKC: the α, the δ and the η/L isoenzymes (Fig. 2). Previous observations in rat epidermis reported the expression of PKCδ and PKCη/L (31,32). PKCα mRNA was found by some Authors (31) but not by Others (32) in cultured mouse KC and in mouse epidermis. In human KC, PKCδ mRNA levels are less abundant than either PKCη/L or PKCα mRNA (Fig. 2). We did not find any PKCβI, -βII, -γ, -ε, and ζ mRNA even loading up to 20 μg poly(A)+ RNA on agarose gels (data not shown).

As observed in many different cells (28,33), the treatment of NCC with PMA (100 ng/ml for 20 min) leads to a ~70% reduction of cytosolic PKC activity (fractions 5-30 in Fig. 3A) concomitant to a significant increase of the activity of a Ca^{2+}- and lipid- independent protein kinase (fractions 80-120 in Fig

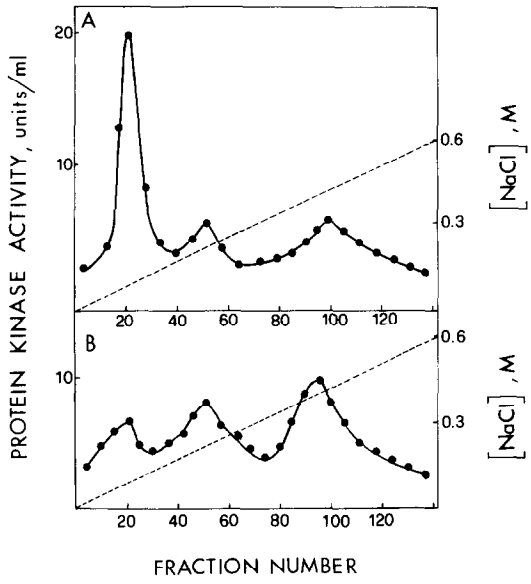

Figure 3. Effect of PMA on the levels of PKC activity.
Keratinocytes (6 x 10^7 cells) were collected fro NCC previously cultured for 20 min in the presence of 0.01% dimethylsulfoxide (DMSO, the solvent of PMA) (panel A) or in the presence of 100 ng/ml PMA. The cytosolic fractions were prepared and chromatographed on DEAE-chromatography as reported under Experimental Procedures. The eluted fractions (100 µl) were assayed for PKC activity in an assay mixture containing all PKC effectors (see legend to Fig. 1)

3B). This kinase activity could correspond to the so-called PKM, previously described in neutrophils (28) and other cell types (33) after cell activation by phorbol esters.

Recently, a formal evidence for an involvement of a PKC isoform, namely PKCβ, in the rate of differentiation of murine erythroleukemia cells has been provided (34). The induction of features of terminal differentiation by either PMA or synthetic diacylglycerols in KC (13,14) supported the hypothesis that PKC activation is involved in keratinocyte differentiation. Moreover, early differentiation of KC is accompained by an increase in total phorbol esters binding activity (15). We found that the total activity of cytosolic PKC is ~65% higher in differentiated KC than in non differentiated cells (compare panels A and C in Fig. 1 and see also Table III). On the contrary, the Ca^{2+}-independent PKC activity is similar in ME and in NCC (Table III). Finally, the PKC activity associated to the particulate fraction of KC was similar in NCC and ME (compare panels B and D in Fig. 1). Comparable results were obtained by Makowske et al. studying PKC compartimentation during HL60 cells differentiation (30). The peak of kinase activity, eluted from DE-52 column in fractions 10-25 (Fig. 1, panel D) and present exclusively in the particulate fraction of ME, is not inhibited by staurosporine and could correspond to a protein kinase different from PKC. Cell diffrentiation produces discordant effects on mRNA levels of the three PKC isoforms expressed in keratinocytes. In fact, we found that PKCα and PKCδ mRNA levels are reduced in ME while PKCη/L mRNA levels are slightly increased, when compared to NCC (Fig. 2). Superimposable results were obtained using cultures derived from three different keratinocyte strains (data not shown). Taking into account the opposite effects of keratinocyte differentiation on PKCδ and η/L mRNA levels, we investigated the effects of PMA-induced keratinocyte differentiation on the mRNA levels of these two Ca^{2+}-independent

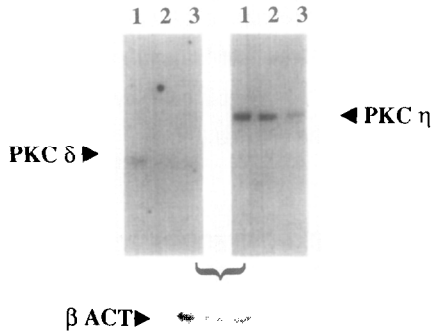

Figure 4. Effect of PMA treatment of non confluent keratinocyte colonies on PKCδ and PKCη/L mRNA levels.

Non confluent colonies of keratinocytes were treated with 10 ng/ml (lanes 2) or 100 ng/ml (lanes 3) PMA or with 0.01% DMSO for 16 h at 37°C. 10 μg of poly(A)+ RNA were electrophoresed and transferred to Hybond-N membranes. Filters were hybridized with radiolabeled PKCδ, and -η/L specific oligonucleotides, or with β actin radiolabeled cDNA probe (as indicated), washed and autoradiographed. Figure shows representative autoradiograms (exposed 6 days at -80°C for PKC oligonucleotides or 16 h at -80°C for β actin) of three performed. A single blot was hybridized with PKCδ, PKCη/L, and β actin probes.

isoenzymes. PMA treatment (100 ng/ml for 16 h) of NCC produces a concentration-dependent down-regulation of PKCδ, and η/L mRNA (Fig. 4). Also PKC activity is significantly reduced in NCC treated with PMA in comparison to control cultures (data not shown). A previous report indicated that mRNA levels of conventional PKC are not affected by prolonged treatment of cells with phorbol esters (35). To our knowledge, this is the first demonstration of an *in vivo* effect of phorbol esters on PKCδ and PKCη/L mRNA levels.

CONCLUSIONS

Data here reported indicate that either Ca^{2+}- dependent (α) or Ca^{2+}- independent (δ and η/L) PKC isoforms are expressed in normal human KC. Keratinocyte differentiation is accompained by a decrease in the mRNA levels of PKCα and PKCδ and by a slight increase in PKCη/L mRNA. A differential regulation of PKC isoenzymes has been observed in many cellular systems in response to various agents (36). Our findings further support the hypothesis that different isoenzymes may perform unique functions in the same cell.

The total PKC activity is ~65% higher in differentiated cultures in comparison to non-differentiated ones without any enzymathic redistribution between cytosolic and particulate fractions. Recent evidences obtained with several cell systems suggest that sustained activation of PKC is needed for long-term cellular responses such as proliferation and differentiation (37).

Although phorbol esters prevalently cause, in our culture system, keratinocyte differentiation (20), PMA induces modifications of both PKC activity and mRNA levels which are completely different from those observed in "spontaneous" KC differentiation in culture. Our results are reminiscent of the data obtained by Dotto et al. (38) studying c-fos and c-myc expression in mouse primary KC. Keratinocyte differentiation induced by Ca^{2+} or by phorbol esters was accompained by divergent changes in the mRNA levels of these two proto-oncogenes (38). This finding led to the hypothesis that Ca^{2+} and phorbol esters,

although inducing a similar differentiation program, elicit very different molecular effects on the primary KC cultures. Similarly, Aihara et al. observed that "natural" signals, such as diacylglycerol, differently from PMA, induce terminal differentiation of HL60 cells by causing a sustained activation of PKC without producing any down-regulation of the enzyme (39).

ACKNOWLEDGMENTS

We thank Prof. Edon Melloni for many helpful discussions and for reviewing the manuscript and Dr. Giovanni Melioli for assistance in cytofluorometric analysis. R.G. is grateful to Prof. Giorgio Corte for sharing many reagents and laboratory facilities. The work of R.G. has been supported by a grant from Consiglio Nazionale delle Ricerche, Comitato Nazionale Biologia Molecolare e Biotecnologie

REFERENCES

1. Nishizuka, Y. (1984) Nature *308*, 693-698.
2. Nishizuka, Y. (1986) Science *233*, 305-312.
3. Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1989) Annu. Rev. Biochem. *58*, 31-44.
4. Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., and Hidaka, H. (1987) Nature *325*, 161-166.
5. Ohno, S., Kawasaki, S., Konno, Y., Inagaki, M., Hidaka, H., and Suzuki, K. (1988) Biochemistry *27*, 2083-2087.
6. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1988) J. Biol. Chem. *263*, 6927-6932.
7. Ohno, S., Akita, Y., Konno, Y, Imajoh, S., and Suzuki, K. (1988) Cell *53*, 731-741.
8. Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T., and Ohno, S. (1990) J. Biol. Chem. *265*, 22434-22440.
9. Bacher, N., Zisman, Y., Berent, E., and Livneh, E. (1991) Mol. Cell. Biol. *11*, 126-133.
10. Fournier, A., and Murray, A.W. (1987) Nature *330*, 767-769.
11. Gupta, A.K., Fisher, G.J., Elder, J.T., Nickoloff, B.J., and Voorhees, J.J. (1988) J. Invest. Dermatol. *91*, 486-490.
12. Griffiths, R.J., Li, W.S., and Blackham, A. (1988) Agents Actions *25*, 344-350.
13. Yuspa, S.H., Ben, T., Hennings, H., and Lichti, U. (1982) Cancer Res. *42*, 2344-2349.
14. Isseroff, R.R., Stephens, L.E., and Gross, J.L. (1989) J. Cell. Physiol. *141*, 235-242.
15. Dunn, J.A., Jeng, A.Y., Yuspa, S.H., and Blumberg, P.M. (1985) Cancer Res. *45*, 5540-5546.
16. Snoek, G.T., Boonstra, J., Ponec, M., and De Laat, S.W. (1987) Exp. Cell Res. *172*, 146-157.
17. Rheinwald, J.G., and Green, H. (1975) Cell *6*, 331-344.
18. Compton, C.C., Gill, J.M., Bradford, P.A., Regauer, S., Gallico, G.G., and O'Connor, N.E. (1989) Lab. Invest. *60*, 600-612.
19. Green, H. (1980) Harvey Lect. *74*, 101-139.
20. Gherzi, R., Melioli, G., DeLuca, M., D'Agostino, A., Distefano, G.F., Guastella, M., D'Anna, F., Franzi, A.T., and Cancedda, R. (1992) J. Cell. Physiol., in press.
21. Briata, P., Briata, L., and Gherzi, R. (1990) Biochem. Biophys. Res. Commun. *169*, 397-405.
22. Gherzi, R., Melioli, G., DeLuca, M., D'Agostino, A., Guastella, M., Traverso, C.E., D'Anna, F., Franzi, A.T., and Cancedda, R. (1991) Exp. Cell Res. *195*, 230-236.
23. Wallace, R.B., Johnson, M.J., Suggs, S.V., Miyoshi, K., Bhatt, R., and Itakura, K. (1981) Gene *16*, 21-28.
24. Briata, P., Radka, S.F., Sartoris, S., and Lee, J.S. (1989) Proc. Natl. Acad. Sci. U.S.A. *86*, 1003-1007.
25. Feinberg, A.P., and Vogelstein, B. (1983) Anal. Biochem. *132*, 6-13.
26. Chirgwin, J.M., Przybyla, A.E., McDonald, R.J., and Rutter, W.J. (1979) Biochemestry *24*, 5294-5299.
27. Jakobsen, K.S., Breivold, E., and Hornes, E. (1990) Nucleic Acids Research *18*, 3669.
28. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F., and Horecker, B.L. (1985) Proc. Natl. Acad. Sci. U.S.A. *82*, 6435-6439.
29. Chambers, T.C., Chalikonda, I., and Eilon, G. (1990) Biochem. Biophys. Res. Commun. *169*, 253-259.
30. Makowske, M., Ballester, R., Cayre, Y., and Rosen, O.M. (1988) J. Biol. Chem. *263*, 3402-3410.
31. Dlugosz, A.A., Knopf, J.L., and Yuspa, S.H. (1991) J. Invest. Dermatol. *96*, 566 (abstract).
32. Koyama, Y., Hachiya, T., Hagiwara, M., Kobayashi, M., Ohashi, K., Hoshino, T., Hidaka, H., and Marunouchi, T. (1990) J. Invest. Dermatol. *94*, 677-680.

33. Kishimoto, A., Kajikawa, N., Shiota, M., and Nishizuka, Y. (1983) J. Biol. Chem. *258*, 1156-1164.
34. Melloni, E., Pontremoli, S., Sparatore, B., Patrone, M., Grossi, F., Marks, P.A., and Rifkind, R.A. (1990) Proc. Natl. Acad. Sci. U.S.A. *87*, 4417-4420.
35. Young, S., Parker, P.J., Ullrich, A., and Stabel, S. (1987) Biochem. J. *244*, 775-779.
36. Kiley, S.C., Parker, P.J., Fabbro, D., and Jaken, S. (1991) J. Biol. Chem. *266*, 23761-23768.
37. William, F., Wagner, F., Karin, M., and Kraft, A.S. (1990) J. Biol. Chem. *265*, 18166-18171.
38. Dotto, G.P., Gilman, M.Z., Maruyama, M., and Weinberg, R.A. (1986) EMBO J. *5*, 2853-2857.
39. Aihara, H., Asaoka, Y., Yoshida, K., and Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. *88*, 11062-11066.