CELL STRUCTURE AND FUNCTION 17: 151–155 (1992)

Two Phosphorylation Reactions Induced by Murine Beta Interferon in BALB/c-3T3 Cells

Shin-ichi Tominaga

Department of Biochemistry II, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan

Key words: interferon/cell proliferation/phosphorylation/adenylate kinase

ABSTRACT. **Treatment with 1000 units/ml of murine beta-interferon enhanced an adenylate kinaselike activity and markedly increased the level of L-alpha-phosphatidyl inositol 4-monophosphate in quiescent BALB/c-3T3 cells. The addition of platelet-derived growth factor (22 units/ml) or poly(I).poly(C) (0.3–1 microgram/ml) to the phosphorylation reaction mixture did not alter this interferon action.**

The antiviral effect of IFN had been explained by the activities of IFN-induced enzymes, such as 2′-5′ oligoadenylate synthetase(s) and a double-stranded RNA-dependent protein kinase (1). On the other hand, the mechanism(s) of the antiproliferative effect of IFN remains obscure. The inhibitory effect of murine β-IFN on PDGF-induced proliferation of BALB/c-3T3 cells has been reported previously (2). To elucidate the mechanism of IFN-action, the phosphorylation reaction, using [γ-^{32}P]ATP as a substrate, of IFN-treated cells with that of untreated BALB/c-3T3 cells was compared. The results indicated the presence of two distinct phosphorylation reactions induced by the treatment with β-IFN.

MATERIALS AND METHODS

Materials. The cell culture media, DME and calf serum were obtained from Gibco. Samples of [γ-^{32}P]ATP (specific activity, 6,500 Ci/mmol), and [2-^{3}H]ADP (specific activity, 28 Ci/mmol) were from Amersham. AMP, ADP, ATP, PI, DPI, TPI, pyruvate kinase, PEP, aprotinin, and PMSF were purchased from Sigma. Poly(I).poly(C), nuclease P1, and phosphodiesterase 1 (snake venom) were from P.L. Biochemicals. Polyethyleneimine (PEI) thin layer plates were from Macherey Nagel (Germany). Silica gel 60 plates were from Merck. Bacterial alkaline phosphatase was from Worthington. BALB/c-3T3 (clone A31) cells and partially purified PDGF (4,500 units/ml, 800 ng/ml) were provided by Dr. C. Stiles (Harvard Medical School) (3). Platelet poor plasma (4) and pure murine β-IFN (specific activity, 5×10^8 units/mg protein) (5) were prepared according to published procedures.

Cell fractionation. The cells were treated with β-IFN and PDGF as previously described (2). In short, after the treatment with 1,000 units/ml of murine β-IFN for 48 hours in DME plus 5% platelet-poor plasma, quiescent BALB/c-3T3 cells in a 150 cm^2 tissue culture flask were further cultured in the presence or absence of PDGF (22 units/ml) for 6 hours, and then harvested using a rubber policeman. After centrifugation, the packed cells were added with 0.1 ml of 10 mM Tris-HCl (pH 7.5 at 10°C), 0.15 M NaCl, 1 mM magnesium chloride, 10 μg/ml aprotinin, 10 μM PMSF, and 0.075% (w/v) Triton X-100, and kept on ice for 10 min. After centrifugation at 10,000 rpm for 10 min at 4°C by a Sorvall SS 34 rotor with adaptors for microfuge tubes, the supernatant was collected (T fraction). The resultant pellet was added with 0.1 ml of lysis buffer, which contained the same components as above except for 1% (w/v) NP-40 instead of 0.075% (w/v) Triton X-100, mixed vigorously, kept on ice for 10 min, and then centrifuged. The supernatant was collected (N fraction), and then the pellet was subjected to other extraction procedures overnight at 4°C, followed by collection of the supernatant after centrifugation (O fraction).

Phosphorylation reaction. Each 30-μl reaction mixture contained 3 μl of the sample, 30 mM Tri-HCl (pH 7.5 at 10°C), 0.12 M KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 0.1 mM ATP and 25 μCi of [γ-^{32}P]ATP (6). Each tube was kept at 30°C for 5 min, and then added with 30 μl of the sample buffer for SDS-polyacrylamide gel electrophoresis, which contained 0.1 M dithiothreitol instead of 2-mercaptoethanol (7), and then immediately heated at 100°C for 5 min to terminate the reactions.

The abbreviations used are: DME, Dulbecco's modified Eagle's medium; IFN, interferon; PDGF, platelet-derived growth factor; PI, L-alpha-phosphatidyl inositol; DPI, L-alpha-phosphatidyl inositol 4-monophosphate; TPI, L-alpha-phosphatidyl inositol 4,5-diphosphate; PEP, phosphoenolpyruvate; PMSF, phenylmethyl sulfonyl fluoride; NP-40, Nonidet P-40; PEI, polyethyleneimine; poly(I).poly(C), poly(riboinosinic).poly(ribocytidylic) acid; Tris, tris(hydroxymethyl)aminomethane.

PEI thin layer chromatography. A 2-μl aliquot of the (heat-denatured) sample was spotted on a PEI thin layer plate, together with 10 nmol each of the unlabeled marker nucleotides. The thin layer plate was washed once with 100% methanol for 10 min, dried, and then developed with either 0.75 M potassium phosphate buffer (pH 3.5) or 1 M LiCl. The plate was processed for autoradiography.

Assay for adenylate kinase activity. The composition of the reaction mixture was the same as that for the phosphorylation reaction, except for the substitution of either 2.3 μM [2-^{3}H]ADP (28 Ci/mmol) or 102 μM [2-^{3}H]ADP (0.65 Ci /mmol) for [γ-^{32}P]ATP. Each sample was heat denatured and then spotted on a PEI thin layer plate, which was developed with 0.75 M potassium phosphate buffer (pH 3.5) and processed for fluorography (8).

Phospholipid analysis. The phosphorylation reaction was carried out for 5 min at 30°C; thereafter, 50 μl of the reaction mixture was added with 150 μl of chloroform/methanol, 1 : 2 (v/v), and 50 μl of 35% (w/v) HCl, and immediately mixed. To the upper layer, 50 μl of chloroform was added, mixed well, and this chloroform layer was combined with the original lower layer. The pooled extracts were washed three times with 200 μl of methanol/1 M HCl, 1 : 1 (v/v), evaporated under a stream of nitrogen, and then resuspended in methanol /chloroform/water, 20 : 9 : 1 (v/v). High-performance thin layer chromatography was performed as described (9), using two different solvent systems: chloroform/methanol/4 M NH_4OH, 9 : 7 : 2 (v/v) and 1-propanol/4 M NH_4OH, 13 : 7 (v/v) (10).

RESULTS AND DISCUSSION

Quiescent BALB/c-3T3 cells were first treated with β-IFN for 48 hours, then stimulated to proliferate by the addition of PDGF, and harvested at 6 hours after stimulation, as described in Materials and Methods. Since it was rather difficult to obtain strict subcellular fractionation of cultured fibroblasts by the methods used for organs such as the liver, a subfractionation method using detergents was employed in these experiments. The T fraction contained the crude cytoplasmic components (11), and the N fraction corresponded to crude membrane and nuclear materials (12). To extract firmly attached nuclear materials, the O fraction was collected.

Phosphorylation reactions of the subfractionated lysates were carried out as described in Materials and Methods, and then the resultant samples were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 1. Radioactive spots just beyond the bromophenol blue dye front were detected by short exposure. Those spots were clearly different from the front line which consisted of [γ-^{32}P]ATP. The intensities of the spots of the N fraction from IFN-treated cells were stronger than those of the untreated cells. The differences ranged from 1.9- to 2.5-fold in 6 experiments, as judged by densitometry. Treatment with PDGF (22 units/ml) for 6 hours before harvesting the cells or the addition of 0.3–1 μg/ml of poly(I).poly(C) into the reaction mixture did not alter the intensity of the spots. The difference between the IFN-treated cells and the untreated ones was noted only in the N fraction but not in the T and O fractions. Furthermore, the reaction mixture containing both T and N fractions showed only an additive effect (data not shown).

Since these spots may correspond to low molecular weight compounds, 2-μl aliquots of the heat-denatured samples in the SDS sample buffer were directly loaded on a PEI thin layer plate, developed and analyzed by autoradiography, as described in Materials and Methods.

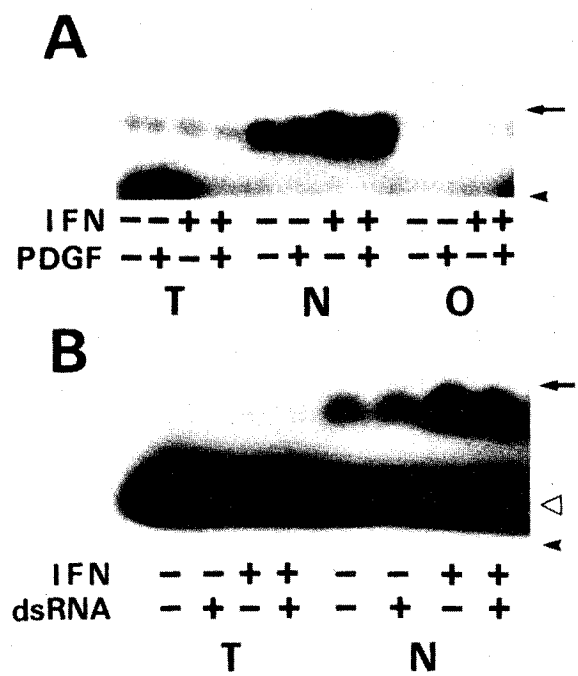

Fig. 1. Effect of IFN on the production of phosphorylated substance(s) that appeared near the front of the SDS-polyacrylamide gel. *Panel A.* Subfractionated cell lysates (T, N, and O fractions) were prepared from BALB/c-3T3 cells, untreated or treated with PDGF (22 units/ml) in the presence or absence of IFN (1000 units/ml), as described in Materials and Methods. For each fraction, the phosphorylation reaction was performed using [γ-^{32}P]ATP as a substrate, as described in Materials and Methods. Heat-denatured samples in SDS sample buffer were fractionated by gel electrophoresis in 10 to 20% linear gradient SDS-polyacrylamide gels and visualized by autoradiography (2).

The arrow indicates the position of bromophenol blue which has been included in the sample buffer. The arrowhead indicates the margin of the gel.

Panel B. The phosphorylation reaction was performed in either the presence or absence of 0.3 μg/ml of poly(I).poly(C) (indicated as dsRNA in the figure).

Arrow and arrowhead are as described in *Panel A*. Open triangle indicates the position of [γ-^{32}P]ATP.

The N fraction from the IFN-treated cells showed a dark spot which migrated to the position of authentic ADP in two different solvent systems (Fig. 2). The difference in the intensity of the spots was at least 56-fold by densitometry. Again, this phenomenon was unaffected by either the treatment with PDGF or the addition of poly(I).poly(C) (data not shown).

For further identification, the material on the PEI plate was eluted and analyzed, as shown in Fig. 3. Bacterial alkaline phosphatase cleaved the labeled phosphate completely, indicating that the phosphate was linked at the 5′ or 3′ free end. Nuclease P1 treatment did not alter the mobility of the material, showing that there was no 3′ linkage of the labeled phosphate. Most significantly, addition of PEP and pyruvate kinase completely transferred the spot from the position of ADP to that of ATP. Therefore, the spot likely corresponded to ADP whose labeled phosphate was transferred from [γ-^{32}P]ATP. Because such a reaction can be explained by adenylate kinaselike activity, the reverse reaction using [2-^{3}H]ADP was studied, as shown in Fig. 4. At two different substrate concentrations (2.3 μM and 102 μM ADP), the N fraction from IFN-treated cells produced more ATP and AMP than that from the untreated cells. The difference of the intensity in the area of ATP was 8.3-fold in the case of the low substrate concentration (2.3 μM) and 2.0-fold in the case of the high substrate concentration (102 μM), as judged by densitometry of the fluorogram. This result suggested that adenylate kinaselike activity was induced by the treatment with β-IFN. Although the functional importance of this phenomenon must await further study, a report describing the increase of Okazaki fragments in IFN-treated cells (13), suggesting the enhanced turnover of nucleotides, is intriguing in relation to this phenomenon.

However, questions still remain about whether or not the spot originally found in the SDS-polyacrylamide gel was ADP, since the position of the spot was so distinct from that of [γ-^{32}P]ATP (Fig. 1). Therefore, the region of the polyacrylamide gel corresponding to the spot was cut out, macerated in triethylamine bicarbonate buffer (pH 9 at 20°C), passed through a disposable syringe

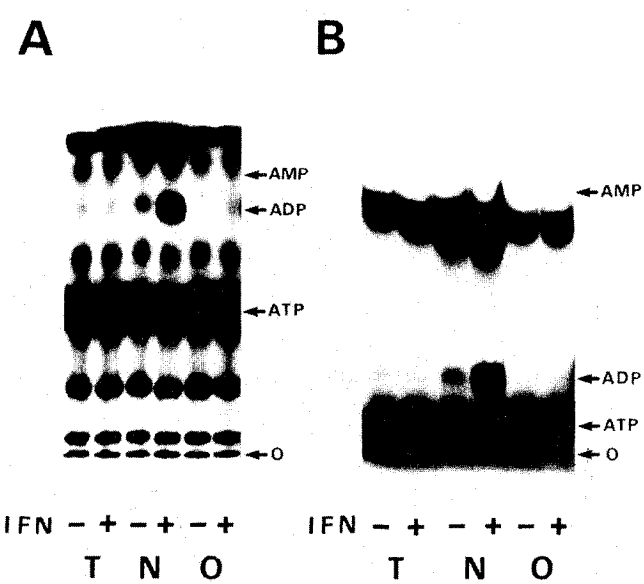

Fig. 2. PEI thin layer chromatography of phosphorylated materials.
The heat-denatured reaction mixture containing subfractionated cell lysates, T, N, and O fractions, in SDS sample buffer were analyzed by PEI thin layer chromatography and subsequent autoradiography, as described in Materials and Methods. Development was carried out either with 0.75 M potassium phosphate buffer (pH 3.5) (*Panel A*) or 1 M LiCl (*Panel B*). O indicates the origin of the chromatogram. Other arrows indicate the positions of authentic marker nucleotides.

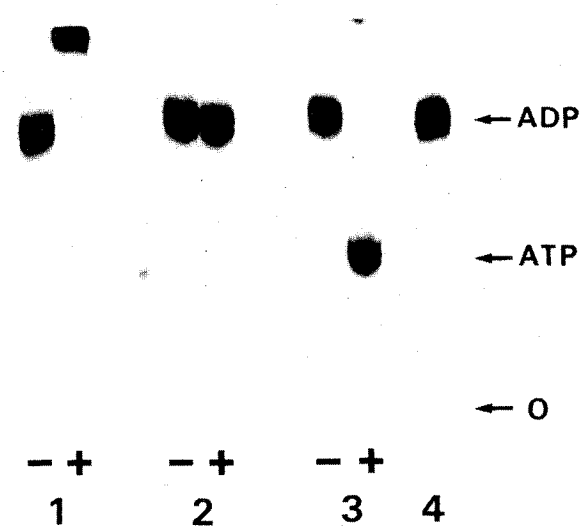

Fig. 3. Properties of the phosphorylated material induced by IFN.
The region of the thin layer plate corresponding to the labeled material in the lane of the IFN-treated N fraction in Fig. 2 was cut out, and then the material was eluted with triethylamine bicarbonate buffer (pH 9 at 20°C), evaporated and resuspended in water.
The eluted material was analyzed by three enzymatic reactions.
Lane 1: Ten μl of the reaction mixture containing 20 mM Tris-HCl (pH 8 at 20°C) either without (−) or with 1.4 μg/ml bacterial alkaline phosphatase (+) was incubated for 60 min at 37°C. After heating at 100°C for 5 min, 5 μl of each sample was analyzed by PEI thin layer chromatography as described in Materials and Methods.
Lane 2: Ten μl of the reaction mixture containing 20 mM sodium acetate buffer (pH 5.5 at 20°C) either without (−) or with 0.5 mg/ml nuclease P1 (+) was incubated as described above. Samples were analyzed as in *Lane 1*.
Lane 3: Ten μl of the reaction mixture containing 20 mM Tris-HCl (pH 8 at 20°C), 5 mM magnesium acetate, 10 mM KCl, 5 mM 2-mercaptoethanol either without (−) or with 2 mM PEP and 150 μg/ml pyruvate kinase (+) was incubated as described above. Samples were analyzed as in *Lane 1*.
Lane 4: Original untreated material.
O indicates the origin of the chromatogram. Other arrows indicate the positions of authentic marker nucleotides.

and needle, evaporated and redissolved in water. Further characterization revealed that this material remained at the origin of the PEI thin layer chromatogram in several different solvent systems, and that it was also resistant to the treatments with bacterial alkaline phosphatase, nuclease P1, snake venom phosphodiesterase, and acid (95°C for 15 min in 10% trichloroacetic acid), but labile to alkaline treatment (55°C for 2 hours in 1 M KOH) (data not shown). These characteristics were clearly different from the properties of ADP. Surprisingly, this material flowed through a series of gel filtration chromatographies using Bio-Gel P-4, P-10 (Bio Rad), and even Sephacryl S-200 (Pharmacia) (data not shown). The properties described above prompted the study of the phospholipid distribution of the samples using high-performance thin layer chromatography (9). As shown in Fig. 5, the material eluted from the SDS-polyacrylamide gel and passed through a Sephacryl S-200 gel filtration column migrated to the position of L-alpha-phosphatidylinositol 4-monophosphate (DPI) in two different solvent systems. Next, lipid extraction was performed from the original phosphorylation reaction mixture before the addition of SDS sample buffer as described in Materials and Methods. In three experiments, a 3.5- to 16-fold difference of the intensity was detected by densitometry at the position of DPI between the N fraction of the IFN-treated cells and that of untreated cells (Fig. 5B). TPI and PI were undetectable under these conditions.

Phosphatidyl inositol turnover was reported to be very important in the signal transduction from the cell surface to the interior (14). Because the pathway of phosphatidyl inositol turnover is complex, the precise point of IFN action and its functional importance is difficult to specify at present. However, the effect of IFN on the signal transduction system, if any, would be intriguing.

Further efforts should be made to study these phe-

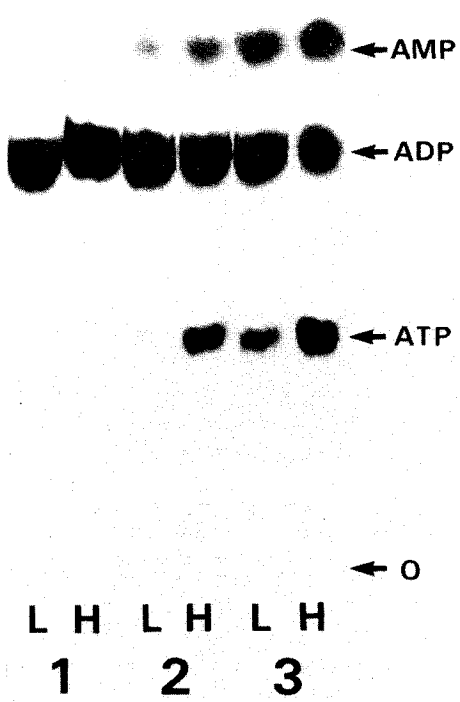

Fig. 4. Enhancement of adenylate kinaselike activity by the treatment with IFN.
The N fraction from IFN-treated or untreated cells were assayed for adenylate kinase activity as described in Materials and Methods.
Lane 1: Control experiment without cell lysate.
Lane 2: The N fraction from untreated cells, in the presence of 2.3 μM ADP (L) or 102 μM ADP (H) as a substrate.
Lane 3: The N fraction from IFN-treated cells in the presence of a low (L) or high (H) concentration of ADP as described above.
O indicates the origin of the chromatogram. Other arrows indicate the positions of authentic marker nucleotides.

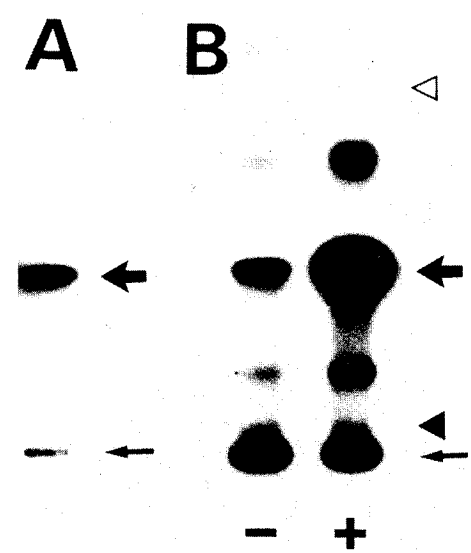

Fig. 5. Enhancement of DPI production by IFN-treated cell lysate.
Panel A. The labeled material produced by the IFN-treated cell lysate (Fig. 1) was eluted from the SDS-polyacrylamide gel as described in the text and loaded onto a Sephacryl S-200 gel filtration column. The single radioactive peak in the void volume was analyzed by high-performance thin layer chromatography using silica gel 60 with chloroform/methanol/4 M NH_4OH, 9 : 7 : 2 (v/v), as described in Materials and Methods.
The thin arrow indicates the origin of the chromatogram. The thick arrow indicates the position of DPI.
Panel B. Phospholipid extraction was performed as described in Materials and Methods on the reaction mixture containing the N fraction from the untreated (−) or IFN-treated (+) cells. The samples were analyzed by high-performance thin layer chromatography using the same solvent systems as described in *Panel A*.
The thin arrow indicates the origin of the chromatogram. The positions corresponding to TPI (closed triangle), DPI (thick arrow), and PI (open triangle) are indicated. The other labeled compounds were not identified.

nomena, because they may be part of the intracellular pathway(s) which IFN activates to carry out its biological actions, such as in antiviral and antiproliferative effects.

Acknowledgements. I would like to thank Drs. C. Stiles for the BALB/c-3T3 (clone A31) cells and PDGF preparation; T. Ariga and R.K. Yu, for teaching me the excellent techniques of lipid analysis and for helpful discussion; and P. Lengyel, for suggestions and encouragement during the study.

REFERENCES

1. LENGYEL, P. (1982). Biochemistry of interferons and their actions. *Annu. Rev. Biochem.*, **51**: 251–282.
2. TOMINAGA, S. and LENGYEL, P. (1985). Beta-interferon alters the pattern of proteins secreted from quiescent and platelet-derived growth factor-treated BALB/c-3T3 cells. *J. Biol. Chem.*, **260**: 1975–1978.
3. ANTONIADES, H.N., SCHER, C.D., and STILES, C.D. (1979). Purification of human platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA*, **76**: 1809–1813.
4. PLEDGER, W.J., STILES, C.D., ANTONIADES, H.N., and SCHER, C.D. (1977). Induction of DNA synthesis in BALB/c-3T3 cells by serum components: Reevaluation of the commitment process. *Proc. Natl. Acad. Sci. USA*, **74**: 4481–4485.
5. JAYARAM, B.M., SCHMIDT, H., YOSHIE, O., and LENGYEL, P. (1983). Simple procedure for the isolation of mouse interferon-beta. *J. Interferon Res.*, **3**: 177–180.
6. SEN, G.C., TAIRA, H., and LENGYEL, P. (1978). Interferon, double-stranded RNA, and protein phosphorylation. *J. Biol. Chem.*, **253**: 5915–5921.
7. LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.
8. TOMINAGA, S. and KAZIRO, Y. (1983). Adenosine triphosphatases associated with bovine brain microtubules. *J. Biochem.*, **93**: 1093–1100.
9. MACALA, L.J., YU, R.K., and ANDO, S. (1983). Analysis of brain lipids by high performance thin-layer chromatography and densitometry. *J. Lipid Res.*, **24**: 1243–1250.
10. SUGIMOTO, Y., WHITMAN, M., CANTLEY, L.C., and ERICKSON, R.L. (1984). Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc. Natl. Acad. Sci. USA*, **81**: 2117–2121.
11. FARRELL, P.J., BROEZE, R.J., and LENGYEL, P. (1979). Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. *Nature*, **279**: 523–525.
12. OLASHAW, N.E. and PLEDGER, W.J. (1983). Association of platelet-derived growth factor-induced protein with nuclear material. *Nature*, **306**: 272–274.
13. MOORE, G. and CLEMENS, M.J. (1983). Inhibition of cell proliferation by human interferons associated with turnover of newly replicated DNA. *Biochem. Soc. Trans.*, **11**: 361–362.
14. NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**: 693–698.

(Received for publication, February 20, 1992
and in revised form, March 14, 1992)