Multiple Doses of Diacylglycerol and Calcium Ionophore Are Necessary to Activate AP-1 Enhancer Activity and Induce Markers of Macrophage Differentiation*

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In contrast to phorbol esters, multiple doses of diacylglycerols are needed to differentiate U937 human monoblastic leukemic cells to a macrophage-like phenotype. Although both of these agents similarly activate protein kinase C in vitro, it is not known why these agents appear to have differing biologic effects. One possibility is that they regulate gene transcription in slightly different ways. Regulation of gene transcription by phorbol esters is complex and involves the stimulation of the transactivating proteins Jun and Fos which form dimers and bind to the AP-1 enhancer elements (5'-TGAGTCA-3'). To understand whether diacylglycerols regulate gene transcription similarly to phorbol esters and to examine whether activation of AP-1 enhancer activity is correlated with differentiation, we have treated U937 human monoblastic leukemic cells with these agents and examined activation of transcription from AP-1 enhancer elements. We find that, although a single dose of diacylglycerol, like phorbol esters, is sufficient to elevate mRNA levels of both the c-jun and c-fos protooncogenes, in contrast to phorbol esters there is no increase in either Jun protein or activation of AP-1 enhancer activity. However, multiple doses of this agent given over 24 h stimulate repeated elevations in c-jun and c-fos mRNA, increases in Jun protein, and enhancer activation. Treatment of U937 cells with ionomycin, a calcium ionophore, also activates AP-1 enhancer activity nor stimulates differentiation with 18 U.S.C. Section 1734 solely to indicate this fact.

The addition of phorbol esters to both fresh samples of human leukemic cells and leukemic cell lines, HL-60 and U937 cells, induces these cells to differentiate into macrophages (Kraft et al., 1989; Harris and Ralph, 1989). This activation of differentiation is mediated at least in part by phorbol ester activation of protein kinase C and translocation of this enzyme to the cell membrane (Kraft et al., 1987; Ebeling et al., 1985). Unlike phorbol esters (Morin et al., 1987), a single dose of a diacylglycerol which activates protein kinase C in vitro (Sharkey et al., 1984) has little effect on the phenotype of these cells. Although identical results have not been reported by all laboratories (Kreutter et al., 1985; Yamamoto et al., 1986), multiple doses of diacylglycerols administered to leukemic cells over 24 h appear to induce changes in surface markers and a decrease in c-myc transcription characteristic of differentiated leukemic cells (Ebeling et al., 1985; Salehi et al., 1988). Since diacylglycerols and phorbol esters activate protein kinase C in a similar fashion (Sharkey et al., 1984), it is unknown why multiple doses of diacylglycerol are needed to mimic the effects of phorbol esters in vitro. In addition, it is not clear why calcium ionophores, which have no effect on differentiation of these cells alone (Morin et al., 1987), enhance the biologic activity of diacylglycerols.

Diacylglycerols are important intracellular second messengers (Rink et al., 1983; Rittenhouse-Simmons, 1979; Rittenhouse, 1982). Increases in diacylglycerols and intracellular calcium induced by the binding of polypeptide growth factors activate protein kinase C (Nishizuka, 1984) and stimulate the transcription of specific gene products (Kaibuchi et al., 1986; Salehi et al., 1988). Like diacylglycerols, phorbol esters bind to the regulatory portion of protein kinase C; however, although both activate protein kinase C similarly in vitro (Sharkey et al., 1984), they regulate this enzyme differently in vivo. For example, prolonged incubation of cells with phorbol esters causes the eventual degradation of all cellular protein kinase C (Kraft et al., 1987); this does not occur when diacylglycerols are used (Ways et al., 1987). Also, the addition of diacylglycerols to cells labeled with [32P]orthophosphate does not stimulate protein phosphorylation identical with that induced by phorbol esters (Morin et al., 1987, Strulovic et al., 1989, and, unlike phorbol esters, the application of diacylglycerols to U937 human monocytic leukemia cells was unable to induce interleukin 1β mRNA (Strulovic et al., 1989). It has been suggested (Strulovic et al., 1989) that the differences between diacylglycerols and phorbol esters may reside in the inability of diacylglycerols to translocate protein kinase C to the membrane for prolonged periods, to activate specific isoforms of protein kinase C, or to stimulate the activation of specific mRNAs.

Phorbol ester induction of gene transcription has been shown to be mediated by consensus DNA sequences AP-1-2-3 recognized by phorbol ester-modulated transacting factors.
The AP-1 enhancer sequence, 5'-TGGTACA-3', binds the proteins encoded by the c-jun and c-fos protooncogenes (Angel et al., 1987a, 1988a, Lee et al., 1987). The addition of phorbol esters to both quiescent fibroblasts and epidermal cells (Berstein and Colburn, 1989; Lamph et al., 1985; Quantin and Breathnach, 1988) stimulates an increase in the transcription of mRNA for both of these genes. Although Jun is capable of binding to this sequence as a homodimer, the formation of a Jun/Fos heterodimer (Halazonetis et al., 1988; Kouzardes and Ziff, 1989; Neuberg et al., 1989) greatly enhances the ability of these proteins to stimulate transcription from the AP-1 enhancer sequence. Because both Jun and Fos are phosphorylated proteins (Barber and Verma, 1987; Boyle et al., 1989), it is possible that post-translational modifications of these proteins may play an important role in the regulation of AP-1 enhancer activity. Not only phorbol esters but epidermal growth factor (Kerr et al., 1989) and tumor necrosis factor α (Brenner et al., 1989) appear to regulate gene transcription through AP-1 enhancer elements. Recent evidence demonstrates that the addition of differentiating agents, phorbol esters, bryostatin, and vitamin D3 to human myeloid leukemic cells stimulates increases in c-jun RNA (Sherman et al., 1990; Aureux et al., 1990) and suggests that c-jun levels may be correlated with differentiation.

To determine whether diacylglycerols activate AP-1 enhancer activity in a fashion identical with phorbol esters and whether the activation of the AP-1 enhancer activity is correlated with differentiation, we have compared the effects of diacylglycerols with or without calcium ionophore and phorbol esters on U937 human monoblastic leukemia cells. We find that although a single dose of diacylglycerol (DiC8) induces elevations in both c-jun and c-fos RNA, unlike phorbol esters, it neither activates the AP-1 enhancer to stimulate transcription nor induces the differentiation of these cells. In contrast, multiple doses of DiC8 given at a 2-h intervals stimulate (1) repeated elevations in the messenger RNA levels for these protooncogenes, (2) increases in Jun protein, (3) activation of transcription from the AP-1 enhancer element, and (4) the induction of markers of differentiation. These data demonstrate that increases in c-jun and c-fos RNA, unlike phorbol esters, do not necessarily correlate with activation of AP-1 enhancer elements, suggesting a complex post-transcriptional control of AP-1 enhancer activity in these hematopoietic cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The diacylglycerol dioctanoylglycerol (DiC8) was purchased from Avanti (Birmingham, AL). We procured ionomycin from Calbiochem, dichloroacetyl[1-14C]chloroamphenicol and [35S]methionine from Amersham. All other chemicals were reagent grade from Sigma. U937 cells obtained from the ATCC were cultured under an atmosphere of 5% CO2 in Dulbecco’s minimal essential medium (DMEM) containing 10% iron-supplemented calf serum.

**Northern Blot Hybridization**—Total cellular RNA was extracted from approximately 2 X 107 cells. At each time point, cells were quick frozen in liquid nitrogen, stored at −70 °C, and at a later time, detested and RNA-isolated using guanidinium thiocyanate/phenol/chloroform (Kraft et al., 1989). Approximately 20 μg of RNA from each time point was loaded onto a 1.5% agarose/formaldehyde gel. After electrophoresis, the RNA was transferred to nitrocellulose and cross-linked with UV light, and the nitrocellulose blot was incubated with labeled probes (Kraft et al., 1987). Purified fragments of c-jun (Angel et al., 1988a), c-fos (Miller et al., 1984), and α-tubulin (Cleveland, 1980) were radiolabeled with [α-32P]dCTP using nick-transla-

1 The abbreviations used are: DiC8, dioctanoylglycerol; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; PMA, phorbol myristate acetate; AP-1, 5'-TGGTACA-3', AP-1 enhancer; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; CMV, cytomegalovirus.

**Electroporation**—Electroporation was done (Potter et al., 1984) in phosphate-buffered saline at 250 V and 250 μF in a Bio-Rad Electroporator. U937 cells were washed twice with Dulbecco’s phosphate-buffered saline and resuspended in culture medium at 106 cells/ml. Routinely, 0.4 ml of cells was transfected with 10 μg of plasmid DNA. Cells were then divided into equal aliquots and resuspended in DMEM and calf serum. Treatments were begun either immediately or after 16 h with no difference in results.

**Immunoprecipitation**—2 X 106 cells were incubated in methionine-free medium for 1/2 h prior to the addition of 150 μCi/ml of [35S]methionine for 1 h. Immunoprecipitation was then carried out as previously described (Chiu et al., 1988). Cells were lysed in 1 ml of ice-cold radioimmunoprecipitation buffer (RIPA), and the lysates were clarified by centrifugation at 15,000 × g for 30 min at 4 °C. 2 μl of anti-trpE-c-jun antisera were added to the lysate and incubated at 4 °C overnight. Antibody-antigen complexes were precipitated with Staphylococcus aureus protein A (Calbiochem), the pellet was washed, and the complex was dissociated by boiling in SDS buffer. The labeled proteins were run on a 10% SDS-polyacrylamide gel, the gel was impregnated with ENHANCE, and fluorography was done for 72 h.

**CAT Assays**—U937 cells in the logarithmic phase of growth were electroporated as described with expression vectors containing the chloramphenicol acetyltransferase (CAT) gene (see “Results”). 24 h after treatment, cells were pelleted, washed twice with Dulbecco’s phosphate-buffered saline, and suspended in 100 μl of a buffer containing 250 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM EGTA, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cells were froze-thawed six times, and the homogenate was spun for 10 min in a microcentrifuge. The supernatant was removed and protein content was determined. 100 μg of protein from each time point was added to 200-μl reaction containing 250 mM Tris-HCl, pH 7.8, 0.5 mM acetyl-CoA, and [35S]chloramphenicol (0.95 μCi). The reaction mixture was incubated for 1 h at 37 °C and then extracted with 1 ml of ethyl acetate. The majority of the ethyl acetate was removed by centrifugation under vacuum, and the remaining material was spotted onto a silica thin layer plate. After chromatography in chloroform/methanol (95:1), the plate was dried, and autoradiography was done for 24 h.

**RESULTS**

Adding phorbol esters to U937 leukemic cells stimulates an increase in c-jun and c-fos RNA levels starting within 15 min of addition and decreasing after 1–2 h (Fig. 1). Changes occur in both the 2.7- and 3.4-kb c-jun mRNA over a similar time course. Likewise, both the HL-60 and PLB-985 leukemic cell lines (Tucker et al., 1988) which are induced to differentiate into macrophages by phorbol esters yielded similar changes in both of these mRNAs (data not shown) when treated with phorbol esters. To examine the phorbol ester induction of Jun protein synthesis, we labeled the U937 cells with [35S]methionine and immunoprecipitated equivalent numbers of [35S]methionine counts at various times after phorbol ester treatment. Immunoprecipitation of Jun protein (Fig. 2) demonstrates that it is transiently induced, peaking at 6 h, and by 24 h decreases to baseline. At all times examined, small amounts of Jun protein were present in these cells. Similar results were obtained when equivalent numbers of cells were subjected to this immunoprecipitation procedure. To evaluate the activation of the AP-1 enhancer element by Jun and Fos during differentiation of U937 cells, we electroporated chimeric plasmids containing a single copy of the phorbol ester responsive element (AP-1 site) (−75/+63) within the collagenase promoter (CollagenaseCAT) (Angel et al., 1987b) gene. The addition of PMA (+) to these cells stimulated CAT activity from the construct containing an AP-1 site but not from one lacking an AP-1 site (−60/+63) (Fig. 3). Likewise, the addition of PMA to these cells stimulates the activity of constructs containing five copies of the AP-1 site upstream of the herpes simplex virus-tk promoter (5 × AP-1), but not the activity of tk-CAT (−enh) lacking this enhancer element. A construct containing the CMV promoter was used as a control. However, phorbol
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FIG. 1. Northern blot analysis of the effects of PMA on c-jun and c-fos RNA levels. U-937 cells in the log phase of growth were treated with PMA (0.1 μM) for varying time periods and Northern blot hybridization carried out as described under "Experimental Procedures".

FIG. 2. Immunoprecipitation of Jun protein from U937 cells. U-937 cells were treated with 0.1 μM PMA in DMEM medium containing 10% calf serum. One-half hour before the times listed, cells were washed in methionine-free medium and incubated with 150 μCi/ml of [35S]methionine. The control cells were not treated with PMA. Normal rabbit serum (NRS) was also used to immunoprecipitate the 6-h time point. Equivalent numbers of counts per min were loaded in each lane.

FIG. 3. The effect of PMA on the chloramphenicol acetyltransferase activity of transiently transfected U937 cells. Supercoiled plasmids containing tk-CAT (Enh−), −73/+63 collagenase-CAT, −60/+63 collagenase-CAT, 5× AP-1 CAT, and CMV-CAT (CMV) were transfected into U937 cells by electroporation. The cells were incubated with 0.1 μM PMA for 24 h and assayed for CAT activity. The data shown are representative of experiments performed in triplicate. The average fold activation of triplicate experiments ± S.D. in which PMA-treated cells are compared to control untreated is as follows: Enh, 1 ± 0.2; −73/+63, 4.8 ± 0.5; −60/+63, 1.1 ± 0.4; 5× AP-1, 12.4 ± 3.1; CMV, 2.4 ± 0.6. The percent acetylation of each treatment was divided by the untreated paired control value to obtain the fold activation.

FIG. 4. The effect of ionomycin and DiC8 on the cellular levels of c-jun RNA. Time course of activation of c-jun and c-fos RNA by DiC8 and ionomycin. U937 cells were treated with 1 μM ionomycin (C,D) or 100 μM DiC8 and total RNA isolated at the times indicated. Blots were probed with either c-jun (A,C) or c-fos (B,D).

The addition of a single dose of either ionomycin (1 μM) or DiC8 (100 μM) to U937 cells induces increases in total cellular c-jun and c-fos RNA over a similar time course as that induced by phorbol esters (Figs. 1 and 4). Densitometry of Northern blots and the tubulin controls has allowed us to quantify the changes induced by this agent. Maximal doses of DiC8 induce a 5-fold change in relative c-jun levels while ionomycin stimulated a 4-fold change. The 5.8-fold change induced by PMA was at a level similar to these other agents. The time course of elevation of c-jun and c-fos by lower doses of both iono-
mycin and DiC8 was identical with that at higher concentra-
tions.

Because the Fos and Jun proteins interact to form a more
effective transcriptional activator than Jun alone operating
through the AP-1 site (Chiu et al., 1988; Halazonetis et al.,
1988; Rauscher et al., 1988), we determined the effect of DiC8
and ionomycin on 5 × AP-1 CAT activity. This construct was
electroporated into U937 cells, and the effect of either iono-
mycin alone or in combination with DiC8 was tested. Neither
a single dose of ionomycin given over a broad concentration
range (Fig. 5A) nor a single dose of 100 μM DiC8 was capable
of activating 5 × AP-1 CAT (Fig. 5B). Ionomycin alone
appeared to slightly inhibit the 5 × AP-1 activity without
effecting cell viability. However, the addition of a single dose
of ionomycin and DiC8 together stimulated a 4.5-fold increase
in CAT activity. Multiple repeat doses of ionomycin and DiC8
given at 2-h intervals caused marked increases in activity,
with 12 doses stimulating enzyme activity to approximately
the same level as a single dose of PMA (53.5 versus 72.6) (Fig.
5B). These data demonstrate that a single dose of ionomycin
or DiC8 is sufficient to induce an elevation in c-jun RNA but
that multiple doses of both agents together are necessary to
maximally stimulate 5 × AP-1 CAT activity.

To explain why a single dose of ionomycin or DiC8 failed
to stimulate 5 × AP-1 CAT activity, we labeled U937 cells
with [35S]methionine and immunoprecipitated Jun protein
after the addition of various combinations of DiC8 and iono-
mycin or PMA alone. Immunoprecipitation was done at 6 h
because Jun protein levels were maximal at this time point
(Fig. 3). In comparison to PMA, little change was seen in Jun
protein levels with either a single dose of DiC8 or ionomycin.
Also, multiple doses of ionomycin had little effect on the
levels of this protein. However, when U937 cells were treated
with multiple doses of DiC8 with or without ionomycin,
marked increases in Jun protein occurred (Fig. 6). The promi-

nent 43-kDa protein seen in both Figs. 6 and 3 was not
presented with trp-Jun fusion protein. Control immunopre-
cipitations performed at time points earlier than 6 h after
treatment exclude the possibility that DiC8 and ionomycin
stimulated changes in Jun protein over a different time course
than PMA (data not shown). These results demonstrate that
activation of the 5 × AP-1 CAT construct correlates with the
ability of these agents to increase immunoprecipitable Jun
protein.

To understand why multiple doses of DiC8 are necessary
to elevate Jun protein, we examined changes in c-jun and c-
fos RNA levels after multiple DiC8 treatments. We found
that, when DiC8 was given at 2-h intervals, each dose of DiC8
induced a repeated increase in c-jun (Fig. 7) and c-fos messen-
ger RNA levels (identical data not shown). With each dose of
diacylglycerol, c-fos RNA levels were rapidly induced and then
decayed over 2 h toward baseline. Densitometry of the c-jun
and tubulin Northern blots and calculation of relative changes
in c-jun mRNA levels demonstrates a 4–8-fold induction of
cellular levels of c-jun by a single dose of DiC8 and ionomycin
which returns toward baseline by 2 h and is again induced by
subsequent doses of DiC8. A third dose of DiC8 again induces
an increase in c-jun mRNA.

The addition of phorbol esters to U937 cells (Harris and
Ralph, 1985) induces increases in 1) superoxide anion pro-
duction, 2) responsiveness to chemotactic peptide, 3) expres-
sion of Leu-11 surface receptors, and 4) a marked decrease
in cell surface OKT9 (May et al., 1989) with little effect on
surface IgG. Examination of these surface markers of differ-
entiation 24 h after treatment with DiC8 and/or ionomycin

demonstrates that the addition of a single dose of either agent
is not sufficient to induce changes in Leu-11 or OKT9. How-
ever, the addition of multiple doses induces a fall in the
percentage of cells positive for OKT9 (86% to 44%) and a rise
in the percentage of cells positive for Leu-11 (2.0% to 39%)
(Fig. 6B). The reduction in OKT9 and the increase in Leu-11
expression induced by three doses of these agents was not as
dramatic as the changes seen after PMA treatment. The
surface levels of IgG, or OKM-1 which are unaffected by PMA
treatment are also unchanged by these treatments. The acti-
vation of AP-1 enhancer activity and increases in Jun protein
appear to correlate with the induction of differentiation mark-
ers in U937 cells.

**DISCUSSION**

Activators of leukemic differentiation including bryostatin,
vitamin D3, and phorbol esters induce similar increases in c-
jun mRNA (Sherman et al., 1990; Auwerx et al., 1990). By
comparing the effects of PMA and DiC8 (with or without
ionomycin) on U937 cells, we found that a single dose of

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**Fig. 5.** Activation of CAT activity by single and multiple
doses of ionomycin and DiC8. A, the effect of ionomycin on CAT
activity. U937 cells were electroporated with 5 × AP-1 CAT
and divided into 7 aliquots. One aliquot remained untreated. Each of the
remaining aliquots was treated with varying doses of ionomycin, and
one also with PMA. After 24 h, cells were homogenized, and CAT
activity was determined. Data presented are representative of exper-
iments performed in triplicate. The average of the fold activation of
the triplcate determinations ± S.D. is as follows: 5, 0.8 ± 0.4, 1, 1 ±
0.5, 0.5, 0.9 ± 0.6; 0.05, 0.7 ± 0.6; 0.005, 0.8 ± 0.5. The percent
acetylation of each treatment was divided by the control (C) value to
obtain the fold activation. B, the effect of combined ionomycin and
DiC8 on CAT activity. U937 cells were electroporated with 5 × AP-1
CAT activity. The addition of phorbol esters to U937 cells (Harris and
Ralph, 1985) induces increases in 1) superoxide anion pro-
duction, 2) responsiveness to chemotactic peptide, 3) expres-
sion of Leu-11 surface receptors, and 4) a marked decrease
in cell surface OKT9 (May et al., 1989) with little effect on
surface IgG. Examination of these surface markers of differ-
entiation 24 h after treatment with DiC8 and/or ionomycin

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ever, the addition of multiple doses induces a fall in the
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(Fig. 6B). The reduction in OKT9 and the increase in Leu-11
expression induced by three doses of these agents was not as
dramatic as the changes seen after PMA treatment. The
surface levels of IgG, or OKM-1 which are unaffected by PMA
treatment are also unchanged by these treatments. The acti-
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fig. 6. The effect of multiple treatments with DiC8 and ionomycin on the induction of Jun protein and differentiation of U-937 cells. A, immunoprecipitation of Jun protein in DiC8 or ionomycin-treated U-937 cells. U937 cells were treated with 1 μM ionomycin, alone or in combination with 100 μM DiC8, either once (1x) or three times (3x). Control cells were either untreated or treated with 0.1 μM PMA. After treatment, cells remained in DMEM medium plus 10% calf serum for 6 h. They were then spun down and placed in methionine-free medium for ½ h with 150 μCi/ml of [35S]methionine. Cells were then spun down again, homogenized and immunoprecipitated as described under “Experimental Procedures.” An aliquot of the radioimmune precipitation buffer homogenate of PMA-treated cells was immunoprecipitated with normal rabbit serum (NRS). Equivalent numbers of counts per min were loaded in each lane. B, the effect of DiC8 and ionomycin treatments on the induction of cell surface markers of differentiation. U937 cells were treated as described above and at 24 h were sent for FACS analysis of surface markers. The number shown is the percentage of cells expressing each marker and is the average of triplicate experiments carried out over a 3-month period. N.D., experiment not done.

diaclylglycerol stimulated an elevation of both c-jun and c-fos RNA over a similar time course and to the same extent as phorbol esters, but, unlike PMA, did not induce an increase in AP-1 enhancer activity or differentiation markers. In contrast, multiple doses of DiC8 which induce the differentiation of U937 cells cause an elevation both in Jun protein levels and an activation of AP-1 enhancer activity. In addition, we have also shown that other hematopoietic differentiating agents (Harris et al., 1985), including interferon γ, granulocyte-macrophage colony stimulating factor, and tumor necrosis factor α, all stimulate AP-1 enhancer activity after treatment of U937 cells. These results demonstrate that the induction of RNA encoding transcription factors does not correlate with the induction of differentiation. Instead, activation of transcription from the plasmid containing the 5X AP-1 enhancer element linked to the CAT gene correlates with the induction of macrophage-like differentiation by a wide range of agents. This work suggests a complex regulation of AP-1 enhancer activity.

Like diacylglycerols, the calcium ionophore ionomycin also stimulates increases in c-jun messenger RNA levels. Our results are similar to those found in a wide range of hematopoietic cell lines using the calcium ionophore A23187 (Auwerx et al., 1990). This increase can be maximally induced at nanomolar concentrations of ionomycin suggesting that changes in intracellular calcium concentrations are sufficient to induce changes in c-jun mRNA levels. However, although the increases in mRNA occur over a similar time course and to a similar extent as after PMA treatment, there is no activation of either Jun protein synthesis or activation of AP-1 enhancer activity. The addition of ionomycin in combination with DiC8 enhances the ability of the latter agent both to induce Jun protein and AP-1 enhancer activity. The effects of calcium ionophores closely parallel the biologic activity of this compound. Alone, calcium ionophores have little effect on the differentiation of human leukemic cells, but greatly enhance the ability of DiC8 to induce differentiation markers (Morin et al., 1987), and, although diacylglycerols induce the translocation of protein kinase C to the membrane calcium ionophores, they cause little effect on this enzyme (Morin et al., 1987). Increases in intracellular calcium may be sufficient to activate c-jun mRNA synthesis, but activation of protein kinase C may be necessary to allow for both Jun protein synthesis and activation of AP-1 enhancer activity.

Transcriptional and post-translational mechanisms appear to play a role in control of Jun activity. Jun protein activates its own transcription through an AP-1 sequence in the Jun promoter (Angel et al., 1988a). Because this increase occurs rapidly in the presence of cycloheximide, existing cellular Jun protein is likely post-translationally modified to induce changes in transcription (Angel et al., 1988a). Evidence demonstrates that dephosphorylation of the Jun protein may enhance the ability of this transactivating protein to bind the AP-1 sequence (Boyle et al., 1986), suggesting that this is the mechanism for control of c-jun transcriptional activation. In U937 cells, nuclear run-off experiments (data not shown)

![Figure 6](https://example.com/fig6.png)

![Figure 7](https://example.com/fig7.png)

**Fig. 6.** The effect of multiple treatments with DiC8 and ionomycin on c-jun and c-fos RNA levels in U937 cells. U937 cells were treated with DiC8 (100 μM) and ionomycin (1 μM) at zero time and with DiC8 (100 μM) at 2 and 4 h. At each time point, total RNA was isolated as described under “Experimental Procedures.” The Northern blot was probed with c-jun and tubulin and scanned on a densitometer. The c-jun levels were divided by the α-tubulin amounts and expressed as relative RNA levels. The baseline is given an arbitrary value of 1.0. The time at which cells were treated is shown by arrows.

**Fig. 7.** The effect of multiple doses of DiC8 and ionomycin on c-jun and c-fos RNA levels in U937 cells. U937 cells were treated with DiC8 (100 μM) and ionomycin (1 μM) at zero time and with DiC8 (100 μM) at 2 and 4 h. At each time point, total RNA was isolated as described under “Experimental Procedures.” The Northern blot was probed with c-jun and tubulin and scanned on a densitometer. The c-jun levels were divided by the α-tubulin amounts and expressed as relative RNA levels. The baseline is given an arbitrary value of 1.0. The time at which cells were treated is shown by arrows.

"S. E. Adunayh, T. M. Unlag, F. Wagner, and A. S. Kraft, unpublished results."
demonstrate that both phorbol esters and diacylglycerols stimulate a large increase in c-jun transcription. Since this increase occurs rapidly, it is possible that post-translational modification of existing low levels of Jun (Fig. 2) in untreated cells may also play a role in activation of transcription in this cell line by ionomycin and DiC8.

Although the induction of c-jun mRNA by PMA and DiC8 occurs to a similar level and over a similar time course, the amount of Jun protein synthesized by a single dose of each of these agents differs greatly. PMA stimulates a large increase in Jun while a single dose of DiC8 combined with ionomycin causes a small but measurable increase in both Jun protein levels and activation of the AP-1 enhancer element. Multiple doses of DiC8 induce repeated elevations in c-jun mRNA leading to an incremental accumulation of Jun protein and increased activation of 5 × AP-1 CAT. This incremental increase in Jun protein may be secondary to its half-life of at least 6 h (Angel et al., 1988a) which allows for accumulation of protein with each dose of DiC8. The low level of Jun protein synthesis induced by each dose of DiC8 may reflect the status of protein kinase C in the cell. Phorbol esters permanently translocate protein kinase C to the particulate fraction of the cell. In contrast, the addition of a single dose of ionomycin and DiC8 causes only transient association (less than 5 min) of protein kinase C with the particulate fraction. Our experiments suggest a complex regulation of AP-1 enhancer transcriptional or -translational control mechanisms. Our importance of this enhancer element in mediating the differentiation of hematopoietic cells.

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REFERENCES

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987a) Cell 49, 729–739

Angel, P., Baumann, L., Stein, B., Delius, H., Rahmsdorf, H. J., and Herrlich, P. (1987b) Mol. Cell. Biol. 7, 2256–2266

Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988a) Cell 55, 975–985

Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T., and Karin, M. (1988b) Nature 332, 166–171

Aureus, J., Stuebs, B., and Sassone-Corsi, P. (1990) Nucleic Acids Res. 18, 221–228

Barber, J. R., and Verma, I. M. (1987) Mol. Cell. Biol. 7, 2201–2211

Bell, R. M. (1986) Cell 45, 631–632

Bernstein, L. R., and Colburn, N. H. (1989) Science 244, 566–569

Boyle, W. J., Woodgett, J. R., Karin, M., and Hunter, T. (1989) J. Cell. Biochem. Suppl. 13b, 46

Brenner, D. A., O'hara, M., Angel, P., Chojkier, M., and Karin, M. (1989) Nature 337, 661–663

Chiu, R., Imagawa, M., Imbra, R. J., Bockoven, J. R., and Karin, M. (1987) Nature 329, 648–651

Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) Cell 54, 541–552

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirschen, M. W. (1980) Cell 20, 95–105

Ebeling, J. G., Vandenbark, G. R., Kuhn, L. J., Ganong, B. R., Bell, R. M., and Niedel, J. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 815–819

Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E., and Leder, P. (1988) Cell 55, 917–924

Harris, P., and Ralph, P. (1985) J. Leuk. Biol. 37, 407–422

Harris, P. E., Ralph, P., Grabriolove, J., Welte, K., Karmali, R., and Moore, M. A. S. (1985) Cancer Res. 45, 3090–3095

Kabuchi, K., Tsuda, T., Kikuchi, A., Tamimoto, T., Yamashita, T., and Takai, Y. (1986) J. Bioi. Chem. 261, 1187–1192

Kerr, L. D., Holt, J. T., and Matrisian, L. M. (1989) Science 242, 1424–1427

Kreutler, D., Caldwell, A. B., and Morin, M. J. (1985) J. Biol. Chem. 260, 5979–5984

Kourzarides, T., and Ziff, E. (1989) Nature 340, 568–571

Kraft, A. S., Baker, V. V., and May, W. S. (1987) Oncogene 1, 111–118

Kraft, A. S., William, F., Pettit, G. R., and Lilly, M. B. (1989) Cancer Res. 49, 1287–1293

Lamp, W. W., Wamsley, P., Sassone-Corsi, P., and Verma, I. M. (1988) Nature 334, 629–631

Lee, W., Mitchell, P., and Tijan, R. (1987) Cell 49, 741–752

May, W. S., Jr., Sahyoun, N., Wolf, M., and Cuatrecasas, P. (1989) Nature 317, 549–551

Miller, A. D., Curren, T., and Verma, I. M. (1984) Cell 36, 51–60

Morin, M. J., Kreutler, D., Rasmussen, H., and Sartorelli, A. C. (1987) J. Biol. Chem. 262, 11758–11763

Neuberg, M., Schuermann, M., Hunter, J. D., and Muller, R. (1989) Nature 339, 589–590

Nishizuka, Y. (1984) Nature 308, 693–697

Paller, H., Weir, L., and Leder, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1716–1719

Rauscher, F. J., Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohrmann, D., Tijan, R., and Franza, B. R. (1988) Science 240, 1010–1016

Rink, T. J., Sanchez, A., and Hailam, T. J. (1983) Nature 303, 317–319

Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580–587

Rittenhouse, S. E. (1982) Cell Calcium 3, 311–322

Ryder, K., and Nathans, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8464–8467

Ryder, K., Lau, L. F., and Nathans, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1487–1491

Ryseck, R. P., Hirai, S. I., Yaniv, M., and Bravo, R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1898–1903

Sharkey, N. A., Leach, K. L., and Blumberg, P. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 607–610

Sherman, M. L., Stone, R. M., Datta, R., Bernstein, S. H., and Kufe, D. W. (1990) J. Biol. Chem. 265, 3220–3227

Strulovici, B., Daniel-Issakani, S., Oto, E., Nestor, J., Chan, H., and Tsur, A. P. (1989) Biochemistry 28, 3569–3576

Tucker, K. A., Lilly, M. B., Heck, L., and Rado, T. A. (1988) Blood 70, 372–378

Waxman, D. K., Dodd, R. C., and Earp, H. S. (1987) Cancer Res. 47, 815–819

Yamamoto, S., Gotoh, H., Aizu, E., and Kato, R. (1985) J. Biol. Chem. 260, 14930–14934
Multiple doses of diacylglycerol and calcium ionophore are necessary to activate AP-1 enhancer activity and induce markers of macrophage differentiation.
F William, F Wagner, M Karin and A S Kraft

*J. Biol. Chem.* 1990, 265:18166-18171.

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