J. Biochem. 128, 989–998 (2000)

Phorbol Ester-Potentiated Liposomal Transfection to Monocytic PLB-985 Cells[1]

Li Jun Hao,* Dan Yang,* Yoshito Fujii,* Akira Yamauchi,* Norio Suzuki,† Hiroshi Kikuchi,† Yasufumi Kaneda,‡ and Michio Nakamura*,[2]

Department of Host-Defense Biochemistry, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523; †Pharmaceutical Formulation Research Laboratory, Daiichi Pharmaceutical Co., Ltd., Tokyo 134-8630; and ‡Department of Gene Therapy Science, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871

Received July 31, 2000; accepted September 27, 2000

In order to improve transient gene transfer into PLB-985 cells, we treated cells with 12-tetradecanoylphorbol 13-acetate (TPA) 4 h before transfection, and increased by 540-fold the reporter activity of the firefly luciferase gene transfected by TFL-01, a cationic liposome. Dioctanolyglycerol added before TPA addition inhibited the TPA-dependent increase in activity, suggesting it to be a TPA competitor for PKC binding. H7, staurosporine, GO 6976, and H8, but not GF 109203X and forskolin, inhibited the TPA-dependent increase in reporter activity when added 8 h after TFL-01/gene addition. Forskolin and GF 109203X also inhibited the increase when added before TPA. Therefore, for the potentiation of transfection by the TPA/TFL-01 method, conventional PKC activity with significant but low protein kinase A (PKA) activity are first required, and then a novel PKC activity with significant PKA activity. TPA enhanced the uptake of FITC-labeled phosphorothioated oligonucleotides and their prolonged maintenance by cells, suggesting increased TFL-01–assisted plasmid uptake and its stabilization in TPA-treated PLB-985 cells. This method was used successfully for the sensitive analysis of the promoter function of the gp91^{phox} gene, implying the method to be generally useful for promoter analyses of various genes expressed in differentiated human monocytic cells.

Key words: PKC, PLB-985 cells, TFL-01, TPA, transfection.

The cellular mechanisms involved in gene transfer to eukaryotic cells seem to be specific for cell types, since one transfection technique efficiently applicable to certain cell lines is frequently ineffective for other cell lines. Primary cells, some highly transformed cells, and non-adherent cells including myeloid cell lines such as PLB-985 cells are poorly transfected. For these cells, various methods have been developed to improve the efficiency of gene transfer. These methods include the use of calcium-phosphate coprecipitation (*1*), liposome- or proteoliposome-mediated delivery (*2*), microinjection (*3*), electroporation (*4*), and retroviruses (*5*). However, these methods have one or more problems such as cellular toxicity, poor reproducibility, or a sophisticated procedure. Because PLB-985 cells can be induced to differentiate into granulocytes and monocyte/macrophages by certain reagents, these cells are indispensable for the analysis of cell-type–specific transcriptional regulation in myelogenous cells. For these cells, an electroporation method has been used to transiently transfect reporter genes, but the general use of the method is hampered by at least two factors. One is the severe damage to cell viability so that the results obtained might be particular to extremely strong cells. The other problem is the low transfection efficiency, resulting in the requirement for huge numbers of cells for one assay.

We developed a simple lipofection method for PLB-985 cells, the sensitivity of which was increased by almost three orders of magnitude by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) through the activities of conventional protein kinase C (cPKC) and novel PKC (nPKC) in this sequence. Previous studies (*6*) showed that TPA increases transfection into other cells, but the extent was less than one order.

MATERIALS AND METHODS

Chemicals—TPA, 4α-phorbol 12-myristate 13-acetate (4αTPA), phorbol 12,13-diacetate (PDA), 1,2-dioctanoyl-*sn*-glycerol (DiC8), 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine 2HCl (H7), *N*-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H8), forskolin, and GF 109203X were purchased from Sigma (St. Louis, MO). GO 6976 and staurosporine were purchased from Calbiochem-Novabiochem (La

[1] This research was supported in part by Grants-in-Aids for Scientific Research from Monbusho and delivered by the Japanese Society for the Promotion of Sciences, and by institutional grants for collaboration research.

[2] To whom correspondence should be addressed. Phone: +81-95-849-7848, Fax: +81-95-849-7805, E-mail: nakamura@net.nagasaki-u.ac.jp

Abbreviations: TPA, 12-tetradecanoylphorbol 13-acetate; 4αTPA, 4α-phorbol 12-myristate 13-acetate; PDA, phorbol 12,13-diacetate; DiC8, 1,2-dioctanoyl-*sn*-glycerol; H7, 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine 2HCl; H8, *N*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide; PKC, protein kinase C; cPKC, conventional protein kinase C; nPKC, novel PKC; PKA, protein kinase A; FBS, fetal bovine serum; S-oligo, double-stranded phosphorothioated oligonucleotide; CGD, chronic granulomatous disease.

Jolla, CA). TPA, 4αTPA, forskolin, GF 109203X, GO 6976, and staurosporine were dissolved in DMSO to make stock solutions of 160 μM, 160 μM, 25 mM, 0.10 mM, 0.10 mM, and 0.25 mM, respectively. DiC8 was dissolved in 100% ethanol to 200 mM. PDA, H7, and H8 were dissolved in water to 16, 25, and 25 mM, respectively. TFL-01, a cationic liposome, containing *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine chloride and cholesterol, was prepared as reported previously (*7*), dried and kept in vials at 4°C. The PKC assay kit and PKA assay kit were purchased from Upstate Biotechnology (Lake Placid, NY) and GibcoBRL Lifetechoriental (Tokyo), respectively.

Cell Cultures—PLB-985 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS)(Asahi Technoglass, Funabashi) and 2 mM L-glutamine (Wako Life Science, Tokyo) were cultured in a CO_2 incubator under 5% CO_2–95% air at 37°C. In a typical transfection assay, 1 μl of 160 μM TPA was added to 1 ml of cells (5×10^5/well) incubated in a well of a 12-well multiwell-plate (Cat. No. MS-80120, Sumitomo Bakelite, Tokyo) 4 h before transfection. The cells were usually incubated for 16–20 h.

Luciferase Reporter System—We used a pGVC2 firefly luciferase plasmid with SV40 promoter as an experimental reporter, and pRL-CMV renilla luciferase plasmid with CMV immediate-early enhancer promoter (Promega, Madison, WI) as an internal control. The original concentrations of these plasmids were adjusted to 1 μg/μl. In each experiment, varying amounts of the former plasmid and a fixed amount (50 ng) of the latter plasmid were applied to PLB-985 cells.

TFL-01 Transfection—The stock solution of TFL-01, was prepared by suspending TFL-01 in distilled water so that the concentration of *O,O'*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine chloride was 1 M. Then, 50 μl of plasmid solution and 50 μl of TFL-01 suspension, both adjusted to appropriate concentrations in serum-free RPMI-1640 medium and preincubated for 15 min at room temperature, were added to wells containing TPA-treated or untreated 5×10^5 PLB-985 cells in 1 ml of culture medium containing 10% FBS.

Electroporation—TPA-treated or nontreated PLB-985 cells were transiently transfected by electroporation using a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA). 5×10^6 cells in 300 μl of HEPES-buffered (25 mM, pH 7.4) serum-free RPMI 1640 medium were put in a 4 mm–gapped electroporation cuvette (Bio-Rad) containing 10 μg of pGVC2 firefly luciferase plasmid and 50 ng of pRL-CMV renilla luciferase plasmid. The cells in the cuvette were mixed by gentle pipetting, kept for 15 min at room temperature, and electroporated at 260 V with a capacitance of 950 μF. The cells were kept on ice for 15 min, and incubated in 6 ml of culture medium for 20–22 h.

Luciferase Assays—After various incubation times, the cells were transferred into 1.5-ml Eppendorf tubes, washed twice with divalent cation–free PBS (PBS^-), lysed in 100 μl (electroporation method) or 50 μl (TFL01 transfection) of 1 × PicaGene Dual Cell Culture Lysis Reagent (Toyoink, Tokyo), vortexed for 3 min, and rotated for 10 min at room temperature. Aliquots (10 μl) of clarified cell lysate were used for the assays of firefly and renilla luciferase activity with a PicaGene Dual SeaPansy Luminescence Kit (Toyoink) as reported previously (*8*). In the experiment shown in Fig. 7, firefly luciferase activities were normalized to the simultaneously assayed renilla luciferase activities.

Assay of PKC Activity—PKC activities of untreated and TPA-treated PLB-985 cells were assayed with a PKC assay kit (Upstate Biotechnology) at various times after TPA addition according to the instructions of the manufacturer. In brief, 5×10^5 PLB-985 cells cultured in 1 ml culture medium were centrifuged, washed twice with PBS^-, and lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg/ml leupeptin, and 25 μg/ml aprotinin. Postnuclear supernatants were obtained by centrifugation for 15 min at 1,000 ×*g*. The phosphorylation of a specific substrate (QKRPSQRSKYL), namely the transfer of the γ-phosphate from [γ-^{32}P]ATP to the substrate, was assumed to be completely dependent on PKC activity. The phosphorylated substrate was separated from the residual [γ-^{32}P]ATP on P81 phosphocellulose paper and quantified by a scintillation counter.

Assay of PKA Activity—PLB cells (5×10^5 cells/ ml in a well) either treated or untreated with TPA were harvested at the times indicated in Fig. 3, A and B, centrifuged, and washed two times with PBS^-. The cells were lysed in extraction buffer (pH 7.5) containing 5 mM EDTA and 50 mM Tris, and cellular debris was removed from the lysates by centrifuging for 15 min. The PKA activity in the lysates was determined using a PKA assay kit (GibcoBRL Lifetechoriental). Differences between the phosphorylation of the consensus sequence (X-Arg-Arg-X-Ser-X), a specific PKA substrate, and that of the pseudosubstrate sequence (X-Arg-Arg-X-Ala-X), a potent and specific inhibitor of PKA activity, were assumed to be specific to PKA activity.

Annealing of FITC-Phosphorothioate Oligodeoxynucleotides—S65/41wF: 5′-CTGTTTTCATTTCCTCATTGGAAGA-3′; S65/41wRf: 5′-fitcTCTTCCAATGAGGAAATGAAAACAG-3′ were purchased from Hokkaido System Science (Sapporo). These two single strands, 5 nmol each, were annealed to duplex (S-oligo) in 400 μl of annealing reaction solution containing 40 μl of 10-times high annealing buffer.

Fluorescence Detection of FITC of S-Oligo by FACScan and Fluorescent Microscopy—Annealed FITC-S-oligo (2 μg /well) was introduced into untreated and TPA-treated PLB-985 cells (5×10^5) by 40 nmol of TFL01. PLB-985 cells were treated with various reagents at the times specified in Fig. 6. Cells cultured for various times were harvested into 1.5-ml Eppendorf tubes by microfuge centrifugation at 6,000 rpm for 90 s, and washed twice with PBS^- containing 0.5% BSA, 10 mM NaN_3, and 0.5 mM EDTA. The green fluorescence intensities of the cells were detected by FACScan (Becton-Dickenson) and an Axiovert 135M, an inverted fluorescent microscope (Carl Zeiss Japan, Tokyo) with a 40× objective lens.

Statistics—Figures 1, 2, 4, 5, and 7 show typical results obtained in two or three experiments performed in triplicate. Figure 3 and Fig. 6, A and C, show typical results obtained in each of two or three experiments performed in duplicate, individual values. Statistical differences were determined by paired (Fig. 5, B and C) or unpaired (others) Student's *t*-test, and *p* values lower than 0.05 were considered significant.

RESULTS

TPA Greatly Enhances the TFL-01–Assisted Transfection of Firefly Luciferase Reporter Gene into PLB-985 Cells—

Most cell lines so far examined (U937, HEL, Sultan, COS 7, mGH_2) are significantly transfected with luciferase reporter genes by TFL-01, a cationic liposome (data not shown). PLB-985, a promonocytic cell line, could also be transfected with reporter genes by the liposome (compare the first column with the fifth column in Fig. 1, $p < 0.001$), but the extent is low. We, therefore, tried to increase the sensitivity to TFL-01 using TPA because TPA increases cell adherence (*9*) and transfection in other cells (*10*). TPA produced a 540-fold increase in the reporter activity of the firefly luciferase gene transfected with TFL-01 (compare the fifth and sixth columns in Fig. 1, $p < 0.005$). Reporter activity was also increased by TPA when the gene was transfected by electroporation, but the increase was only 8-fold greater than the activity obtained in the absence of TPA (compare the third and fourth columns in Fig. 1, $p < 0.005$). The reporter activity obtained by the TPA-potentiated and TFL-01–assisted gene transfection (TPA/TFL-01) method was about 600-times greater than that obtained by the conventional electroporation method. One assay by the TPA/TFL-01 method requires one-fifth the number of plasmids and one-tenth the number of cells required for an assay by the electroporation method. Thus the TPA/TFL-01 method is highly sensitive compared to conventional electroporation for transfection. Moreover, the method is rapid and easy to perform.

We confirmed that PLB-985 cells treated with TPA are committed more to monocytic lineage, as reported previously (*9*); one and two days after treatment, CD14 and $gp91^{phox}$ were expressed on the surface 1.5 times ($p < 0.05$) and 2 times more ($p < 0.001$), respectively, in treated cells than in un-treated cells. Therefore, transcriptional reporter activity in the treated cells may reflect the activity in monocytically differentiated cells.

Optimization of the TPA/TFL-01 Gene Transfection System—The TPA concentration was optimized to 160 pmol/5 $\times$ 10^5 cells in 1 ml of 10% FBS-containing RPMI-1640 medium (Fig. 2A), and this concentration was used in all experiments. In contrast to most other liposomes, TFL-01 should be applied to cells in the presence of serum, otherwise the reporter activity decreases to 20% the activity obtained in the presence of serum. In order to obtain reproducibly high reporter activity of transfected luciferase genes in the TPA/TFL-01 system, we varied the contents of TFL01 and pGVC2 firefly luciferase plasmid and maintained a fixed amount (50 ng) of pRL-CMV renilla luciferase plasmid, which is used as an internal control in the dual luciferase assay system. Both renilla and firefly luciferase activities were maximal when the amounts of liposome and pGVC2 plasmid were 40 nmol and 2 μg, respectively (Fig. 2, B and C). Under these conditions, the reporter activity became maximal 16 h after plasmid addition and remained constant for the following 6 h (Fig. 2D). The transfection was dramatically potentiated when TPA was added 2–8 h (especially 4 h) before plasmid addition (Fig. 2E). We, therefore, routinely added TPA 4 h before transfection and assayed luciferase activities 16–22 h after transfection. Under these conditions, the firefly luciferase activity was proportional to the amount of pGVC2 firefly luciferase reporter plasmid (Fig. 2F), indicating the system could be used for the quantitative analyses of the promoter functions of various genes.

PKC and PKA Activities of PLB-985 Cells Are Modulated by TPA—As shown in Fig. 2E, the highest reporter gene activity was obtained by the TPA/TFL-01 system in cells treated with TPA 4 h before transfection. This 4-h lag time is consistent with the time that the PKC activity remains significantly high (Fig. 3A). One or more TPA-sensitive PKC, namely conventional and/or novel PKC, may accordingly play a role in the TPA/TFL-01 system. In contrast, the activity of PKA is low during this period (Fig. 3B). PKA has been reported to be directly or indirectly regulated by PKC in signal transduction pathways (*11–17*).

TPA Is the Only Potentiator of TFL-01–Assisted Gene Transfection to PLB-985 Cells—Because PKC is supposed to be a target of TPA for potentiating the TFL-01–assisted transfection of the reporter gene to PLB-985 cells (Fig. 3A), we examined the effect of other PKC activators (DiC8 and PDA) and 4αTPA, an inactive steric analogue of TPA (Fig. 4A). DiC8 slightly increased firefly reporter activity by 2.7-fold compared with an ethanol solution (Eth) ($p < 0.05$), but the extent was only about 3% that achieved by TPA. Additional DiC8 added 8 h after transfection increased the efficiency more, but the total extent remained less than 5% of that obtained by TPA (data not shown). Therefore, DiC8 mimics TPA only slightly. Neither PDA nor 4αTPA potentiated transfection. As shown in Fig. 4B, DiC8 added before, but not after, TPA addition significantly inhibited the TPA/TFL-01–assisted transfection by 52% ($p < 0.025$), suggesting it to be a relative competitor against TPA for the potentiation of TFL-01–assisted gene transfection. DiC8 may occupy the TPA-binding site of the PKC causing slight activation as though it were actually a dominant-negative form of TPA.

The Activities of Both PKC and PKA Are Required for Optimal TPA/TFL-01–Enhanced Transfection—Because our data (Fig. 3) suggested that the TPA/TFL-01 gene transfection system involves increased PKC and decreased

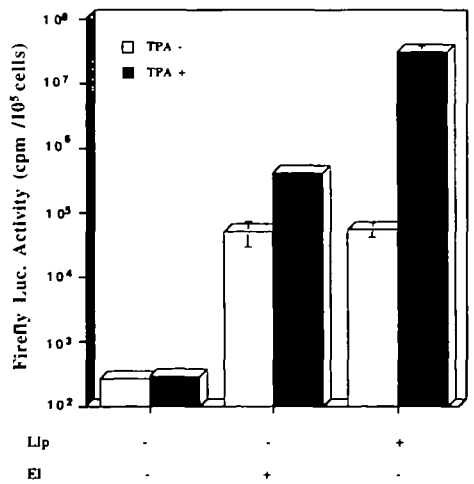


Fig. 1. **TPA effects on reporter activity of the firefly luciferase gene transfected by TFL-01 or electroporation.** TPA (160 nM) was added (closed columns) or not added (open columns) to PLB-985 cells 4 h before transfection by 40 nmol TFL-01 (Lip) or electroporation (El) of pGVC2 firefly luciferase plasmid (2 μg/5 $\times$ 10^5 cells and 10 μg/5 $\times$ 10^6 cells, respectively) with 50 ng renilla luciferase plasmid. Cells were harvested 20 h after transfection, lysed and used for the reporter assays (see "MATERIALS AND METHODS"). The endogenous nonspecific luciferase activity of cells was 215 $\pm$ 57 cpm/10^5 cells ($n = 3$).

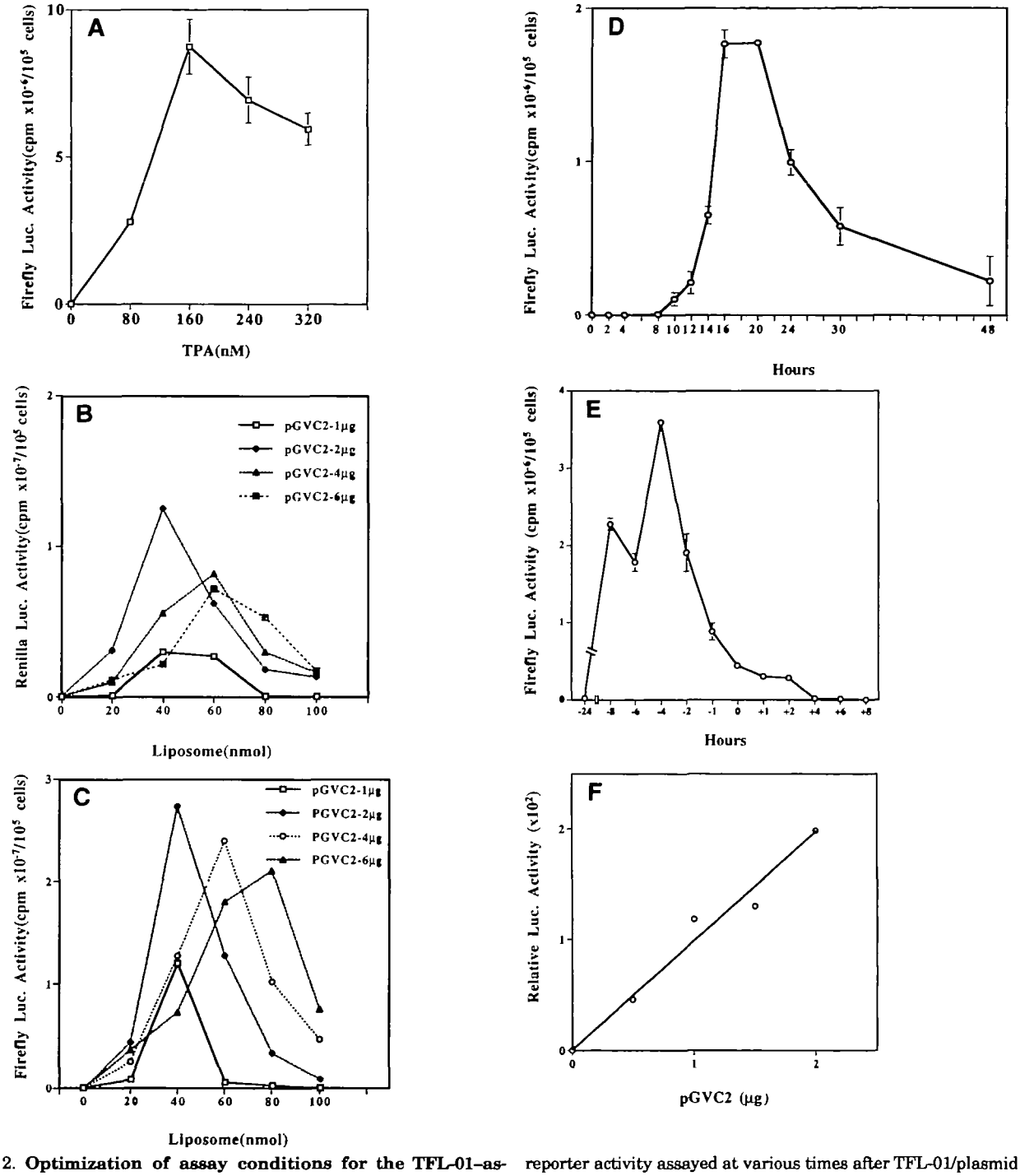


Fig. 2. **Optimization of assay conditions for the TFL-01-assisted transfection of firefly luciferase reporter plasmid into PLB-985 cells.** A: Dose response to TPA. B: Reporter activity of the renilla luciferase plasmid (50 ng/5 $\times 10^5$ cells) transfected by various amounts of TFL-01 into TPA-treated cells with various amounts of pGVC2 firefly luciferase plasmid (1–6 μg). C: Reporter activity of firefly luciferase plasmid transfected as B. Various amounts of pGVC2 firefly luciferase plasmid (1–6 μg) with a fixed amount of renilla luciferase plasmid (50 ng) were mixed with a specified amount of liposome and the mixture was added to cells. D: Time course of firefly reporter activity assayed at various times after TFL-01/plasmid addition. E: Effect of the time of TPA addition. TPA (160 nM) was added to cells at various times before (–) and after (+) TFL-01/plasmid addition. F: Dose response of the pGVC2 firefly luciferase plasmid to the reporter activity. The amount of pGVC2 plasmid was adjusted to 2.0 μg by vector plasmid in each assay. Firefly luciferase activity was normalized to the activity of the simultaneously transfected renilla luciferase plasmid (50 ng). Conditions other than variables were essentially the same as those for the last column of Fig. 1.

PKA activities, we attempted to clarify the period they are involved in the system (Fig. 5A). H7, a PKC inhibitor, strongly inhibited the appearance of the reporter activity of the transfected firefly luciferase gene regardless of the time

when it was added to the system, and the inhibition produced was significantly greater when TPA was added early to PLB cells ($p < 0.005$ for differences between the –5 and 8 h values, and the 8 and 10 h values). Therefore, PKC exerts its positive effect on the TPA-dependent potentiation of TFL-01–assisted transfection to PLB cells throughout the period of incubation. H8, a PKA inhibitor, unexpectedly also inhibited the appearance, but to the lesser extent than that achieved by H7. In contrast to H7, no difference was observed between the two values for the effect of H8 at 8 and 10 h after the addition of the firefly luciferase gene, suggesting no significant contribution of PKA to the process (such as vivid transcription) ongoing between these two times. Forskolin (FK, an indirect PKA activator) inhibited the appearance only when added prior to gene addition. In conjunction with the results obtained with H8, this result suggests that PKA activity should be significant but not high around the time of gene addition, and rather high during the late phase of transfection.

cPKC and nPKC Are Critical in the Early Phase and Late Phase, Respectively, of the TPA/TFL-01 Transfection System—To identify PKC isoforms involved in the increase in the reporter activity of the firefly luciferase gene, we applied group-specific PKC inhibitors to the TPA/TFL-01 transfection system 8 h after gene addition (Fig. 5B) or 1 h before TPA addition (Fig. 5C). GO 6976 (GO, an inhibitor of PKCs-α, β1, and μ), but not GF 109203X (GF, an inhibitor of all cPKCs), added after the addition of the firefly luciferase gene significantly decreased the TPA-potentiated reporter activity of the gene by 34% ($p < 0.001$), suggesting that PKC-μ, but not cPKC, shares one-third in the late potentiation of transfection. Because staurosporine, which inhibits all members of both the cPKC and nPKC families, reduced the activity by more than 90%, about two-thirds of the late phase TPA-dependent potentiation can be attributed to nPKC members other than PKC-μ. On the other

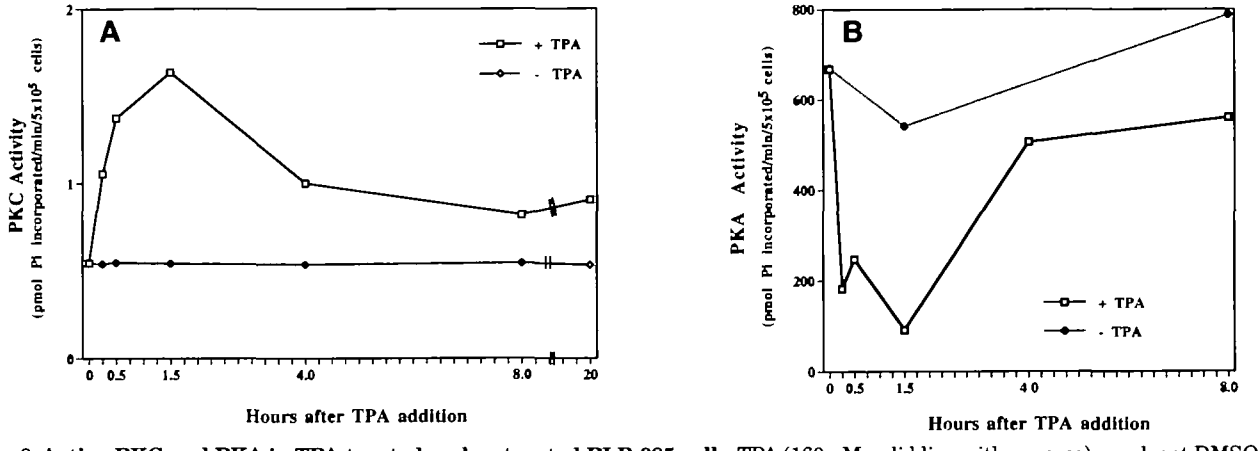


Fig. 3. **Active PKC and PKA in TPA-treated and untreated PLB-985 cells.** TPA (160 nM, solid line with squares) or solvent DMSO (dotted line with diamonds) was added to the cells (5×10^5/ml) at 0 time. At each time, the activities of PKC (A) and PKA (B) were assayed as described under "MATERIALS AND METHODS." Data represent averages of duplicate samples obtained in one of two similar experiments.

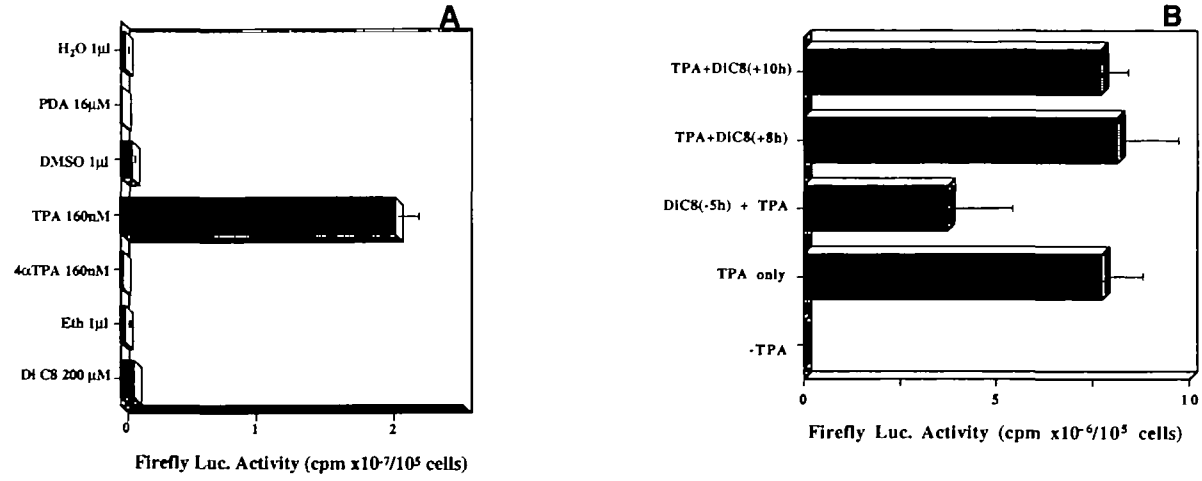
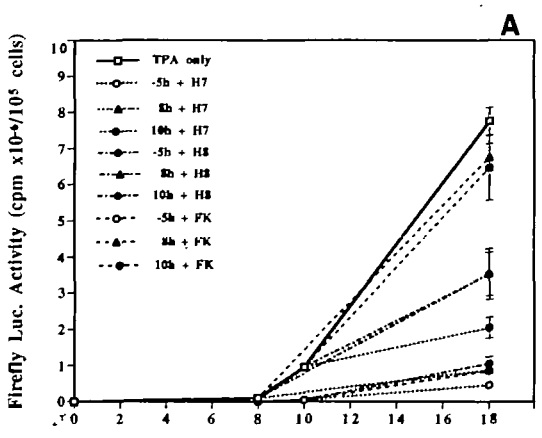
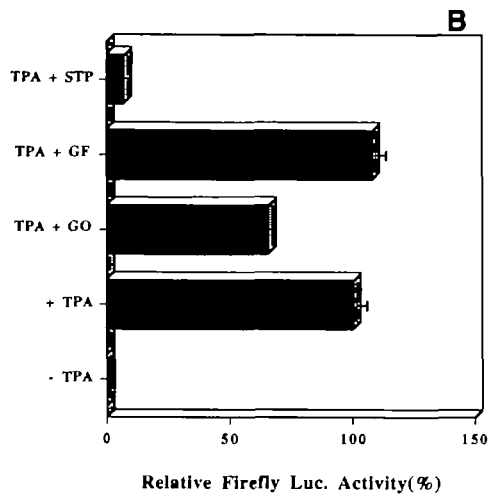


Fig. 4. **Effects of PKC activators on the reporter activity of firefly luciferase gene transfected into PLB-985 cells by TFL-01.** Reporter activities were assayed as in Fig. 1 (last column). A: One of following reagents was added to the cells (5×10^5/ml) 4 h before TFL-01/plasmid addition: PDA, TPA, 4αTPA, DiC8, or the corresponding carrier solvent, H_2O, ethanol (Eth), or DMSO. B: Inhibition of TPA-potentiated transfection by DiC8. DiC8 (200 μM) was added 1 h before TPA addition [DiC8(–5) + TPA], or 8 h [TPA + DiC8(+8)], or 10 h [TPA + DiC8(+10)] after the addition of TFL-01/plasmid. DiC8 added before TPA significantly inhibited the TPA-potentiated reporter activity ($p < 0.025$).





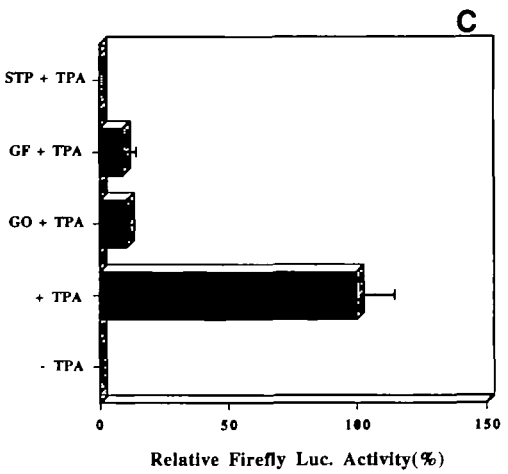
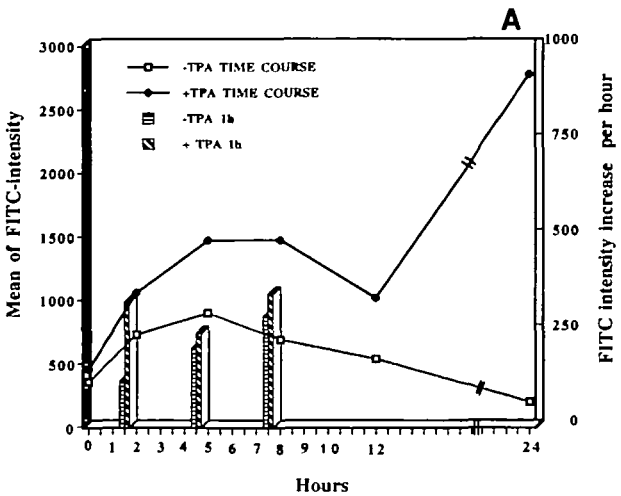


Fig. 5. **Effects of various inhibitors of PKC and PKA and an activator of PKA on the reporter activity of firefly luciferase gene transfected into PLB-985 cells by the TFL-01/TPA method.** A: Effects of H7, H8, and forskolin (FK), added 5 h before, or 8 or 10 h after TFL-01/plasmid addition. No addition (black, solid line with squares) or 25 μM H7 (red, dotted lines), H8 (green, semi-dot lines), or FK (blue, dashed lines) was added to the standard TPA-potentiated TFL-01–transfection system 5 h before (open circles), or 8 h (triangles) or 10 h (crossed circles) after TFL-01/plasmid addition. B: Effects of GO 6976, GF 109203X, and staurosporine added 8 h after TFL-01/plasmid addition. GO 6976 (GO), GF 109203X (GF), or staurosporine (STP) was added to the TPA-potentiated TFL-01 transfection system. C: Effects of GO, GF, and STP added 1 h before TPA addition.



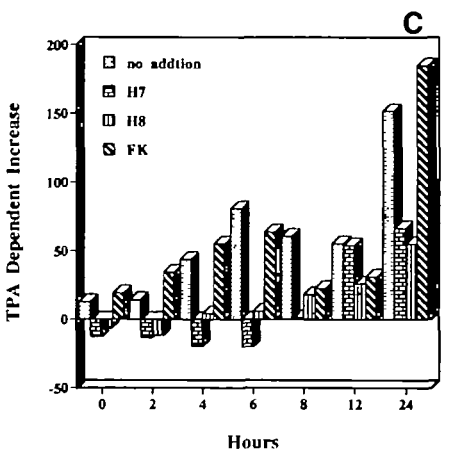


Fig. 6. **Effects of TPA on the uptake of S-oligo into PLB-985 cells and its storage.** A: FITC-labeled S-oligo was added to TPA-treated (solid line) or -untreated (dotted line) PLB-985 cells with TFL-01, and the fluorescence intensities associated with the cells were monitored by FACScan. S-oligo uptake in 1 h was monitored between 1 and 2 h, 4 and 5 h, and 7 and 8 h after the addition of TFL-01/FITC–labeled S-oligo as increases in FITC intensity in PLB-985 cells treated with (diagonally striped columns) or without (horizontally striped columns) TPA. B: Phase contrast (a and b) and fluorescence pictures (c–f) of TPA-treated (b, d, and f) or -untreated (a, c, and e) PLB-985 cells transfected with TFL-01/FITC–labeled S-oligo. Phase contrast pictures at 6 h after transfection (a and b) and fluorescence pictures at 6 h (c and d) and 22 h (e and f) after TFL-01/FITC-S-oligo addition are shown. C: Effects of H7 (bricked columns), H8 (vertically striped columns), and forskolin (FK, diagonally striped columns) on the TPA-dependent increase in FITC-S-oligo fluorescence. The difference between the fluorescence intensity of TPA-treated cells and untreated cells is shown as a TPA-dependent increase. One of these reagents at a final concentration of 25 μM was added to PLB-985 cells 1 h before TPA-addition.

hand, GF 109203X added before TPA dramatically decreased the TPA-potentiated appearance of reporter gene activity to the same extent as GO 6976 and staurosporine (STP) ($p < 0.001$, Fig. 5C). These results suggest that the PKC that plays a role prior to transfection and in the early phases of the transfection process is a cPKC, and the PKC that cats during the late phases of the process is an nPKC. Even if an nPKC plays a significant role during the early phase, its extent might be as great as 10%, because the residual activity after GF 109203X treatment was 10%, and this was completely abolished by staurosporine (Fig. 5C).

TPA-Treated PLB-985 Cells Incorporate DNA Fragments and Keep Them Longer than Untreated Cells—As the intracellular events caused by TPA treatment are essentially the same regardless of the method of transfection, mobilized PKC and PKA activities may have contributed to the one-order increase in reporter activity by the TFL-01–assisted transfection as in electroporation (Fig. 1). The additional 1.5-order increase should be attributable to at least one TPA-dependent factor that is specific to the TPA/TFL-01 transfection system. We, therefore, suspected that TPA increases the incorporation of plasmids into PLB-985 cells, and monitored the uptake and accumulation of FITC-labeled S-oligo, as a model plasmid, into the cells (Fig. 6). In the early stages, S-oligo accumulated more in TPA-

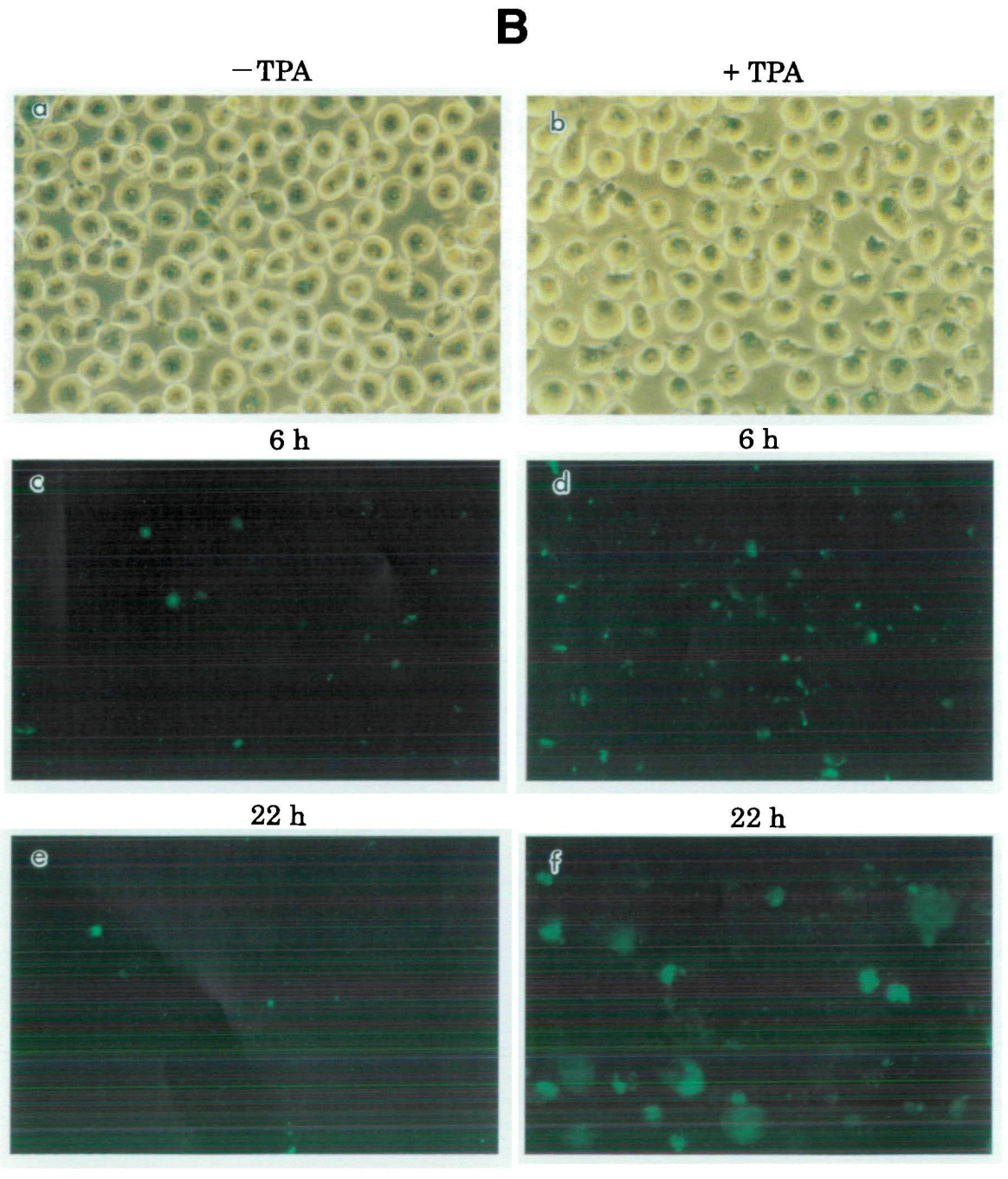


Fig. 6B.

treated cells than in untreated cells, and the content of S-oligo increased in the former cells, but decreased in the latter cells, 22–24 h after nucleotide addition. The rate of S-oligo uptake by TPA-treated cells was especially high in the initial phase (1–2 h) (Fig. 6A). The amounts of S-oligo fragments specifically accumulated in cells were about 3.5 times and 10 times higher than those accumulated in untreated cells 8 and 24 h, respectively, after the addition of the fragments (Fig. 6A). These data are consistent with the results obtained by fluorescence microscopy (Fig. 6B). Many more bright fluorescent spots were present in TPA-treated cells than in untreated cells. These results suggest that TPA increases the amount of active plasmid in PLB-985 cells, accounting at least in part for the additional increase specific to the TFL-01–assisted transfection system.

The TPA-dependent accumulation of S-oligo was significantly decreased by H7 and H8 in the early and late phases of transfection (Fig. 6C), suggesting that PKC and PKA activities are involved in increasing the uptake of plasmids and their stabilization in cells. Forskolin failed to inhibit the TPA-dependent increase in S-oligo accumulation in either the early or late phases of transfection. Forskolin added at the same time, however, almost completely abolished the increase in transfection (Fig. 5A). Accordingly, an initially high PKA activity must have made normally incorporated plasmids ineffective. It should be noted that the early increase, but not late increase, in the TPA-dependent uptake of plasmids is especially important because plasmids incorporated for 8 h just before assay contributed little to reporter activity (Fig. 2A). These results suggest that the relatively long-lasting plasmid uptake specific to the TPA/TFL-01 transfection system is also dependent on PKC and PKA activities, and contributes to the TPA-potentiated increase in the reporter activity of the transfected firefly luciferase gene.

Application of the TPA/TFL-01 Transfection Method to the Assay of the gp91^{phox} Promoter in PLB-985 Cells—Because TPA activates various transcriptional activators, TPA-potentiated transfection with TFL-01 is possibly exceptional for the standard promoters of plasmids used so far (SV-40 and CMV promoters). We have, however, succeeded in applying the TPA/TFL-01 method to the assay of the promoter activity of phagocyte-specific gp91^{phox} after optimizing the conditions in preliminary experiments. The promoter was inserted into the 5′ upstream region of the firefly luciferase gene in pXP2 vector (Fig. 7). Renilla luciferase plasmids were cotransfected into the cells as an internal control. The relative luciferase activity dependent on –105W, a wild gp91^{phox} promoter, was 2.29 (± 0.28), 5 times higher than the activity in promoter-free plasmids (0.45 ± 0.03). The introduction of a single base pair mutation (–53C/T) observed in a CGD patient (*8*) decreased the promoter activity by 92% in contrast to 40% observed in HEL cells transfected by electroporation. The present promoter assay reflects faithfully the *in vivo* activity of the patient's monocytes because the monocytes expressed about 5% of the normal content of gp91^{phox} mRNA (*18*). Two other single base pair mutations of the gp91^{phox} promoter (–55T/C and –57A/C) reported in other CGD patients also exhibited low reporter activities (8–9%), corresponding to the extents of gp91^{phox} mRNA expression and the superoxide generating activity (5–10%) of the peripheral blood monocytes (*19*, *20*) (Fig. 7). Therefore, the promoter activities of these mutants were concluded to reflect the *in vivo* activities of the peripheral monocytes. The present TPA/TFL-01 method is now concluded to provide a reliable, rapid and sensitive method for the analysis of promoter function in monocytically shifted PLB-985 cells. We have now successfully reduced the assay volume to one-fourth, providing a significant savings of all reagents, plasmids, and cells (data not shown).

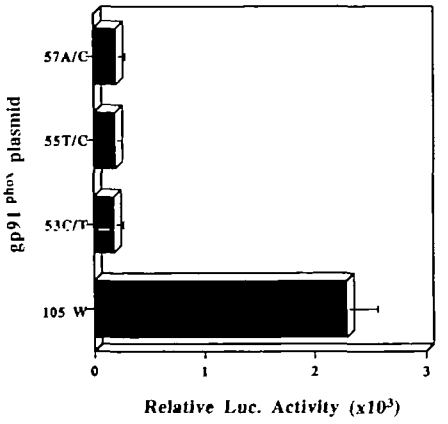


Fig. 7. **Application of TPA-potentiated transfection by TFL-01 to the analysis of gp91^{phox} promoter in PLB-985 cells.** Preliminary experiments showed the optimal assay conditions to use (60 nmol TFL-01, 4 μg of pXP2N firefly luciferase plasmid containing –105/+ 12 bp gp91^{phox} promoter, and 50 ng of renilla luciferase plasmid). Single mutations reported in CGD patients were introduced into the wild promoter (105W) at bps –53(53C/T), –55(55T/C), or –57(57A/C). The relative luciferase activity of the pXP2N vector plasmid was 0.45 ± 0.03.

DISCUSSION

The results presented in this paper demonstrate the great enhancement of TFL-01–assisted transient transfection of reporter genes into PLB-985 cells by TPA, which can contribute greatly to rapid and reliable analyses of transcription mechanisms in differentiated human monocytic cells. This enhancement is attributed, at least in part, to two mechanisms, one common to the electroporation system and which should involve intracellular events, and the other specific to liposomal transfection.

The common mechanism may be primarily evoked by TPA-activated cPKC prior to gene transfection and in the early phases of the transfection proccess and by nPKC in the late phases. DiC8 can bind to these PKCs in competition with TPA but fails to activate them. The inefficient potentiation by DiC8 is consistent with the finding that TPA-activated PKC can phosphorylate proteins partially distinct from those phosphorylated by DiC8-activated PKC (*21*). Contrary to PKC, the PKA activity required for the potentiation seems to be significant but low in the early phases of transfection and rather high in the later phases. The decrease in PKA activity observed after the addition of TPA is a mirror image to the increase in PKC activity, suggesting a correlation between these two activities. This is consistent with the finding that PKC phosphorylates the N-terminal segment of type VI adenylyl cyclase, an enzyme upstream to PKA, and inhibits its activity (*14*). PKC, however, dynamically modulates PKA activities dependent on

its isoform and the types of PKA (*15–17*). The further decrease by H8 and increase by forskolin (Fig. 5A) in the activity inhibits the potentiation, suggesting a sophisticated contribution of PKA activity to TPA potentiation. It should be noted that the significant role of PKC but not PKA during the 2 h period from 8 h to 10 h after plasmid transfection (Fig. 5A) suggests, at least in part, their individual contributions of PKC and PKA to potentiation. The combination of these kinase modulations must contribute to the overall processes of the increased transfection including the stabilization of incorporated plasmids (Figs. 5 and 7). These processes may include transcription and translation processes. NF-κB, a transcription factor existing in an inactive form with an inhibitor protein (IκB) in the cytoplasm, actually translocates to the nucleus, leaving IκB in the cytosol, upon TPA treatment, and activates various genes (*22*). Although PKC is found primarily in the cytoplasm, evidence suggests its nuclear and perinuclear location (*23*), which is induced by TPA (*24*, *25*). Other studies have shown that TPA stimulates the transcription of exogenous genes driven by certain promoters (*26*), which is consistent with the inhibitory effect of H7 added even in the later stages of our TFL-01–assisted transfection (Fig. 5).

Other mechanisms specific to TFL-01 liposomal transfection should contribute greatly to the potentiation by TPA. One is the increased uptake of the exogenous gene into cells (Fig. 6A), which, interestingly, is not affected by forskolin modulation, namely increased PKA activity (Fig. 6C). A novel PKC, PKCε, has been reported to increase the expression of receptors for retroviruses (*27*). An epidermal growth factor receptor has been reported to be phosphorylated and activated in response to TPA addition (*28*). These observation suggest that the introduction of a certain ligand to TFL-01 increases transfection efficiency more. Our present results do not exclude the contribution of additional mechanisms, which, regardless of what they are, should be mostly specific to liposomal treatment and dependent on TPA.

It should be noted that neither TPA nor TFL-01 worked significantly in HL-60 cells, which are myelogenous in origin, as in PLB-985 cells, suggesting the existence of cell type–specific mechanisms. Differences in PKC isoforms and/or signal transduction pathways may produce the difference between these two cell lines, a hypothesis that should be analyzed in detail in the future.

At present, TFL01-assisted reporter gene transfection into TPA-treated PLB-985 cells can be successfully applied to the analysis of the transcriptional regulation of genes that are predominantly expressed in human monocyte / macrophage lineages.

REFERENCES

1. Van der Eb, A.J. and Graham, F.L. (1980) Assay of transforming activity of tumor virus DNA. *Methods Enzymol.* **65**, 826–839
2. Mannino, R.J. and Gould-Fogerite, S. (1988) Liposome mediated gene transfer. *BioTechniques* **6**, 682- 690
3. Graessmann, M. and Graessmann, A. (1983) Microinjection of tissue culture cells. *Methods Enzymol.* **101**, 482–492
4. Machy, P., Lewis, F., MacMillan, L., and Jonak, Z.L. (1988) Gene transfer from targeted liposomes to specific lymphoid cells by electroporation. *Proc. Natl. Acad. Sci. USA* **85**, 8027–8031
5. Varmus, H. (1988) Retroviruses. *Science* **240**, 1427–1435
6. Reston, J.T., Gould-Fogerite, S., and Mannino, R.J. (1993) Differential effects of the phorbol ester TPA on DNA-mediated transfection in a variety of cell lines. *Biochim. Biophys. Acta* **1177**, 49–53
7. Kikuchi, A., Aoki, Y., Sugaya, S., Serikawa, T., Takakuwa, K., Tanaka, K., Suzuki, N., and Kikuchi, H. (1999) Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor. *Hum. Gene Ther.* **10**, 947–955
8. Suzuki, S., Kumatori, A., Haagen, I. A., Fujii, Y., Sadat, M.A., Hao, L.J., Tsuji, Y., Roos, D., and Nakamura, M. (1998) PU.1 as an essential activator for the expression of gp91^{phox} gene in human peripheral neutrophils, and B lymphocytes. *Proc. Natl. Acad. Sci. USA* **95**, 6085–6090
9. Tucker, K.A., Lilly, M.B., Heck, L., Jr., and Rado, T.A. (1987) Characterization of a new diploid myeloid leukemia cell line (PLB-985) with granulocytic and monocytic differentiating capacity. *Blood* **70**, 372–378
10. Reston, J.T., Gould-Fogerite, S., and Mannino, R.J. (1991) Potentiation of DNA mediated gene transfer in NIH3T3 cells by activator of protein kinase C. *Biochim. Biophys. Acta* **1088**, 270–276
11. Mukhopadhyay, A.K. and Schumacher, M. (1985) Inhibition of hCG-stimulated adenylate cyclase in purified mouse Leydig cells by the phorbol ester PMA. *FEBS Lett.* **187**, 56–60
12. Kassis, S., Zaremba, T., Patel, J., and Fishman, P.H. (1985) Phorbol esters and β-adrenergic agonists mediate desensitization of adenylate cyclase in rat glioma C6 cells by distinct mechanisms. *J. Biol. Chem.* **260**, 8911–8917
13. Heyworth, C.M., Whetton, A.D., Kinsella, A.R., and Houslay, M.D. (1984) The phorbol ester, TPA inhibits glucagon-stimulated adenylate cyclase activity. *FEBS Lett.* **170**, 38–42
14. Lai, H.L., Lin, T.H., Kao, Y.Y., Lin, W.J., Hwang, M.J., and Chern, Y. (1999) The *N* terminus domain of type Vi adenylyl cyclase mediates its inhibition by protein kinase C. *Mol. Pharmacol.* **56**, 644–650
15. Zimmermann, G. and Taussig, R. (1996) Protein kinase C alters the responsiveness of adenylyl cyclases to G protein α and βγ subunits. *J. Biol. Chem.* **271**, 27161–27166
16. Bol, G.F., Hulster, A., and Pfeuffer, T. (1997) Adenylyl cyclase type II is stimulated by PKC via C-terminal phosphorylation. *Biochim. Biophys. Acta* **1358**, 307–313
17. Ebina, T., Kawabe, J., Katada, T., Ohno, S., Homcy, C.J., and Ishikawa, Y. (1997) Conformation-dependent activation of type II adenylyl cyclase by protein kinase C. *J. Cell. Biochem.* **64**, 492–498
18. Kuribayashi, F., Kumatori, A., Suzuki, S., Nakamura, M., Matsumoto, T., and Tsuji, Y. (1995) Human peripheral eosinophils have a specific mechanism to express gp91^{phox}, the large subunit of cytochome b_{558}. *Biochem. Biophys. Res. Commun.* **209**, 146–152
19. Newburger, P.E., Skalnik, D.G., Hopkins, P.J., Eklund, E.A., and Curnutte, J.T. (1994) Mutations in the promoter region of the gene for gp91^{phox} in X-linked chonic granulomatous disease with decreased expression of cytochome b_{558}. *J. Clin. Invest.* **94**, 1205–1211
20. Eklund, E.A. and Skalnik, D.G. (1995) Characterization of a gp91^{phox} promoter element that is required for interferon gamma-induced transcription. *J. Biol. Chem.* **270**, 8267–8273
21. Issandou, M., Bayard, F., and Darbon, J.M. (1988) Inhibition of MCF-7 cell growth by 12-O-tetradecanoylphorbol-13-acetate and 1,2-dioctanoyl-*sn*-gycerol: Distinct effects on protein kinase C activity. *Cancer Res.* **48**, 6943–6950
22. Baeuerle, P.A. and Baltimore, D. (1988) I kappa B: a specific inhibitor of the NF-κB transcription factor. *Science* **242**, 540–546
23. Masmoudi, A., Labourdette, G., Mersel, M., Huang, F.L., Huang, K.P., Vincendon, G., and Malvieya, A.N. (1989) Protein kinase C located in rat liver nudei. Partial purification and biochemical and immunochemical characterization. *J. Biol. Chem.* **264**, 1172–1179
24. Girard, P.R., Stevens, V.L., Blackshear, P.J., Merrill, A.H., Jr.,

Wood, J.G., and Kuo, J.F. (1987) Immunocytochemical evidence for phorbol ester-induced directional translocations of protein kinase C in HL60, K562, CHO, and E7SKS cells: possible role in differentiation. *Cancer Res.* **47**, 2892–2898
25. Thomas, T.P., Talwar, H.S., and Anderson, W.B. (1988) Phorbol ester-mediated association of protein kinase C to the nuclear fraction in NIH 3T3 cells. *Cancer Res.* **48**, 1910–1919
26. Lebkowski, J.S., McNally, M.A., Okarma, T.B., and Lerch, L.B. (1987) Inducible gene expression from multiple promoters by the tumor-promoting agent, PMA. *Nucleic Acids Res.* **15**, 9043–9055
27. Jobbagy, Z., Olah, Z., Petrovics, G., Eiden, M.V., Leverett, B.D., Dean, N.M., and Anderson, W.B. (1999) Up-regulation of the Pit-2 phosphate transporter/retrovirus receptor by protein kinase C epsilon. *J. Biol. Chem.* **274**, 7067–7071
28. Davis, R.J. and Czech, M.P. (1984) Tumor-promoting phorbol diesters mediate phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* **259**, 8545–8549