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Tumor promoters alter gene expression and protein phosphorylation in avian cells in culture

(phorbol esters/*src* gene/34- to 36-kilodalton protein/phosphotyrosine)

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ABSTRACT **We have investigated the effect of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on the synthesis and modification of polypeptides in normal avian cells and cells infected by wild-type and temperature-sensitive Rous sarcoma virus (RSV). Using two-dimensional gel electrophoresis, we have detected alterations in both the abundance of cellular polypeptides and in their phosphorylation that seem unique to TPA treatment. However, the state of phosphorylation of the major putative substrate for the action of the *src* gene-associated protein kinase, the 34- to 36-kilodalton protein, was not altered. Moreover, examination of the phosphorylated amino acid content of total cellular phosphoproteins revealed that the response to TPA was not associated with detectable increases in their phosphotyrosine content. These results make it unlikely that TPA acts by the activation of the phosphorylating activity of the cellular *proto-src* gene or by the activation of other cellular phosphotyrosine-specific kinases. We have shown previously that temperature-sensitive RSV-infected cells at nonpermissive temperature demonstrate an increased senstivity to TPA treatment [Bissell, M. J., Hatie, C. & Calvin, M. (1979) *Proc. Natl. Acad. Sci. USA* 76, 348–352]. Our present results indicate that this is not due to reactivation of the phosphorylating activity of the defective *src* gene product or to its leakiness, and they lend support to the notion of multistep viral carcinogenesis.**

A major achievement of tumor virology in the last few years has been the identification and characterization of the products of viral "oncogenes," which are responsible for the initiation and maintenance of malignant transformation (1). In the case of Rous sarcoma virus (RSV) the product of its transforming gene, *src*, has been shown to be tightly associated with a protein kinase (2, 3) that specifically phosphorylates tyrosine (4). Several putative substrates of this activity have been identified, including the 34- to 36-kilodalton (kDal) protein (5–7) and vinculin (8). However, the link between the function of the *src* gene and the pleiotropic changes in cellular phenotype that are associated with oncogenic transformation by RSV has remained elusive.

Uninfected normal vertebrate cells contain loci related to every retrovirus oncogene identified so far (1, 9). In the case of RSV, the cellular *sarc* (*proto-src* gene) has been shown to code for a product highly similar to that of the viral *src* gene (10, 11). Such results have led to proposals that malignant transformation of cells by retroviruses may be a consequence of gene dosage: By providing an efficient promoter for transcription, viruses overload cells with gene products that are similar to cellular gene products normally under strict quantitative control (3, 9). These proposals rest on the assumptions that the substrate specificity of the viral and cellular gene products are similar and that the products of the proto-oncogenes play an important role in cell differentiation and growth control (9).

One way to obtain insights into the possible role that these gene products and their substrates play in normal cellular functioning and how alterations in their activities bring about malignant transformation would be to identify modulators of expression or modification of such gene products other than tumor viruses. Treatment of cells in culture with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent tumor promoter in the two stage carcinogenesis system, induces pleiotropic changes similar to those associated with transformation by RSV (12). These observations have led a number of investigators to propose that the action of tumor promoters may be modulated via the product(s) of the *proto-src* gene (13, 14). Some aspects of this proposal have been tested previously (15).

In this paper we have investigated the effect of the TPA treatment of chicken embryonic fibroblasts (CEF) on polypeptide synthesis and protein phosphorylation and compared these changes to those that are characteristic for RSV-induced transformation (5). We also asked whether such effects are accompanied by alterations in two cellular parameters directly related to the action of the *src* gene—i.e., the alteration of the state of phosphorylation of the 34- to 36-kDal protein and an increase in phosphotyrosine content of cellular proteins.

MATERIALS AND METHODS

Cell Culture and Virus Infection. CEF were prepared as described (16, 17). The wild-type (wt) and the temperature-sensitive (ts) mutant of the Prague (PrA) strain of RSV, LA-24 (18), were focus purified in our laboratory. Secondary cultures were prepared on day 5 after seeding, and cultures were kept at 39°C. Most experiments were performed on secondary cells, seeded in medium 199 (17) supplemented with 2% tryptose phosphate broth and 0.5% heat-inactivated chicken serum. LA-24-infected cells were moved to 41.5°C 5–8 hr after secondary seeding. TPA (Consolidated Midland, Brewster, NY) was freshly diluted in medium 199 (1 μg/ml) from a concentrated stock solution in methanol (1 mg/ml). Cells treated with the equivalent amounts of methanol were used as controls.

Radiolabeling and Two-Dimensional Polyacrylamide Gel Electrophoresis. These techniques were as described (5), except that the labeling with [^{35}S]methionine was done in 35-mm-diameter dishes, in methionine-free medium 199 supplemented with 2% tryptose phosphate broth. TPA was present in the medium when TPA-treated cells were labeled. Cell lysates were subjected to nonequilibrium pH gradient electrophoresis followed by electrophoresis in the presence of sodium dodecyl sulfate as described (5).

Biochemical Assays. Glucose transport was measured by the uptake of ^{3}H-labeled 2-deoxy-D-glucose (New England Nu-

Abbreviations: kDal, kilodalton(s); TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CEF, chicken embryonic fibroblasts; RSV, Rous sarcoma virus; ts, temperature-sensitive; wt, wild-type; PrA, Prague strain of RSV, subgroup A; pp60^{src}, phosphorylated polypeptide product of the *src* gene.

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clear) as described (17). The phosphotyrosine content of phenol-soluble cellular polypeptides was determined according to the published methods (4) with slight modifications (19). Unlabeled marker phosphotyrosine was synthesized according to ref. 20.

RESULTS

Effect of TPA Treatment on Synthesis of Methionine-Containing Polypeptides. We examined the nature of TPA-induced changes in gene expression by comparing the patterns of polypeptides synthesized in TPA-treated and control cells. Examination of these patterns (Fig. 1) indicated that we could detect on the order of 900 polypeptides. The same basic pattern was found in several different experiments. TPA treatment did not result in the appearance or disappearance of any polypeptides that could be resolved by this technique.

TPA treatment did, however, lead both to apparent increases and decreases in the abundance of several polypeptides, in normal and wt-RSV-infected cells (Fig. 1 *B* and *D*). The most dramatic increases in abundance in TPA-treated cells were observed in the spots marked with circles. In addition to a drastic increase in the abundance of doublet at 72–74 kDal, there were increases in the abundance of a group of polypeptides at 47–52 kDal, near actin (a), and a spot at 42 kDal. Treatment of normal uninfected cells with TPA led to decreases in the abundance of several polypeptides, marked with squares (Fig. 1*B*). A doublet at 92–94 kDal and spots at 190 kDal, 70 kDal, and 58 kDal all showed a marked decrease in TPA-treated normal CEF. Most of the changes induced by TPA treatment were also observed in untreated wt-RSV-transformed cells (compare Fig. 1 *B* and *C*). It is difficult to ascertain whether TPA treatment of wt-RSV-infected cells led to decreases in abundance of the same polypeptides as in TPA-treated normal cells, because the abundance of these polypeptides is already decreased in wt-RSV-infected cells (squares, Fig. 1*C*). In addition, TPA treatment reproducibly induced changes in the abundance of several other polypeptides, not marked in these figures. Quantitation of these spots by liquid scintillation spectrometry (data not shown) confirmed our visual impressions. The time course of these changes was established by the analysis of cells treated with TPA for 4, 8, and 24 hr. This analysis indicated that the maximal changes in abundance occur somewhere between 8 and 24 hr of treatment (data not shown). When cells were treated with 4-*O*-methyl-TPA, a derivative of TPA that is not effective as a tumor promoter in whole animals (12), no changes in abundance of any of these polypeptides were observed (data not shown).

Effect of TPA Treatment on Phosphorylation of Cellular Polypeptides. It is currently thought that changes in cellular parameters associated with RSV-induced transformation are

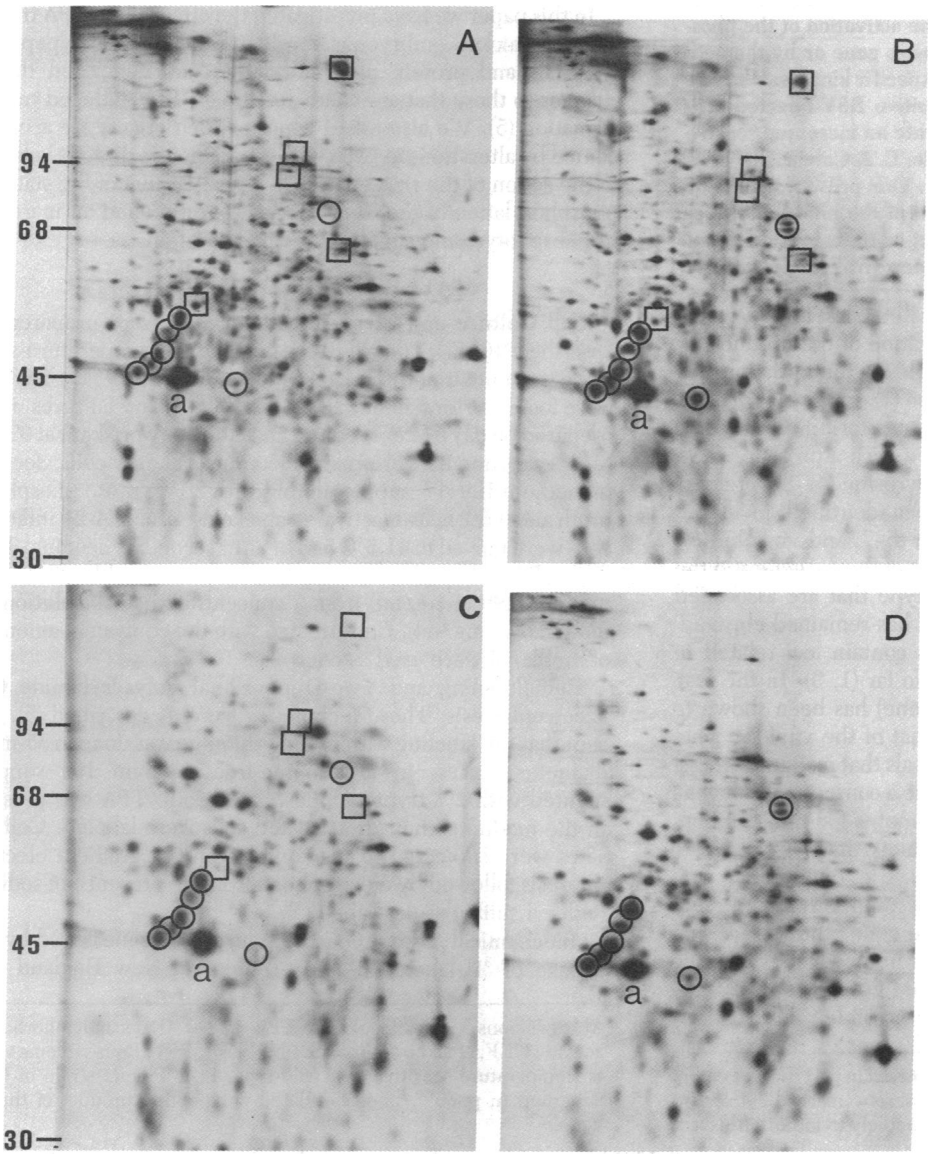

FIG. 1. Two-dimensional electrophoretic analysis of [^{35}S]methionine-labeled polypeptides synthesized in normal CEF (*A*), TPA-treated normal CEF (*B*), PrA-RSV-infected CEF (*C*), and TPA-treated PrA-RSV-infected CEF (*D*). Treatment with TPA was at 50 ng/ml for a total of 8 hr. Nonequilibrium pH gradient electrophoresis of 1.5×10^6 dpm of trichloroacetic acid-precipitable material in the horizontal dimension, with the acidic end at the left, followed by electrophoresis in the presence of sodium dodecyl sulfate in the vertical dimension, from top to bottom, were performed as described (5). The molecular weights of protein standards $\times 10^{-3}$ are indicated on the left. Circles mark polypeptides with increased abundance in TPA-treated cells, and squares mark polypeptides with decreased abundance in TPA-treated cells. The location of actin is marked *a*. The figure represents autoradiograms obtained after a 7-day exposure.

elicited by the action of the phosphorylated polypeptide product of the *src* gene (pp60^{src})-associated protein kinase on its cellular targets (21). It was therefore of interest to determine whether or not alterations in the state of phosphorylation of cellular proteins that are associated with transformation by RSV occur in TPA-treated cells as well.

We examined the pattern of protein phosphorylation in TPA-treated normal CEF and ts-RSV-infected CEF at nonpermissive temperatures (Fig. 2). Although treatment of normal CEF with TPA led to apparent increases in the state of phosphorylation of several polypeptides, marked by circles (Fig. 2*B*), none of these apparent changes were as dramatic as those observed in the pattern of [^{35}S]methionine-labeled polypeptides. Most significantly, we could not detect any changes in the state of phosphorylation of the 34- to 36-kDal polypeptide, the major identified substrate of the *src*-associated protein kinase (5–7). In addition, we could not detect any changes in the state of phosphorylation of any spots migrating in the region where the *proto-src* gene product would migrate (around 60 kDal). At the same time, we easily could detect the change in the state of phosphorylation of both pp60^{src} and the 34- to 36-kDal protein in cells infected with a ts mutant of RSV, LA-24, when the cells were shifted from nonpermissive (41.5°C) to permissive (35.0°C) temperatures for the duration of the pulse (Fig. 2*D*).

Under the conditions of these experiments, we observed significant changes in morphology (data not shown) and glucose transport in parallel cultures treated with TPA. These TPA-induced alterations were enhanced in ts-RSV-infected cells kept at nonpermissive temperatures as reported (16) and were found to be sensitive to cycloheximide in both types of cells (Fig. 3). Because the ts-RSV cells infected at nonpermissive temperatures constitute a more sensitive assay for the effect of TPA, they may allow the detection of minor changes in protein phosphorylation that could have gone unnoticed in TPA-treated normal CEF. We analyzed the pattern of protein phosphorylation in ts-RSV-infected cells at nonpermissive temperatures (Fig. 2*C*). The overall effects of TPA treatment are similar to those observed in TPA-treated normal CEF (Fig. 2*B*). Again, we could not detect any significant change in the state of phosphorylation of either the 34- to 36-kDal protein or pp60^{src}. The slight phosphorylation of the pp60^{src} spot in these cells is expected, because the inactive *src* product synthesized at nonpermissive temperatures is known to be phosphorylated at a serine residue (21).

Upon close examination of the phosphoprotein patterns, we detected a new spot in normal and ts-RSV-infected cells at non-

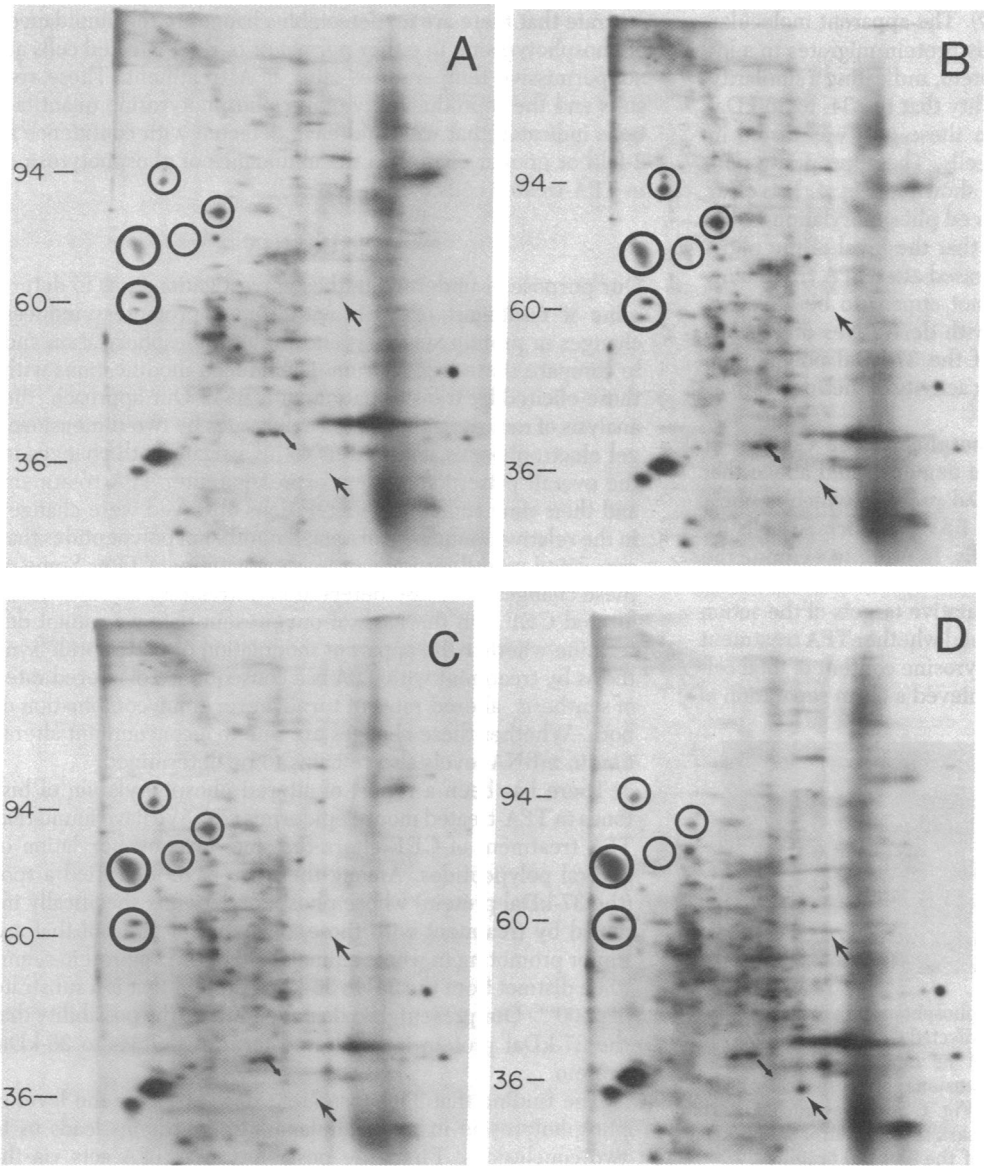

FIG. 2. Two-dimensional electrophoretic analysis of [^{32}P]orthophosphate-labeled polypeptides of normal CEF (*A*), normal CEF treated with TPA at 50 ng/ml (*B*), and ts-RSV-infected CEF treated with TPA at 50 ng/ml (*C*), all at 41.5°C, and untreated ts-RSV-infected CEF, shifted from 41.5°C to 35.0°C for 4 hr (*D*). Electrophoresis was performed as described for Fig. 1. Approximately 5 × 10^5 dpm of trichloroacetic acid-precipitable material was loaded on each gel. The figures represent autoradiographs obtained after a 3-day exposure. Upward-pointing arrows mark the location of pp60^{src} and the 36-kDal protein; the downward-pointing small arrow marks the location of the 37-kDal protein. Circles indicate polypeptides whose phosphorylation was altered in TPA-treated cells.

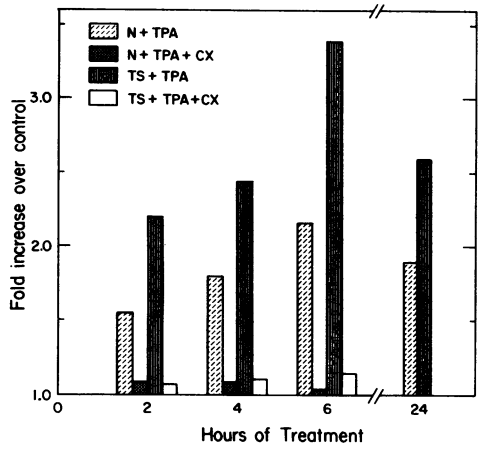

FIG. 3. Effect of TPA on glucose transport in normal (N) and ts-RSV (TS)-infected CEF at 41.5°C. Cells were treated with TPA at 50 ng/ml in the presence or absence of cycloheximide (CX) at 5 μg/ml. The inhibitor was added 45 min prior to treatment with TPA; under these conditions, there was a 97% inhibition of protein synthesis. For the 24-hr time point, only data from cells treated with TPA are shown because inhibitor-treated cells did not survive.

permissive temperatures, after treatment with TPA (small arrow, Fig. 2 *B* and *C*), but not in untreated ts-RSV-infected cells at permissive temperatures (Fig. 2*D*). The apparent molecular mass of this material is 37 kDal. This protein migrates to a location close to the 34- to 36-kDal protein, indicating a similarity in both size and charge. The possibility that the 34- to 36-kDal protein was migrating aberrantly in these gels was tested in TPA-treated wt-PrA-RSV-infected cells. The appropriate portions of the phosphoprotein gels are shown in Fig. 4. It is clear that the putative target of TPA-induced phosphorylation is distinct from the 36-kDal protein and that the level of the phosphorylation of 37-kDal protein increased after TPA treatment, while that of 36-kDal protein did not appear to be affected. Examination of this phenomenon with derivatives of TPA indicated that the phosphorylation of the 37-kDal polypeptide correlated with the tumor-promoting activity of such derivatives (data not shown).

Effect of TPA Treatment on Phosphotyrosine Content of Cellular Phosphoproteins. We have demonstrated above that the phosphorylation of 34- to 36-kDal protein, which is phosphorylated at a tyrosine residue in RSV-transformed cells (6, 7), is not changed in TPA-treated cells. In order to examine the possibility that the effect of TPA is associated with increased tyrosine phosphorylation of other putative targets of the action of the *src* kinase (22), we have examined whether TPA treatment leads to an increase in the phosphotyrosine content of total cellular phosphoproteins. We have achieved a clean separation of

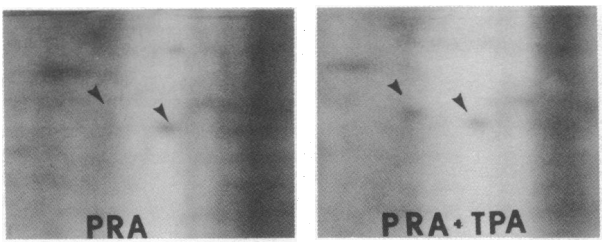

FIG. 4. Appearance of the 37-kDal phosphoprotein in TPA-treated wt-PrA-RSV-infected cells. PrA-RSV-infected CEF were treated with TPA at 50 ng/ml for a total of 24 hr and pulse labeled with [^{32}P]orthophosphate for 4 hr. Two-dimensional electrophoresis of cell lysates was performed as described for Fig. 1. The relevant sections of the gels are shown. Right arrowheads mark the location of the 36-kDal protein; left arrowheads, the position of the 37-kDal protein.

Table 1. Abundance of phosphotyrosine in total cellular phosphoproteins

Cells	Temp, °C	Phosphotyrosine, % Exp. 1	Exp. 2	Exp. 3	Exp. 4
Uninfected	41.5	0.03	0.04	0.05	0.04
Uninfected + TPA*	41.5	0.04	0.04	0.06	0.03
ts-RSV-infected	41.5	0.03	0.05	0.04	0.05
ts-RSV-infected + TPA*	41.5	0.03	0.04	0.05	0.05
ts-RSV-infected	35.0	0.31	0.27	0.26	0.17
ts-RSV-infected, 41.5°C	35.0†	—	—	—	0.18
ts-RSV-infected, 35.0°C	41.5†	—	—	—	0.03

Cells were labeled with [^{32}P]orthophosphate for 18 hr in the presence and absence of TPA. The three phosphorylated amino acids—phosphoserine, phosphothreonine, and phosphotyrosine—were separated and identified by ninhydrin staining of unlabeled markers. The spots were scraped off the plate, eluted, and assayed for radioactivity in an aqueous scintillator. The numbers in the table represent the percentage of total phosphorylated amino acid radioactivity found in the phosphotyrosine spot.

* Final concentration, 50 ng/ml.

† Shifted to this temperature for 2 hr after labeling at the temperature indicated in the first column for 16 hr.

the three types of phosphorylated amino acids in partial hydrolysates (not shown). The data summarized in Table 1 demonstrate that there are no detectable changes in the abundance of phosphotyrosine in either normal or ts-RSV-infected cells at nonpermissive temperatures after TPA treatment. These results and the reproducibility of our phosphotyrosine quantitations indicated that we could have detected with confidence a 2-fold or greater change in the abundance of phosphotyrosine in TPA-treated cells.

DISCUSSION

Our purpose in undertaking these investigations was to determine if treatment of CEF with tumor promoters induces changes in protein synthesis and protein phosphorylation and to compare these tumor promoter-induced modifications with those elicited by transformation with RSV. Our approach, the analysis of radioactively labeled cell lysates by two-dimensional gel electrophoresis, allowed us to investigate both changes in the overall pattern(s) of gene expression after TPA treatment and their time course. The alterations observed were changes in the relative abundance of a small number of polypeptides that correlated with the tumor-promoting activity of TPA. Some of these changes were also detected in untreated PrA-RSV-transformed CEF. On the basis of our present data we cannot determine whether the apparent modulation of polypeptide synthesis by treatment with TPA is a consequence of altered rates of synthesis, altered rates of turnover, or some combination of both. Whether these changes are due to a concomitant alteration in mRNA levels also remains to be determined.

There has been a report of altered phosphorylation of histones in TPA-treated mouse epidermis (23). We have found that TPA treatment of CEF alters the state of phosphorylation of several polypeptides. Among these we have identified a spot (the 37-kDal protein) whose phosphorylation is specifically induced by treatment with those analogues of phorbol that are tumor promoting in whole animals. This 37-kDal protein seems to be distinct from the 34- to 36-kDal protein that is a substrate of pp60^{src}. Our present data do not eliminate the possibility that the 37-kDal protein is an altered form of the 34- to 36-kDal protein.

The finding that TPA treatment does not alter the level of phosphotyrosine in total cellular phosphoproteins leads us to two conclusions. First, the possibility that TPA acts via the

modification of the state of phosphorylation of tyrosines in other, hitherto-unidentified, substrates of the *src* associated protein kinase appears unlikely. In addition, this result suggests that TPA does not act through the activation of other cellular phosphotyrosine-specific protein kinases (24, 25). It is still possible, however, that TPA treatment may lead to minor alterations in the phosphotyrosine content of a number of polypeptides, or a major alteration in the phosphotyrosine content of a few polypeptides with low abundance in the cell. Such changes would be below our limit of resolution. In addition, a transient increase in the phosphotyrosine content of proteins in TPA-treated cells would have gone undetected by our methods of analysis, given the known *in vivo* lability of this residue (26).

The results obtained with protein phosphorylation and phosphotyrosine analysis argue against the hypothesis that TPA action is modulated via the activity of the *proto-src* gene (13, 14). A similar conclusion was reached from the analysis of levels of *proto-src*-gene-related activity by the immunocomplex assay (15). However, *in vitro* assays do not always reflect the state of enzymatic activity in the intact cell (27). The approach taken in this investigation, in addition to constituting a more rigorous and sensitive test, allowed us to examine the possibility that TPA may act via alterations of the substrates of $pp60^{src}$ by means of an alternative pathway, a notion that has not received attention so far.

These experiments were designed to extend previous work in our laboratory which demonstrated that TPA-treated ts-RSV-infected cells at nonpermissive temperatures display a more faithful phenocopy of transformed cells than do uninfected cells treated with TPA, with regard to morphology, glucose uptake, inhibition of collagen synthesis, and growth under soft agar (16). The data presented in this paper eliminate the possibility that this effect was merely a consequence of the "leakiness" of the phosphorylating activity of the ts mutant of RSV at the nonpermissive temperature.

Overall consideration of our results leads us to two general conclusions. First, TPA treatment elicits specific changes in both polypeptide synthesis and protein phosphorylation. Some of these alterations are different from those observed in cells transformed by RSV. These latter modifications, effects of TPA treatment of CEF that are not manifest in RSV-transformed cells, may form a basis for an eventual understanding of the mode of action of TPA at the molecular level. Second, the observation that alteration in several transformation parameters can occur without changes in the state of phosphorylation of the substrates of $pp60^{src}$ or an increase in the abundance of total cellular phosphotyrosine has some important implications concerning the mechanism of the $pp60^{src}$-induced transformation in particular, and for the notion of viral carcinogenesis as a multistage process in general. The current notion concerning the mode of action of the *src* gene product is that the alterations in the state of phosphorylation of its putative targets result in a cascade of events that culminate in transformation (21). This cascade, however, may have multiple control points. The observations that the phosphorylation of the 34- to 36-kDal protein is not significantly altered in cells transformed with two other tumor viruses, avian myelocytomatosis virus and avian erythroblastosis virus (5), and that increased levels of phosphotyrosine are not found in all virally or chemically transformed cells (27) are indeed consistent with this notion. TPA treatment elicits changes in several transformation parameters, yet we have shown that this process occurs without the phosphorylation of the 34- to 36-kDal protein. Thus TPA may act at a different control point in the pathway of these pleiotropic changes. An alternative interpretation of our results is that $pp60^{src}$ kinase-induced phosphorylation of the 34- to 36-kDal protein, while specific to the action of the $pp60^{src}$ kinase, may not be involved in the primary control of these transformation parameters. In revertants of RSV-infected vole cells, which are normal by several criteria, a fully active $pp60^{src}$ (28) along with a fully phosphorylated 34- to 36-kDal protein is found (R. Erickson, personal communication). Taken together with the results presented in this paper, these observations suggest that $pp60^{src}$ may have multiple, dissectable specificities and lend strength to the notion that viral carcinogenesis may be a multistep process (16). Recent observations with DNA virus-induced transformation point to similar conclusions (29). The results presented in this paper should stimulate further interest in the dissection of the pathways of action of viral transforming genes and tumor promoters.

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