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Differential expression of an 80-kDa protein kinase C substrate in preneoplastic and neoplastic mouse JB6 cells

(phosphoprotein/tumor promotion/p80 antibody/tumor phenotype)

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ABSTRACT **An 80-kDa protein (p80), previously reported to be a major protein kinase C substrate in preneoplastic JB6 mouse epidermal cells, has been shown to be transiently phosphorylated by phorbol 12-*O*-tetradecanoate 13-acetate. Phosphorylation was maximal at 2 hr of phorbol 12-*O*-tetradecanoate 13-acetate treatment and returned to basal levels by 24 hr. In contrast, using a p80-specific antibody, we found that phorbol 12-*O*-tetradecanoate 13-acetate treatment produced no increase in p80 concentration. p80 showed a progressive decrease in JB6 cells during progression from a preneoplastic to neoplastic phenotype. The lack of p80 expression in neoplastic cells was not attributable to lack of protein kinase C; the protein kinase activity and protein concentration were similar in cells of all three phenotypes. When p80 mRNA was analyzed by hybridization to a putative p80 cDNA clone, its relative concentration paralleled that of p80 protein, with high levels present in preneoplastic JB6 cells, and little or no evidence for p80-hybridizing RNA in transformed cells. Thus, p80 appears to be regulated pretranslationally at the level of mRNA concentration during preneoplastic progression in mouse epidermal JB6 cells.**

Ligand binding to receptor protein kinases activates pathways that begin with protein phosphorylation and culminate in altered programs of gene expression related to growth or differentiation or neoplastic transformation (1–3). For protein kinase C, although certain substrates have been implicated in differentiation responses to phorbol esters (4), no substrates have as yet emerged whose presence, absence, or phosphorylation mediates neoplastic transformation.

The JB6 mouse epidermal cell variants provide a model for studying late-stage tumor promotion (5). These include clonal lines derived from a spontaneously immortalized population that are resistant (P^-) or sensitive (P^+) to tumor-promoter-induced anchorage independence and tumorigenicity as well as tumorigenic derivatives of P^+ cells (6, 7). P^- cells can be converted to P^+ cells by introduction of P^+ active promotion sensitivity genes (8, 9) and P^+ cells can be converted to neoplastically transformed cells by tumor promoter treatment (6, 7) or by introduction of transforming DNA (10). The phenotypic conversion P^- to P^+ to transformed can, therefore, be reasonably regarded as preneoplastic-to-neoplastic progression in JB6 cells.

Comparison of protein kinase C substrate phosphorylation in P^-, P^+, and transformed JB6 cells, after exposure to the protein kinase C-activating tumor promoter phorbol 12-*O*-tetradecanoate 13-acetate (TPA), revealed differential phosphorylation of only one of some 13 substrates—namely, an 80-kDa/pI 4.5 protein (p80) (11, 12). The change observed was a progressive decrease in p80 phosphorylation from P^- to transformed phenotype (11). p80 is a substrate for protein kinase C found in many cells and tissues including JB6 cells (11, 13). Unlike many other kinase substrates, it appears to be an exclusive protein kinase C substrate since no significant phosphorylation of it has been reported to occur in response to other kinases (13–15). Its function in phosphorylated or unphosphorylated form is currently unknown; studies of p80 in cells undergoing growth differentiation, or preneoplastic progression responses to protein kinase C activators are expected to be informative.

The present communication addresses the question of whether the p80 regulation observed during preneoplastic progression involves only phosphorylation or occurs at the level of mRNA and/or protein expression. The results indicate a progressive loss of p80 expression.

MATERIALS AND METHODS

Cells. The JB6 *in vitro* model system was derived from BALB/c mouse primary epidermal cells (5, 16). All cell lines were maintained in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine and 5% (vol/vol) fetal bovine serum. JB6 cells were exposed to TPA (10 ng/ml, 16 nM) for times indicated in each experiment.

Reagents. Antibody to protein kinase C was a gift from Karen L. Leach (Upjohn). Soluble p80 was purified as described (17). Tryptic and chymotryptic peptides (J.P. and D.K., unpublished results) were isolated using reverse-phase HPLC. An oligopeptide derived from the p80 protein sequence, having the sequence NH_2-Glu-Ala-Ala-Glu-Pro-Glu-Gln-Pro-Glu-Gln-Pro-Glu-Gln-Pro-Ala-Ala-COOH described in detail elsewhere (18) was synthesized by solid-phase methods (19). The purity of the synthetic peptide was demonstrated by its elution as a single peak on reverse-phase HPLC and amino acid composition. The peptide was coupled to keyhole limpet hemocyanin at a keyhole limpet hemocyanin/peptide weight ratio of 2:1. New Zealand White rabbits were immunized by intradermal injections of either HPLC-purified whole p80 or keyhole limpet hemocyanin-peptide emulsified in complete Freund's adjuvant. The peptide antibody obtained reacted with p80 antigen purified from rat brain. TPA was supplied by Chemicals for Cancer Research, Eden Prairie, MN. Amersham was the source of [^{32}P]-orthophosphate (carrier free).

***In Vivo* Protein Phosphorylation and Immune Precipitation.** JB6 cells were incubated in phosphate-free EMEM medium (Flow Laboratories), containing 50 μM sodium vanadate and 5% dialyzed fetal calf serum (Flow Laboratories) for 3 hr prior to labeling. Cells were labeled with [^{32}P]orthophosphate (200 μCi/ml; 1 Ci = 37 GBq) for 2 hr. When TPA-exposure times were >2 hr, the ^{32}P-labeling occurred during the

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Abbreviations: p80, 80-kDa protein; TPA, phorbol 12-*O*-tetradecanoate 13-acetate; P^- and P^+, resistant and sensitive cells, respectively.

terminal 2 hr of tumor promoter treatment. Cells were lysed in TNT buffer (20 mM Tris·HCl, pH 7.5/200 mM NaCl/1% Triton X-100) containing 2 mM EDTA, 2 mM EGTA, aprotinin (180 kallikrein units/ml), and 1 mM phenylmethylsulfonyl fluoride. After centrifuging the lysates for 10 min in an Eppendorff microcentrifuge, the supernatants were incubated with p80 peptide antiserum and protein A-Sepharose (Pharmacia). Immune precipitates were collected, washed, and analyzed on 10–20% gradient SDS/polyacrylamide gels.

Immunoblotting of 80 kDa. JB6 cells were lysed by boiling in lysis buffer [40% (vol/vol) glycerol/12% (vol/vol) 2-mercaptoethanol/8% (wt/vol) SDS/0.2 M Tris/1% bromophenol blue). Protein concentrations were determined by using the Pierce BCA protein assay. Proteins (20 μg) were separated by one-dimensional electrophoresis on SDS/10% polyacrylamide gels. The proteins were then transferred to nitrocellulose sheets at 200 mA for 5 hr by using the buffer system of Towbin *et al.* (20). Immunoblotting was performed with ^{125}I-labeled protein A using a 1:500 dilution of p80 peptide antiserum. Filters were washed in blotto buffer [5% (wt/vol) nonfat dry milk/0.2% Nonidet P-40/50 mM Tris·HCl, pH 8.0/2 mM $CaCl_2$/80 mM NaCl], dried, and exposed to Kodak XAR-5 film for 48 hr.

RNA Hybridization. Total cellular RNA was prepared by the guanidine hydrochloride method (21). RNA was fractionated by formaldehyde/gel electrophoresis (22), blotted onto nitrocellulose, and hybridized (23) with a nick-translated (24) putative p80 cDNA clone, designated 3.1.2. Specific activity of the probe was 5.0×10^8 cpm/μg and the probe was used at 1.6×10^7 cpm/ml. Filters were hybridized overnight at 42°C in 5× SSC, and the most stringent wash was 0.5× SSC at 55°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 15 min. Filters were exposed to Kodak XAR-5 film for 2 days.

Densitometry of Autoradiograms. Autoradiograms were scanned for their relative intensities of bands using an LKB Ultrascan XL laser densitometer.

RESULTS

Phosphorylation of p80 After Exposure to TPA. Exposure of JB6 cells to TPA has been shown to produce a transient increase in the phosphorylation of an 80-kDa protein (11). Since these experiments were performed using cell lysates separated by one-dimensional electrophoresis, the degree of p80 phosphorylation was uncertain due to comigration with other phosphoproteins of 80-kDa. To demonstrate that the 80-kDa protein inducibly phosphorylated by TPA was the protein kinase C substrate p80, we immunoprecipitated TPA-treated JB6 cell lysates with a peptide antiserum specific for p80. In the experiments described below, antiserum raised against the whole p80 (17) was also used (data not shown), and the results were identical to those shown. Fig. 1*A* shows JB6 P^- cells metabolically labeled with [^{3}H]lysine and immunoprecipitated with peptide antiserum (lane 2). This experiment demonstrated the specificity of this antiserum for p80 synthesized in JB6 cells. Fig. 1*B* showed the pattern of p80 phosphorylation (migration identical to *A*) in JB6 P^- cells when treated with TPA for 0, 2, 5, 8, and 24 hr. The results from three experiments showed there was an increase in p80 phosphorylation that showed a 6-fold maximum at 2 hr of TPA treatment (lane 4) and persisted until 5 hr of tumor promoter exposure (lane 6) with a return to basal levels by 24 hr (compare lanes 10 and 2). When JB6 P^- cells were labeled with [^{3}H]lysine followed by treatment with TPA, there was no observed increase in the intensity of the 80-kDa protein band (data not shown). If the antibody had recognized only the phosphorylated form, then the band intensity after 2 hr of treatment should have been 6-fold higher than observed in Fig. 1. It appears, therefore, that the antibody recognizes both phosphorylated and nonphosphorylated forms of p80 and can thus detect changes in amount of p80 regardless of whether phosphorylation is altered.

p80 Synthesis Is Not Increased by TPA. To determine whether the observed increase in p80 phosphorylation reflected an increase in synthesis or was strictly posttranslational, we exposed JB6 P^- cells to TPA for 0.5, 1, 4, and 24 hr. Cell lysates were then analyzed by immunoblotting for levels of p80 per 10^5 cells, by using peptide antiserum. The results of this experiment are shown in Fig. 2*A*. It is evident that the level of p80 did not increase after tumor promoter treatment but actually appeared to decrease after prolonged TPA exposure (24 hr). In the event that transiently induced synthesis occurred earlier than 30 min, we tested shorter TPA-exposure times (Fig. 2*B*). The results from this experiment further confirmed that p80 synthesis was not increased by exposure to TPA. In addition total cellular RNA, isolated from P^- cells after TPA treatment for 0, 4, and 24 hr, showed no difference in the level of p80-hybridizing RNA (data not shown) when probed with a putative p80 clone (see below).

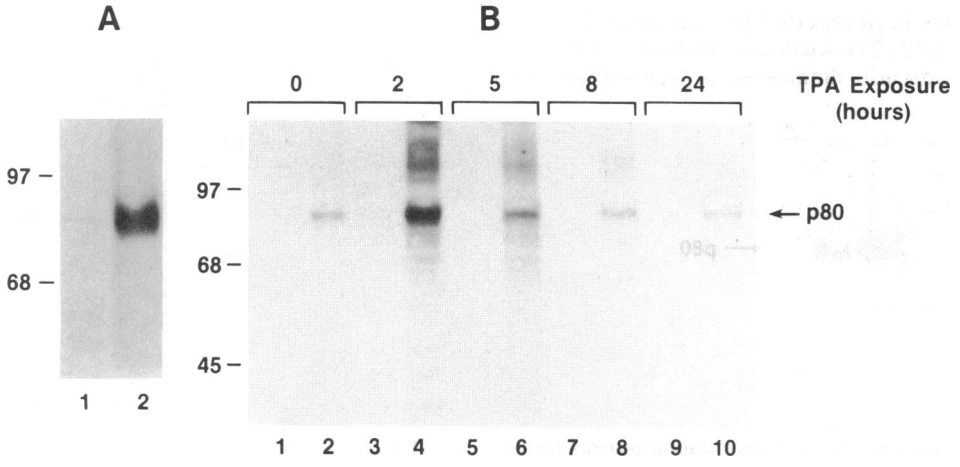

FIG. 1. TPA treatment increases the phosphorylation of p80. JB6 clone 30 cells (P^-) were labeled with either [^{3}H]lysine (100 μCi/ml) for 4 hr or [^{32}P]orthophosphate (200 μCi/ml) for 2 hr. The cells were treated with TPA (10 ng/ml) for various times. The cells were lysed in TNT buffer and immunoprecipitated with either preimmune or p80 peptide antiserum. Samples were loaded onto a SDS/10% polyacrylamide gel. The gel was divided; the [^{3}H]lysine portion was treated with Resolution (EM Laboratories) and then both halves were dried and exposed to Kodak XAR-5 film for 48 hr. All lanes contained an equivalent concentration of cell lysate (from 10^5 cells). (*A*) Cells were labeled for 4 hr with [^{3}H]lysine. Lanes: 1, preimmune; 2, p80 peptide antiserum. (*B*) Cells were labeled with [^{32}P]orthophosphate for 2 hr before harvesting. TPA exposure times (0–24 hr) are indicated at the top of the figure. Lanes: 1, 3, 5, 7, and 9, preimmune; 2, 4, 6, 8, and 10, p80 peptide antiserum. Molecular masses (in kDa) of protein size standards are indicated at the left. p80 is indicated at the right by an arrow.

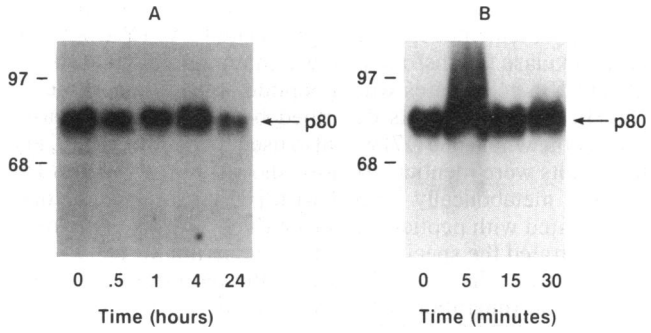

FIG. 2. TPA does not increase p80 levels. JB6 clone 30 cells (P^-) were treated with TPA (10 ng/ml) for various times, then lysed in Laemmli buffer (25), and electrophoresed on an SDS/10% polyacrylamide gel. The gel was then immunoblotted on a nitrocellulose sheet according to the procedure of Towbin *et al.* (20). The filter was incubated with p80 peptide antiserum (diluted 1:500) in blotto buffer followed by incubation with ^{125}I-labeled protein A (5.0×10^5 cpm). Each lane contained an equivalent concentration of cellular protein (20 μg). Filters were exposed to Kodak XAR-5 film for 48 hr. (*A*) TPA treatment of clone 30 cells for various times ranging from no treatment to 24 hr. (*B*) TPA treatment of clone 30 cells for various times ranging from no treatment to 30 min. Molecular masses (in kDa) of protein size standards are indicated at the left and p80 is indicated by arrows. TPA exposure times are indicated at the bottom of *A* and *B*. This experiment was repeated three times with similar results, showing maximal increases at 2 hr of 6-, 6.5-, and 7-fold as analyzed by densitometric analysis.

Differential Expression of p80 During Preneoplastic Progression. Earlier measurements using JB6 cells (11, 12) indicated that the level of phosphorylated p80 decreased when preneoplastic cells became transformed. To ascertain whether this could be attributed to differential expression of p80 in JB6 preneoplastic and neoplastic cells, we analyzed lysates from P^-, P^+, and transformed cells by immunoblotting with p80 peptide antiserum. Fig. 3 shows that there was a differential expression of p80, with a high level expressed in P^- cells, intermediate levels in P^+ cells, and little or no expression in neoplastically transformed cells. This observation has been extended to a second set of independently derived JB6 cell lines of P^-, P^+, and transformed phenotypes, and the results were identical to those shown in this experiment (data not shown). Since a coordinate down-regulation of p80 and protein kinase C has been reported for transformed mouse NIH 3T3 fibroblasts (26, 27), we determined whether protein kinase C protein was also diminished in transformed JB6 cells. Fig. 4 demonstrates that P^- and transformed JB6 cells contained an equivalent amount of protein kinase C. This is consistent with an observation from this laboratory (11) of similar protein kinase C activity in P^-, P^+, and transformed cells. Thus, a progressive decrease in p80 expression, without protein kinase C change, was seen during progression from P^- to the transformed phenotype, with a transformed cell value that was 5% of the P^- value.

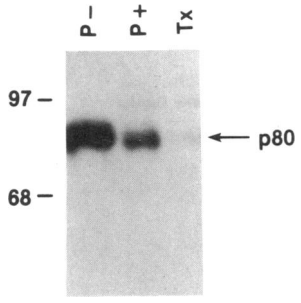

FIG. 3. Level of cellular p80 decreases during progression to tumor cell phenotype. Mouse JB6 cell clones 30 (lanes P^-), 41 (lane P^+), and RT101 (lane Tx) were lysed in Laemmli buffer (25) and loaded onto an SDS/10% polyacrylamide gel. Immunoblotting was then performed as in Fig. 2. Filters were exposed to Kodak XAR-5 film for 48 hr. Molecular masses (in kDa) of protein size standards are indicated on the left. The position of p80 is indicated by an arrow. Densitometric analysis of p80 levels measured in two experiments gave a mean value for P^+ of 53.5 ± 2.0% and a mean value for transformed cells of 5 ± 1.5% of the P^- value.

Hybridization of JB6 RNA to a Putative p80 Clone. To study possible mechanisms involved in the differential expression of p80 during preneoplastic progression of JB6 cells, it was necessary to obtain a DNA clone of the gene encoding p80. JB6 P^- cDNA was cloned into the *Eco*RI restriction site of the vector λ Zap (Stratagene), and the library was then screened with p80 peptide antiserum. One positive cDNA clone (3.1.2) containing a 2.4-kilobase insert was isolated (S.L.S. and N.H.C., unpublished data). To determine the authenticity of the putative p80 clone, we subcloned the 2.4-kilobase insert into the plasmid Bluescript (Stratagene) and expressed it in bacteria as a β-galactosidase fusion protein (S.L.S. and N.H.C., unpublished data). The bacterial fusion protein was analyzed on an SDS/10% polyacrylamide gel and its size appeared >80 kDa, a result expected if the β-galactosidase initiator were used during translation. In addition both the peptide and whole p80 antisera recognized the bacterial protein. Clone 3.1.2 was used as a probe in a hybridization reaction with total RNA isolated from P^-, P^+, and transformed JB6 cells. As shown in Fig. 5, when this putative p80 clone was used as a probe against P^- and P^+ total cellular RNA, a single 2.6-kb band was observed; but little or no hybridization appeared with RNA from transformed cells. Densitometric analysis from three experiments showed the mean value for the hybridizing band in P^+ RNA was 50 ± 2% and transformed RNA was 2.5 ± 0.4% of the P^- RNA value. This pattern was similar to that observed for the differential expression of p80 in these cells, thus suggesting regulation at the level of cytoplasmic mRNA concentration.

DISCUSSION

With the aid of specific p80 antiserum, we have now shown that TPA exposure of JB6 P^- cells caused a transient 6-fold increase in phosphorylation of the p80 protein kinase C substrate. The phosphorylation increase reached maximum

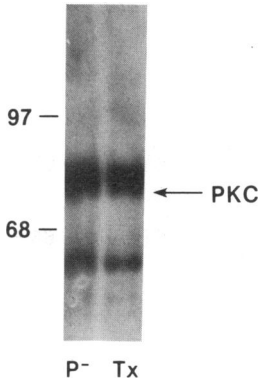

FIG. 4. Level of protein kinase C is similar in P^- and transformed JB6 cells. JB6 clone 30 cells (lane P^-) and RT101 cells (lane Tx) were lysed in Laemmli (25) buffer, boiled, and electrophoresed on an SDS/10% polyacrylamide gel. The gel was immunoblotted as stated in Fig. 2. Each lane contained an equivalent amount of protein (20 μg). The filter was exposed to Kodak XAR-5 film for 48 hr. Molecular masses (in kDa) of protein size standards are indicated at the left. The arrow points to the active form of protein kinase C (PKC), and the lower band is a degraded subunit. This experiment was repeated twice with similar results.

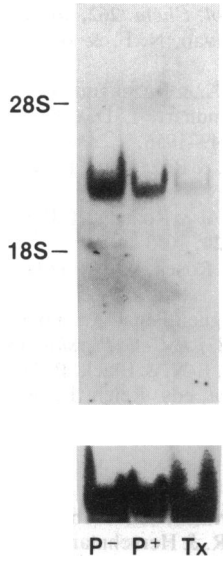

FIG. 5. Differential expression of RNA hybridizing to putative p80 clone 3.1.2 in JB6 P^- and transformed cells. (*Upper*) Clone 30 (lane P^-), 41 (lane P^+), and RT101 (lane Tx) total cellular RNAs were isolated according to the procedure of Deeley *et al.* (21), electrophoresed on a denaturing formaldehyde gel, and Northern blotted in 20× SSC. The nitrocellulose filter was hybridized (5× SSC/40% formamide/1× Denhart's solution) for 2 days at 42°C with a nick-translated clone 3.1.2 probe (10^8 cpm/μg of DNA). (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) The filter was washed according to the procedure of Thomas (23) with the most stringent wash being 0.5× SSC at 55°C. The filter was exposed to Kodak XAR-5 film with a Corning Lightning Plus screen for 2 days. Each lane contained 10 μg of total cellular RNA. Lanes: P^-, clone 30 RNA; P^+, clone 41 RNA; Tx, RT101 RNA. The positions of the 28S and 18S rRNAs are indicated at the left. The band in lanes 1 and 2 correspond to a 2.6-kilobase mRNA. (*Lower*) Hybridization of the same filter with an actin probe (10^8 cpm/μg of DNA) under conditions cited above. Similar results were obtained in three experiments.

at 2 hr then decreased to basal levels. This time course corresponded well with studies done in this laboratory (11) and by others (28) not utilizing p80 antibody. The decrease in p80 phosphorylation (Fig. 1) occurring after 24 hr of TPA treatment was paralleled in JB6 cells by a decrease in protein kinase C activity (29) and concentration (data not shown). This result correlated with findings demonstrating that treatment of cells with phorbol esters leads to progressive down-modulation of phorbol ester receptors (30), followed by a disappearance of protein kinase C activity (31–33).

What has not previously been shown is whether the TPA-induced phosphorylation of p80 in JB6 P^- cells was actually a consequence of an increase in synthesis. This study indicates that TPA treatment, under conditions that increase phosphorylation, produced no increase in the amount of p80. Thus phosphorylation is not driven by substrate concentration. The reason for the slight decrease in p80 expression after 24 hr of TPA treatment is unknown. Prolonged phorbol ester exposure may affect p80 by causing an increase in the synthesis of cellular proteases (34) thereby decreasing the stability of p80.

Our previous findings indicating differential basal and induced phosphorylation of p80 during preneoplastic progression in JB6 cells raised the question of whether this regulation was pretranslational or strictly posttranslational. The above results make it clear that there was not strictly posttranslational regulation: the amount of p80 showed a progressive decrease during progression to tumor phenotype. A putative p80 cDNA has been cloned by p80 antibody screening (S.L.S. and N.H.C., unpublished data) and used to analyze JB6 cell RNA. By using this clone as a probe, we found that when P^-, P^+, and transformed JB6 cells were compared, the level of p80 mRNA paralleled the level of p80, with little or no evidence for p80-hybridizing RNA in transformed cells. These results indicate that p80 is regulated at the level of mRNA concentration. Further studies should clarify whether the regulation of p80 gene expression is transcriptional or posttranscriptional.

Loss of p80 transformed cells has been reported by others; when NIH 3T3 (26) and BALB 3T3 (35) fibroblasts were transformed, either by oncogenes or by chemical carcinogens, the level of p80 was also shown to significantly decrease. In addition, transformed NIH 3T3 cells appeared to have reduced levels of protein kinase C activity compared to nontransformed cells (26, 27). Unlike the results observed in mouse 3T3 fibroblasts, protein kinase C activity (11) and protein concentration (Fig. 4) are similar in uninduced P^-, P^+, and transformed mouse JB6 epidermal cells.

Since neoplastically transformed cells differ from nontransformed cells in parameters related to unrestrained growth, the question might be raised as to whether changes in p80 are related generally to growth rather than specifically to transformation. Whether p80 synthesis can be attributed to noncycling G_0 cells present in the more normal P^- cells and absent in the transformed cells appears unlikely, however. All three cell lines were assayed in logarithmic phase and showed a 100% labeling index when labeled for one doubling time with [^{3}H]thymidine (data not shown). This suggests no G_0 population in P^-, P^+, or transformed JB6 cells. It would be of interest, however, to investigate possible cell cycle phase specificity of expression. Bishop *et al.* (36) have found that variants of 3T3 cells sensitive or insensitive to mitogenic stimulation by phorbol esters exhibit similar levels of p80. This discrepancy may be explained if p80 is on a promotion pathway and not on a mitogenesis pathway. In fact, mitogenesis has been dissociated from promotion of transformation in JB6 cells (7).

Finally, although the biochemical function of p80 is far from clarified by these experiments, the possibility suggests itself that p80 may function as a suppressor of neoplastic transformation that is in some way switched off during the P^- to transformed cell progression.

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