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Effects of TPA, Bryostatin 1, and Retinoic Acid on PO-B, AP-1, and AP-2 DNA Binding During HL-60 Differentiation

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Abstract PO-B was originally characterized as a transcriptional regulatory factor of the pro-opiomelanocortin (POMC) gene; however, it has become increasingly clear that this protein may be active in tissues outside the pituitary, since it is present in diverse cell types, including differentiated HL-60 promyelocytic leukemia cells. We previously showed that PO-B DNA-binding is progressively induced during differentiation of promyelomonocytic leukemic HL-60 cells to the macrophage-like lineage (with phorbol esters). We now report that PO-B DNA-binding in HL-60 cells is similarly induced during differentiation to the granulocytic lineage (with either retinoic acid or dimethylsulfoxide). Either a genetic or pharmacologic blockade of HL-60 differentiation prohibited these inductive effects. These studies have prompted our interest in the dynamics of other transcription factor changes during HL-60 differentiation. Of these, we observed that another transcription factor (AP-1) is also robustly induced at the DNA-binding level during macrophage-like HL-60 differentiation, but not during granulocytic differentiation. Conversely, the DNA-binding of the transcription factor AP-2 was slightly reduced by TPA-induced HL-60 differentiation but unchanged during granulocyte differentiation. From these data, we conclude that the induction of PO-B DNA binding is a general marker of HL-60 myelomonocytic differentiation, but that qualitative aspects of the induction of additional distinct transcription factors, such as AP-1, may contribute to lineage-specific determinants of cell fate. J. Cell. Biochem. 65:308–324.

Key words: PO-B; HL-60; differentiation; AP-1

Complex differentiation processes which contribute to the diversification of cell types within the organism ultimately involve the regulation of expression of a wide variety of genes. Our laboratory has been particularly interested in a transcription factor, PO-B, a protein which was originally identified as a major regulator of basal expression of the pro-opiomelanocortin (POMC) gene [Riegel et al., 1990]. The POMC gene is expressed tissue-specifically, but its restricted expression in the pituitary gland appears not to be mediated through PO-B, but instead through synergistic interactions between a number of regulatory elements present within the POMC promoter [Therrien and Drouin, 1991; Liu et al., 1992].

In contrast, our previous studies have demonstrated that PO-B is more likely a general transcription factor, present in many different cell types [Dobrenski et al., 1993]. Thus, it is likely that PO-B regulates a variety of genes outside the pituitary. Since PO-B activity, or at least DNA-binding ability, is dependent on phosphorylation state of the protein [Wellstein et al., 1991], then it is likely that signalling pathways in different tissues and cell types dictate the avidity of this protein for its DNA-binding element in various genes.

One extra-pituitary cell type in which the regulation of PO-B DNA-binding to its cognate element is apparent is the myelomonocytic precursor cell line HL-60. PO-B DNA binding is undetectable in undifferentiated HL-60 cells, but is induced during terminal differentiation of HL-60 cells to the macrophage-like lineage [Dobrenski et al., 1993]. We postulated that one component of the appearance of PO-B in a DNA-

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binding form was due to the progressive inactivation of MAP kinase (ERK) signalling during HL-60 differentiation [Dobrenski et al., 1993]. Thus, it appears likely that the activity of cellular kinases like ERKs and their cognate phosphatases control aspects of HL-60 differentiation through regulating the activity of transcription factors like PO-B.

HL-60 cells have proved to be a particularly useful model system for investigation of cell growth and differentiation processes since they may be induced to several different myelomonocytic lineages with different agents. In the presence of phorbol esters (TPA), retinoic acid (RA), or dimethylsulfoxide (DMSO), or 1,25-dihydroxyvitamin D3 ($1,25[OH]_2D_3$), HL-60 cells terminally differentiate in culture to the macrophage, granulocyte, or monocyte lineage, respectively [Rovera et al., 1979; Breitman et al., 1980; Miyaura et al., 1981]. Each lineage is readily identifiable by either enzymatic or morphological criteria, but in addition, a plethora of other cellular events have been linked to either the onset or the culmination of the HL-60 terminally differentiated state.

In the case of granulocytic differentiation, it is generally believed that RA-induced differentiation to this lineage is mediated through retinoic acid receptor (RAR) signalling, specifically through RAR-α [Collins et al., 1990; Tsai et al., 1992]. The other HL-60 granulocytic inducer, DMSO, appears to act through a different pathway which ultimately converges with the RA signalling pathway [Van Roozendaal et al., 1990]. However, downstream effects on transcription factors during the granulocytic differentiation program remain less clear.

Biochemical studies have conclusively shown that the HL-60 macrophagic inducer TPA is a potent stimulator of protein kinase C (PKC). Another compound which has gained some attention with regard to the study of HL-60 macrophagic differentiation is bryostatin 1, one of a series of macrocyclic lactones isolated from the marine organism *bugula neretina* [Pettit et al., 1982]. Bryostatin 1 also potently stimulates PKC in these cells but fails to induce their differentiation to this myeloid lineage. In addition, bryostatin 1 possesses the ability to block HL-60 macrophage development. Hence, bryostatin 1 is useful to assess the role of PKC in HL-60 differentiative processes as well as to dissect the potential roles of candidate proteins during HL-60 differentiation.

Molecular sequelae following TPA stimulation have been well studied. In general, TPA stimulates the expression of the early response genes of the c-jun and c-fos families whose protein products combine to form the transcription factor AP-1 [Lee et al., 1987; Angel et al., 1987]. This finding has been substantiated in HL-60 cells; both c-jun and c-fos expression are rapidly induced following TPA treatment [Sherman et al., 1990; Müller et al., 1985]. As a consequence, AP-1 DNA binding is also stimulated as a result of TPA exposure [Szabo et al., 1991; Mollinedo et al., 1993]. Another recent report demonstrated that AP-1 DNA binding is induced concurrent with the onset of ($1,25[OH]_2D_3$)-induced monocytic differentiation of HL-60 cells [Kolla and Studzinski, 1993], which qualitatively resembles TPA-induced macrophagic HL-60 differentiation.

In this report, we present an analysis comparing the DNA-binding properties of three transcription factors, PO-B, AP-1, and AP-2, throughout HL-60 differentiation. Induction and commitment to either the granulocytic or macrophagic differentiation programs in these cells was accompanied by an increase in DNA-binding activity for PO-B and AP-1, although the extent of induction for each lineage differed markedly between the two. Furthermore, either a genetic (differentiation-resistant cells) or pharmacologic (with bryostatin 1) blockade of HL-60 differentiation prohibited the DNA-binding inductions of both PO-B and AP-1. From these data, we conclude that the induction of both transcription factors is positively correlated with the differentiated state in HL-60 cells, presumably heralding the activation of as yet unidentified differentiation-related genes.

MATERIALS AND METHODS

Cell Culture and Extract Preparation

Low passage (<30) HL-60 (human promyelocytic leukemia) cells (from ATCC) were maintained in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL, Gaithersburg, MD), 20 mM glutamine, and 50 U/ml penicillin/streptomycin in a 37°C humidified 7% CO_2 atmosphere. RA/DMSO-resistant HL-60 cells [Gallagher et al., 1985] were maintained under identical conditions. TPA-resistant HL-60 cells [Tonetti et al., 1992] were grown in RPMI 1640 medium as above but supplemented with 15% fetal bovine serum.

HeLa cells (human cervical) were maintained at 37°C, 7% CO_2 in DMEM medium (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM glutamine, and 50 U/ml penicillin/streptomycin.

HL-60 cells (either low passage wild-type or resistant) (10^7) were treated with 100 nM all-trans retinoic acid (Sigma, St. Louis, MO), 1.25% DMSO, or 160 nM phorbol-12-myristate 13-acetate (TPA) (Sigma) in culture medium. For experiments with bryostatin 1, low passage wild-type HL-60 cells were treated with either 10 nM or 100 nM bryostatin 1 in culture medium. Cells were harvested at 24 or 48 h or re-fed with treatment medium after 48 h and then harvested at 96 h following treatment.

Small scale preparation of whole cell extracts from HL-60 cells was performed as described previously [Ausubel et al., 1989]. Briefly, 10,000,000 control or treated cells were centrifuged and washed with PBS. Cells were then washed twice in HEPES (10 mM, pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT, pelleted, and resuspended in 20 µl of 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM EDTA, 0.5 mM phenylmethylsulfonylflouride (PMSF), 0.5 mM DTT, and 0.1% NP-40. The suspension was incubated on ice for 10 min, mixed briefly and pelleted at 14,000 × g. The lysed cell supernatant was diluted with 50 µl of buffer D, containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Nuclear extracts were prepared by the method of Dignam and Roeder [Dignam et al., 1983]. Protein concentrations were determined by the method of Bradford [1976], and extracts were frozen (−70°C).

Western Blot Analysis

Extracts (5 µg) were resolved by SDS-PAGE, using 10% linear polyacrylamide gels. Prior to electrophoresis, proteins were denatured by boiling for 5 min in Tris-SDS-β-mercaptoethanol Seprasol (#SA100052, Integrated Separation Systems, Natick, MA) (0.5% SDS). Following electrophoresis, proteins were transferred to a nitrocellulose membrane by electroblotting for 1 h at 150 V in 25 mM Tris-Cl, pH 8.5, 192 mM glycine, and 20% methanol. The membrane was blocked at room temperature for 60 min in TBS-T (20 mM Tris-Cl, pH 7.5, 138 mM NaCl, 0.1% Tween-20), supplemented with 5% nonfat dried milk and 1% bovine serum albumin (BSA) (#NA934, Amersham, Buckinghamshire, UK). After washing briefly with TBS-T, the membrane was incubated at room temperature for 60 min with primary antibody in TBS-T supplemented with 1% BSA. Primary antibodies used included anti-c-Jun (1:1000), a polyclonal antiserum raised against amino acids 73-87 of v-Jun (Oncogene Science, Uniondale, NY); anti-Fos/Fra (1:2000), a polyclonal antiserum raised against amino acids 127–152 of rat Fos (kindly provided by Dr. M.J. Iadarola); and anti-JunB (1:2500), a polyclonal antiserum raised against amino acids 45–61 of mouse JunB (Santa Cruz Biotechnology, Santa Cruz, CA)). After repeated washes with TBS-T, the membrane was incubated at room temperature for 60 min with the secondary antibody, anti-rabbit Ig-HRP (1:2000) in TBS-T with 1% BSA. The membrane was then washed repeatedly with TBS-T, and immunoreactive species were detected by autoradiography with chemiluminescence, according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Electrophoretic Mobility Shift Analysis

Specific protein/DNA complexes were resolved under low-salt, non-denaturing conditions, as described previously [Fried and Crothers, 1981]. Briefly, whole cell extracts (1–25 µg) were incubated with 1–10 fmol of double-stranded oligonucleotide probe ([^{32}P] end-labeled with T4 polynucleotide kinase) representing the cognate PO-B element (5′-GAGAGAAGAGTGACAGGGA-3′), the AP-2 consensus element [Luscher et al., 1989] (5′-GATCGAACTGACCGCCCCGCGGC-CCGT-3′), or the consensus AP-1 element [Lee et al., 1987] (5′-CTAGTGATGAGTCAGCCG-GATC-3′). Poly (dI/dC) (Sigma) (1 µg) was added as non-specific competitor DNA for experiments with PO-B and AP-1, and poly (dA/dT) (1 µg/ml) was used in experiments with AP-2. For experiments with the PO-B and AP-2 elements, samples were incubated for 20 min at room temperature in 10 mM Tris, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol, and 1 mM DTT. For gel shift experiments with the AP-1 consensus element, samples were incubated for 20 min at room temperature in 20 mM HEPES, pH 7.9, 6 mM $MgCl_2$, 5% glycerol, 100 mM EDTA, 1 mM DTT, and 0.5 mM spermidine. Where indicated, unlabeled specific oligonucleotide was included in the incubation reaction in a 10×-fold or 100×-fold molar excess. Binding reactions were subjected to electrophoresis for

3 hours at 100 V in 12.5 mM Tris borate-3 mM EDTA running buffer. Following electrophoresis, gels were dried onto Whatman 3MM paper, and retarded complexes were detected by autoradiography.

RESULTS

PO-B and AP-1 DNA Binding During HL-60 Differentiation

We have previously described the DNA binding induction of transcription factor PO-B during TPA-induced differentiation of HL-60 cells to the macrophage-like lineage [Dobrenski et al., 1993]. We sought to compare and contrast the DNA-binding profiles of other transcription factors known to be involved in cellular proliferation and differentiation processes, such as AP-1. A previous study reported the induction of AP-1 DNA binding (within a few hours) as a result of TPA treatment [Szabo et al., 1991]. Our comparative analyses of PO-B and AP-1 DNA binding also revealed that certain species of AP-1 were induced relatively early in the differentiation of HL-60 cells to the macrophage-like lineage, but in addition, we observed a clear induction of distinct AP-1 DNA binding species after prolonged exposure (96 h) to TPA (Fig. 1A). In contrast to PO-B, a small amount of AP-1 binding species were present in untreated control cells (Fig. 1A, lane 1), but with TPA-induced differentiation, there was a notable change in both the extent of binding as well as the mobility of the shifted complexes (Fig. 1A, lanes 3–11).

Progressive continual exposure to 160 nM TPA induced at least three species which bound specifically to the AP-1 DNA element. Specificity of binding was confirmed by competition analysis with increasing amounts of added unlabelled AP-1 oligo (indicated by triangles). Repeated analyses of extracts from TPA-treated HL-60 cells have confirmed that AP-1 species 3 (lowest mobility) is clearly induced at 48 h and persists through 96 h TPA exposure, at which point the cells have maximally differentiated (Fig. 1A, lanes 6–10). We observed that following TPA treatment, in addition to complex 3, at least two other shifted protein complexes with less distinct electrophoretic mobilities could be resolved (complexes 1 and 2). Both complexes 1 and 2 were barely detectable, but constitutively present, in undifferentiated HL-60 cell extracts (Fig. 1A, lanes 1 and 2).

RA and DMSO treatment of HL-60 cells induce terminal differentiation to the granulocytic lineage. In contrast to the results with TPA, both RA and DMSO exhibited very little induction of AP-1 DNA binding. Only a slight induction of complexes 1 and 2 were observed in comparison to untreated cells (Fig. 1B, lanes 3–8 and lanes 11–16). For comparison, an equivalent amount of protein from 96 h TPA-treated HL-60 cell extracts is shown (Fig. 1B, lanes 17, 18). Western blot analysis revealed that levels of Fos, FosB, and several Fra protein constituents of the AP-1 complex are elevated in 96 h TPA-treated HL-60 cell extracts, whereas only a small amount of Jun, JunB, and only one to two Fra protein species were detected in 96 h RA- or DMSO-treated extracts (Fig. 1C). Time course analyses with TPA-treated HL-60 cells in fact revealed that while Jun and several Fra proteins were induced at relatively early time points (1–6 h, data not shown), only c-Fos and Fra-1 proteins were apparent with comparable kinetics to complex 3, suggesting that c-Fos/Fra-1 might be constituents of the AP-1 complex 3 that we have observed.

We also observed that PO-B DNA binding was induced during differentiation of HL-60 cells to the granulocytic lineage, with either RA or DMSO (Fig. 1D). In contrast to the lineage-specific extent of induction of AP-1 binding, however, PO-B was induced to approximately equivalent levels upon differentiation to either lineage, and we observed no change in the mobility of the shifted complex (Fig. 1D, lanes 4–12, top panel vs. middle panel vs. lower panel). Thus, while both PO-B and AP-1 binding to each of their cognate elements are induced during HL-60 differentiation, there appear to be major qualitative and quantitative differences in the lineage specificity of the extent of the inductions between these two transcription factors.

The induction of both PO-B and AP-1 DNA binding during HL-60 differentiation does not appear to represent a generalized induction of all transcription factors. Analysis of the DNA-binding properties of another transcription factor, AP-2, during HL-60 terminal differentiation, demonstrated that this transcription factor is down-regulated during HL-60 differentiation. Two specific AP-2 DNA binding species were detectable in untreated HL-60 cells, and both species are down-regulated with the macrophagic inducer TPA (Fig. 2A lane 2 vs. 5, 8,

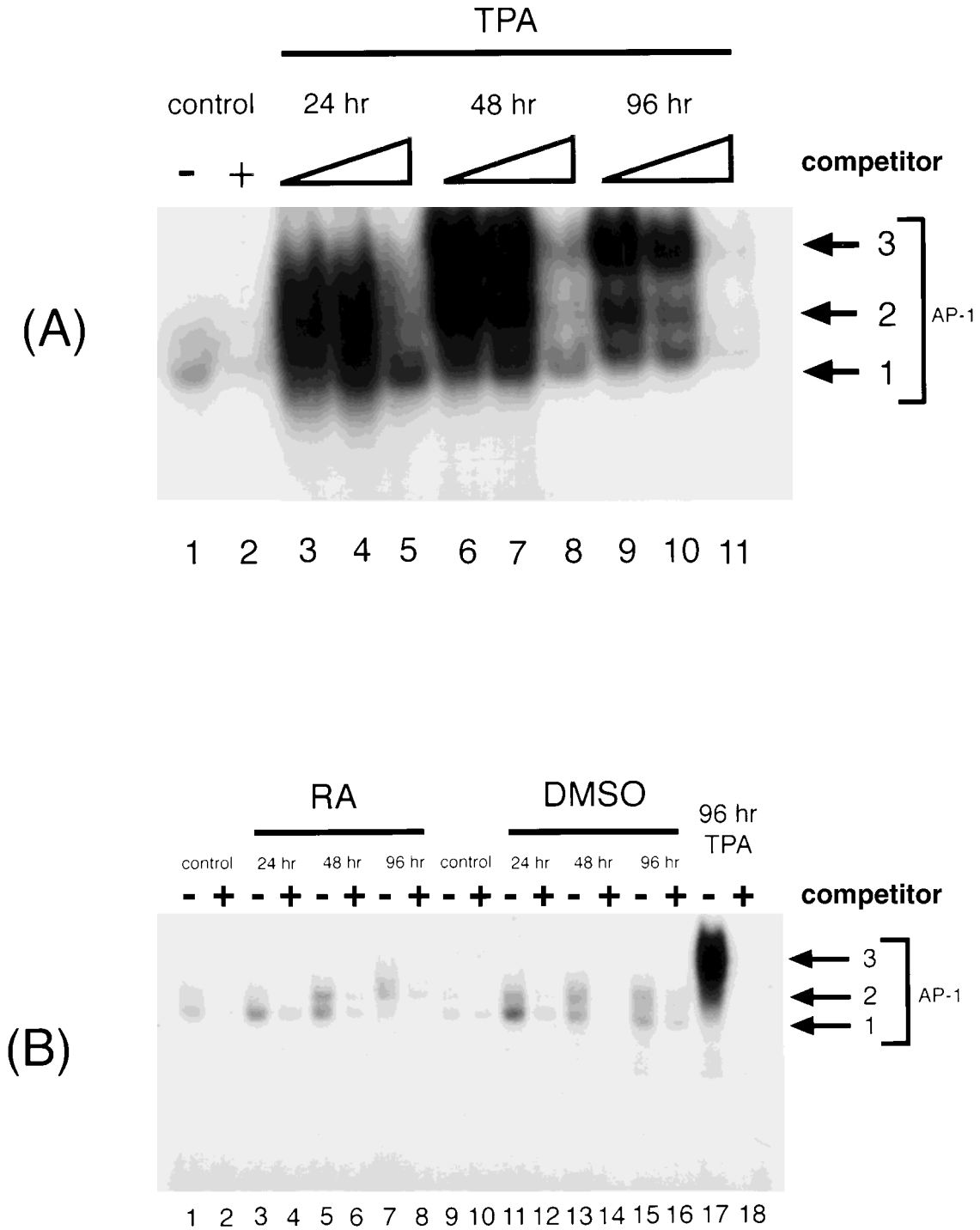
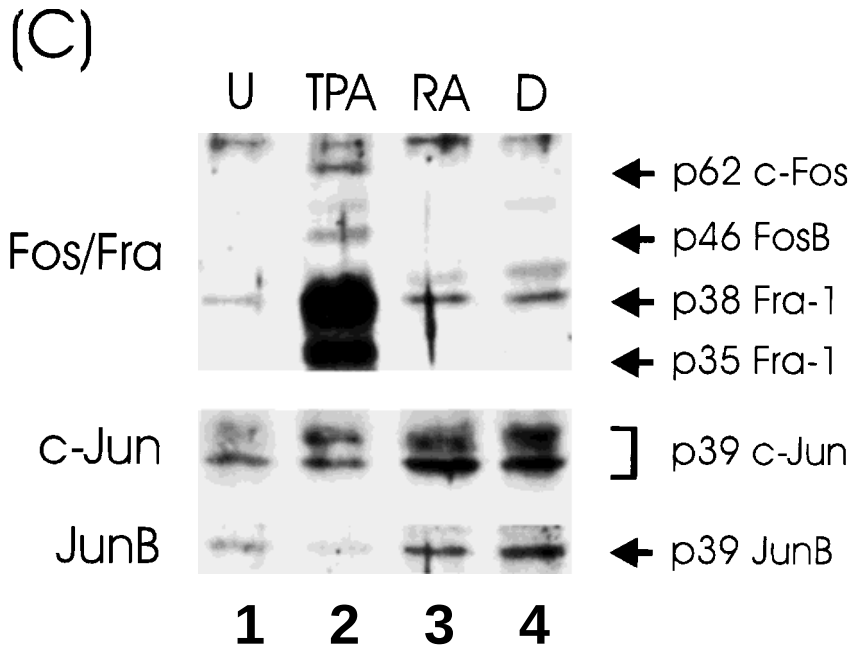


Fig. 1. AP-1 and PO-B DNA binding during differentiation of HL-60 cells with TPA, RA, and DMSO. AP-1 and PO-B DNA binding during HL-60 differentiation was assessed with the electrophoretic mobility shift assay (for details see Materials and Methods). Undifferentiated (control) HL-60 extracts were treated with TPA (A, C, D), RA (B–D), or DMSO (B–D) for 24, 48, or 96 h. Whole cell extracts were incubated with an end-labeled double-stranded PO-B or AP-1 oligonucleotide in the absence or presence of unlabeled competitor DNA, as indicated (−/+, denoting no competitor or 10×-fold molar excess, or triangles, denoting no competitor, 10×-fold, or 100×-fold molar excess unlabeled competitor DNA). **A:** AP-1 DNA binding during TPA-induced HL-60 macrophage-like differentiation (**lanes 3–11**). Specific complexes AP-1 1, 2, and 3 are indicated. AP-1 DNA binding in control (undifferentiated) HL-60 extracts is shown (**lanes 1, 2**). **B:** AP-1 DNA binding during RA- (**lanes 3–8**) or DMSO-induced (**lanes 11–16**) HL-60 granulocytic differentiation. AP-1 DNA binding in control (undifferentiated) HL-60 extracts is shown (**lanes 1, 2**) as is AP-1 DNA binding in 96 h TPA-differentiated HL-60 extracts (**lanes 17, 18**). **C:** Western analysis of AP-1 protein components during HL-60 differentiation. HL-60 cells were treated for 96 h with TPA, RA, or DMSO (**lanes 2–4,** respectively), resolved by SDS-PAGE, and subjected to Western blotting analysis, as described in Materials and Methods. **D:** PO-B DNA binding during TPA-, RA-, and DMSO-induced HL-60 differentiation (top, middle, and lower panels, respectively). AP-1 heterodimer constituents c-Fos, FosB, Fra-1, c-Jun, and JunB are indicated.



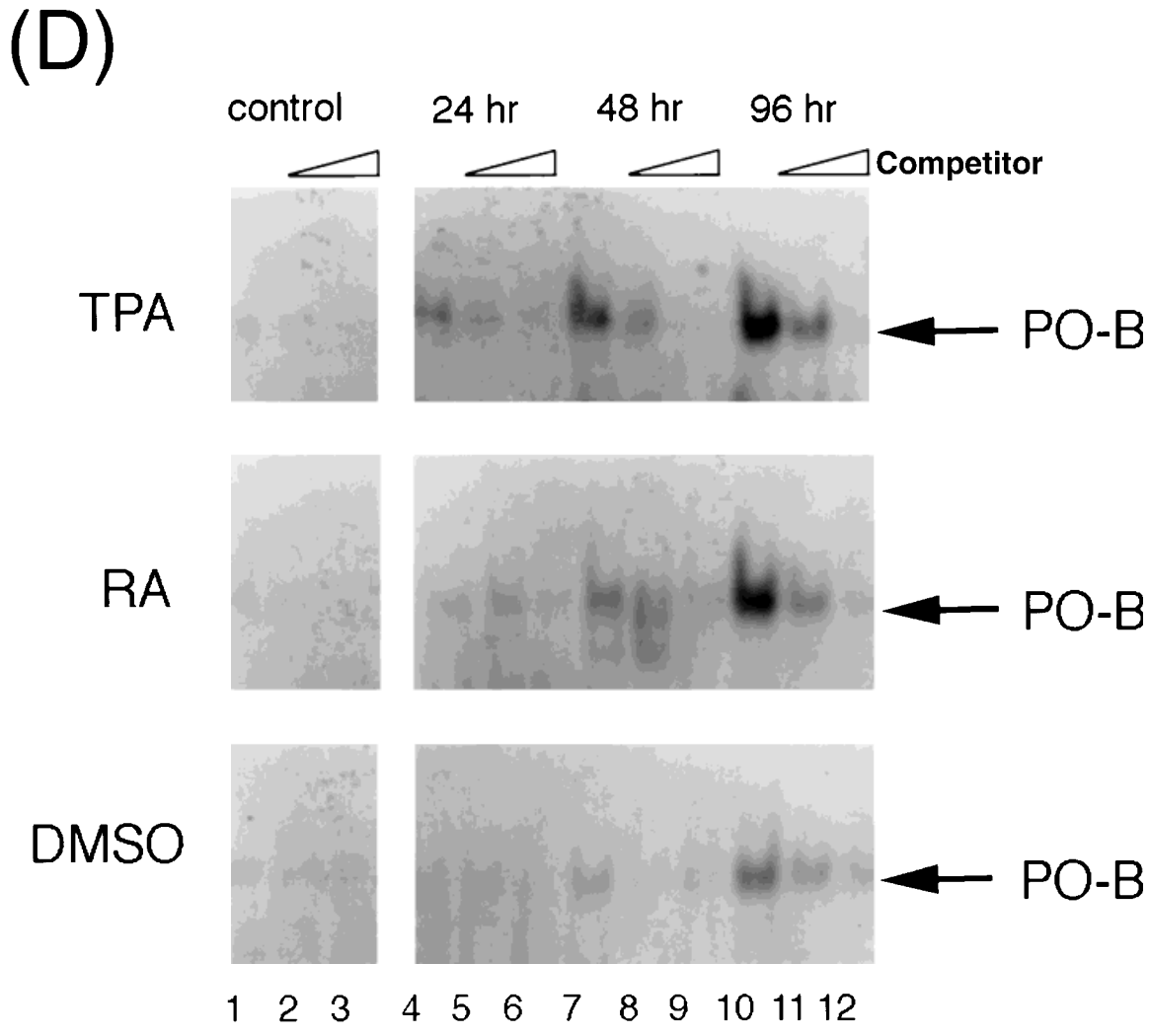


Figure 1. (Continued.)

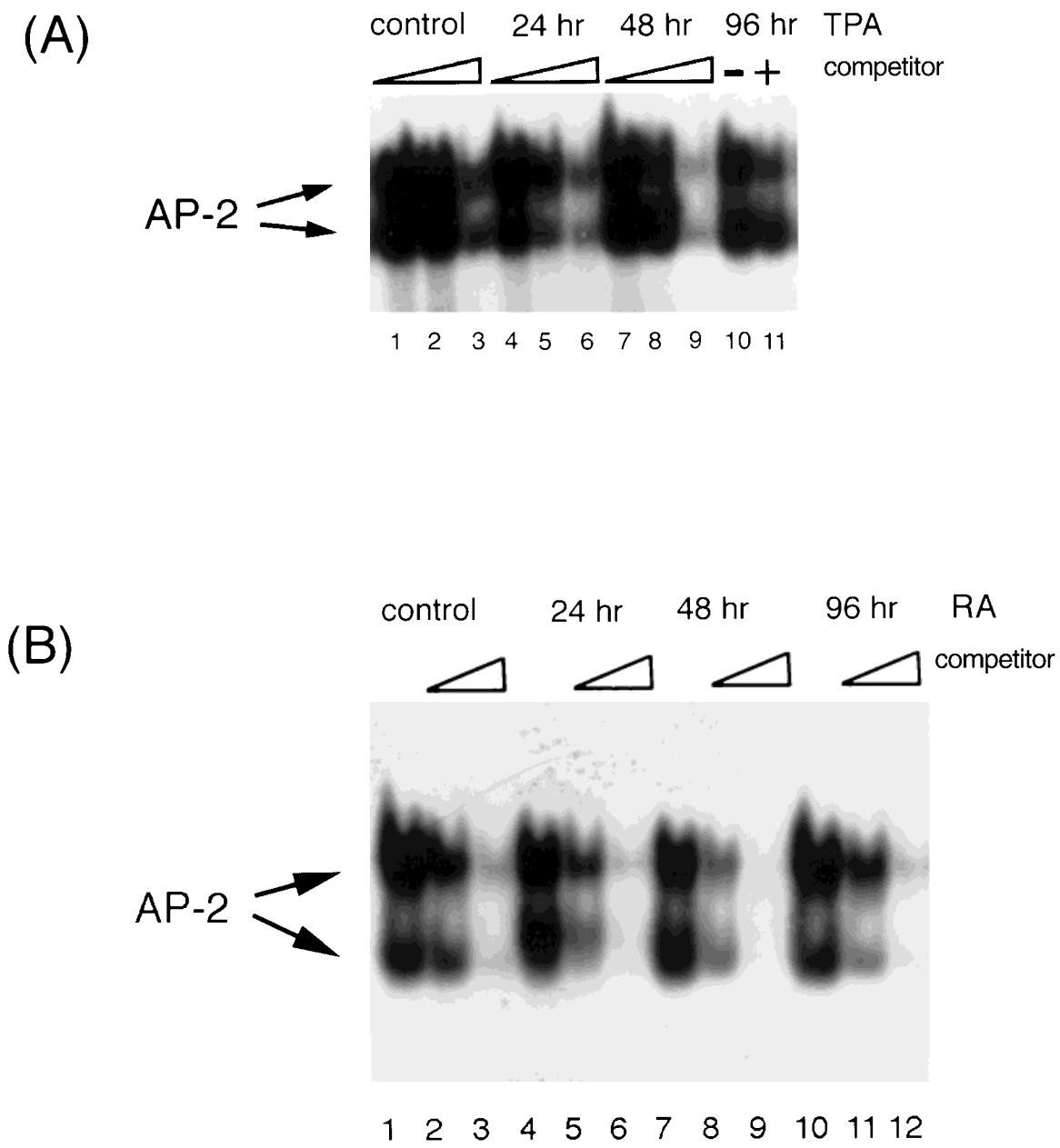


Fig. 2. AP-2 DNA binding during HL-60 differentiation. Electrophoretic mobility shift analysis with HL-60 extracts was performed with an end-labeled double-stranded AP-2 oligonucleotide, essentially as described in the legend to Figure 1. **A:** AP-2 DNA binding during TPA-induced HL-60 macrophage-like differentiation. **B:** AP-2 DNA binding during RA-induced HL-60 granulocytic differentiation. Two specific AP-2 protein-DNA complexes are indicated (arrows).

and 11). However, the granulocytic inducers RA (Fig. 2B) or DMSO (not shown) do not appear to effect AP-2 DNA binding. In addition, the observed changes in both PO-B and AP-1 DNA binding are likely differentiation-related, since treatment of other cell lines (CV-1 and ATt-20) with RA, DMSO, or TPA revealed no changes in the DNA-binding properties of these transcription factors to their cognate elements (not shown).

PO-B and AP-1 DNA Binding is Altered in Differentiation-Resistant HL-60 Cells

Since we observed that both transcription factors PO-B and AP-1 were induced during either granulocytic or macrophagic HL-60 differ-

entiation, we decided to further analyze the correlation between transcription factor induction and differentiation processes per se. As a first step, we obtained differentiation-resistant HL-60 cell lines representative of either lineage, and tested both PO-B and AP-1 DNA binding in these cells before and after treatment with differentiating agents.

HL-60 cells that were resistant to granulocytic differentiation with either RA or DMSO (RA/DMSO-resistant) [Gallagher et al., 1985] were treated with either of these compounds for 96 h and analyzed for both PO-B and AP-1 DNA binding. Treatment with either inducer was incapable of eliciting an induction of either transcription factor binding to its cognate recognition element (Fig. 3A,B). A non-specific species (Fig. 3A, "NS") present in these extracts bound to the PO-B element but was not competed with an unlabelled PO-B oligonucleotide. Low levels of AP-1 DNA binding persisted following treatment of these cells with either RA or DMSO but were indistinguishable from control levels (Fig. 3B, lanes 3,5 vs. lane 1). The robust AP-1 complex induced with 96 h TPA treatment is shown as a positive control for comparison (Fig. 3B, lane 7). Analysis of these mutant cells demonstrates that when granulocytic differentiation is genetically blocked, so is the induction of the transcription factors PO-B and AP-1, and that the defect must occur relatively early, prior to the appearance of the PO-B and AP-1 proteins in a DNA-binding form.

We also obtained TPA-resistant HL-60 cells [Tonetti et al., 1992], which do not fully differentiate to the macrophage-like lineage in response to TPA treatment in culture. Interestingly, we found that these cells exhibited constitutive levels of both PO-B and AP-1 DNA binding (Fig. 3C,D). We observed high levels of PO-B DNA binding in untreated TPA-resistant HL-60 cells which did not significantly change during exposure of the cells to TPA (Fig. 3C), although there was a small decrease in PO-B DNA binding at 48 h TPA treatment which was reversed after 96 h (Fig. 3C, lane 7 vs. lane 10).

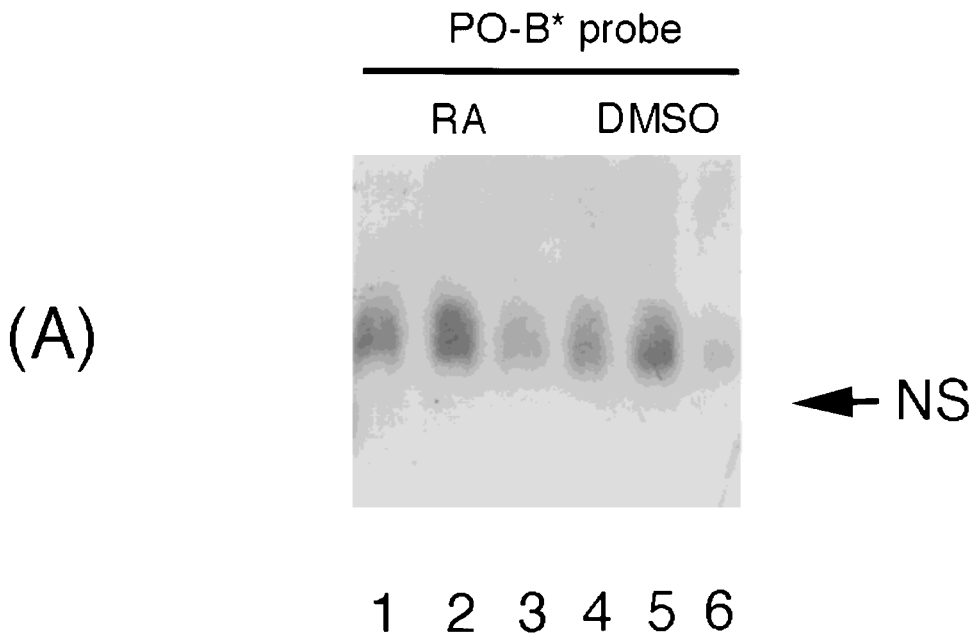
Levels of AP-1 protein were elevated in untreated TPA-resistant HL-60 cells [unpublished observation], and as with PO-B, were not altered during TPA treatment of these cells (Fig. 3D, lanes 4,6,8 vs. lane 1). Interestingly, however, the appearance of the AP-1 complex in the mobility shift gel was considerably different in shape, and more uniform, than the AP-1 complexes induced during either granulocytic or macrophagic HL-60 differentiation of wild-type cells (Fig. 1A). This might be explained by the differential induction of members of the Jun family of proteins in these two cell types. Indeed, it has been reported that the induction of both c-jun and junB mRNA is both temporally and quantitatively distinct in wild-type vs. TPA-resistant HL-60 cells [Tonetti et al., 1992].

Pharmacologic Blockade of HL-60 Differentiation Prevents the Induction of PO-B and AP-1 DNA Binding

Since the mutation in TPA-resistant HL-60 cells appeared to occur after the inductions of both PO-B and AP-1 DNA binding, we next decided to block TPA-induced macrophagic differentiation pharmacologically with bryostatin 1. Bryostatin 1 and phorbol esters such as TPA share several properties but have opposite effects on HL-60 differentiation. Like TPA, bryostatin 1 potently stimulates PKC in these cells but fails to induce their differentiation to the macrophage-like lineage. In fact, bryostatin 1 is capable of preventing TPA-induced macrophage development in HL-60 cells [Kraft et al., 1986], consistent with its general behavior as a TPA antagonist in other cell systems [Dell'Aquila et al., 1987; Mackanos et al., 1991; Dale et al., 1989]. Alone, at high concentrations (100 nM), bryostatin 1 does not promote HL-60 differentiation, but at low doses (10 nM) exerts a mild differentiative response in these cells [Stone et al., 1988].

It has been previously reported that TPA and bryostatin 1 exert similar effects on protein kinase C (PKC), in that they both promote the translocation of this enzyme from the cytosolic to the particulate fraction [Kraft et al., 1986]. Indeed, it has been reported that bryostatin 1 induces this rapid translocation even faster than TPA.

The effects of bryostatin 1 on HL-60 differentiation, as assessed morphologically, were also consistent with previous reports. Treatment of HL-60 cells with 100 nM bryostatin 1 was incapable of inducing macrophage development as assessed by cell adherence and the morphological changes concomitant with differentiation. (Fig. 4, panel d). In contrast, TPA treatment resulted in marked growth inhibition and cell adherence (Fig. 4, panel b). Low doses of bryostatin 1 (10 nM) have been reported to induce a mild differentiative response in HL-60



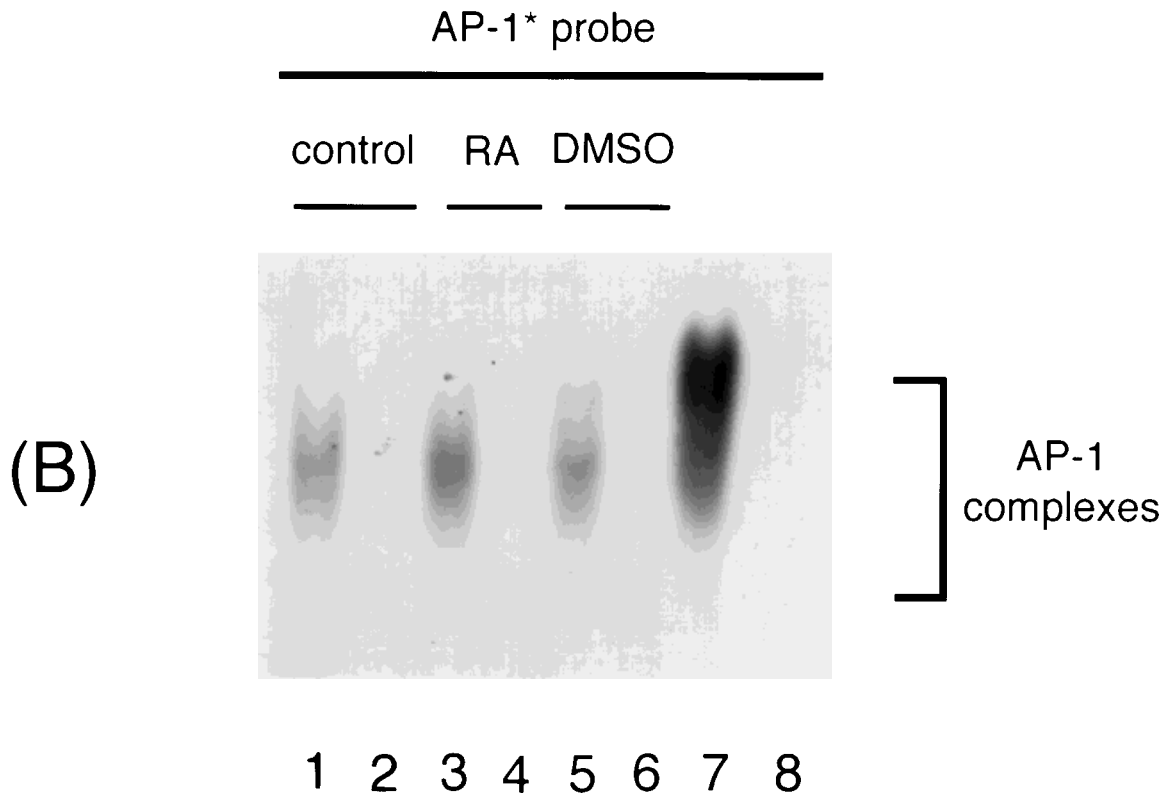


Fig. 3. AP-1 and PO-B DNA binding in differentiation-resistant HL-60 cells. AP-1 or PO-B DNA binding in HL-60 RA/DMSO-resistant (A, B) or TPA-resistant (C, D) was visualized by electrophoretic mobility shift analysis, as described in the legend to Figure 1. **A:** PO-B DNA binding in HL-60 RA/DMSO-resistant cells. Cells were treated with RA (**lanes 1–3**) or DMSO (**lanes 4–6**) for 96 h, as described in Materials and Methods. **B:** AP-1 DNA binding in HL-60 RA/DMSO-resistant cells. Cells were treated with RA (**lanes 3, 4**) or DMSO (**lanes 5, 6**) for 96 h, as described in Materials in Methods. AP-1 DNA binding in control cell extracts as well as in 96 h TPA-differentiated cell extracts are shown for comparison (**lanes 1, 2** and **lanes 7, 8,** respectively). **C:** PO-B DNA binding in TPA-resistant HL-60 cells. Cells were treated for 0–96 h (**lanes 1–12**) with TPA, as described in Materials and Methods. Specific PO-B binding is indicated (arrow) as is a non-specific species (NS). For comparison, PO-B protein in HeLa extracts is shown (**lane 13**). **D:** AP-1 DNA binding in TPA-resistant HL-60 cells. Cells were treated for 0–96 h (**lanes 1–9**) with TPA, as described in Materials and Methods. Specific AP-1 binding is indicated (bracket).

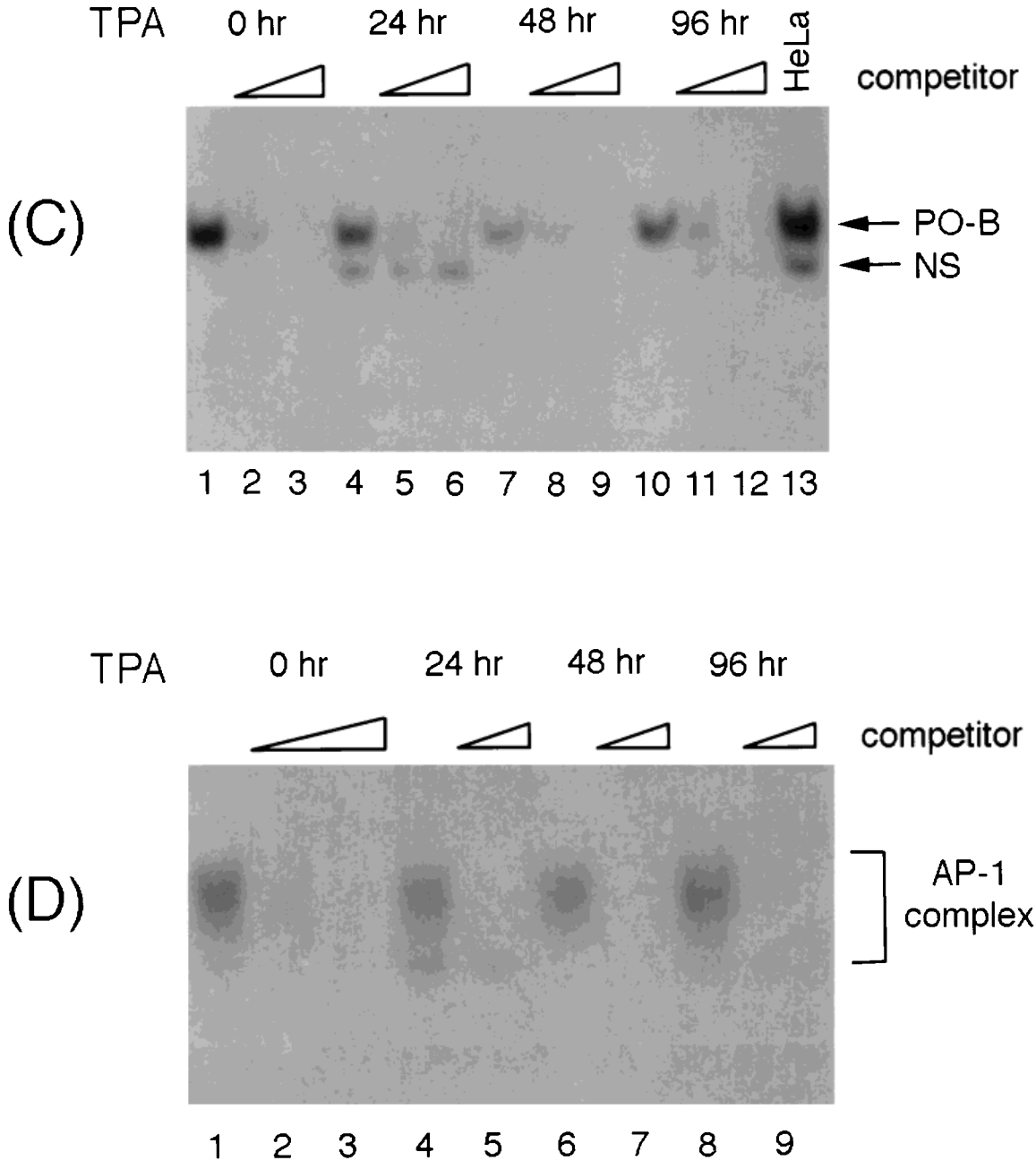


Figure 3. (Continued.)

cells which is characterized by an increase in the number of adhered cells as well as the appearance of some cytochemical markers of macrophagic differentiation [Stone et al., 1988]. Treatment of HL-60 cells with 10 nM bryostatin 1 for 96 h did result in approximately 10% cell adherence compared with about 80% adherence with TPA (Fig. 4, panel c vs. panel b). Co-culture experiments in which HL-60 cells were treated simultaneously with both bryostatin 1 (100 nM) and TPA confirmed that high dose bryostatin 1 was capable of blocking TPA-induced differentiation (Fig. 4, panel e vs. panel b).

Consistent with its ability to block HL-60 macrophagic differentiation, bryostatin 1 blunted the DNA-binding inductions of both PO-B and AP-1. While 100 nM bryostatin 1 did not completely abolish either PO-B or AP-1 DNA binding, the extent of specific binding of

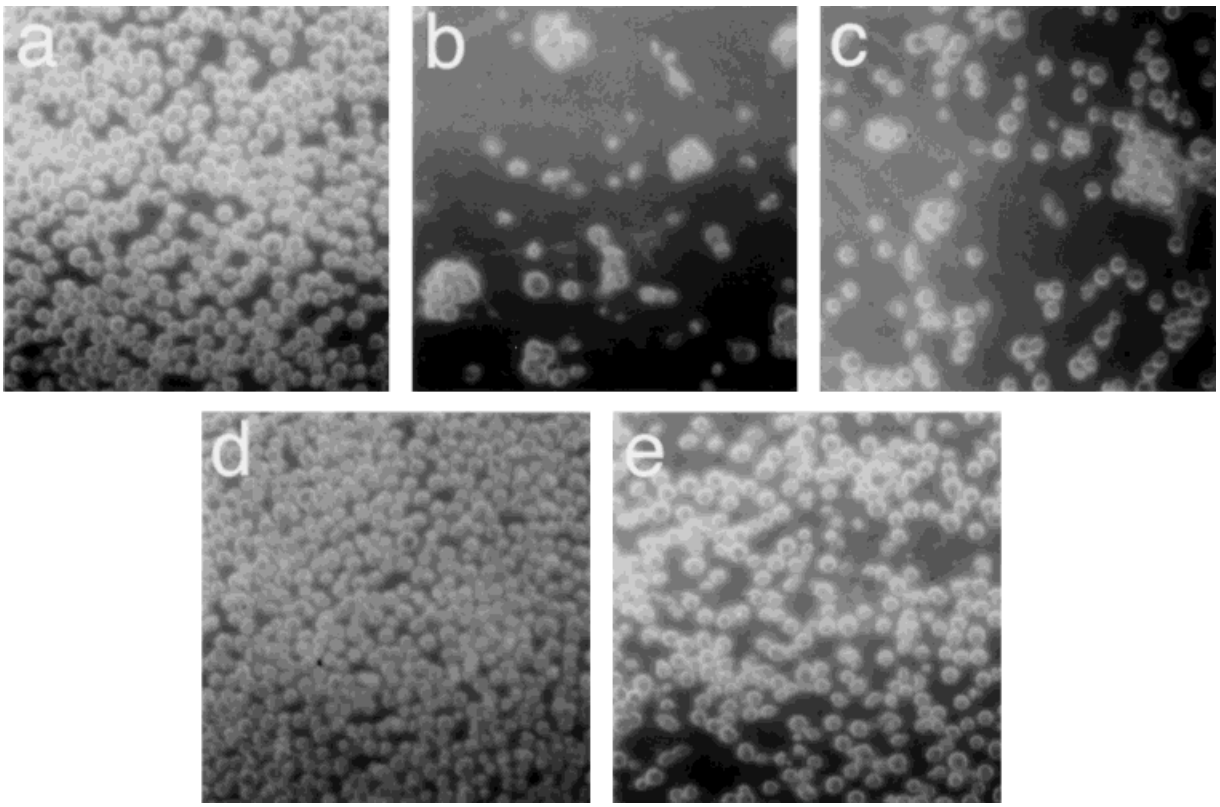


Fig. 4. Morphological effects of TPA and bryostatin 1 on HL-60 cell macrophagic differentiation. Low passage (approx. p30) undifferentiated HL-60 cells were treated in vivo for 96 h with 160 nM TPA (**b**), 10 nM bryostatin 1 (**c**), 100 nM bryostatin 1 (**d**), or 160 nM TPA and 100 nM bryostatin 1 together (**e**), then photographed. Control (undifferentiated) cells are shown for comparison (**a**).

either factor to its cognate element was markedly diminished as a result of exposure to both compounds simultaneously (Fig. 5A, lanes 7, 8 vs. Fig. 1D and Fig. 5B, lanes 13–18 vs. lanes 4–12). Interestingly, AP-1 complex 3 appeared to be the most sensitive to bryostatin 1 inhibition.

The Effects of Bryostatin 1 on PO-B and AP-1 DNA Binding in HL-60 Cells

Consistent with its inability to elicit HL-60 macrophage-like development, 100 nM bryostatin 1 alone was unable to induce either PO-B or AP-1 DNA binding (Fig. 6). A non-specific species which bound to the PO-B element was detectable after 24–48 h bryostatin 1 exposure but consistently disappeared after 96 h treatment (Fig. 6A, lanes 1–6 vs. lanes 7–9). Similar to PO-B, AP-1 DNA binding was unaffected with 100 nM bryostatin 1 treatment (Fig. 6B).

Lower doses of bryostatin 1 (10 nM), which promote a mild differentiative response in HL-60 cells, had variable effects upon PO-B and AP-1 DNA binding (Fig. 6C,D). Similar to the effects we observed with 100 nM bryostatin 1, 10 nM bryostatin 1 was incapable of inducing PO-B DNA binding (Fig. 6C), although the same non-specific species induced by 100 nM bryostatin 1 was apparent. In contrast, AP-1 DNA binding was mildly induced with 10 nM bryostatin 1 exposure (Fig. 6D). Interestingly, however, this dose of bryostatin 1 was only capable of inducing AP-1 complexes 1 and 2, which were apparent 24–48 h following bryostatin 1 exposure (Fig. 6D, lanes 1–6), and the extent of induction was significantly reduced compared to what we observed with TPA treatment (Figs. 1A, 5B). In addition, AP-1 complex 3, which we observed only after 48–96 h exposure to either RA or TPA was clearly not apparent following 10 nM bryostatin 1 treatment. This result raises questions about the significance of AP-1 complex 3, especially its precise relationship to terminal differentiation in HL-60 cells. Furthermore, these experiments demonstrate that there are differences

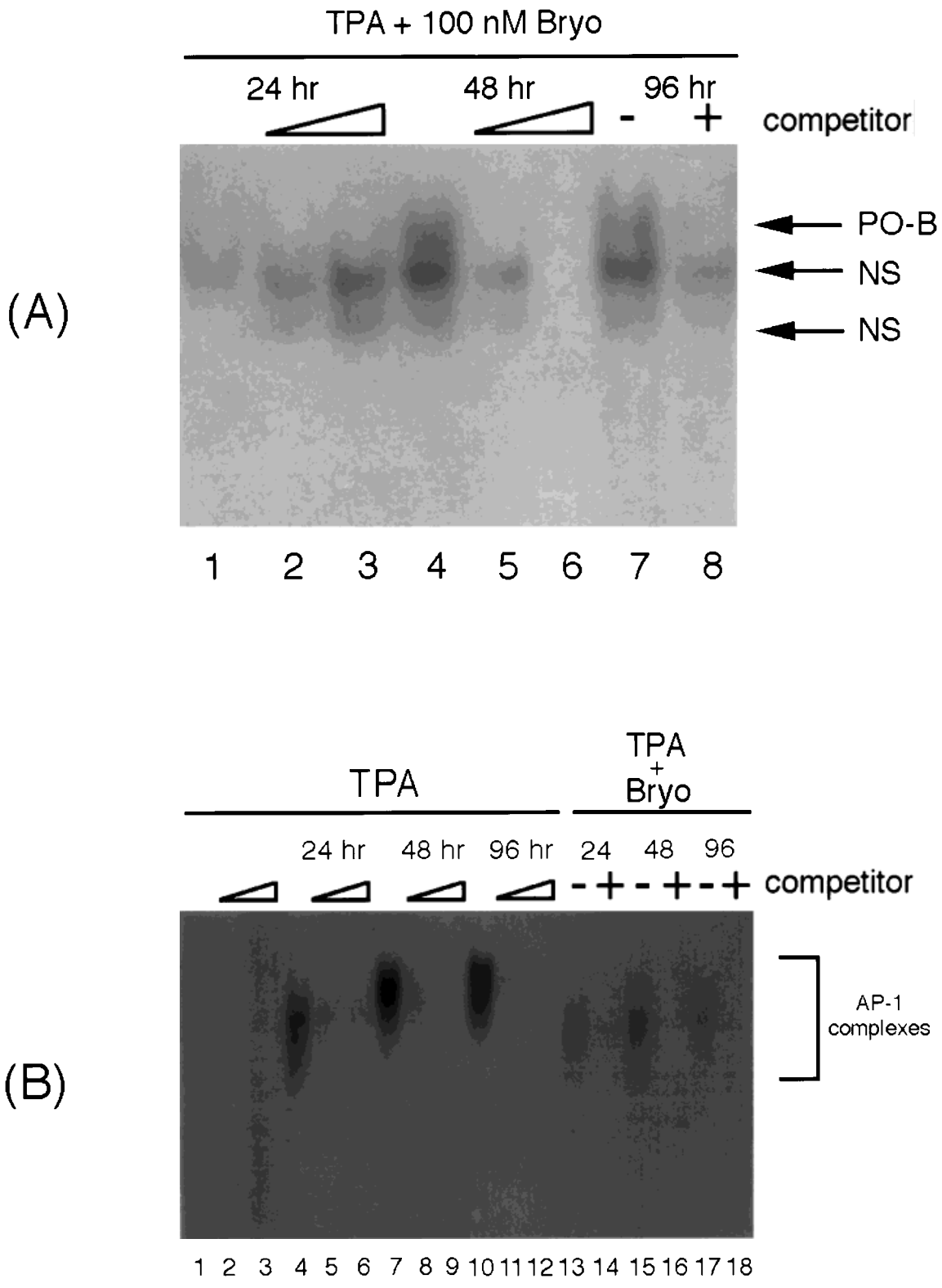


Fig. 5. PO-B and AP-1 DNA binding in HL-60 cells co-cultured with both TPA and bryostatin 1. HL-60 cells were treated for 24–96 h with both TPA (160 nM) and bryostatin 1 (100 nM). Whole cell extracts were prepared from each time point and analyzed with the electrophoretic mobility shift assay (see legend to Fig. 1). **A:** PO-B DNA binding in TPA + 100 nM bryostatin 1-treated cells. PO-B and non-specific (NS) binding species are each indicated. **B:** AP-1 DNA binding in TPA alone (**lanes 1–12**) or TPA + 100 nM bryostatin 1-treated (**lanes 13–18**) cell extracts. AP-1 complexes are indicated (bracket).

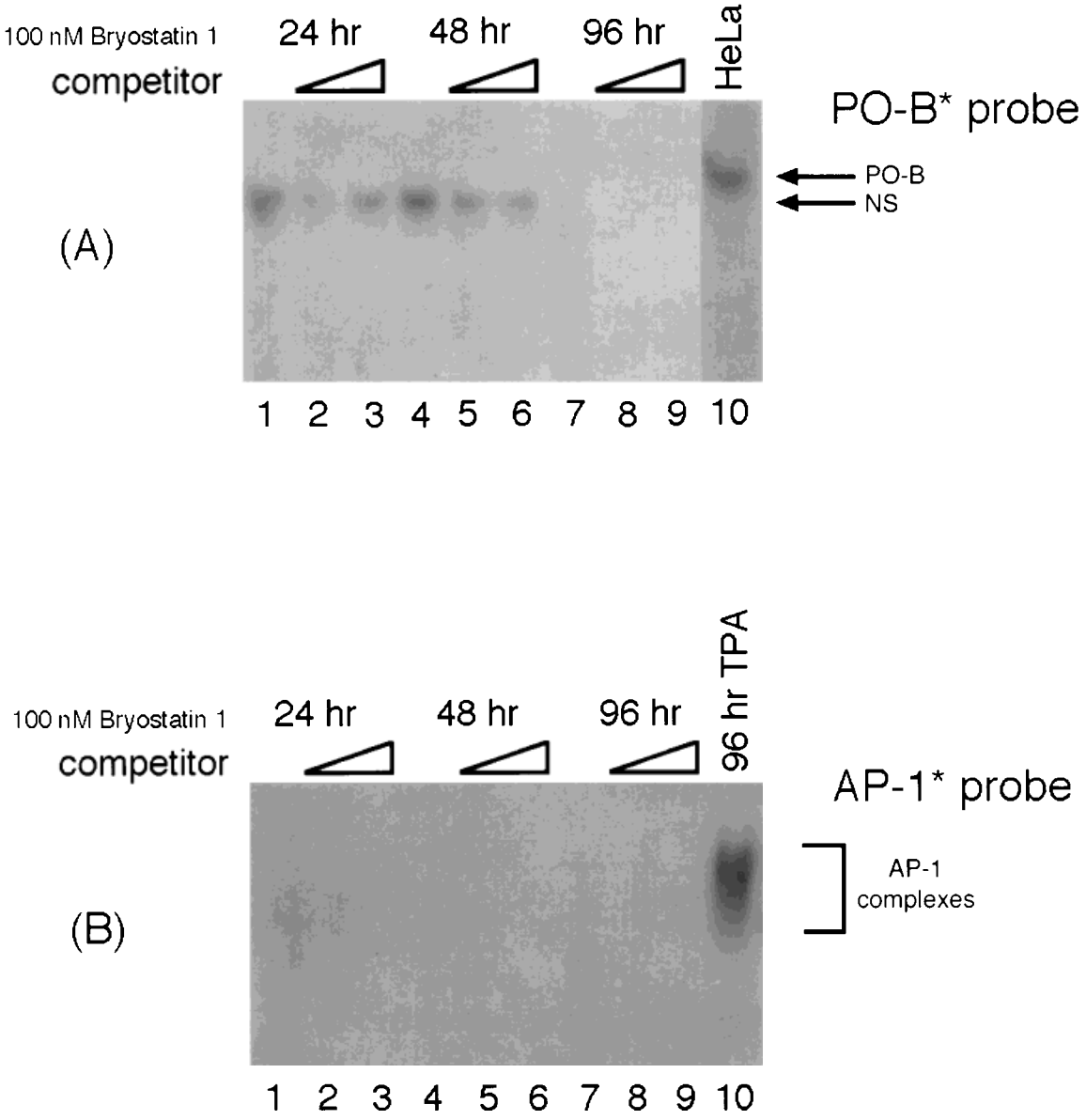


Fig. 6. PO-B and AP-1 DNA binding in bryostatin 1-treated HL-60 cell extracts. HL-60 cells were treated with either 100 nM (A, B) or 10 nM (C, D) bryostatin 1, and PO-B and AP-1 DNA binding was assessed as described in the legend to Figure 1. **A:** PO-B DNA binding in 100 nM bryostatin 1-treated HL-60 cell extracts. Only a non-specific species (NS) was detectable in 24–48 h-treated extracts (**lanes 1–6**). For comparison, PO-B in HeLa cell extracts is shown (PO-B, **lane 10**). **B:** AP-1 DNA binding in 100 nM bryostatin 1-treated HL-60 cell extracts. No AP-1 binding was detectable in treated cell extracts (**lanes 1–9**). AP-1 in 96 h TPA-treated HL-60 extracts is shown for comparison (**lane 10**). **C:** PO-B DNA binding in 10 nM bryostatin 1-treated HL-60 cell extracts. As in (A), only a non-specific species (NS) was detectable in 24–48 h-treated extracts (**lanes 1–9**). **D:** AP-1 DNA binding in 10 nM bryostatin 1-treated HL-60 cell extracts. Note that a small amount of higher mobility AP-1 complexes (1 and 2) was detectable in 24–48 h 10 nM bryostatin 1-treated HL-60 cell extracts (**lanes 1–6**). AP-1 (complex 3) in 96 h TPA-treated HL-60 extracts is shown for comparison (**lane 10**).

in the DNA-binding behaviors of the two transcription factors PO-B and AP-1 during HL-60 differentiation, highlighted by the lineage-specificity of the extent of their inductions.

DISCUSSION

Our previous work has ascertained that PO-B, a transcription factor which regulates the basal expression of the POMC gene [Riegel et al., 1990], is in fact present in many different cell types outside the pituitary, suggesting that this protein may play a role in regulating a diverse set of genes. In particular, we previously determined that PO-B DNA binding to its cognate element is regulated during myelomonocytic differentiation. PO-B DNA binding is gradually

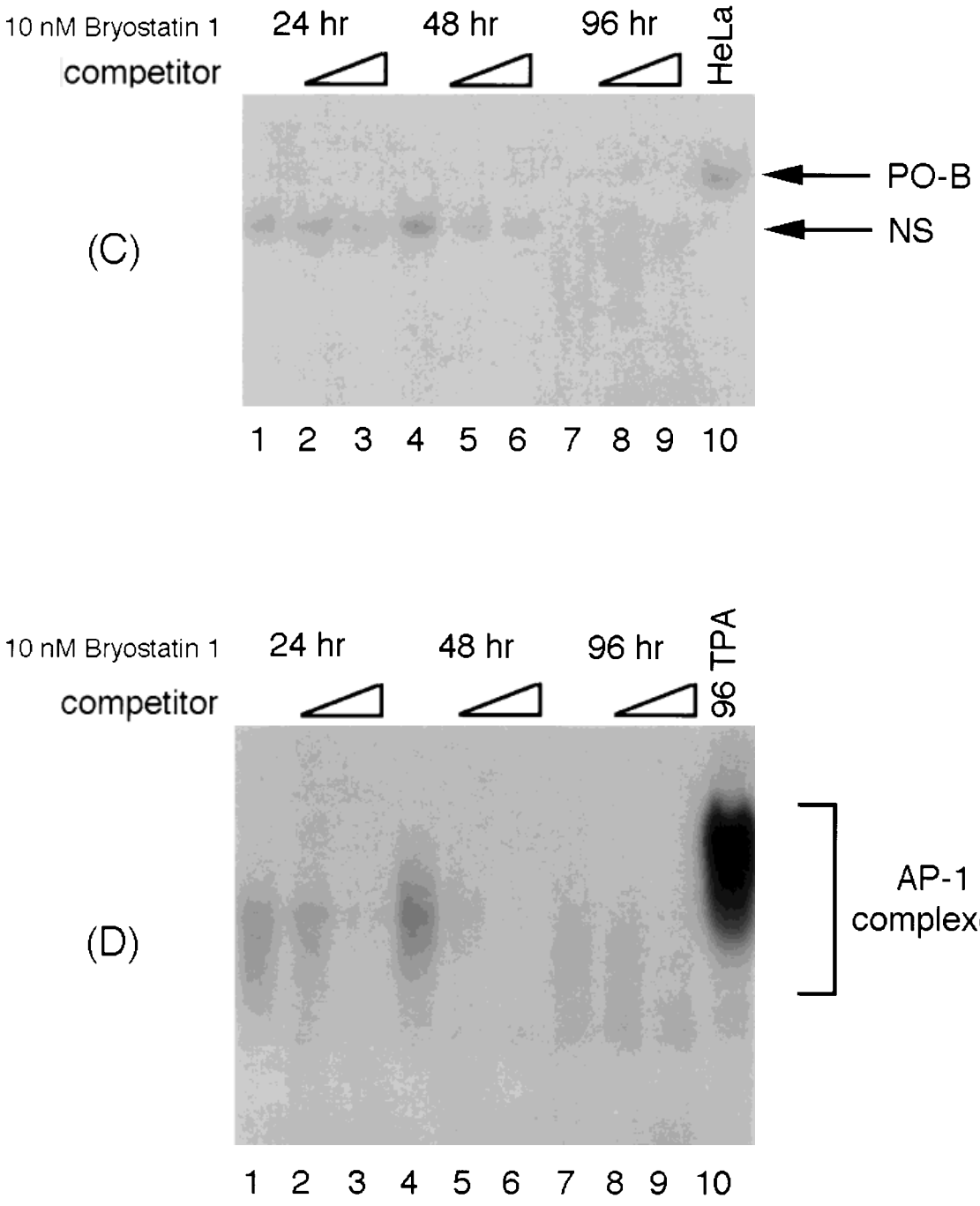


Figure 6. (Continued.)

induced during macrophage-like development of the promyelomonocytic cell line HL-60, possibly due to the progressive inactivation of ERK signalling [Dobrenski et al., 1993].

We now report that PO-B DNA binding is also induced as a consequence of initiating the HL-60 granulocytic differentiation program. Our data indicate that PO-B DNA binding is induced to approximately equivalent levels concomitant with the induction and execution of differentiation to either lineage. In parallel, we monitored the DNA-binding profile of two other transcription factors, the multimeric transcription factor AP-1, which shares a similar recognition element to PO-B, and AP-2, which has been previously associated with the RA-induced differentiation of F9 cells [Luscher et al., 1989]. Analogous to PO-B, AP-1 DNA binding was

induced during HL-60 differentiation; however, we noted a significant difference in the extent of induction with regard to lineage. Interestingly, AP-2 DNA-binding was down-regulated during TPA-induced differentiation of HL-60 cells but unchanged during granulocyte differentiation. Changes in the extent of DNA binding may reflect changes in absolute amounts of the transcription factor or in changes which alter the DNA binding affinity of the protein.

Commitment to the macrophage-like lineage in HL-60 cells occurs within hours after exposure to TPA. The effects of TPA stimulation have been characterized in many cell types; one of the most well documented cellular events following phorbol ester treatment is the stimulation of PKC, the phorbol ester receptor [Ashendel et al., 1983; Kikkawa et al., 1983; Niedel et al., 1983]. In contrast, bryostatin 1 was incapable of eliciting a differentiative response, based both on morphological criteria as well as the lack of induction of either PO-B or AP-1 DNA binding. It is possible, that the differential effects of TPA and bryostatin 1 are due in part to stimulation of distinct isoforms of PKC, which has been demonstrated to result in distinct cellular consequences in some instances [Hocevar and Fields, 1991; Szallasi et al., 1994a,b].

At the molecular level, PKC signalling leads to the induction of the immediate early genes of the jun (c-jun, jun-B, jun-D) and fos (c-fos, fosB, and fra's) families, whose gene products dimerize to form the transcription factor AP-1. Message levels of c-jun, junB, and fos are induced rapidly (within a few hours) following treatment of HL-60 cells with TPA [Sherman et al., 1990; Datta et al., 1991; Szabo et al., 1991; Müller et al., 1985] and AP-1 protein levels become elevated within several hours [Szabo et al., 1991].

Our data are generally consistent with previous data reporting the induction of AP-1 DNA binding during macrophagic or monocytic differentiation [Szabo et al., 1991; Kolla and Studzinski, 1993]. The pattern of AP-1 complexes which we observed after TPA treatment resemble those following exposure of HL-60 cells to ($1,25[OH]_2D_3$). The heterogeneous composition of the AP-1 complexes at different times during HL-60 differentiation was examined previously with antibodies against c-Jun and c-Fos [Kolla and Studzinski, 1993]. In these studies c-Jun appeared to be component of the AP-1 complex in untreated and differentiated HL-60 cells. Whereas c-Fos was a component of the complex in undifferentiated cells but was not present in all AP-1 complexes observed in extracts from differentiated cells. Similar experiments that we have performed have been inconclusive since abolition or supershift of the AP-1 complex has required relatively large amounts of antibody which have non-specific effects on other protein-DNA complexes [unpublished observation]. Studies from other laboratories have monitored changes in AP-1 DNA binding during HL-60 differentiation with either TPA, RA or DMSO, sodium butyrate, or ($1,25[OH]_2D_3$); however, in those studies, the maximum time of exposure to any agent was 24–48 h [Mollinedo et al., 1993; Szabo et al., 1991].

In contrast to the situation with PO-B, which was induced to almost identical levels with any differentiation inducer, we observed that some DNA-binding species of AP-1 (complexes 1 and 2) were slightly induced with the granulocytic differentiation inducers RA and DMSO, but clearly the extent of induction of AP-1 was markedly reduced in comparison to TPA stimulation. Most likely, these differences are attributable to a differential heterodimeric composition of AP-1 as a consequence of either differentiation program. Indeed, our Western blot analysis revealed that in particular, the Fos family members c-Fos and Fra-1 were greatly induced after 96 h TPA exposure, whereas Jun family members (c-Jun and JunB) were only slightly elevated after 96 h TPA.

From our data, it is clear that induction of HL-60 differentiation is positively correlated with the progressive stimulation of PO-B DNA binding to its cognate element. Since either a genetic or pharmacologic blockade of HL-60 differentiation prohibited the induction of PO-B, we consider it unlikely that PO-B is stimulated by TPA, RA, or DMSO alone, independent of differentiation-related processes. In contrast, AP-1 DNA binding appears to be preferentially stimulated with initiation and execution of the terminally differentiated macrophagic or monocytic program. Our data support the notion that the induction of PO-B DNA binding is a general marker of HL-60 myelomonocytic differentiation.

It appears that while the transcription factors AP-1 and PO-B are induced during HL-60 differentiation, this does not appear to represent a generalized induction of all transcription factors, since levels of AP-2 was down-regu-

lated during HL-60 monocyte differentiation. It is also worthy of mention that PO-B DNA binding is constitutive in undifferentiated F9 cells, a murine embryonal carcinoma cell line which can be stimulated to differentiate in culture to primitive endoderm (with RA) or to parietal endoderm (with both RA and cAMP) [Strickland and Mahdavi, 1978]. Furthermore, PO-B DNA binding is not induced any further with RA-induced differentiation of these cells (data not shown). Thus, it does not appear that the induction of PO-B characterizes all differentiation processes.

The precise mechanistic events which lead to transcription factor activation during HL-60 differentiation remain unclear. Our previous work has implicated the modulation of ERK signalling as one potential regulatory cascade affecting proteins like PO-B during HL-60 macrophage development [Dobrenski, et al., 1993]. Interestingly, the inability to detect certain species of AP-1 binding to DNA in undifferentiated HL-60 extracts has been attributed to an inhibitory activity present in unstimulated cells [Kolla and Studzinski, 1993]. Further experiments are required to determine what role protein kinases and phosphatases play in regulating the expression of HL-60 differentiation-related genes through controlling transcription factor binding to DNA. In addition, a number of gene promoters harbor AP-1 or AP-2 sites [Angel et al., 1987; Luscher et al., 1989]. However, the only current defined downstream target of PO-B is the POMC gene [Wellstein et al., 1991]. Clearly it will be important in future work to define the downstream targets of the transcription factors activated during differentiation.

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REFERENCES

Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M (1987): Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49:729–739.

Ashendel CL, Stoller JM, Boutwell RK (1983): Protein kinase activity associated with a phorbol ester receptor purified from mouse brain. Cancer Res 43:4333–4338.

Ausubel FM, Brent R, Kingston RE, Moore DD, Feidman JG, Smith JA, Struhl K (1989): "Current Protocols in Molecular Biology." New York, NY: Greene Publishing Associates.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Breitman TR, Selonick SE, Collins SJ (1980): Induction of differentiation of a human promyelocytic cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 77:2936–2940.

Collins SJ, Robertson KA, Mueller L (1990): Retinoic acid-induced granulocytic differentiation of HL-60 myeloid leukemia cells is mediated directly through the retinoic acid receptor (RAR-α). Mol Cell Biol 10:2154–2163.

Dale IL, Bradshaw TD, Gescher A, Pettit GR (1989): Comparison of effects of bryostatins 1 and 2 and 12-O-tetradecanoylphorbol-13-acetate on protein kinase C activity in A549 human lung carcinoma cells. Cancer Res 49:3242–3245.

Datta R, Sherman ML, Stone RM, Kufe D (1991): Expression of the jun-B gene during induction of monocytic differentiation. Cell Growth Diff 2:43–49.

Dell'Aquila ML, Nguyen HT, Herald CL, Pettit GR, Blumberg PM (1987): Inhibition by bryostatin 1 of the phorbol ester-induced blockage of differentiation in hexamethylene bisacetamide-treated Friend erythroleukemia cells. Cancer Res 47:6006–6009.

Dignam JD, Lebovitz RM, Roeder RG (1983): Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Dobrenski AF, Zeft AS, Wellstein A, Riegel AT (1993): DNA binding of the transcription factor PO-B is regulated during differentiation of HL-60 cells. Cell Growth and Diff 4:647–656.

Fried MG, Crothers DM (1981): Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res 9:6505–6525.

Gallagher RE, Bilello PA, Ferrari AC, Chang CS, Yen RC, Nickols WA, and Muly III EC (1985): Characterization of differentiation-inducer-resistant HL-60 cells. Leuk Res 9:967–986.

Hocevar BA, Fields AP (1991): Selective translocation of B_{II}-protein kinase C to the nucleus of human promyelocytic (HL-60) leukemia cells. J Biol Chem 266:28–33.

Kikkawa U, Takai Y, Tanaka Y, Miyake R, Nishizuka Y (1983): Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. J Biol Chem 257:13341–13350.

Kolla SS, Studzinski GP (1993): Resolution of multiple AP-1 complexes in HL-60 cells induced to differentiate by 1,25-dihydroxyvitamin D_3. J Cell Phys 156:63–71.

Kraft AS, Smith JB, Berkow RL (1986): Bryostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester-induced differentiation of human promyelocytic leukemia cells (HL-60). Oncogene 1:111–118.

Lee W, Mitchell P, Tjian R (1987): Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741–752.

Liu B, Hammer GD, Rubinstein M, Mortrud M, Low MJ (1992): Identification of DNA elements cooperatively activating proopiomelanocortin gene expression in the pituitary glands of transgenic mice. Mol Cell Biol 12:978–3990.

Luscher B, Mitchell PJ, Williams T, Tjian R (1989): Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. Genes Dev 3:507–1517.

Mackanos EA, Pettit GR, Ramsdell JS (1991): Bryostatins selectively regulate protein kinase C-mediated effects on GH4 cell proliferation. J Biol Chem 266:11205–11212.

Miyaura C, Abe E, Kuribayashi T, Tanaka H, Konno K, Nishii Y, Suda T (1981): 1-Alpha, 25 dihydroxyvitamin D_3 induces differentiation of human myeloid leukemia cells. Biochem Biophys Res Commun 102:937–943.

Mollinado F, Gajate C, Tugores A, Flores I, Naranjo JR (1993): Differences in expression of transcription factor AP-1 in human promyelocytic HL-60 cells during differentiation towards macrophages versus granulocytes. Bioch J 294:137–144.

Müller R, Curran T, Müller D, Guilbert L (1985): Induction of c-fos during myelomonocytic differentiation and macrophage proliferation. Nature (London) 314:546–548.

Niedel J, Kuhn E, Vandenbark G (1983): Phorbol diester receptor copurifies with protein kinase C. Proc Natl Acad Sci USA 80:36–40.

Pettit GR, Herald SL, Doubeck DL, Arnold E, Clardy J (1982): Isolation and structure of bryostatin 1. J Amer Chem Soc 104:6846–6848.

Riegel AT, Remenick J, Wolford R, Berard D, Hager G (1990): A novel transcriptional activator (PO-B) binds between the TATA box and CAP site of the pro-opiomelanocortin gene. Nucleic Acids Res 18:4513–4521.

Rovera G, Santoli D, Damsky C (1979): Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol ester. Proc Natl Acad Sci USA 76:2779–2783.

Sherman ML, Stone RM, Datta R, Bernstein SH, Kufe D (1990): Transcriptional and post-transcriptional regulation of c-jun expression during monocytic differentiation of human myeloid leukemic cells. J Biol Chem 265:3320–3323.

Stone RM, Sariban E, Pettit GR, Kufe DW (1988): Bryostatin 1 activates protein kinase C and induces monocytic differentiation of HL-60 cells. Blood 72:208–213.

Strickland S, Mahdavi V (1978): The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell 15:393–403.

Szabo E, Preis LH, Brown PH, Birre MJ (1991): The role of jun and fos gene family members in 12-O-tetradecanoylphorbol-13-acetate induced hematopoietic differentiation. Cell Growth and Diff 2:475–482.

Szallasi Z, Denning MF, Smith CB, Dlugosz AA, Yuspa SH, Pettit GR, Blumberg PM (1994a): Bryostatin 1 protects protein kinase C-δ from down-regulation in mouse keratinocytes in parallel with its inhibition of phorbol ester-induced differentiation. Mol Pharm 46:840–850.

Szallasi Z, Smith CB, Pettit GR, Blumberg PM (1994b): Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. J Biol Chem 269:2118–2124.

Therrien M, Drouin J (1991): Pituitary pro-opiomelanocortin gene expression requires synergistic interaction of several regulatory elements. Mol Cell Biol 11:3492–3503.

Tonetti DA, Horio M, Collart FR, Huberman E (1992): Protein kinase C β gene expression is associated with susceptibility of human promyelocytic leukemia cells to phorbol ester-induced differentiation. Cell Growth and Diff 3:739–745.

Tsai S, Bartelmez S, Heyman R, Damm K, Evans RM, Collins SJ (1992): A mutated retinoic acid receptor-alpha exhibiting dominant-negative activity alters the lineage development of a multipotent hematopoietic cell line. Genes Dev 6:2258–2269.

Van Roozendaal KEP, Darling D, Farzeneh F (1990): DMSO and retinoic acid induce HL-60 differentiation by different but converging pathways. Exp Cell Res 190:137–140.

Wellstein A, Dobrenski AF, Radonovich MN, Brady JF, Riegel AT (1991): Purification of PO-B, a protein that has increased affinity for the pro-opiomelanocortin gene promoter after dephosphorylation. J Biol Chem 266:2234–12241.