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Suppression of Tissue Factor Expression, Cofactor Activity, and Metastatic Potential of Murine Melanoma Cells by the *N*-Terminal Domain of Adenovirus E1A 12S Protein

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Abstract Tissue factor, the cellular initiator of blood coagulation, has been implicated as a determinant of metastatic potential in human melanoma cells. Here, we report that differential expression of tissue factor in murine melanoma cell lines of known metastatic behavior is mediated by AP-1-dependent and 12S E1A oncoprotein-repressible gene transcription. When compared to weakly metastatic C10 cells, highly metastatic M4 cells possessed elevated levels of tissue factor cofactor activity, transfected promoter activity, and heterodimeric AP-1 DNA-binding complexes containing Fra-1. Transient co-expression of the adenovirus E1A 12S oncoprotein strongly repressed transcription of an AP-1-driven tissue factor reporter gene indicating the additional requirement of *N*-terminal E1A-interacting coactivators. Stable expression of E1A mutants defective in CBP/p300-binding failed to suppress tissue factor expression and experimental metastasis by M4 cells while clones expressing wild type E1A exhibited greatly reduced tissue factor cofactor activity and metastatic potential in vivo. Overexpression of functional tissue factor in cells containing wild type E1A failed to restore the highly metastatic M4 phenotype suggesting that additional E1A-responsive and CBP/p300-dependent genes are required to facilitate metastasis of murine melanoma cells demonstrating high tissue factor expression and cofactor activity. J. Cell. Biochem. 85: 54–71, 2002.

Key words: tissue factor; adenovirus E1A; AP-1; Fra-1; melanoma; metastasis

Tissue factor (TF) is a transmembrane glycoprotein that serves as a specific cell surface receptor and essential cofactor for plasma coagulation factor VII/VIIa [Broze, 1982]. The primary function of TF in the adult is to initiate blood coagulation [Bach et al., 1988; Edgington et al., 1991] and constitutively expressed TF serves as a protective hemostatic barrier in organ capsules and blood vessels [Drake et al., 1989]. Inappropriate expression of TF has been associated with several pathologic conditions such as endotoxemia [Taylor et al., 1991], atheroclerosis [Wilcox et al., 1989; Nemerson, 1995] and, in particular, with the development and progression of cancer [reviewed in Callander et al., 1992; Rao, 1992]. Specifically, TF has been implicated as a determinant of metastatic potential in colorectal cancer, non-small cell lung cancer, and malignant melanoma [Mueller et al., 1992; Shigemori et al., 1998; Sawada et al., 1999]. Although the relative importance of the extracellular factor VIIa ligand-binding domain versus the intracellular cytoplasmic domain is controversial, melanoma and other cell lines expressing high levels of

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human TF have been shown to disseminate rapidly, particularly to the lung, when assayed using a mouse model of hematogenous metastasis [Mueller et al., 1992; Bromberg et al., 1995; Mueller and Ruf, 1998]. As a consequence, delineation of the mechanisms that mediate TF gene expression in melanoma cells of defined metastatic potential will likely provide greater insight into the role of TF in melanoma metastasis. Although a number of studies have elucidated transcriptional mechanisms that control TF gene activation in monocytic, epithelial, endothelial, smooth muscle, and fibroblastic cells [reviewed in Mackman, 1997], no laboratory has yet examined TF promoter regulation in melanoma cells of well characterized metastatic potential in a species homogeneous system. In this study, we describe an analysis of the mouse TF promoter in murine melanoma cells of known metastatic behavior in vivo.

Our interest in the regulation of TF expression stems from previous research that focused on identifying the operative cis-acting regulatory elements and trans-acting factors that contribute to activation of the mouse TF promoter in murine AKR-2B fibroblasts, a model for stromal myofibroblast cytodifferentiation. Initial promoter mapping studies established that serum growth factor-induced activation of TF gene transcription is dependent on two activator protein 1 (AP-1) DNA-binding sites spanning −200 to −220 relative to the start site of transcription [Felts et al., 1995]. Biochemical analyses revealed that transcriptional activation is mediated by the rapid recruitment of c-Fos into heterodimeric AP-1 DNA-binding complexes with JunD. Co-stimulation with transforming growth factor β1 (TGF-β1) and serum was subsequently shown to promote synergistic activation of a minimal AP-1 driven TF (AP1-TF) promoter in fibroblasts [Felts et al., 1997]. The molecular basis for this synergy was found to be dependent upon both c-Fos and an unidentified component of the basal TF transcription apparatus, provisionally termed co-activator of c-Fos or CAF. The existence of CAF was established, in part, from co-expression studies in which over-expression of c-Fos suppressed synergistic stimulation of TF promoter activity by transcriptional squelching. Further evidence for a c-Fos-dependent and TF basal promoter-specific coactivator came from additional co-transfection experiments demonstrating that the AP1-TF promoter was strongly repressed by ectopic expression of the adenovirus E1A 12S oncoprotein [Felts et al., 1997].

In a recent study from our laboratory, a panel of E1A mutants defective in their ability to bind and sequester specific cellular proteins were exploited in an attempt to more rigorously define the molecular basis for E1A inhibition of c-Fos dependent TF gene transcription in fibroblasts [Liu et al., 2000]. Consistent with previous results, an *N*-terminal deletion mutant (d2-36) and a more modest point mutant (RG2), which were both defective in binding the related coactivators, CREB-binding protein (CBP) and p300 (together referred to as CBP/p300), retained the ability to repress AP1-TF promoter activity relative to wild type E1A. However, deletion and point mutants deficient in binding to retinoblastoma (RB) family members (pRB, p107, and p130) failed to inhibit AP1-TF promoter activity in fibroblasts stimulated by either serum, serum plus TGF-β1, or by c-Fos overexpression. The involvement of pRB in modulating TF gene transcription in fibroblasts was further implied by the finding that E1A-mediated repression of AP1-TF promoter activity was restricted to the G_0 to G_1 transition of the cell cycle, where hypophosphorylated, and hence, E1A-interacting forms of pRB predominate. However, ectopic expression of pRB (or CBP/p300 and combinations thereof) failed to rescue the AP1-TF promoter from inhibition by wild type E1A suggesting that pRB might be necessary but not sufficient for high level promoter activation. Credence for this hypothesis was supported by the finding that co-expression of pRB completely rescued the AP1-TF promoter from inhibition by the d2-36 mutant but not by the RG2 point mutant. Furthermore, p107, but not p130, could substitute for pRB in relieving repression by E1A d2-36 suggesting that only specific members of the RB tumor suppressor family can cooperate with c-Fos to activate TF gene transcription in fibroblasts. Collectively, these experiments suggested that CAF, previously defined as a c-Fos and E1A-interacting factor mediating synergistic activation of TF transcription in fibroblasts [Felts et al., 1997], likely consists of multiple proteins including pRB and an unidentified *N*-terminal E1A-interacting entity distinct from CBP/p300 [Liu et al., 2000].

In light of our previous work, the aims of the present study were 1) to quantitatively

determine the activity of mouse TF in murine melanoma cells of pre-defined metastatic potential, 2) to characterize the regulation of the mouse TF promoter in highly metastatic cells, and 3) to assess the influence of adenovirus E1A 12S oncoprotein on mouse TF expression and metastatic behavior of stably transfected cells in NOD/SCID mice. Collectively, our data indicate that differential TF expression in murine melanoma cells is primarily mediated at the transcriptional level and is dependent upon specific AP-1 DNA-binding complexes. The relative abundance of AP-1 DNA-binding proteins differ markedly between highly metastatic and weakly metastatic melanoma cells and the heterodimeric AP-1 protein composition of these complexes is distinct from the c-Fos/JunD complex found in fibroblasts. Moreover, the transcriptional activity of the mouse TF promoter in highly metastatic melanoma cells appears to be regulated by a different repertoire of coactivators than previously identified in fibroblasts. Furthermore, we find that high level expression of TF appears to be necessary, but not sufficient, to confer a highly metastatic phenotype on murine melanoma cells in vivo.

MATERIALS AND METHODS

Plasmid Constructs

The TF promoter:chloramphenicol acetyltransferase (CAT) reporter plasmids used in transient transfection experiments were constructed in our laboratory and have been described previously [Felts et al., 1995]. Wild type and mutant E1A 12S expression vectors were also constructed and tested as previously described [Liu et al., 2000]. A CMV enhancer/promoter-driven mouse TF expression vector was created by subcloning the mouse TF cDNA excised from pSSB-c70 [Ranganathan et al., 1991] into pCI (Promega). Briefly, the c70 mouse TF cDNA was released from pSSB by *Bam*HI digestion. The cDNA was blunt-ended with Klenow fragment and *Sal*I linkers were attached. The cDNA was then ligated into *Sal*I-linearized pCI vector. The resultant construct, pCI-mTF, was propagated in *E. coli* strain HB101. Clones containing the mouse TF cDNA insert in the correct orientation were selected by DNA sequencing. All plasmid DNA used for transfection was purified by double cesium chloride gradient centrifugation.

Rabbit Anti-Mouse Tissue Factor Antibodies

Peptides corresponding to amino acids 65–83 (SDRSRNWKNKCFSTTDTEC, MC 90) and 275–294 (CKRRKNRAGQKGKNTPSRLA, MC 93) of the nascent mouse TF polypeptide were synthesized, purified, and coupled to carrier protein as previously described [Kelm et al., 1999]. The extracellular domain of mouse TF [Ranganathan et al., 1991] was expressed in *E. coli* as a His_6-tagged fusion protein and purified using Ni-NTA agarose (Qiagen). Rabbit immunization and antisera production was carried out by a commercial vendor (Cocalico). Polyclonal antibodies were affinity purified from the IgG fraction of rabbit antisera using peptide-coupled agarose columns and screened by Western blotting using established methods [Kelm et al., 1999].

Cell Culture, Transfection, and Reporter Gene Assay

The K1735 melanoma clones M4 and C10 [Fidler et al., 1981] exhibiting strong and weak metastatic potential, respectively, were kindly provided by Leo Furcht (Department of Laboratory Medicine and Pathology, University of Minnesota). Cells were routinely maintained at 37°C, 5% CO_2, and grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing high glucose supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For transient transfections, 90–95% confluent cells were trypsinized and plated at a density of 8×10^5 cells/100 mm dish in complete growth medium. After an overnight incubation, monolayers were washed with MCDB 402 modified medium (JRH Biosciences), and transfected with a total of 10 μg plasmid DNA including 7.5 μg TF-CAT reporter, 0.5 μg of a control β-galactosidase (β-gal) reporter, pCMVβ (Clontech), and varying amounts of pCI-E1A 12S expression vector using Lipofectamine™ reagent (Life Technologies, Inc.) Empty pCI vector was added to maintain a constant amount of transfected DNA. Lipofectamine reagent (1 mg/ml) was combined with DNA at a ratio of 2 μl/μg DNA and the mixture was incubated for 45 min at room temperature to allow formation of DNA/liposome complexes prior to addition to washed cells. Cells were incubated with the transfection mixture for 5 h and then washed twice with

serum free medium before the addition of complete growth medium. Cellular extracts were prepared 48 h after transfection using CAT enzyme-linked immunosorbent assay (ELISA) lysis buffer (Roche Molecular Biochemicals). In experiments evaluating the effects of various transfected E1A constructs, growth medium was replaced after 18 h with serum-free MCDB 402 medium. After 48 h, the cultures were then re-stimulated for 6 h with 20% FBS and 5 ng/ml recombinant human TGF-β1 (Austral) prior to cell lysis. Lysates were assayed for total protein by BCA dye binding assay (Pierce) using bovine serum albumin (BSA) as a standard. CAT and β-gal reporter proteins were quantified by commercial immunoassays (Roche Molecular Biochemicals).

Stable transfections were done using the Lipofectamine system also. M4 melanoma cells, 1×10^6, were plated in growth medium and allowed to adhere to 35 mm 6-well tissue culture dishes overnight. Pre-formed DNA:lipid complexes, prepared at the ratio of 1.5 μg pCI-E1A 12S plasmid and 0.3 μg pRSV-neo to 20 μl of Lipofectamine reagent, were applied to cell monolayers and incubated for 5 h under serum-free conditions. Subsequently, cells were fed with an equal volume of growth medium supplemented with 20% FBS and cultured for an additional 72 h. The cultures were then split (~1:15) and selection agent included in the growth medium. Selection of transfected cells was accomplished at a dose of 1 mg/ml G418 (Life Technologies) and cultures were fed with fresh growth medium every 4 days. Colonies generally appeared 10–14 days after selection was initiated. Individual colonies were expanded and screened for levels of TF and E1A protein expression, and for E1A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and neomycin resistance gene mRNA expression. ME (M4 cells expressing E1A) clones were frozen within 1 week of the start of expansion and new cultures started after 8 weeks of growth. After 4–8 weeks, all cell lines were replaced from frozen stocks in order to prevent phenotypic changes. These and other stable transfectants (Table I) were selected and cultured in the continual presence of doses of antibiotic established as toxic to the parent cell line by MTS cell proliferation assay (Promega). Stable ectopic expression of mouse TF in a selected E1A-expressing cell line (ME16) was accomplished as described above using expression plasmids encoding mouse TF, pCI-mTF, and the hygromycinB resistance gene, pREP4 (Invitrogen).

Immunoblotting of E1A and Tissue Factor

Immunoblot analyses of lysed cell extracts were performed using a mouse monoclonal anti-E1A antibody specific for a C-terminal epitope (M73, Santa Cruz) or a rabbit anti-TF antibody recognizing the extracellular domain of mouse TF (MC 788, prepared as described above). Tumor cells were washed twice in phosphate-buffered saline (PBS) and then scraped in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris pH 8.0) containing the same protease inhibitors as used for the TF activity assay. Cells were sonicated and protein was quantified by DC protein assay (BioRad). Whole cell protein was resolved on a 10 or 12% SDS–polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore). Protein blots were blocked and probed using previously described methods [Liu et al., 2000]. As a loading control, selected blots were reprobed with mouse anti-GADPH (clone 6C5, Research Diagnostics, Inc.)

TABLE I. Generation of Stable Melanoma Cell Lines Ectopically Expressing E1A 12S Proteins or E1A + Mouse TF

Cell line designation	Description	Plasmids	Antibiotic selection	Dose[§]
M4	Highly metastatic			0
C10	Weakly metastatic			0
MEX*	M4 + E1A wild type	pCI-E1A, pRSV-neo	G418	1.0
MRGX*	M4 + E1A RG2	pCI-E1ARG2, pRSV-neo	G418	1.0
MDTX*	M4 + E1A d2-36	pCI-E1Ad2-36, pRSV-neo	G418	1.0
MCX*	Control for E1A-expressing cell lines	pCI, pRSV-neo	G418	1.0
MMTX*	ME16 + mTF	pCI-mTF, pREP4	G418; hygromycin B	1.0; 1.0
MMCX*	Control for MMTs	pCI, pREP4	G418; hygromycin B	1.0; 1.0

[§]Antibiotic dose in mg/ml.
*X designates clone number.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from all cell lines as described previously [Kelm et al., 1997]. An oligonucleotide probe containing the two TF AP-1 elements (5′-TTGAATCACGGTTGAGTCA-3′) was end-labeled with [α-^{32}P]dATP (10 mCi/ml), Klenow fragment (Roche Molecular Biochemicals) and 5 mM dNTP mix, and purified over a G-25 spin column (Amersham Pharmacia Biotech). Binding reactions for EMSAs contained 2–3 μg nuclear protein, 1 μg poly (dIdC), and buffer (20 mM HEPES pH 8, 60 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol). Reactions were incubated with 25,000 cpm of ^{32}P-labeled AP-1 DNA probe for 20 min at room temperature. For supershift assays, 1 μl of Fos or Jun family member-specific antibody (Santa Cruz Biotechnology) was added to the reaction and incubated for one additional hour at 4°C. DNA-protein complexes were separated from unbound probe on a native 6% polyacrylamide gel in Tris:glycine:EDTA buffer as previously described [Felts et al., 1995]. Gels were vacuum-dried and exposed to Kodak BioMax MR film at −70°C.

Immunoprecipitation Assay

M4 cells, seeded at a density of 7×10^5 cells/plate, were transiently transfected with expression vectors encoding wild type or mutant E1A 12S oncoproteins [Liu et al., 2000] as described above using 10 μg of plasmid DNA. Cell extracts were prepared 45 h after transfection with lysis buffer consisting of 50 mM HEPES pH 7.0, 250 mM NaCl, 0.1% (v/v) NP-40. Lysates were assayed for total protein by BCA dye binding assay (Pierce) using BSA as a standard. A total of 100 μg of lysed cell protein pooled from three independent transfectants was combined with 1 μg of anti-E1A monoclonal antibody (M73, Santa Cruz Biotechnology) and immunoprecipitation was carried out as previously described [Liu et al., 2000]. Immunoprecipitates were denatured in reducing SDS sample preparation buffer, resolved on a 6% polyacrylamide minigel, and analyzed by Western blotting for the presence of CBP, p300, pRB, p107, and p130 using the following antibodies diluted to 1–2 μg/ml: mouse anti-pRB (G3-245, Pharmingen), mouse anti-p107 (SD9, Santa Cruz Biotechnology), rabbit anti-p130 (C-20, Santa Cruz Biotechnology), rabbit anti-CBP (A22, Santa Cruz Biotechnology), and rabbit anti-p300 (C-20, Santa Cruz Biotechnology). Western blotting of 3 μg total lysed protein with anti-E1A M73 was used to verify ectopic expression of each mutant E1A protein.

TF Cofactor Activity Assay

TF activity was determined by chromogenic assay as described previously [Vrana et al., 1996]. Briefly, cells were washed twice with PBS, incubated with 5% glycerol in PBS for 5 min, washed again and subjected to hypotonic lysis in water supplemented with protease inhibitors (leupeptin, aprotinin, and pepstatin A at 1.0 μg/ml and phenylmethylsulfonyl fluoride at 0.1 mM). Total protein was quantified by dye-binding assay (DC Protein assay) using BSA as the standard. Whole cell protein, 20 μg, was diluted to 60 μl with 0.02 M HEPES pH 7.5 and added to 0.0075% inosithin diluted in imidazole-buffered saline in 96-well microtiter plates. This mixture was then combined with a solution of 0.125 nM human factor VII (Enzyme Research Labs), 105 nM factor X (Enzyme Research Labs), and 9.4 mM $CaCl_2$. After 10 min, factor X hydrolysis was stopped by addition of EDTA (0.02 M) and the factor Xa chromogenic substrate, S-2222 (0.29 mM, Pharmacia-Chromogenix). Factor Xa was measured by continuously monitoring color development over 10 min at a wavelength of 405 nm using a Vmax spectrophotometer (Molecular Diagnostics). All samples were assayed in triplicate.

Experimental Metastasis Assay

Female 5–9 week old non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice were used in this study for experimental metastasis. Breeding pairs were purchased from Jackson Laboratories (NOD/LtSz-*Prkdc*^{scid}/J). Mice were kept in a barrier facility under pathogen free conditions in filter-top cages with irradiated and filtered drinking water. The age of the mice within one experiment was kept the same. Prior to use, cell lines were screened for common mouse viruses by a commercial vendor (Charles River) using a mouse antibody production test. For experimental metastasis, cells were trypsinized, suspended in growth medium, washed twice, and resuspended in sterile PBS (free of calcium and phosphate). A total of 7×10^5 cells in a volume of 200 μl were injected into the lateral tail vein of the NOD/SCID mice. Mice were checked at least three times per week. As a positive control for

metastasis, M4 cells were injected in each experiment. After 14 days, mice were killed, and all major organs were excised and fixed in 10% formalin for at least 72 h. Lungs were inspected for visible nodule formation and weighed. Visually detectable nodules were counted and divided into two categories (< or ≥ 1 mm in diameter). Because there were instances where the number of large (≥ 1 mm) nodules could not be counted exactly, each mouse was classified into one of two sub categories: 0–10 nodules ≥ 1 mm or more than 10 nodules ≥ 1 mm for statistical analysis. The number of mice from different cell lines, or combinations of cell lines, that fell into each of these two sub categories was compared using Fisher's exact test. The combined groups were (A) M4, (B) ME16 + ME38 + ME43 + ME52 + ME63, (C) MC6 + MC9, and (D) C10 + MMC10 + MMT14 + MMT16. Fisher's exact test was also used to make pair-wise comparisons between groups (A) and (B), M4 vs. ME, and between groups (B) and (C), ME vs. MC. In order to detect micrometastases, selected lungs were paraffin embedded and sectioned for histology. Slides were stained with hematoxylin and eosin and examined microscopically for tumor cells.

RESULTS

Differential Expression of TF Activity in Melanoma Cells is Mediated at the Transcriptional Level and is Dependent Upon Specific AP-1 Binding Complexes

The metastatic potential of the murine melanoma cell clones M4 and C10 was previously evaluated by lung colonization assay [Fidler et al., 1981]. M4 cells were shown to be highly metastatic in mice whereas C10 cells displayed weak metastatic behavior. Since TF expression has been implicated as a determinant of metastatic potential in human melanoma cells [Mueller et al., 1992; Bromberg et al., 1995], we tested these two clonal mouse cell lines for TF cofactor activity. Using a functional chromogenic assay that depends on the ability of a TF/factor VIIa complex to activate factor X, lysates of highly metastatic M4 cells exhibited dramatically increased levels of TF cofactor activity compared to lysates from weakly metastatic C10 cells (Fig. 1A). Comparative immunoblotting of lysates from C10 and M4 cells using a mouse TF-specific antibody confirmed that the observed difference in TF cofactor activity was likely due to differences in TF protein expression and not to a mutation promoting functional TF inactivation (Fig. 1B).

To gain further insights into the mechanisms that contribute to enhanced TF expression in M4 cells, we tested the ability of several fragmented TF promoter constructs to drive transcription of a CAT reporter gene in M4 cells. The promoter constructs used are shown

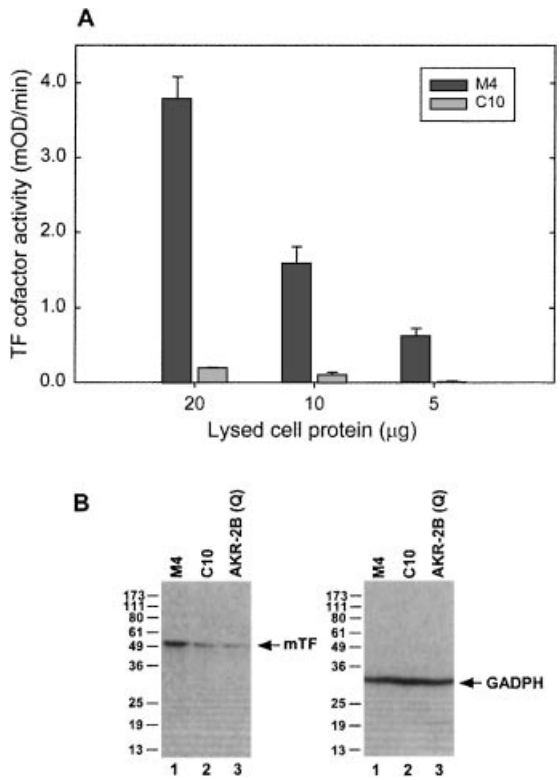

Fig. 1. Tissue factor (TF) cofactor activity and protein levels in murine melanoma cell lines. **A**: Rapidly growing cells were lysed and varying amounts of protein were assayed for TF activity by chromogenic assay measuring factor X activation by factor VIIa/TF. Data are expressed in mOD/min and represent the average ± SD of triplicate samples. The highly metastatic cell line, M4, showed markedly increased TF activity in comparison to the weakly metastatic cell line, C10. **B**: Total cell protein (25 μg/lane) extracted from exponentially growing M4 (**lane 1**), C10 (**lane 2**), or from quiescent AKR-2B cells (**lane 3**) was resolved by SDS–PAGE on a 12% gel and probed by immunoblotting using a rabbit polyclonal antibody (MC 788) raised against the extracellular domain of mouse TF (**left panel**). The electrophoretic mobility and corresponding molecular mass of the predominant mouse TF species (~50 kDa band) detected by MC 788 is consistent with the findings of Toomey et al. [1997] and with fully glycosylated mouse TF [Hartzell et al., 1989]. As a loading control, the TF blot was reprobed with mouse anti-GADPH (**right panel**). The relative abundance of TF protein differed among the cell lines with M4 > C10 > quiescent AKR-2B. Markers are indicated in kilodalton.

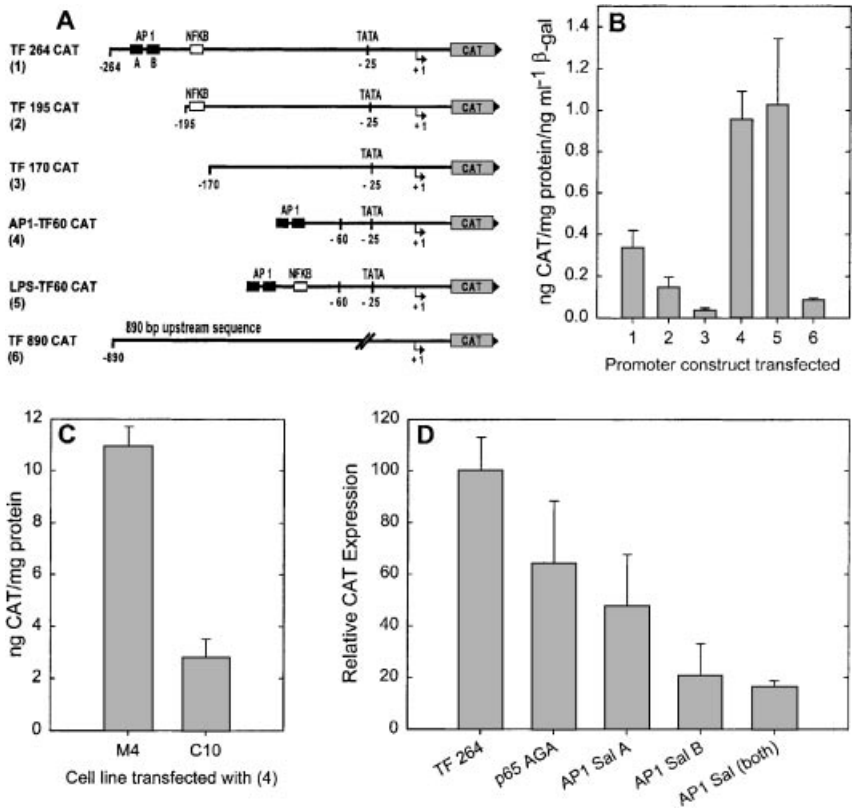

Fig. 2. Dependence of TF promoter activity in murine melanoma cells on AP-1 cis-elements. **A**: Schematic of the different TF-CAT reporter gene constructs tested in M4 cells. **B**: Highly metastatic M4 cells were transiently transfected with 10 μg total plasmid DNA including 7.5 μg TF-CAT reporter (#1, 2, 3, 4, 5, or 6 as in A), 0.5 μg pCMVβ, and 2 μg pCI. After 48 h, cell lysates were prepared and assayed for CAT, β-gal, and total protein. β-gal values were used to correct for transfection efficiency. Data represent the average of triplicate plates transfected ± SD. The two TF AP-1 elements are necessary and sufficient to mediate high level promoter activity in M4 cells as evidenced by the activity of AP1-TF60 (#4) compared to constructs lacking the AP-1 sites, TF195 (#2) and TF170 (#3), or containing additional DNA, TF 890 (#6), TF264 (#1), and LPS-TF60 (#5). **C**: Weakly metastatic C10 cells and strongly metastatic M4 cells were transiently transfected in parallel with AP1-TF60 (#4) and assayed as above. AP1-TF60 shows differential activity in C10 versus M4 cells. **D**: M4 cells were transiently transfected as in (B) with either wild type TF264, or mutant versions in which the NFκB site (p65 AGA), the 5′ AP-1 element (AP1 Sal A), the 3′ AP-1 element (AP1 Sal B), or both AP-1 sites (AP1 Sal both) were mutated. Construction of these mutant reporters was described previously [Felts et al., 1995]. For each mutant reporter, data are expressed as a percentage of the corrected CAT value measured in TF264 (defined as 100%) and represent the average of triplicate plates transfected ± SD. Truncation of both AP-1 sites reduces promoter activity by ~85%.

schematically in Figure 2A and have been described previously [Felts et al., 1995]. As shown in Figure 2B, the chimeric TF promoter (AP1-TF60) that contains only the two TF AP-1 cis-elements (−220 to −200) linked to the TATA-containing TF basal promoter, demonstrated by far the most robust activity in transient transfection experiments. The presence of a nuclear factor κB (NFκB) binding site between the two AP-1 elements and the TATA box did not augment activity of the promoter (Fig. 2B, compare AP1-TF60 (#4) to LPS-TF60 (#5)). It should be noted that the two AP-1 elements together with the NFκB site comprise the so-called lipopolysaccharide (LPS)-response element which is required for LPS-induced and NFκB-dependent activation of TF gene transcription in monocytes and endothelial cells [Mackman, 1997]. Interestingly, the longer wild type TF promoter constructs (TF264 and TF890) displayed lower overall activity relative to the AP1-TF60 and LPS-TF60 chimeras in M4 cells indicating, perhaps, the presence of negative regulatory elements in DNA flanking the AP-1 and NFκB sites. Nevertheless, mutation of both AP-1 elements within the context of the TF264 reporter (AP1 Sal both) reduced promoter activity by ~sixfold while mutation of the NFκB site (p65 AGA) decreased activity by only ~1.5-fold (Fig. 2D). Similar effects were observed when the two AP-1 elements and the NFκB site were sequentially deleted from

TF264 (Fig. 2B, compare TF264 (#1) to TF195 (#2) and TF170 (#3)). Collectively, these results suggest that the two AP-1 elements within the TF 5′-flanking region are necessary and sufficient to mediate activation of TF gene transcription in murine M4 cells.

To evaluate whether differential activation of the TF promoter might underlie the differential expression of TF protein in C10 and M4 cells, we compared the activity of AP1-TF60 in C10 and M4 cells transfected in parallel. As shown in Figure 2C, the activity of AP1-TF60 was ~fourfold lower in C10 relative to M4, suggesting that the differences seen in TF activity are indeed mediated at the transcriptional level. In an attempt to identify the molecular basis for the difference in TF promoter activity in these melanoma cell lines, we carried out band shift assays to analyze the AP-1 protein composition of each cell type. An oligonucleotide probe consisting of the two TF AP-1 sites was incubated with nuclear protein extracted from C10 and M4 cells, respectively. As illustrated in Figure 3, 2 μg of nuclear protein from M4 cells displayed significantly more AP-1 DNA-binding activity than 3 μg of nuclear protein from C10 cells suggesting a dramatic difference in the relative abundance of AP-1 proteins in C10 versus M4 cells. Since AP-1 complexes consist of either Jun/Jun homodimers or Jun/Fos heterodimers [Karin et al., 1997], we also hypothesized that the composition of the AP-1 DNA-binding complexes might differ between C10 and M4 cells. For that reason, we performed supershift assays with antibodies specifically directed against members of the Jun and Fos families, respectively. As shown in Figure 3, both cell lines displayed a quantitative supershift with a pan Fos antibody (lanes 3 and 13) indicating that the TF AP-1 DNA-binding complexes in these cell lines are composed of Fos/Jun heterodimers. In M4 cells, the dominant Fos family member appears to be Fra-1 (Fig. 3, lane 16), while in C10 cells, a Fra-1 supershift is barely detectable (lane 6). In terms of the Jun family, JunB and JunD supershifted species are clearly evident in M4 cells but are dramatically reduced in C10 cells (Fig. 3, compare lanes 18 and 19 to lanes 8 and 9, respectively). Collectively, these results suggest that the difference in transfected TF promoter activity between M4 and C10 cells is likely due to a difference in the relative abundance of Fra-1/JunB or Fra-1/JunD heterodimers but is not due to an overt cell type-specific difference in AP-1 DNA-binding protein composition.

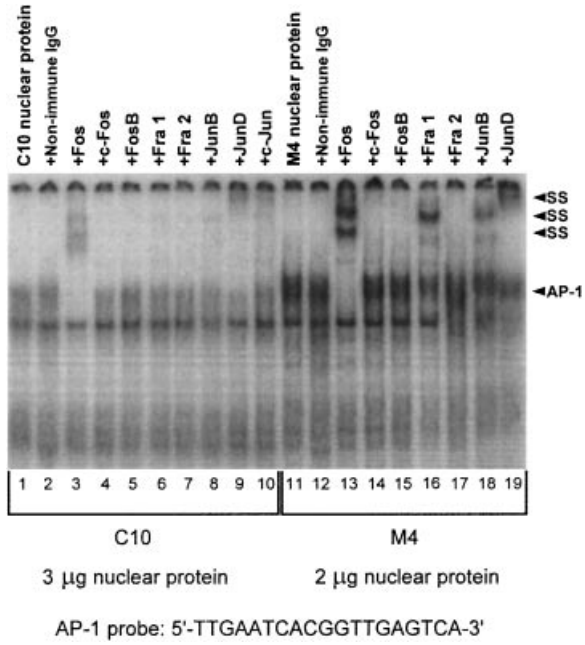

Fig. 3. Abundance and composition of AP-1 DNA-binding complexes in C10 versus M4 melanoma cells. Band shift analysis of nuclear protein extracted from melanoma cell lines of differing metastatic potential was conducted using an AP-1 probe derived from the TF 5′-flanking region and AP-1 specific antibodies. In C10 (weakly metastatic clone) lanes, 3 μg of nuclear protein was tested, while in M4 (highly metastatic clone) lanes, 2 μg protein was used. Nuclear protein from M4 cells (**lanes 11–19**) shows a greater abundance of AP-1 DNA-binding activity (AP-1 labeled arrowhead) relative to C10 (**lanes 1–10**). A pan Fos antibody generated a complete supershift of nucleoprotein complexes formed with both C10 and M4 protein (lanes 3 and 13, respectively) while Fra-1, JunB, and JunD-specific antibodies produced detectable supershifts with M4 protein (lanes 16, 18, and 19, respectively). Antibody supershifted complexes are denoted with SS labeled arrowheads.

Adenovirus E1A 12S Oncoprotein Represses an AP-1-Dependent TF Promoter in Transiently Transfected M4 Cells

We have previously shown that AP1-TF promoter activity is repressed by the E1A 12S oncoprotein in serum or serum plus TGF-β1 stimulated AKR-2B fibroblasts by a mechanism that involves sequestration of a c-Fos-dependent coactivator [Felts et al., 1997]. This coactivator likely consists of multiple proteins and includes the retinoblastoma tumor suppressor protein, pRB [Liu et al., 2000]. Thus, we were interested in determining whether the same was true in melanoma cells where the activity of the AP-1 cis-elements in the mouse TF promoter

does not appear to depend on c-Fos binding/recruitment. Initially, to test the effect of the E1A 12S oncoprotein on TF promoter activity in melanoma cells, several different TF reporter gene constructs were transiently cotransfected with different amounts of E1A expression vector into highly metastatic M4 cells. As shown in Figure 4, the E1A 12S oncoprotein was capable of repressing the activity of the full-length promoter (TF890), a truncated promoter (TF264), and importantly, the chimeric TF promoter that contains only the two TF AP-1 elements upstream of the TATA-containing TF basal promoter (AP1-TF60). The activity of the TF basal promoter (TF60) was not altered by E1A (Fig. 4). These data suggest that the AP-1 sites are necessary and sufficient to mediate the repression by E1A in M4 cells. Moreover, despite the difference in activity of AP1-TF60 in C10 versus M4 cells (Fig. 5A), this promoter construct was similarly repressed by the E1A 12S gene product in both cell lines (Fig. 5B).

We have recently characterized a panel of cytomegalovirus (CMV) enhancer/promoter-driven expression vectors harboring cDNAs encoding E1A deletion and point mutants specifically defective in the binding of CBP/p300 (d2-36, RG2) or the pocket proteins, pRB, p107, and p130 (dCR2, CG124, YH47/CG124) in AKR-2B fibroblasts [Liu et al., 2000]. To confirm that these mutant proteins behaved similarly in M4 cells, we analyzed the pocket protein- and CBP/p300-binding characteristics of wild type and mutant E1A proteins ectopically expressed in M4 cells. Western blot analyses of anti-E1A immunoprecipitates showed that wild type E1A was capable of interacting with p300, CBP,

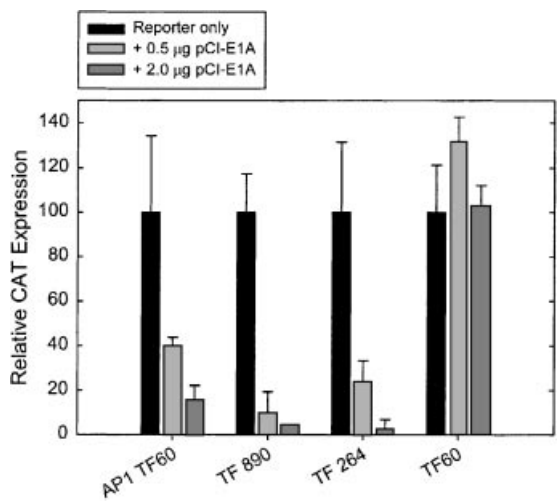

Fig. 4. Repression of AP-1-dependent TF promoter activity in M4 cells by adenovirus E1A 12S oncoprotein. M4 cells were transiently transfected with a total of 10 μg plasmid DNA including 7.5 μg of a selected TF-CAT reporter (AP1-TF60, TF890, TF264, see Fig. 2A, or the minimal TF promoter, TF60), 0.5 μg pCMVβ, and varying amounts of pCI E1A 12S expression vector (pCI-EIA). Empty pCI vector was added to equalize transfected DNA. After 48 h, cell lysates were prepared and assayed for CAT, β-gal, and total protein. For each TF-CAT reporter, data are expressed as a percentage of the corrected CAT value measured in the reporter only control (defined as 100%) and represent the average of triplicate plates transfected ± SD. The activity of the basal TF promoter (TF60) is unaffected by E1A due to the absence of linked AP-1 cis-elements.

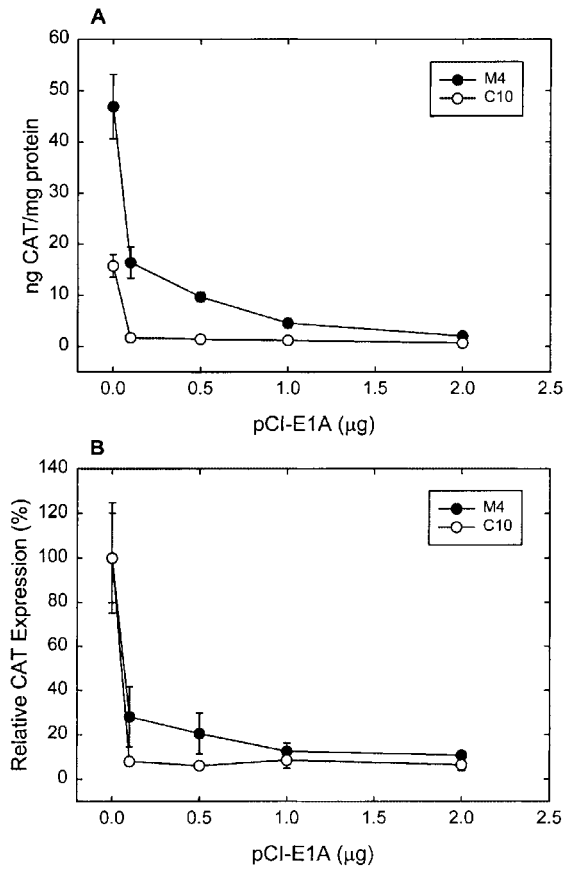

Fig. 5. Repression of AP1-TF60 promoter activity by E1A 12S oncoprotein in melanoma cells of differing metastatic potential. **A**: Weakly metastatic C10 cells and highly metastatic M4 cells were transiently transfected with a total of 10 μg plasmid DNA including 7.5 μg AP1-TF60 CAT reporter, 0.5 μg pCMVβ, and varying amounts of pCI E1A 12S expression vector (pCI-EIA). Empty pCI vector was added to equalize transfected DNA. After 48 h, cell lysates were prepared and assayed for CAT and total protein. Data represent the average of triplicate plates transfected ± SD. Representing the raw data in this fashion emphasizes the difference in relative AP1-TF promoter activity in C10 versus M4 cells. **B**: Normalizing the data by setting the reporter only value to 100% for each cell line indicates that E1A is equally efficient at repressing TF promoter activity in both cell lines.

p130, p107, and pRB (Fig. 6, lane 2). The *N*-terminal deletion mutant, d2-36 (lane 3), and the amino acid 2 point mutant, RG2 (lane 8), were specifically deficient in CBP/p300-binding. Similarly, the conserved region 2 deletion mutant, dCR2 (lane 6), and the double point mutant, YH47/CG124 (lane 10), were each defective in p130, p107, and pRB-binding. The single point mutant, CG124 (lane 9), and the double mutant, d2-36/CG124 (lane 11), failed to bind pRB but retained the ability to interact with p107 and p130. The d2-36/CG124 double mutant was also unable to bind CBP/p300. To test the effects of E1A mutations on AP1-TF promoter activity in melanoma cells, M4 cells were co-transfected with AP1-TF60 and wild type or selected mutant E1A expression vectors. As shown in Figure 7, wild type E1A dramatically repressed promoter activity when compared to control cells cotransfected with empty expression vector. Mutants defective in binding of pRB, p107, and p130 retained the ability to repress promoter activity. These included the pRB-specific single point mutant, CG124, and the double point mutant, YH47/CG124. Curiously, mutants deficient in CBP/p300 binding were generally ineffective, or less effective than wild-type E1A in repressing promoter activity. These included the deletion mutant d2-36 and the double mutant d2-36/CG124 that additionally lacks the ability to bind pRB. The RG2 point mutant demonstrated relatively weak and inconsistent expression in transiently transfected cells and thus was excluded from this analysis. Nonetheless, the fact that the 2–36 region of E1A is essential for inhibition of AP1-TF

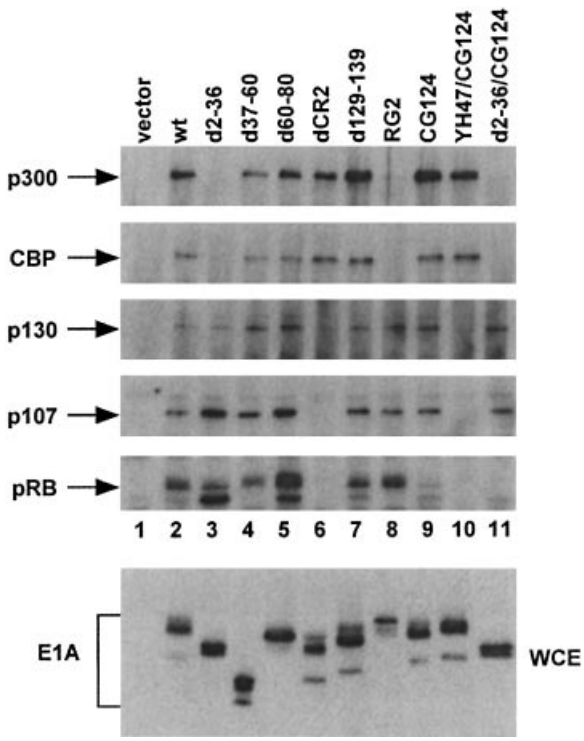

Fig. 6. Pocket protein- and CBP/p300-binding properties of mutant E1A 12S proteins expressed in M4 melanoma cells. M4 cells were transiently transfected with 10 μg wild type or mutant E1A expression vectors. After 45 h in growth medium, whole cell lysates were prepared, and E1A was immunoprecipitated from 100 μg lysed cell protein with an anti-E1A monoclonal antibody, M73. Immunoprecipitates were denatured and protein components separated on a 6% SDS–polyacrylamide gel. E1A-associated proteins including p300, CBP, p130, p107, or pRB were detected by immunoblotting using commercially available antibodies described in Materials and Methods. (**Bottom panel**) Ectopic expression of wild type and mutant E1A proteins was confirmed by Western blotting of 3 μg total lysed cell protein resolved on a 10% SDS–polyacrylamide gel. Mouse anti-E1A M73 was used to probe the blot. WCE, whole cell extract.

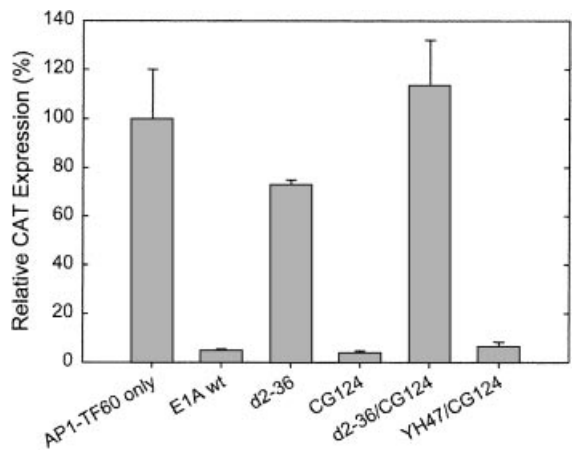

Fig. 7. Differential effects of E1A deletion and point mutants defective in either CBP/p300 or pocket protein binding on AP1-TF60 promoter activity in transiently transfected M4 cells. Highly metastatic M4 melanoma cells were transiently transfected with a total of 10 μg plasmid DNA including 7.5 μg AP1-TF60 CAT reporter, 0.5 μg pCMVβ, and 1 μg of expression vector encoding either wild type (wt) or mutant (d2-36, CG124, d2-36/CG124, or YH47/CG124) E1A 12S proteins. Empty pCI vector was added to equalize transfected DNA. Following an 18-h recovery period in complete growth medium, cells were serum-starved for 48 h then stimulated for 6 h with 20% FBS + 5 ng/ml TGF-β1. Cell lysates were prepared and assayed for CAT, β-gal, and total protein. CAT values were corrected for total protein and β-gal expression. To account for variability in E1A mutant protein expression, corrected CAT values were also normalized for E1A protein detected in cell extracts by immunoblotting and quantified by densitometry. Data are expressed as a percentage of the reporter only control (defined as 100%) and represent the average of triplicate plates transfected ± SD.

promoter activity in M4 cells (Fig. 7) suggests that, in contrast to AKR-2B fibroblasts [Felts et al., 1997], the coactivator proteins, CBP/p300, may be key players in TF promoter activation in melanoma cells.

E1A 12S Oncoprotein Represses Endogenous TF Activity in M4 Cells and Effectively Reduces Experimental Hematogenous Metastasis to Lung

To further investigate the effect of the adenovirus E1A 12S oncoprotein and the importance of its *N*-terminal CBP/p300-interacting region on endogenous TF function in murine melanoma cells, we derived stable transfectants of M4 cells that express either wild type E1A or the *N*-terminal mutants, RG2 and d2-36 (Table I). Representative cell lines were assayed for TF cofactor activity and E1A protein expression after clonal selection. As shown in Figure 8A, M4 cells stably expressing wild type E1A (ME16) demonstrated very little TF cofactor activity relative to control cells transfected with empty expression vector (MC6). The marked reduction, and in some cases, absence of TF cofactor activity was observed in multiple independent cell lines expressing wild type E1A. These results suggest that the E1A 12S oncoprotein is capable of repressing endogenous TF expression/activity in M4 melanoma cells. Importantly, stable expression of the *N*-terminal mutants, RG2 and d-26, did not substantially impair TF cofactor activity relative to control cells (Fig. 8A, compare MRG6 and MDT20 to MC6). Differences in functional TF cofactor activity among the cell lines tested were not attributable to overt differences in E1A expression (Fig. 8B, lanes 2, 4, and 7) although it should be noted that some other clones showed only very weak E1A expression (lane 5).

Since it has been reported that TF contributes to the metastatic potential of melanoma cells [Mueller et al., 1992; Bromberg et al., 1995], we were interested in determining whether expression of E1A could inhibit or reduce experimental metastasis of murine melanoma cells by downregulating TF expression/activity. Therefore, clonal cell lines expressing either wild type E1A (ME), the RG2 point mutant (MRG), or d2-36 deletion mutant (MDT) were tested for metastatic potential in mice. To eliminate the possibility that differences in metastatic potential could result from differential antigenicity of the tumors, NOD/SCID mice were chosen as test

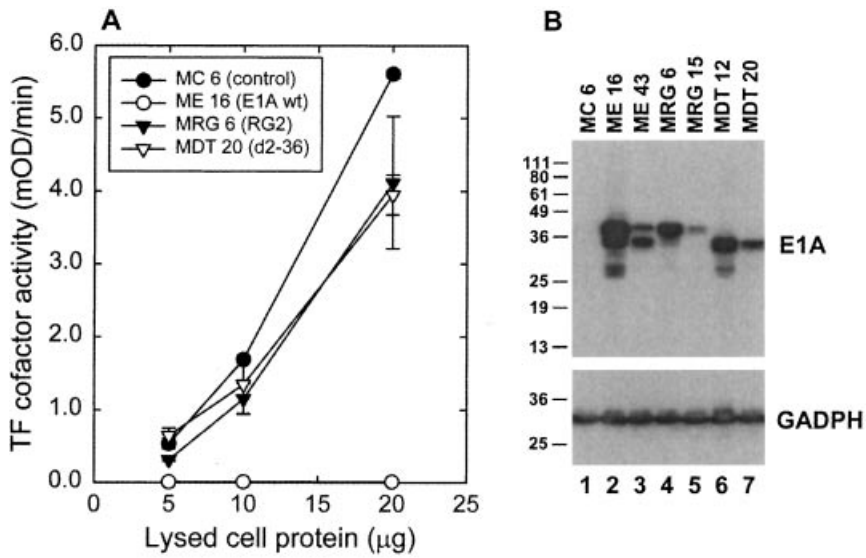

Fig. 8. TF cofactor activity of murine melanoma cell lines stably expressing wild type E1A (ME) or *N*-terminal E1A mutants defective in CBP/p300 binding (MRG and MDT). **A**: M4 cells were stably transfected with expression vectors encoding either wild type E1A or E1A mutants (RG2 or d2-36) as described in Materials and Methods. Rapidly growing cells from selected clones were lysed and TF cofactor activity was assayed using varying amounts of protein as described in Figure 1. Cells stably expressing wild type E1A 12S (ME16) exhibited markedly reduced TF activity compared to a control cell line (MC6) and to cell lines expressing *N*-terminal E1A mutants (MRG6 or MDT20). **B**: To evaluate the expression of E1A protein, 25 μg of whole cell protein from the indicated cell lines was resolved by SDS–PAGE and probed by immunoblotting using the mouse anti-E1A monoclonal antibody, M73 (**top panel**). As a loading control, the E1A blot was reprobed with mouse anti-GADPH (**bottom panel**).

animals. As a control, we first confirmed that M4 cells were indeed highly metastatic compared to C10 cells. Lungs excised from mice following i.v. injection and growth of M4 cells showed numerous nodules on the organ surface whereas comparable nodules could not be found on the surface of lungs from mice injected with the same number of C10 cells (Table II). Interestingly, clonal ME cell lines expressing wild type E1A and corresponding low TF activity showed distinctly different metastatic behavior as evidenced by the markedly reduced size and number of tumor nodules (Table II) and weight of lungs post fixation (Fig. 9). Lungs of animals injected with ME16, for example, did not reveal the extent of nodule formation when compared to M4 although a few small nodules were detected on the surface that indicated limited metastasis (Table II). On the other hand, lungs of mice injected with MRG or MDT cells were similar to M4 lungs in terms of the number and size of tumor nodules (Table II) and lung weight (Fig. 9). These results suggest that E1A 12S protein significantly reduces the metastatic potential of murine melanoma cells by a mechanism which involves the *N*-terminal CBP/p300-binding domain of E1A, the same region implicated in the modulation of TF promoter and cofactor activity.

To test whether reduction of TF expression was the principal cause for the impaired

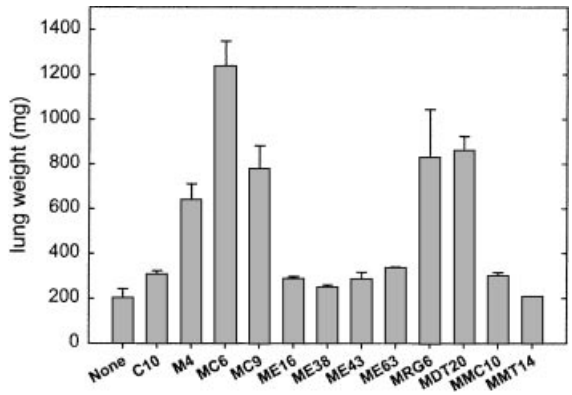

Fig. 9. Wet weight of lungs excised from NOD-SCID mice injected with murine melanoma cells. The indicated cell lines, at 7×10^5 cells/animal, were injected into the lateral tail vein of NOD-SCID mice. After 14 days, mice were killed, and lungs were excised and fixed in 10% formalin. The lungs of animals injected with cells expressing wild type E1A (ME) weighed significantly less than the lungs of animals injected with control cells (MC) or cells expressing *N*-terminal E1A mutants (MRG and MDT). ME lungs were comparable to weakly metastatic C10 lungs.

metastatic behavior of ME cell lines, we stably transfected the poorly metastatic cell line, ME16, with a mouse TF expression vector. As shown in Figure 10, several cell lines were derived that exhibited TF cofactor activity which matched (MMT16) or exceeded (MMT11, 13, 14) that of M4. Injections of NOD/SCID mice with MMT cells were performed to determine

TABLE II. Experimental Hematogenous Metastasis to Lung of Melanoma Cell Lines Ectopically Expressing Wild Type or Mutant E1A 12S Proteins

Cell line	Mice injected	Nodules < 1 mm	Nodules ≥ 1 mm
M4	12	N.D.	14, 15, 16, 44, >100 (8)
C10	3	0, 0, 0	0, 0, 0
ME16	8	0, 0, 0, 4, 7, 8, 8, 9	0, 2, 5, 0, 0, 0, 0, 0
ME38	3	0, 10, 14	0, 2, 1
ME43	3	5, 7, 14	0, 0, 0
ME52	2	0, 0	0, 0
ME63	2	9, 13	1, 1
MC6	3	N.D.	85, 96, 100
MC9	1	N.D.	>100
MRG6	9	N.D.	>100
MRG15	3	N.D.	>100
MMC10	4	0, 0, 0, 0	0, 0, 0, 0
MMT14	3	0, 0, 0	0, 0, 0
MMT16	3	0, 0, 0	0, 0, 0

NOD-SCID mice were injected i.v. with 7×10^5 cells/mouse using selected clones of cell lines described in Table I. Two weeks later, lungs were excised, fixed, and pulmonary nodules estimated as ≥ 1 mm and < 1 mm were counted. Cells expressing wild type E1A 12S protein (ME) produced markedly fewer ≥ 1 mm nodules in comparison to control vector transfected cells (MC) or to the parent cell line (M4). Using Fisher's exact test, pair-wise comparison between the M4 and ME groups resulted in a *P*-value of (< 0.0001) and pair-wise comparison between the ME and MC groups resulted in a *P*-value of (< 0.0002). These results show that there are statistically significant differences in the occurrence of large nodules among these groups of cell lines. MRG6 and MRG15, which express an *N*-terminal E1A mutant defective in CBP/p300-binding, were strongly metastatic and did not differ from M4 and MC clones. Overexpression of mouse TF in ME16 (see MMT14 and MMT16) did not reverse the metastasis-inhibiting effect of wild type E1A. N.D., not detectable because of the large number of ≥ 1 mm nodules.

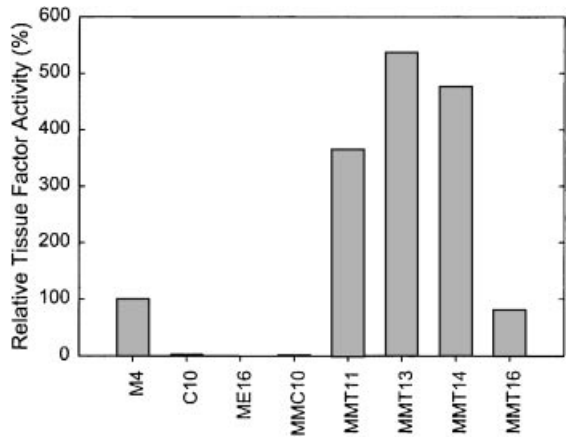

Fig. 10. TF cofactor activity of murine melanoma cell lines stably transfected with both E1A and mouse TF (MMT). The high EIA-expressing clonal cell line, ME16, was stably transfected with a mouse TF expression vector to generate MMT clones 11 to 16. Rapidly growing cells were lysed and an equivalent amount of protein was assayed for TF cofactor activity as described in Figure 1. Data are expressed as a percentage relative to the ME16 parent cell line, M4 (defined as 100%). MMT clones demonstrated markedly enhanced TF activity when compared to the parent cell line, ME16, and to a control cell line which was transfected with empty pCI vector only (MMC10).

if ectopic expression of TF in E1A-expressing melanoma cells would enhance metastatic potential. However, no significant increase in either the size or abundance of lung nodules was observed when compared to the parent cell line ME16 (compare MMT14 and MMT16 to ME16, Table II) indicating that the highly metastatic phenotype of M4 cells does not result solely from high level TF expression/activity. Hence, it is likely that a more complex set of interactions involving multiple E1A-responsive and CBP/p300-regulated genes, including tissue factor, must occur in order to manifest a pro-metastatic phenotype in this model system.

DISCUSSION

Adenovirus E1A (Early region 1A) gene products are nuclear phosphoproteins whose primary function is to trans-activate other viral early genes required for adenovirus replication by co-opting the transcriptional apparatus of a host cell during infection (reviewed by Berk, 1986). Transcription and processing of the E1A gene of the group C adenoviruses (Ad2 and Ad5) gives rise to two major mRNA transcripts, designated 12S and 13S, which are translated into proteins of 243 and 289 amino acids, respectively (reviewed in Moran and Mathews, 1987). The E1A 12S and 13S proteins possess multiple independent domains responsible for mediating host cell immortalization and/or transformation by modulation of host cell DNA synthesis, gene transcription, and differentiation status (reviewed in Berk, 1986; Nevins, 1995; Condorelli and Giordano, 1997; Flint and Shenk, 1997). Somewhat paradoxically, E1A proteins have also been implicated as suppressors of tumorigenicity and metastatic potential (reviewed in Chinnadurai, 1992; Mymryk, 1996; Yu and Hung, 1998; Hung et al., 2000). The E1A 12S protein lacks a 46 amino acid sequence, designated conserved region (CR3), that is essential for activation of viral early genes by the 13S protein [Moran and Mathews, 1987]. However, the E1A 12S protein possesses two other conserved regions (CR1 and CR2) required for mediating transcriptional activation or repression of host cell genes involved in cell proliferation and differentiation [Flint and Shenk, 1997]. Mechanistically, E1A 12S oncoprotein has been shown to interact with a number of cellular proteins including the transcriptional coactivators, CBP/p300, pRB and the related pocket proteins, p107 and p130 ([Wang et al., 1993; Bishopric et al., 1997] and references therein), and the TATA-binding protein [Song et al., 1997]. Specific E1A amino acids that mediate binding of CBP/p300 and the pRB family have been mapped to the *N*-terminus and to CR1 and CR2, and mutants deficient in binding have been characterized ([Wang et al., 1993] and references therein). Because of its unique ability to specifically interact with a variety of different transcriptional regulatory proteins, E1A is commonly used as a tool to identify cofactors involved in the activation or repression of eukaryotic promoters.

In the present study, we have exploited the functional attributes of the E1A 12S protein to gain a better understanding of the control of TF gene expression at the molecular level and of the role of TF protein in contributing to the metastatic potential of murine melanoma cells in vivo. Although TF expression has been investigated extensively in monocytes, endothelial cells, smooth muscle cells, epithelial cells, and fibroblasts [reviewed in Camerer et al., 1996; Mackman, 1997], little was known about the regulation of TF in melanoma cells. Previous studies have shown that metastatic potential of human melanoma cell lines is strongly

correlated with TF activity and TF expression [Mueller et al., 1992; Bromberg et al., 1995]. In support of this notion, we found that highly metastatic M4 murine melanoma cells possessed elevated TF cofactor activity (and TF protein) compared to weakly metastatic C10 cells (Fig. 1). Moreover, we also verified that TF activity in murine melanoma cells is strongly correlated with metastatic behavior since stable expression of wild type E1A 12S oncoprotein in M4 cells resulted in both dramatically reduced TF cofactor activity (Fig. 8) and impaired metastatic potential (Fig. 9, Table II). Since C10 and M4 melanoma cells were derived from the same primary tumor [Fidler et al., 1981], we reasoned that these cell lines would also serve as useful models to study the regulation of the TF gene at the molecular level in relation to metastatic potential.

In order to characterize the cis-elements and trans-acting factors that contribute to regulating TF gene expression in melanoma cells, we studied the transcriptional activity of various TF promoter constructs in transiently transfected C10 and M4 cells. Promoter mapping studies revealed that two AP-1 binding sites upstream of the minimal TF promoter were required to mediate both high level transcriptional activity (Fig. 2) and repression by E1A 12S protein (Figs. 4 and 5). Importantly, comparison of the activity of an AP-1-dependent TF promoter in C10 versus M4 cells suggested that the differential expression of TF cofactor activity in these two cell lines was attributable to differences in transcriptional capacity (Fig. 2C). These results do not necessarily exclude the possibility that there might be differences at other levels too, since TF gene expression can also be regulated by mechanisms involving mRNA stabilization [Crossman et al., 1990; Brand et al., 1991; Ahern et al., 1993]. However, our finding that M4 cells appeared to contain a greater quantity of Fra-1/JunB and Fra-1/JunD heterodimers than C10 cells (Fig. 3), underlines the significance of AP-1-dependent transcription in contributing to the expression of TF in melanoma cells. Interestingly, the composition of the TF AP-1 DNA-binding complexes observed in M4 cells is somewhat unique in that Fra-1 appears to be the dominant Fos family member. In previous studies that have evaluated AP-1 proteins in TF gene regulation in cell types such as fibroblasts, endothelial cells, and monocytes, c-Fos and Fra-2 have been implicated as the functionally relevant components of AP-1 [Felts et al., 1995; Hall et al., 1999; Liu et al., 2000].

The predominance of Fra-1, as opposed to other Fos family members, in AP-1 complexes from M4 cells, may also explain the results of the E1A 12S protein expression studies. Unlike AKR-2B fibroblasts, where c-Fos/JunD complexes dominate [Felts et al., 1995, 1997], repression of AP1-TF promoter activity in M4 cells by E1A is mediated by a mechanism that does not involve the pRB protein family (Figs. 6 and 7). This is quite different from AKR-2B cells and primary mouse embryo fibroblasts where pRB appears to be a major player in c-Fos-dependent TF transcription [Liu et al., 2000]. On the contrary, sequestration of an E1A *N*-terminal-interacting factor(s), most likely CBP/p300, appears to be responsible for the repressive effect of E1A on TF promoter activity in M4 cells. Moreover, stable expression of E1A in M4 cells did not impair AP-1 DNA-binding capacity when compared to the parental M4 cell line suggesting that repression by E1A does indeed occur via sequestration of a coactivator rather than inhibiting the expression of an essential AP-1 DNA-binding protein such as Fra-1. However, because elevated Fra-1 expression has been implicated in enhancing the invasiveness of epithelial tumor cell lines [Kustikova et al., 1998], we cannot discount the latter possibility.

In an attempt to manipulate TF expression and, perhaps, metastatic behavior of M4 melanoma cells, we derived transfectants that stably expressed wild type E1A or mutants defective in CBP/p300-binding and compared their TF cofactor activity in vitro with their ability to form metastatic lung nodules in vivo. This strategy differed from several previous studies where the complete E1A gene, which encodes both 12S and 13S splice variants, was utilized to evaluate the tumor and/or metastasis-suppressing function of E1A [Frisch et al., 1990, 1991; Yu et al., 1991, 1992, 1993b; Deng et al., 1998]. Clones expressing wild type E1A 12S protein exhibited both low TF cofactor activity and drastically reduced metastatic potential in keeping with evidence that the E1A 12S protein can function as a suppressor of tumorigenic growth and metastasis in certain tumor cell lines [Pozzatti et al., 1988; Frisch and Dolter, 1995]. In contrast, clones expressing the RG2 or d2-36 mutant forms of E1A showed high TF cofactor activity and were strongly metastatic in vivo (Figs. 8, 9, and Table II). These results

establish a strong correlation between TF expression/activity, CBP/p300-binding ability of E1A, and in vivo metastatic potential in this model system. However, other findings suggest that the reduction in metastatic potential conferred by E1A cannot be attributed exclusively to a decrease in TF cofactor activity since stable ectopic expression of mouse TF failed to reverse the metastasis-suppressive effect of wild type E1A (Fig. 10 and Table II). A similar finding was made by Yu et al. [1993a] who found that re-expression of HER-2/*neu* oncoprotein did not counteract the metastasis-suppressing function of full-length E1A in *neu*-transformed NIH-3T3 cells despite restoration of tumorigenicity. Such results should not be surprising since E1A has been shown to repress expression of a variety of other genes implicated in metastasis and/or progression to a malignant phenotype ([Mymryk, 1996; van Groningen et al., 1996] and references therein) including some AP-1-dependent genes that encode secreted proteases [Frisch et al., 1990; Offringa et al., 1990; Hagmeyer et al., 1993]. Taken together, our data suggest that TF is one member of a class of E1A-responsive and CBP/p300-regulated genes that putatively cooperate to regulate metastatic potential in murine melanoma cells. In this regard, further insights into the mechanism by which E1A 12S "reprograms" global melanoma gene expression may be obtained by evaluating gene expression profiles of stable E1A 12S transfectants versus the M4 parent cell line using DNA microarray technology [Clark et al., 1999]. This experimental strategy coupled with an in vivo assessment of the metastatic potential of melanoma cells stably expressing mutant forms of E1A defective in binding to specific regulatory proteins (e.g., CBP/p300, pRB, p107, p130, or C-terminal binding protein) might permit the categorization of cofactor-dependent gene families whose differential expression might facilitate progression to a metastatic phenotype.

The mechanism(s) by which TF may contribute to enhancing the metastatic potential of melanoma cells is an issue that has not yet been resolved. While several studies have indicated the importance of TF procoagulant activity, and hence, the extracellular ligand-binding domain in the metastatic process [Mueller et al., 1992; Fischer et al., 1995], Bromberg et al. [1995] reported that a TF mutant (Lys165, Lys166 $\rightarrow$ Ala) defective in procoagulant activity but unimpaired in factor VII/VIIa binding was still able to promote experimental metastasis in mice when expressed in a human melanoma cell line. However, it was later shown that this particular extracellular domain TF mutant, while demonstrating very low clotting activity in human plasma, retained significant clotting activity when expressed in Chinese hamster ovary (CHO) cells and assayed in mouse plasma [Mueller and Ruf, 1998]. Thus, the relative utility of such a mutant in evaluating the contributions of TF procoagulant activity to TF-dependent metastasis in mice was deemed questionable [Mueller and Ruf, 1998]. Importantly, studies of other TF mutants suggested that the prometastatic function of TF in stably transfected CHO cells requires not only the binding of proteolytically active factor VIIa to the extracellular domain, but also cooperating, and as yet unknown functions of the cytoplasmic domain [Mueller and Ruf, 1998].

In the experimental model of hematogenous metastasis employed here and in the studies cited above, at least three distinct molecular events are required in order to generate pulmonary nodules. These include tumor cell residency, tumor cell implantation, and tumor cell growth within the lung parenchyma. It is not difficult to imagine a role for TF in tumor cell adhesion and implantation given that the extracellular, factor VIIa-binding domain has been implicated in mediating interaction with matrix-associated tissue factor pathway inhibitor-1 [Fischer et al., 1999] and in generating protease activity required for tumor cell invasion [Mueller et al., 1992]. The role of the TF cytoplasmic domain is less established but likely involves signaling events modulated by serine phosphorylation [Zioncheck et al., 1992] and/or actin-binding protein 280 (ABP-280) interaction [Ott et al., 1998]. In this regard, it is intriguing that Abe et al. [1999] reported that stable over-expression of TF in a low tumor-producing melanoma cell line resulted in increased vascular endothelial growth factor (VEGF) expression with dependence on the TF cytoplasmic domain. This observation is consistent with an earlier study reporting that VEGF expression could be induced by stable transfection of murine fibrosarcoma cells with TF cDNA [Zhang et al., 1994]. Moreover, because inactivation of the TF gene leads to embryonal death of mice due to abnormal circulation [Carmeliet et al., 1996; Toomey et al., 1996] and loss of

vascular integrity [Bugge et al., 1996], TF has been implicated in both tumor-associated angiogenesis and blood vessel development. However, the overall necessity of TF in these processes is likely to be influenced by genetic background [Toomey et al., 1997]. Since angiogenesis is a requisite event in the progression to metastasis, the potential to induce VEGF-mediated neovascularization is one mechanism by which TF, and in particular, its cytoplasmic and presumptive signaling domain, may augment the metastatic potential of melanoma cells. This concept raises several experimentally testable questions relevant to our studies. For example, does E1A expression in this murine melanoma cell system also impair VEGF expression, and if so, does ectopic expression of TF restore VEGF production in E1A-expressing clones or can E1A modulate VEGF expression directly by a TF-independent pathway? The answers will likely reveal new insights into the significance of TF expression on VEGF production in malignant melanoma cells [Abe et al., 1999], and, perhaps, how these two distinct gene products and others cooperate to facilitate melanoma metastasis.

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