Melanotransferrin gene expression in melanoma cells is correlated with high levels of Jun/Fos family transcripts and with the presence of a specific AP1-dependent ternary complex

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The involvement of the transcription factor AP1 in the regulation of melanotransferrin (MTf) gene expression was investigated. MTf, also known as p97, is a tumour-associated antigen that is overproduced in most melanomas. Its gene expression is under the control of an enhancer element containing two AP1 binding sites. By Northern analysis, we demonstrate that MTf mRNA is detected at various levels in melanoma SK-MEL-28 cells and that its greatest expression coincides with the presence of large amounts of *jun* and *fos* transcripts. Gel retardation assays revealed that the induction of expression of these proto-onco-

genes is correlated with increased AP1 binding activity and that a region of the MTf enhancer is involved in the formation of a ternary AP1-dependent complex, implicating a second nuclear factor whose binding characteristics are similar to those of nuclear factor of activated T cells (NF-AT). In transient expression experiments, the activity resulting from ternary complex formation was high and specific to melanoma cells. These data provide a possible explanation for the mechanisms of AP1 factor family involvement in MTf up-regulation in melanoma cells.

INTRODUCTION

Members of the Jun and Fos families of transcription factors regulate a large number of genes and have been implicated in cellular processes including proliferation and differentiation (reviewed in [1]). AP1 activity results from the dimerization of Jun and Fos proteins by interaction of their leucine-zipper domains. The relative amounts of the different forms resulting from both homo- and hetero-dimerization may explain the varying abilities of these factors to regulate gene transcription through their recognition site, called the TRE [TPA-responsive element, where TPA is another name for phorbol 12-myristate 13-acetate (PMA)] (reviewed in [1,2]). AP1 family members are tightly regulated by mechanisms that include post-transcriptional modifications such as phosphorylation, which influences their binding and trans-activating activity [3–5]. Regulation by phosphorylation results from the activation of transduction pathways for which Jun and Fos are at the end-point. Their corresponding immediate-early genes transmit to the nucleus a variety of external signals driven by growth factors and other compounds such as the phorbol ester PMA. AP1 activity can also be modulated by interaction with other nuclear factors, e.g. the glucocorticoid receptor, which acts as a negative regulator (reviewed in [6]).

Composite DNA elements, where TRE sites are juxtaposed with motifs recognized by other transcription factors, illustrate the means by which AP1 co-operates with specific partners to control gene expression in response to specific conditions. This is exemplified in the case of the Ets and nuclear factor of activated T cells (NF-AT) transcription factor families, which are characterized respectively by their Ets and Rel DNA binding domains. The control regions of several genes that are highly expressed in

transformed cells carry composite sites at which AP1 and Ets factors co-operate to induce transcription in response to oncogenes; the genes for collagenase and urokinase-type plasminogen activator are well known examples [7,8]. Thus this association may contribute to the up-regulation of some AP1-regulated genes in transformed cells. In the second example, co-operation between AP1 and NF-AT members occurs after T cell activation and turns on a set of genes coding for cytokines (reviewed in [9]).

Little is known about gene regulation by AP1 in melanocytes and melanomas. To our knowledge, no AP1 target sites have been found in the control regions of the few melanocyte-specific genes studied. A variety of experimental evidence suggests, however, that AP1 factors are important in this cell lineage. First, AP1 is activated after UV irradiation [10,11], a suspected risk factor for the occurrence of melanomas [12]. Secondly, melanocytes divide poorly and require the presence of phorbol esters and cAMP inducers in order to be maintained in culture; melanoma cells grow independently of these agents, a feature that has been associated with changes in the protein kinase C pathway [13–15].

The melanoma antigen melanotransferrin (MTf) is a transferrin-related protein whose function in iron internalization has been demonstrated recently [16]. Due to its high expression at the surface of most melanoma cells [17], it represents a useful model with which to study gene regulation in this type of tumour cell. The MTf enhancer is a 166 bp element located 2 kb upstream from the promoter. This element is the target for the basic helix–loop–helix upstream stimulatory factor and for AP1 at two binding sites 130 bp apart [18,19]. We report here an analysis at the RNA level of the expression of MTf and of members of the Jun and Fos families in melanoma cells, and show that their levels are modulated by similar culture conditions. Furthermore,

Abbreviations used: MTf, melanotransferrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FCS, fetal calf serum; NF-AT, nuclear factor of activated T cells; NF- κ B, nuclear factor- κ B; PMA, phorbol 12-myristate 13-acetate ('TPA'); TRE, TPA-responsive element.

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accumulation of *jun* and *fos* mRNAs coincided with increased binding activity at the two AP1 sites of the MTF enhancer. One AP1 binding site is involved in the formation of a ternary complex implicating a second nuclear factor. This factor appears to be related to NF-AT proteins, and its association with AP1 may contribute to the specificity of expression of the MTF antigen in melanoma cells.

EXPERIMENTAL

Oligonucleotides

The sense-strand sequences of wild-type and mutated oligonucleotides from the MTF enhancer region corresponding to the two previously described AP1 sites (underlined) [18] were as follows: I, 5'-CAAGTTCAGTGAGTCACAGCTGAATGACA-3'; II, 5'-TTGCCAAAGGAAGGACTTAGTCATCAAAC-3'; IImutAP1, 5'-TTGCCAAAGGAAGGACTTcGatATCAAAC-3'; IImutGGA, 5'-TTGCCAAAGtAAGGACTTAGTCATCAAAC-3'. Other oligonucleotides used were the AP1 site from the collagenase promoter [9] (col; 5'-AGCTTCATGAGTCAGCCGGATC-3'), the NF-ATp site corresponding to the GM-430 element of the granulocyte-macrophage colony-stimulating factor gene [20] (NF-AT; 5'-CACACATCTTTCTCATGGAAAGATGA-3') and the nuclear factor- κ B (NF- κ B) site of the κ light chain enhancer (Promega) (NF- κ B; 5'-AGTTGAGGGGACTTTCACAGGC-3').

Constructions

Oligonucleotides I, II, IImutAP1 and IImutGGA were inserted 5' to the thymidine kinase promoter in pBLCAT5 [21]. Phosphorylated double-stranded oligonucleotides were ligated to pBLCAT5 digested with *Xba*I, filled with Klenow fragment and dephosphorylated. Constructs containing two copies of each oligonucleotide were selected and checked by sequencing using the Sequenase kit (Amersham).

Cell culture, treatments and preparation of conditioned media

SK-MEL-28 and HeLa cell lines were grown in Dulbecco's modified Eagle's medium (Boehringer Mannheim) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were treated with 5-azacytidine (Sigma) as follows: 5×10^5 cells were plated in 100 mm Petri dishes on day 0, treated with 10 μ M 5-azacytidine the next day and collected 48 or 72 h later. Cells collected on day 8 were treated for 48 h with 5-azacytidine, washed and cultured in medium containing 10% FCS for 6 days, with an additional change of medium on day 5. Control cells were subjected to the same protocols but with 5-azacytidine-free medium. Conditioned media were prepared from cells plated at 10^6 per 100 mm Petri dish and cultured for 6 days, with a change of medium on day 3. The culture supernatants were collected and centrifuged for 5 min at 1500 g.

Treatments with PMA (Sigma) and ionomycin (Sigma) were realized by adding 100 ng/ml or 0.5 mg/ml of each compound respectively, 3 h before cell collection.

RNA preparation and Northern experiments

Total RNA was extracted from cultured cells using RNA-PLUS® (Bioprobe Systems) as described by the manufacturer. Samples of 20 μ g of RNA were resolved by electrophoresis through 1% agarose/formaldehyde gels and transferred by capillarity to a Hybond N⁺ nylon membrane (Amersham). After heating for 2 h at 80 °C, blots were hybridized to ³²P-labelled

probes in 50% formamide at 42 °C in a hybridization oven (Robbins Scientific). The MTF probe was a 189 bp fragment corresponding to exon 15 obtained by PCR amplification with primers complementary to positions 1941–1959 and 2112–2130 of the published nucleotide sequence [22]. The *c-jun* probe was a 800 bp fragment of the human cDNA isolated by a *Hind*III–*Pst*I digestion of plasmid RSV-cJ [23], the *junB* probe was a 1200 bp fragment of the mouse cDNA obtained by a *Hind*III–*Xho*I digestion of plasmid RSV-JunB [24], and the *c-fos* probe was a 950 bp fragment of the mouse cDNA obtained by a *Bgl*II digestion of plasmid RSV c-fos [25]. The intensities of bands on Northern blots were measured with a PhosphorImager (Molecular Dynamics).

Gel retardation assays

Nuclear extracts were prepared by the microprep preparation technique described by Schreiber et al. [26] from cells plated at 10^6 per 100 mm Petri dish on day 0 and collected after various times in culture [(2–4) $\times 10^6$ cells]. The protein concentration was determined by a Bradford assay (Bio-Rad). Binding reactions were performed in a volume of 20 μ l containing 1 ng of 5' ³²P-labelled double-stranded oligonucleotide (5000 c.p.m.), 2 μ g of poly(dI-dC), 4 mM MgCl₂, 50 mM KCl, 0.125 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 10 mM Hepes, pH 7.9, 0.05% Nonidet P40, 10% glycerol and 5 μ g of nuclear protein. After 15 min at 4 °C, the mixtures were loaded on to a 6% polyacrylamide gel. For competition experiments, a 100-fold molar excess of the unlabelled competitor oligonucleotide was added to the binding reaction.

Transient expression assays

For transient assays, 5×10^5 cells were plated in 60 mm Petri dishes 24 h prior to transfection with calcium phosphate-precipitated plasmid DNA as previously described [18]. The medium containing the precipitate was removed 16 h later and the cells were cultured for another 48 h before harvesting. Plasmid DNAs were prepared using the Qiagen plasmid kit (Qiagen, Düsseldorf, Germany). Chloramphenicol acetyltransferase activity was determined using a phase-extraction assay with [¹⁴C]chloramphenicol and butyryl-CoA. Transfection experiments were repeated at least three times with two different DNA preparations for each construct.

RESULTS

Induction of MTF mRNA in melanoma SK-MEL-28 cells

A 4 kb MTF mRNA was easily detected in the total RNA from SK-MEL-28 melanoma cells but not in that from HeLa cells (Figure 1). This is consistent with the amount of protein on the surface of these cell types [17,27]. To test whether this difference in expression was the result of gene methylation, SK-MEL-28 and HeLa cells were incubated with the demethylating agent 5-azacytidine for various times. The MTF mRNA level increased substantially on day 8 in both treated and untreated SK-MEL-28 cells (Figure 1, lanes 4 and 5); this increase was thus not due to the 5-azacytidine treatment. In HeLa cells, 5-azacytidine did not induce MTF RNA to a detectable level (Figure 1, lanes 7, 8 and 10). It is, therefore, unlikely that methylation is a key event in the regulation of the MTF gene.

Co-ordinate modulation of MTF with *jun* and *fos* mRNA expression

To investigate the up-regulation of MTF mRNA observed on day 8, SK-MEL-28 cells were plated at a fixed density on day 0 and

Figure 1 Northern blot analysis of total RNA prepared from SK-MEL-28 and HeLa cells

RNA was prepared from cells treated (+) or not (–) with 5-azacytidine and cultured for 2, 3 or 8 days. The 8-day cultured cells were treated with 5-azacytidine for the first 48 h and allowed to recover for the subsequent 6 days in drug-free medium. Hybridization was to MTF and GAPDH probes.

Figure 2 Northern blot analysis of total RNA from SK-MEL-28 melanoma cells cultured for 3–8 days

Sequential hybridizations were to MTF and GAPDH, *c-fos*, *c-jun* and *junB* probes. Exposure times were 1 day for MTF, 2 days for *c-fos* and 7 days for *c-jun* and *junB*. (A) Cells cultured without changes in medium; (B) medium renewals on days 2 and 5; (C) medium renewals on days 3 and 6. Arrows indicate the timing of medium renewals.

cultured over a period of 8 days with or without replacement of medium (Figure 2). RNA was prepared at 24 h intervals and analysed by Northern blotting. In cells grown in unchanged medium (Figure 2A), the amount of 4 kb MTF mRNA peaked on day 6 and then declined on days 7 and 8. After quantification and normalization of the differences in RNA loading to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, the amount of MTF mRNA on day 6 was 6-fold greater than that on day 3. A renewal of the cell culture medium down-regulated the MTF mRNA level, with a decrease on day 6 when medium was replaced on day 5 (Figure 2B) and on day 7 when medium was replaced on day 6 (Figure 2C). The down-regulation was transient and followed by a new rise in the MTF mRNA level on the subsequent days.

We then analysed the expression of AP1 family members in these cells by sequentially probing the blots with sequences corresponding to *jun* and *fos* genes. As shown in Figure 2, *c-jun*, *c-fos* and *junB* mRNA levels in melanoma cells were highly variable depending on the culture conditions and, interestingly, there was a similarity between the pattern of expression of the members of the AP1 family and that of MTF. The *c-jun* probe hybridized with transcripts of approx. 2.6 and 3.4 kb, as previously observed [28]. *fosB* and *fra-1* mRNA levels also showed a similar pattern, whereas *junD* and *fra-2* mRNAs were not

Figure 3 Analysis of MTF and *c-fos* mRNAs after addition of SK-MEL-28 and HeLa conditioned media

SK-MEL-28 cells (10^6) were plated on day 0 and cultured with a medium change on day 3. RNA was prepared from cells cultured until day 7 without further treatment (lane 1) or with the following medium replacements on day 6: lane 2, 10% FCS medium; lane 3, 0% FCS medium; lane 4, SK-MEL-28 conditioned medium; lane 5, SK-MEL-28 conditioned medium heated for 5 min at 65 °C; lane 6, HeLa conditioned medium. Conditioned media were prepared from cells grown under the same conditions as the recipient cells.

detected at a significant level (results not shown). The similarity was particularly notable in the absence of medium replacement for MTF and *c-fos* transcripts, whose levels peaked with a similar time course on day 6 (Figure 2A). Also, the transient down-regulation of the MTF mRNA after replacement of the medium was associated with the disappearance of the three AP1 mRNAs (Figures 2B and 2C; see particularly day 7 in Figure 2C).

We investigated whether the down-regulation was due to the removal of a stimulatory factor which could be present in the culture supernatant of SK-MEL-28 melanoma cells. The effect of adding fresh medium on day 6 was compared with that of adding conditioned media obtained from SK-MEL-28 or HeLa cells grown in the same conditions. RNAs were prepared on day 7 and analysed by Northern blotting successively with MTF and *c-fos* probes. Addition of fresh medium with or without serum resulted in decreases in the MTF and *c-fos* mRNA levels (Figure 3, lanes 2 and 3). In contrast, with either SK-MEL-28 or HeLa cell conditioned media, there was no down-regulation of MTF and *c-fos* mRNAs (Figure 3, lanes 4 and 6). Furthermore, heat-treated SK-MEL-28 cell conditioned medium similarly did not alter the levels of MTF and *c-fos* mRNAs (Figure 3, lane 5). This suggests either the presence of a heat-resistant stimulatory activity in both SK-MEL-28 and HeLa culture supernatants or the existence of an intrinsic stimulatory activity in cells that is inhibited by fresh medium, an inhibition which disappears with time. In either case, a stimulatory activity appears to be necessary for the accumulation of both *c-fos* and MTF mRNAs.

Analysis of AP1 binding activity in nuclear extracts prepared on various days of culture

We next analysed the relationship between the level of *jun/fos* mRNAs and the presence of nuclear factors which bind to the two AP1 binding sites in the MTF enhancer. Gel shift experiments were performed with oligonucleotides corresponding to MTF AP1 sites I and II (see the Experimental section for sequences) and SK-MEL-28 nuclear extracts prepared from cells collected after various times of culture in the absence of any medium replacement. The binding activity at both sites increased as a function of time and was particularly high from day 5 (Figure 4A, lanes 3 and 8). This agrees with the time course of accumulation of *jun/fos* mRNAs in cells cultured under the same conditions (see Figure 2A). Thus the accumulation of the proto-oncogene mRNAs coincides with an increase in AP1 binding activity recognizing both sites of the MTF enhancer. Note also that the patterns of retardation for the two AP1 sites were different.

Ternary complex formation at AP1 binding site II

We investigated the reasons for the difference in retardation patterns. The retardation patterns for sites I and II were compared with that for the collagenase promoter TRE site [9]. Oligonucleotides I and col gave similar retarded complexes (Figure 4B, lanes 1 and 3), different from that for site II (Figure 4B, lane 5). No complex was formed on site II in the presence of excess col TRE or site I competitor (Figure 4B, lanes 7 and 8); furthermore, mutation of the TRE motif in site II abolished complex formation (IImutAP1; Figure 4B, lane 9). Thus AP1 is involved in formation of the complex at this site. We then investigated whether a second nuclear factor contributes to the complex generated at site II. In addition to the TRE, the sequence surrounding site II harbours a GGA motif reminiscent of the core binding site for the Ets transcription factor family. Interestingly, replacement of one G residue of this GGA motif resulted in a retardation pattern similar to that of the col TRE (IImutGGA; Figure 4B, lane 10); the generated complex disappeared in the presence of col TRE competitor (Figure 4B, lane 11). We concluded that site II is involved in ternary complex formation involving AP1, which causes a second nuclear factor to interact with the GGA motif adjacent to the TRE.

Presence of a melanoma NF-AT-like protein in the ternary complex

With the goal of identifying the nuclear factor present with AP1 in the ternary complex, competition experiments were performed in gel shift assays (Figure 5A). Whereas no competition could be obtained with known binding sites for Ets factors (results not shown), ternary complex formation was abolished after addition of an NF-AT oligonucleotide (Figure 5A, lane 7) and partially displaced by addition of an NF- κ B oligonucleotide (Figure 5A, lane 8). The NF-AT oligonucleotide (GM-430 NF-ATp) was chosen because of its ability to bind efficiently NF-ATp, a member of the NF-AT family, in the absence of AP1 [20]. Indeed, both NF-AT and NF- κ B oligonucleotides had no effect on AP1 complex formation, as shown by the absence of competition with the col probe (Figure 5A, lanes 3 and 4) and by the presence of the remaining AP1 complex on site II (Figure 5B, lane 7). This was important to control because of the absence of ternary complex formation after addition of an AP1 oligonucleotide (Figure 5A, lane 6). Thus the factor interacting with AP1 appears to belong to the Rel/NF- κ B family and to have binding characteristics similar to those of NF-AT members.

Finally, we investigated whether ternary complex formation could be influenced by treating melanoma cells with PMA and ionomycin, a combination of compounds which leads to productive NF-AT–AP1 co-operation in T cells. The results are presented in Figure 5(B) and compared with those obtained in HeLa cells. Two interesting observations were made. First, ternary complex formation on site II was not observed in HeLa nuclear extracts (Figure 5B, lanes 5–8), whereas its presence was detected in a panel of five different human melanoma cell lines studied (results not shown). Secondly, ionomycin treatment led to a specific increase in ternary binding activity in melanoma extracts (Figure 5B, lane 3) but had no influence on binding activity in HeLa extracts (Figure 5B, lane 7). In contrast, PMA treatment led to an increase in binding activity in both SK-MEL-28 and HeLa nuclear extracts (Figure 5B, lanes 2 and 6), a result which most probably reflects the increase in the AP1 protein fraction. Treatment with both compounds led to a further increase in binding activity in SK-MEL-28 extracts (Figure 5B, lane 4) and to a result identical to that obtained with PMA alone in HeLa extracts (Figure 5B, lane 8). These results

Figure 4 AP1 binding activity and ternary complex formation in SK-MEL-28 nuclear extracts

(A) Gel shift analysis of binding activities in nuclear extracts prepared from SK-MEL-28 cells cultured for 3–7 days with the AP1 site I or site II probes. Oligonucleotides I and II correspond to the Mf enhancer sequence from position –2025 to –1994 and –1899 to –1871 respectively [18]. An equal amount of each nuclear extract was used for each assay. The arrows indicate specific complexes. (B) Comparison of the retarded complexes generated on oligonucleotides col and I with those generated on wild-type and mutated oligonucleotide II. — indicates the absence of competitor.

Figure 5 Gel shift characterization of the nuclear factor involved with AP1 in the ternary complex

(A) Competition experiments with oligonucleotides bearing known binding sites for NF-AT and NF- κ B factors. (B) Comparison of the site II binding activities present in nuclear extracts of SK-MEL-28 and HeLa cells prepared in the absence of treatment (—), and after addition of PMA (= TPA), ionomycin (iono) or both compounds together.

reinforce the NF-AT-like nature of the protein interacting with AP1 on composite site II, and give some clues concerning the specificity of the ternary complex which might have functional implications for gene expression in melanoma cells.

Figure 6 Transient expression experiments with AP1 sites I and II cloned 5' to the thymidine kinase promoter

The wild-type and mutated oligonucleotides (see the Experimental section for sequences) are depicted as follows: unshaded boxes correspond to wild-type AP1 sites, and shaded boxes to the wild-type GGA motif. The mutations are represented by crosses. The histogram represents the chloramphenicol acetyltransferase (CAT) activity in transient assays in SK-MEL-28 and HeLa cells relative to that of the pLCAT5 vector containing the thymidine kinase promoter.

Ternary complex formation is associated with a high melanoma-specific trans-activating potential

To evaluate whether ternary complex formation is functionally relevant, AP1 sites I and II of the MTf enhancer were cloned in front of the thymidine kinase promoter and their activities were compared in transient expression experiments (Figure 6). In SK-MEL-28 melanoma cells, two copies of the oligonucleotide containing AP1 site II ($2 \times \text{II}$) led to very high trans-activation (80-fold) of the thymidine kinase promoter. Lower activation (50-fold) was obtained with two copies of site I ($2 \times \text{I}$) although, unlike site II, it is a perfect AP1 consensus sequence. All transactivation was abolished when residues in site I ($2 \times \text{ImutAP1}$) or site II ($2 \times \text{IImutAP1}$) were changed. Interestingly, mutation of the GGA motif near site II reduced activation from 80-fold to 20-fold, suggesting that the higher activity of site II than of site I can be attributed to the formation of the ternary complex. In HeLa cells, both sites I and II poorly trans-activated the minimal promoter (10-fold) and the mutation of the GGA motif did not have any effect. This is in accordance with the absence of the ternary complex in HeLa cells.

DISCUSSION

We report here an investigation of the importance of MTf gene regulation by AP1 factors in the SK-MEL-28 melanoma cell line, which expresses high levels of this tumour antigen at the cell surface [17].

First, we show that the level of MTf mRNA varies considerably as a function of the culture growth conditions in this melanoma cell line. Such growth-related variations in MTf expression have also been observed at the protein level by other authors in a way that is somewhat reminiscent of the regulation of the transferrin receptor in relation to cell proliferation ([16]; reviewed in [29]). Although we did not study in further detail the mechanisms responsible for this variation, we show that high levels of mRNAs for several members of the Jun and Fos families coincide with the peaks of MTf expression and that the same conditions down-regulate both MTf and *jun/fos* mRNAs.

The MTf enhancer harbours two functionally relevant AP1 binding sites [18,19]. A result arguing in favour of increased proto-oncogene expression playing a role in inducing MTf expression is the observation of a correlated increase in AP1 binding activity. Furthermore, AP1 is involved in formation of a

ternary complex targeted to a composite sequence element including the TRE and an adjacent GGA motif in the MTf enhancer. This association of two factors over the TRE site II of the enhancer was previously unnoticed because of the complete dependence of binding of the second factor upon the presence of AP1. Because of its binding characteristics, our first suspicion was that the unknown factor could be a member of the Ets family. This turned out not to be the case, and the factor was shown to be related to NF-AT proteins of the Rel/NF- κ B family. NF-AT has been described as a T-cell-specific transcription complex which results from the association of Jun and Fos together with NF-AT proteins and whose induction after antigenic stimulation up-regulates the expression of a set of cytokines [30–32]. However, a study of the tissue pattern of expression of the four NF-AT members known to date revealed sites of expression outside the immune system [33]. This suggests that the AP1–NF-AT association might regulate a specific set of genes in different cell types by means of distinct NF-AT proteins with restricted distributions and/or by cell-specific modulation of AP1 activity.

In T cells, the NF-AT complex can be induced by co-treating the cells with a combination of a phorbol ester such as PMA and a calcium ionophore such as ionomycin. PMA acts via protein kinase C to activate Jun and Fos, while ionomycin, which raises the level of intracellular free calcium, allows the nuclear translocation of an NF-AT component (reviewed in [9,34]). Interestingly, the protein binding activity corresponding to the ternary complex in melanoma nuclear extracts was significantly increased after cell treatment with PMA and ionomycin added either together or separately. This suggests a similarity between the pathways leading to the induction of the NF-AT complex in T cells and the mechanisms leading to ternary complex formation on the AP1 composite element of the MTf enhancer in melanoma cells. However, induction of these pathways does not appear to be necessary for detection of the AP1–NF-AT binding association in melanoma extracts. This is also the case at the functional level, as shown by the high trans-activating potential resulting from ternary complex formation in the absence of any induction. This raises the possibility that the AP1–NF-AT association observed in melanoma cells occurs as a result of some constitutive pathway activation resulting from the transformation process. This could explain some of our unsuccessful attempts to activate expression through the MTf enhancer AP1 sites via classical ways. Indeed,

we showed in a previous paper that co-transfection with Jun and Fos expression vectors down-regulated a reporter construct containing the MTF enhancer [19]. In addition, we were unable to show an increase in MTF RNA expression after PMA treatment of melanoma cells (results not shown). This suggests that the AP1 protein fraction in melanoma cells might be quantitatively and/or qualitatively appropriate for successful association with NF-AT members present in the nucleus.

To approach the possible role of the AP1–NF-AT association in melanoma transformation, it will be of interest to compare the expression patterns of NF-AT proteins in melanocytes and melanoma and to analyse the effects of immunosuppressive drugs such as cyclosporin or FK506, which block the translocation of NF-AT in the nucleus via inhibition of the phosphatase calcineurin (reviewed in [9,34]). The potential therapeutic activity of such drugs in skin disorders such as psoriasis is already suspected and has been analysed [35].

Recently it was shown that UVB irradiation up-regulates AP1 and NF- κ B binding in human skin [36]. Thus these two transcription factor families may be involved in a number of physiological and pathological mechanisms in the skin. The present work illustrates the case of a melanoma-expressed gene which is regulated through a composite DNA element by AP1 and Rel factors, and which might provide a useful model towards understanding the potential role of the association of these factors in transformation.

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REFERENCES

- Angel, P. and Karin, M. (1991) *Biochim. Biophys. Acta* **1072**, 129–157
- Sassone-Corsi, P. (1994) *EMBO J.* **13**, 4717–4728
- Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. and Hunter, T. (1991) *Cell* **64**, 573–584
- Binetruy, B., Smeal, T. and Karin, M. (1991) *Nature (London)* **351**, 122–127
- Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. and Woodgett, J. R. (1991) *Nature (London)* **353**, 670–674
- Schüle, R. and Evans, R. M. (1991) *Trends Genet.* **7**, 377–381
- Gutman, A. and Wasylyk, B. (1990) *EMBO J.* **9**, 2241–2246
- Nerlov, C., Rorth, P., Blasi, F. and Johnsen, M. (1991) *Oncogene* **6**, 1583–1592
- Rao, A. (1994) *Immunol. Today* **15**, 274–281
- Devary, Y., Gottlieb, R. A., Lau, L. F. and Karin, M. (1991) *Mol. Cell. Biol.* **11**, 2804–2811
- Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R. J. (1994) *Cell* **76**, 1025–1037
- Larue, L., Dougherty, N. and Mintz, B. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9534–9538
- Brooks, G., Wilson, R. E., Doooley, T. P., Goss, M. W. and Hart, I. R. (1991) *Cancer Res.* **51**, 3281–3288
- Yamanishi, D. T., Graham, M., Buckmeier, J. A. and Meskens, Jr., F. L. (1991) *Carcinogenesis* **12**, 105–109
- Brooks, G., Goss, M. W., East, J. A. and Hart, I. R. (1993) *J. Biol. Chem.* **268**, 23868–23875
- Kennard, M. L., Richardson, D. R., Gabathuler, R., Ponka, P. and Jefferies, W. A. (1995) *EMBO J.* **14**, 4178–4186
- Brown, J. P., Woodbury, R. G., Hart, C. E., Hellström, I. and Hellström, K. E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 539–543
- Duchange, N., Ochoa, A., Plowman, G. D., Rozé, A., Amdjadi, M. and Zakin, M. M. (1992) *Nucleic Acids Res.* **20**, 2853–2859
- Brooks, A., Zakin, M. M. and Duchange, N. (1995) *Biochim. Biophys. Acta* **1260**, 301–307
- Cockerill, P. N., Bert, A. G., Jenkins, F., Ryan, G. R., Shannon, M. F. and Vadas, M. A. (1995) *Mol. Cell. Biol.* **15**, 2071–2079
- Boshart, M., Klüppel, M., Schmidt, A., Schütz, G. and Luckow, B. (1992) *Gene* **110**, 129–130
- Rose, T. M., Plowman, G. D., Teplow, D. B., Dreyer, W. J., Hellström, K. E. and Brown, J. P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1261–1265
- Angel, P., Hattori, K., Smeal, T. and Karin, M. (1988) *Cell* **55**, 875–885
- Chiu, R., Angel, P. and Karin, M. (1989) *Cell* **59**, 979–986
- Offringa, R., Gebel, S., van Dam, H., Timmers, M., Smits, A., Zwart, R., Stein, B., Bos, J. L., van der Erb, A. and Herrlich, P. (1990) *Cell* **62**, 527–538
- Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
- Plowman, G. D. (1986) Ph.D. Thesis, University of Washington, U.S.A.
- Angel, P., Allegreto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T. and Karin, M. (1988) *Nature (London)* **332**, 166–171
- Testa, U., Pelosi, E. and Peschle, C. (1993) *Crit. Rev. Oncogen.* **4**, 241–276
- Jain, J., McCaffrey, P. G., Valge-Archer, V. E. and Rao, A. (1992) *Nature (London)* **356**, 801–804
- Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A. and Crabtree, G. R. (1994) *Nature (London)* **369**, 497–502
- McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A. and Hogan, P. G. (1993) *Science* **262**, 750–754
- Hoey, T., Sun, Y.-L., Williamson, K. and Xu, X. (1995) *Immunity* **2**, 461–472
- Nolan, G. P. (1994) *Cell* **77**, 795–798
- Michel, G., Kemény, L., Homey, B. and Ruzicka, T. (1996) *Immunol. Today* **17**, 106–108
- Fisher, G. J., Datta, S. C., Talwar, H. S., Wang, Z.-Q., Varani, J., Kang, S. and Voorhees, J. J. (1996) *Nature (London)* **379**, 335–339