Development 121, 3969-3977 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV3260



# Differential regulation of AP-1 and novel TRE-specific DNA-binding complexes during differentiation of oligodendrocyte-type-2-astrocyte (O-2A) progenitor cells

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## SUMMARY

**AP-1 is an ubiquitous transcription factor which is composed of the Jun and Fos proto-oncogene proteins and is thought to play a role in both cell proliferation and differentiation. We have used an immortal, bipotential oligodendrocyte-type-2 astrocyte progenitor cell line (O-2A/c-myc) which can differentiate into oligodendrocytes or type-2 astrocytes in vitro, to investigate whether AP-1 DNA-binding activity fluctuates during glial cell differentiation. Unexpectedly, DNA-mobility shift assays using a TRE-containing oligonucleotide derived from the promoter of the glial-specific gene, glial fibrillary acidic protein (GFAP/AP-1), revealed that O-2A/c-myc progenitor cells were devoid of conventional AP-1 DNA-binding complexes. O-2A/c-myc cells did however contain several novel GFAP/AP-1-specific DNA-binding complexes, which we have termed $AP_{prog}$. $AP_{prog}$ complexes recognise the TRE consensus motif present in the GFAP/AP-1 oligonucleotide together with adjacent 3′ sequences but do not contain c-Jun or any other known Jun-related proteins. When O-2A/c-myc cells underwent terminal differentiation $AP_{prog}$ complexes were lost and conventional AP-1 DNA-binding activity became evident, particularly in astrocytes. These changes appear to be closely linked to the differentiation process since they did not occur in a derivative of the O-2A/c-myc cell line that contains an activated *v-ras* oncogene and which fails to differentiate under appropriate culture conditions. The inverse regulation of conventional AP-1 and $AP_{prog}$ complexes within the O-2A lineage suggests that these factors may play a role in the regulation of glial cell differentiation or glial cell-specific gene expression.**

Key words: oligodendrocyte-type-2 astrocyte (O-2A) cell lineage, differentiation, AP-1, DNA-mobility shift assays

The abbreviations used are: AP-1, activator protein 1; TRE, 12-*O*-tetradecanoate 13-acetate (TPA) response element (TRE); CRE, cAMP response element; CNS, central nervous system; O-2A, oligodendrocyte type-2 astrocyte; $O\text{-}2A^{prim}$, O-2A cells from primary cultures; Tag, large T antigen; ts, temperature sensitive; PDGF, platelet derived growth factor; bFGF, basic fibroblast growth factor; PF, Sato modified medium containing PDGF and bFGF; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; FCS, foetal calf serum; PBS, phosphate-buffered saline; SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline and Tween-20; PMSF, phenylmethysulphonyl fluoride; PAS, protein A sepharose.

## INTRODUCTION

The identification of genes involved in the regulation of precursor cell differentiation is a long-term goal of developmental biologists. Our studies focus on the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell and its differentiated progeny. The O-2A progenitor cell differentiates according to culture conditions (Raff, 1989) into (i) the myelin forming oligodendrocyte or (ii) the type-2 astrocyte, both in vitro (Raff et al., 1983; Warrington et al., 1992) and in vivo (Barnett et al., 1993). Many of the growth factors that regulate mitogenesis and/or survival of the O-2A lineage have been identified (Barres et al., 1992: Bögler et al., 1990; McKinnon et al., 1990; Mayer et al., 1994) making it a useful system for studying the control of glial cell differentiation. However, less is known about the transcriptional control of differentiation of this lineage.

Previous studies have suggested that the products of the c-Jun and c-Fos proto-oncogenes may play a role O-2A differentiation. In newly formed oligodendrocytes from primary cultures of the rat optic nerve, Jun and Fos protein expression can be upregulated by stimulation with PDGF (Hart et al., 1992) and, in O-2A progenitor cells shaken off from cortical cultures, a rapid increase in *c-jun* and *c-fos* proto-oncogene expression has been demonstrated after treatment with several growth factors (Bhat et al., 1992). Jun and Fos bind to activator protein 1 (AP-1)/12-*O*-tetradecanoate 13-acetate (TPA) response element (TRE) sequence motifs (Bohmann et al.,

1987; Kerppola and Curran, 1991). Recently, AP-1 has been identified as an important transcriptional regulator of the glial-specific gene, glial fibrillary acidic protein (GFAP) in type-1 astrocytes (Masood et al., 1993). These data suggest that AP-1 could be involved in growth regulation and/or differentiation-specific gene expression in the O-2A lineage.

c-Jun is a major component of AP-1 and is one of a family of related proteins (which also includes JunB and JunD) that possess a leucine zipper through which they form homodimers or heterodimers that bind to DNA and enable such complexes to regulate transcription. c-Jun has been shown to heterodimerize with c-Fos, and other Fos-related proteins, and members of the CREB/ATF (cAMP response element-(CRE)-binding proteins /activating transcription factor) family of binding proteins or to form Jun-Jun homodimers (Karin and Smeal, 1992). Recently it has been shown that Jun and Fos can heterodimerize with the Maf family of proteins (Kataoka, K. et al., 1994). These different combinations of proteins can possess distinct DNA-binding specificities, affinities and transcriptional activities. Jun-Fos heterodimers possess both an increased DNA-binding activity and *trans*-activating activity when compared with Jun-Jun homodimers, (Smeal et al., 1989; 1991; Angel and Karin, 1991).

In order to facilitate molecular and biochemical studies on cells of the O-2A lineage, we have established an immortal O-2A cell line that differentiates into oligodendrocytes and type-2 astrocyte in a similar manner to primary cultures. The cell line was generated by infecting mitogen-expanded O-2A progenitor cells from 7-day-old rat optic nerves (Bögler et al., 1990) with a drug-selectable retrovirus encoding the c-*myc* proto-oncogene (O-2A/c-myc). O-2A/c-myc cells display the phenotypic and cell surface markers typical of primary O-2A progenitors, suggesting that they faithfully retain many of the important biological characteristics of this lineage (Barnett and Crouch, 1995).

To address the role of AP-1 in O-2A differentiation, we have carried out DNA-mobility shift assays with a TRE element known to contribute to the glial-specific expression of the GFAP gene (GFAP/AP-1). Surprisingly, O-2A/c-myc progenitor cells lack AP-1 but contain novel DNA-binding complexes that bind to the GFAP/AP-1 oligonucleotide, which we term $AP_{prog.}$ Mutations in the GFAP/AP-1 TRE and its flanking sequences together with competition studies with other TRE-containing oligonucleotides demonstrate that $AP_{prog}$ complexes require the TRE together with the flanking sequences in the GFAP/AP-1 oligonucleotide for optimal binding. $AP_{prog}$ complexes are absent from oligodendrocytes and astrocytes but are present in mitogen-expanded O-2A progenitors from primary cultures. The regulated expression of AP-1 and $AP_{prog}$ suggests that these factors may play a significant role in O-2A lineage differentiation.

## MATERIALS AND METHODS

### O-2A cell culture and in vitro differentiation

The generation and characterisation of the O-2A/c-myc cell line has already been described. (Barnett and Crouch, 1995). Briefly, a retrovirus encoding avian c-*myc* and hygromycin resistance was used to infect O-2A cells taken from the optic nerve of 7- to 9-day-old rats (Barnett et al., 1993). Infected cells were selected with 30 μg/ml hygromycin B, picked as clones and maintained as progenitor cells in 5 ng/ml of both PDGF and bFGF (Bögler et al., 1990). The resulting O-2A/c-myc cell line differentiates into oligodendrocytes in a modified version of DMEM medium lacking growth factors (referred to as Sato medium; Bottenstein and Sato, 1979; Raff et al., 1983; Wolswijk and Noble, 1989) and type-2 astrocytes in DMEM containing 10% FCS (FCS) in a similar manner to O-2A cells taken from primary cultures (Barnett and Crouch, 1995). The cell line is routinely maintained as progenitor cells in culture by the addition of 5 ng/ml of both PDGF (British Biotechnology) and bFGF (Precision: PF) every day, with a replacement of fresh Sato medium every other day.

Differentiated O-2A/c-myc cultures were obtained by plating the same number of O-2A/c-myc progenitor cells into 3 flasks and treating them with PF until the cells were 80% confluent. Two flasks were then transferred to the appropriate culture medium for 4 days; Sato medium (for oligodendrocyte differentiation) and DMEM-FCS (for type-2 astrocyte differentiation) while the third was kept in PF medium (progenitors). In differentiation experiments where O-2A/c-myc progenitor cells are placed on cover slips in the appropriate medium for 5 days and then double immunolabelled with A2B5 and anti-GalC or A2B5 and anti-GFAP, we found that over 80% of the cells differentiated into oligodendrocytes in Sato medium and over 80% of the cells differentiated into astrocytes in DMEM-FCS (Barnett and Crouch, 1995). Approximately 10-20% of these oligodendrocytes express myelin basic protein and proteolipid protein (I. Griffiths personal communications). The O-2A/c-myc progenitor cells maintained in PDGF and bFGF were A2B5+,O4+, GalC–,GFAP– suggesting they were proligodendrocytes (Gard and Pfeiffer, 1990).

### Western blot analysis

O-2A/c-myc progenitor cells, or cultures differentiated to oligodendrocytes or astrocytes (for three days), were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl of sample buffer (120 mM Tris pH 6.8, 20% glycerol, 5% SDS, 2% β-mercaptoethanol, 0.1% bromophenol blue), sonicated and approximately 50 μg of protein loaded on to a 10% SDS-PAGE gel. After electrophoresis, the proteins were electroblotted onto Immobilon membrane (Millipore) and blocked overnight in 5% dried milk in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20 pH 8.0). The membrane was washed thrice in TBST, incubated with 1:2000 dilution of polyclonal anti-c-Jun antiserum (948-4; Frame et al., 1991) for 2 hours at room temperature in 5% dried milk and TBST, washed thrice in TBST and incubated a further 1 hour in anti-rabbit IgG antibody (Southern Biotechniques; 1:2000) conjugated to horse radish peroxidase. The membrane was washed several times over 30 minutes in TBST and then treated with chemiluminescence reagent (Amersham).

### Preparation of cell lysates for DNA-mobility shift assays

Whole-cell extracts were prepared from 25 $cm^2$ tissue culture flasks of O-2A progenitors, oligodendrocytes and type 2 astrocytes at 4°C using the method of Marais et al. (1993). Briefly, the cells were washed twice with ice-cold PBS, drained extensively and lysed directly in the flasks with 75 μl (25 $cm^2$ flask) or 225 μl (75 $cm^2$ flask) of buffer E (20 mM Hepes pH 7.9, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 10% glycerol, 1 mM dithiotreitol (DTT), containing 0.4 M KCL. 0.4% Triton X-100 and the following protease inhibitors: 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 mM benzamidine and 50 μg ml phenylmethylsulfonyl fluoride). The cells were scraped using cell scrapers and the extracts clarified by centrifugation in a microfuge for 15 minutes. Protein content was estimated using the Bio-rad laboratories protein assay kit and the extracts were stored at −70°C.

### Oligonucleotides

Table 1 illustrates the oligonucleotides that were used in this study. The common AP-1(TRE) or CRE consensus motifs are underlined and similarities in flanking sequences are shown in bold type. Bro-

**Table 1. Comparison of oligonucleotide sequence for several TRE elements and one CRE element that contain a AP-1 consensus sequence but differing flanking sequence**

| Oligonucleotide | Sequence |
|---|---|
| GFAP/AP-1 | G CC**C** A**GT** **GAA** TGA CTC ACC **TTG** GCA CA |
| Collagenase/AP-1 | AG**C** T**GT** **G**TC TGA CTC ATG C**T** |
| Stromelysin/AP-1 | AAG CAA TT**A** TGA GTC AGT **TTG** CGG |
| CRE | AG CTC CCG TGA CGT CAC CC**G** |

The common AP-1(TRE) or CRE consensus motifs are underlined.
Bold text indicates sequence similarity of oligonucleotides with GFAP/AP-1.

modeoxyuridine (BrdU)-substituted derivatives of GFAP/AP-1 were synthesised by Cruachem. Double-stranded oligonucleotides used as probes in the mobility-shift assays were produced by annealing the sense and corresponding antisense strands in annealing buffer (67 mM TrisHCl pH 7.3, 13 mM $MgCl_2$, 6.7 mM DTT, 1.3 mM spermidine, 1.3 mM EDTA). Double-stranded oligonucleotides were labelled with $\gamma^{32}$P-ATP (Amersham) using T4 polynucleotide kinase (NBL gene science Ltd) and the probe purified using Biorad Biospin 30 columns (Biorad).

### Gel mobility shift assay

Incubations were carried out in 30 µl final volume. 25 µg of cell extract (volume adjusted with binding buffer: (10× stock: 1 M Hepes, 0.5 M EDTA, 4 M NaCl, 50 mM PMSF, 10% BSA, 0.4% glycerol) were incubated with 0.5 µl polydIdC (1 mg/ml), 0.15 µl/ml DTT (1 M) and 1 µl of $^{32}$P-labelled oligonucleotide on ice for 30 minutes. Samples were then loaded on a 4% TBE (Tris-borate 0.09 M Tris, 0.09 M boric acid, 5 mM EDTA) acrylamide gel, electrophoresed at 150 Volts at 4°C for 2 hours, fixed in 10%:10% (v/v) methanol: acetic acid, dried down and exposed to Fuji X-ray film. Antibody addition experiments were carried out as described above except the lysates were preincubated with antibody (2 µl) for 30 minutes on ice before the addition of the oligonucleotide. The antibodies used in this study were polyclonal rabbit antisera raised against the following bacterial Jun or Fos proteins: 948-4 anti-c-Jun, c-terminal 82 amino acids of chicken c-Jun; 730-5 anti-c-Jun, full length chicken c-Jun; 388-4 anti-Fos, segment of v-Fos spanning the basic region (Vosatka et al., 1989); anti-Jun B and anti-JunD, bacterial segments of murine JunB and JunD proteins as described by (Kovary et al., 1991a).

### UV cross-linking

UV cross-linking experiments to estimate the molecular weight of the individual components of $AP_{prog}$ complexes used a derivative of the GFAP/AP-1 oligonucleotide synthesized with bromodeoxyuridine (BrdU) in place of thymidine, thus conferring increased susceptibility to UV-light-induced protein-DNA cross-linking (Lathe, 1985; Chodosh et al., 1986). The DNA-binding reaction and gel electrophoresis was carried out as described above. The retardation gel was removed from the glass plates, wraped in cling film and placed on a UV transilluminator. The gel was irradiated for 30 minutes at 4°C and areas containing the DNA-binding complexes of interest cut from the gel in longitudinal strips. These gel strips were soaked in SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 30% glycerol, 2.5% SDS, 2% mercaptoethanol, and bromophenol blue) for 15 minutes at 37°C then turned through 90° and placed between the plates on top of the stacker (pH 6.8) of a 10% SDS-PAGE acrylamide resolving gel (pH 8.8) and the proteins electrophoresed from the gel pieces. After electrophoresis the gel was fixed in 10% acetic acid:10% methanol for 10 minutes, dried down for autoradiography and exposed to Fuji X-ray film.

## RESULTS

### O-2A progenitor cells lack conventional Fos/Jun AP-1

To investigate AP-1 DNA-binding activity during glial cell differentiation, we first performed DNA-mobility shift assays on whole-cell extracts prepared from proliferating O-2A/c-myc progenitor cells using a TRE-containing oligonucleotide derived from the promoter of the glial-specific intermediate filament gene, glial fibrillary acidic protein (GFAP). This particular sequence was chosen because previous studies have identified it as an important functional element conferring astrocyte-specific expression on the GFAP promoter (Masood et al., 1990). For comparison, whole-cell extracts were prepared from proliferating chick embryo fibroblasts (CEF) since these cells contain large amounts of AP-1 DNA-binding activity (Suzuki et al., 1991; Hawker et al., 1993). As shown in Fig. 1A, CEF extracts contained a single major complex that bound to the GFAP/AP-1 oligonucleotide, which by several criteria (see below) was identified as a conventional Fos/Jun-containing AP-1 complex. Surprisingly, O-2A/c-myc progenitor cells did not contain any corresponding AP-1-like complex, although they did contain several less abundant complexes of greater electrophoretic mobility that bound to the GFAP/AP-1 oligonucleotide, which we have termed $AP_{prog}$ (Fig. 1A). $AP_{prog}$ consisted of three distinct complexes, the most rapidly migrating of which varied in intensity from extract to extract. Consistent with this apparent lack of conventional AP-1, O-2A/c-myc progenitor cells did not express detectable levels of c-Jun protein in comparison with CEF as determined by western blot (Fig. 1B) or radioimmunoprecipitation analysis (data not shown). The $AP_{prog}$ complexes were unlikely to result from proteolysis of conventional AP-1 in the O-2A/c-myc extract because CEF AP-1 complexes persisted unchanged after mixing and incubation with the O-2A/c-myc extract prior to the addition of the oligonucleotide probe (Fig. 1A). Occasional CEFs lysates were found to contain DNA-binding

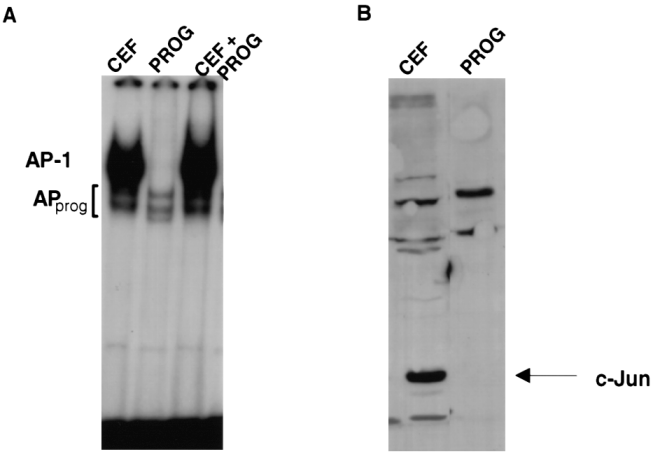


**Fig. 1.** DNA-mobility shift assays were carried out using the GFAP/AP-1 oligonucleotide and lysates from CEFs and O-2A/c-myc progenitor cells (Prog). (A) To ensure $AP_{prog}$ is not a degradation product of AP-1, the CEF and Prog lysate were incubated together on ice for 30 minutes prior to incubation with the oligonucleotide. (B) Western blot analysis using the c-Jun antisera on CEF and Prog lysates.

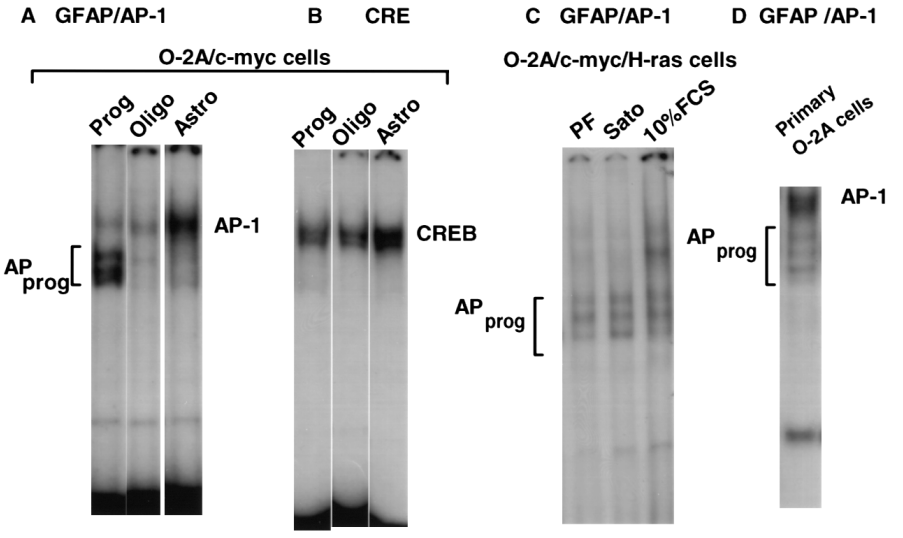


**Fig. 2.** $AP_{prog}$ but not CRE-binding proteins are differentially expressed during O-2A progenitor cell differentiation. DNA-mobility shift assays were carried out using the (A) GFAP/AP-1 oligonucleotide and (B) CRE oligonucleotide with lysates from O-2A/c-myc progenitor cells and its differentiated progeny, oligodendrocytes and astrocytes. All of the lanes shown in A and B come from the same exposure of the same gel. (C) DNA-mobility shift assay carried out using the GFAP/AP-1 oligonucleotide with lysates from the O-2A/c-myc/Ha-ras cell line that does not differentiate under all culture conditions. (D) Primary O-2A cells contain $AP_{prog}$.

proteins of similar electrophoretic mobility to $AP_{prog}$, although UV cross-linking studies suggested that these proteins were distinct (see below).

### Differential expression of AP-1 and $AP_{prog}$ during O-2A differentiation

To determine whether the pattern of DNA-binding complexes specific for GFAP/AP-1 persisted when O-2A/c-myc progenitor cells were induced to differentiate, extracts were prepared from differentiated cultures of oligodendrocytes and astrocytes, and analysed by DNA-mobility shift assay. As shown in Fig. 2A, analysis of these extracts revealed that oligodendrocytes and astrocytes contained little or no $AP_{prog}$ complexes when compared to O-2A/c-myc progenitor cells. Furthermore, in astrocytes, particularly, the decrease in $AP_{prog}$ complexes was accompanied by appearance of increased levels of DNA-binding complexes with similar electrophoretic mobility to conventional AP-1. These fluctuations in binding activity were specific for the GFAP/AP-1 oligonucleotide, since a CRE-containing oligonucleotide bound a distinct pattern of DNA-binding complexes that was invariant in progenitor cells, oligodendrocytes and type-2 astrocytes (Fig. 2B). It was also apparent from this that the $AP_{prog}$ complexes were electrophoretically distinct from the CRE-specific complexes, suggesting that the former were unlikely to be composed of CREB or ATF family proteins (Brindle and Montminy, 1992).

To investigate whether the down-regulation of $AP_{prog}$ complexes was linked to terminal differentiation in the O-2A lineage or simply a consequence of different culture conditions, we made use of a derivative of the parental O-2A/c-myc cell line that contains several copies of the activated viral Ha *ras* oncogene and which fails to differentiate under appropriate conditions (Barnett and Crouch, 1995). Extracts were prepared from cultures of O-2A/c-myc/Ha-ras cells maintained in either PF, Sato, or 10%FCS and analysed using the GFAP/AP-1 probe. As shown in Fig. 2C, $AP_{prog}$ complexes persisted in O-2A/c-myc/Ha-ras cells under all growth conditions, although, as in parental O-2A/c-myc cells, a modest increase in conventional AP-1-binding activity became evident in cells cultured in 10% FCS. We conclude that the down-regulation of $AP_{prog}$ complexes correlates more closely with the terminal differentiation process itself than with culture conditions. $AP_{prog}$ complexes were also detected in extracts prepared from primary cultures of O-2A progenitor cells grown in PF (Fig. 2D), indicating that $AP_{prog}$ complexes were not confined to the immortal O-2A/c-myc cell line. These primary cultures of O-2A progenitor cells also contained low levels of AP-1, probably due to the presence of differentiated oligodendrocytes and/or type-1 or 2 astrocytes that are present in non-clonal rat optic nerve preparations.

### $AP_{prog}$ complexes do not contain Jun-related polypeptides

In order to gain insight into the composition of the $AP_{prog}$ complexes, antibodies against various candidate proteins were preincubated with the extract in the DNA-binding assay prior to addition of the GFAP/AP-1 probe. Under these conditions, a specific interaction between the test antiserum and a component of a DNA-binding complex is indicated by disruption or further retardation (supershifting) of the protein-DNA complex. We first tested antisera specific for individual members of the Jun family (c-Jun, JunB and JunD) and a more broadly reactive anti-Fos family antiserum for their capacity to disrupt and/ or supershift $AP_{prog}$ complexes using CEF AP-1 for comparison.

As shown in Fig. 3A,B, addition of anti-c-Jun, anti-JunB, or anti-JunD antisera had no significant effect on $AP_{prog}$ complexes, while anti-c-Jun antisera were able to disrupt and/or supershift most or all of the CEF AP-1. The JunB and JunD antisera did not affect CEF AP-1 because AP-1 in this particular cell type is composed predominantly or solely of p39 c-Jun complexed with a Fos-related antigen (Fra's; most likely Fra-2; Suzuki et al., 1991; A. Kilbey and DAFG, unpublished results). That the anti-JunB and anti-JunD antisera were in fact effective in the mobility shift assay was confirmed by control experiments that showed that both antisera could disrupt and/ or supershift a proportion of the AP-1 complexes in extracts of Swiss 3T3 cells, which express significant levels of JunB and JunD (data not shown; Kovary and Bravo, 1992). We conclude from these experiments that the $AP_{prog}$ complexes do not contain subunits that are immunologically related to any of the known Jun family proteins. In contrast, the more slowly

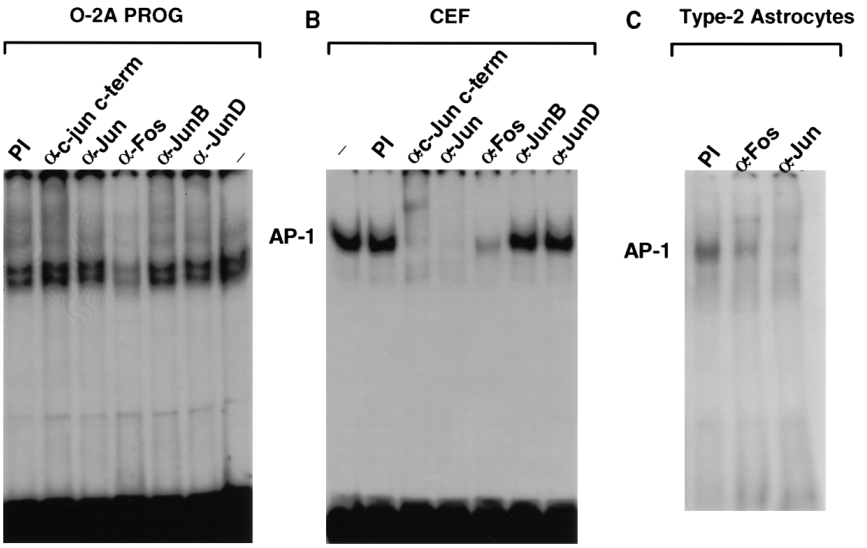


**Fig. 3.** $AP_{prog}$ does not contain a Jun family member. Supershift DNA-mobility shift assays were carried out with O-2A/c-myc progenitor cell lysates (Prog) incubated with antibodies to c-Jun (730, 948), Jun B, Jun D and v-Fos prior to incubation with the oligonucleotide (A). CEFs were used as positive controls (B). Type-2 astrocytes have 'conventional' Jun/Fos containing AP-1. Supershift DNA-mobility shift assays were carried out with type-2 astrocytes, induced to differentiate in DMEM-10% from O-2A/c-myc progenitors and incubated with antibodies to c-Jun and v-Fos (C).

migrating complex, which appeared upon differentiation to type-2 astrocytes, was substantially disrupted and/or supershifted by both anti-c-Jun and anti-Fos antisera, confirming that this was 'conventional' AP-1 (Fig. 3C).

Other candidate transcription factors whose DNA-binding specificity is known to overlap that of AP-1 include members of the CREB/ ATF family and the p45 and p18 (Maf) components of the NF-E2 transcription factor (Kataoka, K., et al., 1994; Igarashi et al., 1993; Andrews et al., 1993). Supershift experiments using antisera specific for p45 and p18 demonstrated that $AP_{prog}$ did not contain any detectable p45- or Maf-related components (data not shown). We did not test for CREB/ATF-related components using specific antisera since, as described above, $AP_{prog}$ complexes did not bind to a CRE-containing oligonucleotide indicating that they are unlikely to consist of proteins related to CREB or ATF.

Interestingly, in several experiments the $AP_{prog}$ complexes were weakly diminished by incubation with anti-Fos antiserum (Fig. 3A), although to a much lesser extent than the CEF AP-1 complex, which was almost completely disrupted. This suggests that the $AP_{prog}$ complexes contain a component that is immunologically related to Fos; however, we were unable to establish the nature of this component more precisely because this antiserum cross-reacts with c-Fos and several other members of the Fos family (Vosatka et al., 1989).

### $AP_{prog}$ complexes require TRE motifs for binding

To investigate the sequence requirements for binding of $AP_{prog}$ complexes, we first compared the complexes in O-2A/c-myc cells which bound to two other TRE-containing oligonucleotides (derived from the collagenase and stromelysin genes; colTRE and stromTRE; Table 1) with GFAP/AP-1. As shown in Fig. 4A, colTRE and stromTRE did interact with DNA-binding complexes of similar electrophoretic mobility to $AP_{prog}$, although, in each case, the pattern was subtly different from the complexes detected by GFAP/AP-1. Whereas GFAP/AP-1 detected three electrophoretically distinct complexes, the colTRE bound predominantly to the most rapidly migrating two of these, whilst the stromTRE was more similar to GFAP/AP-1. In contrast, each of these TRE-containing oligonucleotides bound indistinguishable c-Jun-containing AP-1 complexes in CEF extracts (data not shown). As before, (Fig. 2A), low levels of a slower mobility complex migrating at a similar position to AP-1 was also detected using the GFAP/AP-1 and stromTRE probes. This complex bound more strongly to the colTRE probe and may reflect the presence of low levels of AP-1 due to contaminating oligodendrocytes and astrocytes that are present in PF maintained O-2A/c-myc progenitors. At all events, these results suggested that the colTRE was capable of binding only a subset of the

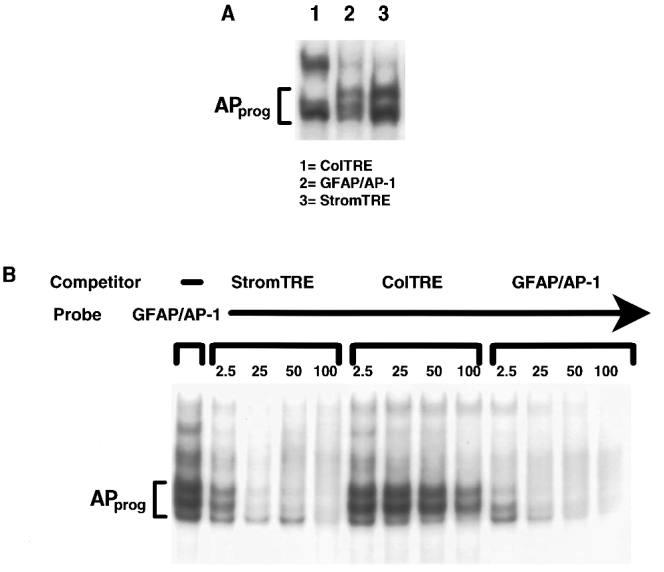


**Fig. 4.** The pattern of $AP_{prog}$ DNA-binding varies depending on the TRE-containing oligonucleotide used as a probe. (A) Lysates from O-2A/c-myc progenitor cells were incubated with the colTRE, GFAP/AP-1 and StromTRE in DNA-mobility shift assays. (B) DNA-binding heterocompetition analysis of $AP_{prog}$ in O-2A/c-myc progenitor cell lysates. All binding reactions were performed with the GFAP/AP-1 oligonucleotide as probe in the presence of increasing amounts (μg) of indicated unlabelled competitor.

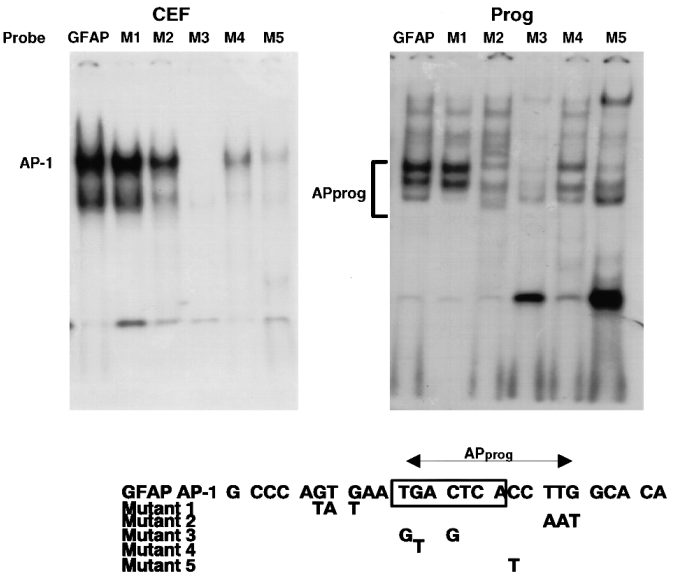


**Fig. 5.** Mutations in the core TRE consensus motif and flanking sequences of GFAP/AP-1 oligonucleotide impairs binding of AP-1 and $AP_{prog}$ complexes. DNA-mobility shift assays were carried out using CEF and O-2A/c-myc progenitor cell lysates with oligonucleotides mutated as summarised in the accompanying table.

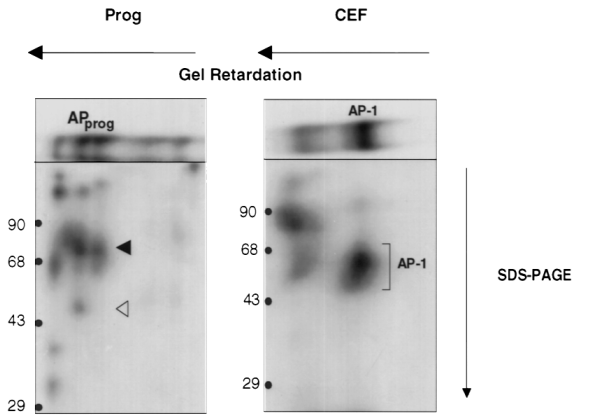


**Fig. 6.** The three bands in $AP_{prog}$ are composed of a complex of proteins. DNA-mobility shift assays were carried out using O-2A/c-myc progenitor cell and CEF lysates incubated with a bromodeoxyuridine-substituted GFAP/AP-1 oligonucleotide as probe. The resulting gel was exposed to UV, the area containing AP-1 and $AP_{prog}$ cut out of the gel, placed at 90° on top of a stacker and electrophoresed on a 10% SDS-PAGE gel.

$AP_{prog}$ complexes that form on the GFAP/AP-1 probe. To confirm this interpretation, the capacity of unlabelled colTRE and stromTRE oligonucleotides to compete for binding of radioactive GFAP/AP-1 probe was tested. As shown in Fig. 4B, while homologous competition with cold GFAP/AP-1 and stromTRE led to a similar and relatively equal reduction in binding of all complexes, the colTRE competed the most rapidly migrating complex preferentially.

One possible explanation for these results was that sequences necessary for optimal $AP_{prog}$ binding included not only the TRE motif, which is common to all three oligonucleotides, but also flanking nucleotides, which differ in each case (see Table 1). To investigate this possibility, mutant derivatives of the GFAP/AP-1 oligonucleotide with specific alterations in either the core TRE motif or in flanking sequences were tested for their capacity to bind $AP_{prog}$ complexes in O-2A/c-myc extracts and AP-1 in CEF extracts. The results of this analysis are shown in Fig. 5. Alterations to nucleotides flanking the core TRE motif to the 5′-side (M1) had no perceptible effect on binding of either CEF AP-1 or $AP_{prog}$. In contrast, mutations that altered one or more nucleotides in the core TRE (M3, M4) impaired AP-1 binding and, in each case, these mutations also impaired binding of $AP_{prog}$ complexes as did mutations affecting sequences 3′ to the core TRE motif (M2, M5). One of these in particular, M2, appeared to impair $AP_{prog}$ binding more severely than AP-1, and it is interesting to note that this mutation alters a TTG triplet that is shared between the GFAP/AP-1 and stromTRE oligonucleotides but absent from colTRE (Fig. 5; Table 1).

These results demonstrate that, as with bona-fide AP-1, $AP_{prog}$ complexes recognise primarily the core TRE motif in GFAP/AP-1 but that flanking sequences, particularly to the 3′ side, are also likely to play a role in determining the affinity of the interaction.

## UV cross-linking studies

In order to estimate the approximate molecular mass of the protein components of $AP_{prog}$ complexes, DNA mobility shift assays were performed using a derivative of the GFAP/AP-1 oligonucleotide in which thymidine was substituted with BrdU, followed by UV cross-linking and a second SDS-PAGE electrophoresis step. As shown in Fig. 6, these experiments revealed that the composition of each $AP_{prog}$ complex was distinctive and readily distinguished from the CEF AP-1 constituents (p39 c-Jun/ Fra heterodimers). For example, the most slowly migrating $AP_{prog}$ complex released a predominant species of approximately 75-80×$10^3$ $M_r$, which was also present in the complex of intermediate mobility (solid arrow), although the latter complex also contained a unique species of approximately 45×$10^3$ $M_r$ (open arrow). In contrast, the most rapidly migrating complex released three unique species of approximately 30, 38 and 68×$10^3$ $M_r$. Smaller amounts of cross-linked species with apparent molecular weights in excess of 200×$10^3$ $M_r$ were also seen in each case; however, these were thought likely to represent DNA molecules that had been cross-linked to more than one protein component.

Since two of the $AP_{prog}$ complexes at least contain more than one subunit capable of cross-linking to DNA, these results suggest that they may be heterodimeric or oligomeric in nature. If so, then the most slowly migrating complex could represent a homo-oligomer of the 75-80×$10^3$ $M_r$ species. Further work will be required to evaluate this possibility; however, the heterogeneity of the $AP_{prog}$ complexes is consistent with their distinctive DNA-binding properties described above.

The appearance of $AP_{prog}$ in progenitor cells and absence in its differentiated progeny suggested that these complexes may be related to the proliferative status of the cells. We therefore compared the profile of complexes in O-2A/c-myc/Ha-ras cells (that do not differentiate) and the rat glioma, C6 cells. As expected from the DNA-mobility shift experiments (Fig. 2C) O-2A/c-myc/Ha-ras cells in all culture conditions had exactly the same profile after UV cross-linking and SDS-PAGE electrophoresis as O-2A/c-myc progenitor cells (data not shown).

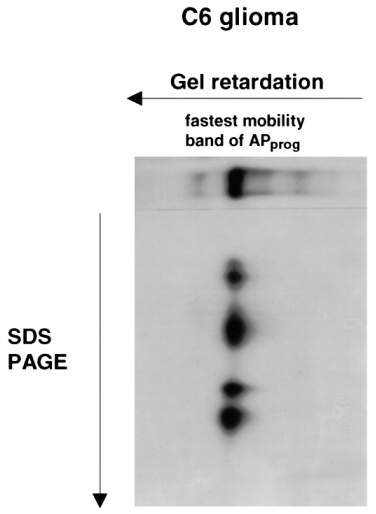


**Fig. 7.** C6 rat glioma cells contain the fastest migrating band of the $AP_{prog}$ complexes. Lysates from the C6 glioma cells were treated as described in Fig. 6.

However, the rat glioma cells expressed only one band in the DNA-mobility shift assay that had exactly the same profile as the fastest migrating $AP_{prog}$ complex after UV cross-linking and SDS-PAGE electrophoresis (Fig. 7).

## DISCUSSION

In this study, we demonstrate that a bipotential O-2A/c-myc progenitor cell line lacks conventional Fos/Jun-containing AP-1 DNA-binding activity. In addition, we have identified novel TRE-specific DNA-binding complexes, termed $AP_{prog}$, that are differentially expressed during differentiation of O-2A glial cells. Although $AP_{prog}$ complexes require a TRE-containing motif for binding, it is clear that $AP_{prog}$ does not contain any of the Jun family of proteins, the major component of AP-1 in most cells. We have also demonstrated that $AP_{prog}$ does not contain any other DNA-binding proteins known to bind to the AP-1 consensus motifs. $AP_{prog}$ complexes were not simply an artefact induced in the cell line by immortalisation with c-myc, since primary cultures of PF expanded O-2A progeitor cells also contain $AP_{prog}$. From UV cross-linking studies, it is clear that the subunit composition of the multiple $AP_{prog}$ complexes is heterogeneous and complex.

Although it is believed that the expression of c-Jun in cells is relatively ubiquitous, some exceptions are known. For example, embryonic stem cells and F9 cells both lack c-Jun (Hilberg and Wagner, 1992; Diccianni et al., 1992; deGroot et al., 1990). Thus, the lack of detectable c-Jun expression in O-2A progenitors, although unusual, is not unique. In many cell types, c-Jun is thought to play a crucial role in the regulation of cell proliferation, in particular the progression through the cell cycle (Carter et al., 1991; Kovary and Bravo, 1991a and b). Jun together with Fos are believed to be downstream of the Ras effector pathway and are activated in response to growth factors binding to their receptors (reviewed by Egan and Weinberg, 1993; Medema and Bos, 1993). Interestingly, in the differentiation-inhibited O-2A/c-myc/Ha-ras cell line that contains activated Ras, there appears to be continual expression of $AP_{prog}$.

Despite this, it has also been demonstrated in other cell types that Jun and Fos expression increases during differentiation when proliferation ceases. For example, in haemopoietic cells and neural PC12 cells, during the development of photoreceptor cells in *Drosophila* and in antigen-mediated T cell activation (Auwerx et al., 1990, Schlingensiepen et al., 1993: Lord et al., 1993: Williams et al., 1990; Angel and Karin, 1991; Bengal et al., 1992; Jain et al., 1992; Bohmann et al., 1994), AP-1 function appears to be necessary for cell differentiation and/or determination. In this study, it was seen that AP-1 was absent prior to, and became expressed during, O-2A differentiation and that there was an inverse relationship between $AP_{prog}$ and AP-1 during differentiation.

Clearly the different functional activities of Jun and Fos indicates that the control of the expression of these genes and protein activity is very complex and involves transcriptional, combinatorial, temporal and posttranslational mechanisms that vary between different cell types (see review Karin and Smeal, 1992; Jackson, 1992; De Felipe and Hunt, 1994). However, it is still not clear what the target genes are for these transcription factors, nor what mechanisms regulate AP-1 expression.

Our findings of increased AP-1 activity during differentiation of O-2A progenitor cells into the type-2 astrocytes is consistent with the current understanding of the regulation of the expression of the GFAP gene from type-1 astrocytes. GFAP is expressed abundantly and almost exclusively in astrocytes and therefore is a good marker for astrocytic differentiation (Bignami et al., 1972). Recently it has been shown the GFAP gene promoter contains a AP-1 element, which binds Fos and Jun and is important in the regulation of GFAP expression (Masood et al., 1993). Therefore, the increase in AP-1 activity that we detect on differentiation of O-2A/c-myc progenitors into type-2 astrocytes and the accompanying expression of GFAP (Barnett and Crouch, 1995) is consistent with the hypothesis that AP-1 has a role in regulating GFAP expression. Our studies also confirm recent findings that CREB is present in oligodendrocytes (Sato-Bigbee et al., 1994); however, this study did not examine expression of CREB during differentiation. Here, we demonstrate that CREB-binding activity is present not only in oligodendrocytes but also in O-2A progenitor cells and type-2 astrocytes.

In the study by Masood et al. (1993), DNA-mobility shift assays were carried out using the GFAP/AP-1 probe with lysates from a human astrocytoma cell line U251, known to express GFAP filaments. In these experiments, AP-1 was present but a second band (band B: Masood et al., 1993), which ran with a faster electrophoretic mobility than AP-1, was also detected. The significance of this band B was unclear to the authors. Comparing our results with those of Masood et al. (1993), it seems possible that band B may correspond to one of the $AP_{prog}$ complexes. We also found that a human glioma T98G, and a rat glioma cell line C6, contained an $AP_{prog}$ complex that migrated in a similar manner to this band B. Due to the possible correlation of these DNA-binding complexes with tumourgenicity, it is tempting to speculate that a component of $AP_{prog}$ may persist in or be activated in gliomas.

Our findings of the lack of c-Jun protein and corresponding lack of detectable AP-1 activity in O-2A/c-myc progenitor cells correlates with a recent hypothesis for the mechanism of glial differentiation. O-2A progenitor cells possess an inherent 'clock' that times differentiation in vivo (Temple and Raff, 1986, Raff et al., 1985); however, progenitor cells maintained in the combined presence of PDGF and bFGF (Bögler and

Noble, 1994), or in the presence of PDGF, insulin, neurotrophin factor-3 (NT-3), forskolin and ciliary neurotrophic factor (CNTF; Barres et al., 1994), are inhibited from differentiating and lose this inherent clock (Bögler and Noble, 1994). This hypothesis (Barres et al., 1994; reviewed by ffrench-Constant, 1994) suggests that primary cultures of O-2A progenitor cells would have high levels of AP-1 while they are dividing but that this is switched off concurrently with differentiation and the loss of the clock. It follows that O-2A progenitor cells that have been grown in growth factors beyond their normal mitotic lifespan ie. reached the end of their 'biological clock', would be predicted to contain low or negligible amounts of AP-1 (Barres et al., 1994; ffrench-Constant, 1994). Thus, our data supports this hypothesis since O-2A/c-myc progenitor cells that have been maintained in PF for many months differentiate rapidly on removal of growth factors and contain low or neglible amounts of AP-1 (Barnett and Crouch, 1995).

These data taken together suggest that $AP_{prog}$ is a novel TRE-specific DNA-binding complex that has a important function in the regulation of O-2A glial cell growth, differentiation and transformation. To confirm this hypothesis, we are currently attempting to clone the components of $AP_{prog}$ from cDNA expression libraries constructed from the O-2A/c-myc cell line.

We would like to also thank the following people for gifts: JunB and Jun D antibodies from R. Bravo, USA; p42 and p18 antibodies from S. Orkin and N. Andrews, USA. This work was funded by the Cancer Research Campaign, A. L. holds a training fellowship from the MS society of Scotland, A. D. was a Wellcome Trust vacation student, and M. R. was a Glasgow University honours student.

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(Accepted 17 August 1995)