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Novel Jun- and Fos-related proteins in *Drosophila* are functionally homologous to enhancer factor AP-1

Karen K.Perkins, Gina M.Dailey and Robert Tjian

Howard Hughes Medical Institute, Department of Biochemistry, University of California, Berkeley, CA 94720, USA

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A homolog of mammalian enhancer binding factor AP-1 was detected in *Drosophila* and was purified from embryo nuclear extracts by sequence-specific DNA affinity chromatography. The purified fraction, dAP-1, displays the sequence specificity as well as transcriptional activation properties of mammalian AP-1 and consists of two major proteins of mol. wts 40 and 70 kd. Antibody cross-reactivity experiments suggest that these proteins are *Drosophila* homologs of proto-oncogene products, Jun and Fos. The *Drosophila* Jun- and Fos-related antigens, when separated, are individually capable of sequence-specific DNA binding, and the Jun-related antigen activates transcription *in vitro*.

Key words: AP-1/*Drosophila*/Fos/Jun/transcription

Introduction

Mammalian activator protein-1 (AP-1) was originally identified in HeLa cells as a factor that enhances transcription from the human metallothionein IIA (hMTIIA) and SV40 early genes via its interaction with the recognition element TGACTCA (Lee *et al.*, 1987a,b). This sequence acts as an enhancer element capable of regulating promoter activity in response to tumor promoting agents such as TPA (Angel *et al.*, 1987; Lee *et al.*, 1987a). The purification of AP-1 by sequence-specific DNA affinity chromatography revealed a heterogeneous population of proteins some of which were subsequently identified as the c-*jun* proto-oncogene product (Jun; Bohmann, *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988), and various Fos-related antigens (FRAs) that cross react with a Fos peptide antibody (Curran *et al.*, 1985; Franza *et al.*, 1987). The copurification of Jun and at least some of the FRAs by DNA affinity chromatography is thought to result from direct binding of these proteins to AP-1 sites (Franza *et al.*, 1988; D.Bohmann and Tjian, unpublished data).

Interestingly, the c-*fos* proto-oncogene product (Fos), which does not appear to bind AP-1 sites directly, is also present in affinity purified AP-1 preparations (Franza *et al.*, 1988; Rauscher *et al.*, 1988a,b). The c-*fos* proto-oncogene has been termed an immediate early response gene on the basis of its rapid and transient induction by various mitogenic agents in the presence of protein synthesis inhibitors (Lau and Nathans, 1987). It has long been observed that Fos exists as part of a nuclear complex with a protein of 39 kd (p39; Curran *et al.*, 1985), and recently p39 was demonstrated to be the product of the *jun* proto-oncogene (Rauscher *et al.*, 1988a). Thus, Fos may copurify with AP-1 during sequence-specific DNA affinity chromatography by virtue of its protein-protein complex formation with Jun (Franza *et al.*, 1988; Rauscher *et al.*, 1988a,b). It has been suggested that the Fos-Jun oligomer acts as a transcriptional regulatory complex responsive to serum, calcium ionophores and other mitogenic stimuli (Distel *et al.*, 1987; Franza *et al.*, 1987, 1988; Rauscher *et al.*, 1988b). Although these studies implicate the inducible Fos-Jun protein complex in the regulated expression of genes containing AP-1 recognition elements, the mechanism by which these proteins act remains to be determined.

Here we describe the identification of Fos- and Jun-related antigens in *Drosophila*. These AP-1-like proteins were first detected during DNase I footprinting analysis of proteins in early *Drosophila* embryos that recognize transcriptionally important sequences of the Antennapedia promoter (Perkins and Tjian, in press). We demonstrate here that affinity purified human and *Drosophila* AP-1 preparations display comparable DNA binding and transcriptional activation properties, and that they consist of factors antigenically related to mammalian Jun and Fos. However, in contrast to mammalian AP-1, the *Drosophila* AP-1 family (dAP-1) appears to consist of fewer polypeptide species, possibly not more than two, that have been biochemically isolated. For this reason it may be simpler to study the biochemical function of *Drosophila* Fos- and Jun-related proteins as well as the role of these factors during growth and development.

Results

Detection of a Drosophila homolog of mammalian AP-1

The presence of enhancer factors in *Drosophila* that might bear functional similarity with mammalian factors was initially observed in transient transfection assays of SV40 enhancer/promoter templates in *Drosophila* Schneider line 2 cells (Courey and Tjian, in preparation). These studies demonstrated that the SV40 enhancer element was active in *Drosophila* cells. Since the SV40 enhancer region contains binding sites for several distinct mammalian activator proteins (APs; reviewed in Jones *et al.*, 1988), it was important to determine which of these sites was responsible for enhancer activity in *Drosophila* cells. To address this question, we examined the binding specificity of proteins present in crude *Drosophila* embryo extracts for sites within the SV40 enhancer region by DNase I footprinting (Figure 1A). These experiments revealed the presence of *Drosophila* factors that bind to mammalian AP-1 (Figure 1A, lanes 2 and 3), but not to AP-2 or AP-3 recognition elements (data not shown). Interestingly, we also observe factors binding to AP-4 and octamer recognition elements of the SV40 enhancer (K.K.Perkins and R.Tjian, unpublished observations).

We next tested the *in vivo* enhancer activity of individual

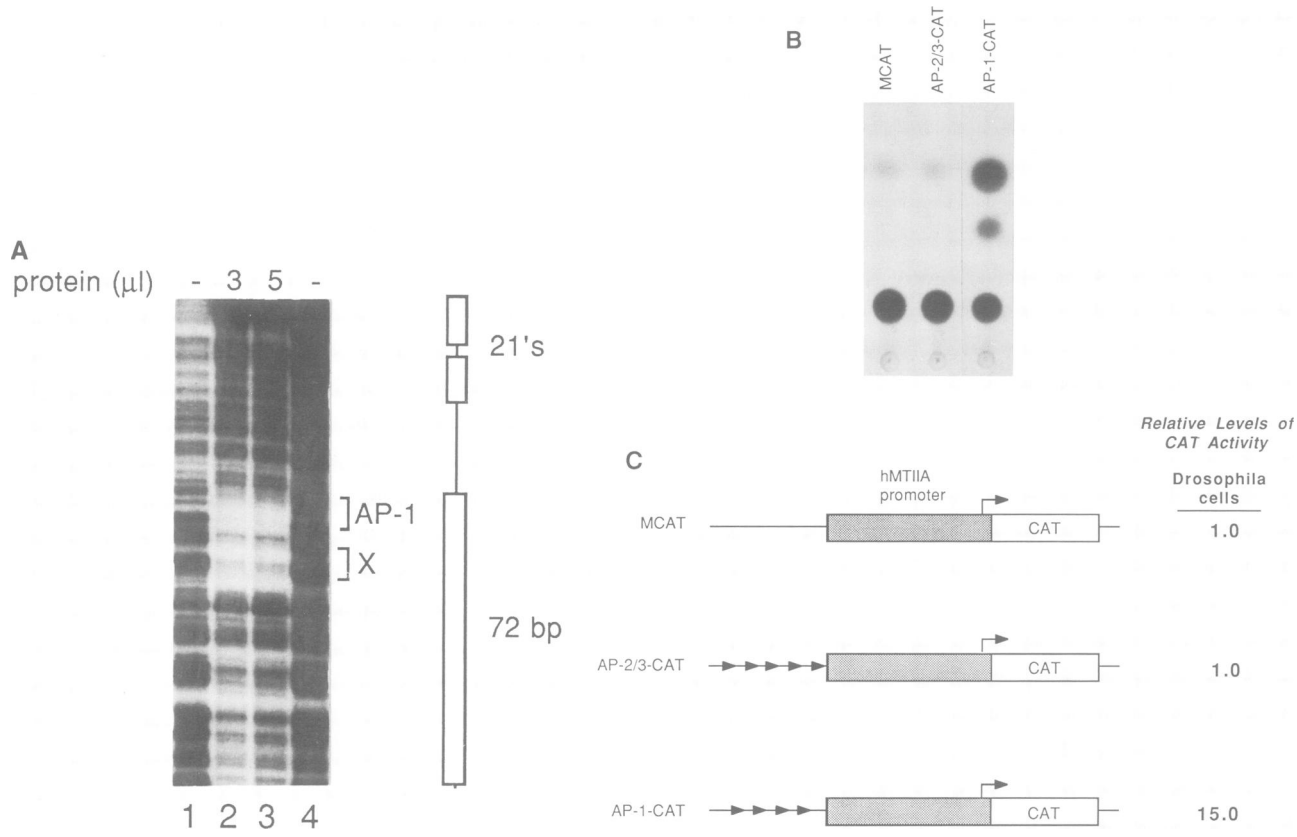

Fig. 1. Detection of dAP-1. (**A**) DNase I footprinting of a *Drosophila* embryo nuclear extract on the SV40 enhancer region. The footprinting probe was an *Eco*RI−*Hin*dIII fragment (6 fmol, spanning the distance between −350 and +454 bp with respect to the SV40 early start site) from plasmid pSVGCO (Gidoni *et al.*, 1985). The probe was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at the *Eco*RI site. End-labeled DNA was incubated either in the absence of protein (lanes 1 and 4) or in the presence of 3 μl or 5 μl (lanes 2 and 3, respectively) of a standard ammonium sulfate (10−60%) precipitated *Drosophila* embryo nuclear extract (25 mg of protein/ml). DNase I footprinting reactions contained nonspecific DNA (1 μg of poly(d[I−C])) and were carried out as described (see Materials and methods); reactions were analyzed by 6% polyacrylamide sequencing gel electrophoresis (Heberlein *et al.*, 1985; Jones *et al.*, 1985) and visualized by autoradiography. The relative positions of the regions protected by DNase I digestion are indicated by the brackets and are taken from Lee *et al.* (1987a). AP-1 refers to the position footprinted by HeLa AP-1. X refers to the site recognized by the HeLa octamer protein. The drawing on the right depicts various control regions of the SV40 promoter (pSVGCO), including the 21s (21 bp repeats that are recognized by SP1), and the single 72 bp enhancer. (**B**) Detection of dAP-1 *in vivo*. Transient transfection assays in *Drosophila* Schneider line 2 cells (Schnieder, 1972) were carried out with plasmids containing the enhancerless human metallothionein promoter (−69 to +37 bp surrounding the start site; gray box) inserted into the *Hin*dIII site immediately upstream from the CAT gene in pUC118 (pMCAT).

Four copies of the AP-1 consensus sequence (sequence denoted by arrowheads; AP-1−CAT) or five copies of the SV40 core element (sequence denoted by arrowheads; AP-2/3−CAT) were inserted into the *Bgl*II site within the hMTIIA promoter (P.Mitchell and R.Tjian, to be published elsewhere). These constructs are depicted in (C). Approximately 5 μg of each plasmid construct were transfected into *Drosophila* Schneider line 2 cells with 15 μg of carrier pUC118 plasmid DNA as described under Materials and methods. (**C**) Relative levels of CAT activity were measured as described (Gorman *et al.*, 1982), then visualized by autoradiography and quantitated by densitometric scanning.

AP recognition elements in *Drosophila* cells (Figure 1B). For these experiments, multiple synthetic copies of either the AP-1 binding site, or the SV40 core element, which contains both AP-2 and AP-3 sequences (as described in the figure legends) were inserted in tandem into the plasmid pMCAT, which contains the hMTIIA promoter, but lacks upstream enhancer sequences (P.Mitchell and R.Tjian, unpublished data). This promoter is linked to the reporter gene encoding chloramphenicol acetyl transferase (CAT). Therefore, transcription from the hMTIIA promoter will result in expression of CAT enzyme activity. Constructs containing multiple AP-1 sites (AP-1−CAT) or SV40 core elements (AP-2/3−CAT) have already been shown to induce transcription at least 15-fold compared to the control plasmid (pMCAT) when transfected into HeLa cells (P.Mitchell and R.Tjian, unpublished data). In contrast, when we transfected these plasmids into *Drosophila* Schneider cells, only AP-1−CAT directed the expression of high levels of enzyme activity, whereas pMCAT and AP-2/3−CAT yielded barely detectable levels of CAT activity (Figure 1B). Furthermore, in a separate series of experiments, tandem AP-1 sites were inserted either 5′ or 3′ of an enhancerless SV40 promoter linked to the CAT gene (pUCAT; Lee *et al.*, 1987b). When these constructs were transfected into *Drosophila* Schneider cells, they both yielded high levels of CAT enzyme activity, in contrast to barely detectable enzyme levels from pUCAT (data not shown). These results demonstrate that the AP-1 recognition element acts as an enhancer in *Drosophila* cells, and that one or more *Drosophila* factors function in a manner analogous to mammalian AP-1. In contrast, no functional equivalents of AP-2/3 appear to be present in *Drosophila* cells and thus, the SV40 core enhancer is quiescent in *Drosophila*.

Purification of Drosophila AP-1

To establish that the AP-1-like factor was a genuine *Drosophila* homolog of mammalian AP-1, it was necessary to define its biochemical properties. We therefore purified

Table I.

Fraction	Total protein (mg)	Total units[a] ($\times 10^3$)	Specific activity (units $\times$ 10^3/mg)
Nuclear extract	1560	17.0[b]	0.010
Ammonium sulfate (10–40%)	768	17.0[b]	0.022
Heparin	363	17.0	0.044
S-300	372	16.0	0.043
1st affinity	0.64	16.4	25.0
2nd affinity	0.02	6.6	330.0

[a]1 unit of footprinting activity is defined as the minimal amount of protein required to protect completely the AP-1 binding site on the Antp P2 promoter in a standard DNase I protection reaction using ~3 fmol of probe DNA.
[b]This value is an estimate assuming 100% recovery at the heparin agarose step.

dAP-1 from nuclear extracts prepared from *Drosophila* embryos collected between 0–12 h of development as summarized in Table I (see Materials and methods). These extracts have been demonstrated to be active for RNA polymerase II-mediated transcription from a number of promoters (Heiermann and Pongs, 1985; Biggin and Tjian, 1988; Heberlein and Tjian, 1988; Soeller *et al.*, 1988). At each stage of the preparation, fractions were assayed for AP-1 binding activity by DNase I footprinting. The first step in the purification procedure involved ammonium sulfate fractionation of the nuclear extract. The 10–40% ammonium sulfate fraction contained 100% of the estimated footprinting activity. The activity in this fraction was further purified by heparin agarose and Sephacryl S-300 gel filtration chromatography. S-300 chromatography does not result in a substantial purification. This step does, however, remove contaminating activities such as nucleases and phosphatases that affect subsequent stages of the purification procedure. Fractions from the S-300 column that contained AP-1 binding activity were pooled, mixed with non-specific competitor DNA, and subjected to two consecutive cycles of sequence-specific DNA affinity chromatography (Kadonaga and Tjian, 1986). Active affinity column fractions were then analyzed by SDS–PAGE (Figure 2A). We estimate that from 250 g of *Drosophila* embryos we obtain 20 μg of *Drosophila* AP-1. The isolated dAP-1 fraction contained two polypeptide species of ~40 and 70 kd, and we purified ~33 000-fold.

Comparison of DNA-binding specificities of Drosophila AP-1 and human AP-1

The DNA-binding specificities of *Drosophila* AP-1 and human AP-1 were compared by DNase I footprinting analysis of three different templates containing either the SV40 promoter, the human proenkephalin promoter, or the hMTIIA promoter (Figure 2, B–D). Each of these templates has previously been demonstrated to contain binding sites for human AP-1 (Lee *et al.*, 1987a,b; Mermod *et al.*, 1988). We chose these templates because they contain human AP-1 binding sites with distinct AP-1 recognition sequences ranging in affinity from very weak to very strong (as described in the legend to Figure 2).

To compare directly the specificity of each protein for the different binding sites, human AP-1 and *Drosophila* AP-1 were titrated within the linear range of binding affinity for each site (Figure 2, B–D). For example, the SV40 promoter construct we used contains three distinct human AP-1 binding sites. The strongest site is located within the 72 bp element; the second site occurs more proximal to the start site in the A/T-rich region, and the third, weakest binding site lies greater than 250 bp upstream of the start site. Our titration experiments to measure *Drosophila* AP-1 binding to SV40 indicate that dAP-1 recognizes each of these sites with the same relative affinity and degree of sequence specificity as human AP-1, with minor differences in the enhanced bands surrounding the footprinted region (Figure 2B). *Drosophila* AP-1 and human AP-1 also bind with similar specificity to the enkephalin and hMTIIA promoters (Figure 2C and D). In both cases, the resulting enhanced band is identical, although the intensity of this band varies. Interestingly, human AP-1 binds to a site within the A/T-rich region of the enkephalin promoter that does not contain an AP-1 consensus sequence. However, *Drosophila* AP-1 only weakly recognizes this site. Taken together, these results demonstrate that *Drosophila* AP-1 and human AP-1 recognize the same binding sites with similar affinities.

Detection of Jun- and Fos-related antigens in purified Drosophila AP-1

Mammalian AP-1 comprises a family of proteins that co-purify by virtue of their sequence-specific DNA-binding properties. Included in this family are the proto-oncogene products Jun and Fos (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988), the product of immediate early gene, *jun*-B (Ryder *et al.*, 1988; Y.Hu and R.Tjian, unpublished data) and various Fos-related antigens (FRAs). By contrast, Fos is present in purified mammalian AP-1 preparations by virtue of its complex formation with Jun (Franza *et al.*, 1988; Rauscher *et al.*, 1988a; R.Turner and R.Tjian, unpublished observations). In light of this it was of interest to determine whether any of the proteins present in the affinity purified *Drosophila* AP-1 were similar to mammalian Jun- and Fos-related proteins. Therefore, affinity purified *Drosophila* AP-1 was subjected to SDS–PAGE, transferred to nitrocellulose, and treated with either affinity purified Jun peptide-specific antibody (Figure 3A; Bohmann *et al.*, 1987), or affinity purified Fos peptide-specific antibody (Figure 3B; Curran *et al.*, 1985). Cross-reactivity was then visualized by treatment of the complexes with alkaline phosphatase-conjugated goat antiserum to rabbit IgG (Blake *et al.*, 1984). As demonstrated in Figure 3, the Jun antibody cross-reacts with the 40 kd protein, but not the 70 kd protein (Figure 3A), whereas the Fos peptide antibody cross-reacts only with the 70 kd protein (Figure 3B). These results indicate that the 70 kd is a *Drosophila* Fos-related antigen, and that the 40 kd protein is a *Drosophila* Jun-related antigen.

Separation of the two dAP-1 polypeptides

To determine whether the Jun- or Fos-related antigen was responsible for the observed AP-1 DNA-binding activity, it was necessary to separate the two proteins. For this purpose, we employed reverse phase high performance liquid chromatography (HPLC). SDS–PAGE and silver staining of the material eluted from HPLC revealed that the 40 and 70 kd proteins had been separated into two discrete fractions (Figure 4A, fractions 10 and 13). Interestingly, when the HPLC fractions were analyzed by DNase I footprinting, we

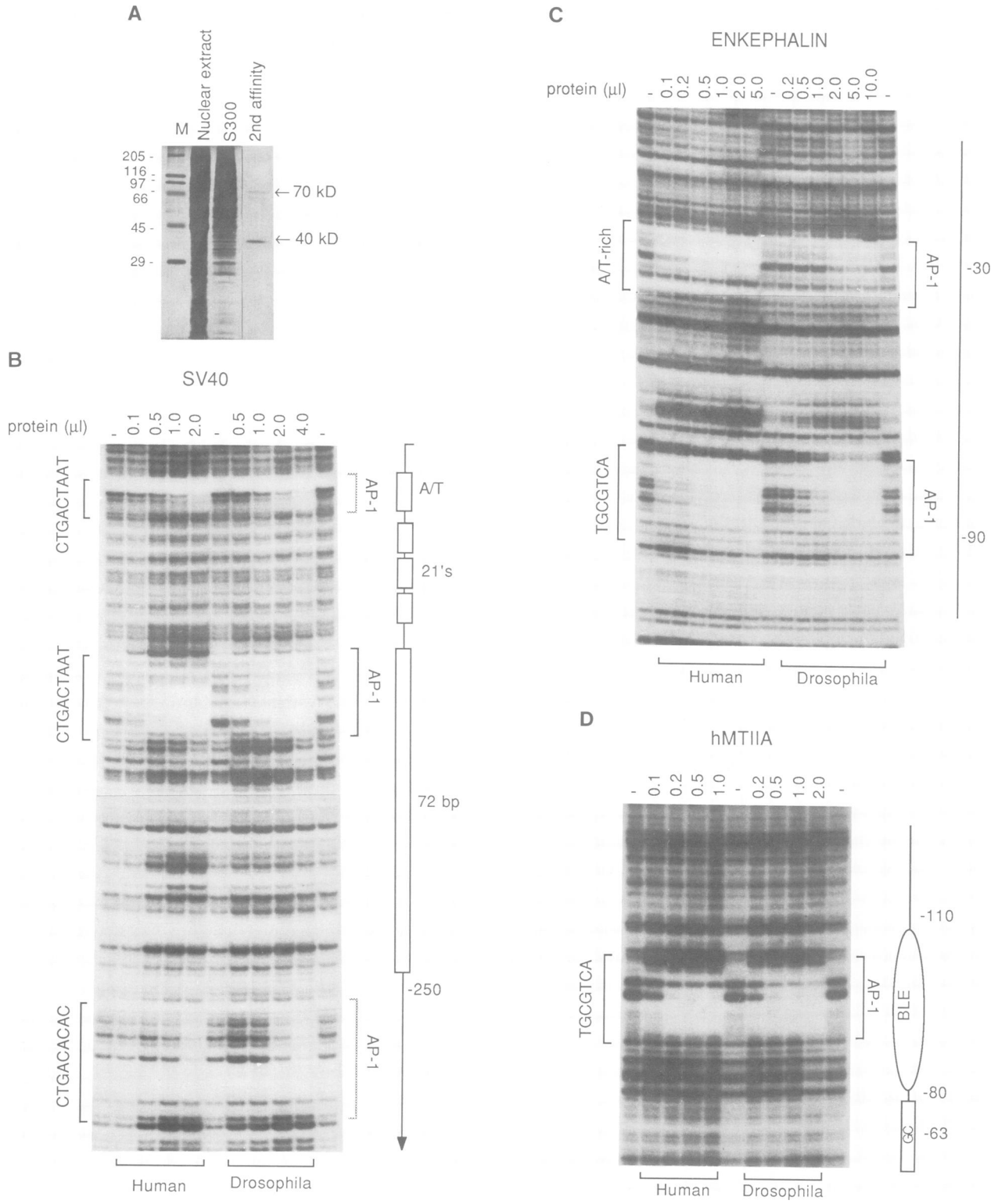

Fig. 2. Sequence specificities of *Drosophila* and human AP-1. (**A**) Protein fractions from various stages of the purification were subjected to 10% SDS−PAGE and visualized by silver staining. The left lane displays mol. wt standards (**M**). The amount of protein in each subsequent lane is as follows: nuclear extract, 20 μg of protein, 0.2 footprinting units; S300, 6 μg of protein, 0.4 footprinting units; second affinity, 0.2 μg of protein, 300 footprinting units. (B−D) DNase I footprinting reactions were carried out as described under Materials and methods. Reactions contained the indicated amount of either dAP-1 or hAP-1 purified as described (see Materials and methods). (**B**) SV40. The footprinting probe was an *Eco*RI−*Hin*dIII DNA fragment (6 fmol, spanning the distance between −350 bp and +454 bp with respect to the SV40 early transcriptional start site) from plasmid pSVGCO (Gidoni *et al.*, 1985). Probe was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at the *Eco*RI site. (**C**) Enkephalin. The footprinting probe was an *Eco*RI−*Hin*cII DNA fragment (3 fmol, spanning the distance between −190 bp and 214 bp with respect to the start site) in plasmid pENKAT-12 (Comb *et al.*, 1986). Probe was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase at the *Eco*RI site. (**D**) hMTIIA. The footprinting probe used was a *Bam*HI−*Hin*dIII DNA fragment (6 fmol, spanning the distance between −764 bp and +72 bp with respect to the hMTIIA start site) in plasmid HSI (Haslinger and Karin, 1985). The probe was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase at the *Bam*HI site. The drawings to the right of each figure depict the relative regions of control elements on each promoter, with respect to the transcription start site.

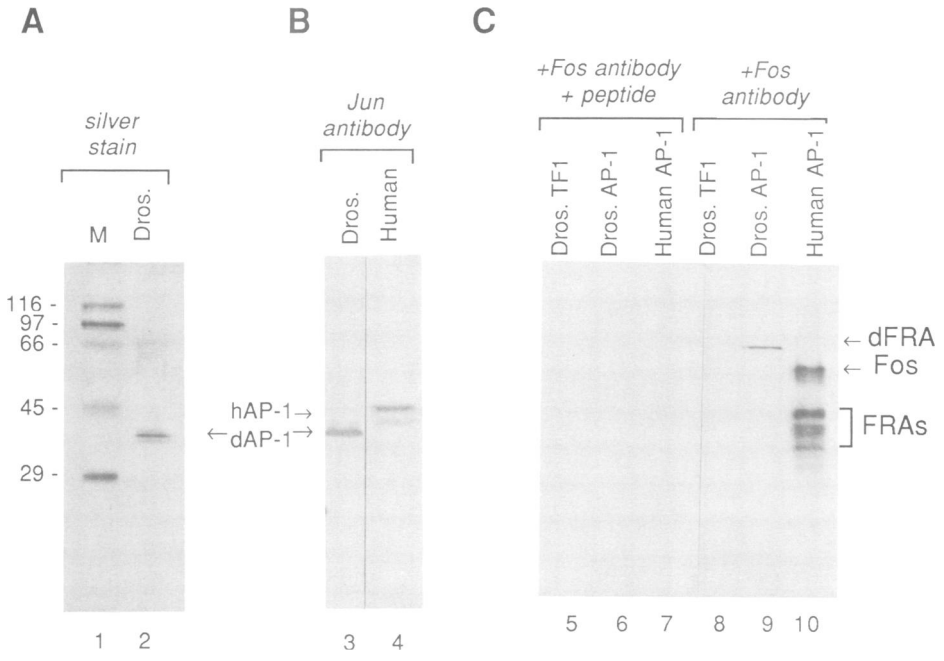

Fig. 3. *Drosophila* AP-1 proteins are antigenically related to the mammalian AP-1 family. (**A**) Protein mol. wt standards (**M**, lane 1) and purified dAP-1 (100 μl, second pass affinity fraction, lane 2) were subjected to SDS−PAGE and silver staining. (**B**) Purified dAP-1 (100 μl, second pass affinity fraction, lane 3) or hAP-1 (30 μl, first pass affinity fraction, lane 4) were subjected to SDS−PAGE and transferred to nitrocellulose by electroblotting. Filters were treated with affinity purified Jun PEP1-specific antisera (USC-4; Bohmann *et al.*, 1987; Bos *et al.*, 1988) in blocking solution that contained 10 mM Tris−HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20, 3% bovine serum albumin, and 10% calf serum (lanes 3 and 4). (**C**) *Drosophila* transcription factor I [50 μl, second pass affinity fraction (4 μg of protein/ml), K.K.Perkins and R.Tjian, to be published elsewhere, lanes 5 and 8], or *Drosophila* AP-1 (100 μl, second pass affinity fraction, lanes 6 and 9) or human AP-1 (30 μl, first pass affinity fraction, lanes 7 and 10) were subjected to SDS−PAGE and transferred to nitrocellulose. Filters were treated with affinity purified Fos M peptide-specific antisera (0.7 μg of protein; lanes 8−10; Curran *et al.*, 1985) in blocking solution, or with the same Fos antisera (0.7 μg of protein) that was preincubated with peptide antigen (20 μg) at room temperature for 30 min in blocking solution.

found that both polypeptides are independently capable of site-specifically binding to the AP-1 recognition element (Figure 4B, fractions 10 and 13). These results indicate that both proteins copurify by virtue of sequence-specific DNA binding to the AP-1 site directly.

In vitro transcription of hMTIIA with purified dAP-1

We tested the ability of the Jun- and Fos-related antigens to activate transcription *in vitro*. As templates for these *in vitro* experiments, we used the same constructs previously employed to measure AP-1 activity *in vivo* (see Figure 1). Transcription of these templates was carried out in a *Drosophila* embryo nuclear extract and was analyzed by primer extension as described (see Materials and methods). The results in Figure 5A demonstrate that the human metallothionein promoter can be accurately transcribed by a *Drosophila* extract. The presence of four AP-1 sites upstream of the hMTIIA promoter in AP-1−CAT resulted in 5-fold more transcription than from the enhancerless control (pMCAT) (Figure 5A, lanes 1 and 2). This result also confirms the interchangeability of the *Drosophila* and human transcriptional apparatus previously observed by others (Heberlein *et al.*, 1985).

The transcriptional activity of the purified dAP-1 species was then measured by reconstitution with a *Drosophila* extract that had been depleted of these proteins using the AP-1 sequence-specific affinity resin (see Materials and methods). When incubated with the dAP-1-depleted extract, the level of transcription from both AP-1−CAT and pMCAT is very low (Figure 5B, lanes 1 and 2). Addition of either second pass affinity purified dAP-1 (Figure 5B, lanes 3 and 4) or HPLC-purified 40 kd protein (Figure 5B, lanes 5 and 6), resulted in a substantial increase (4- to 5-fold) in the level of transcription from the AP-1−CAT construct. Addition of the HPLC-purified 70 kd protein alone (Figure 5B, lanes 7 and 8), or in the presence of the 40 kd Jun-related antigen (Figure 5B, lanes 9 and 10) had only a marginal effect if any on the level of transcription from either template. It is important to note that the footprinting activity of the 70 kd Fos-related antigen was substantially lower than that of the 40 kd Jun-related antigen. Consequently, the transcription experiment was carried out using protein that possessed ~10-fold more Jun-related antigen footprinting activity than Fos-related antigen footprinting activity. Using our present purification scheme, we are unable to concentrate and add sufficient quantities of the Fos-related antigen to the reaction to determine whether it would play a direct role in transcription. We therefore conclude that at least the Jun-related antigen possesses AP-1 transcriptional activity. We cannot exclude the possibility that the Fos-related antigen also possesses AP-1 transcriptional activity.

Discussion

Functional and structural similarities between Drosophila and human AP-1

Human transcription factor AP-1 consists of multiple proteins that include the proto-oncogene products c-Jun and c-Fos (Bohmann *et al.*, 1987; Angel *et al.*, 1988), immediate early gene product, Jun-B (Ryder *et al.*, 1988; Y.Hu and R.Tjian, unpublished observations), and various Fos-related antigens that are identified by their cross-reactivity with c-Fos

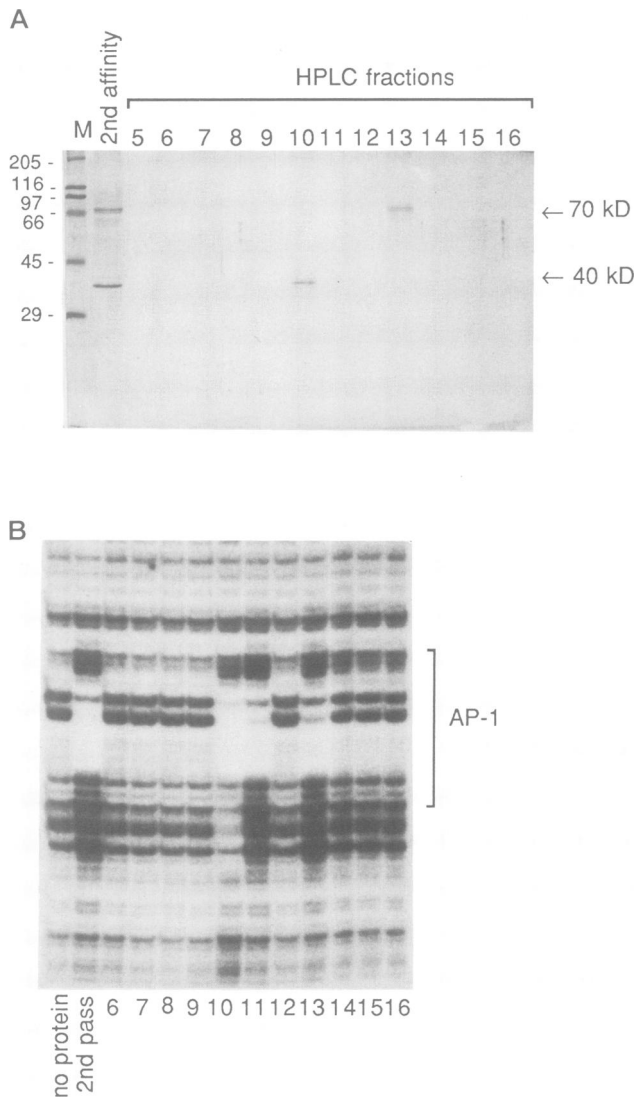

Fig. 4. Separation of dAP-1 proteins. HPLC was performed as described in Materials and methods. Protein was eluted from a C8 column (1 ml) with a linear gradient of acetonitrile and ~15 fractions were collected surrounding the protein peaks. (**A**) The samples were subjected to SDS–PAGE and silver staining. (M) Mol. wt standards; fractions 8 and 10 contain ~0.2 μg protein each. (**B**) DNase I footprinting analysis of HPLC fractions. Aliquots of each HPLC-derived fraction were mixed with 0.1 M HEMG (50 μl) and lyophilized to remove acetonitrile as described under Materials and methods. Each fraction (5 μl) was then assayed for site-specific DNA-binding activity in a standard DNase I footprinting reaction (Heberlein *et al.*, 1985; Jones *et al.*, 1985). The amount of HPLC fraction used in the footprinting reactions represents ~10% the amount of protein visualized by silver staining in (A). The lane designated second pass represents the footprinting of ~2% the amount displayed on the protein gel. The footprinting probe used is described in the legend to Figure 2D.

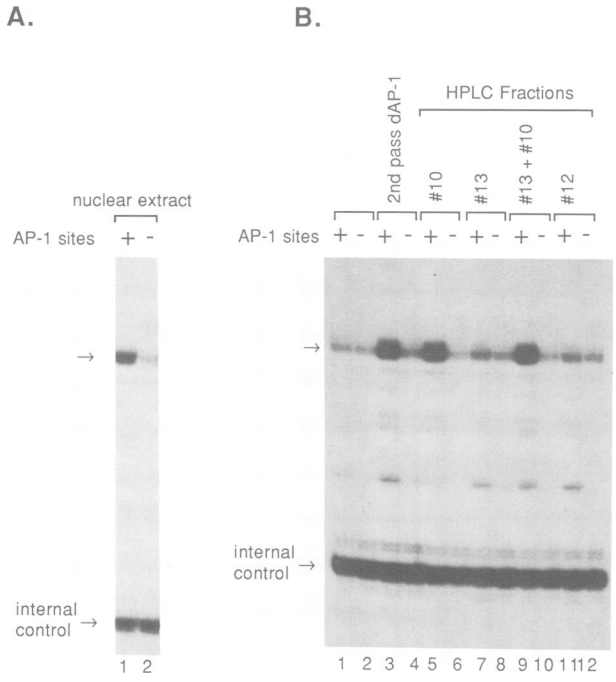

Fig. 5. Stimulation of hMTIIA transcription by purified dAP-1. (**A**) Transcription reactions (25 μl) contained either AP-1 CAT supercoiled DNA (0.2 μg, lane 1) or pMCAT supercoiled DNA (0.2 μg, lane 2), *Drosophila* embryo extract (100 μg of protein), and pHpHL supercoiled DNA (30 ng) as an internal control. pHpHL is a plasmid containing the *Drosophila* alcohol dehydrogenase (Adh) proximal promoter that has been deleted internally between −386 bp and −56 bp upstream of the start site, leaving the TATA box intact (Heberlein *et al.*, 1985). This plasmid is transcribed at a basal level and does not respond to the addition of promoter selective factors. (**B**) Transcription reactions (25 μl) contained AP-1 CAT supercoiled DNA (0.2 μg, lanes 1, 3, 5, 7, 9 and 11) or pMCAT supercoiled DNA (0.2 μg, lanes 2, 4, 6, 8, 10 and 12), pHpHL (30 ng) as an internal control, and *Drosophila* embryo nuclear extract (~100 μg) that had been depleted of dAP-1 using a sequence-specific affinity resin (see Materials and methods). Reactions were supplemented with either second pass affinity purified dAP-1 (7.5 μl; 25 footprinting units; lanes 3 and 4), HPLC fraction 10 (7.5 μl; 25 footprinting units; lanes 5 and 6), HPLC fraction 13 (7.5 μl; 2.5 footprinting units, lanes 7 and 8), a mixture of HPLC fractions 10 and 13 (3.5 μl of each, lanes 9 and 10) or fraction 12 (7.5 μl, lanes 11 and 12). Transcription reactions and primer extension analysis were carried out as described (see Materials and methods). Reaction products were subjected to 8% polyacrylamide sequencing gel electrophoresis and visualized by autoradiography.

antibodies. Here we have shown that *Drosophila* AP-1 possesses DNA-binding and transcriptional activation properties in common with human AP-1, and consists of two major proteins of mol. wts 40 and 70 kd that have been identified as Jun- and Fos-related antigens (dJRA and dFRA) on the basis of their cross-reactivity with antibodies. The Jun-specific antibody is directed against a peptide within the DNA-binding domain of Jun. The amino acid sequence within the Jun DNA-binding domain is also conserved in Jun-B (Ryder *et al.*, 1988), and in the yeast transcriptional activator, GCN4 (Vogt *et al.*, 1987). Thus, the *Drosophila* AP-1 proteins appear to be members of a family that are highly conserved between different organisms.

Biochemical properties of Drosophila Fos- and Jun-related antigens

Separation of dFRA and dJRA by HPLC enabled us to analyze the biochemical properties of these proteins. Both *Drosophila* Fos- and Jun-related antigens specifically recognize the AP-1 enhancer element. It has been suggested that at least some of the Fos-related antigens from HeLa cells also recognize the AP-1 sequence element, whereas Fos is associated with the AP-1 site indirectly, by virtue of its protein–protein complex formation with Jun (Rauscher, 1988a). The *Drosophila* Fos-related antigen is therefore more functionally similar to the HeLa Fos-related antigens rather than to Fos itself, since dFRA binds the TGACTCA sequence directly. However, it is not clear from our results

whether the dFRA and dJRA are also physically associated.

The separation of dFRA and dJRA has allowed us to test directly their transcriptional activation potential. We found that the purified 40 kd Jun-related antigen from *Drosophila* is indeed a sequence-specific transcriptional activator. By contrast, we were unable to detect significant transcriptional activity using the 70 kd dFRA protein. Human c-Jun has been demonstrated to recognize the TGACTCA sequence element (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988) and to activate transcription *in vitro* (D.Bohmann and R.Tjian, unpublished observations). In addition, v-Jun has been reported to activate transcription in F9 embryo carcinoma cells (Angel *et al.*, 1988; Imler *et al.*, 1988). Several studies have suggested that Fos participates in transcription (Setoyama *et al.*, 1986; Lech *et al.*, 1988) and that the Fos–Jun oligomer in HeLa cells is a transcriptional regulatory complex (Distel *et al.*, 1987; Franza *et al.*, 1987; Franza *et al.*, 1988; Rauscher *et al.*, 1988a,b). It is possible that the *Drosophila* Fos- and Jun-related antigens function in a similar manner, although independently of one another and in response to different types of stimuli, providing an additional level of regulation. At present, however, our studies indicate that the Jun-related antigen is the transcriptional activator and that, although the Fos-related specifically recognizes the AP-1 site it does not appear to stimulate transcription on its own.

Several explanations could account for the fact that the dFRA did not activate transcription. First, the dFRA may not act as a transcription factor. Secondly, the putative transcriptional activation domain of dFRA may have been denatured by HPLC. Alternatively, higher levels of dFRA may be required to stimulate transcription than is presently possible to obtain by our current purification procedure. Our results indicate that the DNA-binding activity of the Fos-related antigen is ~10-fold lower than that of the Jun-related antigen, suggesting that the Fos-related antigen might be present at relatively low levels in *Drosophila* cells. Finding a method to induce high levels of the Fos-related antigen in *Drosophila*, or cloning the gene, would provide a more direct means of elucidating the transcriptional activation properties of this protein.

AP-1 is a highly conserved family of transcription regulators

Recently, AP-1-like proteins have been detected in both *Saccharomyces cerevisiae* and *S.pombe* that possess DNA binding and transcriptional activation properties related to but distinct from GCN4 (Harshman *et al.*, 1988; Jones *et al.*, 1988). Like mammalian AP-1, the yeast factor appears to consist of multiple proteins. Unlike mammalian AP-1 and yeast AP-1, *Drosophila* AP-1 appears to consist of only two polypeptide species. One possible explanation for this is that other *Drosophila* AP-1 proteins exist, but are low in abundance, and/or are not detectable by silver staining of SDS–polyacrylamide gels. Alternatively, we may have already detected the entire repertoire of *Drosophila* AP-1 proteins and found that is is a much simpler family than that of either yeast or humans. The simplicity of this system could facilitate analysis of the biochemical mechanisms by which these proteins function.

A blastoderm-specific locus (bsg25D) has been cloned from *Drosophila* that possesses a low level of structural similarity to Fos (Boyer *et al.*, 1987). It is unlikely that the Fos-related antigen we have identified is the product of this gene since the only apparent region of similarity between Fos and bsg25D is distinct from the Fos peptide antigen. At present the 40 and 70 kd proteins described here represent the only examples of Jun- and Fos-related antigens in *Drosophila*.

Drosophila provides an ideal system for studying the roles of Jun- and Fos-related antigens in development. A number of *Drosophila* homologs of mammalian oncogenes have been detected whose function during development has been established. For example, the maternal effect locus, *dorsal*, that is required for establishing dorsal–ventral polarity in the *Drosophila* embryo is highly homologous to proto-oncogene product c-Rel (Steward, 1987). The *Drosophila* segment polarity gene, *wingless* is homologous to the mouse mammary oncogene *int*-1 (Cabrera *et al.*, 1988; Rijsewijk *et al.*, 1987). In addition, the tyrosine kinase domain of the *Drosophila sevenless* homeotic gene is similar to that of the proto-oncogene product c-Ros (Birchmeier *et al.*, 1986; Hafen *et al.*, 1987; Bowtell *et al.*, 1988). It is likely that identification of the genes encoding the *Drosophila* Jun- and Fos-related antigens will lead to an understanding of their roles in *Drosophila* development. Fos is known to be transiently expressed in several neuronal and non-neuronal cell types in response to a variety of stimuli, and has therefore been implicated in the coupling of short-term events to long-term adaptive responses such as cellular development and differentiation (Curran and Morgan, 1985; Morgan *et al.*, 1987; and reviewed in Goelet *et al.*, 1986; Curran and Morgan, 1987; Hanley, 1988). Detection of genes that are activated by the Jun-related antigen and possibly the Fos-related antigen will provide important clues concerning the roles of dJRA and dFRA in oncogenesis in *Drosophila*. *Drosophila* should therefore provide a useful system in which to study the mechanistic roles of Fos and Jun in signal transduction pathways and in development.

Materials and methods

Drosophila embryo nuclear extracts

Canton-S wild-type flies were maintained in population cages at 25°C. Embryos were collected on yeasted molasses–agar trays between 0 and 12 h of development and stored for up to 3 days at 4°C as described (Biggin and Tjian, 1988). Nuclear extracts were typically prepared from 125 g of embryos essentially as described (Soeller *et al.*, 1988) except that nuclei were not pelleted by centrifugation in sucrose. The final ammonium sulfate pellet was resuspended in 25% the weight of the starting material (31 ml) of buffer containing 25 mM Hepes–KOH, pH 7.6, 40 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT and 10% glycerol (0.04 M/HEMG). The resulting suspension was centrifuged at 10 000 r.p.m. for 10 min in a Sorvall SS34 rotor. The supernatant was initially dialyzed against 0.04 M/HEMG with several buffer changes, and then finally against HEMG containing 0.1 M KCl (0.1 M HEMG) until the conductivity was equivalent to that of 0.1 M HEMG. The nuclear extract (~24 mg of protein/ml) was stored at −70°C and retained 100% of the activity for one year.

Nuclear extracts utilized in reconstituted transcription reactions were depleted as follows. An aliquot of extract (500 μl) was mixed in a 1 ml plastic tube with AP-1 sequence-specific affinity resin (250 μl; described below) that had been previously equilibrated with 0.1 M/HEMG, and then gently compacted by centrifugation at 3000 *g* for 2 min at 4°C. The extract/resin suspension was mixed by rotation at 4°C for 30 min, and then centrifuged at 12 000 *g* for 2 min at 4°C. The supernatant was removed and reapplied to fresh resin and the procedure was repeated. The depleted extract was then stored at −70°C.

Drosophila AP-1 purification

All steps were carried out at 4°C.

To purify dAP-1, the nuclear extract was prepared from 0 to 12 h embryos (125 g of embryos) as described above except that the supernatant from

the ammonium sulfate nuclear lysis was brought to 40% saturation by the addition of 0.17 g/ml of solid ammonium sulfate. The suspension was stirred for 15 min at 4°C and then centrifuged at 15 000 r.p.m. for 20 min in a Sorvall SS34 rotor. The pellet was resuspended in 12.5% the weight of the starting material (16 ml) of 0.04 M/HEMG, and dialyzed as described above. This fraction (10–40% AS, 24 mg of protein/ml) was stored at −70°C and retained 100% of the dAP-1 footprinting activity for at least one year.

Two 10–40% AS fractions were pooled (32 ml, 24 mg of protein/ml) and applied to a 25 ml heparin agarose column (2.6 × 5.9 cm; Heberlein *et al.*, 1985) that had been previously equilibrated with 0.1 M/HEMG. The column was washed with three volumes of the same buffer, and then eluted with three volumes of 0.4 M HEMG. Protein-containing fractions of the 0.4 M eluate were pooled (heparin fraction, 33 ml, 11 mg of protein/ml) and applied to a S-300 Sephacryl gel filtration column (5.9 × 48.1 cm) previously equilibrated with 0.1 M/HEMG; 8 ml fractions were collected and stored at −70°C. Fractions containing dAP-1 footprinting activity were pooled (S-300 fraction, 310 ml, 1.2 mg of protein/ml), supplemented with Nonidet P-40 (NP-40, 0.1%) and poly(dI:dC) (3.9 mg), stirred for 10 min at 4°C, and then centrifuged at 10 000 r.p.m. for 10 min in a Sorvall SS34 rotor. The supernatant was applied to three DNA affinity columns (1 ml each; Kadonaga and Tjian, 1986) that had previously been equilibrated with 0.1 M/HEG (25 mM Hepes–KOH, pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol, 0.1 M KCl) containing 0.1% NP-40. All first affinity column buffers contained NP40. The complementary oligodeoxynucleotides used for this column were, 5′-GATCAGTGAATCACAATGA-3′ and 5′-GATCTCATTGTGATTCACT-3′. The column was washed with 10 volumes of 0.1 M/HEG, and then step eluted with HEG containing 0.2, 0.5 and 1.0 M KCl (3 ml each step). The dAP-1 footprinting activity was detected in the 0.5 M eluate; activity was pooled (first affinity, 8 ml, 80 μg of protein/ml) mixed with dI:dC (16 μg) as described above, and applied to one second DNA affinity 1 ml column previously equilibrated with 0.1 M/HEG containing 0.1% LDAO. All second affinity column buffers contained 0.1% LDAO. The complementary oligodeoxynucleotides used to prepare the resin were 5′-GATCGTGACTCAGCGCG-3′ and 5′-GATCCGCGCTGAGTCA-3′. The column was washed with 10 volumes of 0.1 M/HEG, step eluated with HEG containing 0.2 M and 0.5 M KCl (3 ml each step), and then eluted with a step gradient containing 1 ml each of 0.6, 0.7, 0.8, 0.9 and 1 M KCl in HEG. dAP-1 footprinting activity was obtained in the 0.7 and 0.8 M KCl eluates (second affinity, 2 ml, 10 μg of protein/ml). The protein concentration in affinity purified fractions was estimated by SDS–PAGE and silver staining. The fraction was stored at −70°C.

Purification by HPLC was carried out as follows. All steps were carried out at 25°C. An aliquot (200 μl) of a second affinity fraction was incubated in the presence of 10 mM DTT and 5 M guanidine–HCl at 37°C for 20 min and then applied directly to an HPLC C8 column that had previously been equilibrated with 0.1% trifluoroacetic acid. The column was eluted with a linear gradient containing 0–100% acetonitrile. Fractions were collected, lyophilized to dryness and analyzed directly by SDS–PAGE. For DNase I footprinting or transcription analysis the acetonitrile containing fraction was supplemented with 0.1 M/HEMG (50 μl) and lyophilized to a volume of 50 μl. The volume was then increased by the addition of 100 μl of 0.5 mM DDT and again lyophilized to a 50 μl volume. This procedure was repeated 4 to 5 times to efficiently remove the acetonitrile, and then the pH was adjusted to pH 7.5 by the addition of 1–2 μl of 1 M Hepes–KOH, pH 8.5.

Purification of human AP-1

Human AP-1 was purified through the first affinity column as described (Lee *et al.*, 1987b) except that the affinity column was loaded in buffer Z (25 mM Hepes–KOH, pH 7.8, 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% NP-40, and 0.1 μM $ZnCl_2$) containing 0.1 M KCl and step eluted with three column volumes of buffer Z containing 1.0 M KCl.

In vitro transcription and DNase I footprinting

In vitro transcription and primer extension reactions were performed as described (Heberlein *et al.*, 1985). The probe used for primer extension was an oligonucleotide of sequence 5′-CCACCGTTGATATATCCCA ATGGC-3′ that hybridizes within the CAT gene. Using this primer, accurate initiation from the hMTIIA promoter in a HeLa cell nuclear extract gives rise to a 120 nucleotide extension product (P.Mitchell and R.Tjian, unpublished results). DNase I footprinting reactions were carried out as previously described (Heberlein *et al.*, 1985; Jones *et al.*, 1985).

DNA transfection and transient expression assays

Schneider line 2 (SL2) cells were plated in M3 medium containing 2 mM glutamine and 10% fetal calf serum at a density of 10^7 cells per 100 mm Petri dish at ~24 h prior to transfection. Cells were transfected with CAT plasmids (5 μg) in the presence of pUC118 carrier DNA (15 μg) by the Ca-phosphate method (DiNocera and Dawid, 1983). The plates were incubated for 48 h at 25°C and then harvested.

Cells were harvested by scraping the bottom of the petri plate. They were then washed once with PBS and resuspended in 0.25 M Tris–HCl, pH 7.9 (200 μl) and transferred to a 1 ml plastic tube. Cells were disrupted by quick-freezing (−70°C) and thawing (37°C) and sonication for 1 min using a Sonifier Cell Disrupter cup sonicator set on the highest setting. The extracts were then incubated at 65°C for 5 min, followed by centrifugation at 15 000 *g* for 10 min at 4°C. The supernatants (CAT extracts) were typically 1–2 mg/ml. CAT assays were performed in the linear range of CAT activity according to Gorman *et al.* (1982). Equivalent amounts of protein were added to each CAT assay. Acetylated chloramphenicol species were separated by thin layer chromatography, visualized by autoradiography and quantitated by densitometry.

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