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Functional conservation of the cell cycle-regulating transcription factor DRTF1/E2F and its pathway of control in *Drosophila melanogaster*

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SUMMARY

The cellular transcription factor DRTF1/E2F is implicated in the control of early cell cycle progression due to its interaction with important regulators of cellular proliferation, such as pocket proteins (for example, the retinoblastoma tumour suppressor gene product), cyclins and cyclin-dependent kinase subunits. In mammalian cells DRTF1/E2F is a heterodimeric DNA binding activity which arises when a DP protein interacts with an E2F protein. Here, we report an analysis of DRTF1/E2F in *Drosophila* cells, and show that many features of the pathway which regulate its transcriptional activity are conserved in mammalian cells, such as the interaction with pocket proteins, binding to cyclin A and cdk2, and its modulation by viral oncoproteins. We show that a *Drosophila* DP protein which can interact co-operatively with E2F proteins is a physiological DNA binding component of *Drosophila* DRTF1/E2F. An analysis of the expression patterns of a *Drosophila* DP and E2F protein indicated that *DmDP* is developmentally regulated and in later embryonic stages preferentially expressed in proliferating cells. In contrast, the expression of *DmE2F-1* in late stage embryos occurs in a restricted group of neural cells, whereas in early embryos it is widely expressed, but in a segmentally restricted fashion. Some aspects of the mechanisms which integrate early cell cycle progression with the transcription apparatus are thus conserved between *Drosophila* and mammalian cells. The distinct expression patterns of *DmDP* and *DmE2F-1* suggest that the formation of DP/E2F heterodimers, and hence DRTF1/E2F, is subject to complex regulatory cues.

Key words: DP/E2F, Rb, cell cycle, transcription

INTRODUCTION

In mammalian cells the cellular transcription factor DRTF1/E2F plays a crucial role in co-ordinating early cell cycle progression through its cyclical interactions with key regulators of cellular proliferation (La Thangue, 1994). For example, the retinoblastoma tumour suppressor gene product (pRb), which has an established role in negatively regulating early cell cycle progression from G_1 into S phase and is frequently mutated in human tumour cells (Cobrinik et al., 1992), binds to and inactivates the transcriptional activity of DRTF1/E2F (Hiebert et al., 1992; Zamanian and La Thangue, 1992). The other pRb-related proteins p107 and p130 (generically known as pocket proteins) also bind to DRTF1/E2F (Shirodker et al., 1992; Schwarz et al., 1993; Cobrinik et al., 1993), and are believed to regulate its transcriptional activity at distinct points during cell cycle progression (Schwarz et al., 1993; Zamanian and La Thangue, 1993; Zhu et al., 1993).

Another important property concerns cyclins A and E, which, together with the cdc2-related catalytic subunit cdk2, bind to DRTF1/E2F in a cell cycle-regulated fashion (Bandara et al., 1992; Devoto et al., 1992; Lees et al., 1992). Although the role of these cyclin-dependent kinases (cdk) in DRTF1/E2F has not been established, it is likely that they are involved in regulating transcriptional activity, either indirectly by modulating the activity of the pocket protein or directly by altering the properties of the DNA binding components in DRTF1/E2F.

The nature of the genes regulated by DRTF1/E2F has provided some insight into its role in co-ordinating cell cycle progression, since most of the identified genes have been implicated in promoting cellular proliferation, particularly through S phase (Nevins, 1992). For example, the E2F binding sites in the dihydrofolate reductase (DHFR) and B-*myb* promoters are important for the induction of transcription towards the end of G_1 (Means et al., 1992; Lam and Watson, 1993); a similar role having been suggested for the E2F sites in a variety of other promoters. Indeed, for *DHFR* and B-*myb* the induction of the transcriptionally active form of DRTF1/E2F during cell cycle progression correlates with

increased transcription of these genes (Slansky et al., 1993; Lam and Watson, 1993).

In mammalian cells DRTF1/E2F is regulated by viral oncoproteins (Nevins, 1992). Adenovirus E1a, for example, releases the transcriptionally active form of DRTF1/E2F by sequestering proteins, such as pocket proteins, which inactivate its transcriptional activity (Heibert et al., 1992; Zamanian and La Thangue, 1992). Since this effect requires regions in E1a which are also necessary for E1a to transform mammalian cells (Bandara and La Thangue, 1991), it is believed that the de-regulation of DRTF1/E2F is important in the transformation process, thus overcoming the mechanisms which normally restrain cellular proliferation.

Some of the DNA binding components of DRTF1/E2F have recently come to light, and it is now clear that DRTF1/E2F DNA binding activity results from a group of heterodimeric transcription factors, each heterodimer being composed of a DP and an E2F protein (La Thangue, 1994). The first DP protein to be identified, DP-1, is a frequent component of DRTF1/E2F in many different mammalian cell types, such as F9 embryonal carcinoma, 3T3 and HeLa cells (Girling et al., 1993; Bandara et al., 1993, 1994). In contrast, E2F family members, such as E2F-1 (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992), appear to be somewhat less frequent (Chittenden et al., 1993). Of the E2F family members so far characterised, E2F-1, E2F-2 and E2F-3 can bind pRb through a C-terminal region, an interaction which is dependent upon the integrity of the pocket-region in pRb (Ivey-Hoyle et al., 1993; Lees et al., 1993), whereas E2F-4 and E2F-5 bind to p107 and p130 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Buck et al., 1995). Thus, in the context of the DP/E2F heterodimer, it is likely that the E2F partner dictates the nature of the pocket protein which binds to and regulates its transcriptional activity.

Recent studies have characterised in detail several members of the DP family (Girling et al., 1994; Ormondroyd et al., unpublished data). The DP proteins are similar, particularly across their DNA binding regions and several domains located in their C-terminal halves, and each DP protein is able to interact combinatorially with members in the E2F family of proteins (Girling et al., 1994). Although the precise roles of different DP proteins in regulating the cell cycle have yet to be resolved it would appear possible, from the fact that their expression is tissue-restricted (Girling et al., 1994), that they function in particular cell types.

One of the more genetically manipulatable organisms with a significant level of understanding of the mechanisms which control the cell cycle is the fruitfly *Drosophila melanagaster*. Considerable information is available regarding the regulation of mitosis (Gonzalez et al., 1994; Orr-Weaver, 1994), but less is known about the processes which control early cell cycle progression. Thus, we sought to identify and characterise DRTF1/E2F and its pathway of control in *Drosophila* cells.

Here, we report an analysis of DRTF1/E2F in *Drosophila melanogaster*. We show that many features of the pathway which controls its transcriptional activity are conserved in mammalian cells, such as the interaction with pocket proteins, binding to cyclin A/cdk2, and its modulation by viral oncoproteins. A *Drosophila* DP protein, which co-operates with E2F proteins, is a physiological DNA binding component of *Drosophila* DRTF1/E2F. A comparison of the expression of *DmDP* and *DmE2F-1* indicates that both genes have distinct expression patterns. The expression of *DmDP* is restricted to proliferating cells. In contrast, in late embryos, *DmE2F-1* expression occurs in a restricted group of neural cells, whereas in early embryos it is widely expressed but in a segmentally restricted fashion. A functional equivalent of the DRTF1/E2F pathway suggests that some aspects of the mechanisms which control early cell cycle progression are conserved between flies and mammals.

MATERIALS AND METHODS

Preparation of extracts from cell lines, gel retardation, and immunochemical techniques

Extracts from F9 EC and SL2 cells were prepared as previously described (Partridge and La Thangue, 1991). *Drosophila* embryo extracts were obtained from Promega. Gel retardation reactions were performed using either an oligonucleotide which contained the distal E2F binding site taken from the adenovirus type 5 E2a promoter (nucleotides −71 to −50; La Thangue et al., 1990) or the distal E2F binding site from the *Drosophila* DNA polymerase α promoter (nucleotides −358 to −337; Hirose et al., 1993). Immunoblotting and gel retardation were performed by standard procedures using two different rabbit antisera called anti-DmDP (C). Immunoblotting was performed with the addition of the appropriate competing peptides (about 2 nM). Peptide C represents the C-terminal 23 residues of DmDP.

Fusion proteins and in vitro translation

Cyclin A (PA-CA) and cdk2 (GST-cdk2) have been described previously (Bandara et al., 1992). Ad5 E1a coding sequences were transcribed and translated using rabbit reticulocyte lysate (Promega). For in vitro transcription of DmDP, the 2.3 kb insert from λDm3.3 (encoding the complete DmDP protein) was subcloned into pBS to create pBS Dm3.3; transcription was performed by standard procedures. To prepare the GST-DmDP fusion protein, the 1.3 kb insert from λDm 2.1 was subcloned into pGEX-2T to create pGEX-Dm2.1, and contains coding information from residue 179 to 445. Expression and purification of GST-DmDP (2.1) was as previously described (Girling et al., 1993).

Isolation of DmDP cDNA clones

A *Drosophila melanogaster* (Canton S) cDNA library, cloned into λgt11 (Clonotech), was screened (about 5×10^5 plaques) with a radiolabelled mouse DP-1 probe (DP-1/1.1; Bandara et al., 1994) in 50% formamide, 5× Denhardt's solution, 5× SSC, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA. Filters were washed to a stringency of 0.5× SSC, 0.1% SDS at 55°C. Two independent clones were isolated, which were repeatedly rescreened and sequenced, referred to as λDm3.3 and λDm2.1, containing a 2.3 kb and 1.3 kb insert, respectively.

In situ hybridisation

In situ hybridisation for *DmDP* and *DmE2F-1* localisation with embryos and polytene chromosomes was performed as described previously after labelling the pBS Dm3.3 or DmE2F-1 insert (Alphey et al., 1992).

RESULTS

A DRTF1/E2F DNA binding activity in *Drosophila* cells interacts with an Rb-like protein

The presence of a DRTF1/E2F DNA binding activity in

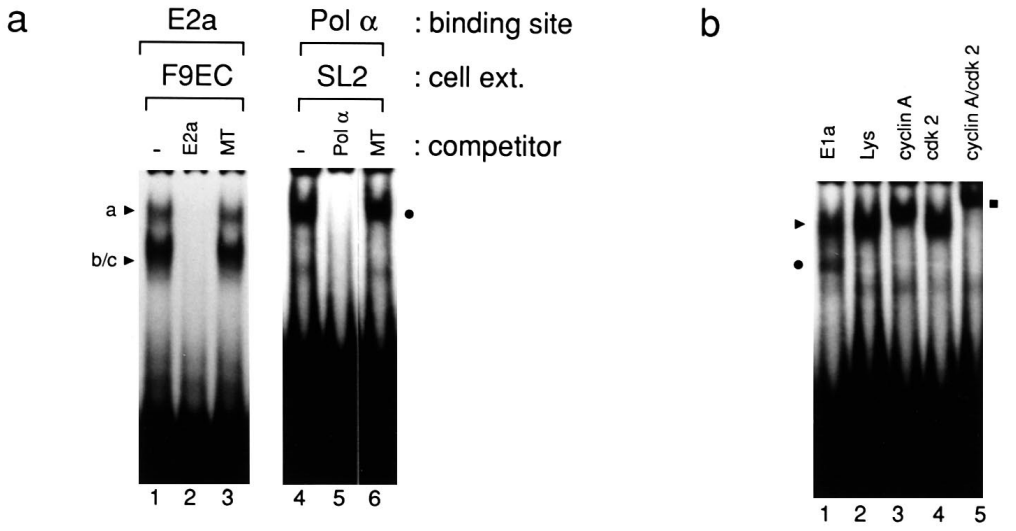

Fig. 1. (a) DRTF1/E2F in *Drosophila* SL2 cell extracts: DRTF1/E2F was assessed by gel retardation in either F9 EC (tracks 1, 2 and 3) or SL2 (tracks 4, 5 and 6) using either the E2F site taken from the adenovirus E2a promoter (E2a site) or the *Drosophila* DNA polymerase α promoter (Pol α site) in the presence of competing wild-type (tracks 2 and 5) or mutant (tracks 3 and 6) E2F sites. In F9 EC extracts, the DRTF1a and b/c complexes are indicated by ►, and in SL2 extracts the specific E2F site complex by ●. (b) Effect of adenovirus E1a, cyclin A and cdk2 on the SL2 cell DRTF1/E2F complex: DRTF1/E2F was assessed by gel retardation using the Pol α site in the presence of the adenovirus 12S E1a protein (track 1) or control treated reticulocyte lysate (track 2); complexed and free DRTF1/E2F are indicated by ● and ►, respectively. Note that the level of free DRTF1/E2F (►) is enhanced in the presence of lysate containing the E1a protein. Cyclin A (track 3), cdk2 (track 4) or both together (track 5) were added as fusion proteins (about 50 ng cyclin A and 100 ng cdk2). The cyclin A/cdk2 induced shift is indicated by ■.

extracts prepared from *Drosophila* SL2 cells was assessed by gel retardation using an E2F binding site taken from the *Drosophila* DNA polymerase α promoter (referred to as the Pol α E2F site; Hirose et al., 1993). The Pol α E2F site bound a sequence-specific activity in SL2 cells (Fig. 1a, track 4, indicated by ●), since the DNA binding activity was efficiently competed by the wild-type Pol α site but not a mutated site (Fig. 1a, compare tracks 5 and 6). Thus, a DNA binding activity with the sequence specificity of DRTF1/E2F exists in *Drosophila* SL2 cells.

The transcriptionally active form of DRTF1/E2F, which when bound to the E2F site produces a characteristic migration in the gel retardation assay, is abundant in F9 embryonal carcinoma (EC) cells (La Thangue and Rigby, 1987). It was apparent that the migration of the DNA binding complex formed in SL2 cell extracts was slower than the F9 EC cell form of DRTF1/E2F (Fig. 1a, compare tracks 1 and 4; uncomplexed DRTF1/E2F indicated by b/c and complexed as 'a'). Indeed, previous studies have indicated that in mammalian cells the association of proteins, such as pRb and related proteins, cyclins A and E, and cdk2 with DRTF1/E2F is responsible for the retarded mobility (La Thangue, 1994). We were therefore interested to establish whether the migration of the SL2 activity was because similar proteins interact with *Drosophila* DRTF1/E2F. We took several approaches towards addressing this question.

In mammalian cell extracts the complexed form of DRTF1/E2F is sensitive to the action of viral oncoproteins, such as the adenovirus E1a protein (Bandara and La Thangue, 1991). The effect of adenovirus E1a is to release the active transcription factor by sequestering pRb and related proteins from DRTF1/E2F (Bandara and La Thangue, 1991; Zamanian and La Thangue, 1992, 1993). We tested whether treating SL2 cell extracts with the adenovirus E1a protein affected SL2 DRTF1/E2F. For this, we used in vitro transcribed and translated E1a, which is capable of releasing the active form of mammalian DRTF1/E2F (Bandara and La Thangue, 1991). The control reticulocyte lysate had little effect on SL2 DRTF1/E2F, in contrast to E1a, which released the faster migrating form of DRTF1/E2F (Fig. 1b, compare tracks 1 and 2, faster migrating form indicated by ► and slower by ●). Although the efficiency of the E1a protein is less than previously observed in mammalian cells (Bandara and La Thangue, 1991), it was nevertheless significant and not apparent in the control lysate. This result suggests that either pRb or a related protein interacts with *Drosophila* DRTF1/E2F.

In mammalian cells, cyclin A can interact with pocket protein-complexed DRTF1/E2F and subsequently recruit the catalytic subunit cdk2 (Bandara et al., 1992; Cao et al., 1992; Devoto et al., 1992). We thus assessed whether the form of DRTF1/E2F present in SL2 cell extracts can, likewise, interact with cyclin A and cdk2. As in mammalian cells, cyclin A bound to SL2 DRTF1/E2F, since a slower migrating DRTF1/E2F DNA binding complex was apparent upon addition of a cyclin A fusion protein to SL2 cell extracts (Fig. 1b, compare tracks 2 and 3). Alone cdk2 was not able to bind (Fig. 1b, compare track 2 to 4) unless cyclin A was simultaneously added, in which case an even slower migrating complex (relative to cyclin A alone) was apparent (Fig. 1b, compare tracks 4 and 5, complex indicated by ■), events which again are similar to the behaviour of these proteins in mammalian cells (Bandara et al., 1992). These data suggest that a *Drosophila* protein with structural features in common with a generic pocket protein associates with DRTF1/E2F in SL2 cells, which, furthermore, is able to interact with cyclin A and cdk2. We conclude that a DNA binding activity with many of the properties of mammalian DRTF1/E2F is present in *Drosophila* SL2 cells.

Isolation and characterisation of cDNAs encoding a *Drosophila* DP protein

In mammalian cells DRTF1/E2F DNA binding activity arises

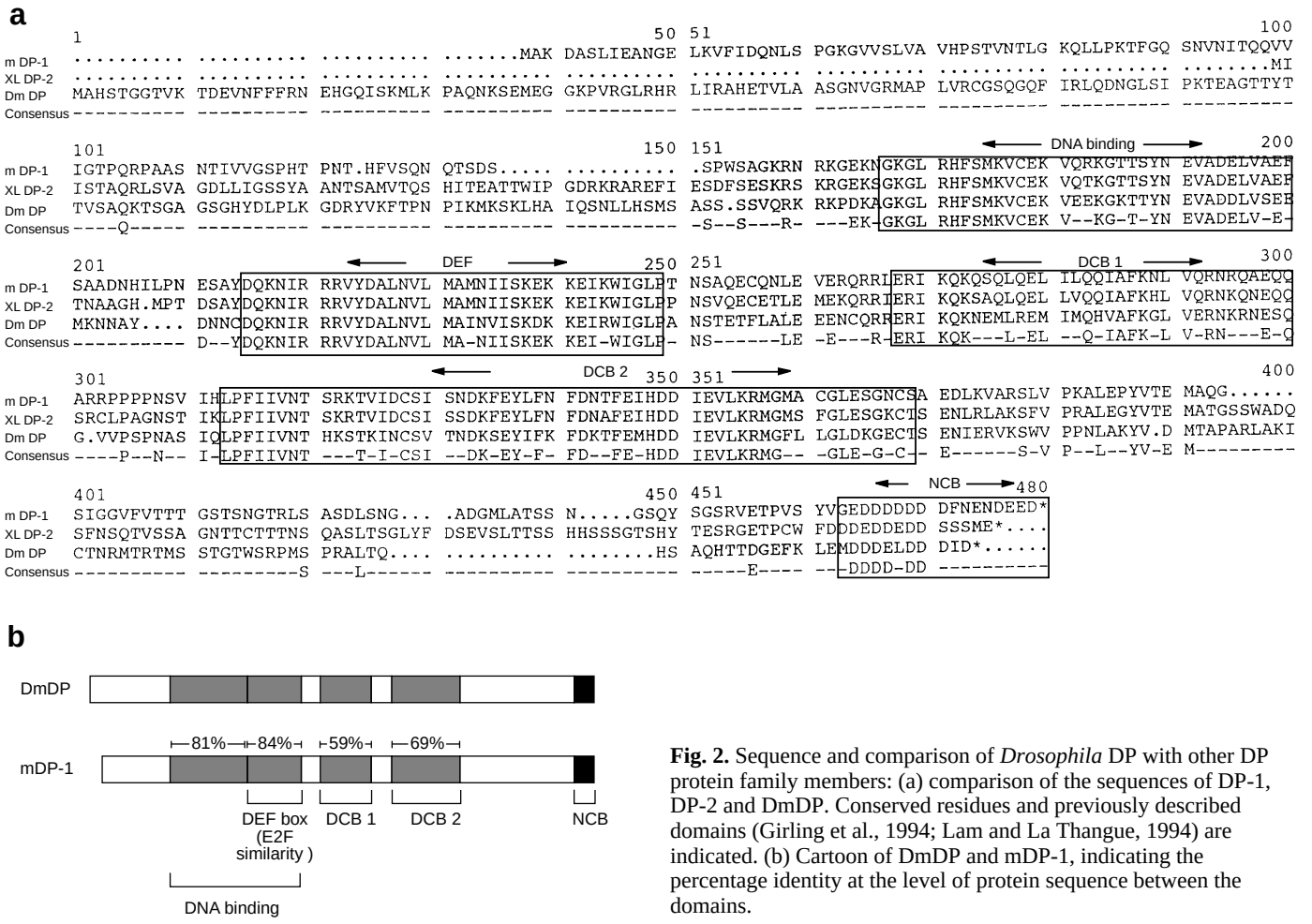

Fig. 2. Sequence and comparison of *Drosophila* DP with other DP protein family members: (a) comparison of the sequences of DP-1, DP-2 and DmDP. Conserved residues and previously described domains (Girling et al., 1994; Lam and La Thangue, 1994) are indicated. (b) Cartoon of DmDP and mDP-1, indicating the percentage identity at the level of protein sequence between the domains.

when a DP protein binds to an E2F protein (Bandara et al., 1993; Girling et al., 1994), both types of protein being encoded by distinct gene families (Girling et al., 1994; La Thangue, 1994). We were interested to determine if the DP component of DRTF1/E2F was present in *Drosophila* cells. To this end, we screened a *Drosophila* embryo cDNA library with a murine DP-1 probe. Several overlapping cDNA clones were isolated which encoded a protein identical to a previously isolated *Drosophila* DP protein (Dynlacht et al., 1994).

Based on the first potential initiating methionine, the complete open reading frame of DmDP encodes a 445 residue protein, in contrast to mammalian DP-1, which has 410, and *Xenopus laevis* DP-2, with 376 residues (Fig. 2a; Girling et al., 1993; Helin et al., 1993; Girling et al., 1994). The sequence conservation between DmDP and the vertebrate DP proteins is particularly well conserved across the region required for DNA binding activity (Bandara et al., 1993), which shows greater than 80% identical residues (Fig. 2a and b). A small region of sequence similarity between mammalian DP and E2F proteins, located in the C-terminal region of the DNA binding domain (Fig. 2a; Girling et al., 1993), is necessary for the formation of a DP/E2F DNA binding heterodimer (Bandara et al., 1993). This region, which is known as the DEF box (Girling et al., 1994; Lam and La Thangue, 1994), is also very conserved between DmDP, DmE2F-1 (Ohtani and Nevins, 1994), and the vertebrate DP and E2F proteins (Fig. 2b), suggesting that it performs a similar functional role in allowing DmDP and DmE2F-1 to interact as a DNA binding heterodimer. The N-terminal region of DmDP is not particularly well conserved with vertebrate DP family members, and several gaps have to be introduced to obtain the greatest level of similarity (Fig. 2a). In contrast, the region C-terminal to the DNA binding domain, which contains two domains conserved between vertebrate DP proteins, called DCB1 and DCB2, is well conserved (Fig. 2a and b). Furthermore, like the other known DP proteins, DmDP has an acidic C terminus (referred to as the NCB; Fig. 2a and b). Overall DmDP has a greater level of identity and similarity with DP-2 (49% identity and 66% similarity) compared to DP-1 (45% identity and 60% similarity).

A variety of biochemical and functional experiments indicated that DmDP requires an E2F partner to form a high-affinity DNA binding heterodimer and to activate transcription through the E2F binding site (data not shown), thus confirming the results from previous studies (Dynlacht et al., 1994).

DmDP is a physiological DNA binding component of *Drosophila* DRTF1/E2F

To characterise DmDP we prepared anti-DmDP sera raised against a peptide derived from the C-terminal region, referred to as anti-DmDP (C). Western blotting with DmDP expressed

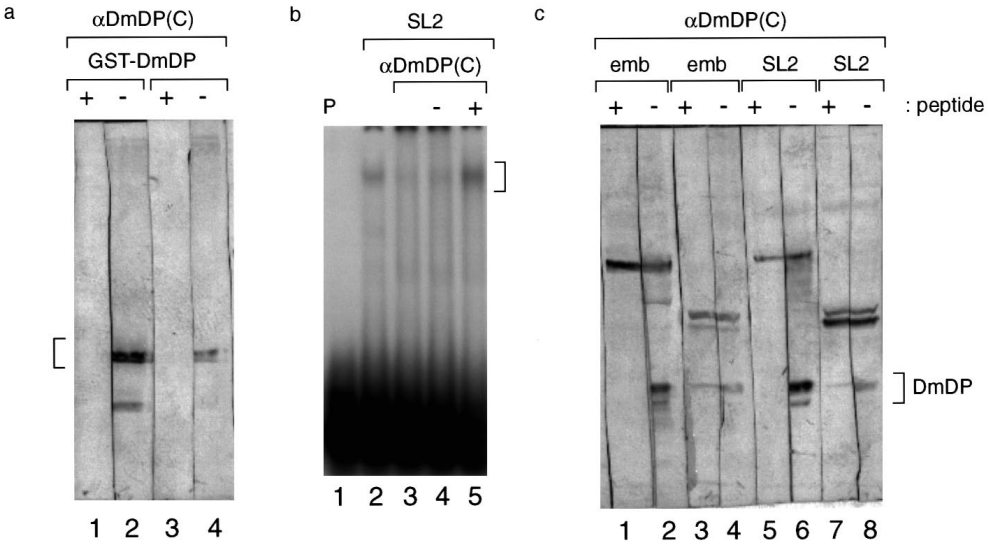

Fig. 3. Characterisation of DmDP: (a) anti-DmDP (C) was assessed by immunoblotting with the purified GST-DmDP fusion protein in the presence of either the homologous (+; tracks 1 and 3) or heterologous (–; tracks 2 and 4) peptide. The position of the fusion protein is indicated by the bracket. Each pair of tracks was treated with a different anti-DmDP (C) antiserum. The position of GST-DmDP is indicated. (b) DRTF1/E2F was assessed by gel retardation assay in extracts prepared from asynchronously growing SL2 cells in the presence (tracks 3, 4 and 5) or absence (track 2) of anti-DmDP (C), which was further incubated with either the homologous (+; track 5) or heterologous (–; track 4) peptide. Track 1 shows the probe alone. The DRTF1/E2F DNA binding complex is indicated by the bracket. (c) The anti-DmDP (C) sera were assessed by immunoblotting on *Drosophila* embryo (tracks 1, 2, 3 and 4) or SL2 cell (tracks 5, 6, 7 and 8) extracts in the presence of either the homologous (+; tracks 1, 3, 5 and 7) or heterologous (–; tracks 2, 4, 6 and 8) peptide. The polypeptides specifically recognised by anti-DmDP (C) are indicated by the bracket.

as a GST fusion protein, in the presence and absence of the homologous peptide, confirmed the specificity of the antisera (Fig. 3a, compare tracks 1, 2, 3 and 4).

To determine if DmDP is a physiological DNA binding component of *Drosophila* DRTF1/E2F, we assessed the effect of anti-DmDP(C) on the DRTF1/E2F DNA binding activity in SL2 cell extracts. Both antisera raised against the C-terminal peptide disrupted SL2 DRTF1/E2F (Fig. 3b, compare tracks 2 and 3 and data not shown). This effect was specific, since it was not apparent when the homologous peptide was included in the reaction (Fig. 3b, compare tracks 4 and 5). That anti-DmDP (C) reacted with most of the DRTF1/E2F in extracts prepared from asynchronous cultures of SL2 cells suggests that DmDP is a constitutive DNA binding component of DRTF1/E2F; in this respect DmDP is similar to mammalian DP-1 (Bandara et al., 1993, 1994).

Next, we determined the size of DmDP in SL2 cell and *Drosophila* embryo extracts. By western blotting the antisera resolved DmDP as a polypeptide of about 50,000 M_r (Fig. 3c, tracks 2, 4, 6 and 8). The addition of either the homologous or heterologous peptide confirmed the specificity of anti-DmDP (C) for these polypeptides (Fig. 3c, compare tracks 1, 3, 5 and 7 with 2, 4, 6 and 8). Although both anti-DmDP (C) antisera recognised a common 50,000 M_r polypeptide, one of the antisera defined several small polypeptides (Fig. 3c, track 2 and 6). This may reflect differences in the quality of the antisera.

Expression of *DmDP* during *Drosophila* embryogenesis

We next assessed the temporal and spatial expression of *DmDP* by in situ hybridisation to *DmDP*-encoded RNA during embryonic development. In syncytial embryos DmDP RNA was loosely clumped around nuclei migrating to the surface (Fig. 4a), a feature previously observed for other maternal RNAs such as cyclin A (Raff et al., 1990). Upon cellularisation, DmDP RNA became concentrated below the nuclei (Fig. 4b), probably because it was being excluded from the new cells as they form. Similar effects on *string* and *twine* RNA have been previously noted (Alphey et al., 1992). At a slightly later time soon after cellularisation and just as the embryo began to gastrulate (between stage 5 and 6), DmDP RNA was detectable within cells (Fig. 4c), probably resulting from transcription of the zygotic genome. In early gastrulation (stage 6), DmDP RNA was relatively uniform (Fig. 4d) although, during the later stages of germ band elongation (stage 9), expression of the RNA was apparent in regions occupied by dividing cells (Fig. 4e). In older embryos (stage 13), the expression of DmDP was restricted to the central nervous system (Fig. 4f, g and h), to which proliferating cells are essentially confined at this stage of development. This in situ analysis indicates that the message for DmDP is maternally stored and, furthermore, during embryogenesis its expression is predominantly restricted to proliferating cells.

In later development the expression of *DmDP* was still restricted to proliferating tissues. In the third instar larval brain *DmDP* was expressed at high levels in the optic lobes and less so in the ventral ganglion (Fig. 5a). Leg discs also expressed high levels of *DmDP* (Fig. 5b) but not salivary glands or fat bodies (Fig. 5c). In the eye disc, *DmDP* was expressed anterior to the morphogenetic furrow, in a stripe just posterior to it, with a lower level of expression over the rest of the disc (Fig. 5d); generalised expression was apparent in the antennal disc (Fig. 5d). The expression of *DmDP* predominantly in the anterior pre-furrow region of the eye disc again correlates with regions of cellular proliferation. In the testis, *DmDP* RNA accumulated through the spermatocyte growing stages and was degraded after meiosis (data not shown).

Expression of *DmE2F-1* during *Drosophila* embryogenesis

We examined the expression pattern of *DmE2F-1* by in situ hybridisation. In a similar fashion to *DmDP*, in syncitial

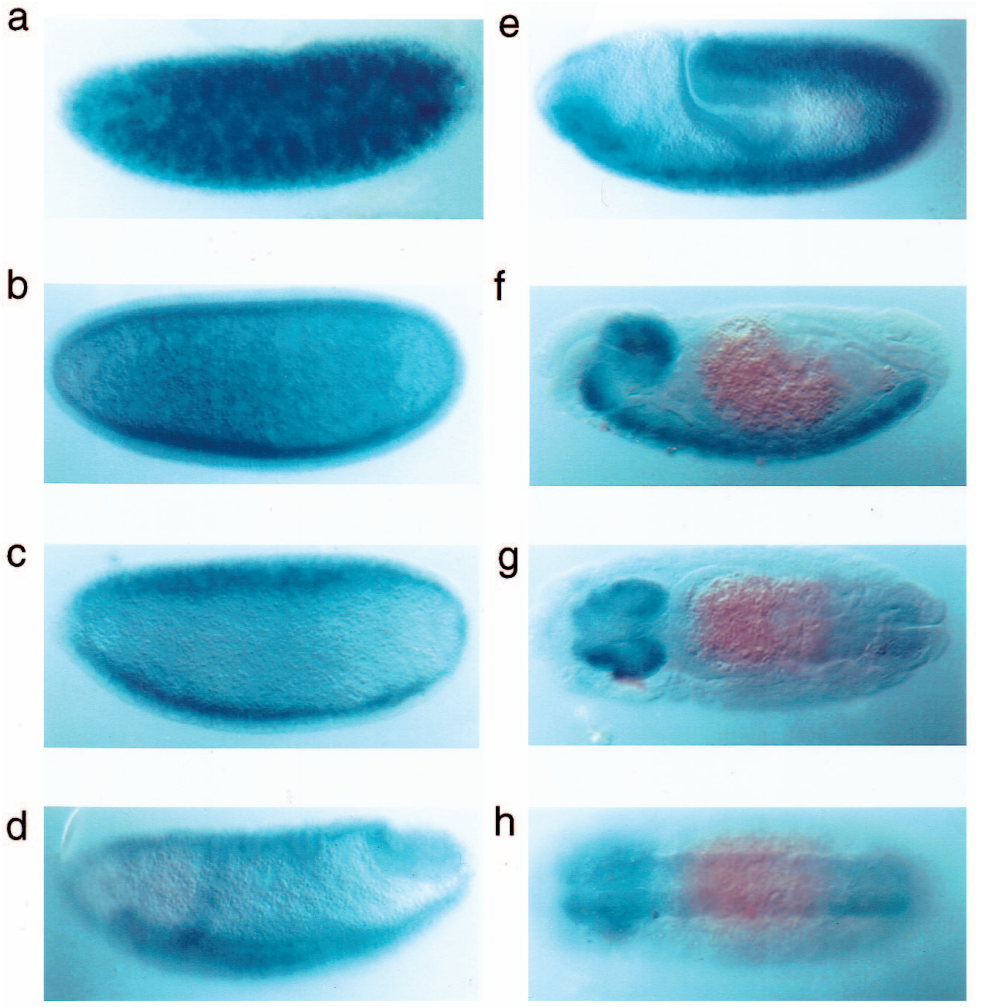

Fig. 4. Expression of *DmDP* during *Drosophila* embryonic development. The level and distribution of *DmDP* during embryogenesis was assessed by in situ hybridization in: (a) syncitial embryos; (b) early cellularisation (stage 3); (c) cellularisation (between stages 5 and 6); (d) early gastrulation (stage 6); (e) late gastrulation (stage 9) and; for (f), (g) and (h), in a series of older embryos (stage 13); (g) and (h) show different focal planes of the same embryo, and (f) shows the same embryo rolled 90°. Note that the developing lobes of the brain at the front of the embryo and the rest of the central nervous system express *DmDP*.

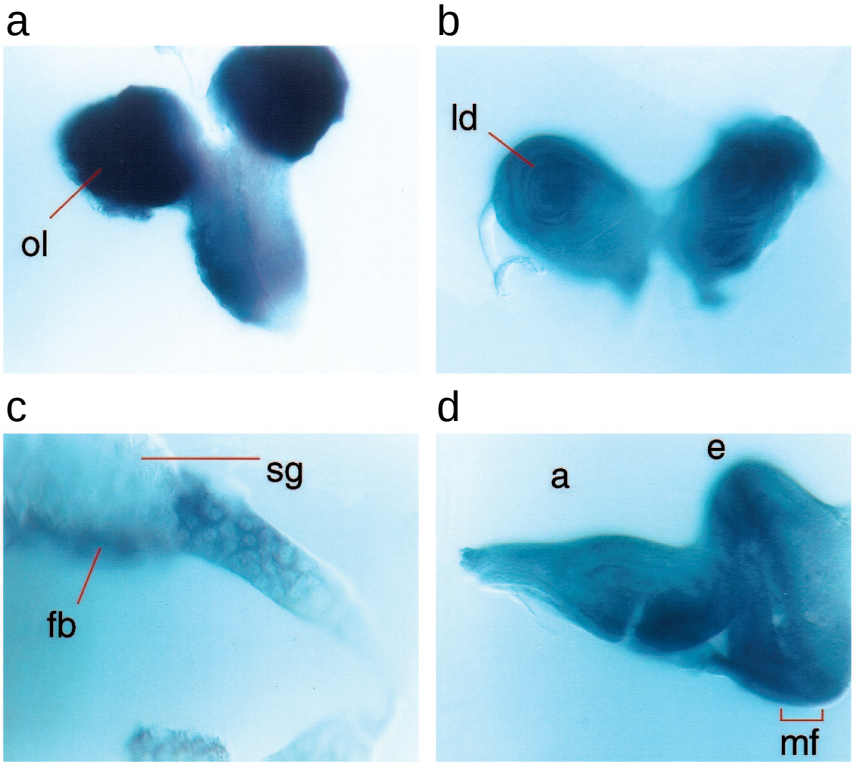

Fig. 5. Expression of *DmDP* in third instar larvae. The level and distribution of *DmDP* in third instar larvel brains was assessed in: (a), leg discs (b), salivary gland (c) and antennal/eye discs (d). Note that the salivary gland shows a low level of expression; ol, optic lobe; ld, leg disc; sg, salivary gland; a, antennal disc; e, eye disc; mf, approximate position of morphogenetic furrow.

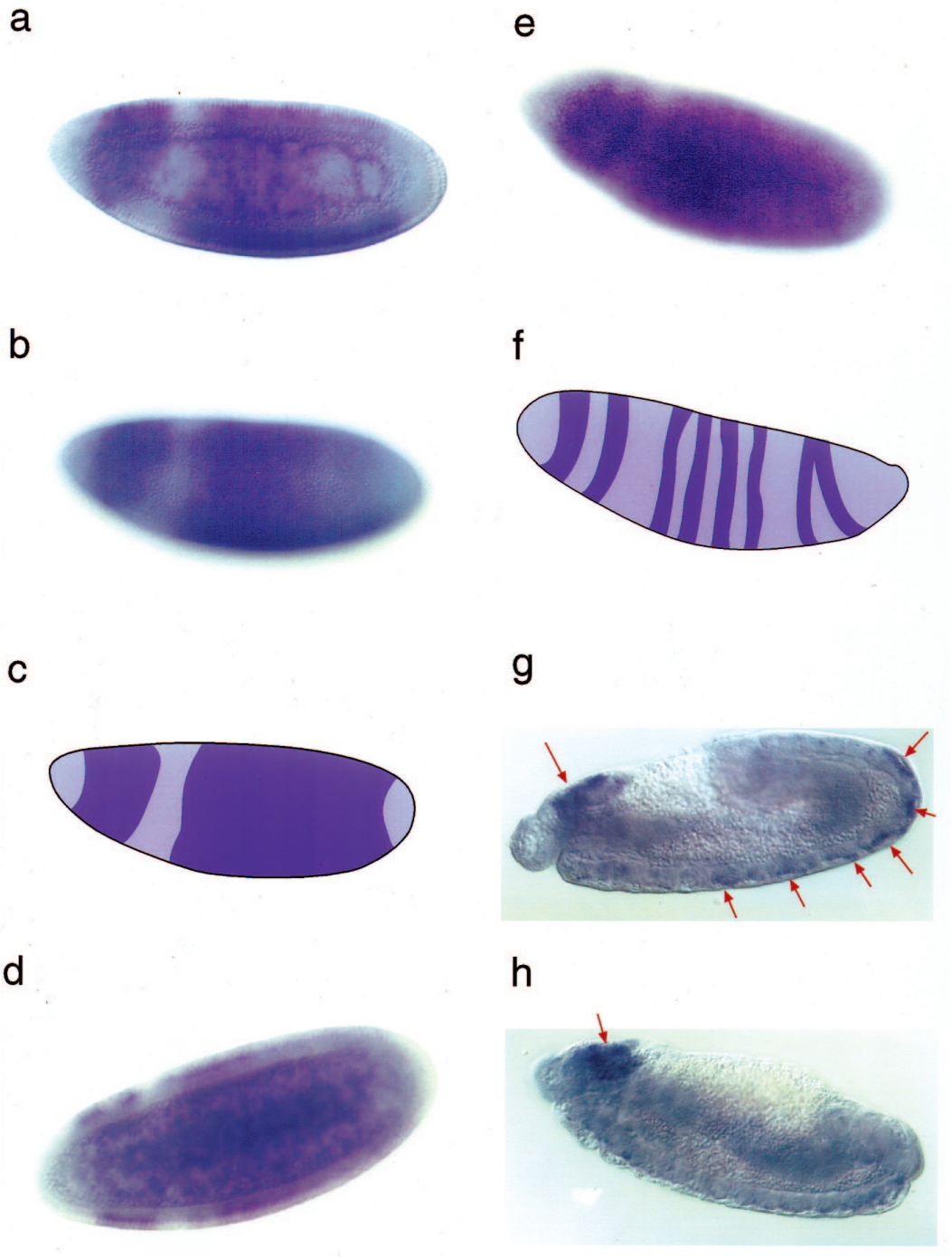

Fig. 6. Expression of *DmE2F-1* during embryonic development. The level and distribution of *DmE2F-1* during embryogenesis was assessed by in situ hybridisation in embryos undergoing cellularisation (stage 4). (a and b) A focal plane through and on the surface of the embryo, respectively. (c) A diagrammatic representation of the expression pattern in b. (d and e) Early gastrulating embryos (stage 6); (d) a focal plane through the embryo, (e) a surface plane. (f) A diagrammatic representation of the expression pattern in e. (g and h) The expression in older embryos at stages 11 and 12, respectively, is shown; arrows indicate areas of greatest expression.

embryos *DmE2F-1* was uniformly distributed and loosely clumped around migrating nuclei (data not shown). However, around the time of cellularisation (stage 4) and activation of zygotic gene expression, DmE2F-1 RNA became distributed into two regions of greatest expression: a broad band at the posterior end and a narrower (and sharper) band at the anterior end of the embryo (Fig. 6a, b, and c). The narrow anterior band coincided approximately with the pro-cephalic neuroregion, which is destined to become brain tissue, and the region with reduced expression (between the two bands) overlapped with the cephalic furrow. By early gastrulation (stage 6), the anterior bands had become more restricted to two narrower regions and the broader posterior bands into a number of individual stripes (Fig. 6d, e and f). There was significant expression between the stripes; the stripes representing areas with the greatest level. By stage 6, the expression of DmE2F-1 thus occurs in a segmentally restricted and repeated fashion. By stage 12, *DmE2F-1* was in scattered cells in the ventral nerve cord, the greatest level occurring in the posterior brain region (areas of expression indicated by arrow in Fig. 6g). By stage 13, the expression was almost exclusively restricted to the brain (indicated by arrows in Fig. 6h). This pattern of expression is in contrast to that for *DmDP* which, at this stage of development, is expressed throughout

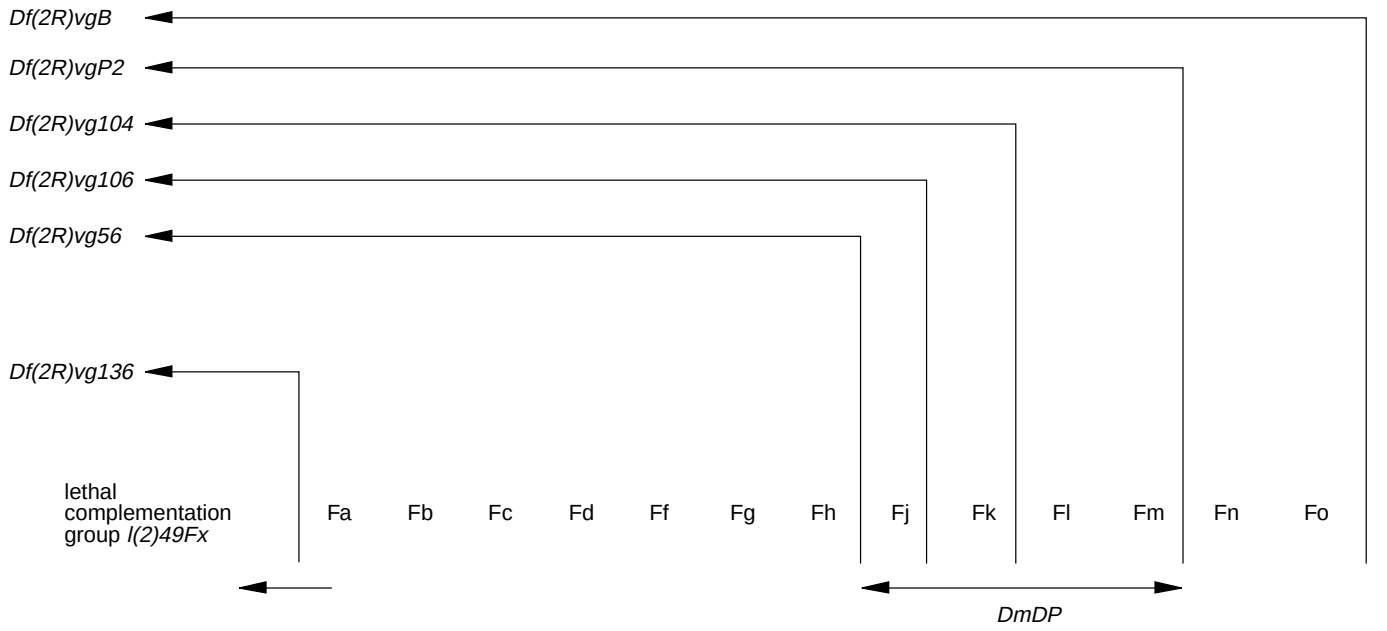

Fig. 7. Cytogenetic location of *DmDP*. In situ hybridisation to deficiency stocks indicated that *DmDP* maps within *DF (2R) vg*-P2, but outside *DF (2R) vg*-56. Deficiency break points and lethal map are taken from Lasko and Pardue (1988).

the central nervous system (Fig. 4f). The expression of *DmE2F-1* is thus more restricted at this stage of development relative to *DmDP*.

Chromosome location of *DmDP*

In situ hybridisation to polytene chromsomes gave a single signal at 50A1-4. The location is on the edge of a region around *vestigial wing* (*vg*), which has been extensively mutagenised and deficiency mapped (Lasko and Pardue, 1988). *DmDP* was found to be the deficiency within *Df (2R) vg-B*, thus refining its chromosomal location. Further in situ analysis of the deficiencies *Df (2R) vg-56* and *Df (2R) vg-P2* indicated that *DmDP* lies outside *vg-56* but within *vg-P2*. These data are summarised in Fig. 7. We are currently determining which complementation group corresponds to *DmDP*.

DISCUSSION

Conservation of the DRTF1/E2F cell cycle-regulating pathway in *Drosophila* cells

In mammalian cells, the activity of DRTF1/E2F is regulated through a complex array of afferent signals. For example, pocket proteins repress its transcriptional activity through regulatory processes which are integrated with the cell cycle due to the upstream activity of cdks (La Thangue, 1994). Indeed, our analysis of DRTF1/E2F in SL2 cells suggests that analogous afferent signalling proteins may integrate their activities with DRTF1/E2F in *Drosophila* cells. This idea is based on several pieces of data presented in this study. Particularly, in SL2 cell extracts DRTF1/E2F resolves as a DNA binding complex which co-migrates with DRTF1a (La Thangue et al., 1990), the form in mammalian cells which contains associated pocket proteins, such as pRb and p107 (Bandara and La Thangue, 1991; Bandara et al., 1992). The SL2 DRTF1/E2F complex is sensitive to the action of the adenovirus E1a protein, which causes the appearance of a faster migrating DNA binding complex, an effect analogous to that seen in mammalian cells where E1a releases transcriptionally active DRTF1/E2F from pocket protein complexes (Bandara and La Thangue, 1991). Finally, the SL2 DRTF1/E2F DNA binding complex is further retarded upon the addition of cyclin A and cdk2, again reflecting the properties of these proteins in mammalian cells (Bandara et al., 1992). These data therefore argue that in SL2 cells a pocket protein-like molecule associates with DRTF1/E2F, and furthermore that this protein can interact with cyclin A and cdk2.

We believe that our data suggest that *Drosophila* DRTF1/E2F is regulated through a pathway which involves a protein sharing considerable similarity with the pocket proteins of mammalian cells. Of course, we do not wish to imply identity, but rather a generic similarity. However, given the common behaviour of E1a, cyclin A and cdk2 in mammalian and *Drosophila* cells, then it would seem likely to be a protein which shares a reasonable degree of structural similarity with the known mammalian pocket proteins.

Furthermore, E1a proteins which are mutated in CR1 (conserved region 1) or CR2, which in mammalian cells fail to bind pRb, cannot dissociate *Drosophila* DRTF1/E2F (Bandara and La Thangue, data not shown), providing further support for the idea of fly pocket-protein homologues.

In this respect, it is relevant that the *Drosophila* E2F-1 protein contains a significant level of similarity with the mammalian E2F-1, -2 and -3 proteins, extending into the C-terminal region (Ohtani and Nevins, 1994). In the mammalian E2F proteins this region is necessary for forming a stable physical complex with pocket proteins, such as pRb (Flemington et al., 1993; Helin et al., 1993), which, given the sequence simiarlity, argues that the C-terminal region of *Drosophila* E2F-1 is likely to possess a similar property.

Indeed, this implication is compatible with the evidence reported in this paper for a pocket-like protein in SL2 cells, underscoring the possiblity that the mechanisms involved in regulating the activity of DRTF1/E2F, and hence early cell cycle control, are shared between mammals and flies.

In mammalian cells, functional E2F binding sites occur in the control regions of genes which are necessary for cell cycle progression, such as *DHFR* and *DNA polymerase α* (Nevins, 1992). Similarly, in *Drosophila* the DNA polymerase α gene is induced towards the end of G_1 and into S phase (Hirose et al., 1993), which, combined with the fact that the promoter contains a series of E2F sites responsive to DmE2F-1 (Ohtani and Nevins, 1994), supports the idea that DRTF1/E2F performs a role in integrating cell cycle events with the transcription apparatus during the fly cell cycle.

The isolation and characterisation of a functionally and structurally related DP protein from *Drosophila*, DmDP, indicates that DP genes have been conserved during evolution. However, a comparison of DmDP with the available vertebrate DP proteins, notably DP-1, -2 and -3, indicated that DmDP has a significantly greater level of identity with DP-2 relative to DP-1 and DP-3 (Fig. 2 and data not shown). It is unclear whether this has any bearing on the functional role of DmDP in the *Drosophila* cell cycle and whether, like vertebrate DP proteins, *DmDP* is also representative of a gene family.

The expression of *DmDP* and *DmE2F-1* during *Drosophila* embryogenesis

In situ analysis of DmDP RNA during embryonic development indicated that *DmDP* encodes a maternally stored RNA and that, once transcription from the zygotic genome is activated, its expression correlates relatively well with cellular proliferation. For example, in the later stages of embryogenesis the nervous system (particularly the brain) is the predominant proliferative tissue. Indeed, in late embryos the expression of *DmDP* was restricted, almost exclusively, to the developing nervous system. In third instar larval brains and a variety of discs the expression of *DmDP* was high. Furthermore, the anterior pre-furrow cells of the eye disc, which contain dividing cells (less frequent in the posterior region), expressed *DmDP*.

An analysis of *DmE2F-1* indicated that its expression during embryogenesis was distinct from that of *DmDP*. In early embryos (stage 4) it was expressed at the greatest levels in two bands, one in the anterior and the other in the posterior region of the embryo. As embryogenesis continued to stage 6, these areas became divided into a greater number of stripes. At stage 4 the embryo exists as a syncitial blastoderm with nuclei undergoing nuclear divisions without the imposition of G_1 or G_2 phases. After cellularisation, most cells remain in a G_2 state, S phase and mitosis beginning during stage 8 (Orr-Weaver, 1994). During these stages the expression of *DmE2F-1* overlapped with *DmDP*, and thus may result in transcriptionally active DRTF1/E2F. Since many of the target genes encode proteins required for progression through S phase, transcriptional activation by DRTF1/E2F may contribute to these early cell cycle transitions.

It is possible that the segmentally repeated pattern of *DmE2F-1* is involved in determining the mitotic domains which subsequently occur during later stages of embryogenesis (during stages 7, 8, 9 and 10; Orr-Weaver, 1994). However, since *DmDP* did not follow a similar expression pattern, quantitative differences in *DmE2F-1* expression may not translate into quantitative differences in functional DRTF1/E2F, since for this to occur it would be necessary for DmDP to be present in excess. Alternatively, it is possible that different populations of E2F site DNA binding activities occur in the highly expressing areas, such as DP/E2F heterodimers and E2F homodimers, which may be important in allowing the activity of target genes to be fine-tuned.

During later embryonic stages the expression of *DmE2F-1* became more restricted than *DmDP*, particularly in the central nervous system where *DmE2F-1* was expressed in scattered cells in the brain, in contrast to *DmDP*, which was more uniformly expressed (compare Figs 4f and 6h). Since the central nervous system is the predominant proliferative tissue at this stage of development, this implies that DmE2F-1 performs a more specialised role than DmDP. The expression of *DmDP* and *DmE2F-1* in the same cells could result in DRTF1/E2F, whereas in the absence of DmE2F-1 it is possible that the activity of DmDP is integrated with other pathways. Indeed, a similar situation may occur in mammalian cells where a considerable amount of DP-1 occurs in a non-DNA binding complex (Sørensen and La Thangue, unpublished data).

In conclusion, this study shows that many of the properties and characteristics of DRTF1/E2F are conserved between *Drosophila* and mammalian cells, such as the interaction with similar afferent signalling proteins and the molecular composition of DRTF1/E2F from DP/E2F heterodimers. In addition, the distinct and novel expression patterns of *DmDP* and *DmE2F-1* suggest that the formation of DP/E2F heterodimers, and hence DRTF1/E2F, is subject to complex regulatory cues.

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