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S Amero

Sarah C.R. Elgin
Washington University in St. Louis, selgin@wustl.edu

A Beyer

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A unique zinc finger protein is associated preferentially with active ecdysone-responsive loci in *Drosophila*

Sally A. Amero,[1] Sarah C.R. Elgin,[2] and Ann L. Beyer[1]

[1]Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908 USA; [2]Department of Biology, Washington University, St. Louis, Missouri 63130 USA

Using an immunochemical approach, we have identified a unique antigen, PEP (protein on ecdysone puffs), which is associated in third-instar larvae with the active ecdysone-regulated loci on polytene chromosomes; PEP is not associated with most intermolt puffs and is found on some, but not all, heat shock-induced puffs. The distribution pattern changes with changing puffing patterns in the developmental program. We have screened an expression library and recovered a cDNA clone encoding PEP. PEP possesses multiple potential nucleic acid- and protein- binding regions: a glycine- and asparagine-rich amino terminus, four zinc finger motifs, two very acidic segments, two short basic stretches, and an alanine- and proline-rich carboxyl terminus. The *Pep* gene maps by in situ hybridization to the cytological locus 74F, adjacent to the early ecdysone-responsive region; however, the gene is not regulated by ecdysone at the level of transcription. The pattern of *Pep* expression through development suggests that maternal *Pep* gene transcripts are supplied to the embryo, and that the abundance of *Pep* gene transcripts decreases to a lower, fairly constant level thereafter. This unusual protein may play a role in the process of gene activation, or possibly in RNA processing, for a defined set of developmentally regulated loci.

The giant polytene chromosomes of *Drosophila melanogaster* provide a unique opportunity to assay the protein interactions at defined genetic loci, because specific proteins can be localized on the chromosomes through indirect immunochemical assays (e.g., Elgin et al. 1988). Analyses of this type have provided a general picture of transcriptional activity in puffs and interbands from the distribution patterns of RNA polymerase II (Sass 1982; Weeks et al. 1982) and topoisomerase I (Fleischmann et al. 1984). The presence of heterogenous nuclear ribonucleoproteins, (hnRNPs) (S.A. Amero and A.L. Beyer, unpubl.) and small nuclear ribonucleoproteins (snRNPs) (Sass and Pederson 1984; S.A. Amero and A.L. Beyer, unpubl.), which are involved in packaging and processing of nascent RNA molecules (Dreyfuss 1986; Zieve and Sauterer 1990), is also observed at these sites.

A coordinated program of developmental gene expression is visible as a reproducible, temporal pattern of puffing activity on the polytene chromosomes (Ashburner 1970). Three sets of chromosomal loci are included in the program, which is initiated in response to ecdysteroid hormones (Hodgetts et al. 1977). The existing intermolt puffs (including loci 25AC, 68C, and 90BC) regress; several "early" puffs (including loci 2B, 22B, 23E, 63F, 74EF, and 75B) appear quickly and then regress. Subsequently, >100 "late" puffs appear in a temporal sequence (Ashburner and Berendes 1978; Pongs 1988). A number of early genes have been cloned (Burtis et al. 1990; Segraves and Hogness 1990; Thummel et al. 1990), and their products may participate in activation of the late genes (Urness and Thummel 1990; for review, see Ashburner 1990); the products of these late genes are thought to initiate metamorphosis.

A different program of gene expression, and a different puffing pattern, ensues from heat shock or a number of other stresses (Ritossa 1962; Ashburner and Bonner 1979). Nine puffs, the sites of the genes encoding the heat shock proteins (Spradling et al. 1977), are rapidly induced at 37–38°C; concomitantly, the developmental puffs regress as transcription at these loci is repressed and splicing of pre-mRNAs is inhibited (Yost and Lindquist 1986). At least one specific factor, the heat shock transcription factor, is required in addition to the basal transcriptional machinery for this induction process (Parker and Topol 1984; Wu 1984).

Using monoclonal antibodies to nuclear proteins from *Drosophila* embryos, we have identified an unusual protein, PEP (protein on ecdysone puffs), which possesses a distinctive distribution pattern on polytene chromosomes: PEP is associated preferentially with activated, hormone-responsive loci, including both early and late genes but is not found on most intermolt puffs, which

regress in response to hormone. The pattern of PEP localization reflects the progression of the developmental program. In addition, PEP is associated with some, but not all, heat shock-induced puffs. Cloning of the *Pep* gene product has revealed the presence of numerous putative nucleic acid- and protein-binding motifs in the unique PEP sequence. In situ hybridization using the cDNA probe places the gene at locus 74F, adjacent to an early ecdysone-responsive region, but *Pep* gene expression is not regulated at the level of transcription by ecdysone. Thus, PEP is likely to be an important component of selected active loci, recruited in larval stages to complement the transcriptional machinery or splicing apparatus for ecdysone-mediated gene expression.

Results

PEP is associated preferentially with active, ecdysone-induced puffs

Monoclonal antibodies to nuclear proteins from *Drosophila* embryos were screened for interesting distribution patterns on polytene chromosomes by indirect immunofluorescence, and mAbs Y1D2 and Y2A11 were chosen for further study. These monoclonal antibodies gave an immunofluorescence distribution pattern covering most visible puffs but few band and interband regions, and no nucleoplasmic or cytoplasmic debris (Fig. 1, B). Remnants of the nucleolus are not stained. mAbs Y1D2 and Y2A11 produce identical staining patterns on the two salivary glands from the same larva (not shown). Subsequent experiments described below show that the two monoclonal antibodies recognize separate epitopes in the PEP antigen.

Fine mapping of the distribution pattern of PEP by the immunofluorescence assay over two larval and two prepupal developmental stages (Table 1) reveals a tight correlation between visible puffing of the developmentally regulated loci and the presence of PEP. Excluding the intermolt puffs, >90% of the visible developmental puffs are stained. The few visible puffs that are not stained occur either in the very early or very late stages; the correlation is perfect in the middle stages. Only in several cases is staining detected prior to visible puffing (loci 48B, 52C, and 82F) or following regression (locus 47A). The protein is not associated with the intermolt puffs at loci 25AC, 68C, and 90BC in the early puffing stages but is associated with the intermolt puff at locus 3C (not shown). Particularly noteworthy is the intense staining of the ecdysone-responsive puffs at loci 74EF and 75B at all stages examined, as these puffs are thought to undergo early activation and late regression in response to ecdysone. Locus 71DE, on the other hand, is puffed throughout these developmental stages but is associated with PEP only in the middle stages. On any given chromosome, the developmental loci listed in Table 1 constitute ~50% of the total staining pattern for the PEP antigen; the additional sites may represent developmental loci not scored in the original list prepared by Ashburner and Berendes (1978). However, some of

these additional sites are consistently stained, such as loci 50CD, 52F, 61E, 70A, and 70D (not shown), and staining at these sites shows no developmental response to ecdysone.

One instructive example of the distribution pattern of PEP is presented in Figure 1, C and D, using mAb Y1D2. Most of the developmental (hormone-responsive) loci that are puffed at stage 2 are stained (loci 22B, 62E, 63F, 69A, 74EF, and 75B) while the intermolt puff at locus 68C and the puffs at loci 21C, 23E, and 71DE are not. Some of the latter puffs do become stained at later stages, however. Two other developmental loci (63E and 66B) that are induced later in development differ in their staining characteristics on this chromosome; locus 66B is stained rather brightly, while locus 63E is stained at a much lower level. It is clear by comparison to the distribution pattern of RNA polymerase II on the same chromosome (Fig. 1E) that the sites associated with PEP are a small subset of the loci associated with RNA polymerase and potentially engaged in transcription. In sum, the general features of the distribution pattern of PEP conform to the classical models for a factor involved in expression of the hormone-induced loci. However, the additional sites stained indicate that PEP most likely is involved in other activation systems in addition to that triggered by ecdysone.

The distribution pattern of PEP on chromosomes from heat-shocked larvae also demonstrates a selectivity for certain active loci. Of the nine major heat shock-induced sites, PEP is found at loci 87A, 87C, 93D, and 63BC but not at loci 95D, 64F, and 70A (Fig. 2B,D). Low-level staining is observed at locus 67B (Fig. 2D), and locus 33B was not scored. The heat shock puffs that are associated with PEP do not strictly correspond to those heat shock genes that are also induced by ecdysone, such as the small heat shock protein genes at locus 67B (Zimmerman et al. 1983) or with heat shock genes containing introns, the *hsp83* gene at locus 63BC (Yost and Lindquist 1986), and the *hsr93D* gene at locus 93D (Ryseck et al. 1985; Garbe and Pardue 1986). Thus, PEP is not required for the processes of puffing, transcription, or RNA processing per se. The protein is associated, however, with a particular subset of heat shock-induced loci, as well as a group of developmentally regulated genes and, thus, overlaps two programs of gene expression.

The Pep gene encodes the Y1D2 and Y2A11 epitopes

Western blots of proteolytic digests of chromosomal proteins showed that mAbs Y1D2 and Y2A11 recognize different epitopes in the PEP antigen. A nuclear extract from *Drosophila* embryos was digested with increasing amounts of trypsin and fractionated by electrophoresis in an SDS–polyacrylamide gel, and the peptides were transferred electrophoretically to a nitrocellulose filter for Western blot analyses. Identical filters were probed with either TBS (not shown), mAb Y1D2 (Fig. 3A), or mAb Y2A11 (Fig. 3B). Both monoclonal antibodies recognize the 110-kD nuclear PEP antigen; often, but not always, mAb Y1D2, but not mAb Y2A11, recognizes sev-
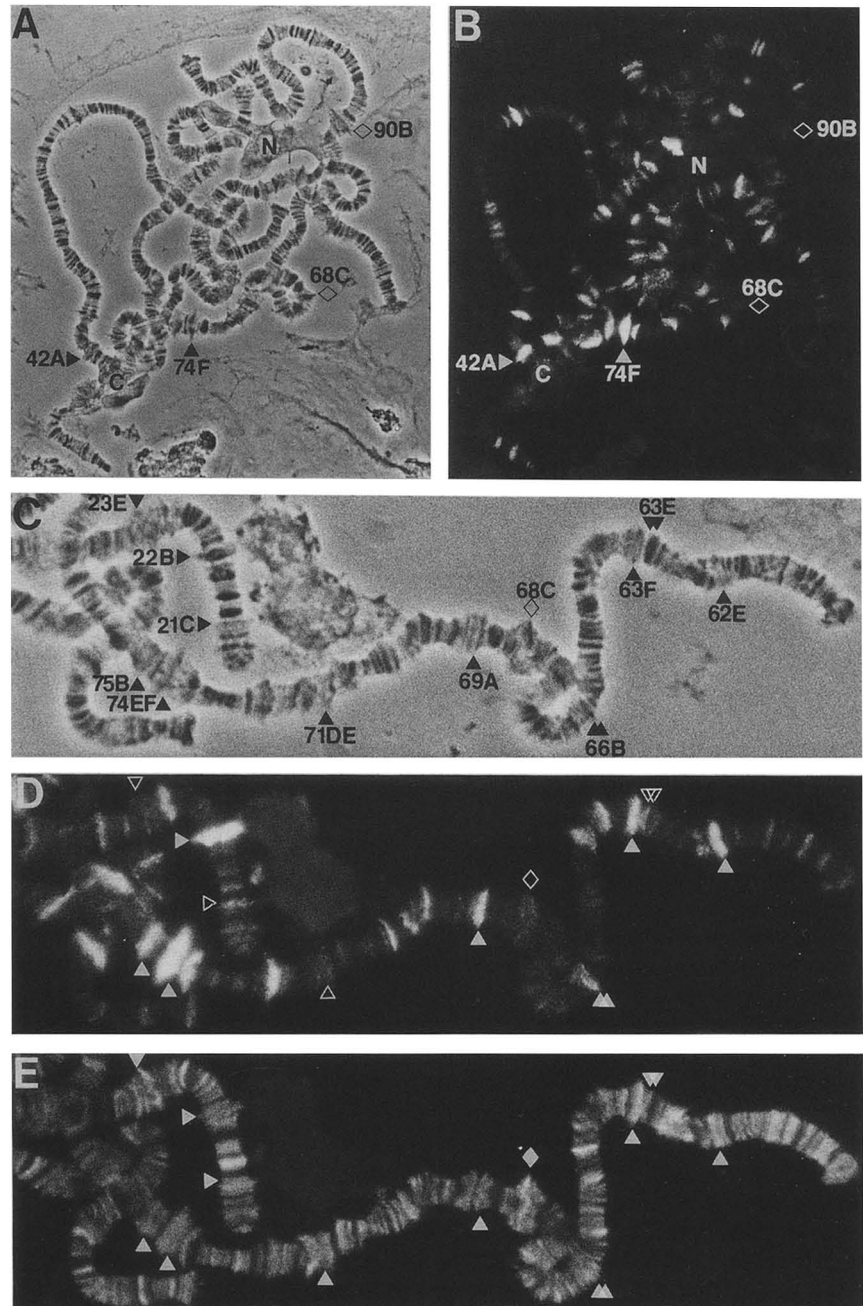
Figure 1. Distribution of PEP on polytene chromosomes from control larvae. Polytene chromosomes were squashed in an acetic acid–formaldehyde fixative solution onto a microscope slide and stained with mAb Y1D2, using an indirect immunofluorescence method (James and Elgin 1986). The chromosomes were photographed under phase-contrast and fluorescence illumination and mapped according to the designations of Lefevre (1976). (A) The phase-contrast picture of chromosomes from a puff stage 2 larva, where the intermolt puffs 68C and 90BC, (◇), as well as the ecdysone puffs 42A and 74EF(▲), are clearly visible. The nucleolus (N) and chromocenter (C) are also marked. (B) The fluorescence picture of the same chromosomes shown in A, stained using mAb Y1D2. (▲) The stained developmental loci; (◇) those not stained. (C) The phase-contrast picture of chromosome arm 3L at puff stage 2. (▲) The developmental loci that are active at this stage; (◇) the intermolt puff 68C; (overlapping ▲ ▲) developmental puffs that will become active at later developmental stages (66B and 63E). (D) The fluorescence picture of the chromosome arm 3L (shown in C) stained with mAb Y1D2. The symbol designations are described in the legends B and C and denote the level of fluorescence due to PEP-specific staining. (E) The fluorescence picture of the chromosome arm 3L shown in C and D, restained with affinity-purified antibodies specific for the carboxy-terminal domain of the largest subunit of RNA polymerase II (Fisher et al. 1989).

eral smaller polypeptides in the original extract, which are likely to be degradation products. The difference between the two epitopes is evident from the retention of the Y1D2 epitope on several smaller tryptic fragments, while in the same samples, the Y2A11 epitope is found only in the parental PEP antigen. Two prominent signals (20 and 24 kD) appear in all lanes containing trypsin, even in the absence of nuclear extract; these most likely arise from nonspecific recognition of the vast excess of trypsin in these samples. The filter incubated in TBS produced no signal.

The mAb Y1D2 was used to isolate a 1.8-kb *Droso-*

phila DNA fragment from a λgt11 expression library representing cDNAs from 0- to 3-hr embryos (M. Phillip and D. Brutlag, pers. comm.). The partial cDNA fragment was shown to encode both the Y1D2 and Y2A11 epitopes from the PEP protein in the Western blot analysis shown in Figure 4. Bacterial lysates from a λ lysogen strain carrying the 1.8-kb cDNA fragment, both before and after induction, as well as *Drosophila* nuclear proteins and lysate from a lysogen carrying the unrelated HP1 gene (James and Elgin 1986), were fractionated in an SDS–polyacrylamide gel (Fig. 4A), blotted to nitrocellulose, and probed with mAb Y1D2 (Fig. 4B). The monoclonal

Table 1. *Binding of the PEP protein to developmentally regulated puffs*

Arm	Locus	Stage 2		Stage 6		Stage 12		Stage 18	
		P	B	P	B	P	B	P	B
2L	21C	−	−	−	−	−	−	+	+
	21F	−	−	±	±	−	−	−	−
	22A	−	−	−	−	−	−	−	+
	22B**	+	+ +	+ +	+ +	−	−	−	−
	22C	−	−	+	+	+	+	±	±
	23E**	+	−	+	+	+	+	+	±
	25AC*	+	−	±	±	+	±	−	±
	34A	−	−	+	+	X	X	+	+
2R	42A	+	+ +	+	+	+ +	+ +	+ +	+ +
	46A	−	−	+	+ +	X	X	−	−
	46F	−	−	−	−	±	±	+	±
	47A	−	−	−	−	+	±	−	±
	48B	−	±	±	+	−	−	±	±
	52A	−	−	±	+	−	−	X	X
	52C	−	±	±	±	+	+	X	X
	55E	X	X	+	+ +	+	+	+	+
	58BC	±	+	+	+ +	−	−	X	X
	60B	−	−	−	−	X	X	−	−
3L	62E	+	+	+ +	+ +	+	+	±	+ +
	63E	−	−	−	−	−	+	±	±
	63F**	+	+	+ +	+	±	+	+ +	+
	66B	+	+	±	±	+	+	+	+
	68C*	+ +	±	+ +	±	+	−	+	−
	69A	+	+	X	X	−	+	+	+
	71DE	+	±	+	+ +	X	X	+	−
	74EF**	+ +	+ +	+ +	+ +	+	+	+ +	+ +
	75B**	+ +	+ +	+ +	+ +	+	+	+ +	+
	75CD	−	−	X	X	X	X	±	−
	78D	−	−	+ +	+ +	+	+	+	+
3R	82F	−	+	+	+	+	+	+ +	+ +
	85D	+	±	+ +	+/+	X	X	+	+/+
	85F	+	+ +	+ +	+ +	+	+	+ +	+ +
	90BC*	+ +	−	−	−	+ +	−	+ +	−
	91D	−	−	±	+	±	±	+	−
	93F	−	−	−	−	+	+	−	−
	95F	−	−	+	+	X	X	+	±
	98F	+	−	+	+	−	−	−	−
	100DE	−	+	−	−	±	+	+	+

(*) Intermolt puffs; (**) early puffs; all others are late puffs. The puffing status of the developmental puffs used in this analysis is presented under the columns marked P; puffs were scored as very prominent (+ +), clearly puffed (+), slightly puffed (±), or not puffed (−). An X denotes a puff that was not visible in the field. Two nuclei were averaged for stages 2 and 6. Puffing stages were determined by comparison of the puffing patterns to the tabulations presented by Ashburner and Berendes (1978). The binding of PEP (columns marked B), or the level of fluorescence at a puff, was scored as intense (+ +), clearly above background (+), barely detectable above background (±), or not detectable (−). The +/+ designation in the binding column for locus 85D indicates that two distinct fluorescence signals were visible at this locus. The X chromosome was not scored.

antibody recognizes one major protein species of 110 kD, the PEP antigen, in the nuclear extract (Fig. 4B, lane 1), and three large polypeptides in the PEP lysogens (Fig. 4B,

lanes 3 and 5), but nothing in the HP1 lysogen (Fig. 4B, lane 4). Although some low-level expression of the β-gal/PEP fusion protein occurs in the absence of IPTG (Fig. 4B, lane 3), the induction by IPTG is apparent by comparison (Fig. 4B, lane 5). The same nuclear and PEP lysogen extracts were probed also with mAb Y2A11 (Fig. 4C). The Y2A11 epitope is present in the 110-kD nuclear PEP antigen (Fig. 4C, lane 1) and in the largest of the induced β-gal/PEP fusion proteins, but not in the two smaller induced polypeptides (Fig. 4C, lane 3). Therefore, the 1.8-kb DNA sequence encodes two epitopes from the PEP antigen, confirming its origin in the *Pep* gene. This partial cDNA fragment was used to isolate a 2.7-kb cDNA *Drosophila* clone from a second library made to optimize synthesis of full-length copies of mRNAs (Brown and Kafatos 1988). This longer clone was used for the analyses described below.

The Pep *gene maps to the cytological locus 74F*

The chromosomal site of the *Pep* gene was determined by in situ hybridization to polytene chromosomes of a biotin-labeled 2.5-kb *HindIII* fragment from the cDNA clone. Signal was detected within the proximal portion of the puff at the cytological locus 74F (Fig. 5), adjacent to the early ecdysone-responsive region. The puffing at this locus appears to be due to the vigorous ecdysone induction of the neighboring E74 early gene (Thummel et al. 1990), rather than the *Pep* gene (see below). Genomic Southern blot analyses (not shown) suggest that there is a single copy of the *Pep* gene covering no more than 8 kb of the chromosome at this site.

The Pep *gene is not regulated by ecdysone*

Transcripts from the *Pep* gene were examined at different developmental stages by probing a Northern blot containing poly(A)$^+$ RNA from staged organisms with the cDNA probe used for the in situ hybridizations above. In every developmental stage, a single signal representing a transcript of ~2.8 kb is visible (Fig. 6); the signal is enhanced, however, in the lanes containing samples from ovaries and 0- to 3-hr embryos. The loading controls for this filter representing transcripts from the ribosomal protein gene *rp49* (O'Connell and Rosbash 1984) indicate overloading in the lanes containing RNA from 4- to 12-hr embryos and third-instar larvae. From this analysis we conclude that maternal *Pep* gene transcripts are supplied to the embryo, and the level of *Pep* gene transcripts decreases to a relatively constant level following embryogenesis. The abundance of *Pep* gene transcripts does not reflect the pulses of ecdysone that occur in mid-embryogenesis (Kraminsky et al. 1980) and in each larval instar (Hodgetts et al. 1977) but may be correlated with the presence of ecdysone in ovaries and the syncytial zygote (Richards 1981). In an additional experiment, a Northern blot filter containing RNA samples from isolated larval organs that had been exposed in vitro to ecdysone or to ecdysone plus cycloheximide was
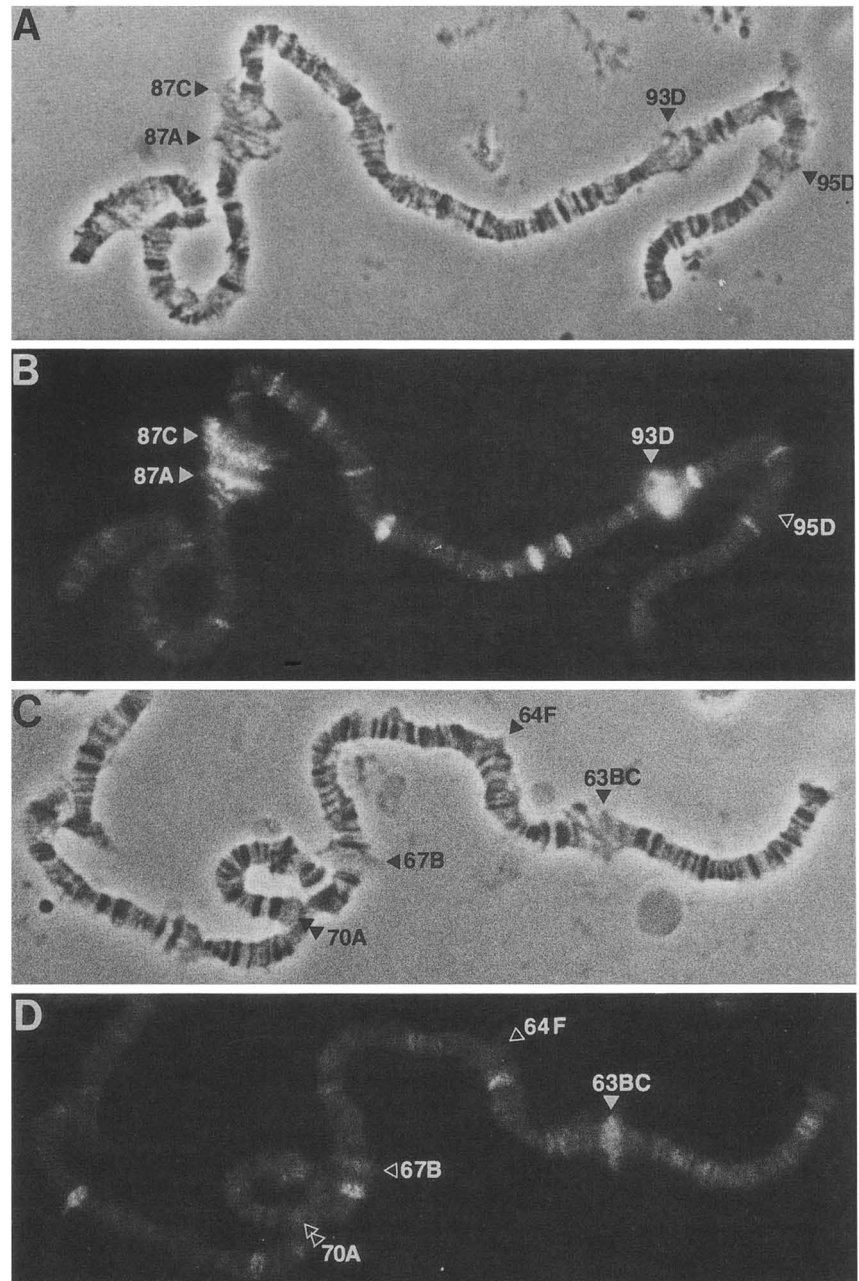
Figure 2. Distribution of PEP on polytene chromosomes from heat-shocked larvae. Larvae were maintained at 38° for 40 min to induce the heat shock response before squashing and staining of their polytene chromosomes. The chromosomes were photographed and mapped as described in the legend to Fig. 1. (*A*) The phase-contrast picture of chromosome arm 3R, where the heat shock puffs at loci 87A, 87C, 93D, and 95D are clearly visible. (*B*) The fluorescence picture of the same chromosome shown in *A*, stained using mAb Y1D2. (△) Puffs lacking PEP-specific staining; (▲) symbols mark puffs displaying significant PEP-specific staining. (*C*) The phase-contrast picture of chromosome arm 3L, where the heat shock puffs at loci 63BC, 64F, 67B, and 70A are clearly visible. (*D*) The fluorescence picture of the same chromosome shown in *C*, stained using mAb Y1D2. Symbols denote the level of fluorescence due to PEP-specific staining, as described in the legend to *B*.

probed with the same *Pep* cDNA fragment; the treatments had no effect on *Pep* gene expression (not shown).

The PEP antigen contains numerous motifs suggesting nucleic acid interactions

For sequence analysis, the 2.7-kb cDNA was subcloned into the BlueScript phagemid (Stratagene) in two overlapping pieces, one 2.5-kb *Hind*III 5′-terminal fragment and one 300-bp *Eco*RI 3′-terminal fragment. The nucleotide sequence was determined on each strand using nested deletions; an average of six readings per base pair position was compiled. The amino acid sequence derived

from the primary nucleotide sequence predicts a molecular mass of 77,942 daltons for PEP (Fig. 7A), rather than the 110-kD figure deduced from its electrophoretic mobility. The discrepancy may arise as a consequence of the highly charged domains found in the sequence (see below) and/or PEP may be modified post-translationally. Because both ends of the clone contain only closed reading frames, the clone probably covers the entire protein-coding sequence, that is, to the amino terminus of the protein, but does not necessarily extend to the transcription start site (Hultmark et al. 1986). Several potential translation start sites are found within the first 200 nucleotides of open reading frame; of these, only the first
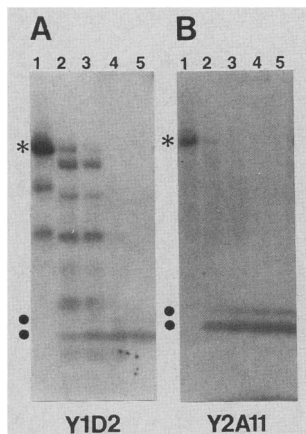
Figure 3. Epitope mapping of mAbs Y1D2 and Y2A11. *Drosophila* nuclear extract was digested with increasing amounts of trypsin, and identical samples were fractionated by electrophoresis in an SDS–polyacrylamide gel; the polypeptides were transferred to nitrocellulose and probed with either TBS (not shown), mAb Y1D2, or mAb Y2A11. (*A*) Western blot analysis of the polypeptides probed with mAb Y1D2. (Lane *1*) *Drosophila* nuclear proteins; (lanes *2–4*) the same proteins digested for 45 min with 10, 25, or 50 μg of trypsin/ml; (lane *5*) trypsin alone (50 μg/ml). (*) The position of the nuclear PEP antigen; (●) two nonspecific signals that may arise from the excess of trypsin in these samples. (*B*) A duplicate Western blot analysis using mAb Y2A11.

and in some RNA-binding proteins (Miller et al. 1985; Joho et al. 1990). The PEP fingers are distinctive in possessing five amino acid residues between the histidine residues rather than four (Fig. 7B), a motif found predominantly in *Drosophila* finger proteins (Wingender 1988); they are not of the type found in nuclear hormone receptors (Wingender 1988). The spacing of the fingers in PEP is unusual; most repetitive fingers in other proteins are separated by only 7–10 amino acid residues (Berg 1988; Lee et al. 1989).

3. The segment from residues 396–630 in the PEP protein spans two exceptionally acidic regions (boxed in Fig. 7A), which surround a fourth zinc finger of the type mentioned above (double underline in Fig. 7A). Acidic region 1 (79 residues in length) possesses 24 glutamic acid (30%) and 11 aspartic acid (14%) residues, with a net charge of -31. Immediately preceding acidic region 1 there is a basic sequence, Lys-Arg-Lys-Lys-Lys-Pro-Val (overlined in Fig. 7A) similar to the nuclear localization signal found in the SV40 large T antigen, Pro-Lys-Lys-Lys-Arg-Lys-Val (Dingwall and Laskey 1986). Throughout most of this acidic region, alanine residues appear every five to six residues. Zinc finger 4 occurs midway between the acidic regions. Acidic region 2 (90 residues in length) possesses 34 glutamic acid (38%) and 9 aspartic acid
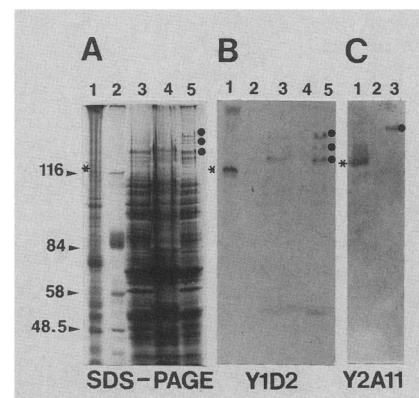
two, centered at nucleotides 218 and 297, possess flanking sequences that conform to the *Drosophila* start site consensus sequence, (A/C)AA(A/C)AUGG (Cavener 1987). The scanning model for translation (Kozak 1989) would suggest that the first AUG in the sequence, which represents a poor, but acceptable, match to the consensus, is likely to be the initiator codon for synthesis of PEP; however, the second site, in phase with the first, presents a perfect match to the consensus sequence and might be used. It is possible that multiple sites are functional, as has been shown for the glucocorticoid receptor (Miesfeld et al. 1986).

A number of interesting motifs are discernible within the deduced sequence for PEP:

1. The amino terminus of the protein consists of a region 166 amino acid residues in length, of which 40 residues (24%) are glycine and 33 residues (20%) are asparagine (both underlined in Fig. 7A). This segment is reminiscent of the carboxy-terminal glycine-rich regions that exist in the rat hnRNP A1 protein (Cobianchi et al. 1986), the *Drosophila* Hrb98DE protein (Haynes et al. 1990), and nucleolin from several species (Caizergues-Ferrer et al. 1989), proteins all known to bind to RNA.

2. A second putative nucleic-acid binding segment, commencing with amino acid 215, contains three zinc finger motifs (double underline in Fig. 7A) of the X_2-Cys-X_2-Cys-X_{10-12}-His-X_5-His type, motifs found in many eukaryotic transcriptional activators (Johnson and McKnight 1989; Struhl 1989; Berg 1990)

Figure 4. Western blot analysis of fusion proteins produced in λgt11 lysogens. The 1.8-kb *Drosophila* DNA fragment identified from a λgt11 expression library with mAb Y1D2 was used to produce an IPTG-inducible β-gal/PEP fusion protein in *E. coli* strain Y1089. A similar λ lysogen containing the gene for a fusion protein with the unrelated *Drosophila* heterochromatin protein HP1 (James and Elgin 1986) was used as a control. (*A*) SDS–polyacrylamide gel electrophoresis of *Drosophila* nuclear proteins (lane *1*), molecular weight markers (lane *2*), uninduced PEP lysogen proteins (lane *3*), induced HP1 lysogen proteins (lane *4*), and induced PEP lysogen proteins (lane *5*), stained with Coomassie blue. The sizes of the molecular mass markers are given in kilodaltons (*left*), asterisks mark the position of the nuclear PEP protein, and dots mark the positions of induced fusion proteins. (*B*). Western blot analysis of the proteins in the gel in *A*, probed with mAb Y1D2. (*C*) Western blot analysis of *Drosophila* nuclear proteins (lane *1*), molecular mass markers (lane *2*), and proteins from the induced PEP lysogen (lane *3*), using mAb Y2A11.

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Figure 5. Localization of the *Pep* gene on poly-
tene chromosomes. Polytene chromosomes
from third-instar larvae were squashed onto a
microscope slide in acetic acid and probed with
the 2.5-kb *Hind*III fragment from the 2.7-kb
cDNA clone. The only signal on the chromo-
somes was mapped to the proximal portion of
the ecdysone-responsive puff at locus 74F, by
reference to the designations of Lefevre (1976).
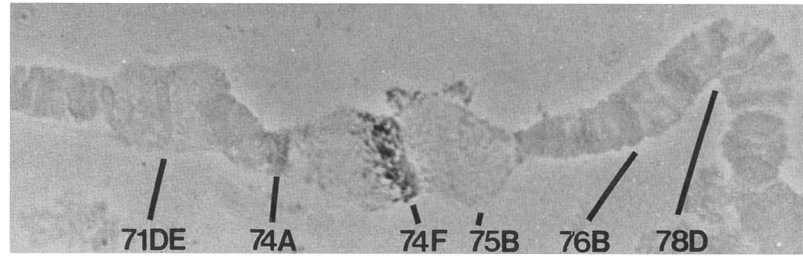
(10%) residues, with a net charge of −43; interest-
ingly, a second stretch of basic residues, Arg-Lys-Lys-
Arg-Lys-Val, (overlined in Fig. 7A) resides next to
acidic region 2, in a position analogous to the basic
sequence preceding acidic region 1. Several functions
have been observed for highly acidic domains in other
chromosomal proteins (Earnshaw 1987), including
transcriptional activation (Ptashne 1988), recognition
of histones for polyubiquitination (Sung et al. 1988),
or nucleosome assembly/disassembly (Dingwall et al.
1987).

4. The carboxy-terminal 80 amino acids of PEP are rich
in alanine (34%), proline (24%), and serine and thre-
onine (15% combined).

5. The *Pep* cDNA clone contains 377 nucleotides be-
yond the first termination codon, ending in the se-
quence AAUAAA, the consensus polyadenylation
signal (Proudfoot and Whitelaw 1988). Because the
cDNA library was constructed by primer extension
from the poly(A) tails of mRNAs, the existence of this

canonical sequence at the very end of the clone,
rather than 20–30 nucleotides upstream, suggests
that the clone arose by priming from an adjacent A/
T-rich sequence. Within the 3′-untranslated sequence
is found a nearly perfect match to a 450-bp genomic
sequence reported previously from locus 74F; the ge-
nomic sequence was reported to hybridize to a 2.8-kb
ecdysone-inducible transcript in KC cells (Moritz et
al. 1984). That genomic sequence contains 29 nucle-
otides not found in our cDNA, suggesting strain-spe-
cific polymorphism. The 74F genomic sequence was
cloned from Canton-S flies, while the cDNA library
used here was prepared from the Oregon-R strain. The
genomic sequence also contains an A-rich stretch
downstream of the AAUAAA motif, which may have
served as the start of the primer extension reaction
during construction of the cDNA library. The overlap
with the 74F sequence and the identity of the tran-
script sizes suggest that the gene fragment reported
by Moritz et al. (1984) is from the gene we have iso-
lated. Thus, from the prior localization of the 74F
sequence on the chromosomal walk from this region
(Thummel et al. 1990), we know that the *Pep* gene
probably resides 15–20 kb in the proximal direction
from the ecdysone-responsive E74 gene.

6. It is interesting to note the linear duplication of seg-
ments in the carboxy-terminal half of PEP, that is, the
repetition of zinc finger, nuclear localization signal,
and acidic domain. A search of Genbank version 63
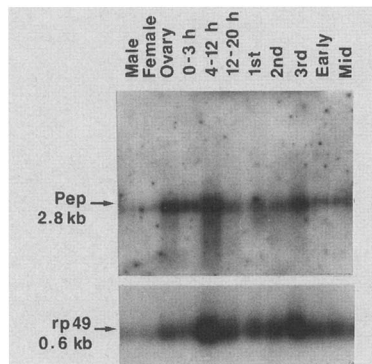for proteins found that the PEP sequence is unique.

Figure 6. Northern blot analysis of PEP expression during de-
velopment. Samples of poly(A)+ RNA (1 μg) from the stages
indicated (adult males and female carcasses, ovaries, 0- to 3-hr
embryos, 4- to 12-hr embryos, 12- to 20-hr embryos, first-instar
larvae, second-instar larvae, third-instar larvae, early pupae, and
mid-stage pupae) were fractionated electrophoretically and
transferred to a nylon membrane as described by Haynes et al.
(1990). The RNA was probed with a labeled 2.5-kb *Hind*III frag-
ment from *Pep* cDNA. The size of the *Pep* transcript was de-
duced by comparison to RNA size markers. The loading con-
trols for this filter represent transcripts from the ribosomal pro-
tein gene *rp49* (O'Connell and Rosbash 1984) and indicate
overloading of the samples from 4- to 12-hr embryos and third-
instar larvae.

Discussion

The chromosomal distribution of the PEP antigen sug-
gests a tight correlation between the presence of this
protein and transcription at a defined subset of active
loci. The pattern changes with changes in the develop-
mental program or following heat shock. The pattern is
more selective than others reported at active loci [topoi-
somerase I (Fleischmann et al. 1984), RNA polymerase II
(Sass 1982; Weeks et al. 1982), hnRNP proteins (S.A.
Amero and A.L. Beyers, unpubl.), and snRNP proteins
(Sass and Pederson, 1984; S.A. Amero et al., in prep.)], as
staining does not appear at most intermolt puffs or in-
terbands, or at certain heat shock-induced puffs. The
strong correlation between PEP and active hormonally
regulated loci argues for a role for the protein in the
process of ecdysone-mediated transcriptional activation.

A 1
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       ►
       GGTTCGGATCGGAGCGTAATATTGGATAGGCGGAGTGTTTAGACAAGGGCGGCCATCTTTCTGAAGTGAGGAAAACTGTAGTCCAAGTAGTCCAGTCATT

 101   TTGACAAATCGCCGTTGCAACACCTTGTTTAAATAATTTCCTCGCTTAAACACAAACCCTACATTACACATCCCATAAACACTCCCAAAAACAGCAAAAAAA

                M  V  S  V  K  V  N̲  G̲  N̲  P  Q  N̲  R  L  V  N̲  N̲  A  K  V  N̲  G̲  N̲  M  A  F  R  G̲  N̲
 203   AAACCCATCAAAATTATGGTCAGCGTAAAGGTCAACGGAAATCCGCAGAATCGTTTAGTGAATAACGCAAAGGTCAACGGCAACATGGCTTTCCGTGGTAAC

        Q  N̲  R  N̲  R  N̲  F  G̲  G̲  G̲  N̲  N̲  N̲  Y  G̲  G̲  P  M  G̲  A  N̲  R  M  G̲  G̲  M  N̲  M  S  P  W  E  S  Q
 305   CAGAACCGCAACCGCAATTTTGGAGGTGGCAACAACAACTATGGTGGACCCATGGGCGCCAACCGCATGGGCGGTATGAACATGTCGCCTTGGGAATCGCAG

        N̲  P  G̲  G̲  G̲  Q  F  G̲  N̲  N̲  M  R  Q  G̲  G̲  G̲  Q  M  N̲  A  Q  A  I  N̲  L  A  N̲  N̲  L  L  N̲  N̲  L  F
 407   AATCCCGGCGGCGGACAGTTTGGTAACAACATGCGACAGGGTGGCGGTCAGATGAACGCCCAGGCCATCAACTTGGCCAACAATCTGCTGAACAATCTGTTC

        R  N̲  Q  N̲  P  P  S  L  L  D  L  P  R  G̲  G̲  G̲  G̲  M  G̲  N̲  R  N̲  Q  R  G̲  G̲  P  M  V  S  R  G̲  G̲  G̲
 509   AGGAACCAGAATCCACCATCGCTTCTCGACTTGCCCCGCGGCGGCGGTGGCATGGGAAATCGCAACCAACGTGGTGGCCCGATGGTCAGTCGCGGCGGCGGT

        A  G̲  N̲  R  L  N̲  N̲  R  R  G̲  Q  G̲  G̲  G̲  F  Q  N  R  G̲  A  T  G̲  S  G̲  P  K  P  P  P  K  Q  G̲  G̲  G̲
 611   GCCGGCAATCGTCTCAATAACCGTCGTGGCCAGGGAGGTGGCTTCCAAAATCGTGGCGCCACTGGTAGTGGACCCAAGCCCCCGCCAAAGCAGGGCGGCGGC

        G̲  I  R  K  Q  N  A  F  D  R  A  K  K  L  L  A  K  N  A  N  H  K  K  K  E  P  T  P  G  E  K  K  I  E
 713   GGTATTCGCAAGCAAAATGCTTTTGATCGTGCCAAGAAACTTTTGGCTAAAAATGCCAACCATAAAAAGAAGGAACCCACTCCTGGCGAAAAGAAAATCGAG

        S  P  T  K  E  S  P  Y  A  S  V  P  N  D  M  F̲̲  Y̲̲  C̲̲  H̲̲  L̲̲  C̲̲  K̲̲  K̲̲  H̲̲  M̲̲  W̲̲  D̲̲  A̲̲  N̲̲  S̲̲  F̲̲  E̲̲  N̲̲  H̲̲
 815   AGCCCTACCAAGGAGTCTCCATACGCTAGTGTGCCGAACGACATGTTCTACTGTCATCTGTGCAAGAAGCACATGTGGGATGCCAACTCTTTCGAGAACCAC

        I̲̲  K̲̲  G̲̲  R̲̲  T̲̲  H̲̲  L  M  M  R  E  G  I  E  E  S  Y  R  L  K  A  N  M  I  R  Q  E  A  K  I  A  E  Q  L
 917   ATCAAGGGCCGCACCCATCTGATGATGCGTGAGGGCATTGAGGAGAGCTATCGCCTCAAGGCCAACATGATCCGTCAGGAGGCTAAGATCGCAGAGCAGCTC

        K  S  I  E  F  D  R  L  K  R  M  G  K  S  K  Q  R  Q  L  D̲̲  Y̲̲  C̲̲  T̲̲  M̲̲  C̲̲  D̲̲  L̲̲  N̲̲  F̲̲  H̲̲  G̲̲  H̲̲  I̲̲  S̲̲
1019   AAGTCGATCGAGTTTGACCGCTTGAAGCGCATGGGCAAAAGCAAGCAGCGTCAGCTGGACTACTGCACCATGTGCGACCTGAACTTCCATGGTCACATCTCG

        T̲̲  H̲̲  R̲̲  K̲̲  S̲̲  E̲̲  G̲̲  H̲̲  L  Q  L  K  K  F  L  H  P̲̲  K̲̲  C̲̲  I̲̲  E̲̲  C̲̲  N̲̲  K̲̲  E̲̲  F̲̲  A̲̲  T̲̲  R̲̲  I̲̲  D̲̲  Y̲̲  D̲̲  T̲̲
1121   ACCCATCGCAAGTCGGAGGGACATTTGCAGCTGAAGAAGTTCTTGCACCCCAAGTGCATTGAGTGCAACAAGGAGTTCGCCACTCGCATTGACTACGATACT

        H̲̲  L̲̲  L̲̲  S̲̲  A̲̲  E̲̲  H̲̲  L  K  K  A  A  E  N  N  T  K  V  G  E  R  K  R  Q  T  L  P  I  S  T  E  E  E  E
1223   CATCTGCTGTCCGCCGAGCATCTGAAGAAGGCTGCCGAGAACAACACCAAGGTGGGTGAGCGCAAGCGCCAGACCTTGCCCATCAGCACCGAAGAAGAGGAG

        T  R  D  L  R  L  P  Q  K̲̲  R̲̲  K̲̲  K̲̲  K̲̲  P  V  K  K │ E  G  E  A  A  D  G  E  A  K  K  E  G  A  G  D  G
1325   ACCCGCGATTTGCGCCTGCCCCAGAAGCGCAAGAAGAAGCCGGTCAAGAAGGAGGGCGAAGCAGCCGATGGCGAGGCTAAGAAGGAGGGTGCCGGCGATGGC

      │ E  G  A  E  G  D  E  A  G  E  E  A  K  E  G  E  E  A  A  D  E  T  K  E  G  D  E  L  N  E  S  Q  E
1427   GAGGGTGCCGAAGGTGATGAAGCCGAGGGAGAAGAGGCCAAGGAGGGTGAGGAAGCTGCTGACGAGACAAAGGAGGGCGATGAGCTAAACGAGAGCCAAGAG

      │ E  E  E  V  A  L  P  V  D  P  E  D  C  I  L  D  F  N  D  G  D  E  I  P  S  E  V  D │ T  R  L  P  K  Y
1529   GAAGAGGAAGTGGCACTGCCCGTGGACCCCGAGGACTGCATCCTTGACTTCAACGACGGCGATGAGATCCCCAGCGAGGTGGATACCCGCCTACCTAAGTAC

        N  W  Q  R  A  V  G  P  G  L  I  S  K  L  E  C  Y̲̲  E̲̲  C̲̲  S̲̲  V̲̲  C̲̲  S̲̲  K̲̲  F̲̲  F̲̲  D̲̲  T̲̲  E̲̲  V̲̲  T̲̲  A̲̲  E̲̲  I̲̲
1631   AACTGGCAGCGTGCTGTCGGTCCCGGTCTGATCTCCAAGCTGGAGTGCTATGAGTGCTCGGTGTGCAGCAAGTTCTTCGACACCGAGGTGACCGCCGAGATT

        H̲̲  S̲̲  R̲̲  T̲̲  A̲̲  T̲̲  H̲̲  H  R  N  F  L  K  F  I  N  E  K  S  S  D  T  K  I  A  Q  K  R  A  A  A  A  L  E
1733   CACTCCCGTACGGCGACTCATCACCGCAACTTCTTGAAGTTTATCAACGAGAAATCAAGCGATACCAAGATCGCACAGAAGCGCGCAGCTGCCGCCCTGGAG

        E  N  E  R  K̲̲  K̲̲  R̲̲  K̲̲  V │ E  E  A  E  A  P  A  A  E  G  A  A  E  E  T  T  E  G  A  E  G  E  L  Y  D
1835   GAGAATGAGCGTAAGAAGCGCAAGGTTGAGGAGGCAGAGGCTCCGGCTGCCGAGGGCGCAGCCGAGGAAACCACCGAAGGCGCTGAGGGTGAGCTGTACGAT

      │ P  S  E  A  T  G  D  D  E  D  V  E  M  V  D  D  N  A  E  G  E  G  E  G  E  G  D  E  E  A  E  A  E  V
1937   CCATCGGAAGCCACTGGCGATGACGAGGACGTGGAGATGGTGGATGATAATGCTGAGGGCGAGGGCGAAGGCGAAGGTGACGAGGAAGCCGAGGCTGAGGTC

      │ E  E  D  G  A  G  Q  D  N  G  E  E  E  M  E  A  Q  E  E  E  G  Q  E  G  E  Q  E  P  E  P  E │ P  A  P
2039   GAGGAAGATGGCGCCGGCCAGGACAACGGCGAAGAGGAGATGGAAGCCCAGGAGGAAGAGGGTCAGGAGGGCGAGCAAGAGCCCGAACCTGAGCCAGCTCCA

        V  Q  T  P  A  P  A  E  P  A  P  P  A  K  T  P  A  K  T  P  T  K  A  A  A  P  A  A  V  A  S  P  A  A
2141   GTCCAAACTCCTGCCCCGGCTGAACCTGCACCACCAGCCAAGACCCCAGCCAAGACTCCGACCAAGGCAGCTGCTCCAGCCGCTGTTGCCAGTCCCGCAGCT

        A  A  T  S  A  D  A  S  P  S  P  A  K  K  A  T  P  A  R  A  A  A  G  A  K  A  T  P  Q  R  Q  R  A  R
2243   GCGGCAACGTCGGCAGACGCCCTCTCCATCTCCGGCCAAGAAGGCAACGCCTGCTCGCGCTGCCGCCGGAGCCAAGGCCACGCCGCAGCGACAACGCGCCCGC

        G  R  Y  N  R  Y                                        ◄
2345   GGTCGCTACAATCGCTACTAAGTAGACGTCGAAGGAATTCGTAATTTAACATAACATAAACCTTAATATACAAGAAAAAAATTTACATTCACGCCCAGGAGGA
```

Figure 7. (*See following page for legend.*)

2447 GGATGAAGAAAGAACAGATCAAATCGATCTCTTCCCGCAATTTGACATTTGCATCCCAATTTGCATATACACTGAATGAATGGAAACTGAGAAGAAGACGGT

2549 CAACTTTTAGTTGAAACAGTTGAATATATTGTACGACGAAGCTTCTTGAAACTGACGAGGGAGAACACAAACAAGTTAATTGAATCGAGAATCGTATTTCCT

2651 ACCCGATTCCCCCCTCACAGAACGCATACCATGCCAGCCAGCGAACCAACCATTCAACTTATGTAATCATATAAATTCTATAATAAAC

B PEP FINGER MOTIFS:

FYCHLCKKHMWDANSFENHIKGRTH

DYCTMCDLNFHGHIST--HRKSEGH

PKCIECNKEFATRIDYDTHLLSAEH

YECSVCSKFFDTEVTAEIHSRTATH

DROSOPHILA CONSENSUS:

```
                           L
F . C . . C . K . F . . . . . F . . H .   . . . H
                           Y
```

Figure 7. Sequence of the *Pep* cDNA and protein. (*A*) The sequence of the 2.7-kb cDNA from the *Pep* gene was compiled using the ODBUTIL program (Staden 1980) and translated using the DM program (Mount and Conrad 1986). Within the deduced protein sequence are found the following motifs: a glycine (G)- and asparagine (N)-rich segment (glycines and asparagines are underlined), four zinc finger motifs (double-underlined), two acidic regions (boxed areas), two nuclear localization-type signals (overlined), and an alanine (A)- and proline (P)-rich carboxyl terminus. The overlap with the 74F genomic sequence (Moritz et al. 1984) is underlined in the 3'-untranslated *Pep* cDNA sequence. The probe used for the in situ hybridization and Northern blot analyses is indicated between the arrowheads. (*B*) Alignment of zinc finger motifs in PEP with the consensus finger sequence from *Drosophila* proteins (Harrison and Travers 1990). The conserved pairs of cysteine (C) and histidine (H) residues are shaded and underlined.

The additional sites in the PEP distribution pattern suggest a function(s) for the protein in other activation systems as well.

Our observations complement the regulatory model proposed by Ashburner and his colleagues to accommodate the salient features of the larval/prepupal ecdysone system (Ashburner et al. 1974; Ashburner 1990). The model suggests that the genes within the early puffs are directly activated by ecdysone in conjunction with the ecdysone receptor. Activation of the early puffs does not require de novo protein synthesis but is strictly dependent on the presence of hormone (Ashburner 1974). Several of the early genes have been cloned and shown to respond directly to ecdysone (Burtis et al. 1990; Segraves and Hogness 1990; Thummel et al. 1990), as expected. Our finding of PEP on the early puffs, in the early puffing stages, introduces an additional factor to this process and suggests that PEP is recruited into early gene activation or expression. Second, in the Ashburner model, activation of the late genes is proposed to involve the products of the early genes (Ashburner 1974); this process is dependent on de novo protein synthesis. This aspect of the model has been confirmed recently, since the E74 protein, the product of the E74 early gene, has been localized on the late puffs (Urness and Thummel 1990). Our studies add a new component to this level of ecdysone-mediated gene expression as well, because PEP is not the product of a classic early gene but is found on the late puffs. Thus, the evidence suggests that PEP is involved in ecdysone-mediated gene expression of both the early and late genes in the Ashburner model, and that PEP binding at these sites is regulated, perhaps indirectly, by ecdysone.

The known properties of the *Pep* gene and its protein product—its pattern of expression, sequence motifs, and recruitment to active complexes—would be expected of a "coactivator" transcription factor, as described by Lewin (1990), a protein that adapts the transcriptional apparatus to specific induction mechanisms, in this case, the ecdysone induction mechanism. In this scenario,

PEP may communicate between the basal transcription factors and the ecdysone receptor, or early gene products, using zinc fingers to contact specific DNA sequences and acidic domains for protein–protein contacts. The protein may perform a similar function in other activation systems, possibly at the minor sites in the PEP distribution pattern. Our observations to date—the tight coupling with transcription, intense staining within a puff, and certain sequence motifs—are also consistent with the possibility that PEP is a component of a ribonucleoprotein complex, and we note the ability of the transcription factor IIIA, also a zinc finger protein, to contact both DNA and RNA (Miller et al. 1985). In this context, PEP may communicate between the hormonally activated transcription complexes, containing the ecdysone receptor or the early gene products, and the RNA packaging and processing machinery, containing the transcripts of the early and late genes. It will clearly be exciting to explore further the functions of this unique protein.

Methods and materials

Generation of monoclonal antibodies

Drosophila melanogaster (Oregon-R) strains were maintained as a laboratory population in a 25°C growth chamber, as described by Elgin and Miller (1977). Embryos collected at staged intervals were frozen and stored at −80°C. Nuclei were isolated according to the procedures described by Mayfield et al. (1978) including centrifugation through a sucrose cushion. Nuclear proteins from ~50 grams of embryos (6–12 hr) were extracted by homogenization in a Thomas size B homogenizer in 0.2 mM EDTA (pH 8.0), and preboiled RNase A was added to a final concentration of 1 μg/ml. The suspension was incubated at 37°C for 30 min; nuclei were then pelleted by centrifugation at 16,500g in a Sorvall HB-4 rotor for 10 min at 4°C. All buffers used for homogenizing embryos, isolating and digesting nuclei, and extracting nuclear proteins contained 5 μg leupeptin/ml, 0.2 Ti units aprotinin/ml, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (all from Sigma) to inhibit endogenous proteases. The supernatant containing EDTA-soluble nuclear pro-

teins was dialyzed against 3 liters of 0.2 mM EDTA (pH 8.0), 0.1 mM PMSF for 3 hr in the cold room and lyophilized.

Nuclear proteins were fractionated in Triton/acetic acid/ urea–polyacrylamide gels, and proteins in gel bands were used for immunization of BALB-c mice as described by Amero et al. (1988). Fusions of spleen cells and myeloma cells, production of hybridoma cell lines, and screening for positive hybridomas were conducted as described by James and Elgin (1986), following the procedures of Galfré and Milstein (1982).

Epitope mapping and Western blot analyses

Peptide mapping experiments were conducted by incubating 120 μl of EDTA nuclear extract in the presence of increasing concentrations of trypsin (Boehringer Mannheim Biochemicals), either 10, 25, or 50 μg/ml in 125 mM Tris-HCl (pH 6.8), 1% SDS, and 7.5% glycerol (after the procedure of Cleveland et al. 1977) at 37°C for 45 min. The digestions were stopped by the addition of SDS to a final concentration of 2% and of β-mercaptoethanol to 166 mM; the samples were boiled, and identical aliquots were loaded onto 12% SDS–polyacrylamide gels (30% : 0.4%), as described by Laemmli (1970). For Western blot analyses, proteins were transferred from gels to nitrocellulose filters and probed as described by James and Elgin (1986). Clarified ascites fluid diluted 1 : 20 with blocking solution was used as the primary antibody, and ^{125}I-labeled anti-mouse secondary antibody [F(ab')$_2$] fragment (Amersham Corp.) was used at a dilution of 1 : 500 in blocking buffer. The filters were exposed overnight to preflashed Kodak X-Omat film at −80°C with an intensifying screen.

Screening cDNA libraries

The λgt11 cDNA expression library, subcloned from the original λgt10 library at the EcoRI sites (M. Philip and D. Brutlag, pers. comm.), was screened with mAb Y1D2, using essentially the procedure of Huynh et al. (1985), as described by James and Elgin (1986). However, repeated screening attempts suggested an instability of the Pep clone in this vector, so the primary screen was performed at a dilution of 9×10^5 plaques on ten 150-mm Luria broth (LB)/ampicillin plates, using Escherichia coli strain Y1088. Twenty-five positive plaques from the primary screen were rescreened by Western blot analysis of the proteins produced in a high-titer 2-ml minilysate of E. coli strain Y1088 grown at 37°C in the presence of IPTG for 6 hr (Silhavy et al. 1984); three isolates produced positive signals. One phage with a 1.8-kb insert was chosen for further analysis.

A β-galactosidase–PEP fusion protein was expressed in E. coli strain Y1089 lysogenized with the phage insert, following the procedures of Huynh et al. (1985). Following temperature shock and induction with IPTG, the bacterial cells (from 100-ml cultures) were collected by centrifugation, resuspended in 10 mM Tris-HCl (pH 7.5), and frozen in liquid nitrogen. For protein analysis, the samples were fractionated in a 7% SDS–polyacrylamide gel for Western blot analysis.

The 1.8-kb Pep insert was gel-purified by GeneClean (BIO 101), labeled with [α-^{32}P]dATP (Amersham Corp.) by random priming (Feinberg and Vogelstein 1983), and used to screen a second cDNA library from 8- to 12-hr embryos (Brown and Kafatos 1988). One-tenth the complexity of the library (and therefore 3×10^4 colonies) was screened, and seven positive clones were isolated. The inserts in the pNB40 vector ranged in size from 1.7 to 2.7 kb; the clone containing the 2.7-kb insert, designated p3221, was chosen for further analysis.

In situ hybridization

Salivary glands were dissected from third instar larvae in 45% acetic acid, and polytene chromosomes were squashed onto subbed microscope slides as described by Pardue (1986). The 2.5-kb HindIII fragment from p3221 was isolated by GeneClean (BIO 101), labeled with biotin–UTP (Bethesda Research Laboratories) by random priming (Feinberg and Vogelstein 1983), and hybridized as described by Pardue (1986). The signal was visualized using the DNA Detection System from Bethesda Research Laboratories.

Northern blot analysis

The developmental Northern filter reported by Haynes et al. (1990) was reprobed with the 2.5-kb HindIII fragment from the Pep cDNA.

The fragment was purified from agarose gels by GeneClean (BIO 101) and labeled with [α-^{32}P]dATP (Amersham Corp.) by random priming (Feinberg and Vogelstein 1983). A total of 1×10^9 cpm was included in the hybridization, performed in the solutions recommended by Maniatis et al. (1982). The hybridization reaction was allowed to incubate for 24 hr at 65°C.

The filter was washed twice in 2× SSC, 0.1% SDS, at room temperature, and twice in 0.2×SSC, 0.1% SDS, at 60°C. The filter was air-dried for 15 min, covered with plastic wrap, and exposed to Kodak X-Omat AR film at −80°C with an intensifying screen.

Sequence analysis

The insert in p3221 was subcloned in two pieces into the BlueScript phagemid for sequence determination. One 2.5-kb HindIII fragment covers the 5′ end of the gene, extending into the pNB40 vector, and one 300-bp EcoRI fragment overlaps ~50 bp of the HindIII fragment and extends to the EcoRI site 20 bp into the vector. These insert fragments were gel-purified by GeneClean (BIO 101) and cloned into the appropriate sites of BlueScript Plus phagemid (Stratagene, La Jolla, CA) in E. coli BB4 strain. Each orientation of each insert was isolated.

A series of nested deletions in each strand was created using exonuclease III and S1 nuclease (both from Boehringer Mannheim Biochemicals) for both the p3221 and λgt11 plasmids. Prior to ligation with T4 DNA ligase (Boehringer Mannheim), the ends of the deletions were filled in using Klenow fragment (Bethesda Research Laboratories) and nucleoside triphosphates. VCS-M13 helper phage for making single-stranded templates, dideoxynucleotides, primers, and Sequenase enzyme for sequencing reactions were all obtained from Stratagene. dATP labeled with ^{35}S was purchased from Amersham. Several primers were synthesized by the University of Virginia Protein and Nucleic Acid Research Facility.

The contiguous sequences were assembled, and consensus sequences were computed using the ODBUTIL program (Staden 1980). The consensus sequence was translated into protein sequence using the DM program (Mount and Conrad 1986). The PEP protein sequence was compared to Genbank using the FASTP program (Pearson and Lippman 1988). The sequences obtained for the λgt11 and the p3221 inserts are identical, except for the presence of 58 bp on the amino-terminal side of the λgt11 insert, which is missing from the p3221 insert.

Immunofluorescence assays

Immunofluorescence analyses of polytene chromosomes were conducted by the methods of Silver and Elgin (1977), as modi-

fied by James and Elgin (1986). Polytene chromosomes were squashed in a 45% acetic acid–3.7% formaldehyde fixative solution. Antibody Y1D2 was used as a neat hybridoma supernatant; affinity-purified goat antibodies specific for the carboxy-terminal domain of the largest subunit of RNA polymerase II (Fisher et al. 1989) were diluted 1 : 100 in blocking buffer. Anti-mouse FITC-conjugated antibody and anti-goat FITC-conjugated antibody were both obtained from ICN Immunobiologicals and diluted 1 : 1000 and 1 : 300, respectively. For restaining, the coverslips were flipped off the chromosomes with a razor blade, and the slides were washed three times in TBS at room temperature.

The chromosomes were photographed through a Leitz Orthomat fluorescence microscope using Kodak Tri-X film. Pictures were printed using high-contrast filters on Kodak Polycontrast paper. Developmental stages were determined by comparison of the puffing patterns to those in Table 1 of Ashburner and Berendes (1978).

Acknowledgments

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S A Amero, S C Elgin and A L Beyer

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