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A Unique Ribonucleoprotein Complex Assembles Preferentially on Ecdysone-Responsive Sites in *Drosophila melanogaster*

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The protein on ecdysone puffs (PEP) is associated preferentially with active ecdysone-inducible puffs on *Drosophila* polytene chromosomes and contains sequence motifs characteristic of transcription factors and RNA-binding proteins (S. A. Amero, S. C. R. Elgin, and A. L. Beyer, Genes Dev. 5:188–200, 1991). PEP is associated with RNA in vivo, as demonstrated here by the sensitivity of PEP-specific chromosomal immunostaining in situ to RNase digestion and by the immunopurification of PEP in *Drosophila* cell extract with heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. As revealed by sequential immunostaining, PEP is found on a subset of chromosomal sites bound by the HRB (heterogeneous nuclear RNA-binding) proteins, which are basic *Drosophila* hnRNPs. These observations lead us to suggest that a unique, PEP-containing hnRNP complex assembles preferentially on the transcripts of ecdysone-regulated genes in *Drosophila melanogaster* presumably to expedite the transcription and/or processing of these transcripts.

Developmentally regulated gene expression in *Drosophila melanogaster* is visible cytologically as a predictable series of puffing patterns on the giant polytene chromosomes found in third-instar larval salivary glands (6). This gene activity is regulated primarily at the level of transcription by the steroid hormone ecdysone (7, 20), culminating in metamorphosis (44). As originally predicted by Ashburner and colleagues (5, 7), the major regulatory proteins in the ecdysone cascade appear to be ecdysone-regulated transcription factors (9, 12, 14, 16–18, 22, 42, 51, 52), but unforeseen accessory factors also are necessary for the proper function of certain isoforms of the ecdysone receptor (25, 55). Central to the transcriptional cascade model is the accurate temporal appearance of these major regulatory factors (3, 5, 50), which is controlled by the threshold responses of the different regulatory genes to different titers of ecdysone (23) and by these factors' appropriate combinatorial interactions with the ecdysone-inducible genes (19).

The *Drosophila* protein on ecdysone puffs (PEP) binds preferentially to the subset of chromosomal sites that are activated by ecdysone, as visualized by chromosomal immunostaining (1). Within the PEP polypeptide sequence exist multiple, potential nucleic acid-binding motifs and putative protein-protein interaction domains that might be expected for an accessory regulator in the hormone cascade (1), but the binding characteristics and function of the protein are not known. Our new observations show that PEP associates with RNA at chromosomal ecdysone puffs to form transcript-specific heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. No other known protein possesses the characteristic of being an hnRNP found preferentially on coordinately regulated genes. We suggest that PEP may function preferentially in the generation or maturation of transcripts from ecdysone-induced genes.

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MATERIALS AND METHODS

Immunofluorescence assays. Immunofluorescence analyses of polytene chromosomes were conducted by the methods of Silver and Elgin (46), as modified by James and Elgin (21) and Amero et al. (1). Salivary glands were dissected from third-instar larvae in Cohen and Gotchell medium G containing 0.5% Nonidet P-40 and incubated in a formaldehyde fixative solution; chromosomes were then squashed in 45% acetic acid (4). Hybridoma cell supernatant containing monoclonal antibody (MAb) Y1D2 was incubated on slides for 2 h at 23°C, the slides were rinsed in Tris-buffered saline (TBS) (50 mM Tris-HCl [pH 7.6], 150 mM NaCl) containing 0.05% Tween 20, and fluorescein isothiocyanate-conjugated goat anti-mouse Ab (ICN Immunobiologicals) diluted 1:1,000 in TBS was subsequently incubated on the slides for 1 h at 23°C. The chromosomes were photographed through a Leitz Orthomat fluorescence microscope using Kodak Tri-X film. For sequential stainings, the chromosomes were washed with TBS containing 0.05% Tween 20 and incubated with HRB-specific rabbit antiserum diluted 1:500 in blocking buffer. The rabbit Abs were detected by using rhodamine-conjugated sheep anti-rabbit Ab (ICN Immunobiologicals) diluted 1:300 in TBS, and the photography was repeated with different optics. Control experiments confirmed that these secondary antibodies and optical systems do not overlap (2).

For immunofluorescence staining of intact tissues, salivary glands were dissected in Cohen and Gotchell medium G containing 0.5% Nonidet P-40 (4). For RNase treatments, approximately half of the glands were incubated in TBS and the other half were incubated in TBS plus 50 μg of preboiled RNase A (Sigma) per ml for 30 min at room temperature. For

DNase treatments, approximately half of the glands were incubated in TBS plus 10 mM $MgCl_2$ and the other half were incubated in TBS plus 10 mM $MgCl_2$ containing 50 U of RNase-free DNase I (Promega) per ml. The glands were transferred to TBS plus 0.05% Tween 20 for 5 min and were treated with formaldehyde fixative (as described above) for 25 min at room temperature. To permeabilize the glands, they were treated with TBS plus 1% Triton X-100 for 15 min at room temperature and subsequently washed in TBS plus 0.05% Tween 20. Finally, the glands were immunostained by incubation in hybridoma cell supernatant containing the PEP-specific MAb Y2A11 (1) for 2 h at room temperature, rinsed in TBS plus 0.05% Tween 20, and incubated in the secondary antibody described above. Glands were washed again in TBS plus Tween 20 and mounted under a siliconized coverslip in GelMount (BioMeda). The control and nuclease-treated glands were photographed as described above.

Chromosomes from nuclease-treated salivary glands were squashed in 45% acetic acid following the formaldehyde treatment, stained with MAb Y1D2 culture supernatant as described above, and treated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary Ab (Cappel Laboratories) diluted 1:500 in TBS containing 10% normal goal serum (Gibco/BRL).

Cell culture. *Drosophila* Schneider II (S2) cells were grown to a density of $\sim 2 \times 10^6$ cells per ml in spinner culture in *Drosophila* Schneider's medium (Gibco/BRL), 10% characterized fetal bovine serum (HyClone), and 1% penicillin-streptomycin (GIBCO). The initial volume of the spinner cultures (100 ml) was doubled daily for 2 days prior to harvest to reach a final total volume of 500 ml.

Preparation of nuclear extract. Nuclei were isolated and nuclear extract was prepared according to the procedures of Risau et al. (38); as described in the legend to Fig. 3, washed cells were homogenized in a buffer containing Triton X-100 and then vortexed and washed in TEN buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1.5 mM Na_2EDTA). The final nuclear pellets were resuspended in 100 μl of cold TMN buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1.5 mM $MgCl_2$) per 10^8 cells. Following sonication on ice (10 successive 10-s pulses with 50-s pauses at setting 14 on a Microson sonicator), the suspension was centrifuged in a Sorvall HB4 rotor at 10,000 × *g* for 10 min at 4°C. The supernatant was saved, and the pellets were resuspended, sonicated, and recentrifuged. The two supernatants were pooled to constitute working nuclear extract.

All buffers for preparation of nuclear extract and analyses of proteins contained fresh 1 mM phenylmethylsulfonyl fluoride (Sigma) and were prepared from double-distilled, autoclaved water that had been treated with 0.1% diethyl pyrocarbonate (Sigma). All glassware was baked at 200°C and autoclaved.

Immunopurification of hnRNP complexes. hnRNP complexes were immunopurified from S2 cell extracts as previously described (29). Cell extracts were incubated with the MAb 8G6 (prebound to protein-A sepharose [Pharmacia LKB Biotechnology, Piscataway, N.J.]) for 15 min at 4°C. The protein-A sepharose was recovered by centrifugation, washed four times with buffer containing 0.5% Triton X-100 and protease inhibitors (0.5% aprotinin, 2 mg of leupeptin per ml, and 2 mg pepstatin A per ml), and the beads were resuspended directly in sample buffer. Antibody specificity was demonstrated by similar immunopurifications from cell extracts in the presence of the ionic detergent Empigen BB, which disrupts protein-protein and protein-RNA interactions.

Protein analyses. Protein samples were mixed with equal volumes of sodium dodecyl sulfate (SDS)-gel loading buffer (4% SDS, 10% β-mercaptoethanol, 62.5 mM Tris-HCl [pH 6.8], 16% sucrose, 0.002% bromphenol blue). Protein samples were boiled for 5 min prior to loading onto SDS-polyacrylamide gels (26) and run at 100 V (constant voltage) until the marker dye reached the bottom of the gels. Molecular masses were estimated from the migrations of precolored standard protein markers (Amersham).

Two-dimensional nonequilibrium pH gel electrophoresis was performed essentially as described by O'Farrell et al. (33) with an ampholine gradient of pH 3 to 10 separated for 4 h at 400 V in the first dimension. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension and stained with silver (53) or transferred to nitrocellulose for Western blot (immunoblot) analysis.

For immunoblotting, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) and probed with the indicated MAbs. Bound antibodies were detected by using either ^{125}I-labeled goat anti-mouse $F(ab')_2$ or biotinylated secondary Abs (anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G from Vector Laboratories) prepared according to the manufacturer's recommendations. Biotinylated antibodies were detected using the VectaStain system (Vector Laboratories).

ssDNA chromatography. S2 cell extracts were fractionated by single-stranded DNA (ssDNA) affinity chromatography essentially as described previously (29). Proteins were bound to ssDNA-cellulose (United States Biochemical Corp., Cleveland, Ohio) in 0.1 M NaCl. Columns were washed with 1 mg of heparin per ml in 0.1 M NaCl and eluted with 2 M NaCl and with 4 M guanidine hydrochloride.

RESULTS

We have undertaken combined cytological and biochemical studies to investigate the unique PEP from *D. melanogaster*. This protein is associated preferentially with active, ecdysone-responsive sites on polytene chromosomes, as demonstrated by an indirect immunofluorescence assay (Fig. 1A and B); more than 95% of the mapped, ecdysone-regulated puff sites are bound by PEP (1). The role of PEP at these sites is not known, although sequence motifs in PEP are appropriate for a role in sequence-specific binding to either DNA or RNA (1). Candidate recognition motifs include a glycine- and asparagine-rich segment and four zinc finger motifs of the C_2H_2 type (Fig. 1C).

Additional cytological observations demonstrated the inclusion of PEP in an RNase-sensitive complex in intact salivary glands (Fig. 2). Control glands immunostained with the PEP-specific MAb Y2A11 displayed very bright nuclear fluorescence and little to no cytoplasmic staining (Fig. 2A and B). By contrast, salivary glands incubated with RNase A—but treated identically to control glands for fixing and staining—failed to display nuclear fluorescence (Fig. 2C and D). Nuclear staining with antiserum specific for RNA polymerase II was only slightly diminished by the RNase treatment (not shown), and salivary glands treated with RNase-free DNase I retained the bright, PEP-specific nuclear fluorescence (not shown). Therefore, the RNase treatment probably allows PEP to leave the nucleus by disrupting PEP-containing RNP complexes. Alternatively, RNase treatment could affect the Y2A11 epitope and disrupt staining. In either case, intact RNA is specifically required for the visualization of PEP in these polytene nuclei.

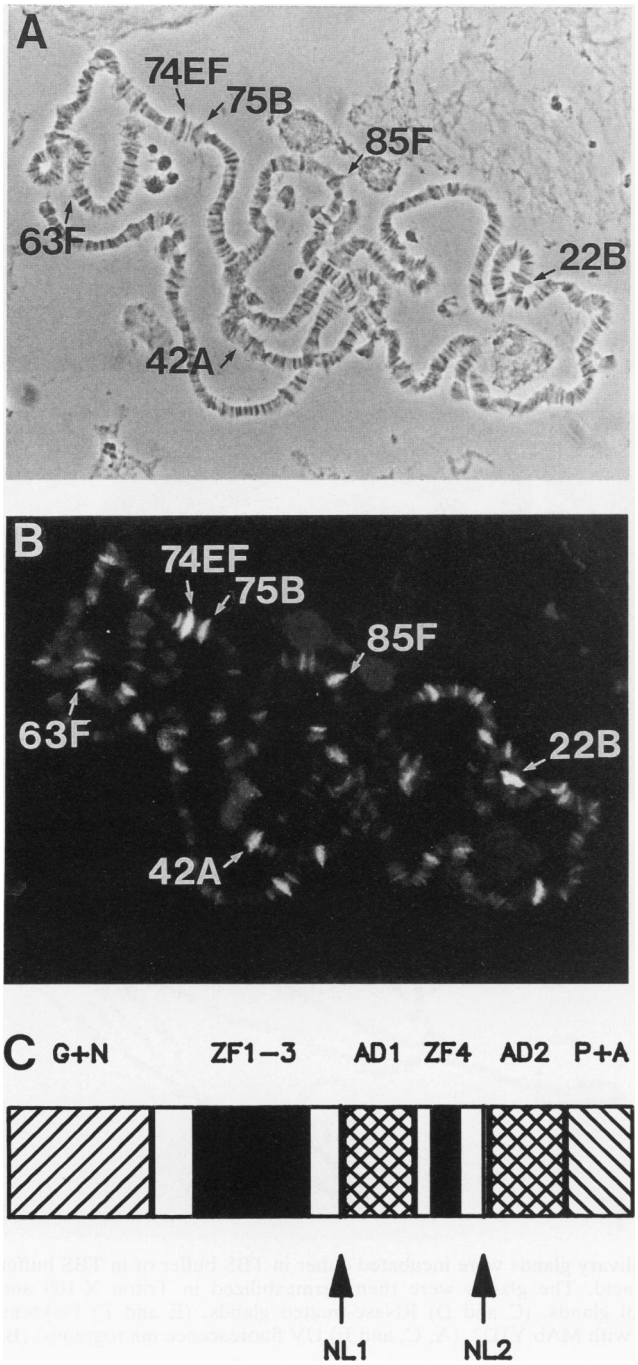

FIG. 1. PEP is a unique zinc finger protein found preferentially on ecdysone-induced chromosomal puffs. Polytene chromosomes from a third-instar larval salivary gland were fixed in a formaldehyde fixative solution, squashed in acetic acid onto a microscope slide, and immunostained with MAb Y1D2, which is specific for PEP. The chromosomal positions of known, prominent ecdysone-responsive loci are indicated. (A) Phase contrast micrograph. (B) UV fluorescence micrograph. (C) Discernible motifs within the 715-residue PEP sequence are depicted, including the N-terminal glycine- and asparagine-rich region (G+N), zinc fingers 1 through 4 (ZF1–3 and ZF4), putative nuclear localization signals (NL1 and NL2), acidic domains (AD1 and AD2), and the C-terminal proline- and alanine-rich segment (P+A). Lengths and positions of segments are drawn to scale.

The effects of RNase treatment on PEP-specific chromosomal immunostaining were examined further by squashing the polytene chromosomes from RNase-treated salivary glands and staining them with a second MAb (Y1D2) specific for PEP. The MAbs Y2A11 and Y1D2 recognize different epitopes in PEP but produce identical staining patterns (1). Following the RNase treatment, the characteristic bright staining on puffs was virtually eliminated (Fig. 2E and F). We conclude that RNA is integral to a PEP-containing complex on the ecdysone-induced puffs, since the integrity of an RNA component is necessary for retention of the PEP at these sites on the chromosomes.

To complement our cytological studies, we turned to cultured *Drosophila* cells to biochemically characterize the putative PEP RNP complex. First, however, the cellular distribution of PEP in salivary glands was compared with that in the *Drosophila* S2 cells used for these subsequent analyses. Immunostaining of a fixed, intact polytene nucleus with the PEP-specific MAb Y1D2 revealed a close association of the protein with the visible chromatin and its absence from nonchromosomal nuclear space and the discernible nucleolar region (Fig. 3A and B). In addition, the distribution of PEP was determined in cellular fractions generated during the preparation of nuclear extract from *Drosophila* S2 cells by the procedures of Risau et al. (38). Proteins in aliquots from each supernatant and from the final pellet were fractionated by SDS-PAGE and also analyzed by Western blotting from identical gels. As shown in Fig. 3C and D, PEP was detected only in the nuclear pellet and the nuclear extract and not in the cytoplasmic fractions. These observations demonstrate that PEP is a nuclear protein, probably restricted to chromosomes; no other site of PEP localization has been detected.

The possibility that PEP may bind RNA prompted an examination of the protein's chromatographic properties on single-stranded DNA cellulose, since the hnRNPs are known to bind single-stranded DNA (29). *Drosophila* S2 cell extract was first treated with micrococcal nuclease and then applied to the column. Retained proteins that either did not bind to the column or were eluted first in heparin and then in higher-salt buffers (2 M NaCl and then 4 M guanidine HCl) were analyzed by SDS-PAGE and Western blotting. PEP was retained quantitatively by the column and eluted cleanly by the heparin wash (Fig. 4), suggesting primarily an electrostatic interaction with the column. A few known hnRNPs share these chromatographic properties, including the human 120-kDa U hnRNP (24) and the human and *Drosophila* PABP proteins (29). As a control, the Western filter was reprobed with MAb 8G6, specific for the hrp40 protein; the hrp40 proteins comprise approximately eight members of *Drosophila* hnRNP complexes in the 38-to-40 kDa size range (29) (see Fig. 6). As expected from previous studies (29), retention of the hrp40 proteins on the column was resistant to the heparin wash but sensitive to 2 M NaCl (Fig. 4). Thus, although PEP qualifies from these observations as an ssDNA-binding protein, its interaction with ssDNA is different from that of many of the other hnRNPs (29).

Immunoprecipitation experiments were performed to test directly whether PEP is associated with *Drosophila* hnRNP complexes. Using hrp40-specific MAb 8G6, complexes in *Drosophila* S2 cell extract were precipitated either in the presence of Triton X-100 (a nonionic detergent) or Empigen BB, an ionic detergent that disrupts protein-protein and protein-RNA interactions (29). Proteins in the immunoprecipitates were analyzed first by one-dimensional SDS-PAGE and Western blotting. PEP was recovered quantitatively in

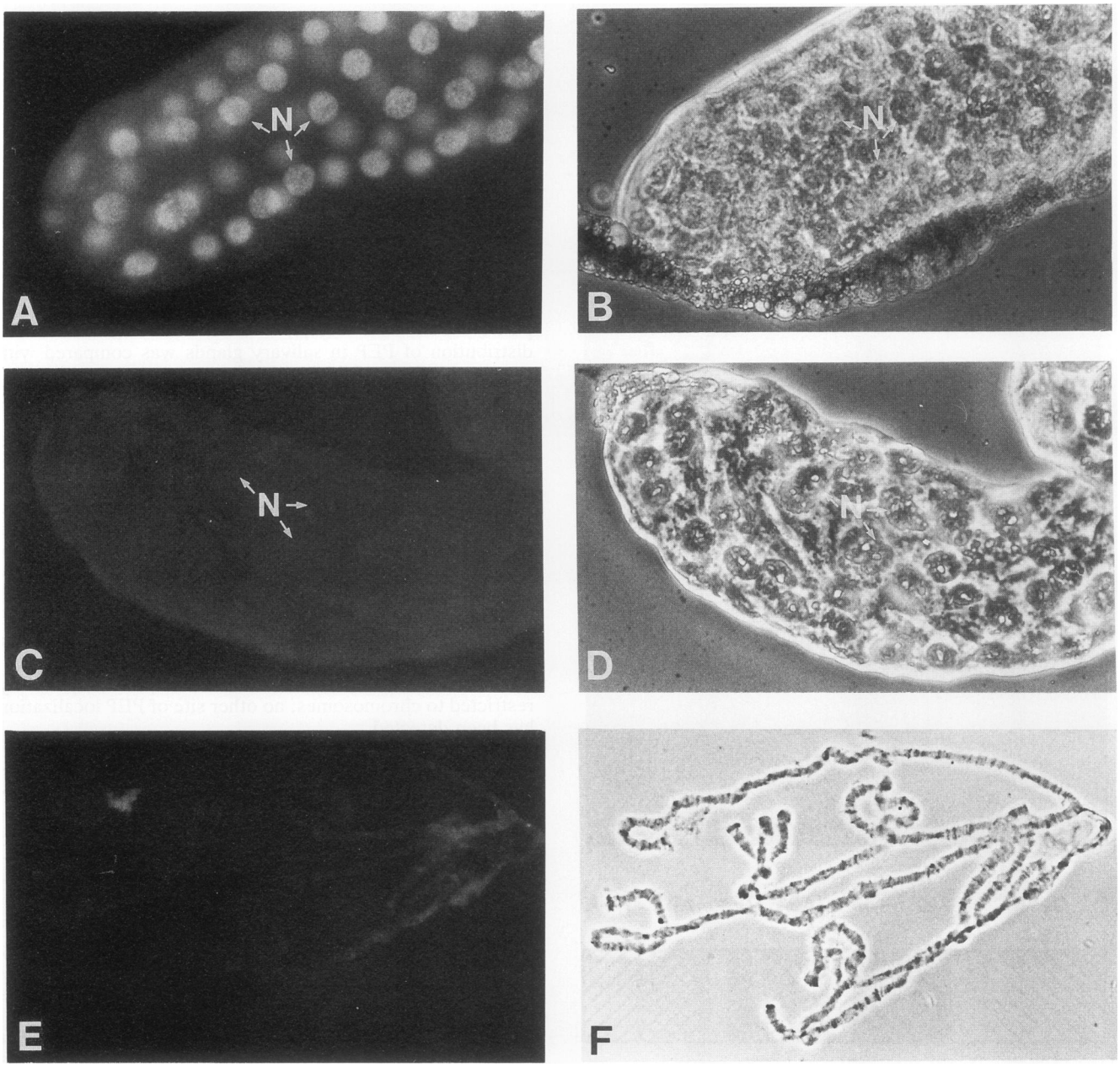

FIG. 2. PEP exists in a chromosomal RNP complex in vivo. Intact salivary glands were incubated either in TBS buffer or in TBS buffer containing RNase A prior to fixation with formaldehyde and acetic acid. The glands were then permeabilized in Triton X-100 and immunostained with the PEP-specific MAb Y2A11. (A and B) Control glands. (C and D) RNase-treated glands. (E and F) Polytene chromosomes from the RNase-treated glands were squashed and stained with MAb Y1D2. (A, C, and E) UV fluorescence micrographs; (B, D, and F) phase contrast micrographs. N, polytene nuclei.

the presence of Triton but not in Empigen (Fig. 5), showing that PEP is indeed associated with *Drosophila* hnRNP complexes. Affinity chromatography with PEP-specific MAbs also indicated an association between PEP and the HRB proteins (data not shown).

Drosophila hnRNP complexes contain over 20 different polypeptides, and many have been identified (28). To identify PEP in hnRNP complexes, the immunoprecipitated proteins were analyzed further by two-dimensional SDS-PAGE followed by either silver staining or Western blotting. In the hnRNP complexes, one polypeptide spot that migrated electrophoretically at the position of ≈100 kDa (as expected for PEP [1]) corresponded to the PEP Western signal (Fig. 6). This spot also migrates in the isoelectrofocusing direction as expected for PEP, since the protein possesses two extremely acidic regions (1). This analysis revealed that PEP is one of the largest, most acidic, and more abundant members of hnRNP complexes.

Through a sequential immunostaining technique, we have shown previously that the PEP distribution comprises a specific subset of chromosomal sites bound by RNA polymerase II and is thus a subset of the sites potentially engaged

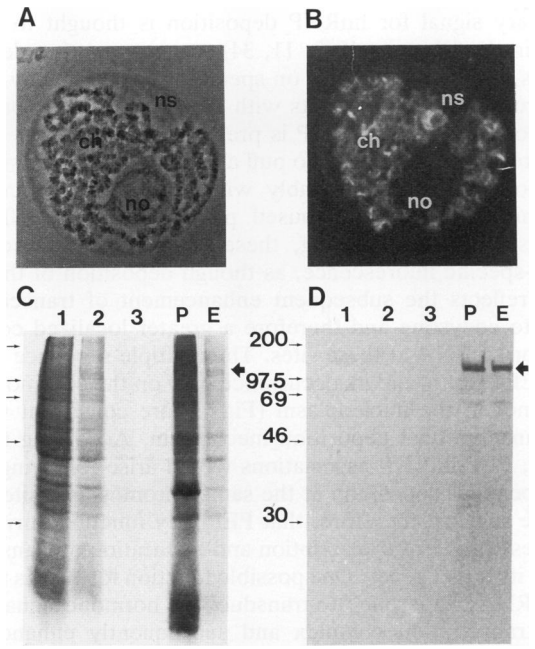

FIG. 3. PEP is found only on chromosomes. A polytene nucleus was squashed from a larval salivary gland, fixed to a microscope slide, and immunostained with MAb Y1D2, which is specific for PEP. (A) The nucleus was photographed under phase-contrast illumination. (B) The nucleus was rephotographed under fluorescence illumination. no, nucleolus; ns, nonchromosomal space; ch, polytene chromosomes. (C and D) Nuclear extract was prepared from *Drosophila* S2 cells according to the procedure of Risau et al. (38): (i) homogenization of S2 cells and centrifugation produces supernatant 1 (cytoplasmic); (ii) resuspension of pellet in TEN buffer and recentrifugation produces supernatant 2 (cytoplasmic); (iii) repetition of incubation in TEN buffer produces supernatant 3 (cytoplasmic); and (iv) resuspension of pellet, sonication in TMN buffer, and recentrifugation produces nuclear pellet and nuclear extract. Aliquots of all supernatants and pellets generated were analyzed for the presence of PEP by Western blotting. (C) SDS-PAGE of proteins in supernatants 1 through 3 (lanes 1 through 3); the nuclear pellet (lane P); the nuclear extract stained with Coomassie blue (lane E). (D) Western blot analysis of proteins in a gel identical to that in panel C, probed with MAb Y1D2, which is specific for PEP. Thick arrows mark the band and signal corresponding to PEP, and thin arrows mark the positions of molecular weight markers.

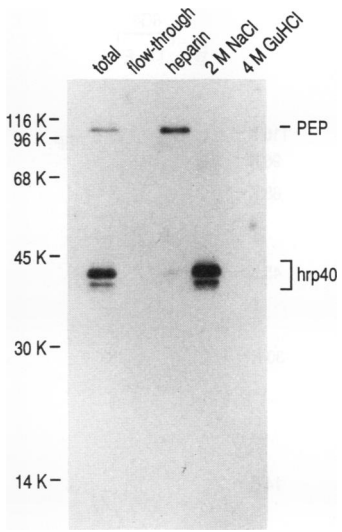

FIG. 4. The interaction of PEP with ssDNA is heparin sensitive. S2 cell extract was bound to an ssDNA-cellulose column at 0.1 M NaCl. The column was washed with 1 mg of heparin per ml and eluted with 2 M NaCl and with 4 M guanidine hydrochloride (GuHCl). Proteins in each fraction were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with the PEP-specific MAb Y1D2 and the hrp40-specific MAb 8G6. Molecular weight standards are indicated on the left, and positions of PEP and hrp40 are indicated on the right.

in transcription (1). Because we found PEP in hnRNP complexes, the PEP chromosomal distribution was compared with that of the *Drosophila* HRB proteins (36), which are basic hnRNPs, by using this same technique. For these experiments, a rabbit antiserum that recognizes three to four major hnRNP isoforms in the 38- to 40-kDa range was used (36), since this technique requires the use of primary antibodies from different species. PEP and the HRB proteins were found to coexist at most active sites on *Drosophila* polytene chromosomes (Fig. 7B and C). Both patterns display bright fluorescence over most of the visible puffs, but the HRB pattern (Fig. 7C) is noticeably brighter on interband regions than is the PEP pattern (Fig. 7B). Numerous loci are stained noticeably by the anti-HRB serum but only weakly by the anti-PEP MAb (Fig. 7, horizontal arrows), but no sites with bright PEP staining and weak HRB staining were found. Care has been taken to normalize staining on prominent puffs. Thus, PEP exists at a subset of the chromosomal sites occupied by hnRNP complexes. Interestingly, numerous neighboring sites at which the intensity of staining was not constant between the PEP and HRB patterns were visible (Fig. 7, paired arrows), indicating that PEP and the HRB proteins may not exist in a fixed stoichiometry at these sites.

DISCUSSION

The results of this study indicate that PEP exists in an RNA-containing complex and is found primarily at chromosomal sites of ecdysone-induced transcription. The abundant RNP complexes that are deposited cotranscriptionally on nascent transcripts (2) fall into two broad classes: the small nuclear RNP particles (snRNPs) that function in pre-mRNA processing events, primarily in intron removal (48), and the hnRNPs that have been considered traditionally to be RNA-packaging proteins (11, 27) but may also participate actively in the splicing reaction (10, 30, 32, 37, 45, 49). The core hnRNPs are thought to associate with all polymerase II transcripts (2, 31), possibly with transcript-specific (8, 35) or sequence-specific (49) preferences, and the snRNPs probably associate with all intron-containing polymerase II transcripts (2, 15, 39, 41, 54). Thus, both are associated with transcripts from members of coordinately regulated gene families such as those activated in response to ecdysone.

In contrast, our observations indicate that PEP assembles preferentially as part of the RNP complex on transcripts from ecdysone-regulated genes, unlike any other known hnRNP. We question, however, whether PEP and the hnRNPs share the same signals for their chromosomal deposition, given the more restricted localization of PEP and quantitative differences in the immunostaining of PEP and hnRNPs at neighboring chromosomal sites. Whereas the

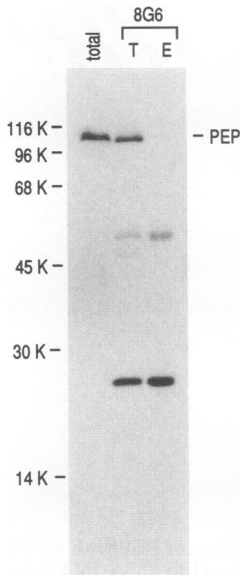

FIG. 5. PEP is associated with immunopurified hnRNP complexes. *Drosophila* hnRNP complexes were immunopurified from S2 cell extract in the presence of Triton X-100 (lane T) with the hrp40-specific MAb 8G6. The specificity of 8G6 is demonstrated by immunopurification in the presence of the ionic detergent Empigen BB (lane E). Immunopurified proteins and total cell lysate (lane total) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the PEP-specific MAb Y1D2. Molecular weight standards are indicated on the left, and the position of PEP is indicated on the right. Low-molecular-weight bands correspond to the heavy and light chains of the antibody used for the immunopurification.

primary signal for hnRNP deposition is thought to reside within the RNA itself (2, 11, 34), we suggest that determinants for PEP deposition on specific transcripts may reside in protein-protein contacts with the transcription complex. The observation that PEP is present at some of the ecdysone-regulated sites prior to puff appearance at those sites (1) supports an early assembly with the basal transcription machinery or with a paused polymerase (40); following ecdysone-induced puffing, these sites become filled with PEP-specific fluorescence, as though deposition of the protein reflects the subsequent enhancement of transcription due to ecdysone and therefore a greater localized concentration of RNA at these sites. The multiple sequence motifs in PEP (Fig. 1) and its occurrence only on the chromosomes and not in the nucleoplasm (Fig. 3) are consistent with a cotranscriptional deposition mechanism. According to this view, PEP-hnRNP associations would arise following their independent deposition at the same chromosomal sites.

We suggest, therefore, that PEP may function during the earliest stages of transcription and maturation of transcripts from its target genes. One possible function for PEP is that of an "RNA chaperone" to transduce the hormone signal from the transcription complex and subsequently enhance the efficiency of processing and/or transport of ecdysone-regulated transcripts in the nucleus. It is note-worthy, however, that PEP is found on puffs, which are sites of vigorous transcription (13, 47), and not on interbands, which are sites of low-level transcription. Therefore, an alternative possibility is that PEP contributes to ecdysone-mediated transcriptional enhancement from a transcript-engaged position (43), either by acting directly on the transcription complex or by expediting transcript removal and processing at these sites. In terms of the Ashburner model for ecdysone-mediated gene regulation, either proposed function for PEP would contribute to the proper temporal appearance of the major regulatory proteins in the cascade. If so, we expect PEP

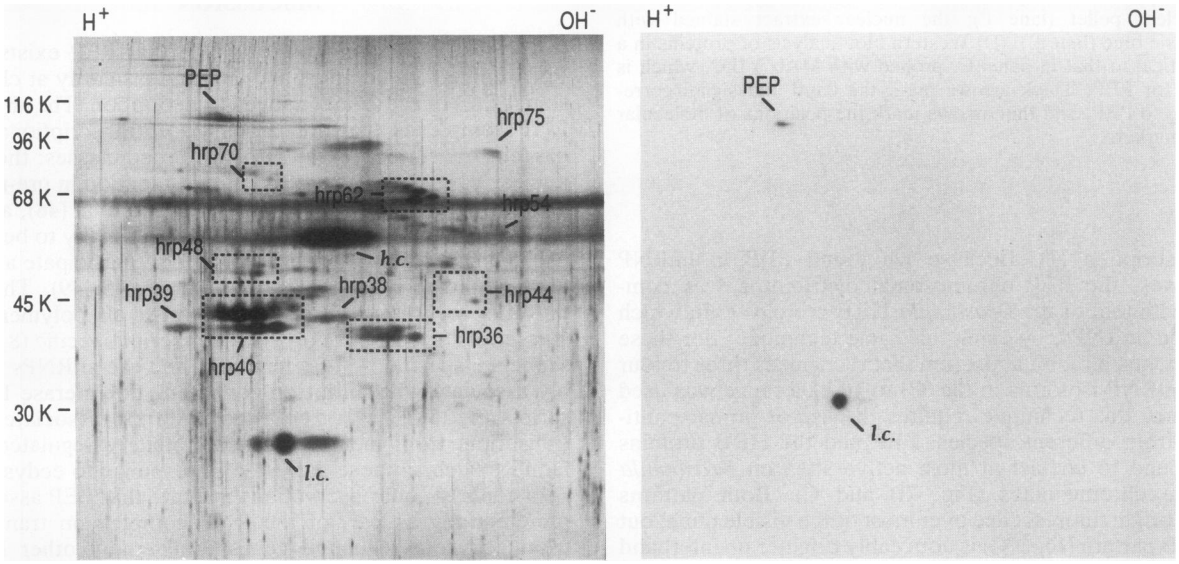

FIG. 6. PEP is a major component of immunopurified hnRNP complexes. Proteins in hnRNP complexes immunopurified with the hrp40-specific MAb 8G6, as described above, were resolved by two-dimensional gel electrophoresis and either detected by silver stain (left panel) or transferred to nitrocellulose membrane and probed with the PEP-specific MAb Y1D2 (right panel). The position of PEP is indicated, and the other immunopurified proteins are labeled as previously described (29). The antibody heavy chain (h.c.) and light chain (l.c.) are also indicated. Molecular weight standards are indicated on the left.

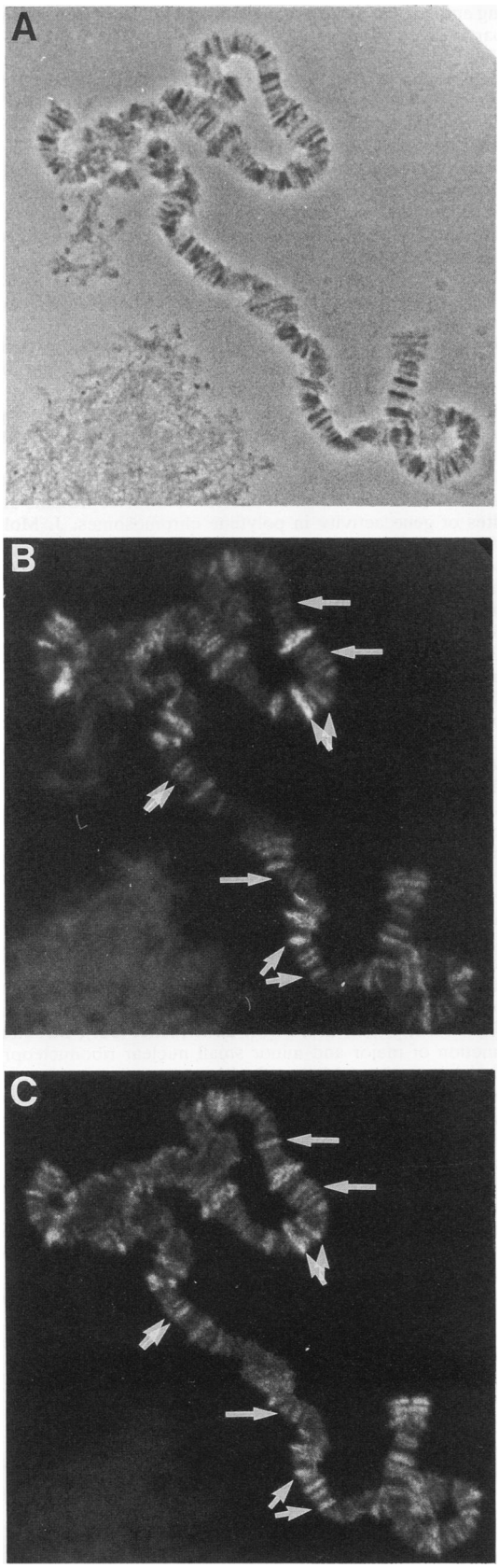

FIG. 7. PEP exists at a subset of chromosomal sites bound by hnRNP complexes. Polytene chromosomes from *Drosophila* third-instar larvae were fixed in formaldehyde, squashed in acetic acid, and stained sequentially with the PEP-specific MAb Y1D2 and HRB-specific antiserum (36). (A) A polytene chromosome photographed under phase-contrast illumination; (B) the chromosome shown in panel A stained with MAb Y1D2 and rephotographed with fluorescence illumination; (C) the same chromosome restained with antiserum specific for the *Drosophila* HRB protein and rephotographed with nonoverlapping fluorescence illumination. Horizontal arrows indicate chromosomal loci that are detected by the HRB-specific antiserum but not by PEP-specific antibodies; paired arrows indicate neighboring sites where the staining intensity is not constant between the two patterns.

function to be important for proper development in *D. melanogaster* and we are anxious to test these possibilities.

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