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Phosphorylation, expression and function of the *Ultrabithorax* protein family in *Drosophila melanogaster*

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Summary

Alternative splicing of the *Ultrabithorax* homeotic gene transcript generates a family of five proteins (UBX isoforms) that function as transcription factors. All isoforms contain a homeodomain within a common 99 aa C-terminal region (C-constant region) which is joined to a common 247 aa N-terminal (N-constant) region by different combinations of three small optional elements. Unlike the UBX proteins expressed in *E. coli*, UBX isoforms expressed in *D. melanogaster* cells are phosphorylated on serine and threonine residues, located primarily within a 53 aa region near the middle of the N-constant region, to form at least five phosphorylated states per isoform. Similar, if not identical states can be generated *in vitro* from purified *E. coli* UBX protein by a kinase activity in nuclear extracts from *D. melanogaster* cells. Temporal developmental profiles of UBX isoforms parallel those for the respective mRNAs, and all isoforms are similarly phosphorylated throughout embryogenesis. Analysis by cotransfection assays of the promoter activation and repression functions of mutant UBX proteins with various deletions in the N-constant region shows that repression is generally insensitive to deletion and, hence, presumably to phosphorylation. By contrast, the activation function is differentially sensitive to the different deletions in a manner indicating the absence of a discrete activating domain and instead, the presence of multiple activating sequences spread throughout the region.

Key words: homeotic genes, bithorax complex, alternative splicing, protein isoforms.

Introduction

The generation of the highly organized body pattern of *Drosophila melanogaster* involves a complex developmental hierarchy of gene activities. Several classes of maternally and zygotically acting genes direct the formation of the body axes and divide the embryo into a series of reiterated segments or metameres (reviewed in Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). The action of the homeotic genes found within the Antennapedia and bithorax complexes (ANT-C and BX-C) then specifies the unique features that distinguish the individual segments. Many of the genes of the hierarchy, including all the homeotics, encode proteins with a homeodomain, a conserved 61 amino acid sequence which has been shown to be sufficient for DNA binding (Desplan *et al.* 1985; Muller *et al.* 1988; Mihara and Kaiser, 1988). The ability of mutations in these genes to affect the expression of each other and of other genes in the hierarchy (Akam, 1987; Scott and Carroll, 1987; Ingham, 1988), and the sequence-specific DNA-binding properties of their protein products (reviewed in Scott *et al.* 1989) indicate that these genes encode transcriptional regulatory molecules. For some, this function has been demonstrated directly by assaying for activity after transient transfection into cultured cells (Jaynes and O'Farrell, 1988; Thali *et al.* 1988; Driever *et al.* 1989; Han *et al.* 1989; Krasnow *et al.* 1989; Struhl *et al.* 1989; Winslow *et al.* 1989), by injection into embryos (Driever *et al.* 1989), and by *in vitro* transcription (Biggin and Tjian, 1989; Ohkuma *et al.* 1990; Johnson and Krasnow, 1990). Several of these homeodomain proteins have been shown to be phosphorylated (Gay *et al.* 1988; Krause *et al.* 1988; Driever and Nusslein-Volhard, 1989) but the significance of this modification is not known.

The homeotic gene *Ultrabithorax* (*Ubx*) primarily specifies the identities of parasegments 5 and 6 (PS5, PS6; the region extending from the posterior compartment of the second thoracic segment to the anterior compartment of the first abdominal segment) (Lewis, 1978; Morata and Kerridge, 1981). *Ubx* also plays a

minor role in PS7 through PS13 of the larval abdomen, where it acts in conjunction with the other two homeotic genes of the BX-C, *abdominal-A* and *Abdominal-B* (Lewis, 1978; Sanchez-Herrero *et al.* 1985; Struhl and White, 1985). These identity functions are required throughout most of development for the specification of embryonic, larval and adult structures (Lewis, 1978). While *Ubx* specifies the identities of epidermal and neural structures in thoracic and abdominal parasegments (Lewis, 1978; Teugels and Ghysen, 1985), it acts in the somatic mesoderm only in the abdominal segments (Hooper, 1986) and in the visceral mesoderm only in PS7 (Bienz and Tremml, 1988).

The ~77 kb *Ubx* transcription unit is alternatively spliced to yield at least 5 different mRNAs whose relative abundance varies throughout development (Beachy *et al.* 1985; O'Connor *et al.* 1988; Kornfeld *et al.* 1989). These mRNAs encode a family of closely related proteins, UBX Ib, Ia, IIb, IIa and IVa which we refer to as UBX isoforms. All have in common an N-terminal region of 247 amino acids (N-constant region) and a 99 amino acid C-terminal region (C-constant region) containing the homeodomain, but differ with respect to three optional elements: one of 9 amino acids and two of 17 amino acids, each of which is located between the constant regions (Fig. 1A). The isoforms display distinct temporal and tissue-specific patterns of expression (J. Lopez and D.S.H., in preparation). Furthermore, UBX Ia and IVa differ functionally in their abilities to transform the peripheral nervous system of thoracic segments when expressed ectopically in transgenic flies (Mann and Hogness, 1990).

It has been hypothesized that the specification of metameric identity by the products of the *Ubx* gene results from their regulation of the expression of target or 'effector' genes (Lewis, 1964; Garcia-Bellido, 1975). *Antennapedia* (*Antp*), a homeotic gene within the ANT-C, appears to be a target for repression by UBX proteins because *Antp* products accumulate to abnormally high levels in Ubx^- mutant embryos in those regions where UBX proteins are normally expressed (Hafen *et al.* 1984; Carroll *et al.* 1986). *Ubx* itself is likely to be subject to autoregulation. High activity of the *Ubx* promoter (P_{Ubx}) in the embryonic visceral mesoderm requires the presence of the endogenous UBX proteins (Bienz and Tremml, 1988), whereas negative regulation of P_{Ubx} by UBX proteins occurs in the embryonic epidermis and larval imaginal discs (K. D. Irvine, J. Botas, R. S. Mann, and D. S. H., unpublished). More direct evidence confirms a functional interaction of UBX proteins with these promoters. UBX Ib, purified from an overproducing *E. coli* strain, binds specifically to sequences near P_{Ubx} and the P1 promoter of the *Antp* gene (P_{AntpP1}; Beachy *et al.* 1988). UBX proteins can activate transcription from P_{Ubx} linked to a reporter gene as well as repress transcription of a P_{AntpP1}–reporter construct after cotransfection into cultured *Drosophila* cells (Krasnow *et al.* 1989). The activation function of UBX proteins appears to require one or more domains not necessary for repression since a protein with most of its N-constant region deleted exhibited repression but not activation activity in these cotransfection experiments. In addition, purified UBX Ib can stimulate transcription *in vitro* from a heterologous promoter fused to UBX binding sites (Johnson and Krasnow, 1990). The regulatory targets thus far identified for UBX proteins have, however, been limited to homeotic genes; indeed targets that more directly effect metameric identities (i.e. the hypothesized 'effector' genes) have not yet been identified for any homeotic gene.

We have been interested in how UBX proteins marshal the multiple genetic functions required for effecting metameric identities during the development of different tissues. We focus here on the question of whether post-translational modification plays a role in modulating UBX activity. To that end, cultured *Drosophila* cells were transformed with isoform-specific expression plasmids to yield stable lines in which individual isoforms are produced in response to heat shock. Examination of these isoforms revealed that each is highly modified by phosphorylation to produce at least five phosphorylated states and we have localized the phosphorylated serine and threonine residues to particular parts of the N-constant region. We present the first evidence for the existence, in the animal, of the individual UBX protein forms, which are also modified to these states throughout embryonic development. Transient cotransfection assays of the promoter-activating functions of UBX proteins bearing various deletions of the N-constant region indicated that this region contains multiple activating sequences rather than a single activation domain. Finally, we have shown that a kinase activity present in nuclear extracts of cultured *Drosophila* cells can phosphorylate purified UBX protein *in vitro*.

Materials and methods

Plasmids

The *Ubx* Ib cDNA sequence had previously been placed downstream of the hsp70 promoter (P_{hs}) in the vector pcopneo (Rio and Rubin, 1985) for expression in cultured *D. melanogaster* cells (Beachy *et al.* 1988). This plasmid, referred to here as pP_{hs}UBX Ib, contains sequences encoding 610 bp of the 5′ untranslated leader, the coding region, ~1 kb of the 3′ untranslated region, and the polyadenylation signal of the *Ubx* Ib mRNA. To express the other UBX isoforms, pP_{hs}UBX Ia, pP_{hs}UBX IIa, pP_{hs}UBX IIb, and pP_{hs}UBX IVa were generated by replacing the 900 bp *Stu*I–*Xho*I fragment spanning the optional elements of the *Ubx* Ib cDNA with the equivalent *Stu*I–*Xho*I fragments isolated from cloned cDNAs encoding each of these proteins (Fig. 1B). The *Ubx* Ia fragment was derived from pφ3602, IIa from EC13, and IVa from EC1 (Kornfeld *et al.* 1989). The *Ubx* IIb fragment was derived from cDNA E6–16T#5 (O'Connor *et al.* 1988), kindly provided by M. O'Connor.

Plasmids designed to express *Ubx* cDNA sequences under the control of the actin 5C promoter (P_{Ac}) after transient transfection are described by Krasnow *et al.* (1989). In-frame deletions of *Ubx* coding sequences were constructed in pP_{Ac}UBX IVa and are designated by the amino acids included

in the deletion (Fig. 5A). *Ubx* sequence coordinates correspond to those of Kornfeld *et al.* (1989). IVa$^{\Delta 39-130}$ was generated by the removal of the 280 bp *Not*I–*Nae*I fragment and ligation of the *Nae*I and filled *Not*I ends. IVa$^{\Delta 131-183}$ resulted from removal of the 159 bp *Nae*I fragment. IVa$^{\Delta 179-226}$, which has a histidine to glutamine substitution at codon 178, was generated by removal of the 141 bp *Bgl*I–*Not*I fragment, followed by ligation of recessed *Bgl*I and filled *Not*I ends. (The *Bgl*I site, not shown in Fig. 5A, is nearly adjacent to the rightmost *Nae*I site.) IVa$^{\Delta 39-183}$ was generated by removal of the 439 bp *Not*I–*Nae*I fragment and ligation of the *Nae*I and filled *Not*I ends. IVa$^{\Delta 37-225}$ resulted from removal of the 567 bp *Not*I fragment. This deletion corresponds to the sequences removed in pP$_{Ac}$UBX Ib$^{\Delta N}$ of Krasnow *et al.* (1989) and UBX-ΔNN of Mann and Hogness (1990).

Plasmids containing P$_{Ubx}$ and P$_{Antp\ P1}$ fused to the bacterial CAT gene (P$_{Ubx}$CAT and P$_{Antp\ P1}$CAT) are described by Krasnow *et al.* (1989).

Cell culture and transfections

Schneider line 2 (S2) cells (Schneider, 1972) were maintained at densities of 2×10^6 to 8×10^6 cells ml^{-1} in T flasks as described by Krasnow *et al.* (1989). To generate stable lines carrying UBX expression constructs, cells were transfected with 20 μg of supercoiled plasmid DNA by calcium phosphate precipitation (Wigler *et al.* 1979) as described by Krasnow *et al.* (1989) and stable lines consisting of mixed clones were selected with G418 according to Rio and Rubin (1985). Cells were subsequently maintained under selection at 25°C. For CAT activity assays, cells were transfected with 1 μg of reporter plasmid combined with 9 μg of expression plasmid and 10 μg of pUC18 DNA and incubated with the precipitates for 60 h as described by Krasnow *et al.* (1989). For analysis of proteins expressed after transient transfection, 20 μg of UBX expression plasmid DNA was used and the cells were harvested 36 h after transfection.

Protein expression and in vivo *labeling*

For induction of UBX protein expression, S2 cells were passaged at least twice without selection and then heat shocked at densities of $2.5–5\times10^6$ cells ml^{-1} by submersion of T flasks in a water bath at 36–36.5°C. Cells were then resuspended, washed with ice cold PBS (5.1 mM NaH_2PO_4/4.9 mM Na_2HPO_4 [pH 6.8]/150 mM NaCl), quick frozen in liquid nitrogen and stored at −70°C.

For labeling of proteins in S2 cells, M3 media (Shields and Sang, 1977), as modified by Lindquist *et al.* (1982), were prepared with the following changes: bactopeptone and yeast extract were always omitted; for [^{32}P]orthophosphate ($^{32}P_i$) labeling, NaH_2PO_4 was replaced by 10 mM Hepes and for [^{35}S]methionine/cysteine ([^{35}S]MET) labeling, L-cysteine and L-methionine were omitted. Cells were washed twice with the appropriate media, resuspended in the same at a density of 1×10^6 cells ml^{-1}, and incubated for 1 h at 25°C. Either 0.5 mCi of $^{32}P_i$ (HCl-free, carrier-free; Amersham) or 0.3 mCi of [^{35}S]MET (Translabel, ICN) was added per ml of cells and the cells were heat shocked for 4 h. The cells were resuspended and washed twice in ice-cold PBS prior to lysis for immunoprecipitation of UBX proteins as described below.

Immunoprecipitation

Cells were lysed in ice-cold RIPA buffer (PBS/5 mM Na_2EDTA/1% Triton X-100/1% sodium deoxycholate/0.1% sodium dodecyl sulfate [SDS]) containing 0.25 mM phenylmethylsulfonylfluoride (PMSF; Sigma), 10 μg ml^{-1} each of leupeptin and pepstatin, and 0.4 units ml^{-1} aprotinin (Boehringer-Mannheim). UBX proteins were immunoprecipitated from cleared lysates at 4°C with either an anti-UBX polyclonal antiserum (Beachy *et al.* 1985) followed by *Staphylococcus aureus* cells (Pansorbin, Calbiochem) or with anti-IgG-linked *Staphyloccus aureus* cells (Tachisorb, Calbiochem) which had been preabsorbed with anti-UBX monoclonal antibodies (J. Lopez and D.S.H., unpublished). Immunoprecipitates were washed 4 times with RIPA buffer and once with PBS except that 40 mM Pipes (pH 6.0) replaced PBS in the final wash of samples prepared for phosphatasing. Pellets were either resuspended immediately for gel analysis or stored at −70°C.

Phosphatase digestion

Immunoprecipitates containing labeled UBX protein from $\sim2\times10^7$ cells were resuspended to a slurry in 125 μl of buffer containing 40 mM Pipes (pH 6.0), 10 μg ml^{-1} each of leupeptin and pepstatin, 0.4 units ml^{-1} aprotinin and digested with either 0 or 0.5 units (7 μg) of potato acid phosphatase (PAP, Sigma) for 30 min at 30°C as described by Cooper and King (1986). Reactions were terminated by washing the immunoprecipitates twice with RIPA buffer and once with PBS.

Isolation of nuclei from Drosophila *embryos*

Adult flies were fed for 2–3 h prior to egg collection to minimize withholding of eggs. Eggs were collected on yeasted grape agar plates (Elgin and Miller, 1978) for 3 h intervals and allowed to develop at 25°C for the appropriate periods of time. Nuclei were isolated according to Heberlein and Tjian (1988) except that all buffers were supplemented to 2 μg ml^{-1} with each of the protease inhibitors leupeptin and pepstatin (Boehringer Mannheim) and to 20 mM with each of the phosphatase inhibitors NaF and β-glycerophosphate. Nuclear pellets were stored at −70°C.

Electrophoresis and immunoblotting

One dimensional gels

S2 cells were lysed by boiling for 2 min in 2×SDS sample buffer (Laemmli, 1970), then sonicated for 30–60 s at ~90 watts in a cup horn sonicator (Branson Sonifier Cell Disrupter, Model W185) and centrifuged for 10 min in an Eppendorf microfuge at room temperature. The equivalent of $\sim3\times10^5$ or $\sim8\times10^5$ cells/lane for stable lines or transiently transfected cells, respectively, were loaded on SDS–polyacrylamide gels (Laemmli, 1970). For resolution of the phosphorylated species of wild-type proteins, we routinely used SDS–polyacrylamide gels containing glycerol which had been poured with a gradient of 8–12% polyacrylamide and 0–10% glycerol. Embryo nuclei were lysed as described above and material isolated from ~5 mg (wet weight) of embryos was loaded per lane on 10% SDS–polyacrylamide gels. Intact, dechorionated embryos, larvae, pupae and adult flies were homogenized at 100°C in 2×SDS sample buffer containing 4 M deionized urea and the equivalent of ~20 mg of whole tissue was analyzed as described for the embryo nuclei.

Two dimensional gels

Nonequilibrium pH gradient electrophoresis (NEPHGE) was carried out for 4.5 h at 400 V according to O'Farrell *et al.* (1977) using solutions containing 1% pH 6–8, 1% pH 8–10.5 ampholines (Pharmacia). The equivalent of $4–6\times10^5$ S2 cells from stable lines or $2–3\times10^6$ transiently transfected cells were loaded per tube gel; for embryo nuclei, material from 20–30 mg of embryos was loaded. Proteins were separated in the second dimension on 8–12% SDS–polyacrylamide gradient gels except that 12% gels were used in the analysis of UBX IVa deletion mutants.

Following SDS–PAGE, gels were transferred to nitrocellulose (Burnette, 1981) or stained with Coomassie and dried for autoradiography. Gels containing ^{35}S-labeled proteins were fluorographed with 22% PPO in dimethylsulfoxide prior to drying. Immunoblotting was carried out using 1:50–1:200 dilutions of the monoclonal antibody FP3.38 (White and Wilcox, 1984) or 1:50 dilutions of the monoclonal antibodies J1.1 and 3F.6 (J. Lopez and D.S.H., unpublished) followed by alkaline phosphatase conjugated secondary antibody (BioRad) in PBS/2% bovine serum albumin/0.05% Tween 20, supplemented with calf serum to 2% for S2 proteins and to 10% for embryo nuclei or whole animal proteins. Proteins were detected by developing with alkaline phosphatase immunochemistry (Blake *et al.* 1984) except that Tris–HCl (pH 9.6) replaced veronal acetate (pH 9.6).

Phosphoaminoacid analysis

UBX proteins labeled *in vivo* with $^{32}P_i$ were purified by immunoprecipitation with the polyclonal antiserum followed by SDS–PAGE. After autoradiography of dried gels, the labeled UBX bands were excised, and protein was recovered by the method of Beemon and Hunter (1978). Proteins were hydrolyzed in 6 N HCl for 2 h at 110 °C as described in Cooper *et al.* (1983) and phosphoaminoacids were separated by thin layer electrophoresis at pH 3.5 in pyridine:acetic acid:water (5:50:945) for 50 min at 1500 V. Unlabeled phophoaminoacid standards added to each sample were visualized with ninhydrin as described and labeled residues were visualized by autoradiography and quantitated by scintillation counting.

Chemical and protease cleavage

UBX Ia labeled *in vivo* with ^{35}S[MET] or $^{32}P_i$ was purified by immunoprecipitation with the monoclonal antibody J1.1 followed by SDS–PAGE and the labeled UBX Ia bands were excised. Partial proteolysis of protein in gel slices (~50–100 ng/slice) was achieved by the method of Cleveland *et al.* (1977) using 1–2 μg *Staphylococcus aureus* V8 protease (Miles), or 8 μg chymotrypsin (Worthington).

Cleavage with *N*-chlorosuccinimide (NCS) was carried out according to Lischwe and Ochs (1982) using 15 mM NCS for 30 min with the modification that after treatment, slices were soaked for 20 min in two changes of 1 M Tris–HCl (pH 8.0) followed by 20 min in two changes of 2×Laemmli sample buffer. Gel slices were loaded directly into the wells of 15% SDS–polyacrylamide gels. Gels containing proteins cleaved by either proteases or NCS were fluorographed with 1 M sodium salicylate, dried and exposed for autoradiography.

In vitro *kinasing*

Kinasing reactions were performed in a total volume of 50 μl containing 25 mM Hepes(Na) (pH 7.3), 40 mM NaCl, 10 mM NaF, 100 μM ATP, 0.5 μCi [γ-^{32}P]ATP (6000 Ci mmole^{-1}, Amersham), 1 mM DTT and 50–100 ng UBX Ia protein purified from *E. coli* (~80% pure, gift of J. Lopez). 0 or 2.5 μg of Kc cell nuclear extract (gift of B. Johnson, described in Johnson and Krasnow, 1990) was added and the reactions were incubated for 30 min at 30 °C. UBX protein was immunoprecipitated and analyzed on standard or 2D gels.

CAT Activity Assays

Cell extract preparation and CAT activity assays were performed as described by Krasnow *et al.* (1989). All assays contained 50 μg of protein extract and were carried out for 30–60 min.

Results

UBX proteins expressed in cultured cells display electrophoretic heterogeneity

Plasmids encoding each of the five *Ubx* cDNAs under inducible control of the hsp70 promoter were constructed using the vector pcopneo (Rio and Rubin, 1985), which carries the bacterial neomycin resistance gene (see Fig. 1 and Materials and methods). *Drosophila* Schneider line 2 (S2) cells (Schneider, 1972), which do not normally express the *Ubx* gene (Beachy, 1986), were transfected with these plasmids by calcium phosphate precipitation and stable lines were established for each construct by selection with the neomycin analog G418. UBX protein expression was induced after heat shock in all but the control cell line (carrying the pcopneo vector), as detected by immunoblotting of proteins in crude cell lysates with the monoclonal antibody FP3.38 (White and Wilcox, 1984) that recognizes all UBX isoforms (Fig. 2A). Maximal production was achieved between 3 and 4 h after initiation of heat shock (Fig. 2B). Indirect immunofluorescence of fixed, permeabilized, heat shocked cells with FP3.38 revealed accumulation of UBX protein exclusively in the nucleus (data not shown). A small region within the nucleus, which may represent the nucleolus, failed to stain, as previously described for UBX protein in cells of the embryonic nervous system and imaginal discs (Beachy *et al.* 1985). UBX protein was completely nuclear by 30 min after initiation of heat shock (the earliest time examined) and remained nuclear at least two hours after cessation of heat shock.

Unlike UBX Ib expressed in *E. coli*, which migrates as a single band of ~43×10^3 M_r with some lower molecular weight degradation products (Fig. 2A, lane 1), each construct generated at least three bands on immunoblots of SDS–polyacrylamide gels (Fig. 2A, lanes 3–7). Similar patterns were observed after transient transfection of S2 cells with plasmids that express these proteins under control of the actin 5C promoter (Krasnow *et al.* 1989), demonstrating that heat shock itself was not required to produce multiple bands (data not shown). Neither could use of multiple AUGs found downstream of the initiation codon account for this heterogeneity since protein was not detected in cells transfected with a construct containing a frameshift at the eighth codon (data not shown). A time course of induction suggested a possible precursor–product relationship between the bands. For an individual isoform, the band with the greatest mobility appeared first and was followed by bands of decreasing mobility as shown for UBX IIa in Fig. 2B. In addition, pulse-chase experiments with [^{35}S]methionine and inhibition of protein synthesis with cycloheximide indicated that most of the more slowly migrating bands arose post-translationally (data not shown).

The class A UBX proteins (i.e. Ia, IIa, IVa) form a consistent set in which the most rapidly migrating band for each isoform exhibited an M_r corresponding to that deduced from its amino acid sequence. This was not, however, the case for the class B UBX proteins (Ib,

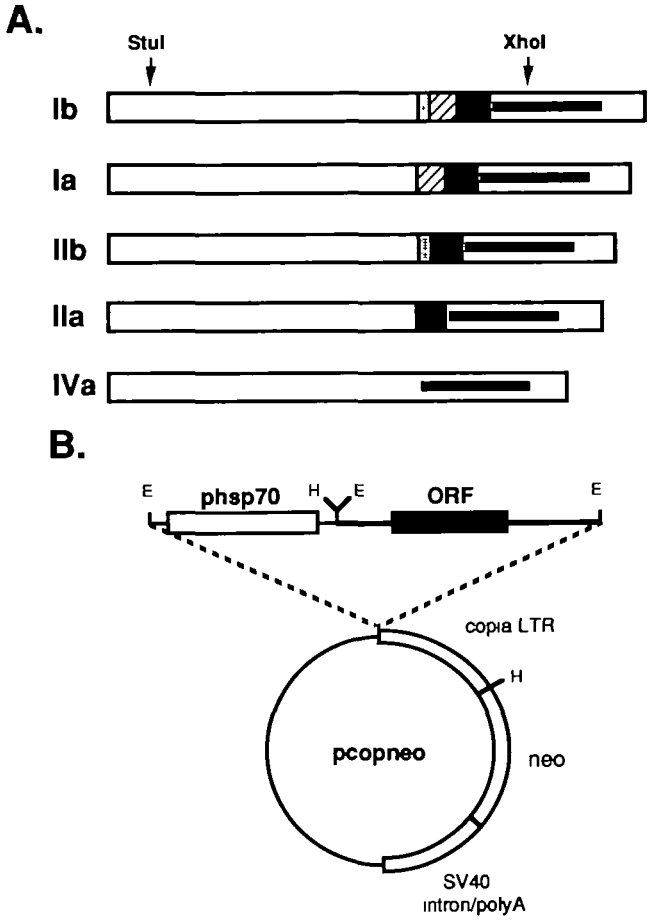


Fig. 1. Structure of *Ubx* cDNAs and plasmids for expression of UBX proteins in cultured cells. (A) UBX proteins or isoforms encoded by 5 different classes of cDNAs are designated UBX Ib, Ia, IIb, IIa, and IVa according to the nomenclature of Beachy *et al.* (1985) and Kornfeld *et al.* (1989). The 'b' designation refers to the presence of the 'b' optional element (9 aa; lightly stippled box) whose use depends on the choice between two splice donor sites separated by 27 bp at the end of the 5′ exon. Isoforms designated I contain both of the other optional elements (each of 17 aa; slashed and darkly stippled boxes), those designated II contain only the more C-terminal of these elements, and IV lacks both. The N-constant (left) and C-constant (right) regions are indicated by open boxes. The homeodomain is indicated by the filled bar. Restriction sites in the cDNA sequence for *Stu*I and *Xho*I, used to construct the expression plasmids described in B, are indicated. (B) The *Ubx* cDNAs including coding (ORF; filled box) and untranslated sequences (thick black lines) were inserted downstream of the hsp70 promoter (open box) and cloned into the pcopneo vector for introduction into S2 cells. The components of pcopneo required for selection in cultured cells – the bacterial neomycin gene (neo) under control of the promoter of the copia LTR and the SV40 intron and polyadenylation signals – are indicated. E, *Eco*RI; H, *Hin*dIII.

IIb), where the bands generated by each isoform migrated overall more rapidly than their smaller class A counterparts (Fig. 2A; compare lanes 3 and 4, and 5 and 6). This anomalous behavior of the class B proteins results from the aberrant secondary splicing of the class B mRNAs that are transcribed from the respective cDNAs in these cell lines, as determined by S1 nuclease protection analyses (K. Kornfeld, E.R.G., and D.S.H., unpublished). This aberrant splicing is class B-specific because the splice donor site is the upstream 5′ exon donor site normally used in the generation of class A mRNAs and therefore retained only in class B mRNAs. Cryptic splice acceptor sites, located quite close to the 3′ ends of the first and second microexons, act in conjunction with this common donor site to convert >50 % of the Ib and IIb mRNAs to mRNAs designated IIa* and IVa*, respectively, because of their close structural similarity to the normal IIa and IVa mRNAs. Furthermore, the proteins encoded by the IIa* and IVa* mRNAs should be almost identical to the IIa and IVa proteins because the aberrant splicing is predicted to retain the original reading frame. Consequently, the expected electrophoretic distributions of UBX proteins from the Ib and IIb cell lines should each represent the sum of the two distributions: Ib+IIa* and IIb+IVa*, respectively. The results shown in Fig. 2A are consistent with this expectation. We have also shown that an expression construct, UBX Ib^{-B}, which contains a frameshift mutation within the first microexon that prevents Ib, but not IIa*, protein expression, generates an electrophoretic pattern virtually identical to that for the IIa protein (data not shown).

We conclude that the heterogeneity observed for class A proteins on SDS–polyacrylamide gels results from post-translational modification of polypeptides with mobilities appropriate to their predicted M_rs, whereas both aberrant splicing and post-translational modification contributes to the class B protein patterns. It should be emphasized that the aberrant secondary splicing of class B mRNAs and the consequent generation of IIa* and IVa* proteins has been observed only when these mRNAs are transcribed from their respective cDNAs, whether in cell lines or in embryos. When class B mRNAs are formed normally in the animal by splicing of the 77 kb *Ubx* transcript, this aberrant secondary splicing has not been observed, as if only one chance is given to splice from one of the two donor sites of the 5′ exon. Because we have not yet obtained expression systems for class B mRNAs and proteins that are uncontaminated with the IIa* or IVa* forms, we have elected to focus here on the class A proteins.

UBX proteins are modified by phosphorylation

To evaluate further the heterogeneous mobility on SDS–PAGE with respect to post-translational modifications, we used a two-dimensional (2D) gel system (O'Farrell *et al.* 1977) in which proteins were first separated according to charge by nonequilibrium pH gradient electrophoresis (NEPHGE) and then according to size by SDS–PAGE. Immunoblots of crude cell lysates resolved on 2D gels showed a series of spots with concomitantly increasing negative charge and apparent molecular weight for each isoform (Fig. 2C). Each

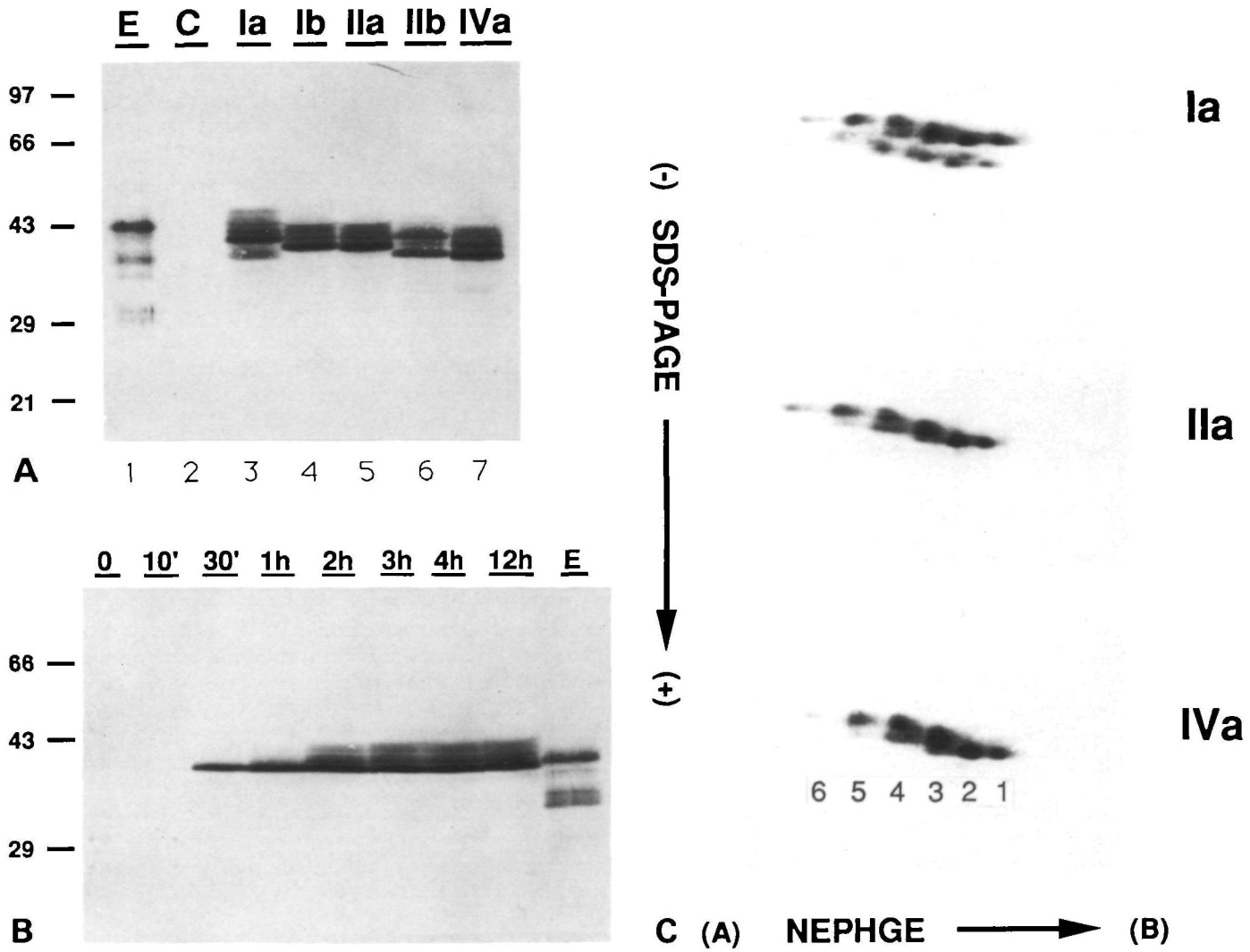


Fig. 2. Analysis of UBX proteins expressed in stable lines of cultured *D. melanogaster* cells. (A) UBX protein expression was induced by heat shock at 36°C for 4 h. Proteins in cell lysates were separated on 8–12% SDS–polyacrylamide gradient gels and UBX isoforms were detected by immunoblotting with the monoclonal antibody FP3.38. Each lane contains an equivalent amount of total protein from one transfected cell line as determined by Coomassie staining of gels and Ponceau-S staining of the nitrocellulose blot. The isoform (UBX Ia, Ib, IIa, IIb, IVa) expressed in each cell line is indicated above the corresponding lane; E, UBX Ib purified from *E. coli* (Beachy *et al.* 1988); C, control cell line transfected with pcopneo plasmid DNA. The extra two weak bands lying below the major set in the Ia lane appear to represent degradation products, which are also visible in Fig. 2C (see text). The positions of the relative molecular mass markers (phosphorylase b, 97×10^3; bovine serum albumin, 66×10^3; ovalbumin, 43×10^3; carbonic anhydrase, 29×10^3; soybean trypsin inhibitor, 21×10^3) are indicated. (B) Cells from the S2 line carrying the UBX IIa expression construct were heat shocked at 36°C and harvested at the indicated times after induction. Proteins were analyzed as described in (A) with equivalent amounts of total cell protein loaded for each time point. Cells harvested prior to induction are designated as '0'; E, UBX IIa expressed in *E. coli*. (C) Lysates of UBX Ia, UBX IIa, and UBX IVa shown in A were analyzed by non-equilibrium pH gradient electrophoresis (NEPHGE) from pH 6–10.5 followed by SDS–PAGE on 8–12% SDS–polyacrylamide gradient gels and immunoblotting with FP3.38. The horizontal arrow indicates the direction of migration of proteins in the first dimension, with more positively charged or basic species (B) migrating farther to the right and more negatively charged or acidic species (A) remaining near the origin. The direction of migration toward the positive electrode in the second dimension is indicated by the vertical arrow. The isoform represented in each immunoblot is indicated to the right. Each spot that can be resolved in all three isoforms is numbered.

group of spots with the same mobility in the second dimension corresponded to one of the bands visualized on standard SDS–polyacrylamide gels (e.g. spots 1 and 2 with the most rapidly migrating band, spots 4, 5, 6 with the most slowly migrating band). At least six spots were easily resolved for each isoform; a seventh more acidic spot could be detected when large quantities of protein were loaded. In addition, the smear below spot

4 often resolved as a discrete spot when less protein was loaded. (On standard SDS–PAGE, UBX Ia showed two additional weak bands that appear to be degradation products that resolve into a second series of spots below the major ones on 2D gels.)

The series of spots showing incremental increases in negative charge suggested phosphorylation as a likely modification. Cells were therefore labeled *in vivo* with either [^{35}S]methionine/cysteine ([^{35}S]MET) or [^{32}P]orthophosphate ($^{32}P_i$) and their UBX proteins analyzed by 2D electrophoresis after purification by immunoprecipitation with a polyclonal antibody (Fig. 3). Autoradiography of ^{35}S-labeled protein revealed the same pattern of spots as detected by immunoblotting of crude cell lysates (upper left panel). ^{32}P labeling demonstrated that all but one of the spots contained phosphate (lower left panel); the unlabeled spot, the presumptive unphosphorylated species (open triangle), migrated at the most basic position and with the greatest mobility. To determine whether phosphorylation alone could account for the shifts in mobility, the labeled, immunoprecipitated proteins were treated with potato acid phosphatase (PAP). Digestion with PAP converted nearly all of the protein to the unphosphorylated position (upper right panel) and removed nearly all of the labeled phosphate (lower right panel). Although some of the phosphate remained under these conditions (filled triangle), it appears that this resulted from antibody protection against PAP digestion since all phosphate residues could be removed if the immunoprecipitation was performed with a monoclonal antibody (J1.1; J. Lopez and D.S.H., unpublished) that recognizes an epitope located in the C-terminal half of the C-constant region (data not shown). We conclude that the observed heterogeneity results from phosphorylation of UBX proteins.

The similarity in the patterns observed for the UBX Ia, IIa, and IVa proteins on 2D gels suggests that they are modified on the same residues. Addition of phosphate alone does not necessarily alter the mobility of the protein on SDS–PAGE (Fig. 2C, spots 1 and 2) and above a certain number of phosphates, the mobility no longer shifts (spots 4, 5, and 6). This pattern suggests that the phosphates may be added in an ordered sequence so that a mobility shift occurs when particular residues are modified. It is possible, however, that each spot represents the accumulation of a certain number of phosphates, regardless of which residues are modified, with the mobility shifting when that number reaches some critical value.

UBX proteins are phosphorylated on serine and threonine residues in specific parts of the N-constant region

To identify the type of amino acid side chain(s) that were phosphorylated, ^{32}P-labeled protein, that had been immunoprecipitated from lysates of S2 cells and gel purified, was acid hydrolyzed. Analysis of the hydrolysis products by thin layer electrophoresis revealed that UBX proteins are phosphorylated on serine

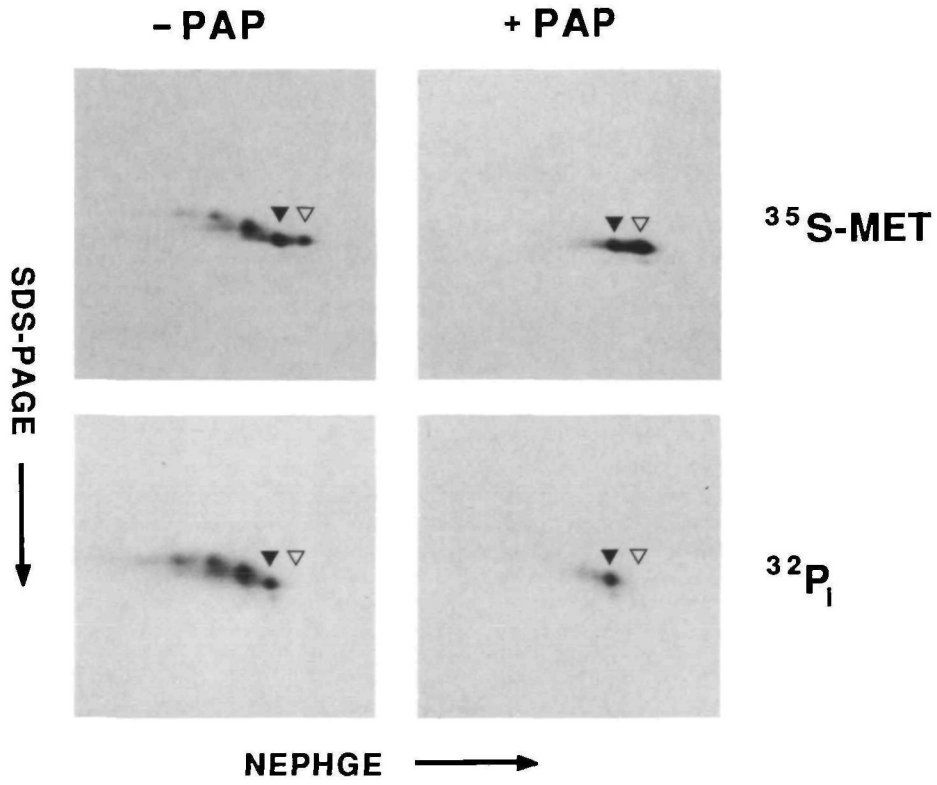


Fig. 3. *In vivo* labeling and phosphatase treatment of UBX IIa. UBX IIa was labeled *in vivo* with either [^{35}S]methionine ([^{35}S]MET) or [^{32}P]orthophosphate ($^{32}P_i$). Labeled UBX IIa was purified by immunoprecipitation with anti-UBX polyclonal antiserum and the immunoprecipitates were treated with (+) or without (−) potato acid phosphatase (PAP). After separation on 2D gels as described in Fig. 2C, proteins were visualized by autoradiography. The open triangles indicate the position at which the unphosphorylated species (spot 1 in Fig. 2C) migrates; the filled triangles indicate the position after the addition of the smallest increment of phosphate (spot 2 in Fig. 2C). For both the −PAP and +PAP experiments, the panels are aligned so that the triangles of the bottom panels can be superimposed on those of the top panels. The +PAP/[^{35}S]MET panel is underexposed relative to the −PAP/[^{35}S]MET panel to permit visualization of both spots. At the appropriate exposure, it is evident that the total amount of radioactivity in the spots prior to treatment with PAP is the same as that present in the two spots after treatment.

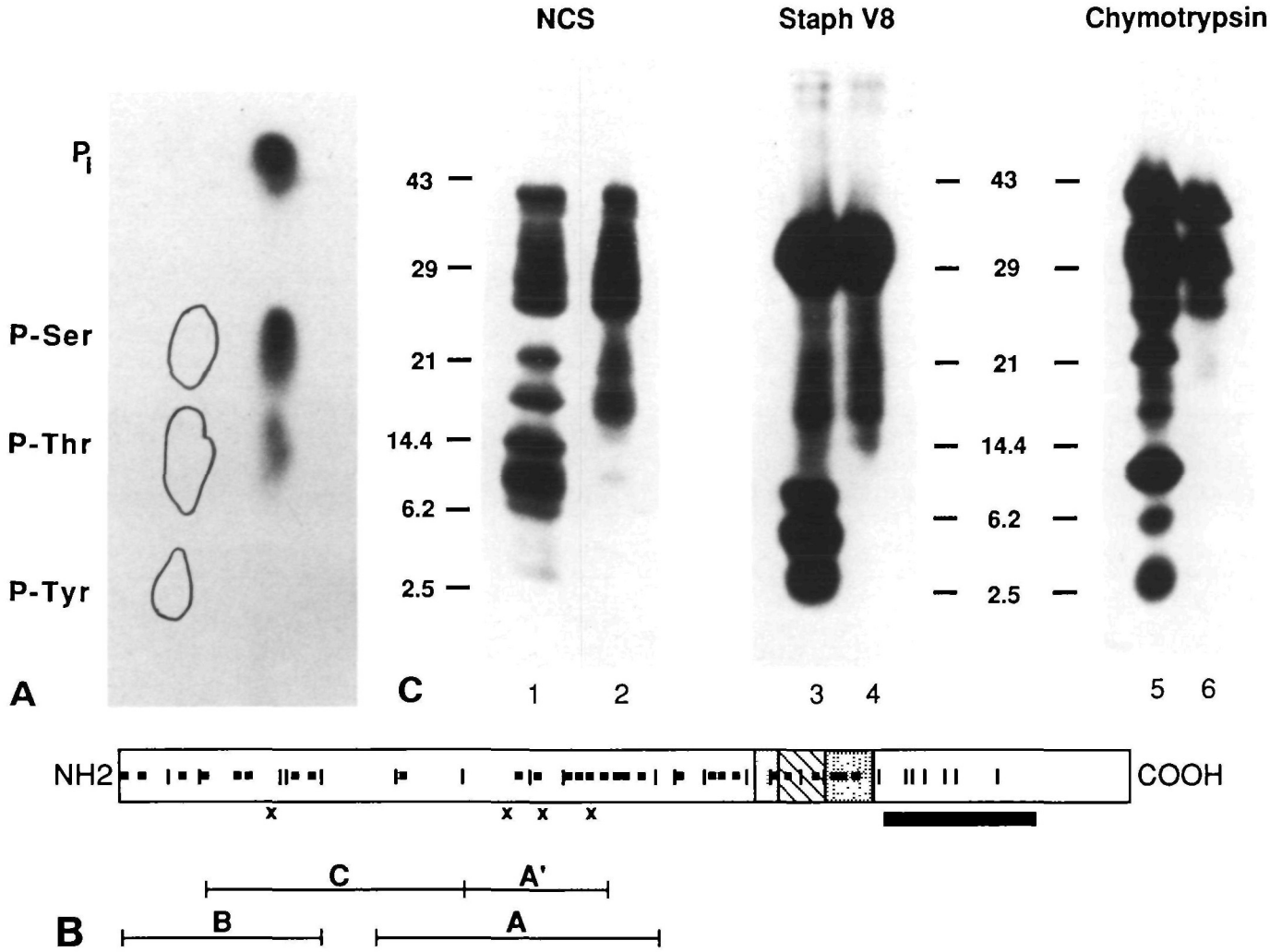


Fig. 4. Phosphoaminoacid analysis and localization of phosphorylated residues. (A) UBX proteins, labeled *in vivo* with $^{32}P_i$, were subjected to acid hydrolysis and separated by thin layer electrophoresis in the presence of excess unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). Labeled amino acids were visualized by autoradiography as shown for UBX Ia; unlabeled standards were visualized by staining with ninhydrin and their locations are indicated in outline form. The location of free $^{32}P_i$ is indicated. Hydrolysis of the other UBX isoforms produced similar results. That the ^{32}P-labeled phosphoserine and phosphothreonine derive exclusively from UBX proteins was demonstrated by showing that a sample, labeled and immunoprecipitated in the same manner from the control cells, yielded no labeled contaminants comigrating with any UBX isoforms on the SDS–polyacrylamide gels used in the final purification steps for the labeled UBX proteins (see text and Materials and methods). (B) The structure of the longest isoform, UBX Ib is shown. The open boxes represent the coding sequences in the N-constant and C-constant regions common to all isoforms; the lightly stippled box represents the 9 amino acid 'b' region; the slashed and darkly stippled boxes represent the two 27 amino acid elements. The location of the homeodomain is indicated by the filled bar below. Serines and threonines are represented by the solid squares and bars, respectively. The regions A, A', B, and C, within which the phosphorylated residues lie, are indicated. X's represent arginine residues which fall near serines and threonines within the phosphorylated regions. (C) UBX Ia labeled *in vivo* with either $^{32}P_i$ or [^{35}S]MET was subjected to partial cleavage with either *N*-chlorosuccinimide (NCS), *Staphylococcal* V8 protease or chymotrypsin. The products were resolved on 15% SDS–polyacrylamide gels, followed by fluorography and autoradiography. Lanes 1, 3, and 5 contain cleavage products of ^{35}S-labeled protein, lanes 2, 4, and 6 contain cleavage products of ^{32}P-labeled protein. The reagent used to generate the cleavage pattern of each pair of lanes is indicated. Relative molecular mass markers: ovalbumin, 43×10^3; carbonic anhydrase, 29×10^3; soybean trypsin inhibitor, 21×10^3; myoglobin fragments, 14.4×10^3, 6.2×10^3, 2.5×10^3.

and threonine residues but not on tyrosine (Fig. 4A). Although the ratio of labeled serine to threonine varied slightly among the isoforms, in each case 2 to 3 times as many serines are phosphorylated as threonines.

Fig. 4B shows the distribution of the 28 serines and 23 threonines in UBX Ib. Because the C-constant region is devoid of serine residues, their phosphorylation must be restricted to the N-constant region and the optional elements. Given that the isoforms display similar patterns on 2D gels, it would be surprising if residues within the optional elements were phosphorylated. These considerations lead to the expectation that

phosphorylated serines will be restricted to the N-constant region, while phosphorylated threonines could exist in either constant region.

To map the phosphorylated residues, we first analyzed fragments generated by protease or chemical cleavage of UBX protein labeled *in vivo* for the presence of phosphate. Immunoprecipitated, gel-purified UBX Ia labeled with either [^{35}S]MET or $^{32}P_i$ was cleaved in separate experiments at tryptophan residues with the oxidizing agent *N*-chlorosuccinimide (NCS), at aspartate and glutamate with *Staphylococcus* V8 protease, and at tyrosine, tryptophan, and phenylalanine with chymotrypsin. For each reagent, fragments of ^{32}P- and ^{35}S-labeled protein were separated in adjacent lanes of SDS–polyacrylamide gels (Fig. 4C). Because partial cleavages occurred under the conditions used for these experiments, the identities of the proteolytic fragments were confirmed by probing similar digests of unlabeled UBX protein produced in *E. coli* with antibodies against epitopes from different regions of the protein (J. Lopez, E.R.G., and D.S.H, unpublished). Comparison of the subset of ^{32}P-labeled fragments with the full complement of ^{35}S-labeled fragments, and the location of serines and threonines in the protein sequence demonstrated that the phosphorylated residues are restricted to the two parts of the N-constant region labeled A (109 aa) and B (60 aa) in Fig. 4B, with the large majority in region A.

We have also examined the set of UBX IVa deletion derivatives shown in Fig. 5A for their ability to be phosphorylated in S2 cells. After transient transfection into S2 cells, the expression plasmids for these derivatives (see Materials and methods) yielded UBX proteins of the appropriate length and in approximately equal amounts (Fig. 5B). Analysis of these proteins on 2D gels (Fig. 5C) showed that both both IVa$^{\Delta 39-183}$ and IVa$^{\Delta 37-225}$ migrated as single spots, which were not affected by treatment of immunoprecipitated protein with PAP (data not shown). This result indicates that all phosphorylated residues are located between residues 39 and 183 of the N-constant region, which is coextensive with the C+A′ region delineated in Figs 4B and 5A. Consistent with this conclusion, IVa$^{\Delta 179-226}$ displayed a wild-type pattern of at least 5 spots while phosphorylation was significantly reduced for IVa$^{\Delta 39-130}$ (region C) and IVa$^{\Delta 131-183}$ (region A′), with the latter showing the greater reduction as indicated by the very low level of only one phosphorylated species (Fig. 5C).

These results are consistent with the previous finding, given that A′ lies within the A region and C overlaps both the A and B regions (Fig. 4B). Furthermore, the monoclonal antibody 3F.6, whose epitope has been mapped to the A′ region and which reacts with all unphosphorylated UBX proteins (J. Lopez and D.S.H., in preparation), exhibited little if any interaction with the more highly modified protein (Fig. 6). The sum of our results therefore indicates that most of the phosphorylated residues are located within the A region, with the remainder in C.

Promoter activation and repression activities of UBX deletion proteins

We assayed the ability of the UBX IVa deletion mutants shown in Fig. 5 to function as transcriptional regulators by transient cotransfection of S2 cells with UBX expression plasmids and reporter plasmids containing P_{Ubx} or P_{AntpP1} fused to the chloramphenicol acetyl transferase gene (CAT; Krasnow *et al.* 1989). UBX IVa caused an 8- to 12-fold stimulation of P_{Ubx}CAT and a 20-fold repression of P_{AntpP1}CAT (Table 1). UBX IVa$^{\Delta 37-225}$ behaved similarly to the equivalent UBX Ib deletion mutant tested previously (Krasnow *et al.* 1989); i.e. this deletion completely abolished stimulation of P_{Ubx}, showing instead a slight repression of this promoter (1.1- to 1.4-fold), while retaining part, but not all, of the P_{AntpP1} repression activity. None of the other four UBX IVa deletions exhibited a significant loss of P_{AntpP1} repression activity, suggesting that phosphorylation may not be relevant to this function.

The same conclusion might be drawn about the role of phosphorylation in P_{Ubx} activation, given that UBX IVa and the UBX IVa$^{\Delta 39-183}$ mutant, which deletes the entire phosphorylation region (A′+C; Fig. 5A), exhibit little significant difference in the P_{Ubx}CAT activities they induce (Table 1). In the case of activation,

Table 1. *Regulatory effects of UBX IVa and mutant derivatives*

Expression plasmid	P_{Ubx}CAT (A)		P_{Ubx}CAT (B)		P_{AntpP1}CAT	
	Activity	Fold	Activity	Fold	Activity	Fold
Ib$^{S/B}$	40±0.3	1	74±4	1	626±19	1
IVa	482±35	12	564±7	8	29±6	0.05
Δ39–130	85±10	2	173±8	2	29±6	0.05
Δ131–183	271±15	7	327±38	4	34±0.2	0.05
Δ179–226	182±13	4.5	234±44	3	20±0.2	0.03
Δ39–183	226±44	6	449±61	6	20±1	0.03
Δ37–225	35±0.6	0.9	49±2	0.7	218±36	0.35

CAT activity assays were performed on S2 cells cotransfected with UBX protein expression plasmids and either P_{Ubx}CAT or P_{AntpP1}CAT reporter plasmids. CAT activity was assayed 60 h after transfection (Krasnow *et al.* 1989) and is given in pmoles of chloramphenicol acetylated per mg of extract protein per minute, and is the average and standard deviation of duplicate transfections for P_{Ubx}CAT (A) and P_{AntpP1}CAT and for triplicate transfections for P_{Ubx}CAT (B). The values for the control plasmid Ib$^{S/B}$ were used as the basal promoter activity to calculate the fold stimulation of P_{Ubx}CAT or repression of P_{AntpP1}CAT. Each column represents a set of transfections performed on one day. Two experiments (A) and (B), performed on different days, are presented for P_{Ubx}CAT to show the variation in the relative abilities of the different proteins to stimulate transcription. The level of UBX IVa protein and of its deletion derivatives was monitored as shown in Fig. 5B (see text). Measurements of P_{Ubx}CAT stimulation at 36 h after transfection, rather than at the standard 60 h time, indicated that the ability of the Δ39–130 deletion, but not of IVa or any of the other deletions, to stimulate P_{Ubx} decreased with time after transfection. This was a reproducible observation that did not result from loss of the Δ39–130 protein; levels of Δ39–130 and wild-type IVa proteins were constant at 36, 48, and 60 h after transfection, when monitored as indicated in Fig. 5B. Presumably, the Δ39–130 deletion renders this protein uniquely sensitive to some changing components(s) in the S2 cells.

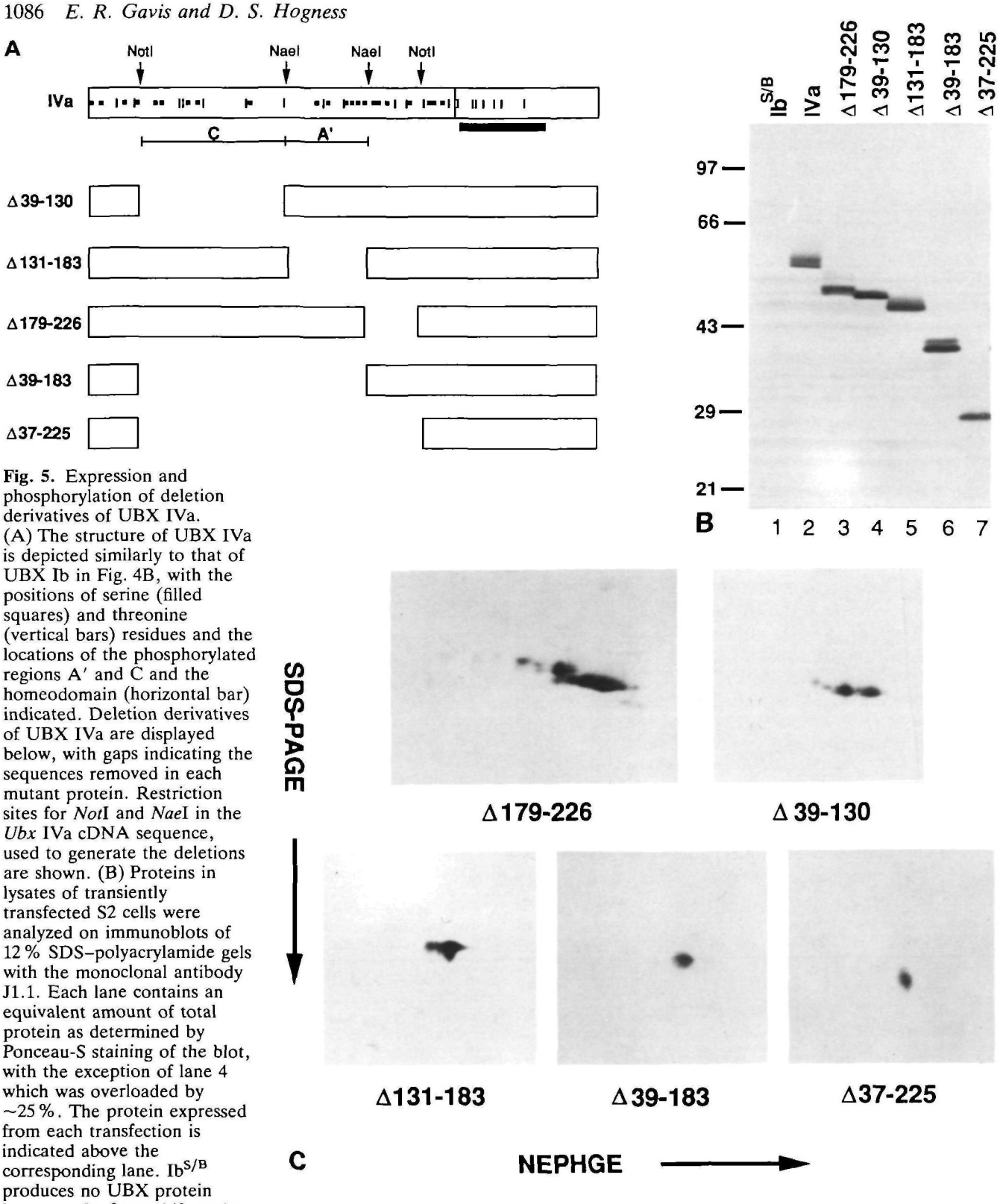


Fig. 5. Expression and phosphorylation of deletion derivatives of UBX IVa. (A) The structure of UBX IVa is depicted similarly to that of UBX Ib in Fig. 4B, with the positions of serine (filled squares) and threonine (vertical bars) residues and the locations of the phosphorylated regions A′ and C and the homeodomain (horizontal bar) indicated. Deletion derivatives of UBX IVa are displayed below, with gaps indicating the sequences removed in each mutant protein. Restriction sites for *Not*I and *Nae*I in the *Ubx* IVa cDNA sequence, used to generate the deletions are shown. (B) Proteins in lysates of transiently transfected S2 cells were analyzed on immunoblots of 12 % SDS–polyacrylamide gels with the monoclonal antibody J1.1. Each lane contains an equivalent amount of total protein as determined by Ponceau-S staining of the blot, with the exception of lane 4 which was overloaded by ~25 %. The protein expressed from each transfection is indicated above the corresponding lane. $Ib^{S/B}$ produces no UBX protein because of a frameshift at the eighth codon of UBX Ib (Krasnow *et al.* 1989) and therefore serves as a control. (C) UBX IVa deletion mutants shown in B were further analyzed on immunoblots of 2D gels with J1.1. Panels are labeled below according to the mutant protein shown in each.

however, deletion of different parts of the A′+C phosphorylated region produced different results. While deletion of A′ (Δ131–183), like deletion of A′+C, had little significant effect on P_{Ubx} activation, deletion of C (Δ39–130) had an obviously significant effect, reducing the P_{Ubx} activation to 24 % ± 7 % (mean

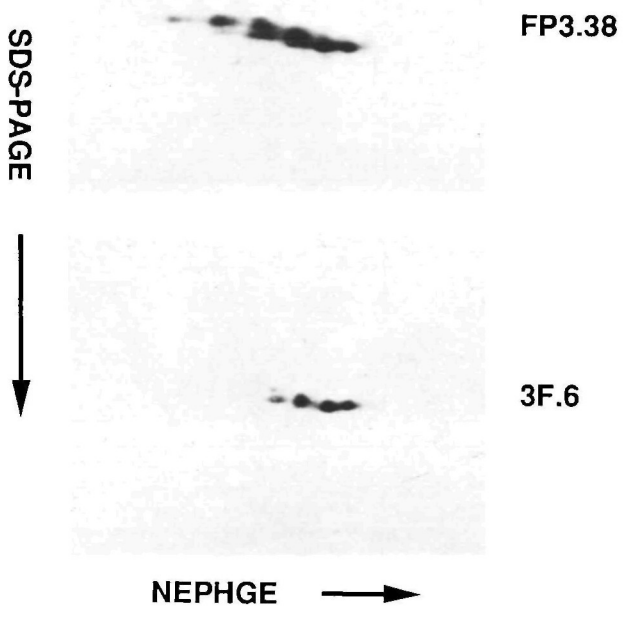


Fig. 6. A monoclonal antibody is sensitive to the phosphorylation state of UBX proteins. Monoclonal antibodies FP3.38 and 3F.6 (J. Lopez and D.S.H, unpublished) were used to probe immunoblots of UBX IIa separated on 2D gels. Identical conditions were used for incubations and developing. The intensities of the signals for spots 1 and 2 (as designated in Fig. 2C) are similar for the two antibodies. No additional spots were detected at higher concentrations of the 3F.6 antibody.

and range of the two experiments in Table 1) of that for UBX IVa. Thus, the loss of P_{Ubx}-activating function occasioned by deletion of C can be regained not only by the return of C, but also by the deletion of A′, or by both. Although one can create by regional deletion a nonphosphorylated protein (UBX $IVa^{\Delta 39-183}$) with the activation function, this evidence for regional interactions makes hazardous the inference that phosphorylation is not required for the P_{Ubx}-activating function of the full-length UBX IVa protein.

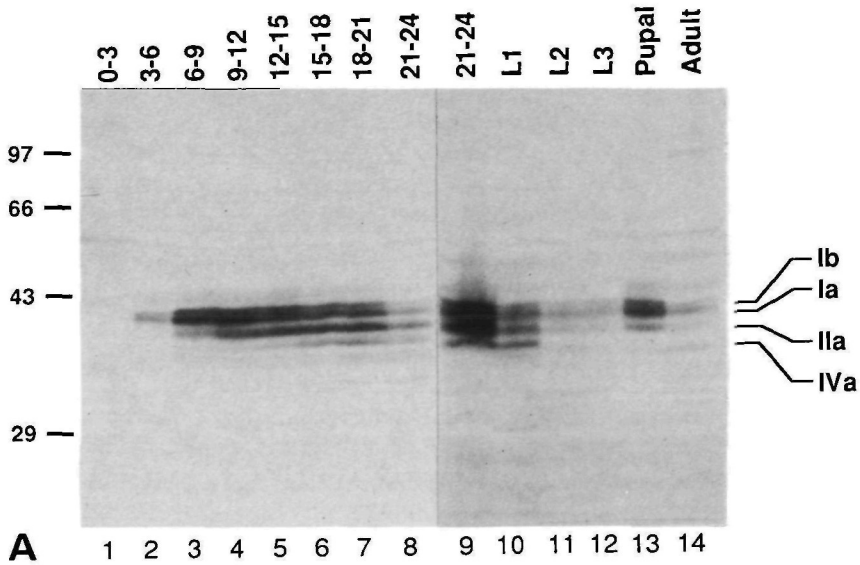
At another level, the P_{Ubx}-activating function that is completely lost as a result of the 189 amino acid Δ37–225 deletion, can be largely, if not entirely, regained by adding back its 42 C-terminal amino acids (as in Δ39–183). However, adding back its 95 C-terminal amino acids (as in Δ39–130) resulted in recovery of only a quarter of that activity, as if the additional 53 amino acids, which comprise the A′ region, have a negative effect on the activation function. Taken together, the data in Table 1 indicate that the N-constant region does not contain a discrete activation domain; rather, interacting activating sequences appear to be distributed over a large part of this region.

Expression and phosphorylation of the endogenous UBX proteins during development

To determine whether the phosphorylation observed in S2 cells occurs during *Drosophila* development, UBX proteins in animals at various developmental stages were examined by immunoblot analyses, with particular detail in embryogenesis. Nuclear lysates were prepared from staged embryos collected at 3 h intervals for analysis of embryonic proteins, whereas each of the major post-embryonic stages were represented by single collections per stage and the proteins derived from whole animal homogenates. (Analysis of whole embryo homogenates proved difficult, but gave results similar to those obtained with nuclear lysates.)

A developmental immunoblot analysis of proteins separated by SDS–PAGE demonstrated the presence of UBX Ib, Ia, IIa, and IVa (Fig. 7A). These proteins could be identified by their M_rs on SDS–PAGE, by their reactivity with antibodies capable of distinguishing between different forms, and by their relative isoelectric points on 2D gels (see below and legend to Fig. 7C). The developmental profile of the UBX proteins closely parallels that of the respective mRNAs (Fig. 7B), suggesting that differential translation of the *Ubx* mRNAs is not an important factor in the regulation of UBX protein expression. In this respect, we note that UBX IIb was not detected on standard or 2D gels. However, since the *Ubx* IIb mRNA is less abundant than the other *Ubx* mRNAs (Kornfeld *et al.* 1989), its protein product may be below our limits of detection. Detection of all UBX isoforms lagged behind that of the respective mRNAs by ~2.5 h.

The electrophoretic heterogeneity of each UBX isoform was not clearly evident on SDS–polyacrylamide gels, in part because of extensive overlap between the modified species of any one isoform with the other isoforms and in part because of significantly lower limits of detection. The embryonic nuclear proteins shown in Fig. 7A were therefore examined on 2D gels to determine whether they are phosphorylated and whether the phosphorylation is developmentally regulated. Fig. 7C shows that the endogenous UBX proteins display similar 2D gel patterns to those of UBX proteins produced in cultured cells. Treatment of immunoprecipitates of embryonic proteins with PAP confirmed that the most basic spot for each isoform was the unphosphorylated species and that, at least for UBX Ia, this species comigrated with the unphosphorylated species of the protein produced in S2 cells when both were resolved on one gel (data not shown). The most highly phosphorylated species were not well resolved, in part because residual yolk protein contaminating the samples obscured that region of the gels and in part because of the limit of detection in these experiments. However, no significant change in the overall extent of phosphorylation was evident during the 0–24 h period. We were unable to employ 2D gel analysis of the proteins at later stages of development because of limitations in the quantity of material that could be analyzed on the NEPHGE gels.


0-3
3-6
6-9
9-12
12-15
15-18
18-21
21-24
21-24
L1
L2
L3
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43
29
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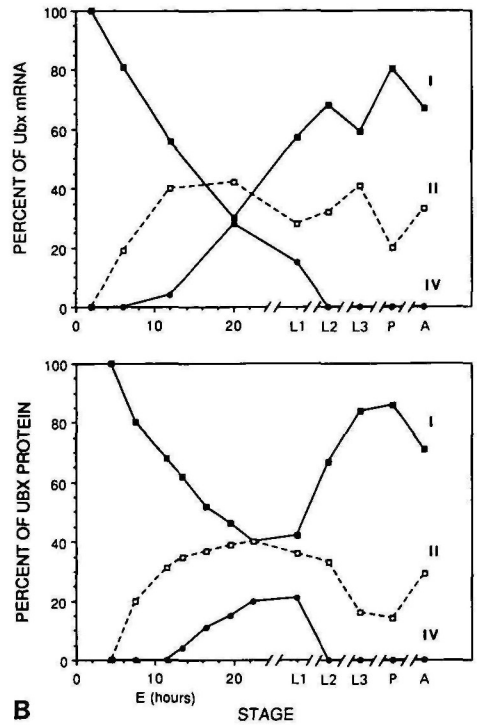
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14


PERCENT OF Ubx mRNA
PERCENT OF UBX PROTEIN
100
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60
40
20
0
I
II
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0 10 20 L1 L2 L3 P A
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STAGE
B

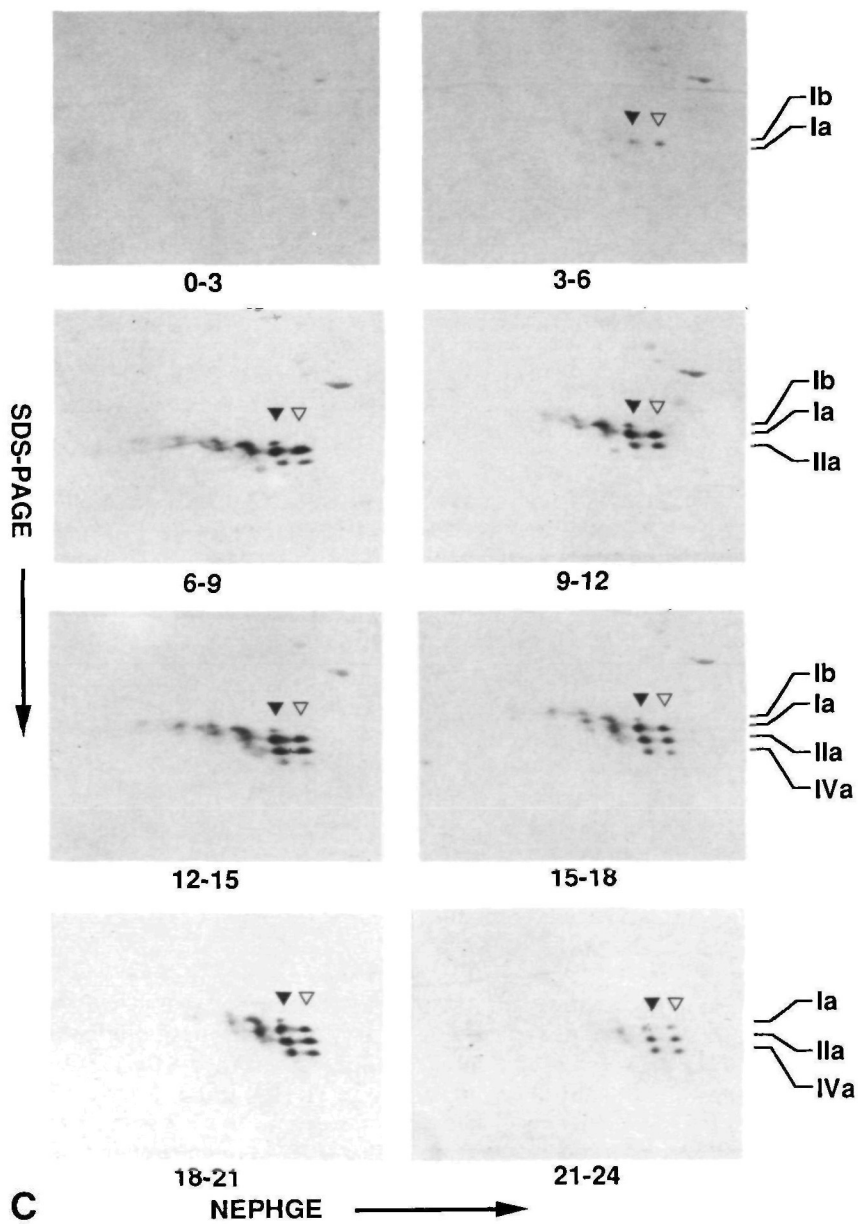
SDS-PAGE
Ib
Ia
IIa
IVa
0-3
3-6
6-9
9-12
12-15
15-18
18-21
21-24
C
NEPHGE

Fig. 7. Developmental analysis of expression and phosphorylation of endogenous UBX proteins. (A) UBX proteins in lysates of nuclei isolated from staged embryos and homogenates of whole larvae, pupae, and adults were analyzed on immunoblots of 10% SDS–polyacrylamide gradient gels probed with the FP3.38 antibody. Equivalent amounts of total protein were loaded for each embryonic time period (lanes 1–8) as determined by Coomassie staining of gels or Ponceau-S staining of nitrocellulose blots. For the larval, pupal and adult time periods (lanes 10–14), equivalent amounts of protein were also loaded but significantly greater quantities were required for detection than for the embryonic samples. To allow comparison with the level of expression in embryos, protein from a homogenate of intact 21–24 h embryos (lane 9) was loaded in an equivalent amount to that in the lanes 10–14. Embryonic time periods are indicated in hours above each lane. Larval time periods correspond to animals harvested during the first (L1), second (L2) and third (L3) larval instars as indicated above each lane. Pupal and adult samples correspond to animals collected throughout these periods. The positions of bands corresponding to UBX Ib, Ia, IIa, and IVa are indicated to the right. (B) Signal intensities of the UBX bands on the immunoblots in A were quantitated by scanning densitometry. Analysis of *Ubx* mRNAs by densitometry measurements of S1 protection experiments was described previously (Kornfeld *et al.* 1989) and the data shown is derived from that study. For each time period, the relative abundance of each mRNA or protein isoform is expressed as a percent of the sum of all *Ubx* forms present at that time. Ia and Ib are considered in sum (I) and are indicated by the filled boxes; IIa+IIb (II) by the open boxes; IVa (IV) by the filled circles. Embryonic time points are plotted as the midpoint of the collection period. Stages: E, embryonic, with numbers indicating hours after egg deposition; L1, first larval instar; L2, second larval instar; L3, third larval instar; P, pupal; A, adult. Breaks in the X axis indicate a discontinuous time scale at later stages. (C) Nuclear proteins from the embryonic time periods shown in A were separated on 2D gels and UBX proteins were detected by immunoblotting with FP3.38. For each panel, the time period is indicated below. The location of spots corresponding to UBX Ib, Ia, IIa, and IVa are indicated. The open triangles indicate the spots corresponding to the unphosphorylated species of Ia, IIa, and IVa (spot 1 in Fig. 2C), the filled triangles indicate the spots after the smallest increment of phosphate has been added (spot 2 in Fig. 2C). The unphosphorylated species for Ib has a higher pI than those for the other proteins and migrates at the position indicated by the open triangle.

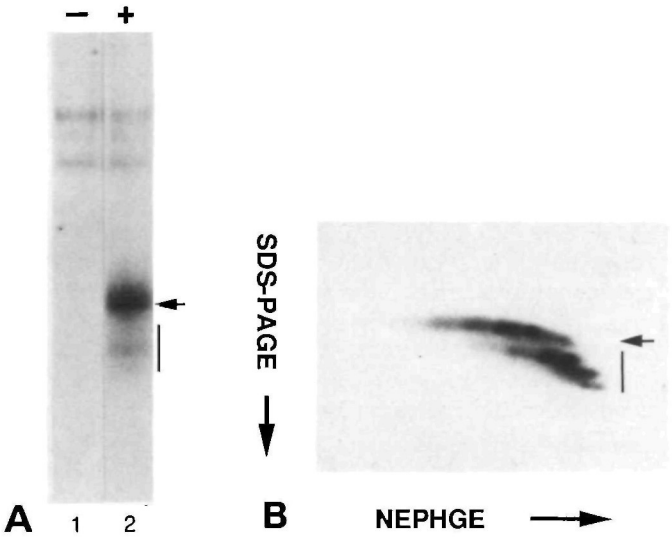

Fig. 8. *In vitro* kinasing of purified UBX protein. (A) UBX Ia protein purified from *E. coli* was incubated with nuclear extract prepared from *Drosophila* Kc cells (Johnson and Krasnow, 1990) in the presence of [γ-^{32}P]ATP (lane 2). A control reaction lacked UBX Ia (lane 1). Following immunoprecipitation, proteins were analyzed on 10% SDS–polyacrylamide gels followed by autoradiography. The location of full-length UBX Ia is indicated by the arrow. Proteolytic fragments generated during the initial purification, which are also labeled, are indicated by the vertical bar. (B) Labeled, immunoprecipitated UBX Ia shown in lane 2 of (A) was analyzed by electrophoresis on 2D gels followed by autoradiography. The arrow indicates the labeled spots resulting from phosphorylation of the full-length protein; the vertical bar indicates phosphorylation of proteolytic fragments.

An activity present in cell extracts can phosphorylate UBX proteins in vitro

UBX proteins expressed in *E. coli* are not phosphorylated as determined by 2D analysis of proteins immunoprecipitated from *E. coli* and treated with or without PAP (data not shown). UBX protein purified from *E. coli* was therefore used as substrate in an *in vitro* assay to search for the activity responsible for phosphorylation *in vivo*. Initial attempts to detect an activity in whole cell extracts of S2 cells demonstrated a low level of incorporation of [γ-^{32}P]ATP into UBX protein (data not shown). However, we were able to achieve substantially higher levels of labeling using nuclear extracts prepared from *Drosophila* Kc cells (Fig. 8A). This difference may reflect the presence of phosphatase activity in the whole cell extracts, which remained despite the addition of phosphatase inhibitors (data not shown), or may indicate enrichment of the kinase activity in the nucleus of Kc cells. 2D gel analysis of the labeled protein revealed a series of labeled spots in a pattern similar to that of the protein expressed in S2 cells and embryos (Fig. 8B). NCS cleavage of this protein demonstrated that the same fragments were phosphorylated as for the UBX protein produced in S2 cells (data not shown), indicating that the activity has the properties expected of the appropriate kinase.

Discussion

We have shown that the naturally occurring UBX proteins as well as UBX proteins expressed in cultured *Drosophila* cells are multiply phosphorylated on serine and threonine residues in the N-constant region. The addition of phosphate produces a characteristic pattern of spots on 2D gels, in which more acidic (i.e. more highly phosphorylated) species show decreased mobility on SDS–PAGE. An effect of phosphorylation on

electrophoretic mobility has been described for a number of other phosphoproteins including histone H1 (Blumenfeld, 1979), the C-terminal domain of RNA polymerase II (Cadena and Dahmus, 1987), the c-*fos* protein (Barber and Verma, 1987), the c-*ets*-1 protein (Pognonec *et al.* 1988), heat shock factor (Sorger and Pelham, 1988), GAL4 (Mylin *et al.* 1989), and retinoblastoma protein (Ludlow *et al.* 1989). It has been suggested that the anomalous behavior may be due to a conformational change of the protein or of a particular domain upon phosphorylation, perhaps altering its ability to be unfolded by SDS (Billings *et al.* 1979). Strikingly, a truncated UBX IVa protein produced by a frameshift within the homeodomain (described in Mann and Hogness, 1990) is multiply phosphorylated but shows no shift in mobility of any of the phosphorylated species (E.R.G. and D.S.H., unpublished). This result suggests that specific sequences near the C terminus may interact with a domain in the N-constant region to produce a phosphorylation-dependent conformational change.

The two regions within which the phosphorylated residues are located are highly conserved at the amino acid level among *Drosophila melanogaster* and its sibling species *Drosophila funebris* and *Drosophila pseudobscura* (Wilde and Akam, 1987). The A′ region (Fig. 4B), which shows 100% conservation, contains 6 serines and 2 threonines. Region C is 80% conserved and contains 5 serines and 5 threonines; all but two serines and one threonine are conserved. Arginines, which identify the sites of a number of serine/threonine kinases (reviewed in Edelman *et al.* 1987), fall within 1–2 residues of three serines located within A′ and of one threonine found within C, and all are conserved. Within A′, one of the arginines lies 2 amino acids C-terminal to serine 177, a position found to be important in peptide substrates of phosphorylase kinase (reviewed in Kemp, 1988). Although the surrounding sequences are highly conserved overall, the conservation of serines and threonines with surrounding arginine residues suggests that they are likely targets for phosphorylation.

A growing number of DNA-binding proteins known or thought to be direct transcriptional regulators have been shown to be phosphorylated on serine and/or threonine residues. These include three *Drosophila* homeodomain proteins in addition to UBX (*engrailed:* Gay *et al.* 1988; *fushi tarazu:* Krause *et al.* 1988; and *bicoid:* Driever and Nusslein-Volhard, 1989), the simian virus 40 large T antigen (Scheidtmann *et al.* 1982), the yeast heat shock factor (HSF; Sorger *et al.* 1987), the cAMP response element binding protein (CREB; Yamamoto *et al.* 1988), the mammalian serum response factor (SRF; Prywes *et al.* 1988), the yeast transcriptional activators GAL4 (Mylin *et al.* 1989) and ADR1 (Cherry *et al.* 1989), the adenovirus-induced transcription factors E2F (Bagchi *et al.* 1989) and E4F (Raychaudhuri *et al.* 1989), the retinoblastoma protein (Ludlow *et al.* 1989), and the c-*myb* proto-oncogene product (c-Myb; Luscher *et al.* 1990).

Phosphorylation has been demonstrated to modulate the activity of some of these proteins. The DNA-binding activity of E2F (Bagchi *et al.* 1989), E4F (Raychaudhuri *et al.* 1989), and SRF (Prywes *et al.* 1988) requires phosphorylation. Reciprocally, phosphorylation decreases the affinity of the c-*ets*-1 protein for DNA (Pognonec *et al.* 1989) and the site-specific DNA-binding activity of c-Myb (Luscher *et al.* 1990). Phosphorylation of CREB by protein kinase C increases its ability to bind DNA by facilitating formation of the higher affinity dimer form (Yamamoto *et al.* 1988). Phosphorylation affects the ability of other proteins to modulate transcription without altering their DNA-binding properties. Temperature-dependent phosphorylation of HSF does not affect DNA-binding but is necessary for transcriptional activation (Sorger and Pelham, 1988). Phosphorylation of residues in a localized region of a protein might help to create an acidic domain or an amphipathic α helix much like that proposed for yeast activators (Ptashne, 1988), permitting these proteins to interact with RNA polymerase or with accessory transcription factors. Alternatively, as proposed for the regulation of CREB activity by protein kinase A, phosphorylation may alter the conformation of the protein allosterically to allow a different region of the protein to interact with the transcriptional machinery (Gonzalez and Montminy, 1989; Yamamoto *et al.* 1990). Direct evidence for the effect of phosphorylation on protein–protein interaction has been demonstrated for CREB as previously described and for the complex between T antigen and the underphosphorylated form of retinoblastoma protein (Ludlow *et al.* 1989).

Phosphorylation-modulated UBX protein activities have not yet been identified. Sequence-specific DNA binding does not require phosphorylation, as UBX Ib purified from *E. coli* exhibits such binding (Beachy *et al.* 1988) and is not phosphorylated (E.R.G. and D.S.H, unpublished). UBX proteins partially purified from isoform-specific S2 cells exhibited DNA-binding specificities similar to that for *E. coli* protein; however, dephosphorylation occurred during purification, and the relative contributions of the phosphorylated and unphosphorylated forms to the binding were not known (E.R.G. and D.S.H., unpublished).

In transient cotransfection experiments, a UBX IVa protein from which the 145 amino acid phosphorylated region has been deleted (UBX IVa$^{\Delta 39-183}$; Fig. 5) did not differ significantly from the full-length UBX IVa in its promoter activation and repression functions (Table 1). While this result demonstrates that phosphorylation is not required for promoter regulation by this deleted UBX protein, it does not eliminate the possibility that phosphorylation of the full-length protein is critical to this regulation (see Results). For example, phosphorylation might neutralize a negatively acting domain, deletion of which would restore the function of the protein and obviate the need for the phosphorylated modification. Models of this sort can be tested by examining the regulatory properties of mutant UBX proteins in which the relevant serine and threonine residues have been individually changed.

In this same context, a deletion derivative of UBX Ia lacking residues 37–225 of the N-constant region (UBX $Ia^{\Delta 37-225}$) has previously been examined for its ability to transform the identities of head and thoracic metameres to that of parasegment 6 – a property exhibited by the parent UBX Ia protein when ectopically expressed (Mann and Hogness, 1990). Ectopic expression of UBX $Ia^{\Delta 37-225}$ effected the same identity transformations as the parent protein, but at reduced efficiency. Like its UBX $IVa^{\Delta 37-225}$ counterpart (Fig. 5), UBX $Ia^{\Delta 37-225}$ is not phosphorylated (E.R.G. and D.S.H., unpublished). Clearly, the same arguments regarding the phosphorylation requirement for the identity functions of the full-length protein as made above for the cotransfection experiments apply here. Finally, unphosphorylated UBX Ib purified from *E. coli* can stimulate transcription *in vitro* (Johnson and Krasnow, 1990); however, under the conditions used in these experiments, the added protein may have been phosphorylated *in vitro*.

While it is possible that phosphorylation serves some other function than direct modulation of UBX regulatory activity (e.g. modulating solubility or stability), we think it more likely that it does indeed alter UBX regulatory activities, but in a manner that our current assays have not been designed to detect. Phosphorylation may, for example, alter the ability of UBX to interact with other proteins to form regulatory complexes that provide further specialization of its cellular functions at the level of metameric position, tissue type, or developmental stage. A possible example of tissue-specific UBX phosphorylation has recently been observed (J. Lopez and D.S.H., unpublished) using *in situ* labeling of UBX proteins with antibodies that are specifically deficient in the recognition of the more highly phosphorylated UBX states, as shown in Fig. 6 for the monoclonal antibody 3F.6. These observations indicate that UBX proteins in the embryonic central nervous system (CNS) are underphosphorylated relative to those in the embryonic epidermis and mesoderm. It is an open and interesting question as to whether this type of tissue specificity is dependent upon or independent of another form of UBX tissue specificity in which isoforms II and IV are localized to the CNS, while isoform I proteins are found in the epidermis and mesoderm but never in the CNS (J. Lopez and D.S.H., in preparation).

Identification and characterization of the kinase that catalyzes UBX phosphorylation, and of its gene, will constitute an important step toward answering this and other questions concerning the developmental role of UBX phosphorylation. The activity that we have identified in nuclear extracts of Kc cells appears likely to be the same as that detected by *in vivo* phosphorylation experiments since it produced a similar pattern of labeled UBX protein on 2D gels as well as similarly labeled NCS cleavage products. It is therefore of considerable interest that we now have a functional assay for the purification of this kinase activity, using UBX proteins purified from *E. coli* as substrates. A number of serine/threonine kinases have been identified in or isolated from *Drosophila* (reviewed in Hunter, 1987; Hanks *et al.* 1988). However, only one of the serine and threonine residues within the phosphorylated regions of UBX is in a context that exhibits specific features of a target of a known kinase; hence it seems likely that the kinase responsible for phosphorylating UBX is not among the *D. melanogaster* kinases whose targets have been characterized. Two segment polarity genes, *zeste-white3* (Siegfried *et al.* 1990) and *fused* (Preat *et al.* 1990), which belong to the same genetic regulatory hierarchy containing *Ubx*, have recently been shown to encode proteins exhibiting sequence homology to serine/threonine kinases. As part of a program to identify the gene(s) encoding the UBX kinase, it clearly would be of interest to examine the effects of mutations in these genes on UBX phosphorylation.

Although initially designed for the analysis of UBX phosphorylation, our examination of UBX deletion mutants in cotransfection experiments (Fig. 5A, Table 1) has extended our understanding of the structural requirements for UBX regulation beyond previous work (Krasnow *et al.* 1989; Samson *et al.* 1989; Mann and Hogness, 1990). In particular, our results indicate that the N-constant region, which had previously been shown to harbor promoter activation sequences, does not contain a discrete activating domain. Rather, multiple activation sequences appear to be distributed throughout the 189 amino acid region defined by the $IVa^{\Delta 37-225}$ deletion mutant that lacks the promoter activation function defined in the cotransfection assays. Given this characteristic and the absence of an acidic region or regions rich in proline or glutamine residues, which have been shown to be important for other eukaryotic activators (Hope and Struhl, 1986; Ma and Ptashne, 1987; Courey and Tjian, 1988; Mermod *et al.* 1989), it would appear that promoter activation by UBX proteins involves mechanisms different from these activators.

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