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Phosphorylation of the *Drosophila* Engrailed Protein at a Site Outside Its Homeodomain Enhances DNA Binding*

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The *engrailed* gene encodes a homeodomain-containing phosphoprotein that binds DNA. Here, we show that engrailed protein is posttranslationally modified in embryos and in embryo-derived cultured cells but is essentially unmodified when expressed in *Escherichia coli*. Engrailed protein produced by bacteria can be phosphorylated in nuclear extracts prepared from *Drosophila* embryos, and phosphotryptic peptides from this modified protein partly reproduce two-dimensional maps of phosphotryptic fragments obtained from metabolically labeled engrailed protein. The primary embryonic protein kinase modifying engrailed protein is casein kinase II (CK-II). Analysis of mutant proteins revealed that the *in vitro* phosphoacceptors are mainly clustered in a region outside the engrailed homeodomain and identified serines 394, 397, 401, and 402 as the targets for CK-II phosphorylation. CK-II-dependent phosphorylation of an N-truncated derivative of engrailed protein purified from bacteria increased its DNA binding 2-4-fold.

Homeodomain proteins represent a large and evolutionarily conserved family of proteins that play critical regulatory roles in the development of most organisms. In *Drosophila*, homeodomain proteins have been shown to bind specific DNA sequences with high affinity (1, 2) and to regulate RNA synthesis *in vitro* (3–5) and *in vivo* (6, 7). Despite these and many other related studies, our understanding of how the activity of homeodomain proteins is regulated is rudimentary.

One important unresolved problem is the manner by which homeodomain proteins select their targets, since most bind DNA *in vitro* with similar affinities and specificities (2, 8–11). It is likely that their specificity for promoter selection is achieved through interactions with other proteins, and examples of interactions with homeodomain proteins that are relevant include the interactions between the α-2 repressor and the MCM-1 and a1 proteins of *Saccharomyces cerevisiae* (12, 13) and the interactions of the *Drosophila* extradenticle protein with the engrailed, Ultrabithorax, Antennapedia, and Abdominal-A homeodomain proteins (14, 15). These interactions appear to involve contacts both within and outside of the homeodomain (16, 17).

An alternative mechanism that might help to regulate the activity of homeodomain proteins is posttranslational modification. Protein phosphorylation has been demonstrated to be an effective means of regulating an increasing number of cellular processes, and a number of homeodomain proteins have been shown to be phosphorylated. These include engrailed (18), fushi tarazu (19), bicoid (6), Hox-1.3 (20), Oct1 (21), and Ultrabithorax proteins (22). Although the function of these modifications has not been established, phosphorylation of homeodomain proteins could in principle affect their interactions with DNA or other proteins.

In the present study, we have analyzed the phosphorylation states of *Drosophila* engrailed protein (En). This homeodomain protein is required for the development of the posterior compartments during embryogenesis and subsequent larval and pupal development, and it is essential to the processes that maintain compartment and segment borders (23, 24). En has 552 amino acids, with a homeodomain near its C terminus and with regions rich in glutamine, alanine, serine, or acidic residues distributed elsewhere in its sequence (25). Here, we show that En is posttranslationally modified by casein kinase II (CK-II),[1] a widely conserved growth-related protein kinase (reviewed in Ref. 26). Furthermore, we identify the major *in vitro* phosphoacceptors and show that phosphorylation by purified CK-II stimulates DNA binding.

MATERIALS AND METHODS

Plasmid Constructions and Polymerase Chain Reaction Mutagenesis—For routine subcloning and plasmid amplification, XL1-Blue (Stratagene) strain was used; for bacterial expression of En and its truncated derivatives, BL21(DE3) (27) strain was used.

To express En derivatives in *Escherichia coli*, the various portions of the *engrailed* coding sequence were inserted into appropriate pAR vectors (54). To express full-length En, the sequence surrounding the ATG of *engrailed* was converted to an *Nde*I site by site-directed mutagenesis, and a 1.76-kilobase *Nde*I fragment (nucleotides 178-1938 of the c-2.4 *en* cDNA (25) containing the entire coding region was inserted into the unique *Nde*I of pAR3038. Most of the En derivatives were constructed from a *Bam*HI partial cleavage of this expression plasmid, pT7En. Expression of these constructs yield fusion proteins with the first 11 amino acids of the gene 10 protein. The expression vector pT7En-1/298 was obtained by subcloning a 888-base pair *Nde*I-*Bam*HI fragment into pAR3040. Note that En-1/298 contains a C-terminal dipeptide (GC) derived from the parental vector. The expression vector pT7En-228/552 was obtained by inserting a blunted 1.23-kilobase *Nae*I-*Eco*RV fragment (obtained from pT7En) into the blunted/dephosphorylated *Bam*HI site of pAR3039.

pHB40P was constructed by digesting pAR3040 with *Xba*I, filling in and redigesting with *Nde*I and *Bam*HI prior to inserting a polylinker using two complementary oligonucleotides: DEL-2 (5′-TATGGTACCTCTAGACTCGAGGG-3′) and DEL-1 (5′-GATCCCCTCGAGTCTAGAGGTACCA-3′). pHB90 was constructed by inserting a 908-base pair *Bam*HI fragment (obtained from a partial *Bam*HI digestion of pT7En) into pHB40P. Nested deletion mutants were generated by exonuclease III followed by S1 nuclease digestions of pHB90 cleaved at the *Kpn*I and

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[1] The abbreviations used are: CK-II, casein kinase II; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.

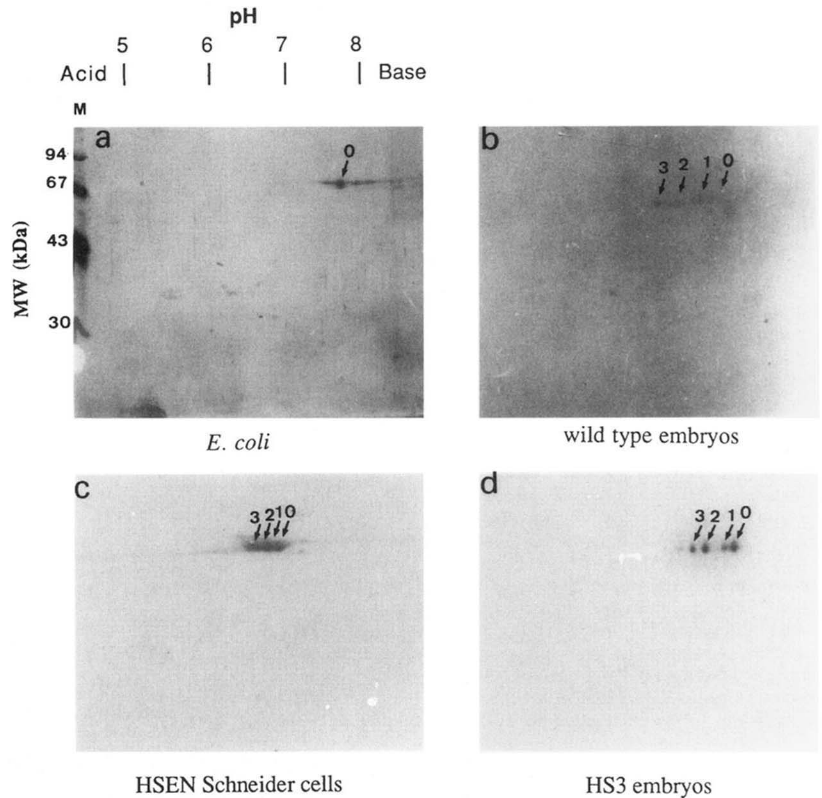


FIG. 1. **Two-dimensional analysis of En modifications in *Drosophila* embryos and tissue culture cells.** Proteins were resolved in the first dimension by isoelectric focusing (*a*, *b*, *d*; separation *right* to *left*) or by non-equilibrium pH gradient electrophoresis (*c*, separation *left* to *right*) and in the second dimension (*top* to *bottom*) by SDS-PAGE (9% gels). Proteins were electrotransferred to nitrocellulose membranes and Western blotted to detect En. *a*, silver staining analysis of a protein extract (1 μg) highly enriched in full-length En expressed in and purified from *E. coli* (as described under "Materials and Methods"). The apparent isoelectric point (7.75) was determined by measuring the pH value of solutions containing pieces of sectioned isoelectric focusing gels. *b* and *d*, Western analysis of En obtained from a nuclear extract prepared from 2–12-h Oregon-R embryos (40 μg of soluble nuclear proteins) or from heat-shocked HS3 embryos of 2–12 h of development (total embryonic proteins from about 300 embryos heat shocked at 37 °C for 1 h), respectively. *c*, Western analysis of En in heat-shocked cultured HSEN cells (50 μg). The relative position of each spot is *marked* and *numbered*. *Lane M*, molecular mass markers. Note that En migrates with an apparent molecular mass of approximately 70 kDa.

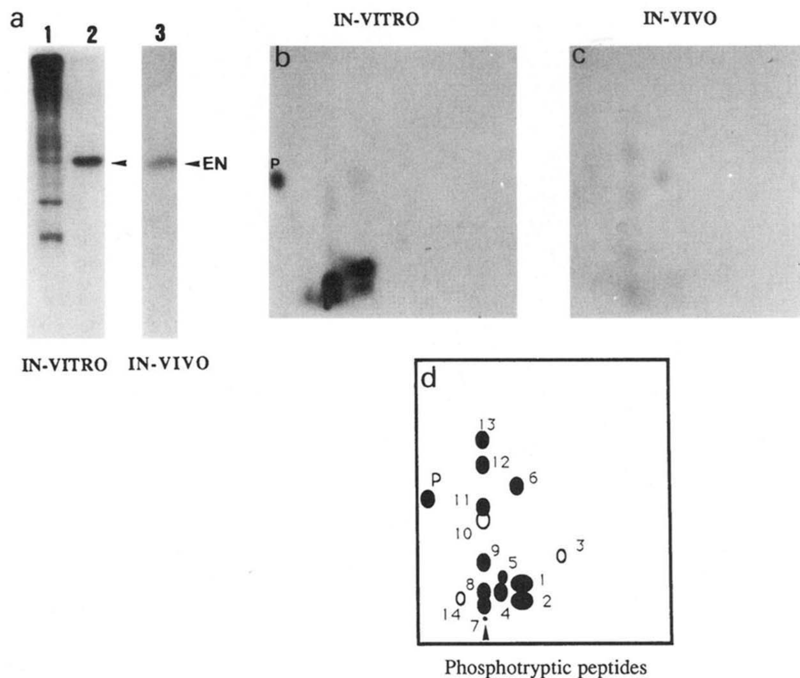


FIG. 2. **Comparative two-dimensional phosphopeptide maps of En phosphorylated *in vivo* and *in vitro*.** The *left part* shows *in vitro* and *in vivo* ^{32}P-labeled En. Samples were analyzed by SDS-PAGE (9% gels) followed by autoradiography. *Lane 2*, partially purified En (1 μg) was incubated with [γ-^{32}P]ATP in presence of 5 μg of soluble nuclear proteins prepared from 2–12-h embryos. *Lane 1*, Control lane analyzing phosphorylated proteins in the nuclear extract (25 μg). *Lane 3*, *In vivo* ^{32}P-labeled En after heat induction of cultured HSEN cells. Metabolically labeled En was immunopurified as detailed under "Materials and Methods." Exposure times were as follows: *lanes 1* and *2*, 10 min; *lane 3*, 2 h. The *arrows* indicate the positions of En. The *right part* shows a comparative fingerprinting analysis of phosphotryptic peptides obtained after *in vitro* or *in vivo* ^{32}P labeling of En. Gel-purified En obtained from either labeling experiment was subjected to tryptic digestion. Phosphotryptic peptides were resolved by electrophoresis at pH 1.9 in the horizontal dimension (*anode* to the *left*) and by chromatography (*bottom* to *top*) in the vertical dimension, as described under "Materials and Methods," and autoradiographed. cpm loaded, as detected by Cerenkov radiation, and exposure time, respectively, were as follows. *a*, bacterially made En phosphorylated *in vitro*, 10,000 cpm, 3 h; *b*, *Drosophila* En phosphorylated *in vivo*, 500 cpm, 3 weeks. Experiments were repeated twice and the same patterns were observed. Origin is indicated by a *vertical arrowhead*. *c*, map position of all the detected phosphotryptic peptides that have been arbitrarily *numbered 1–14*. *Filled* and *open spots* represent comigrating and *in vitro* specific phosphotryptic peptides, respectively. The *spot* marked *P* corresponds to free phosphate.

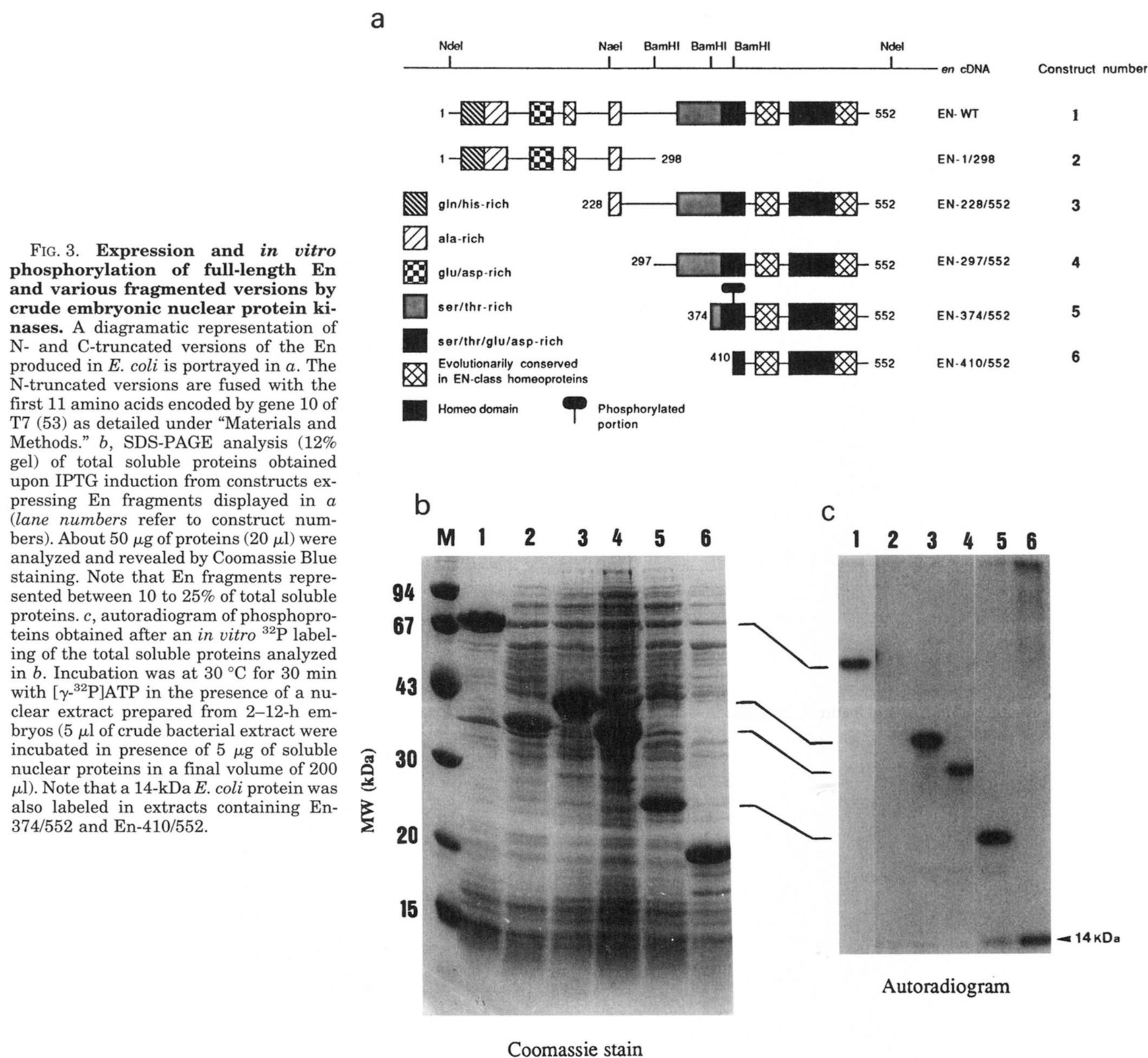


FIG. 3. **Expression and *in vitro* phosphorylation of full-length En and various fragmented versions by crude embryonic nuclear protein kinases.** A diagramatic representation of N- and C-truncated versions of the En produced in *E. coli* is portrayed in *a*. The N-truncated versions are fused with the first 11 amino acids encoded by gene 10 of T7 (53) as detailed under "Materials and Methods." *b*, SDS-PAGE analysis (12% gel) of total soluble proteins obtained upon IPTG induction from constructs expressing En fragments displayed in *a* (*lane numbers* refer to construct numbers). About 50 μg of proteins (20 μl) were analyzed and revealed by Coomassie Blue staining. Note that En fragments represented between 10 to 25% of total soluble proteins. *c*, autoradiogram of phosphoproteins obtained after an *in vitro* ^{32}P labeling of the total soluble proteins analyzed in *b*. Incubation was at 30 °C for 30 min with [γ-^{32}P]ATP in the presence of a nuclear extract prepared from 2–12-h embryos (5 μl of crude bacterial extract were incubated in presence of 5 μg of soluble nuclear proteins in a final volume of 200 μl). Note that a 14-kDa *E. coli* protein was also labeled in extracts containing En-374/552 and En-410/552.

*Xba*I sites (see Fig. 6*a*) as described (55). BL21(DE3) strains freshly transformed with T7 expression vectors expressing N-deleted En versions were selected by immunodetection using mAbs 4D9 (56) as follows. Transformants were directly plated onto nitrocellulose filters on LB/amp plates. Colonies were grown at 37 °C until 1 mm in diameter. From the master filters, two replica nitrocellulose filters (pre-wetted with 10 mM IPTG) were obtained and were placed on LB/amp plate colonies facing upward. Colonies were grown until 1 mm in diameter, filters were washed in 1 × PBS (10 mM sodium phosphate, pH 7.4, 100 mM NaCl) and processed for immunodetection, as described below for Western blots. A number of selected constructs were analyzed by restriction mapping and sequenced using an oligonucleotide corresponding to a portion of the T7 promoter as a primer (oligonucleotide HB-T7, 5′-AATACGACTCACTATAG-3′).

The pHB67 expression vector was constructed by inserting a 677-base pair *Bam*HI fragment (from pT7En) into pHB40P. Expression of this construct yields a fusion protein (En-374/552) with the first 7 amino acids (MVPLDSR) being encoded by the synthetic polylinker of pHB40P. The two mutated forms of En-374/552 were obtained by two successive rounds of polymerase chain reaction amplifications, using appropriated oligonucleotides as primers. First, to construct the pHB67/2A expression vector, two oligonucleotides were used: MUT-1 (5′-GAACGCGTGTCATCCGCGGCCTCCATTCC-3′) and MUT-2 (5′-ATGGTACCTCTAGACGCGAGGGG-3′). Amplified DNA was filled-in, double digested with *Kpn*I and *Mlu*I, gel purified, and inserted into gel-purified pHB67, which was linearized with *Kpn*I and *Mlu*I. Mutated constructs were screened for the presence of a new *Sac*II site (underlined in the MUT-2 sequence) and by DNA sequencing using the HB-T7 oligonucleotide as a primer. Second, to construct the pHB67/4A expression vector, a third oligonucleotide (along with MUT-2) was used, MUT-3 (5′-ATCCGCGGCCTCCATTCCGGCATCTCGGGCAACG-GCGGA-3′). Amplified DNA was filled-in, double digested with *Sac*II and *Kpn*I, gel purified, and inserted into gel-purified pHB67/2A, which was linearized with *Kpn*I and *Sac*II.

Expression and Purification of En—BL21(DE3) cells freshly transformed with pT7En or its derivatives were grown at 37 °C to an A_{600} of 0.8 in the presence of 100 μg/liter ampicillin. IPTG was added to 0.5 mM, and incubation was continued for an additional 3 h. Bacteria were harvested, frozen in dry ice for 30 min, stored at −80 °C, partially thawed on ice (about 15 min), and quickly resuspended in 1/100 of buffer HEDN (25 mM HEPES (pH 7.6), 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 10 μg/ml leupeptin, 0.1 mM benzamidine, 10 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml 1,10-phenanthroline) + 100 mM KCl. 0.8-ml aliquots were subjected to sonication at 4 °C. The bacterial debris and insoluble materials were removed by ultracentrifugation (68,000 rpm, 45 min, 4 °C, in a Beckman TLA100.1 rotor), and supernatants were frozen in dry ice and stored at −80 °C. All subsequent steps were carried out at 4 °C.

To purify cloned full-length En, KCl was added to 225 mM, and

nucleic acids were removed over a DE52 column (Whatman), which was equilibrated with HEDN + 225 mM KCl. bEn was recovered in the flow-through with 2 bed volumes of the same buffer, diluted with buffer HEDN + 150 mM KCl, loaded onto a heparin-Sepharose CL-6B column (Pharmacia Biotech Inc.), and finally eluted with a linear KCl gradient (0.15–1 M KCl in HEDN). Fractions were monitored by SDS-PAGE. bEn eluted with 0.4–0.6 M KCl and was purified to 90% homogeneity as determined by silver staining. 16 mg of partially purified En was obtained from 10 liters of cell culture. The total protein concentration was 0.8 mg/ml, as determined by a dye binding assay (Bio-Rad).

To purify the two N-truncated versions of the En (En-297/552 and En-374/552), supernatants obtained from the high speed centrifugation step were loaded directly onto phosphocellulose columns (Whatman, P11), which were equilibrated with buffer HEDN + 100 mM KCl (flow rate of about 0.4 ml/min). Columns were washed with 4 bed volumes of the same buffer, and the En versions were eluted with a linear KCl gradient (0.1–1 M KCl in HEDN). Fractions were monitored by SDS-PAGE, and fractions containing the En fragment were pooled, diluted 10–20 times with buffer HE (25 mM HEPES, pH 7.6, 0.1 mM EDTA) to bring the KCl concentration to 50 mM, and loaded onto Mono-S columns (HR 5/5, Pharmacia). Fractionations were performed with an HPLC Gold System (Beckman) and were monitored by UV absorption at 280 nm. Columns were extensively washed with buffer HE + 50 mM KCl (flow rate, 2 ml/min), and En fragments were eluted with linear KCl gradients (0.05–1 M KCl in HE (flow rate, 1 ml/min)). 1-ml fractions were collected and monitored by SDS-PAGE. The two En fragments were purified to more than 95%, as determined by Coomassie Blue staining. Protein concentrations were 0.3 and 1.4 mg/ml for En-297/552 and En-374/552, respectively, as determined by UV absorption at 280 nm. For both proteins, 2 mg of highly purified preparation were obtained from 1-liter cell cultures. Aliquots were stored at −80 °C.

For phosphorylation assays, bacterial protein extracts were prepared from 5-ml miniculture as follows. From freshly transformed BL21(DE3) cells, 10 colonies were grown at 37 °C until mid-exponential growth phase (about 2 h) in LB/amp medium. Expression was achieved with 1 mM IPTG, and cells were grown at 37 °C for an additional 3 h. Induced cells were harvested by low speed centrifugation, frozen in dry ice for 30 min, stored overnight at −80 °C, thawed on ice for 5 min, quickly resuspended in 200 μl of buffer HEDN by up/down pipetteing, and finally transferred to 1.5-ml microtubes. Keeping tubes on ice, cells were sonicated 10 times, 1 s each at setting 4 of a Branson Sonifier with a microtip, and bacteria debris was removed by centrifugation for 5 min at 13,000 × *g*; finally, the resulting supernatants (typically containing about 5 μg/μl total protein) were monitored by SDS-PAGE (typically 10 μl of extracts were analyzed) and directly used in our various phosphorylation assays.

Expression, Metabolic Labeling, and Immunopurification of En from Drosophila Tissue Culture Cells—Nuclear extracts were prepared from *Drosophila* embryos or HSEN cells as previously described (Refs. 57 and 58, respectively) and stored in aliquots at −80 °C.

HSEN cells were grown and metabolically labeled with 1 mCi/ml carrier-free [^{32}P]phosphate (ICN Pharmaceuticals) essentially as previously described (18), except that cells were washed twice with 1 × PBS and lysed by three freeze-thaw cycles in 100 μl of 0.25 M Tris-HCl (pH 7.8). The cell lysate was clarified by centrifugation (14,000 × *g*, 5 min, 4 °C) and processed for immunopurification as follows. mAbs 4F11 (18) were linked to antimouse IgG-coated magnetic beads (MagniSort M, DuPont NEN), 1 volume of a 1:1 mixture of mAb 4F11-attached beads, and buffer IM (25 mM HEPES, pH 8, 100 mM KCl, 0.1 mM EDTA, 0.1% (v/v) Nonidet P-40, 1.25 mM $MgCl_2$, 10% glycerol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 10 mM ammonium molybdate) was added and then incubated at 25 °C for 30 min. The beads were washed three times, 10 min each at 25 °C with 2 volumes of buffer IM. Bound En was removed from the beads by boiling for 5 min in 50 μl of SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 4 M urea, 0.0025% bromphenol blue) and finally analyzed by SDS-PAGE and autoradiography.

Two-dimensional Gel Electrophoresis and Immunoblot Analysis of En Modification Patterns—Two-dimensional gels were run following procedures described by O'Farrell *et al.* (59). 10-h-old HS3 embryos were aged for 2 h, washed with 0.04% Triton X-100, 0.7% NaCl onto nitex netting, dechorionated by immersion in 50% commercial bleach for 2 min, and finally extensively washed with Triton X-100, NaCl solution. Embryos were heat shocked at 37 °C for 1 h, suspended in 4 μl/mg of embryos in isoelectric focusing buffer IF (9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, and 2% ampholytes (4 parts of pH 5–8 for 1 part of pH 3.5–10)), supplemented with a protease inhibitor mixture (the same that the mixture included in buffer HEDN), and were homogenized by strokes with a Teflon pestle fitting 1.5-ml microtubes. Samples obtained from soluble extracts were precipitated with 3 volumes of cold acetone and kept on dry ice for 4 h; proteins were recovered by centrifugation (14,000 × *g*, 15 min, 4 °C), ether washed, and solubilized in the buffer IF supplemented with protease inhibitors as above. Samples were warmed 5 min at 42 °C immediately before loading onto isofocusing gels. Isoelectric variants of En were detected by Western blotting as follows. Proteins were transferred electrophoretically to nitrocellulose membranes (HAHY, Millipore), essentially as described by Towbin *et al.* (60), except that 0.1% SDS was added to the transfer buffer. All subsequent incubations were at 25 °C. Nonspecific binding of antibodies was blocked with 3% bovine serum albumin (fraction V from Sigma) in PBS for 30 min, and filters were washed in PTBS (PBS with 0.1% Triton X-100) for 15 min. Primary antibody was added (ascites supernatant from the 4D9 cell line diluted 2-fold in PBS + 3% bovine serum albumin) for 1 h. The filters were washed in PTBS (4 × 5 min) and then incubated for 30 min in PTBS that contained a dilution of 1:2000 of an alkaline phosphatase-conjugated goat anti-mouse IgG (H+L). The filters were washed four times in PTBS as above, washed 1 time in PBS for 5 min, and finally developed with 5-bromo-4-chloro-3-indolyl phosphate + nitro blue tetrazolium following supplier's instructions (Kirkegaard and Perry).

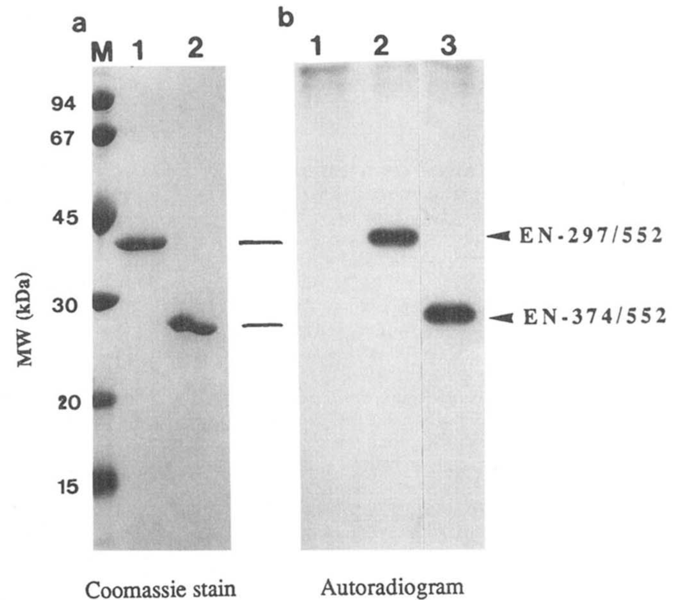


FIG. 4. **Purification and *in vitro* phosphorylation of En fragments.** Two of the En fragments portrayed in Fig. 3*a* (En-297/552 and En-374/552) were purified as detailed under "Materials and Methods." *a*, SDS-PAGE analysis (12% gel) of the most purified protein fraction obtained after separation over a Mono-S HPLC column. *Lane 1*, 10 μg of En-297/552; *lane 2*, 10 μg of En-374/552. The gel was stained with Coomassie Blue. Molecular mass marker sizes are indicated to the *left* in kilodaltons (*M*). *b*, SDS-PAGE analysis of *in vitro* labeled phosphoproteins. Each purified En fragment (5 μg) was incubated in presence of [γ-^{32}P]ATP and 5 μg of soluble nuclear proteins prepared from 2–12-h embryos. An autoradiogram of the dried gel is shown. Exposure was for 30 min. *Lane 1*, nuclear extract incubated alone; *lane 2*, En-297/552; *lane 3*, En-374/552. Note that similar incorporation of [^{32}P]phosphate was observed into either bEn fragment, as determined by trichloroacetic acid precipitation (not shown).

Protein Kinase Assays—Purified En or crude bacterial protein extracts were incubated with crude nuclear protein kinases or purified CK-II (a generous gift from C.V. Glover III). Reactions were carried out for 20 min at 30 °C in buffer P (50 mM Tris-HCl (pH 8), 100 mM NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (ICN Pharmaceuticals, 1 μCi/μl). Typically, 5 μg of purified En or 10 μl of crude bacterial protein extracts were incubated in a final volume of 200 μl in presence of 5 μg of nuclear proteins prepared from embryos as a source of protein kinases. For CK-II-dependent phosphorylation, the purified enzyme was added in the assay at a final concentration of 10 nM. Reactions were stopped with 25 mM EDTA, and phosphoprotein species were analyzed by SDS-PAGE followed by autoradiography of the dried gels. Exposures were at −80 °C with Kodak XAR-5 films and intensifying screens. For quantitation, the phosphorylated products were excised from gels, and Cerenkov radiation was measured.

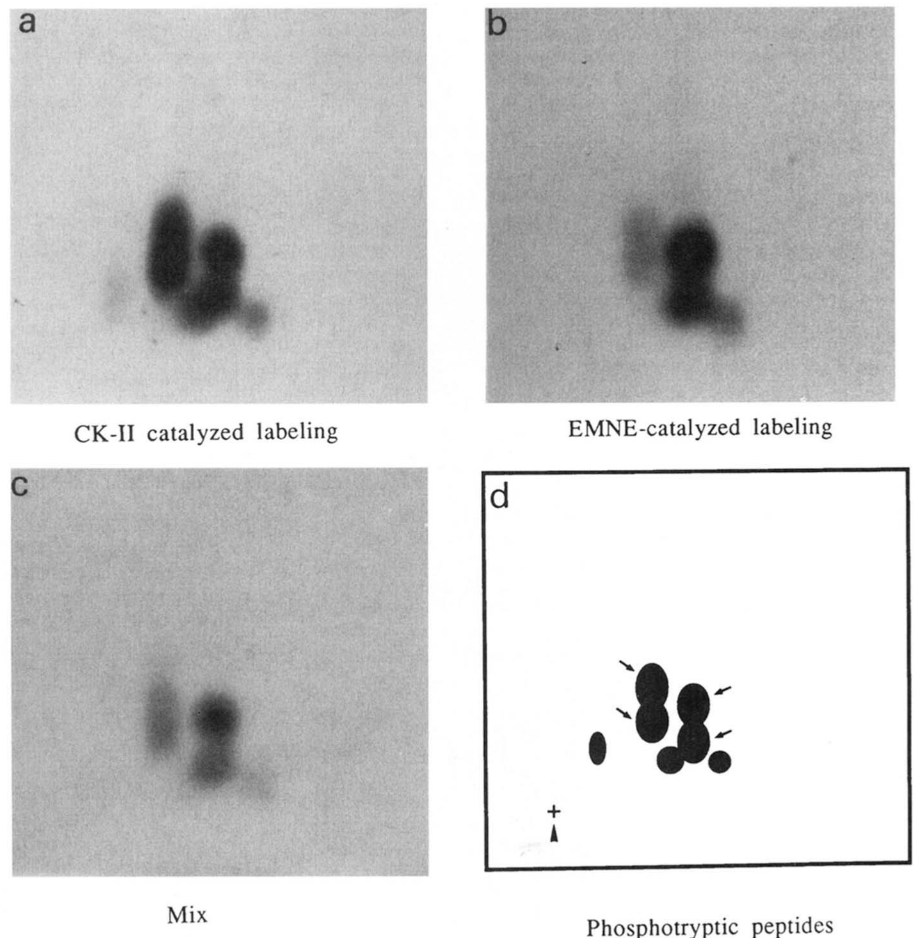


FIG. 5. **Comparative two-dimensional phosphopeptide maps of full-length En phosphorylated *in vitro* by CK-II and nuclear protein kinases prepared from *Drosophila* embryos.** Gel pieces from 9% SDS-PAGE containing either *in vitro* ^{32}P-labeled En were subjected to trypsin digestion, and resulting phosphopeptides were resolved as described in Fig. 2 (*a* and *b*). Phosphotryptic peptides of En phosphorylated *in vitro* by purified *Drosophila* CK-II, and total soluble nuclear kinases were prepared from *Drosophila* embryos, respectively. Experiments were repeated twice. *c*, mixing experiment of either preparation. *d*, map position of all the detected phosphotryptic peptides. *Filled* and *open spots* represent comigrating and CK-II specific phosphotryptic peptides. *Arrows* indicate the phosphotryptic peptides recovered from metabolically labeled En (see Fig. 2; peptides numbered 1, 2, 4, and 5). Origin is indicated by a *vertical arrow*. Cerenkov cpm loaded and exposure time (at −80 °C with intensifying screens) were as follows: *a*, En incubated with CK-II (5600 cpm, 24 h); *b*, En incubated with nuclear protein kinases (2500 cpm, 48 h); *c*, mixture of the phosphotryptic peptides obtained from either labeling (2500 cpm of each, 16 h).

For gel mobility shift experiments, purified En-374/552 (10 μM) was incubated in 50 μl of buffer P with or without purified CK-II (10 nM) for 30 min at 30 °C and directly diluted (a series of 2-fold dilutions) with binding buffer (buffer B) as described below.

Phosphopeptide Mapping—For phosphotryptic peptide analysis, bands corresponding to En were cut from the gels with a razor blade, washed with 25% isopropanol for 1 h to remove SDS, and dried in a vacuum centrifuge (Savant). The gel slices were then incubated with a 50 μg/ml solution of tosylphenylalanyl chloromethyl ketone-treated trypsin (Sigma) in 50 mM ammonium bicarbonate (pH 8) overnight at 37 °C. Trypsin (25 μg) was again added, and samples were incubated for an additional 4 h. Samples were centrifuged to remove particulate material, and the resulting peptides were recovered by lyophilization (four times in H_2O), dissolved in 5% acetic acid, and then were resolved in two dimensions on 100-μm cellulose thin layer plates (Eastman Kodak Co.; chromatogram 13255, 20 × 20 cm) as described by Ward and Kirschner (61). Electrophoresis (pH 1.9) was for 20 min at 1 kV (15 °C). A solution containing 1% acid fuschin (Sigma) was spotted on the plates to monitor the course of electrophoresis and chromatography steps. Exposures were at −80 °C with Kodak X-Omat AR films and DuPont Cronex Lighting Plus intensifying screens.

DNA Binding Assays—Reactions were assembled at 4 °C for 30 min in a final volume of 10 μl of binding buffer B (25 mM HEPES (pH 7.6), 100 mM KCl, 1% (w/v) polyvinyl alcohol, 0.1% Nonidet P-40, 1 mg/ml bovine serum albumin (Life Technologies, Inc.)) supplemented with 1×10^{-10} M ^{32}P-labeled DBEN probe. Complexes were loaded onto 7.5% (29:1 (w/v) acrylamide, *N,N'*-methylenebisacrylamide; Bio-Rad) nondenaturing 0.5 × TBE (90 mM Tris, 90 mM borate, 2 mM EDTA) polyacrylamide gels containing 0.1% Nonidet P-40. Gels (15 cm) were prerun at 4 °C during 2 h at 200 V and run at 4 °C during ⅔ h at the same voltage. Gels were dried onto DE51 paper (Whatman) and exposed to Kodak XAR films at −80 °C.

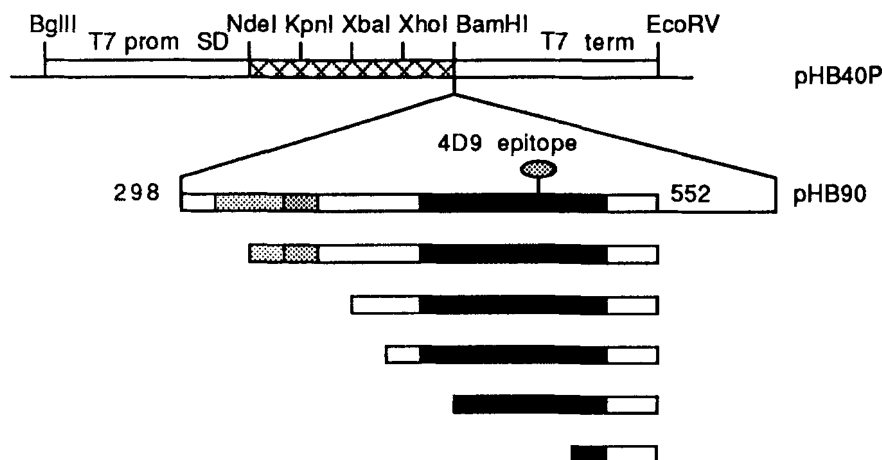
RESULTS

Patterns of Posttranslational Modification of En in Embryos and Tissue Culture Cells—In a previous study, *Drosophila* embryos and cultured cells were labeled with $^{32}PO_4^-$, and phosphoamino acid analysis of immunoprecipitates revealed that En had been modified by a serine-specific protein kinase (18). We have extended these studies, first by determining the fraction of En molecules that are covalently modified and by quantifying the number of modified residues.

To obtain unmodified protein, an *engrailed* cDNA clone was expressed in *E. coli* using a vector with an inducible T7 promoter (27). En accumulated up to 20% of total soluble protein after appropriate induction (see below, Fig. 3*b*) and was partially purified on a heparin-Sepharose column. Two-dimensional polyacrylamide gel electrophoresis of this enriched fraction revealed a major spot of silver staining material with an apparent pI of 7.75 (Fig. 1*a*). This value closely approximates the theoretical value of 7.8 calculated for the engrailed amino acid sequence. Not apparent in this figure are the several degradation products that were revealed by Western immunoblotting (not shown).

In contrast, En extracted from *Drosophila* embryos (2–16 h after egg laying) resolved into an array of four isoforms corresponding to the net addition of zero, one, two, or three negative charges (Fig. 1*b*). More than 75% of the En appeared to be modified. A similar pattern of modifications was observed for protein extracted from embryos of a transgenic line of flies (28) that expresses En uniformly and at high levels under heat shock

a



b

375	394 E/D/S/T-rich 415	422		Construct number (cf Fig.7)
(M)SGVNASSPQPQPIPPPSAV	SRDSGMESSDDTRSETGSTTTE	GGKNEMW--	En-375/552	1
(M)PQPQPIPPPSAV	SRDSGMESSDDTRSETGSTTTE	GGKNEMW--	En-382/552	2
(M)PQPIPPPSAV	SRDSGMESSDDTRSETGSTTTE	GGKNEMW--	En-384/552	3
(M)PPSAV	SRDSGMESSDDTRSETGSTTTE	GGKNEMW--	En-389/552	4
(M)PSAV	SRDSGMESSDDTRSETGSTTTE	GGKNEMW--	En-390/552	5
(M)AV	SRDSGMESSDDTRSETGSTTTE	GGKNEMW--	En-392/552	6
	(M)MESSDDTRSETGSTTTE	GGKNEMW--	En-399/552	7
	(M)GSTTTE	GGKNEMW--	En-410/552	8

FIG. 6. **Strategy for production and selection of bacterial clones expressing En derivatives gradually deleted from the N terminus.** *a*, T7 expression vector used to generate a library of bacterial clones expressing a series of nested En fragments. The vector pHB40P was designed to allow production of non-fused proteins. This vector was constructed from pAR3040 (27), as detailed under "Materials and Methods." Note that a polylinker with single restrictions sites (*Nde*I, *Kpn*I, *Xba*I, *Xho*I, and *Bam*HI) has been inserted between originals *Nde*I and *Bam*HI sites. A 908-base pair *Bam*HI-*en* cDNA fragment (see Fig. 3) was further inserted into the single *Bam*HI site of pHB40P giving rise to pHB90. Nested deletion of the *engrailed* coding sequence was achieved upon a double digestion with *Kpn*I and *Xba*I, consecutively followed by digestions with exonuclease III and S1 nuclease. After Klenow filling in, ligation and transformation of BL21(DE3) competent cells, clones expressing En fragments (corresponding theoretically to ⅓ of the transformants), were selected after transfer to nitrocellulose filters and IPTG induction by immunorevelation with mAbs 4D9, which recognize an epitope within the En homeodomain (located at the C terminus, see Fig. 3). Plasmids from selected clones were purified and sequenced to determine the beginning of the *engrailed* coding sequence. *b*, N-terminal amino acid sequences of a set of eight En fragments. Note that the first residue after the initiator methionine (which is encoded by the *Nde*I site, see *a*) corresponds to a residue naturally present in the primary sequence of the En. This was achieved by placing a *Kpn*I restriction site (which generates a 3′-overhang insensitive to exonuclease III digestion) next to and overlapping the *Nde*I site.

control (Fig. 1*d*). These patterns of isoelectric variants represent the average of all isoforms produced between 2 and 16 h of embryogenesis. These isoforms are acidic with respect to the unmodified protein, and therefore their existence is consistent with the possibility that En is subjected to successive covalent additions of phosphate on at least three serine residues.

A third source of modified En was a Schneider cell line (HSEN) that carries a transfected *engrailed* cDNA under heat shock control (18). After heat shock, En accumulated to high levels in the nuclei of these cells, apparently modified in a manner similar to protein in embryos (Fig. 1*c*). Treating a nuclear extract of these cells with calf intestine alkaline phosphatase eliminated all of the acidic isoforms (not shown), indicating the likely nature of the modifications.

Analysis of in Vivo and in Vitro Phosphotryptic Peptides—To identify the metabolically modified residues, an *in vitro* system capable of phosphorylating En was developed. Sources of unmodified protein and protein kinases were, respectively, En produced in *E. coli* and a nuclear extract prepared from *Drosophila* embryos (EmNE). Highly purified En was readily mod-

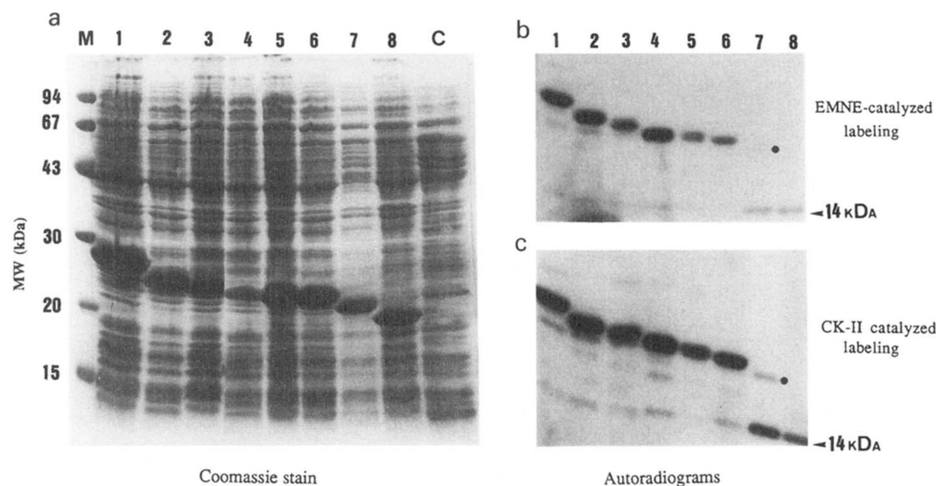


FIG. 7. **Expression and phosphorylation of a set of 8 En fragments.** *a*, SDS-PAGE analysis (12% gel) of total soluble proteins obtained upon IPTG induction of the set of bacterial clones expressing En fragments portrayed in Fig. 6*b*. Identical amounts of total protein were analyzed by Coomassie Blue staining. *Lane numbers* refer to construct numbers shown in Fig. 6*b*. *Lane C* corresponds to an extract obtained upon IPTG induction of a cell line harboring the pHB40P expression vector. Note the high expression level of each En fragment and significant changes in apparent molecular weights, resulting from a deletion of only two residues. *b* and *c*, autoradiograms of phosphoproteins obtained after incubation of each bacterial extract with [γ-^{32}P]ATP and with crude embryonic protein kinases (5 μg) or purified *Drosophila* CK-II (10 nM), respectively. Note that the [^{32}P]phosphate incorporation into the 14-kDa *E. coli* protein (see Fig. 3) is higher with soluble proteins obtained from constructs 7 and 8. The weak [^{32}P]phosphate incorporation into construct 7 obtained from either kinase preparation is indicated by a *dot*.

ified by EmNE, as indicated by [γ-^{32}P]ATP labeling (Fig. 2*a*). Two-dimensional PAGE resolved labeled En into three phosphorylated forms (data not shown).

Phosphotryptic peptides derived from protein phosphorylated *in vitro* and *in vivo* were compared. *In vivo* phosphotryptic peptides were obtained from heat-shocked HSEN cells that had been metabolically labeled with [^{32}P]orthophosphate. En was immunopurified, separated by SDS-PAGE (Fig. 2*a*, *lane 3*), digested with trypsin, and then fractionated, first by high voltage electrophoresis on thin layer cellulose plates and then by chromatography. 11 phosphotryptic peptides were detected by autoradiography, mostly distributed among a set of peptides that were not resolved by electrophoresis (Fig. 2*c*). *In vitro* phosphotryptic peptides were obtained and analyzed in the same manner, and a similar pattern of phosphopeptides was observed (Fig. 2*b*). Mixing experiments (not shown) indicated that the homologous *in vivo* and *in vitro* phosphotryptic peptides comigrated (as symbolized in Fig. 2*d*). However, the relative specific activity of several of the metabolically labeled phosphotryptic peptides (peptides numbered 1, 2, 4, 5, 6, and 7) was not quantitatively reproduced in the *in vitro* reaction, and three peptides (indicated by the *open circles* in Fig. 2*d*) were recovered only after EmNE treatment. We conclude that the *in vitro* reactions reproduce most but not all of the modifications that decorate En *in vivo*.

CK-II Phosphorylates En in a Region Outside of the Homeodomain—To identify the kinase(s) in embryo extracts that modify En and to identify the residues that are its (their) substrates, five N- and C-truncated versions (Fig. 3*a*) were assayed for EmNE-dependent labeling. These protein fragments were efficiently produced in *E. coli* (Fig. 3*b*), and several were good substrates for EmNE-catalyzed phosphorylation in crude bacterial extracts (Fig. 3*c*). Unexpectedly, little interference from soluble bacterial proteins was observed, although a 14-kDa *E. coli* protein was significantly labeled in extracts that contained either of two engrailed fragments (*e.g.* En-374/552 and En-410/552). Two of the five truncated derivatives (En-1/298 and En-410/552, corresponding to the N-terminal 298 and the C-terminal 143 amino acids, respectively) were not significantly labeled. This pattern of labeling suggests that the targets of phosphorylation are between residues 374 and 410.

Two of the En derivatives containing this region (En-297/552 and En-374–552) were purified to apparent homogeneity (see "Materials and Methods"). Preparations of each derivative contained a single species that reacted with Coomassie stain (Fig. 4*a*) or labeled with ^{32}P (Fig. 4*b*). Labeling of either derivative was equivalent. Although ^{32}P labeling was also observed for a protein derivative including residues 297 to 376 (not shown), the 36-amino acid region between residues 373 and 410 apparently includes the major targets of *in vitro* phosphorylation. This portion of En has a high content of acidic residues interspersed with serine and threonine residues (Ref. 25, see also Fig. 3*a*).

Purified En-297/552 was used as a substrate to characterize the enzymatic activity responsible for its modification. In the course of several fractionation steps, it was noted that the principal modifying kinase activity had chromatographic behavior and salt and pH dependence similar to those described for *Drosophila* CK-II (34). Furthermore, Western analysis of our purified fractions indicated that they are enriched with the CK-II α and β peptides, and virtually identical phosphopeptide maps were obtained after *in vitro* labeling full-length En with purified CK-II or EmNE (Fig. 5). Of seven phosphotryptic peptides recovered after CK-II-catalyzed modification, six were also present after reaction with EmNE, and four chromatographed in a manner identical to metabolically labeled phosphotryptic peptides 1, 2, 4, and 5 of Fig. 2*d*. Since most of the *in vivo* phosphoacceptor residues are also modified during EmNE-catalyzed phosphorylation of En (see Fig. 2), we conclude that En is posttranslationally modified by CK-II or CK-II-related enzymes.

To more precisely localize the phosphorylated residues, an extensive series of N-terminal deletions was constructed (see "Materials and Methods"). Eight deleted derivatives with an N terminus between residues 374–409 (Figs. 6 and 7*a*) were characterized. These protein fragments differed as substrates for phosphorylation, but the response of each fragment to CK-II (Fig. 7*c*) and EmNE (Fig. 7*b*) was remarkably similar. Fragments that included residues 389–552 (constructs 1–4) were phosphorylated with equal efficiency. However, efficiency of phosphorylation decreased for construct 4 (En-390/552), slightly further for construct 6 (En-392/552), and almost com-

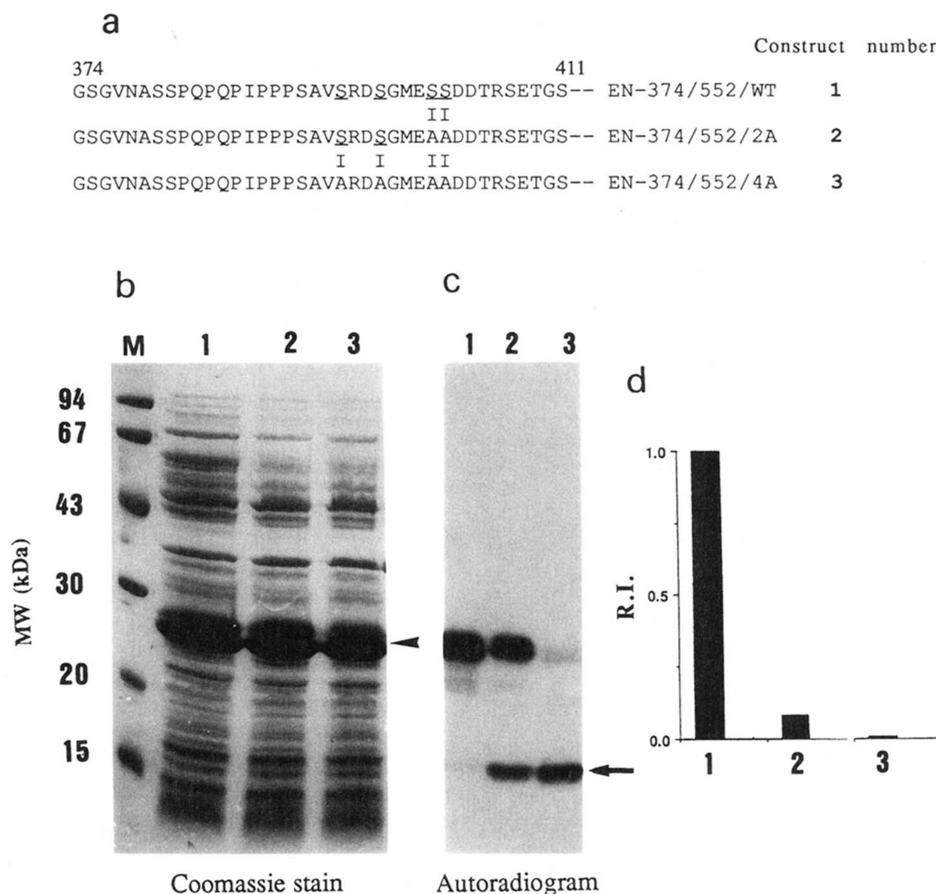


FIG. 8. **Relative CK-II-dependent phosphorylation of wild-type and mutated forms of an extended version of the En homeodomain (En-374/552).** The amino acid sequence of the *in vitro* phosphorylated segment of En is depicted in *a*. Serine residues recognized as potential target residues for CK-II-catalyzed phosphorylation are *underlined*. The two mutated forms of En-374/552 used for phosphorylation assays are indicated *below* the wild-type version. *b*, SDS-PAGE analysis (12% gel) of total soluble proteins obtained upon IPTG induction of each form of En-374/552. *Lane numbers* refer to construct numbers shown in *a*. The *arrow* points out the position of En-374/552 forms. Note that the expression level of each mutated form of En-374/552 is similar to that obtained from the wild-type form. *c*, autoradiograms of *in vitro* phosphorylated En-374/552 forms. Identical amount of total protein prepared from IPTG-induced cultures was incubated with [γ-^{32}P]ATP and purified CK-II (10 nM). The position of 14-kDa *in vitro* phosphorylated *E. coli* protein used as an internal competitor substrate is indicated by an *arrow*. *d*, relative phosphate incorporation into each form of En-374/552. *Column numbers* refer to construct numbers. Quantitation was achieved by measure of the cpm incorporated into each protein and counts of Cerenkov radiation in the corresponding gel slices. Values were scaled relative to the amount of [^{32}P]phosphate incorporated into 14-kDa competitor protein. The ratio obtained (*R.I.*) from the wild-type form of En-374/552 was arbitrarily scaled to 1. To the first approximation, the [^{32}P]phosphate incorporation into each form of En-374/552 relative to the value obtained for the 14-kDa protein should be directly related to the difference of the Michaelis constant of each substrate (the K_m of each form of En-374/552 *versus* the K_m of the 14-kDa protein).

pletely for construct 7 (En-399/552). The fragment beginning with residue 410 was inert (construct 8, En-410/552). This pattern of labeling suggests that the targets of phosphorylation are included among the 18 amino acids from 392 to 409.

Sequence and structural requirements for CK-II phosphorylation have been extensively characterized (29–31). Studies with both natural and synthetic peptides showed that CK-II phosphorylates serine and threonine residues located within or on the N-terminal side of clusters of acidic residues, especially those located three amino acids from a negatively charged residue. In addition, efficient CK-II-dependent phosphorylation has a structural requirement for a β-turn. Five serine/threonine residues between 392 and 409 ($_{392}$AV$\underline{S}$RD$\underline{S}$GME$\underline{SS}$DD$\underline{T}$RSET$_{409}$) of En are embedded in a sequence that fulfills these requirements. Acidic amino acids are at positions 396, 400, 403, 404, and 408. The sequence $\underline{S}$GME$\underline{SS}$ has a high probability of adopting a β-turn structure, and the sequence E$\underline{SS}$DD is positioned next to the predicted turn (32). The combination of structural and sequence considerations, as well as the labeling patterns of deletion mutants, suggests that serines 401/402 are the major targets for CK-II.

This possibility was tested by mutating serines 401/402 and other serine residues. A deletion fragment retaining residues 374–552 (Fig. 8*a*, *construct 1*) was altered (see "Materials and Methods") by changing serines 401 and 402 to alanines (*construct 2*, En-374/552/2A) or by changing serines 394, 397, 401, and 402 to alanines (*construct 3*, En-374–552/4A). All three protein fragments were expressed at equivalent levels (Fig. 8*b*), but labeling of En-374/552/2A and En-374–552/4A by CK-II was reduced by approximately 10- and 100-fold, respectively (Fig. 8, *c* and *d*). Similar results were obtained for these mutant proteins with EmNE kinase (not shown). Since we estimate that the K_m for En-374/552 to be approximately 5 μM for CK-II (data not shown), these results establish that these constructs have mutated the major targets for CK-II-dependent phosphorylation.

DNA Binding and CK-II-dependent Phosphorylation—To determine the functional relevance of the CK-II-dependent phosphorylation, DNA binding of the unmodified and modified forms of En were compared. Binding was monitored by gel retardation as a function of protein concentration. En-374/552 was purified, and a portion was kinased with CK-II. Both forms of the protein bound DNA, but the phosphorylated protein exhibited between 2- and 4-fold greater affinity (Fig. 9, *b* and *c*). Stimulation was observed only after incorporation of more than

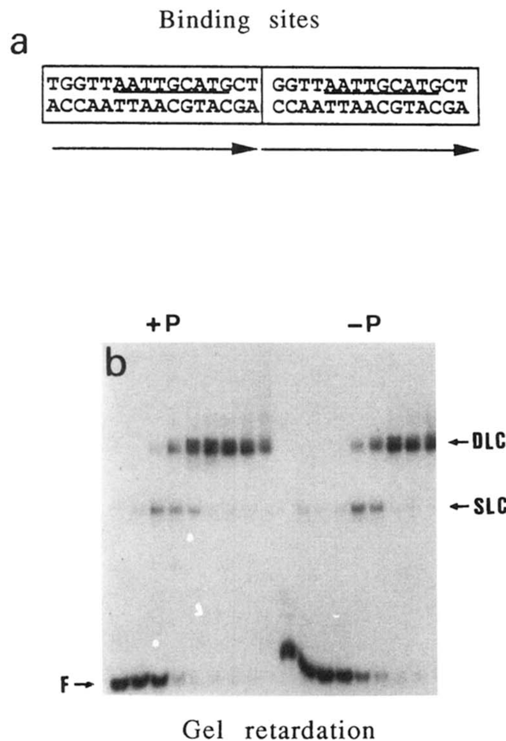


FIG. 9. **CK-II-dependent phosphorylation of an extended version of the En homeodomain (En-374/552) enhances sequence-specific DNA binding.** *a*, double-stranded oligonucleotide used for gel mobility shift assays. The tandemly repeated binding sites recognized by En[2] are *underlined*. *b*, DNA binding assays obtained with purified En-374/552 (see Fig. 4). Identical amounts of En-374/552 (10 μM) were preincubated with or without purified CK-II (10 nM) as described under "Materials and Methods." The stoichiometry of phosphorylation obtained was 3.5 mol of $^{32}PO_4^-$/mol of purified protein. Protein extracts were serially diluted in binding buffer (2-fold increments), and each preparation was reacted with ^{32}P-labeled double-stranded oligonucleotide DNA. Positions of free DNA, single-liganded (*SLC*), and double-liganded (*DLC*) complexes are indicated.

2 mol of phosphate/mol of purified protein (not shown). Equivalent results were obtained using oligonucleotides containing one (not shown) or two (Fig. 9a) binding sites for En. These observations suggest that the ability of En to bind DNA may be regulated by CK-II phosphorylation.

DISCUSSION

The En is a phosphoprotein (18). Here, we use *in vitro* studies to identify casein kinase II as the primary kinase modifying En. We show that CK-II phosphorylates a cluster of target sites located near to the engrailed homeodomain and that several of these sites are also phosphorylated *in vivo*. Finally, we show that CK-II-dependent phosphorylation enhances DNA binding of a truncated portion of En.

States of En Modification in Vivo—En isolated either from wild-type *Drosophila* embryos or from embryos of a heat-shocked transgenic fly strain has at least four isoforms. A qualitatively similar pattern also characterized En induced by heat shock of a stably transformed embryo-derived cell line. The more basic of the isoforms represent less than 25% of the protein in wild-type embryos and has the same apparent pI as unmodified En made in *E. coli*. We assume that the basic isoform is not phosphorylated and that the more acidic isoelectric variants of the modified protein reflect the successive addition of phosphates on at least three serine residues. Indeed, treatment with calf intestine alkaline phosphatase removed most of the acidic isoforms but did not affect the apparently unmodified protein (not shown). We do not know if all the different isoforms exist within each of the embryonic cells that express En or if the different states of modification are cell type specific. Studies of the related Ultrabithorax homeodomain protein indicate that phosphorylation of its multiple isoforms is regulated as a function of cell type and developmental stage (33).

CK-II Phosphorylates En—The relatively low complexity of posttranslational modifications facilitated our efforts to identify the kinases that modify En. Several lines of evidence indicate that CK-II is the embryonic protein kinase primarily responsible for phosphorylating engrailed. (i) Extracts prepared from *Drosophila* embryos generate a population of En modifications that mimic closely the protein forms present *in vivo*. The physical and catalytic properties of this activity in embryo extracts are similar to those described for *Drosophila* CK-II. (ii) The region of En targeted by the embryo extract kinase and CK-II is identical as indicated by analysis of either phosphotryptic peptides or deletion constructs. (iii) Site-directed substitution of four residues whose sequence environment obeys the canonical recognition requirements for CK-II essentially abolished phosphorylation by either kinase preparation. (iv) En is a high affinity substrate for CK-II. (v) CK-II is an abundant kinase in embryos and cultured *Drosophila* cells (34), and immunostaining indicates that it is expressed in most if not all cells during embryogenesis (not shown). Therefore, the apparently normal modifications of En expressed after heat shock induction of a transgenic fly strain is consistent with the major En kinase being widely distributed.

The role of CK-II (or a CK-II-like enzyme with similar substrate specificity) in the post-synthetic modification of En has interesting implications. CK-II has been widely conserved during evolution (35–37), is a nuclear enzyme (38), and in *S. cerevisiae* is essential for viability (37). Although no *Drosophila* CK-II mutants have been described, a variety of data from other animals suggest that the enzyme plays a role in embryogenesis, cell proliferation, and cell differentiation and that its activity is regulated in response to growth factors in a cell cycle-dependent manner (reviewed in Ref. 26). Since growth factor-dependent activation of CK-II correlates with an increase in the phosphoserine/phosphothreonine content of CK-II itself, it is conceivable that CK-II is involved in a signal transduction pathway that involves a cascade of phosphorylation.

engrailed is one of 16 segmentation genes of the segment-polarity class. These genes act coordinately, apparently by means of local cellular interactions and perhaps through a genetic cascade to help establish segment borders and to help assign and maintain the identities of different parts of segments (reviewed in Ref. 39). Although the details of the cellular interactions involved have yet to be delineated, we now know that signals are carried by diffusible ligands such as wingless protein (40–42) and hedgehog protein (43, 44) and that signal transduction in the responding cells involves serine/threonine protein kinases such as shaggy protein (45) and fused protein (46). From our data, it is tempting to speculate that cellular interactions trigger a phosphorylation cascade that ultimately leads to an increase in the activity of CK-II and therefore to an increase in the phosphorylation level of En.

CK-II phosphorylation of a truncated version of the En enhances DNA binding (Fig. 9). Although it would be preferable to test full-length En, due to the presence of contaminating degradation products in our purified preparations of full-length En, the effect of phosphorylation has not been determined for the biologically significant form. However, enhancement of

[2] H.-M. Bourbon, E. Martin-Blanco, D. Rosen, and T. B. Kornberg, unpublished data.

DNA binding after phosphorylation of the truncated version of the En we have characterized, En-374/552, is reproducible but small (2–4-fold). Nevertheless, the higher affinity for DNA of phosphorylated En could lead to the occupancy of a different population or percentage of regulatory sequences than the unmodified isoform. We reserve judgment on its functional significance until active full-length En can be tested with sequences derived from *in vivo* binding sites.

Given that the phosphorylated amino acids of En are about 50 residues to the N-terminal side of the homeodomain (the portion of the protein that directly contacts DNA) (2), the mechanism by which covalently attached phosphates modulate DNA binding is intriguing. It has been proposed, for instance, that CK-II-dependent phosphorylation of MYB and SRF at residues outside their DNA-binding domain causes a conformational change, leading to a change in DNA binding (47, 48). We have observed slight changes in protease sensitivity of En-374/552 after CK-II phosphorylation (not shown), suggesting that the conformation of this protein may also change after phosphorylation.

Lastly, it is relevant to consider that although CK-II is the primary kinase for En, it may account for only a subset of the modifications. Among the segment polarity genes, the proteins produced by *fused* and *shaggy* (*sgg*) are putative serine/threonine protein kinases and so are of particular interest in this context. Sequence comparisons suggest that *sgg* encodes the *Drosophila* homolog of mammalian glycogen synthase kinase 3 (45, 49, 50). Recognition sites for glycogen synthase kinase 3 in the regulatory region of glycogen synthase must be generated posttranslationally by CK-II phosphorylation (51). Phosphorylation by CK-II has no effect on glycogen synthase activity *per se* but potentiates the ability of glycogen synthase kinase 3 to phosphorylate glycogen synthase, which does (reviewed in Refs. 52 and 53). If *sgg* kinase has the same substrate requirements as glycogen synthase kinase 3 (Ser-Thr-Xaa-Ser-Thr (PO_4^-) (51), En would have a suitable substrate at Ser-397 when Ser-401 has been phosphorylated.

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