carbonate-resistant pre-OEP86 does not yield the 52-kD fragment—that is, folding and integration into the envelope seem incomplete at this stage.
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21. Protease treatment of intact chloroplasts yields only one membrane-associated OEP86 breakdown product, namely the 52-kD fragment. The NH_2-terminal portion of OEP86 seems unprotected from proteolysis by association with the outer envelope membrane.
22. A standard pre-OEP86 translocation reaction was separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters (*20*). The filters analyzed by protein immunoblot were with an OEP86 antiserum and stained with the use of the alkaline phosphatase color reaction (*20*). The nitrocellulose filter was subsequently subjected to autoradiography. The band labeled by the OEP86 antiserum coincided completely with the labeled band on the x-ray film.
23. Pre-OEP86 translation product was treated with 1 μg of thermolysin for 10 min on ice (*20*). The radioactive labeled pre-OEP86 and polypeptides of lower molecular mass were completely degraded.
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35. Both i-OEP86 and OEP86-m were constructed from the original clone after restriction with Sac I and Pst I, respectively, and subcloned into the vector pGEM5Zf(+) (Promega). The open reading frame for i-OEP86 contained six additional amino acids (Met, His, Pro, Thr, Arg, and Trp) before the start of the original protein at amino acid 83. OEP86-m started three amino acid positions behind the proteolytic processing site (that is, at amino acid 150).
36. Supported in part by grants from the Deutsche Forschungsgemeinschaft (J.S.) and by a grant from the Swedish Natural Sciences Research Council (G.v.H.).

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Fatty Acylation of Two Internal Lysine Residues Required for the Toxic Activity of *Escherichia coli* Hemolysin

Peter Stanley, Len C. Packman, Vassilis Koronakis, Colin Hughes*

Hemolysin of *Escherichia coli* is activated by fatty acylation of the protoxin, directed by the putative acyl transferase HlyC and by acyl carrier protein (ACP). Mass spectrometry and Edman degradation of proteolytic products from mature toxin activated in vitro with tritium-labeled acylACP revealed two fatty-acylated internal lysine residues, lysine 564 and lysine 690. Resistance of the acylation to chemical treatments suggested that fatty acid was amide linked. Substitution of the two lysines confirmed that they were the only sites of acylation and showed that although each was acylated in the absence of the other, both sites were required for in vivo toxin activity.

Hemolysin (HlyA) secreted by pathogenic *E. coli* binds to mammalian cell membranes, disrupting cellular activities and causing cell lysis by pore formation (*1*, *2*). The toxin is made as an inactive protoxin (proHlyA) that is activated intracellularly by the cosynthesized protein HlyC (*3*). The transformation of proHlyA to mature HlyA toxin is determined by fatty acylation directed by homodimeric HlyC, which uses only acylated acyl carrier protein as a fatty acid donor (*4*, *5*). The mechanism, which is required for the activity of a family of membrane-targeted toxins, including leukotoxins of *Pasteurella* and *Actinobacillus* and the adenylate cyclase–hemolysin of *Bordetella pertussis* (*6*), does not conform to protein maturation processes such as NH_2-terminal myristoylation of glycines and generation of *N*-acyl diglyceride cysteines, acylation of internal residues through ester linkages, or COOH-terminal glypiation (*5*). We have now defined the specific sites of the toxin fatty acylation in vitro and correlate them to the in vivo toxin activity.

During in vitro reactions containing only purified proHlyA, HlyC, and [^{14}C]palmitoylACP (*7*), inactive proHlyA was converted efficiently to mature HlyA toxin (Fig. 1), with hemolytic activity and transfer of labeled fatty acid from ACP increasing in parallel and in direct proportion to the HlyC concentration. Hydroxylaminolysis and alkaline methanolysis of the reaction mixture released the labeled fatty acid from [^{14}C]palmitoylACP but not from [^{14}C]palmitoylHlyA (Fig. 1), and no labeled compounds of small molecular size were detected in the chloroform-methanol phase when active acylated toxin was treated alone (*8*). When [^{14}C]palmitoylHlyA was treated with trifluoromethanesulfonic acid (TFMS), HlyA again remained labeled and again no labeled compounds of small molecular size were found in the extracting organic phase (Fig. 1). The data indicate that the fatty acid was linked covalently, not through an acyl ester bond or sugar linkage but most likely directly by an amide bond.

Three fragments spanning the 1024-residue proHlyA were generated in vivo from T7 expression vectors (*9*–*11*). Fragment N1–520 included the entire hydrophobic membrane-spanning domain, I496–831 included the first 11 out of 13 glycine-rich Ca^{2+}-binding repeats, and C831–1024 contained the final two repeats and the COOH-terminus (Fig. 2). Isopropyl-β-D-thiogalactopyranoside induction of *E. coli* strain BL21 (DE3) produced up to 30 mg of proHlyA fragments per liter of culture, and the three fragments, purified as described (*4*), were individually incubated in vitro with HlyC and [^{14}C]palmitoylACP. One fragment, I496–831, was fatty acylated; the other two were not (Fig. 2). When the three proHlyA fragments were incubated together in equal amounts, I496–831 was still the only fragment labeled (*8*).

In vitro–activated acylated [^{3}H]palmitoylHlyA was digested with endoproteinase Lys-C (*12*). The resulting peptides were resolubilized, first in acidic aqueous acetonitrile, which recovered 15% of the total radioactivity, and then a second time in the presence of guanidinium chloride (GnCl), which recovered all of the remaining 85% (*13*). The two samples obtained were fractionated on a reversed-phase high-performance liquid chromatography (HPLC) C8 column (*13*) (Fig. 3). Each fractionation gave one major ^{3}H peak, the retention times of which differed substantially, which indicated that the peptides were of different size or stoichiometry of substitution or both. Recovery of ^{3}H from the first and second HPLC runs was 78 and 89%, respectively, which confirmed that the relative abundance of the labeled peptides was an approximate reflection of the extent of in vitro labeling of the two sites in the intact protoxin.

Mass spectrometry (*14*) of HPLC peptide peak 1 (two fractions, 184 pmol of ^{3}H) revealed that it contained a predominant species of molecular mass 1630.2 ± 0.8 daltons, termed peptide 1, and that HPLC peak 2 (one fraction, 477 pmol of ^{3}H) con-

P. Stanley, V. Koronakis, C. Hughes, Department of Pathology, Cambridge University, Tennis Court Road, Cambridge CB2 1QP, UK.
L. C. Packman, Department of Biochemistry, Cambridge University, Tennis Court Road, Cambridge CB2 1QW, UK.
*To whom correspondence should be addressed.

tained a predominant species of molecular mass 3808.4 ± 1.4 daltons, termed peptide 2 (Fig. 3). Sequence analysis of the purest fractions (*15*) (Fig. 3) identified the peptide 1 sequence as EQEVSVGXRTEK (*16*), matching HlyA residues 683 to 694, and the peptide 2 sequence as FVTPLLTPGEE-IRERRQSGXYEYITELLVK (*16*), derived from a different region of HlyA, namely residues 545 to 574 (*17*). At positions marked X, no amino acid could be identified by this sequencing, which indicates that in both cases, lysines (Lys^{690} and Lys^{564}, respectively) were modified. Remaining fractions were not studied, as the efficient recovery of tritium suggested that there were no other important sites of labeling. For both peak 1 and peak 2, mass spectrometry of earlier fractions showed the presence of fragments about three times larger. The late retention times of these two hydrophilic sequences indicated that they were substituted with a very hydrophobic group. The masses expected of peptide 1 and peptide 2 with a single palmitoyl group in amide linkage with a lysine were 1628.3 daltons and 3804.8 daltons, respectively, which is consistent with the observed mass within the accuracy of instrumentation (±0.1% with external calibration). In both sequence analyses, the fraction taken for each peak was at least 80% pure with respect to the major species (peptide 1 and peptide 2). Minor contaminating species were detected that arose from segments of HlyA (in peak 1) and HlyC (in peak 2), but their theoretical masses, both with and without palmitoylation, did not correspond to the masses observed. Neither did their sequences contain reactive residues corresponding to the cycles of tritium release.

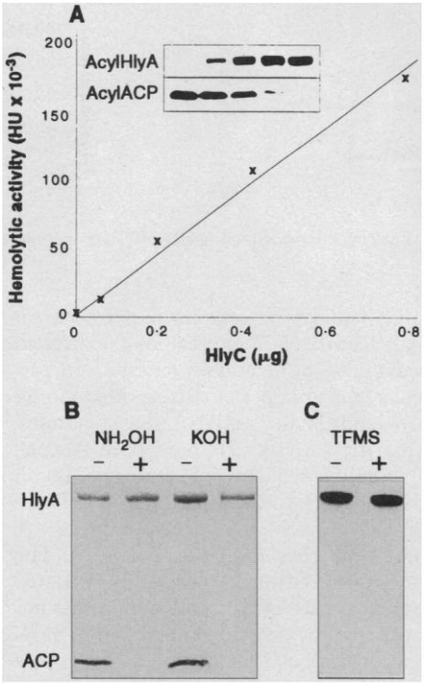

Fig. 1. In vitro HlyC-directed ACP-dependent linkage of palmitic acid to protoxin. (**A**) Reactions containing varying amounts of HlyC (0 to 0.8 μg) and a constant amount of proHlyA (1.5 μg) and of [^{14}C]palmitoylACP (0.3 μg) were incubated in 30 μl of HEDK buffer [25 mM Hepes (pH 8.0), 5 mM EGTA, 1 mM dithiothreitol (DTT), and 100 mM KCl] for 15 min at 37°C. ProHlyA, HlyC, and palmitoylACP were synthesized and purified as described (*7*). Portions of the reaction were either precipitated with trichloroacetic acid and separated by discontinuous tricine SDS-PAGE (*31, 32*) with a 4% stacking gel and 12% separating gel (inset), or diluted in HED buffer [25 mM Hepes (pH 8.0), 5 mM EGTA, and 1 mM DTT] and assayed for hemolytic activity (*33*). Completed in vitro reactions, ethanol precipitated to retain acylACP as control and resuspended in 20 μl of HED–5 M urea, were treated for 1 hour at 23°C with (**B**) 1 M hydroxylamine hydrochloride [150 μl (pH 7.5) with NaOH] (*34*) (NH_2OH, +) or 1 M tris-Cl (pH 7.5) (NH_2OH_1, −) or 0.2 M KOH in 150 μl of methanol (*35*) (KOH, +) or methanol alone (KOH, −). After acidification with 1 M HCl, samples were extracted with chloroform-methanol (2:1) and the aqueous layer was ethanol-precipitated. (**C**) Completed in vitro reactions were also treated with TFMS for 4 hours at −20°C (+) or were untreated (−). In vitro reactions precipitated with isopropanol (25%, on ice for 1 hour) to remove acylACP were resuspended in 20 μl of HED–5 M urea, diluted with three volumes of H_2O, and extracted with chloroform-methanol (2:1). The interphase was incubated for 4 hours at −20°C in TFMS (with the use of a Glycofree deglycosylation kit; Oxford Glycosystems). Precipitate formed after addition of 0.5% ammonium bicarbonate was collected, resuspended in 10 μl of HED–5 M urea, and diluted in 90 μl of H_2O.

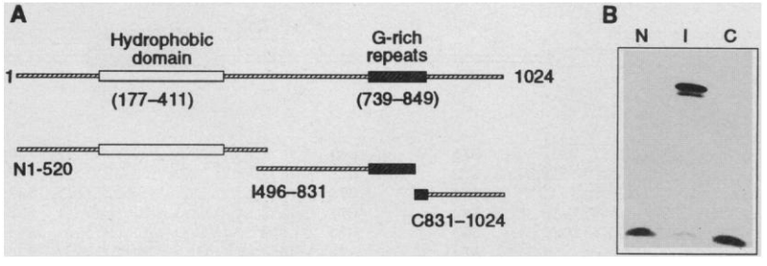

Fig. 2. Localization of modification by in vitro acylation of proHlyA fragments. (**A**) Three fragments of proHlyA (N, NH_2-terminal; I, internal; and C, COOH-terminal) were separately overexpressed from recombinant pAR expression plasmids (*11*) and purified as described for HlyC (*4*). (**B**) In vitro [^{14}C]palmitoylation of 0.5 μg of purified proHlyA fragments (Fig. 1) ethanol precipitated, resuspended in HED-urea, and separated by SDS-PAGE on a two-tier 10:12% gel.

To confirm the positions of the radiolabeled acyl groups, peptides immobilized through their primary amine groups (*15*) [NH_2-terminus and side chain of the COOH-terminal lysine, by diisothiocyanate coupling to polyvinylidine difluoride (PVDF) membrane] were subjected to sequence analysis, and each anilinothiazolinone (ATZ) amino acid was extracted with methanol (Fig. 3). Although counts were released in the first cycle, this was not an indication that the NH_2-terminal residue was labeled, because with peptides that are immobilized through their NH_2-terminal amino acid only, cleavage of the first bond releases the labeled peptide. In subsequent sequence cycles, only background amounts of ^{3}H were seen, except that cycle 8 for peptide 1 and cycle 20 for peptide 2 each gave a large pulse of ^{3}H. The sites of palmitoylation were thus confirmed as Lys^{690} and Lys^{564}.

Mutagenesis was used to substitute the Lys^{564} and Lys^{690} residues of proHlyA with arginine (*18*). The resulting proHlyA proteins $Lys^{564} \rightarrow Arg^{564}$ (K564R) and K690R, and also K564,690R (*16*), in which the two mutations were combined, were subjected to in vitro activation with [^{3}H]palmitoyl-ACP. Proteins K564R and K690R were acylated at levels about 20 and 80%, respectively, of that achieved by the wild-type toxin, in agreement with the relative abundance of acylated peptides 1 and 2 in the HPLC runs, which suggests that acylation at each site was not dependent on the other. No labeling was detected in the doubly substituted K564,690R protoxin, which confirms that HlyA has no other significant HlyC-directed acylated residues (Fig. 4).

When the three mutant protoxins were separately expressed in exponentially growing cultures of *E. coli* carrying *hly*C and the hemolysin secretion genes, all were produced and secreted in amounts indistinguishable from that of the wild-type toxin, but no hemolytic activity was detected in the supernatants (*19*). Furthermore, no hemolytic activity was evident after precipitation of the proHlyA proteins from the supernatant with ammonium sulfate and resolubilization in 6 M GnCl; that is, loss of toxin activity in the mutant proteins was not due to misfolding or aggregation (*20*). These results were confirmed by loss of hemolytic activity in the three toxins acylated in vitro (K564R activity decreased to 0.3% of wild-type activity; K690R and K564,690R activities were undetectable) and also by substitution of the two lysines

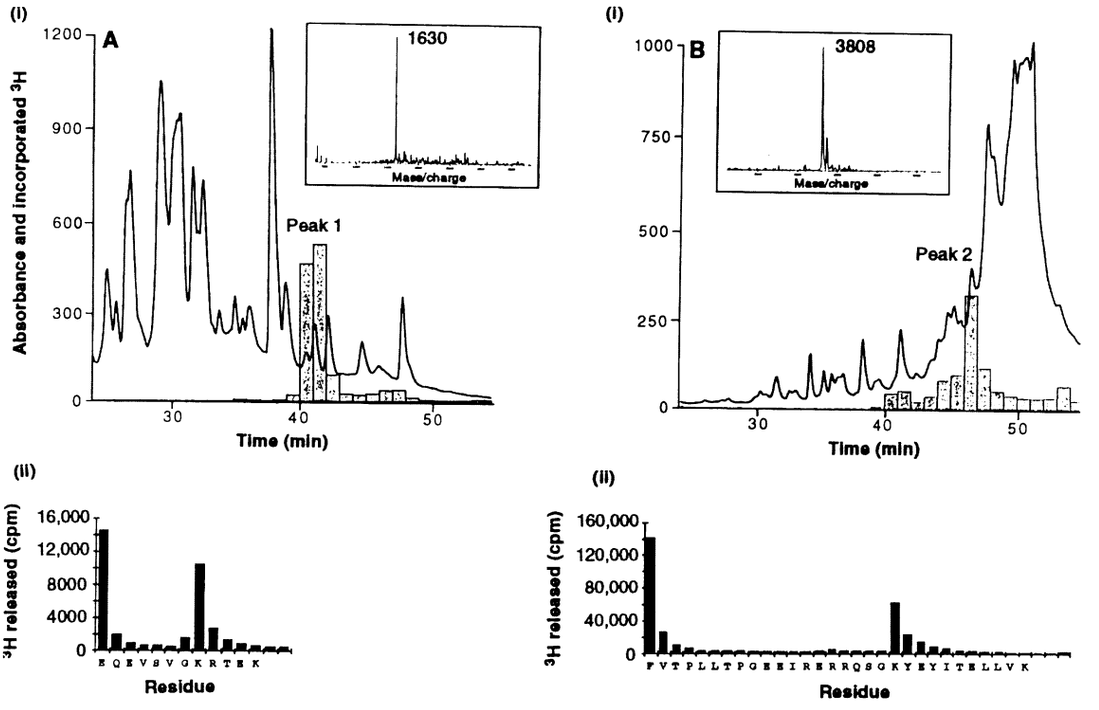

Fig. 3. Mass spectrometry and sequencing of HPLC-separated proteolytic peptides of in vitro ^{3}H-palmitoylated activated intact toxin. In vitro activated ^{3}H-palmitoylated HlyA was completely digested with endoproteinase Lys-C (*12*). Proteolytic peptides were recovered in two stages and separated by reversed-phase HPLC (*13*). The upper panel shows the HPLC separation of peptides solubilized (**A**) by acidic aqueous acetonitrile and (**B**) by subsequent acidic aqueous acetonitrile–GnCl. (i) Partial HPLC profiles at 214 (milliabsorbance units) nm. Fractions (200 μl) were collected and 5-μl aliquots were analyzed by ^{3}H scintillation counting [overlay histogram, cpm × 10^{-2} in (A); cpm × 10^{-3} in (B)]. The two radioactive peaks (1 and 2) are indicated. In sets: mass spectra (*14*) of the purest fractions, used for amino acid sequence analysis. (ii) Radiosequence analysis of peptide 1 and peptide 2 (*15, 16*).

with leucines, neither of the resulting protoxins (K564L and K690L) (*16*) showing any activity in vivo or in vitro.

The discovery of double acylation at Lys^{564} and Lys^{690} and its importance for toxin activity clarifies previous data from in vivo studies of hemolysin function. In particular, the epitopes of two reported monoclonal antibodies (mAbs) that neutralize hemolytic activity (*21*) have been mapped to proHlyA sequences 518 to 598 (mAb B7) and 673 to 726 (mAb D12). These epitopes encompass acylation site A (Lys^{564}) and acylation site B (Lys^{690}). The results also confirm the previously indicated unusual nature of this prokaryotic maturation event (*5*), which recalls internal modification through amide linkages in the mammalian nicotinic acetylcholine receptor, the insulin receptor, and the immunoglobulin heavy chain polypeptide (*22*).

The two acylated residues lie within regions that are very similar in related toxins (Fig. 5). Acylation site A (Lys^{564}) has 65% similarity (belonging to the same class of amino acid) over 22 residues, with 5 residues being identical in six representative toxins (hemolysins, leukotoxins, and cyclolysin), and acylation site B (Lys^{690}) has 59% similarity over 21 residues, with three identical amino acids. Although consensus sequences can be extracted for both acylation sites, apart from the central Gly, Lys motifs, little of the sequence is conserved. Whereas Lys^{564} is one of the few residues present in all protoxins, Lys^{690} is absent from two of the published sequences. The hemolysins of the toxin family exhibit little target cell specificity as compared with the leukotoxins. Substitution of sequence 564 to 739 in *Pasteurella haemolytica* LktA with that from *E. coli* HlyA has been shown to be sufficient to confer in vivo hemolytic activity on the nonhemolytic LktA (*23*). One interpreta-

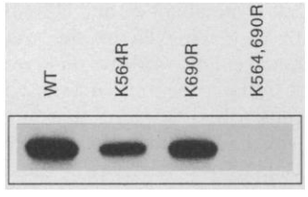

Fig. 4. In vitro acylation of proHlyA derivatives lacking Lys^{564} and Lys^{690}. Wild-type (WT) and mutant proHlyA proteins (0.1 μg) were ^{3}H-palmitoylated in vitro, precipitated with isopropanol, resuspended in HED–5 M urea, separated by SDS-PAGE (10% gel), and visualized by autoradiography (*18*).

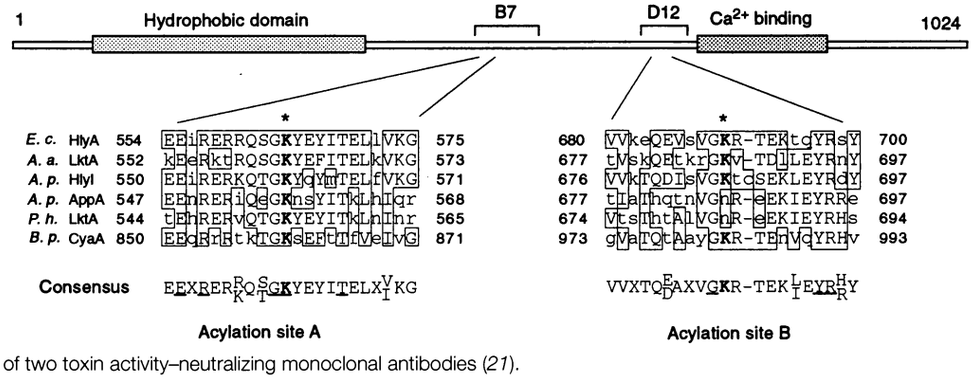

Fig. 5. Alignment of *E. coli* proHlyA acylation sites with sequences of related toxins. Acylation sites A and B are shown in relation to the hydrophobic pore-forming domain and the glycine-rich Ca^{2+}-binding repeats of proHlyA. Underlined residues are common to all six toxins of *Actinobacillus actinomycetemcomitans* (*A. a.*), *A. pleuropneumoniae* (*A. p.*), *Pasteurella haemolytica* (*P. h.*), and *Bordetella pertussis* (*B. p.*) (*6, 17, 36*). Nonconserved amino acids are in lowercase; amino acids belonging to the same class are in uppercase and boxed. Hyphens represent breaks introduced to maximize similarity. Also indicated are the epitope sequences (B7 and D12) of two toxin activity–neutralizing monoclonal antibodies (*21*).

tion of such domain exchange is that separate sequences account for erythrocyte specificity [amino acids 450 to 700 (approximately)] and leukocyte specificity [amino acids 700 to 850 (approximately)] (*23*, *24*), but examination of sites A and B in the different toxins does not support a simple view that their individual acylation might determine erythrocyte and leukocyte binding or that target cell specificity is determined by the HlyC-type protein (*25*, *26*). Nevertheless, it is possible that the major role of acylation, in alliance with Ca^{2+} binding distal to acylation site B, is to initiate binding to the mammalian cell, whereas target cell specificity is determined by a combination of more subtle differences.

Biochemical and physical studies of the pore-forming protein toxins colicin A, aerolysin, and δ-endotoxin have suggested that hydrophobic hairpin structures are central to the transition from the aqueous to the membrane-inserted form, allowing the exposure of hydrophobic domains buried within the secreted toxins when triggered by contact with the target cell (*27*). In hemolysin, the central doubly acylated domain may provide an analogous strongly hydrophobic domain, in this case determined by biochemical modification, as a means of achieving membrane insertion.

Note added in proof: Comparative analysis of tryptic peptides generated from extracellular toxin and protoxin revealed palmitoylation of the ε-amino group of the site B lysine residue (Lys^{983}) in the closely related adenylate cyclase hemolysin (cyclolysin) of *Bordetella pertussis* (*28*). However, cyclolysin sequence 853 to 860 (encompassing the sequence corresponding to acylation site A identified in the *E. coli* hemolysin) was not recovered. Although acylation of cyclolysin Lys^{860} would prevent it acting as a tryptic cleavage site, the toxin population examined was only partially acylated. Thus, the presence of a peptide beginning at residue Ser^{861} may not preclude the existence of an acylated Lys^{860}.

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7. HlyC and proHlyA proteins were purified after expression from recombinant plasmids pT7HlyC (*hlyC* in pAR3039) and pT7PHlyA (*hlyA* in pAR2529) in *E. coli* BL21(DE3)(F-, ompT, recA, rB-) (*9*), as described (*4*, *5*). PalmitoylACP was synthesized from 600 μg of *E. coli* ACP (Sigma) and 0.03 units of acylACP synthetase (Sigma) as described (*5*), with 40 nmol of [^{14}C]palmitic acid (DuPont Biotechnology Systems; specific activity 800 Ci/mol), and purified by chromatography through DEAE-cellulose (Whatman DE52, Whatman Scientific, Maidstone, UK) and octyl-Sepharose CL-4B (Pharmacia) (*5*).
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11. The 1.7 kbp Hind III–Bgl II *hlyA* fragment was mutagenized (*29*) with oligonucleotides to create new restriction sites, in uracil-containing M13mp11 from *E. coli* strain RZ1032 (*dut*, *ung*, *F'*), with a DNA polymerase Klenow fragment (BCL, Boehringer Mannheim, sequencing grade). Phage DNAs transfected into *E. coli* strain TG2 [*supE, hsdΔ5, thiΔ (lac-proAB)Δ(srl-recA)*306::Tn*10 (tet)* F' *(traD36, proAB+, lacZΔM15*] were screened for changes in restriction sites, and all mutations were confirmed by sequencing (*30*) with Sequenase II (U.S. Biochemical). Mutations were introduced either into pAR3040 on a Bam HI–Bgl II fragment (creating proHlyA fragment I496–831) or on a 0.5 kbp Eco RI fragment into pT7PHlyA (creating fragment N1–520). The fragment C831–1024 has been described (*10*). Fragment N1–520 was translated from the *hlyA* ribosome binding site and had no NH_2-terminal fusion sequence but had an additional leucine residue at its COOH-terminus resulting from the fusion. I496–831 and C831–1024 were translated from the T7 gene 10 ribosome binding site and had the gene 10 product NH_2-terminal 11 amino acids. The former also had the tripeptide sequences arginine-glycine-serine and arginine-alanine-serine at the NH_2- and COOH-termini, respectively, and the latter had arginine at the NH_2-terminus.
12. A preparative in vitro reaction {300 μg of proHlyA, 550 μg of HlyC, and 60 μg of [^{3}H]palmitoylACP [synthesized as in Fig. 1, with [^{3}H]palmitic acid (Amersham; specific activity 54 Ci/mol) in 6 ml] generating about 10^8 hemolytic units (HU) (320 HU/ng of HlyA)} was precipitated with isopropanol, resuspended in HED–5 M GnCl, and extracted once with chloroform-methanol (2:1). The interphase was dried under vacuum and resuspended in 30 μl of HED–5 M urea (pH 7.5). Total radioactivity incorporated was 4.8×10^7 cpm. Endoproteinase Lys-C (BCL; sequencing grade) in 25 mM tris-Cl (pH 8.5), 1 mM EDTA, and 20 mM methylamine was added to 5% w/w protein. After three 18-hour incubations at 37°C with fresh enzyme, completion of proteolysis was monitored by SDS–polyacrylamide gel electrophoresis (PAGE) (16% gel) and fluorography. After centrifugation of the completed digestion reaction, the supernatant was dried under vacuum.
13. The peptide mixture was resolubilized in two stages: (i) In 40 μl of 0.5% trifluoroacetic acid (TFA) in 50% aqueous acetonitrile, with mixing and brief sonication, then diluted in 160 μl of H_2O and centrifuged. (ii) The supernatant was loaded directly on to a reversed-phase column equilibrated in 0.1% TFA. The sedimented material from (i) was resuspended in 40 μl of 0.5% TFA in 50% aqueous acetonitrile; 60 μl of H_2O was added, followed by crystals of GnCl (AristaR), to a volume of 200 μl. After centrifugation, the supernatant was loaded onto a reversed-phase column (Aquapore C8, 2 · 1 mm × 100 mm; Applied Biosystems) between 50 μl volumes of 6 M GnCl. In both separations, peptides were eluted over 50 min by a linear gradient of 0 to 60% acetonitrile containing 0.1% TFA. The flow rate was 0.2 ml/min and peptides were detected at 214 nm by a diode array detector (Hewlett Packard).
14. Mass analysis of HPLC peptide fractions was done on a Kratos MALDI III laser (N_2) desorption time-of-flight reflection instrument (Kratos Analytical, Manchester, UK), used in linear mode. Samples (0.2 μl) were mixed with 0.5 μl of matrix (α-cyano-4-hydroxycinnamic acid, 10 mg/ml in 50% ethanol and 0.1% TFA) and applied to a stainless steel slide, air-dried, washed briefly with water, and redried. External calibration over a mass/charge (*m/z*) range of 700 to 5000 D/e was achieved with custom peptides sized by electrospray mass spectrometry. As the resolution of the time-of-flight mass spectrometer was insufficient to resolve ^{3}H and ^{1}H isotopes, the average mass of the ^{3}H palmitoyl group in amide linkage was taken as +238.7 daltons, taking into account the mole percent substitution of the molecule by ^{3}H at this specific radioactivity.
15. Peptide samples (20 to 100 pmol) applied to a glass fiber disc coated with Biobrene Plus, (Applied Biosystems, Warrington, UK) were sequenced on an Applied Biosystems Model 477. No phenylthiohydantoin (PTH) derivative for the palmitoylated residue was detected from the on-line HPLC detector. The position of modified (lysine) residues was indicated by a blank Edman cycle. Equally, no ^{3}H was recovered in the S3 (butyl chloride) solvent in any cycle; counts remained on the disc. To confirm the position of the modified residue, peptide 1 (3×10^5 cpm, 13 pmol) and peptide 2 (1×10^6 cpm, 45 pmol) were applied to a PVDF membrane derivatized with phenylene diisothiocyanate (Sequelon-DITC; Millipore). The efficiency of covalent attachment was 32 and 44%, respectively, determined by assay of unbound peptide extracted by brief washes with 50% aqueous acetonitrile containing 0.1% TFA. Each sample was subjected to automated Edman degradation, and the ATZ amino acid released at each cycle was extracted with methanol and collected and counted for ^{3}H.
16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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18. Plasmids pARAK564R, pARAK690R, and pARAK564,690R were created by introduction of a mutated 0.5-kbp Eco RI–*hlyA* fragment into pT7PHlyA (*11*).
19. For in vivo expression, mutated *hlyA* from pARAK564R, pARAK690R, and pARAK564,690R was introduced on a 3.5-kbp Bam HI fragment into a pEK50 derivative carrying *hlyC* [V. Koronakis, M. Cross, C. Hughes, *Nucleic Acids Res.* **16**, 4789 (1988)]. The genes for the secretion proteins, *hlyB* and *hlyD*, were supplied in trans on a separate plasmid (pEK110) [P. Stanley, V. Koronakis, C. Hughes, *Mol. Microbiol.* **5**, 2391 (1991)].
20. Secreted wild-type and mutant proHlyA proteins were precipitated from cell-free culture supernatant collected at culture absorbance $A_{600} = 0.600$ with ammonium sulphate (40% w/v) and resuspended in 1/50th volume HED-6M GnCl. Samples were diluted in HED and assayed for hemolytic activity [P. Stanley *et al.*, *Mol. Microbiol.* **10**, 781 (1993)].
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32. The deacylation of acylACP seen in Laemmli buffer (*5*) did not occur, and palmitoylACP that had bound to octyl-Sepharose ran as 80% acylACP and 20% free ACP.
33. Hemolytic activity was assayed as released hemoglobin (A543) after incubation with 2% washed equine erythrocytes in 150 mM NaCl and 20 mM $CaCl_2$ [1 HU releases 1 nmol of hemoglobin in 1 hour].

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Coordinate Initiation of *Drosophila* Development by Regulated Polyadenylation of Maternal Messenger RNAs

Fernando J. Sallés,* Marshal E. Lieberfarb,† Christopher Wreden,† J. Peter Gergen, Sidney Strickland

Pattern formation in *Drosophila* depends initially on the translational activation of maternal messenger RNAs (mRNAs) whose protein products determine cell fate. Three mRNAs that dictate anterior, dorsoventral, and terminal specification—bicoid, Toll, and torso, respectively—showed increases in polyadenylate [poly(A)] tail length concomitant with translation. In contrast, posteriorly localized nanos mRNA, although also translationally activated, was not regulated by poly(A) status. These results implicate at least two mechanisms of mRNA activation in flies. Studies with bicoid mRNA showed that cytoplasmic polyadenylation is necessary for translation, establishing this pathway as essential for embryogenesis. Combined, these experiments identify a regulatory pathway that can coordinate initiation of maternal pattern formation systems in *Drosophila*.

In *Drosophila*, four maternal systems define initial embryonic asymmetry: anterior, posterior, dorsoventral, and terminal (*1*). Formation of the anteroposterior axis is guided by the localization of two crucial mRNAs, bicoid in the anterior end (*2, 3*) and nanos in the posterior end (*4*). The dorsoventral and terminal systems each contain a uniformly distributed mRNA—Toll (*5*) and torso (*6*), respectively—encoding a transmembrane receptor (*5, 7*) that responds to localized ligand to create asymmetry (*8*).

These four mRNAs—bicoid, nanos, Toll, and torso—that determine regional specification all exhibit translational activation during early embryogenesis (*3, 6, 9, 10*). Translational activation of maternal mRNAs can be controlled by various processes (*11*), but a predominant mechanism is cytoplasmic elongation of the poly(A) tail (*12, 13*). Cytoplasmic polyadenylation occurs in many species, suggesting it is an ancient and conserved form of mRNA activation during oocyte maturation and embryogenesis. However, it has not been possible to demonstrate an essential role for this regulation in development. To address this question, we have exploited the extensive knowledge of *Drosophila* maternal mRNAs as well as the ability to assess mRNA translation in vivo by injection of mutant embryos.

We used a polymerase chain reaction (PCR)–based assay [poly(A) test (PAT)] to determine whether *Drosophila* mRNAs exhibit changes in poly(A) status upon translational activation (*14*). The size of the PCR products in this assay reflects the poly(A) tail length of the RNA. The assay was applied to four maternal mRNAs—oskar, exuperantia, α4-tubulin, and bicoid—the first three of which are translated during oogenesis (*15*) and were not expected to undergo an elongation of their poly(A) tails during embryogenesis. Bicoid mRNA was a candidate for cytoplasmic polyadenylation because it is translationally regulated (*3*), and in BicD mutant embryos suppression of bicoid mRNA translation is correlated with a short poly(A) tail (*16*).

RNA was isolated from ovaries and embryos at various developmental stages and subjected to PAT analysis (Fig. 1). In oocytes, bicoid mRNA had a poly(A) tail of ~70 nucleotides (nt). Because bicoid mRNA is dormant at this stage, this poly(A) tail length is not sufficient for translation. The length of the poly(A) tail increased within 1 hour after embryo deposition, peaked at ~140 nt in 1 to 1.5-hour embryos, and then progressively shortened (Fig. 1A). The time course of polyadenylation is compatible with a role in translational activation of bicoid mRNA; bicoid protein is first observed in 1- to 2-hour embryos, reaches a maximum at 2 to 4 hours, and then disappears (*3*). Although oskar mRNA contained a poly(A) tail of ~100 nt in oocytes, it was not further elongated during embryogenesis (Fig. 1B); similarly, exuperantia and α4-tubulin mRNAs did not exhibit cytoplasmic polyadenylation. Thus, the increase in poly(A) tail length does not occur in these constitutively translated mRNAs. These data reveal a correlation between cytoplasmic polyadenylation and translational activation during *Drosophila* embryogenesis.

To determine whether polyadenylation controls translational activation of bicoid mRNA, we analyzed the rescuing ability and the poly(A) status of injected bicoid transcripts. Embryos derived from females homozygous for the bcd^{E1} mutation do not produce functional bicoid protein (*17*) or form anterior structures (head and thorax) (*2*). Therefore, the requirements for bicoid mRNA translation can be examined by injecting synthetic transcripts into bcd^{E1} embryos and determining rescue of anterior structures (*18*). Injection of the wild-type bicoid (bcd^{wt}) transcript (Fig. 2) into the anterior region of bcd^{E1} embryos fully rescued the mutant embryos (Fig. 3, A and G), yielding, in some instances, fertile adults. This observation indicated that bcd^{wt} RNA is translated effectively in vivo (*19*).

Determination of the requirement for polyadenylation in translation necessitated the preparation of a nonrescuing bicoid transcript. Because the regulatory sequences for polyadenylation often reside in the 3′ untranslated region (UTR), it was probable that a transcript lacking this region would be inactive. Therefore, an mRNA truncated by 537 nt in the 3′ UTR [at the Sma I site of the complementary DNA (cDNA)] (bcd^{Sma}) was generated (Fig. 2). This shortened bicoid transcript did not rescue mutant embryos (Fig. 3B); even at a 10-fold higher concentration (2 μg/μl), bcd^{Sma} RNA showed essentially no rescue (*20*). The effect of poly(A) tail length on translation was then investigated. bcd^{Sma} RNAs with different poly(A) tail lengths were generated by in vitro polyadenylation (Fig. 2): $bcd^{Sma+A_{50}}$ RNA contained 40 to 60 A residues, which approximates the poly(A) tail on the endogenous, translationally silent, oocyte mRNA; $bcd^{Sma+A_{175}}$ RNA contained 150 to 200 A residues, similar to the poly(A) tail on the endogenous, active, embryonic mRNA. The $bcd^{Sma+A_{50}}$ RNA had no effect on the bcd^{E1} phenotype when injected into mutant embryos (Fig. 3C). In contrast, $bcd^{Sma+A_{175}}$ RNA effectively rescued, with all three thoracic segments often present as well as gnathal and head structures (Fig. 3, D and H).

Although $bcd^{Sma+A_{175}}$ RNA partially

F. J. Sallés, M. E. Lieberfarb, C. Wreden, S. Strickland, Department of Pharmacology, University Medical Center at Stony Brook, Stony Brook, NY 11794–8651, USA. J. P. Gergen, Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794–5215, USA.

*Present address: The Picower Institute for Medical Research, Manhasset, NY 11030, USA.
†These authors contributed equally to this work.