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Messenger RNA for the Insect Storage Protein Calliphorin: *In Vitro* Translation and Chromosomal Hybridization Analyses of a 20 S Poly(A)-RNA Fraction

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A major poly(A)-containing RNA fraction of the approximate size expected of a monocistronic mRNA for the storage protein calliphorin has been isolated from the larval fat bodies of Calliphora vicina *during early instar 3. This 20 S RNA fraction programs the synthesis by cell-free wheat embryo extracts of polypeptides of 86,000 daltons identified by tryptic peptide fingerprinting as precursors of the authentic calliphorin subunits of 83,000 daltons. Complementary DNA synthesized by AMV reverse transcriptase using the same 20 S RNA as template hybridized* in situ *to a single segment of one or two bands in the salivary polytene chromosomes of* C. vicina.

KEY WORDS: *Calliphora*; calliphorin; storage protein; hemolymph; fat body; mRNA; *in vitro* translation; *in situ* hybridization; structural gene.

INTRODUCTION

A major function of the feeding larval stages of holometabolous insects such as the Diptera is the accumulation in the fat body and hemolymph of protein reserves which are subsequently hydrolyzed to provide the amino acids required for development of the pharate pupal and adult stages (reviewed by Thomson, 1975). Munn and his colleagues (Munn *et al.*, 1967, 1971; Munn and Greville, 1969) have isolated one such storage protein, calliphorin, from the late larva of the blue blowfly *Calliphora vicina* Robineau-Desvoidy (= *C.*

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erythrocephala Meigen), when calliphorin composes about 60% of the total protein extractable in aqueous media.

Calliphorin is synthesized in very large amounts in the fat body of actively feeding larvae of *Calliphora* and is secreted into the hemolymph (Price, 1973; Thomson, 1975). In *C. stygia,* the larval fat body consists of about 11,500 cells. During the period of maximum protein synthesis in early third instar, each of these cells produces about 400 ng protein in 24 hr, and of this about 80% is calliphorin (Thomson, 1975). Synthesis of calliphorin stops over the 24-hr period following the cessation of feeding (Kinnear *et al.,* 1971; Thomson, 1975).

Calliphorin consists of a series of closely related but heterogeneous hexamers of about 530,000 daltons (Munn *et al.,* 1971). The subunits from *C. vicina* are, as in *C. stygia,* 83,000 (±5%) daltons and are immunologically homogeneous (Kinnear and Thomson, 1975). Inbred strains breed true for a characteristic number and quantitative pattern of electrophoretically separable calliphorin subunits (Kinnear, 1973), which in both species fall into at least six discrete charge classes. No detailed genetic analysis is available for *Calliphora,* but a similar multiplicity of subunits occurs in the homologous storage protein lucilin in the closely related calliphorid fly *Lucilia cuprina.* In *L. cuprina,* 12 or more structural loci code for subunits differing in charge but having identical molecular weights and immunological determinants (Thomson *et al.,* 1976).

We report here on the isolation of the predominant poly(A)-containing RNA from the fat body of *C. vicina* larvae and show that it has the properties anticipated for an mRNA coding for a polypeptide precursor of calliphorin. *In situ* hybridization of cDNA prepared from this RNA fraction to the salivary polytene chromosomes of *Calliphora* larvae provides an approach to the study of the arrangement within the genome of the structural loci for calliphorin.

MATERIALS AND METHODS

Insects

Mass laboratory cultures of *C. vicina* were established from offspring of field-inseminated females trapped at Canberra, Australian Capital Territory. Larvae were reared on lean bovine muscle at 20–22 C.

Preparation of [^{35}S]Methionine-Labeled Calliphorin

Larvae collected within 6 hr of molting to third instar were washed, anesthetized by chilling in ice water, and injected with 2 μCi L-[^{35}S]methionine

(Radiochemical Center, Amersham, 405 Ci/mmole, in 2 μl water). The larvae were replaced on meat and incubated for a further 48 hr. Healthy, fully mobile larvae with empty crops, composing 85% of those injected, were collected for bleeding into chilled 0.1 M phosphate buffer at *p*H 6.25. The diluted hemolymph was centrifuged to remove hemocytes. Aliquots were subjected to electrophoresis at *p*H 6.25 (Munn *et al.*, 1971) in 0.1 M phosphate buffer in preparative 4% polyacrylamide slab gels run horizontally at 4 C. The position of the calliphorin band (Kinnear and Thomson, 1975) was determined by surface staining of the gels with 8-anilino-1-naphthalenesulfonate (Hartman and Udenfriend, 1969). The calliphorin band was excised and eluted with the phosphate buffer containing 0.15 M NaCl. Aliquots of the eluted calliphorin, precipitated with trichloroacetic acid (TCA) for counting and protein estimation by the procedure of Lowry *et al.* (1951), showed a specific activity of 90 cpm/μg.

Bulk Isolation of Larval Fat Body

Batches of larvae (about 50 g) collected 24 ± 12 hr after molting to instar 3 were washed in chilled water and several changes of saline (Robb, 1969) modified by replacement of sucrose with mannitol (R-M saline). All operations were carried out at 0–4 C. The larvae were drained, mixed with 0.5 vol 30% Ficoll (Pharmacia, Uppsala) in R-M saline, and then crushed between two hand-rotated metal rollers spaced by trial to minimize tissue damage. Aliquots of 20 ml tissue suspension were placed in 50-ml plastic centrifuge tubes, overlaid successively with 20 ml 7% Ficoll in R-M saline and 5 ml R-M saline. After centrifugation for 5 min at 4,000*g* in a swinging bucket rotor, fat bodies were collected with a wide-bore pipette from the top of the 7% Ficoll layer. Contaminating pieces of gut were removed by hand sorting, after which the mixture was diluted by addition of 1 liter R-M saline. Fat bodies (4–5 ml) were collected by centrifugation for 5 min at 2000*g* with very little contamination. Most cells ($>90\%$) were intact when examined by phase contrast microscopy.

Extraction and Purification of Fat Body RNA

The fat bodies collected in bulk were gently resuspended in an equal volume of R-M saline. Aliquots of 2 ml were disrupted by pouring into a vortexing mixture consisting of 5 ml phenol and 5 ml extraction buffer containing 1% (w/v) Sarkosyl, 0.1 M NaCl, 0.1 M tris, 20 mM EDTA at *p*H 8.9. After addition of 5 ml chloroform containing 4% isoamyl alcohol, the upper aqueous phase was removed by brief centrifugation, reextracted with 2.5 ml fresh phenol, and again collected by centrifugation (tube 1). The original phenol phase and the

flocculent interphase were reextracted with 2.0 ml extraction buffer and the aqueous layer was again retained (tube 2). After the phenol phase from the second extraction had been discarded, the interphase was mixed with 5 ml chloroform–isoamyl alcohol and the aqueous phase (tube 3) was retained. The contents of tubes 1–3 were combined and reextracted first with phenol–chloroform–isoamyl alcohol and then with chloroform–isoamyl alcohol. RNA was precipitated by adding 0.1 vol of 1.0 M Na acetate, *p*H 5.0, and 2.5 vol of ethanol. About 35 mg RNA was obtained from each batch of larvae.

For sucrose gradient fractionation, aliquots containing 50 μg RNA were loaded onto linear 10–40% (w/v) sucrose gradients in 10 mM NaCl, 20 mM tris-HCl, 1 mM EDTA at *p*H 7.5. Gradients were centrifuged for 14 hr at 37,000 rpm in a Beckman SW41 rotor at 3 C and were then fractionated by upward displacement with continuous monitoring of absorbance at 254 nm.

Oligo(dT)-cellulose chromatography of the RNA was performed by the procedure of Aviv and Leder (1972), except that the bound RNA was rechromatographed twice on the affinity column.

In Vitro Protein Synthesis

RNA in 0.5-ml fractions from sucrose gradient separations of both total fat body RNA and oligo(dT)-cellulose-bound RNA was precipitated by addition of 50 μl sodium acetate (*p*H 5.0), 1.5 ml ethanol, and 2.5 μg wheat embryo RNA as carrier. After at least 16 hr at −20 C, the RNA was collected by centrifugation at 12,000*g* for 10 min, dried *in vacuo*, and dissolved in 50 μl water. Aliquots were then tested for ability to initiate protein synthesis in the wheat embryo system of Roberts and Paterson (1973), prepared according to Higgins *et al.* (1976*a*). Optimal salt concentrations with *Calliphora* RNA were 3 mM Mg^{2+} and 100 mM K^+. The preincubation step was omitted, and no tRNA and no unlabeled amino acids were added. L-[^{35}S]Methionine was the sole labeled amino acid used. Each incubation mix (50 μl) was allowed to stand for 90 min at 24 C. The acid-insoluble radioactivity in 10-μl aliquots was determined after precipitation onto Whatman GF/C disks with hot TCA (Higgins *et al.*, 1976*b*), and the remainder of each incubation mix was treated with 3 vol acetone to precipitate proteins and polypeptides for further analysis.

The dose–response to each RNA preparation was examined and a subsaturating level of RNA was used subsequently for comparative assays.

SDS-Polyacrylamide Gel Electrophoresis of *in Vitro* Products

Acetone-precipitated products from each *in vitro* incubation were collected by low-speed centrifugation and dissolved in tris–glycine (T-G) buffer (0.05 M

tris, 0.38 M glycine, *p*H 8.3) containing 2% sodium dodecylsulfate (SDS), 1% 2-mercaptoethanol, and 10% sucrose. Samples were agitated strongly, heated 1 min in a boiling water bath, and remixed repeatedly. Aliquots of 10–20 μl were separated by electrophoresis in vertical gel slabs consisting of 4% polyacrylamide in which the sample pockets were cast and a separating gel of 10% polyacrylamide (75 by 75 by 2.5 mm). Half-strength T-G buffer was used in the electrode compartments, with 0.1% SDS in the cathode chamber. Electrophoresis at 20 V/cm for 1.5 hr at 20 C was terminated by fixing and staining in 0.2% Coomassie blue for 30 min at 60 C with continuous agitation. The stain was dissolved in methanol–acetic acid–water with a volume ratio of 5:1:4 and gels were destained in 3:1:6 mixture of the same solvents. Following dehydration in dimethylsulfoxide, gels were impregnated with scintillant, dried down, and fluorographed (Bonner and Laskey, 1974) against Kodak X-OMAT X-ray film.

Tryptic Hydrolysis and Peptide Mapping

The radioactive translation products of five cell-free incubation mixtures programmed with the 20 S fat body RNA fraction were pooled and precipitated with about 6 mg unlabeled calliphorin as carrier. This material and 4.5 mg *in vivo* labeled [^{35}S]calliphorin (4.5×10^5 cpm) were digested separately with trypsin after performic oxidation and used for peptide mapping following the procedures of Higgins *et al.* (1976*a*), except that ascending chromatography was carried out twice in the first dimension, with a drying step between runs. Peptides were located by spraying with 1% ninhydrin spray and radioactivity was detected by autoradiography against Kodak X-OMAT X-ray film.

Synthesis of Complementary DNA and Hybridization with Fat Body RNA

Purified 20 S RNA showing apparent calliphorin messenger activity was used as a template for DNA synthesis by the reverse transcriptase of avian myeloblastosis virus (AMV) as described by Kemp (1975). The cDNA was purified and hybridized to *Calliphora* RNA fractions, also following Kemp's procedures. RNA samples taken up in 0.18 M NaCl, 0.05% SDS, 10 mM tris-HCl, 1 mM EDTA at *p*H 7.5 were diluted serially to give the required concentrations. To each dilution about 10^3 cpm of [^{3}H]20 S cDNA was added. The mixtures were denatured at 100 C for 1 min and then incubated at 60 C for 1 hr. The extent of hybridization was monitored by resistance of the cDNA to digestion by single-strand-specific nuclease. R_0t values were calculated from the RNA concentrations multiplied by the time of incubation according to Britten and Kohne (1968). Results were expressed as percentage resistance of the cDNA to nuclease S_1 after incubation with RNA to the R_0t values specified.

In Situ Hybridization with Salivary Polytene Chromosomes

Wandering larvae of *C. vicina* were dissected in saline (Robb, 1969) about 72 hr after molting to instar 3. Salivary glands were transferred immediately to 45% acetic acid, opened in short pieces, and teased apart in lactic acid–acetic acid (1:1 v/v) on well-cleaned slides. The cells were dispersed by tapping and lightly squashed under a coverglass. After removal of the coverglass in liquid N_2, slides were passed through 95% ethanol, air-dried, treated with 0.2 M HCl (Macgregor and Kezer, 1971) for 10 min at 37 C, washed, dehydrated through an ethanol series, and again air-dried. On each preparation was placed 4 μl of a solution containing 2.2×10^4 cpm [^{3}H] 20 S cDNA in 3 × SSC (1 × SSC contains 0.15 M NaCl, 0.015 M Na_3 citrate, *p*H 7.2), 50% formamide, and 0.01% SDS, under an 18 by 18-mm coverglass ringed with rubber gum. Slides were heated 5 min at 70 C to open any secondary structure in the cDNA and hybridization was allowed to proceed for 12 hr at 40 C, after which the coverglasses were floated off in the incubation solution less cDNA. After two washes in large volumes of this fluid and six washes in 2 × SSC, the preparations were dehydrated through an ethanol series and air-dried.

Slides were dipped in Ilford K2 emulsion diluted 1:1 with water. An exposure of 9 weeks was allowed and autoradiographs were developed in Kodak Dektol (1:1 v/v with water), fixed, washed, stained with Giemsa in phosphate buffer at *p*H 6.8, rinsed, and air-dried.

RESULTS

Characteristics of Fat Body RNA

RNA prepared from bulk-isolated larval fat body was shown on sucrose gradient centrifugation (Fig. 1a) to yield sharp 18 S and 28 S ribosomal RNA peaks without significant amounts of RNA sedimenting between 6 S and 18 S. In addition to the 18 S and 28 S RNA species, another major peak of absorbance was observed to sediment at about 33 S. The large amount of this material present (20–40% of the total absorbance in different preparations) suggests that it may be composed of aggregates of ribosomal RNA, accounting for the observed departure of the molar ratio of 18 S to 28 S RNA from the expected value of 1:2. This explanation is supported by the observation that after heating the RNA at 80 C for 1 min, the 33 S RNA dissociated into material sedimenting between 18 S and 19 S (Fig. 1b). The 28 S RNA also dissociated after heating, as reported for other insect 28 S rRNAs by Shine and Dalgarno (1973).

On passage through oligo(dT)-cellulose, about 1.5% of the total heated fat body RNA bound in the presence of 0.2 to 0.5 M NaCl but could be eluted

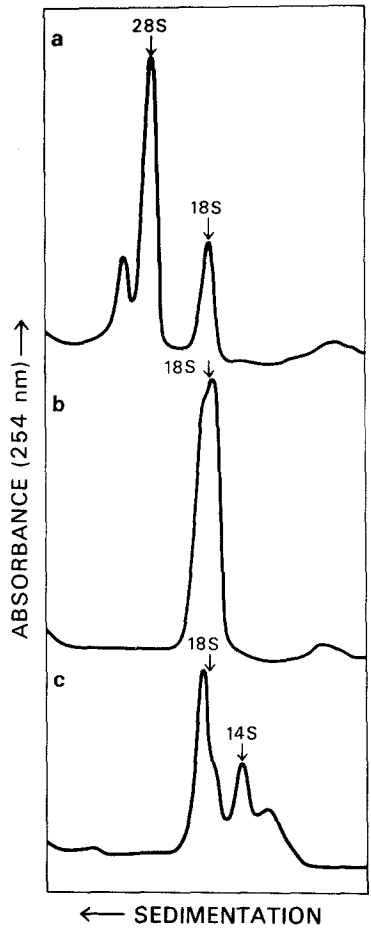

Fig. 1. Sucrose density gradient centrifugation of larval fat body RNA from *Calliphora*. (a) Total RNA, (b) total RNA after heating 1 min at 80 C, (c) oligo (dT)-cellulose-bound RNA.

with water. The fraction binding to oligo(dT)-cellulose was found by sucrose gradient centrifugation (Fig. 1c) to contain broad peaks sedimenting around 9 S and 12 S and sharp peaks sedimenting at 14 S and 20 S, the latter appearing as a faster-sedimenting shoulder on the 18–19 S peak of contaminating ribosomal RNAs. The sedimentation profile of the nonbound fraction (not shown) was indistinguishable from that of the heated, unfractionated RNA.

In Vitro Protein Synthesis Directed by Larval Fat Body RNA

Under subsaturating conditions established for each RNA preparation, incorporation of labeled methionine into acid-precipitable polypeptides was linear with respect to the amount of RNA added to the wheat embryo cell-free

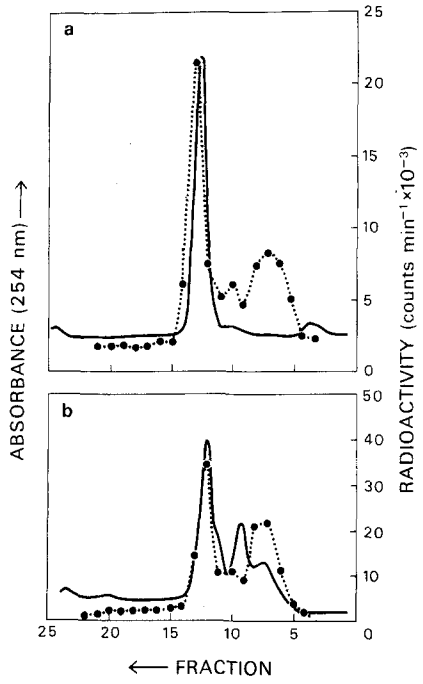

Fig. 2. Translation of fat body RNA in the cell-free wheat embryo system. (a,b) Incorporation into polypeptides directed by RNA precipitated from fractions across the sucrose gradient separations shown. ——, Absorbance; · · · ·, radioactivity. (a) Total RNA, 8-μl aliquots per fraction; (b) oligo(dT)-cellulose-bound RNA, 1-μl aliquots per fraction. (c,d) Fluorographs of the translation products of these fractions analyzed by SDS-polyacrylamide gel electrophoresis (O, origin). (c) Total RNA, gradient fractions 5–14; (d) oligo(dT)-cellulose-bound RNA, gradient fractions 5–14. Arrows indicate position of calliphorin subunit marker.

system. Of the total messenger activity of the whole RNA preparation, 80–90% was recovered after oligo(dT)-cellulose chromatography. About 40–50% of this activity was in the oligo(dT)-bound fraction, representing approximately thirtyfold enrichment of mRNA in this fraction.

The sucrose gradient fractions of total and oligo(dT)-bound RNA were assayed in the wheat embryo translation system (Fig. 2a,b, respectively). The major peak of mRNA activity sedimented at 20 S in both fractions. In addition, there was in each fraction a broader zone of mRNA activities

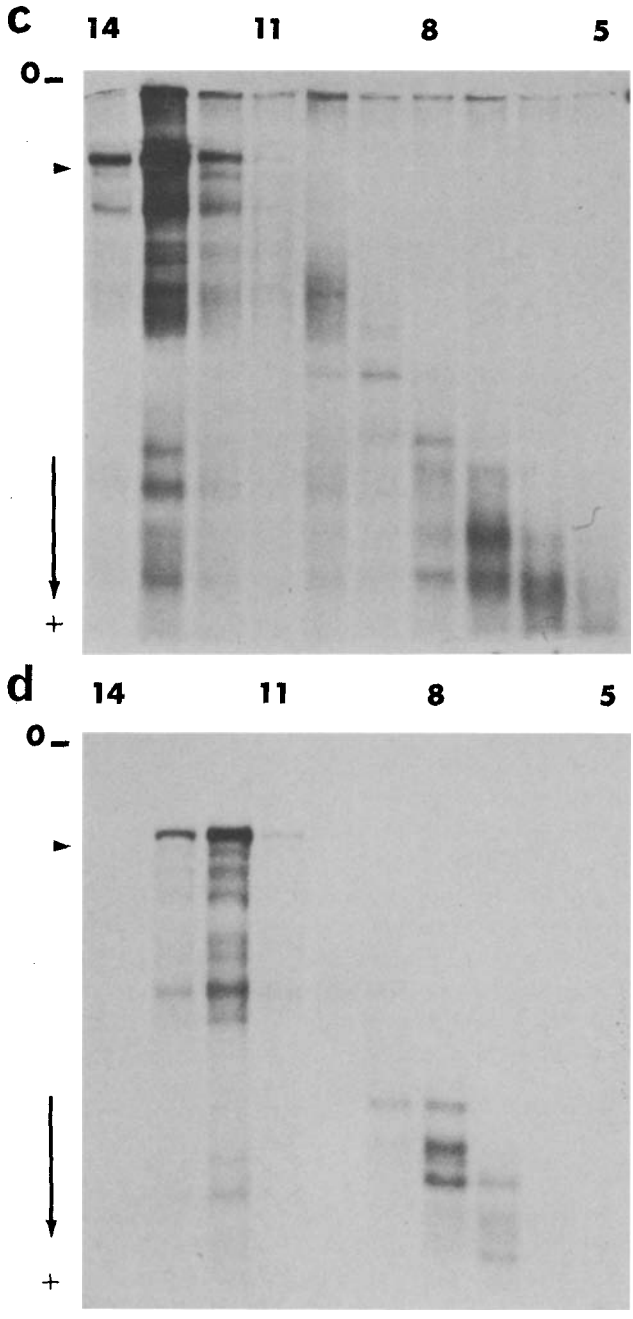

Fig. 2. Continued.

sedimenting between about 7 S and 18 S. The size distributions of mRNAs in the total RNA and oligo(dT)-cellulose-bound fractions were indistinguishable within the degree of reproducibility of this system with different preparations. Fraction 9 (Fig. 2b) showed relatively low efficiency as a template for protein synthesis. This fraction contains 14 S RNA, much of which may be poly(A)-containing mitochondrial rRNA (Kemp and Peacock, to be published). The major mRNA peak at 20 S was purified three- to fourfold with respect to the oligo(dT)-bound RNA by the gradient centrifugation step, giving a final purification of 90- to 120-fold for this component.

The *in vitro* translation products of the RNA fractions from gradient centrifugations of both total and oligo(dT)-bound RNA were examined by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 2c,d). A correlation of size of the polypeptides synthesized with increase in size of the added RNA was evident. The major product translated from the 20 S RNA fraction of total RNA and the oligo(dT)-bound RNA was a polypeptide of about 86,000 daltons, slightly larger than the calliphorin marker (83,000 ± 5% daltons) run on the same gel. In addition, many bands of lower molecular weight were synthesized in lesser amounts from the 20 S RNA. These bands may be incomplete translation products (Burstein *et al.*, 1976) from the same mRNA which codes for the product of 86,000 daltons.

The polypeptides synthesized in the wheat embryo system in response to addition of the 20 S putative calliphorin mRNA were compared with authentic [^{35}S]calliphorin from *C. vicina* by tryptic peptide mapping (Fig. 3). Allowing for small experimental variations in the relative mobility of the tryptic peptides in both chromatographic and electrophoretic dimensions, the products of the wheat embryo system contain [^{35}S]peptides which correspond closely to all of the 40 labeled peptides in the reference calliphorin (Fig. 3a,b). A small number of additional minor radioactive components are probably attributable to peptides derived from the extra 3000 dalton component of the *in vitro* product, and perhaps to incomplete translation products. The distribution of [^{35}S]peptides in the ninhydrin-developed tryptic fingerprint of the reference [^{35}S]calliphorin is shown in Fig. 3c. The labeled fragments are widely distributed among the total of about 110 peptides resolved from calliphorin.

Synthesis and Properties of DNA Complementary to 20 S mRNA

The 20 S fraction of oligo(dT)-bound RNA from the larval fat body was an active template for the AMV reverse transcriptase in the presence of oligo (dT), as found for other poly(A)-containing mRNAs (Kacian *et al.*, 1972; Ross *et al.*, 1972). The 18 S fraction (mainly rRNA) of the nonbound RNA was over 100 times less active as a template. The cDNA obtained using the 20

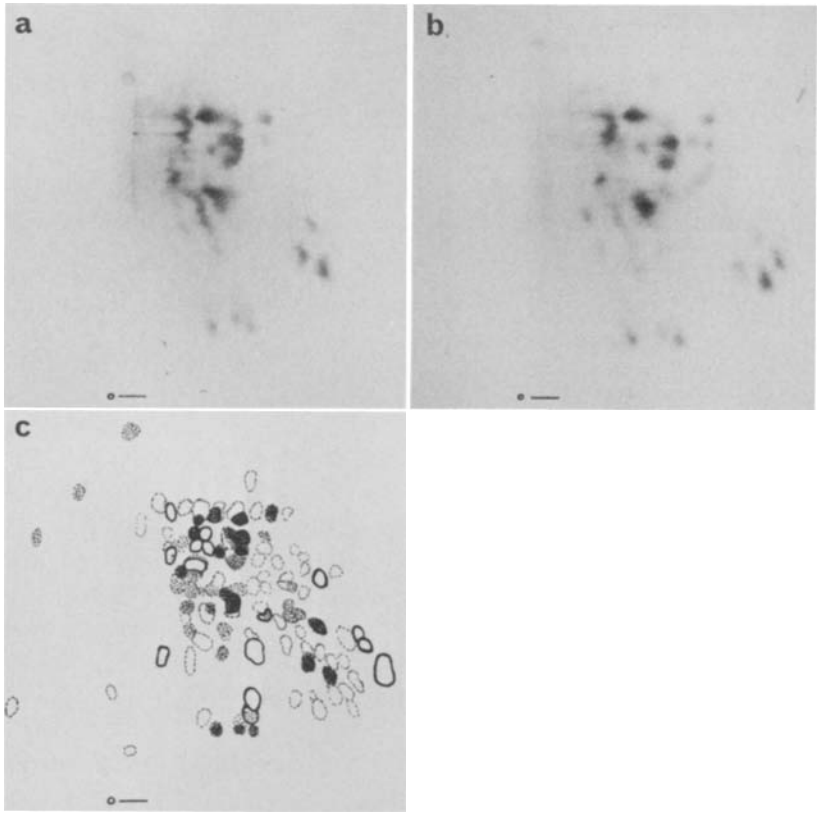

Fig. 3. Tryptic peptide fingerprints. Chromatography bottom to top, electrophoresis left to right; O, origin. (a) Autoradiograph showing [^{35}S]methionine peptides from reference calliphorin. (b) Same from cell-free wheat embryo system programmed with 20 S RNA fraction. (c) Diagram showing distribution of [^{35}S]peptides (stippled) among ninhydrin-detected peptides of reference [^{35}S]calliphorin. Spots which stained heavily with ninhydrin are shown with entire boundary, weaker spots with broken boundary.

S RNA showed an average length of about 500 bases when subjected to alkaline sucrose gradient centrifugation.

As a preliminary test of the specificity of this cDNA ("20 S cDNA"), it was hybridized to total fat body RNA and to the oligo(dT)-bound fraction of the RNA under conditions of great RNA excess (e.g., Bishop and Rosbash, 1973). The rate of hybridization of the 20 S cDNA to the oligo(dT)-bound RNA was about 40 times faster than its hybridization to the total RNA (Fig. 4). This result demonstrates that the 20 S cDNA cannot be complementary to rRNA since the bound RNA fraction is depleted by a factor of more than 100

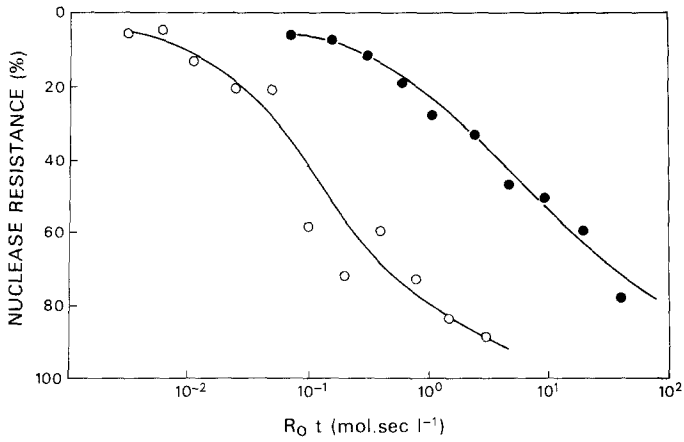

Fig. 4. Hybridization of 20 S cDNA to larval fat body RNA. •, Total RNA: ○, oligo(dT)-bound RNA.

with respect to ribosomal RNA. As the 20 S mRNA codes predominantly for calliphorin, and as the efficiency of cDNA synthesis by reverse transcriptase is similar for a variety of mRNAs (Kacian *et al.*, 1972; Kemp, 1975; Ross *et al.*, 1972), we conclude that the 20 S cDNA formed is primarily a copy of calliphorin mRNA. The degree of purity has not been directly established, since this would require demonstrably pure calliphorin mRNA.

In Situ Hybridization of Calliphorin cDNA to Salivary Polytene Chromosomes

Positive results were obtained in three hybridization experiments with [^{3}H] 20 S cDNA. Where accumulation of silver grains occurred, it was always over a single chromosome region in each nucleus (Fig. 5), recognizable from the pattern of adjacent bands as the same segment in every case. This segment is close to a chromosome end. A mean count of 15.1 grains for the region was obtained from 60 labeled nuclei. Mean background over an area equal to that of the labeled segment ranged from 0.04 to 0.14 grains. In some nuclei the silver grains appeared to lie in two closely spaced zones, apparently covering one, or at the most two, chromosome bands.

Attempts to hybridize 20 S cDNA to salivary polytene chromosomes from *Drosophila* gave negative results in several separate experiments, although immunological cross-reactivity of calliphorin and a component in *Drosophila* has been reported (Munn and Greville, 1969).

DISCUSSION

Calliphorin consists of polypeptides containing about 800 amino acid residues

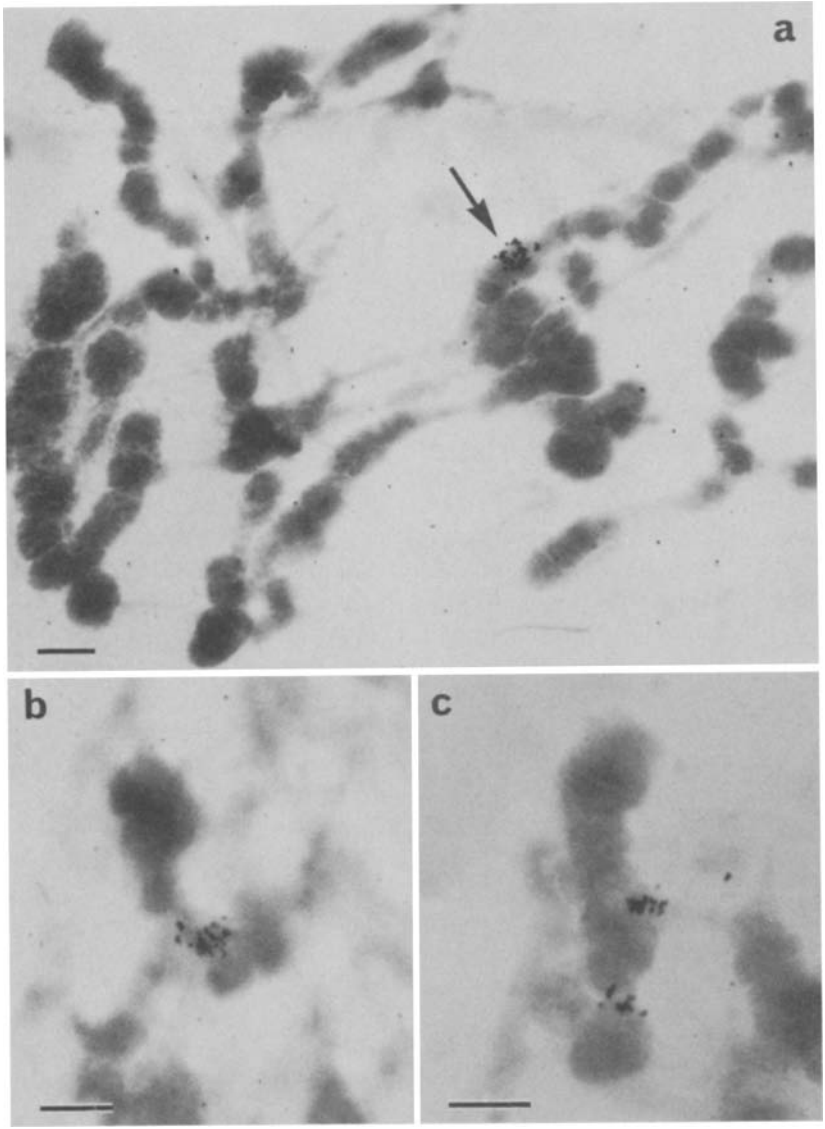

Fig. 5. (a–c) *In situ* hybridization of 20 S cDNA to polytene chromosomes of the larval salivary gland. (a) Note distribution of silver grains in two groups at arrow. (c) Homologues are unsynapsed, twisted, and differentially stretched. Scale bar, 10 μm.

based on subunit sizes given by Munn *et al.* (1971; also Kinnear, 1973). The mRNA for calliphorin must therefore contain at least 2400 bases in the coding region, with additional bases to account for the poly(A) segment, untranslated regions, and/or segments coding for sequences present in precursor polypeptides but not the mature calliphorin subunits. Calliphorin mRNA would therefore be expected to sediment above 18 S. We have no direct measure of homogeneity in the 20 S RNA fraction. However, as the major product of *in vitro* translation of the 20 S fat body RNA is a polypeptide about 3000 daltons larger than authentic calliphorin, and closely related to it as shown by peptide mapping, the 20 S oligo(dT)-bound RNA fraction appears to consist mainly of monocistronic mRNAs coding for precursors of the calliphorin subunits. The synthesis of calliphorin as a larger precursor is similar to the situation holding for a number of other secreted proteins (e.g., Kindås-Mügge *et al.*, 1974; Schmeckpeper *et al.*, 1974). Since calliphorin constitutes about 80% of the protein synthesized *in vivo* by the larval fat body during the feeding stage of instar 3, the high proportion of the 20 S fraction in the oligo(dT)-bound RNA is consistent with its identification as calliphorin mRNA.

[^{35}S]Methionine was chosen as the sole labeled amino acid for *in vitro* protein synthesis in the present experiments because of the unusually high methionine content of calliphorin (Munn *et al.*, 1971), *viz.* about 30 residues in each subunit of 83,000 daltons (Thomson *et al.*, 1976), and the wide distribution of methionine-containing peptides among the total tryptic peptides of purified calliphorin (Fig. 3c). Approximately 110 tryptic peptides are detected with ninhydrin on heavily loaded two-dimensional separations, whereas the total of lysine plus arginine residues is about 76 per calliphorin subunit. Further, purified [^{35}S]methionine-labeled calliphorin gives on tryptic digestion about 40 [^{35}S]peptides, again in excess of the number expected from the subunit amino acid composition. These observations are consistent with limited sequence heterogeneity among calliphorin subunits which are separable on the basis of charge but not molecular weight (Kinnear and Thomson, 1975). The various calliphorin subunits appear likely to be produced from multiple loci similar to those coding for the homologous storage protein lucilin (Thomson, 1975; Thomson *et al.*, 1976). Peptide mapping of individual calliphorin subunits will be needed to examine whether such heterogeneity exists in the *in vitro* products from 20 S mRNA translation, as in the case of feather keratin mRNA (Kemp, 1975). Peptide analysis, or electrophoretic fractionation of the mRNA after deadenylation (Vournakis *et al.*, 1975), would also contribute to assessment of possible contaminating messengers coding for less abundant fat body proteins such as protein II (Munn and Greville, 1969; Kinnear and Thomson, 1975).

The salivary gland chromosomes in *Calliphora* are highly polytenized, although not favorable for chromosome recognition due to the lack of linear

continuity of the banded regions (Thomson, 1969). *In situ* hybridization was, however, attempted using this tissue because of the large number of nuclei available in squash preparations and because of the fact that the salivary glands synthesize no proteins related to calliphorin immunologically or by subunit composition (Kinnear, 1973). The calliphorin cistrons are therefore expected to occur in condensed, banded segments in the salivary gland chromosomes.

The results reported here strongly suggest that the structural genes for the calliphorin subunits are clustered at one site in the genome, involving one, or possibly two, chromosome bands. It is recognized, however, that we have not demonstrated rigorously that the *in situ* data refer to the same sequences as those translated *in vitro*. Hybridization of 20 S cDNA to the polytene chromosomes of pupal trichogen nuclei should now permit precise positioning of the chromosome segment containing the calliphorin cistrons using the maps prepared for *C. vicina* by Ribbert (1967).

Genetic and biochemical evidence suggests that in *Lucilia cuprina* 12 or more closely related structural genes code for the subunits of the calliphorin homologue, lucilin (Thomson *et al.*, 1976). Two of these loci are situated on chromosome 2 and linkage of other structural loci for the lucilin genes to these was inferred. The present evidence that the calliphorin cistrons are clustered together in *C. vicina* is especially interesting in view of the postulated origin of the multiple genes for this major larval storage protein through gene duplication and subsequent mutational divergence (Thomson, 1975; Thomson *et al.*, 1976). A contrasting situation has been described in *Chironomus*, where Tichy (1970) has presented evidence that the cistrons coding for multiple closely related hemoglobins are situated in at least three separate regions on chromosome 3, suggesting that in this case chromosome rearrangement has occurred since the initial gene duplication.

NOTE ADDED IN PROOF

Since this paper was submitted, we have seen the following related article: Sekeris, C. E., Perassi, R., Arnemann, J., Ullrich, A., and Scheller, K. (1977). Translation of mRNA from *Calliphora vicina* and *Drosophila melanogaster* larvae into calliphorin and calliphorin-like proteins of *Drosophila*. *Insect Biochem.* **75**:5.

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