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Purification and Reverse Transcription of the Messenger RNA Coding for the Insect Protein, Calliphorin, Isolated from Larvae of the Blowfly, *Calliphora vicina* R.-D.

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Starting from poly(A)-containing RNA prepared from the fat body of larvae of the blowfly *Calliphora vicina*, we have purified an mRNA coding for the protein calliphorin, which is a major blowfly protein accounting for approximately 9% of the total poly(A)-containing mRNA activity in the fat body of 5-days-old larvae, as demonstrated by translation *in vitro*. The mRNA for this protein was purified by sucrose gradient centrifugation under denaturing conditions and identified by cell-free translation. The peak fraction of the gradient shows three bands of about 20 S after formamide/acrylamide gel electrophoresis. Using reverse transcriptase and *Calliphora* mRNA isolated directly from the acrylamide gel electrophoresis or from sucrose gradients, we synthesized a cDNA probe. This cDNA hybridizes with the template, showing a major kinetic component with a molecular weight of 2.8×10^6, fitting well with the three bands observed in the electrophoresis. Hybridization of the cDNA with total sonicated *Calliphora* DNA shows annealing only at very high c_0t values, indicating that *Calliphora* mRNA is transcribed from the unique portion of the fly genome.

During development of Diptera larvae, a group of closely related proteins are synthesized in the fat body [1–7] and accumulate, making up at a certain stage more than half of the proteins soluble in dilute salt solutions [1,3,5,7]. In the blowfly *Calliphora vicina*, a single protein has been identified, composed of six or seven identical subunits with a molecular weight of approximately 80000 each, known as calliphorin [1,2]; it is immunochemically related to drosophilin and luciphilin, the proteins of *Drosophila* [1,5,6] and *Lucilia* [3] respectively.

It has been demonstrated that the biosynthesis of calliphorin is restricted to 3–5-days-old larvae [7,8]; furthermore, translatable calliphorin mRNA appears in 3-days-old larvae, reaches maximal concentrations in 4–5-days-old animals and then decreases, being absent in pupae or imagos [7]. It is evident that the determination of the mRNA content by means of translational activity encompasses only the functionally active mRNA. We have, therefore, in order to account for all of the RNAs containing the sequences for calliphorin, proceeded to purify the calliphorin mRNA and with the help of reverse transcriptase synthesize its cDNA. The results of these experiments will be presented in this paper.

MATERIALS AND METHODS

Reagents

L-[4,5-$^3H_2$]Leucine (55 Ci/mmol) and L-[^{35}S]methionine (580 Ci/mmol) were obtained from Amersham Buchler (Braunschweig). Poly(U)-Sepharose was purchased from Pharmacia (Uppsala). Fresh wheat germ was a kind gift of GEG (Mannheim). All other chemicals were of analytical grade obtained from Serva (Heidelberg), Merck (Darmstadt) or Boehringer (Mannheim).

Animals

Calliphora vicina R.D. larvae were reared on beef meat at 25 °C and 65% relative humidity. They pupate under these conditions within 7–8 days after egg deposition. Maximal synchronization was ensured by allowing deposition of eggs only for 60 min.

Isolation of Poly(A)-Containing mRNA from the Fat Body

The RNA was prepared from isolated fat bodies, usually of 1000 animals, as described by Scheller and

Karlson [9]. Poly(U)-Sepharose separation was then performed according to Molloy et al. [10]. Two cycles of poly(U)-Sepharose chromatography were performed. The poly(A)-containing RNA was eluted at 40 °C with 0.01 M Tris buffer, pH 7.4, containing 0.2% sodium *N*-laurylsarcosinate but not formamide.

Protein Synthesis in vitro

Protein synthesis was performed in a wheat germ system first described by Roberts and Paterson [11], as used by Roewekamp et al. [12], Sekeris et al. [6], and Sekeris and Scheller [7].

Gel Electrophoresis and Fluorography

Sodium dodecylsulphate/acrylamide gel electrophoresis was performed according to Laemmli and Faure [13] on 7.5% or 10% gels. The proteins were stained with Coomassie blue. Fluorography was performed using the method of Laskey and Mills [14]. The gels were scanned with a Vernon apparatus equipped with an integrator.

Sucrose Gradient Centrifugation

5–20% linear sucrose gradients were prepared in 25 mM Tris-HCl, pH 7.5 containing 100 mM KCl and centrifuged for 6 h at 4 °C in a Beckman SW41 head. The RNA was heated 4 min at 70 °C before centrifugation.

Centrifugation under denaturing conditions was performed in 5–20% sucrose gradients in 70% formamide containing 3 mM Tris-HCl, pH 7.5 and 3 mM EDTA. The time of centrifugation was 24 h at room temperature. The RNA was heated at 70 °C for 5 min prior to centrifugation in the formamide buffer.

Peptide Mapping of Calliphorin Synthesized in vitro

Peptide mapping of the product synthesized *in vitro* by limited proteolysis was performed as described by Cleveland et al. [15]. After sodium dodecylsulphate/acrylamide gel electrophoresis of the synthesized products, the region corresponding to calliphorin, as determined by marker calliphorin, run together with the radioactive products, was cut out and partially digested with chymotrypsin directly in the sample wells of a second gel [15]. The concentration of chymotrypsin was 50 µg/ml and the digestion period 30 min at 24 °C. The concentration of the acrylamide was 15%. The gels were stained with Coomassie blue and then submitted to fluorography.

Electrophoresis of the RNA

RNA was submitted to electrophoresis on 3.2% and 4% polyacrylamide gels prepared in formamide [16]. The gels were either directly scanned at 260 nm in a Gilford apparatus or after staining with toluidine blue and destaining in water. Marker *E. coli* 23-S and 16-S ribosomal RNAs, rat liver 28-S and 18-S ribosomal RNAs and 5-S RNA were also run.

Preparation of cDNA

For the preparation of cDNA, RNA directly extracted from the polyacrylamide gels or RNA from the peak in the preparative sucrose gradients was used. The cDNA was synthesized according to Getz et al. [17]. The final mixture contains 20 µg template RNA, 50 µg/ml $(dT)_{12-18}$, 800 µM deoxyribonucleotides, except dCTP which was present at a final concentration of 500 µM and also radioactively labelled ([^{3}H]-dCTP, 41 Ci/mmol), 100 µg/ml actinomycin D, 50 mM Tris-HCl, pH 8.2, 50 mM KCl, 5 mM magnesium acetate, 10 mM dithiothreitol and 70 units/ml reverse transcriptase (kindly provided by Dr J. Beard, Florida). After a 2-h incubation, the cDNA was separated from the triphosphates on Sephadex G-50 and submitted to an alkaline sucrose gradient centrifugation. Fractions containing cDNA greater than 5 S were pooled, after determining the sedimentation coefficient using a computer program and a single-stranded 5-S [^{3}H]DNA. The pooled cDNA was precipitated and desalted on Sephadex G-25.

RNA · cDNA Hybridization

For the RNA-driven cDNA hybridization, 1000 counts/min cDNA (5×10^6 counts/min per µg cDNA) were mixed with different amounts of RNA and dissolved into 0.24 M phosphate buffer, 0.5% sodium dodecylsulphate, 1 mM EDTA, sealed in glass capillaries, denatured and incubated for the desired time at 60 °C.

To analyse the hybrids, the content of the capillaries was flushed out with 250 µl S1 buffer (0.14 M NaCl, 0.07 sodium acetate, 2.8 mM $ZnSO_4$, 14 µg/ml heat-denatured mouse DNA, pH 4.5) and analysed for double-stranded nucleic acids using 20 units of Sigma S1 nuclease. In all these analyses the ratio between driver and tracer nucleic acids was greater than 10000.

DNA · cDNA Hybridization

DNA from *Calliphora* or from *Drosophila* was isolated, and sonicated in 0.3 M NaCl until the mean sedimentation coefficient reached a value of about 5 S. This DNA was used for the hybridization.

DNA and cDNA were mixed in a ratio of 20000, lyophilised and dissolved in 0.24 M phosphate buffer, as for the RNA · cDNA hybrids. The hybrids were sealed in glass capillaries, denatured for 7 min in a boiling water bath and incubated at 60 °C for the de-

sired time. At the end of the hybridization, the hybrids were challenged with S1 nuclease as described above.

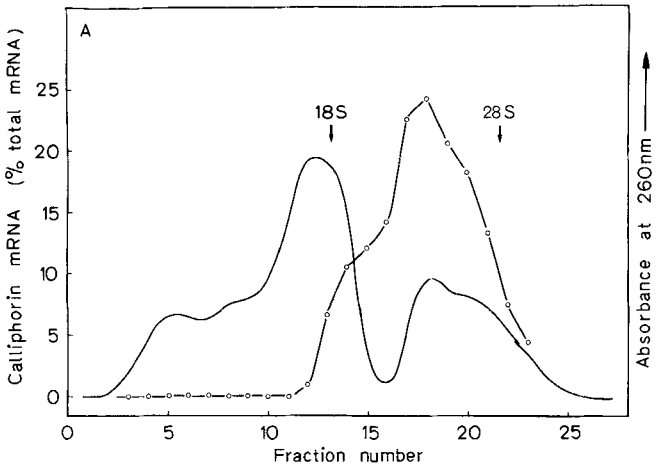
RESULTS

Isolation and Purification of Calliphorin mRNA

Starting material for the purification of calliphorin mRNA was poly(A)-containing RNA isolated from the fat body of 5-days-old blowfly larvae which, as shown previously [7], have large amounts of translatable calliphorin mRNA. As demonstrated previously [7], and confirmed in the present experiments, 6–9% of the polypeptides synthesized *in vitro* and encoded by the mRNA preparation, are calliphorin. This is a minimal estimation, as some of the smaller polypeptides synthesized seem to be incomplete calliphorin chains or products of degradation of this protein, as shown by immunoprecipitation experiments (see also [6]).

The RNA fraction was then submitted to sucrose gradient centrifugation either in the absence of formamide (Fig. 1 A) or, under denaturing conditions, in the presence of 70% formamide. The RNA of the various fractions obtained was precipitated with ethanol, the sediments washed thoroughly with ethanol, dried and dissolved in water, to be assayed for their translational activity in the wheat germ system (Fig. 1 B and 2). In the absence of formamide, calliphorin mRNA activity is distributed in fractions corresponding to the 18–28-S part of the gradient, with two peaks of activity, a prominent one at 24 S and a shoulder at approximately 20 S. In the presence of formamide, calliphorin mRNA activity is distributed over a very restricted part of the gradient, with a maximum at approximately 21 S. The activity at 24 S in the absence of formamide results, apparently, from aggregation of RNA. The relative concentration of calliphorin mRNA at the peak fractions is about 25%. This is a minimal estimate, as some of the smaller polypeptides are precipitated by the antibody to calliphorin.

It is interesting to note that in some experiments (see Fig. 2), two bands can be visualized in the region corresponding to calliphorin, an observation which we have already mentioned in our previous publication [7]. The resolution of the two bands depends solely



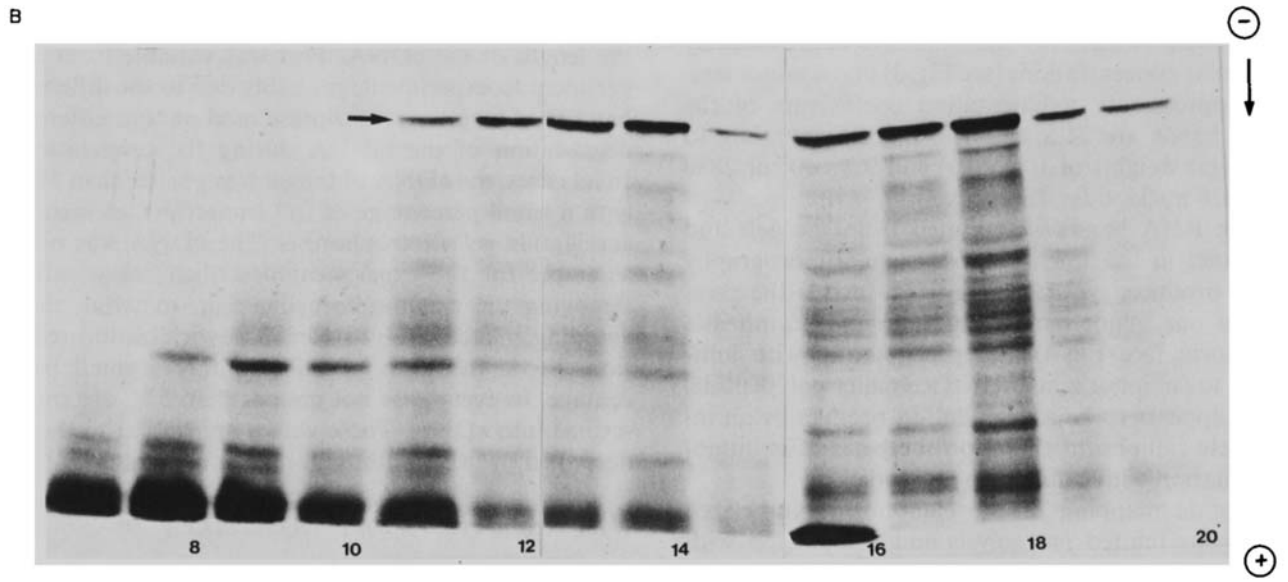


Fig. 1. *(A) Centrifugation of poly(A)-containing RNA from fat body of blowfly larvae; (B) fluorography of the polypeptides synthesized* in vitro *under the influence of RNA fractions from the sucrose gradient.* (A) 5–20% aqueous sucrose gradients as described in Methods. (——) Absorbance at 260 nm, representing 28-S and 18-S ribosomal RNA. O——O relative percentage of calliphorin mRNA to total mRNA as derived from the relative amounts of calliphorin translated *in vitro* (see B). (B) The numbers refer to the fraction numbers of A. The RNA was precipitated and translated as described in Methods. 7.5% polyacrylamide gels were used. The arrows denote the position of marker calliphorin. The gels were scanned in a Vernon apparatus and the relative amount of calliphorin synthesized thus determined

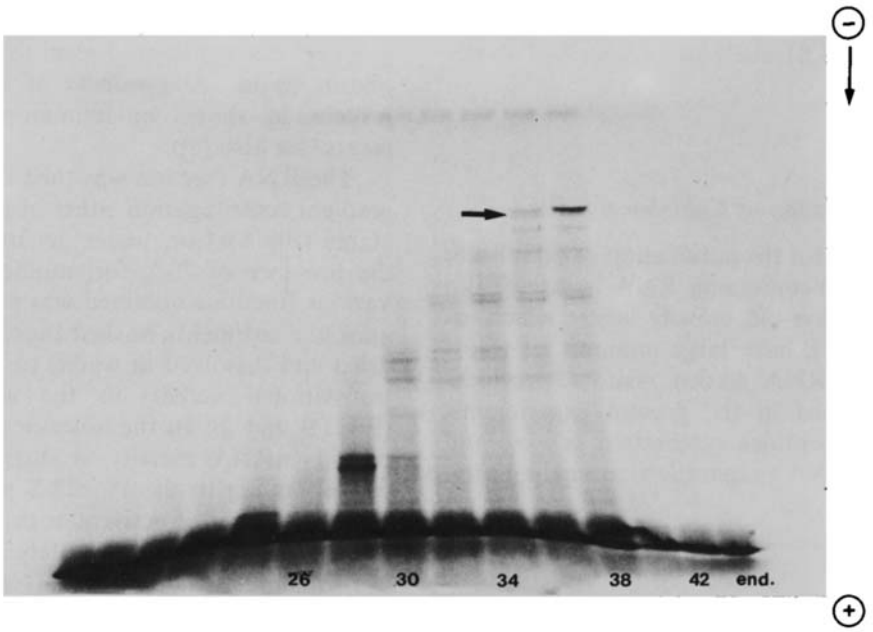


Fig. 2. *Fluorography of polypeptides synthesized* in vitro *under the influence of RNA separated by sucrose gradient centrifugation in formamide.* Conditions of centrifugation, precipitation of RNA, translation, sodium dodecylsulphate/polyacrylamide electrophoresis and fluorography as described in Methods. 10% polyacrylamide gels were used. The numbers correspond to the fraction numbers of the gradient centrifugation. The arrow denotes the position of marker calliphorin

on the conditions of the electrophoresis and the amount of radioactive product applied to the gel and not on the translational system. The nature of the two protein bands will be discussed below.

The fractions of the formamide gradients containing the calliphorin mRNA were pooled and submitted to electrophoresis in formamide/polyacrylamide gels. Both in 3.2% and 4% gels three RNA bands could be seen, two major ones in approximately equimolar concentrations (see Fig. 3) and a minor one. The approximate sedimentation coefficients of the major bands are 21 S and 19.7 S, corresponding to molecular weights of 10.6×10^5 and 9.2×10^5 or 2850 and 2620 nucleotides, respectively.

The RNA bands were eluted from the gels and translated in the wheat germ system. Fluorography of the products synthesized *in vitro* reveals the presence of one major band comigrating with authentic calliphorin (see Fig. 4) and precipitating with antibodies to calliphorin, as well as a smaller polypeptide, which appears to be a degradation product or an incomplete calliphorin chain, on the basis of immunoprecipitation with calliphorin antibodies.

Peptide mapping of the calliphorin synthesized *in vitro* by limited proteolysis and comparison with the peptide pattern of authentic calliphorin (see Fig. 5 A and B) leads to the conclusion that the two proteins are either identical or very similar. Due to the problem of resolution, the high-molecular-weight band appears to be homogenous in the experiment depicted in Fig. 4 (see Discussion).

Synthesis of cDNA from Calliphorin mRNA

Using reverse transcriptase from avian myeloblastosis virus, a cDNA copy from the mRNA was prepared. The mRNA used was eluted from a formamide gel after cutting the 20-S region depicted in Fig. 3 or taken from fractions of sucrose gradient centrifugation, as shown in Fig. 2.

Using an alkaline sucrose gradient, we determined the length of the cDNA. This was variable from experiment to experiment, probably due to the different batches of reverse transcriptase used or to a different degradation of the mRNA during the preparation. In all cases, the cDNA obtained was greater than 10 S with a small percentage of full transcripts, as seen in acrylamide gel electrophoresis. The cDNA was used as tracer for the experiment described below, after removing the small-molecular-weight material, thus avoiding related problems in the hybridization reaction. From the mass of mRNA only a small percentage, in every case not greater than 5%, are transcribed into cDNA. These values are similar to those described by other authors for other templates [18].

Hybridization between mRNA and cDNA

We have hybridized the mRNA with the cDNA in an RNA-driven hybridization, in order to calculate the kinetic complexity of the RNA (Fig. 6). The cDNA hybridizes to only one kinetic component, until 88% of the reactable cDNA has annealed. This component follows second-order reaction kinetics

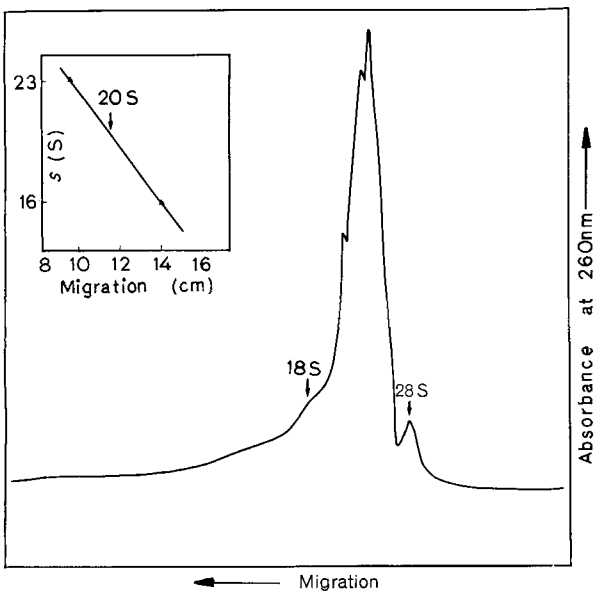


Fig. 3. *Polyacrylamide/formamide gel electrophoresis of mRNA derived from fractions 35–39 of the sucrose gradient centrifugation in formamide of Fig. 2.* The fractions, corresponding to a sedimentation coefficient of approximately 20–21 S were pooled, precipitated with ethanol and submitted to electrophoresis as described in Methods. The gels were scanned in a Gilford spectrophotometer at 260 nm. The arrows denote the position of migration of marker 28-S and 18-S ribosomal RNA. The inset shows the determination of the sedimentation coefficients of the middle peak of the three RNA peaks observed. (——) Absorbance at 260 nm

Fig. 4. *Translation* in vitro *of the RNA isolated from the 19–21-S region of the polyacrylamide/formamide electropherogram of Fig. 3.* The RNA was eluted from the gel electrophoretically. Translation, electrophoresis of the products and fluorography was performed as described in Methods. The arrow denotes the position of marker calliphorin. 7.5% polyacrylamide gels were used. The right slot contained double the amount of radioactivity. F = front

and hybridizes over a range of log r_0t values from 2 to 2.5. Taking this fact into consideration, we can calculate an $r_0t_{1/2}$ value for this component of 1.2×10^{-2} M · s. On the basis of the reaction kinetics, a second component seems to be present, hybridizing more slowly, representing about 10% of the total hybridizable cDNA. This probably represents contaminant poly(A)-containing RNA. The complexity of the faster-reacting population has been calculated to have an M_r of 2.8×10^6. This complexity would represent about three RNA species with a molecular weight of approximately 10^6, corresponding to the three RNA bands depicted in Fig. 3. These values were calculated on the basis of comparison with the kinetic complexity of globin mRNA · cDNA and are therefore only rough estimates. The reaction between globin mRNA and its cDNA shows, under the conditions of our experiment, an $r_0t_{1/2}$ of 1.7×10^{-3} M · s, and the complexity of the globin mRNA was taken as 4×10^5.

Taking the efficiency of the reverse transcriptase to be proportional to the percentage of the mass of the sequences which is transcribed we can assess that our preparation of cDNA contains only a very

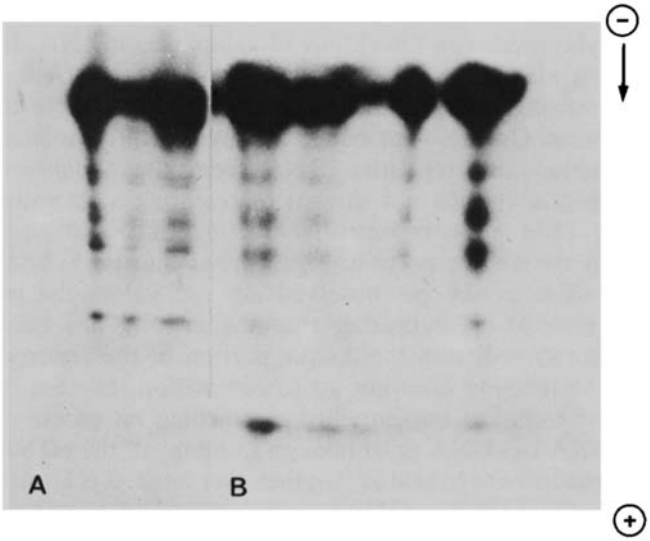


Fig. 5. *Peptide mapping by limited hydrolysis of calliphorin synthesized* in vitro. The part of the gel of Fig. 4 containing the calliphorin synthesized *in vitro* and unlabelled calliphorin marker was excised, placed in a sample well of a second sodium dodecylsulphate/polyacrylamide gel (15%) and treated with chymotrypsin as described in Methods. In a parallel well, unlabelled, marker calliphorin was also submitted to the same procedure. After electrophoresis, the gels were first stained with Coomassie blue (A) and then submitted to fluorography (B)

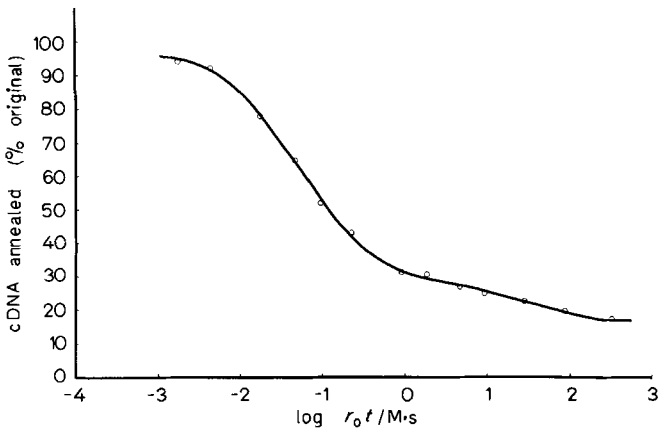


Fig. 6. *Hybridization between cDNA of calliphorin mRNA and its template.* The hybridization conditions are as described in Materials and Methods. The ratio of RNA to cDNA was greater than 20000

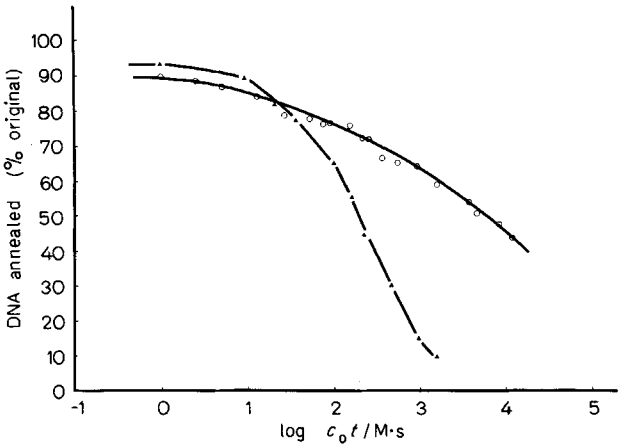


Fig. 7. *Hybridization kinetic between total sonicated* Calliphora *DNA and cDNA of calliphorin mRNA.* The reaction was performed as described in Materials and Methods and the hybrids analysed with nuclease S1. (▲——▲) Reannealing of isolated unique DNA from *Calliphora*; (○——○) annealing between total DNA and cDNA for calliphorin

minor proportion of contaminating, non-calliphorin sequences. This contamination is very much easier to eliminate after separating, on hydroxyapatite, the hybrids formed up to an r_0t value of 10^0 M · s (see Fig. 6).

Hybridization between Total DNA from Calliphora *and* Drosophila *with cDNA for Calliphorin*

Total sonicated DNA from *Calliphora* larvae was hybridized with the cDNA of calliphorin mRNA. In this kind of reaction, the proportion of cDNA hybridizing is dependent on the ratio of the driver to tracer. On the other hand, we have recently isolated unique and repetitive DNA from the *Calliphora* genome (results not shown) representing a c_0t value of 0.8 M · s for the repetitive fraction and 5×10^2 M · s for the unique portion. Fig. 7 shows that no hybridization could be observed at c_0t values below 2×10^2 M · s, indicating that the mRNA has been transcribed from the unique portion of the genome. The reaction does not go to completion, because of the technical impossibility of reaching an excess of DNA to cDNA great enough to allow all the cDNA sequences to hybridize. On the other hand, it is known that in a DNA · cDNA reaction, the driver sequences are reacting somewhat quicker than the driver-tracer sequences, perhaps due to differences in the length of driver to tracer [19]. In any case, these results show clearly that the genes coding for calliphorin mRNA are transcribed from the unique portion of the genome, a situation similar to that found for a great number of genes in different tissues and species. Therefore, a very small number of genes are transcribing the calliphorin mRNA, perhaps one or two per haploid genome.

Hybridization of this cDNA with DNA from *Drosophila* melanogaster embryos fails to reveal hybridization greater than 30%. This could imply that the similarity of the genes coding for drosophilin and calliphorin is not very great. However, the hybridization conditions used in this reaction are relatively stringent, not allowing for the production of hybrids with a high degree of mismatching; the analysis of the hybrids was done using S1 nuclease, an enzyme destroying possibly existing non-hybridized regions interspersed among the hybridized ones. An analysis of the hybrids with hydroxyapatite would give perhaps a totally different picture, as in this kind of assay the non-hybridised regions will be recovered together with the hybridized material.

DISCUSSION

Calliphorin is a major polypeptide of blowfly larvae [1,2,6], appearing at a defined developmental stage and showing age-dependent variations in its content [1,2,7,20]. At the peak of its accumulation, calliphorin makes up approximately 60% of the dilute-salt-soluble protein [7]. Its biological role is still unknown; it has been suggested as being a storage form of amino acids [8], as contributing to the colloid osmotic tension of the hemolymph and as taking part in the tanning of the cuticle [1,7]. Its high tyrosine content (15% of the amino acid residues of this protein are tyrosine) is in favor of the last suggestion. Recent data demonstrating localization of part of the calliphorin in the cuticle of the blowfly, both by immu-

nofluorescence and by chemical means, strongly support the involvement of calliphorin in the cuticular events (Zimmermann, Scheller and Sekeris, unpublished).

Due to the sudden, massive triggering of the synthesis of calliphorin, restricted to a very limited period of blowfly development [7], we have been interested in studying the biosynthesis of this protein, and in learning more about the factors and mechanisms which regulate its synthesis and accumulation. In previous work, we have correlated the degree of biosynthesis of the protein with the amount of calliphorin mRNA, by means of its translation in a wheat germ system [7]. We demonstrated, in accordance with findings in other systems, an increase in the amount of translatable mRNA, paralleling the increased biosynthetic rate. In contrast to other systems, however, we could still detect calliphorin mRNA at periods of development in which biosynthesis of calliphorin was completely shut off.

To study in more detail the quantitative aspect and the intracellular distribution of calliphorin mRNA, as well as its transcription *in vitro*, we deemed it necessary to purify calliphorin mRNA, synthesize its cDNA and with this probe proceed to a physical determination of specific RNA sequences.

Due to the relatively high concentrations of calliphorin mRNA in the fat body of five-days-old larvae [7], we used poly(A)-containing RNA from this source as starting material for the purification. As is well known, the fat body is the site of synthesis of calliphorin [7,8], no other insect tissue examined showing calliphorin mRNA activity [7]. Sucrose gradient centrifugation led to a further enrichment of calliphorin mRNA which, due to its relatively higher molecular weight, is separated from the bulk of the mRNA sequences. In the absence of formamide in the gradient, calliphorin mRNA tends to aggregate with other RNAs, the small-molecular-weight ones in particular, showing two peaks of activity, one at approximately 20 S and another at approximately 24–25 S. In the presence of formamide only the 20-S peak is observed. Analytical electrophoresis of the 20-S RNA fraction in polyacrylamide/formamide gels revealed that it was not homogenous, but was composed of three components, one minor one and two major ones in almost equimolar proportions. It was evident from the results of the translation in the wheat germ system of the 20-S RNA isolated from the formamide gradients, that two polypeptides, both migrating in the region of the calliphorin marker, were formed. A separation of the two polypeptides was observed occasionally depending on the concentration of the polyacrylamide and the amount of labelled proteins.

Peptide mapping by limited proteolysis in sodium dodecylsulphate and subsequent gel electrophoresis [15], and comparison with the peptide pattern of similarly treated calliphorin, revealed a striking identity of the polypeptides to calliphorin. In analogy to conditions prevailing in *Drosophila* [5,21] and *Lucilia* [22], where various polypeptides slightly differing in their electrophoretic characteristics and in their immunochemical behavior are found, we can postulate the existence in *Calliphora* of more than one calliphorin polypeptide, the detection of which has not been yet been technically possible. It is thus tempting to conclude that the two major RNAs of about 20 S code for the two calliphorin polypeptides. The possibility that one of the two mRNAs is a degradation product of the other is very unlikely, as the RNA pattern obtained is constant, independent of the method used for the isolation of the RNA. Lastly, one could postulate a precursor-product relationship between the two polypeptides, in which case we could not account for the existence of the two major mRNA bands.

Considering the molecular weight of the calliphorin mRNAs and the molecular weights of their two respective polypeptides, we conclude that approximately 15% of the nucleotides of the mRNA do not contain information for the protein but must be involved in other, unknown, functions.

The cDNA synthesized according to this RNA reacts with the template with an $r_0t_{1/2}$ of approximately 1.2×10^{-2} M · s, if allowance is made for the percentage of total cDNA reacted. The reaction denotes the presence of a defined component hybridizing between log r_0t values of 2 and 2.5. A similar situation has also been found for other messenger RNAs known to be pure. These results, together with the biological assay of the translation *in vitro*, suggest that our cDNA is a faithful transcript of the mRNA, accepting that reverse transcription is proportional to the amount of sequences present in the original mRNA. This probe could be used to assay the presence of mRNA sequences in a defined population; the very small percentage of hybridization taking place outside the theoretical curve for a second-order reaction could be eliminated using preparative hydroxyapatite chromatography, after incubation of the hybridization components to an r_0t value of 1 M · s. The cDNA separated after this method is currently being employed for the determination of sequences common to calliphorin mRNA during the various developmental stages of the blowfly.

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