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Insect immunity. Isolation of cDNA clones corresponding to attacins and immune protein P4 from *Hyalophora cecropia*

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Diapausing pupae of the Cecropia moth *(Hyalophora cecropia)* respond to an injection of live bacteria by the selective synthesis of certain types of RNA and immune proteins (designated P1 – P9). The *in vitro* translation products of RNA from both injured and infected pupae showed specific patterns with a defined number of extra bands. Some proteins characteristic of the normal RNA were reduced in the immune RNA translation products. Antibody reaction was used to show the selective synthesis of immune proteins P4 and P5 with mRNA from pupae subjected to injury or infection. The protein synthesized *in vitro*, which cross-reacted with P5 antibodies, is most likely a precursor of the attacins described in the preceding paper. A cDNA clone bank was prepared and two clones were isolated and shown to contain 750 bp corresponding to P4 and 250 bp of attacin information. These clones were used to estimate the sizes of the mRNAs by Northern blotting and to estimate, by RNA/DNA hybridization, the levels of P4 and P5 mRNA. *In vivo* incorporation of [^{35}S]methionine into attacins and P4 during different conditions was compared with the levels of the corresponding mRNA.

Key words: insect immunity/*Hyalophora cecropia*/cDNA cloning/attacin/immune proteins P4 and P5

Introduction

The induction of immunity in Cecropia pupae involves a highly selective gene activation (Faye *et al.*, 1975; Boman *et al.*, 1981). When the pupae are challenged by an injection of live, non-pathogenic bacteria they respond by producing ~15 immune proteins, most of them with antibacterial activity. The two major components synthesized are immune proteins P4 and P5 (Rasmuson and Boman, 1979; Pye and Boman, 1977) and these were the first components for which antisera could be produced. P4 is a major protein synthesized in immune hemolymph but it is also detectable in tissue and normal hemolymph by the reaction with antibodies against P4. The function of P4 is not yet known (Rasmuson and Boman, 1979). Recently it was found that P5 is identical to the attacins, a family of six antibacterial proteins described in the preceding paper (Hultmark *et al.*, 1983). Amino acid composition and N-terminal sequences indicate a minimum of two closely related attacin genes originating from a gene duplication.

We have earlier described a method for the purification of mRNA from whole pupae (Boman *et al.*, 1981). To study the organization of the immune genes in Cecropia and the selective induction mechanism we have, as a first step, constructed cDNA clones from mRNA prepared from immunized pupae.

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We report here the identification of clones which cover parts of the mRNA for attacins and immune protein P4. Two clones were used to estimate the amounts of mRNA at two different times and these results were compared with the *in vivo* appearance of attacin and P4 in the hemolymph. Since we cannot ascribe the P5 clones to the different forms of attacin we will in this paper predominantly use 'P5' as a collective designation for the attacins.

Results

Translation of mRNA

We have previously compared the translation products obtained with total RNA prepared from normal, immunized and injured pupae (Boman *et al.*, 1981). Comparative experiments have now been performed with oligo(dT)-purified mRNA preparations. The products obtained were characterized by SDS-PAGE and immunoprecipitation (Figure 1). A comparison of the different lanes gave the following results: (i) the translation products of immune RNA and injury RNA showed 5 – 6 bands which were either weak or absent in the translation product from normal RNA (lanes C – E); (ii) some strong bands present in the translation of normal RNA became weak in the translation products of immune RNA (lanes C and E); (iii) injury RNA gave qualitatively a similar but not identical translation reaction to immune RNA (lanes C and D); (iv) precipitation with antisera showed that both P4 and P5 were synthesized in the presence of immune and injury RNA (lanes F – I); (v) the same

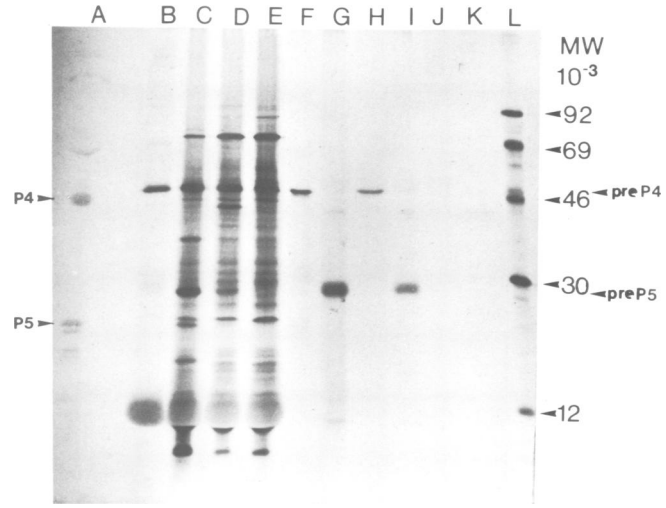

Fig. 1. Translation of mRNA extracted from pupae with different treatments and immune precipitation of translation products. mRNA was translated in the reticulocyte lysate system with [^{35}S]methionine and analyzed by SDS-PAGE before and after immunoprecipitation with antibodies against protein P4 and P5 (see Materials and methods). **Lane (A)** contained [^{35}S]methionine-labeled immune hemolymph. Other lanes are *in vitro* translation products directed by: **(B)** endogenous RNA (H_2O control); **(C)** immune RNA; **(D)** injury RNA; **(E)** normal RNA; **(F)** immunoprecipitation of **C** with anti-P4; **(G)** immunoprecipitation of **C** with anti-P5; **(H)** immunoprecipitation of **D** with anti-P4; **(I)** immunoprecipitation of **D** with anti-P5; **(J)** immunoprecipitation of **E** with anti-P4; **(K)** immunoprecipitation of **E** with anti-P5; **Lane L** contained mol. wt. markers.

method failed to detect these two proteins in the translation products of normal RNA (lanes J–K).

Since the mobilities in Figure 1 for proteins P4 and P5 in hemolymph were higher than the *in vitro* proteins precipitated by the corresponding antisera they are referred to as preP4 and preP5 assuming that they carry a signal sequence (compare lanes A with F and G in Figure 1). In the electrophoresis system used, the preP4 band overlaps with a rabbit protein originating from translation of reticulocyte endogenous RNA (lanes B and F in Figure 1). However, the antibody produced against protein P4 did not react with this rabbit protein in the reticulocyte lysate (lane J in Figure 1).

Construction of a cDNA bank and initial screening of clones

The first strand of cDNA was synthesized (yield 0.85 μg) with avian reverse transcriptase using as template poly(A)-enriched immune RNA (16 μg) with oligo(dT) as primer. The second strand was synthesized with DNA polymerase I. After purification by gel filtration, ~0.37 μg of double-stranded cDNA was obtained. Following treatment with S1 nuclease, the cDNA was tailed with poly(dC). The vector, pBR322, was linearized with *Pst*I and tailed with poly(dG) (see Materials and methods). After annealing of 100 ng of cDNA and 700 ng pBR322 in 300 μl, 40 μl were mixed with 200 μl of competent cells of *Escherichia coli* strain 294. With this procedure an average of ~250 colonies was obtained of which ~75% were ampicillin sensitive. Repeated transformation experiments yielded a total of ~1000 ampicillin-sensitive colonies. Previous work showed that proteins P4 and P5 were the two major proteins synthesized after immunization of Cecropia pupae (Faye *et al.*, 1975). Thus, RNA species for proteins P4 and P5 could be expected to be present in dominant proportion in the immune mRNA preparation. Using ^{32}P-labeled immune RNA as a hybridization probe we therefore screened 400 ampicillin-sensitive colonies for presumptive P4 and P5 clones. The result obtained showed that 30% of the colonies gave strong hybridization with labeled RNA (data not shown).

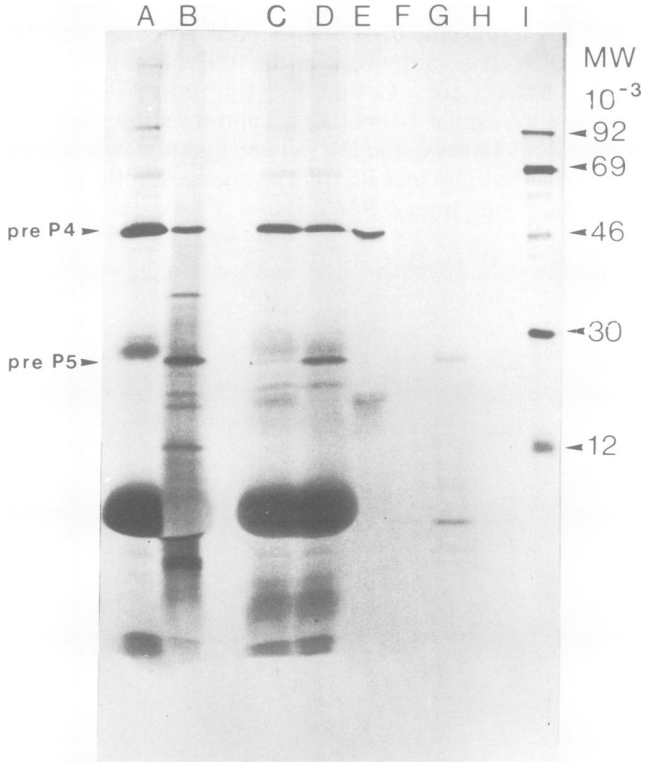

Fig. 2. Identification of clones by hybridization/translation assay. Specific mRNA was selected by hybrid-plasmid DNA bound to nitrocellulose filter and *in vitro* translated (Parnes *et al.*, 1981). The reaction products were analyzed by 10–17% SDS-PAGE, before and after immunoprecipitation with antibodies against protein P4 and P5. The gel was loaded with *in vitro* translation products directed by: **(A)** endogenous RNA control; **(B)** immune mRNA; **(C)** mRNA selected by pCP430 DNA; **(D)** mRNA selected by pCP510 DNA; **(E)** immunoprecipitation of **C** by anti-P4; **(F)** immunoprecipitation of **C** with anti-P5; **(G)** immunoprecipitation of **D** with anti-P5; **(H)** immunoprecipitation of **D** with anti-P4; **(I)** mol. wt. markers.

Identification of clones containing DNA complementary to mRNA for proteins P4 and P5

Strongly positive clones from the colony hybridization with labeled immune mRNA were further screened for P4 and P5 specificity using the mRNA selection method (Parnes *et al.*, 1981). Since only antisera against protein P4 and P5 were available, the search was limited to clones corresponding to these two proteins. Figure 2 shows the identification of two clones for proteins P4 and P5, designated pCP430 and pCP510, respectively. To see if any protein band was intensified in the translation products of mRNA selected by single recombinant DNAs bound to nitrocellulose filters, translation products were analysed without immune precipitation. In lane C, there was no noticeable band intensification. In lane D the band corresponding to protein P5 was clearly intensified over the background. Further evidence for pCP510 as a P5 clone was obtained by immunoprecipitation (lane G in Figure 2). Since the size of preP4 and a rabbit protein (made from endogenous mRNA) are the same (see Figure 1) the identification of pCP430 as a P4 clone depended solely on the immunoprecipitation with P4 antisera (lane E in Figure 2). There was no cross-reactivity between the antisera used and the translation products of the plasmid-selected mRNA (lanes F and H in Figure 2). Thus, we conclude that pCP510 contains part of the cDNA corresponding to P5 mRNA and pCP430 contains cDNA corresponding to P4 mRNA.

The results in Figure 2 were obtained after a number of preliminary screening experiments in which the clones were

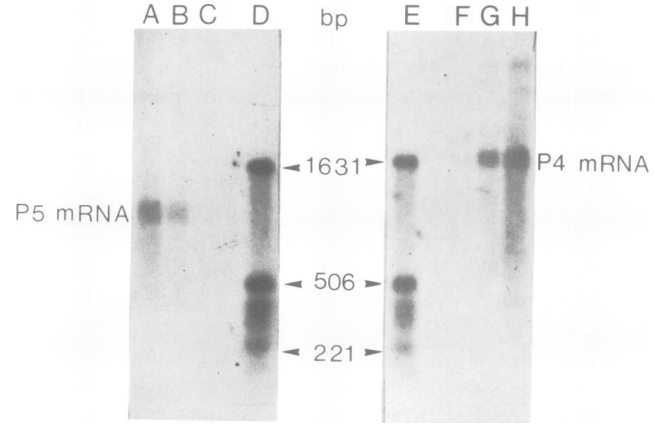

Fig. 3. Detection of Cecropia mRNA for proteins P4 and P5. Poly(A) RNA from immunized, injured and normal pupae were analyzed by electrophoresis in 1.5% agarose gels (Fellous *et al.*, 1982). RNA was transferred to nitrocellulose filter and hybridized to ^{32}P-labeled pCP430 and pCP510 (Northern blotting). **(A** and **H)** immune mRNA; **(B** and **G)** injury mRNA; **(C** and **F)** normal mRNA. The left gel was hybridized with ^{32}P-labeled pCP510, the right gel with ^{32}P-labeled pCP430. *Hinf*1-digested pBR322 was used as mol. wt. marker **(D** and **E)**.

identified. In the screening of clones for proteins P4 and P5, immune precipitation of translation products of the selected mRNA shows, in addition to the main band for preP4 and preP5, two weak additional bands (lanes E and G in Figure 2). The origins of these additional bands were not investigated.

To estimate the size of the cDNA inserted in pCP430 and pCP510, plasmid DNA was prepared from bacteria with these clones. The inserted cDNA was then excised with *Pst*I and the fragments were analyzed by electrophoresis in a 2% agarose gel. Plasmid pCP430 was found to contain a cDNA insert of 750 bp while pCP510 contained only ~250 bp of cDNA. Further screening of clones by colony hybridization with ^{32}P-labeled insert cDNA of both plasmids identified a P4 clone with a 1000 bp insert (pCP445) and two P5 clones containing inserts of ~500 bp (pCP519 and pCP520).

We next estimated the sizes of the mRNA species for P4 and P5 by agarose gel electrophoresis and Northern blotting (Figure 3). The values obtained were ~1650 bases for P4 mRNA and ~950 bases for P5 mRNA. The mRNA sizes estimated by Northern blotting (Figure 3) makes it very unlikely that clones pCP430 and pCP510 are related to the minor protein bands in lane G of Figure 2.

RNA/DNA hybridization by dot-blot

Translation of normal mRNA did not produce any proteins which could be precipitated by antibodies against proteins P4 and P5 (lanes J and K in Figure 1). To examine directly if our different mRNA preparations contained specific mRNA species for P4 and P5, mRNA was spotted onto nitrocellulose paper and hybridized with nick-translated *Pst*I-cleaved DNA from pCP430 and pCP510 (see Materials and methods). The spots were then excised and counted (Figure 4). The results show that preparations of immune and injury RNA both contained mRNA for proteins P4 and P5. Normal RNA from untreated pupae gave only hybridization of the same magnitude as an unrelated RNA preparation used as control. Together with the *in vitro* translation experiment (Figure 1) the result in Figure 4 confirms that an infection or an injury induced *de novo* synthesis of attacin(s) and immune proteins P4 in diapausing Cecropia pupae.

The kinetics of attacin and P4 synthesis

The synthesis of attacins and immune protein P4 was followed *in vivo* by injecting a pupa with [^{35}S]methionine at the onset of an immunization. Hemolymph was withdrawn at different times and the amount of radioactivity in attacins and in P4 was determined after separation of the proteins by SDS-PAGE. Figure 5 shows that up to day 2, radioactivity in attacins increased faster than in P4. There was a short lag period in both the antibacterial activity and the P4 synthesis. The fact that the net incorporation of [^{35}S]methionine at the end was almost the same in attacins and P4 should rule out that the isotope at day 2 became rate limiting for attacin synthesis.

The P4 and P5 plasmids, pCP430 and pCP510, were then used to estimate the levels of the respective mRNA species at two different times after an immunization and an injury (Table I). In the case of immunization, mRNA for protein P4 and P5 was detectable after 2 h. After 48 h, mRNA levels increased 7–10 times. Thus, the difference in response to an injury and an infection seems to be quantitative rather than qualitative. Both Figure 4 and Table I show that the levels for P4 mRNA and P5 mRNA after an injury are ~1/3–1/2 of the corresponding level after an immunization.

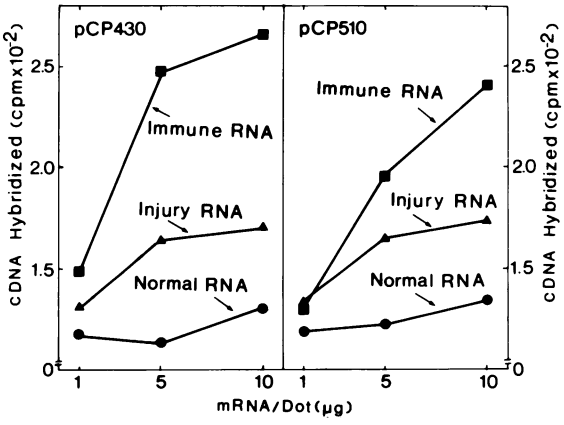

Fig. 4. RNA/DNA hybridization by dot-blot. Immune, injury and normal mRNA were heated at 95°C for 10 min and different amounts were spotted onto the nitrocellulose filter (Thomas, 1980). The cDNA insert was nick-translated as described in Materials and methods. The amount of cDNA added was 1.5 x 10^5 Cerenkov counts/min in 10 ml hybridization buffer and 100 cm^2 nitrocellulose paper.

Discussion

In eucaryotic organisms there are relatively few inducible gene systems so far described (Lewin, 1980). The heat-shock proteins (hsp) have received the most attention (Schlesinger *et al.*, 1982). However, the knowledge accumulated on the hsp

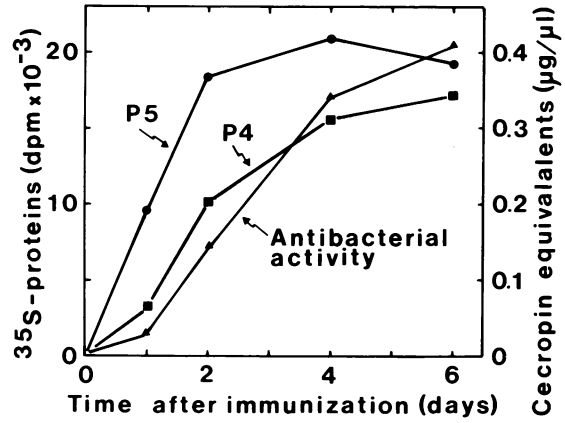

Fig. 5. The appearance of attacins (P5), immune protein P4 and the overall antibacterial activity in the hemolymph of an immunized Cecropia pupa. At the onset of the immunization the pupa was injected with [^{35}S]methionine. Hemolymph samples were withdrawn at the times indicated and the ^{35}S was determined in P4 and P5 after separation by SDS-PAGE. Antibacterial activity was estimated by the inhibition zone assay as described by Hultmark *et al.* (1980). Cecropin A was used as standard.

Table I. Relative concentrations of mRNA for P4 and P5 at two times after immunization and injury of Cecropia pupae

RNA preparation		Hybridization with	
Treatment of pupae	Time (h)	pCP430	pCP510
Immunization	2	43	14
Immunization	48	440	100
Injury	2	20	0
Injury	48	150	46

The plasmids pCP430 and pCP510 were nick-translated and used for dot-blot hybridization (details in Materials and methods). The labeled DNA binding to 5 μg of mRNA is expressed as c.p.m. corrected for background.

system is chiefly at the DNA and RNA levels; a function is not yet known for any of these proteins. In contrast, the function of many of the inducible immune proteins of Cecropia is known and 14 different antibacterial proteins have been isolated. For cecropins A, B and D we have full amino acid sequences and we can assume a minimum of three closely related genes originating from gene duplications (Steiner *et al.*, 1981; Hultmark *et al.*, 1982). Amino acid composition and the N-terminal sequences indicate, for the attacins, a minimum of two very closely related genes, again originating from a gene duplication (Hultmark *et al.*, 1983).

We have shown here that translation of immune RNA gives only 5–6 specific protein bands (Figure 1). This low number depends on the fact that the cecropin family (six proteins with a size of 4 K) move as one band and the same applies to the attacins (six proteins with a size of 20–23 K). In analogy with the hsp system, induction of immunity also gives rise to suppression of normal gene activities, a fact which can be observed both *in vitro* and *in vivo*. The latter is manifested as a delayed development of moths after immunization of pupae.

The present study is a first attempt to work out the control of the Cecropia immunity by the isolation of two plasmids pCP430 and pCP510 which contain cDNA corresponding to immune proteins P4 and P5, respectively (Figure 2). The size of the cDNA fragment in pCP430 is ~750 bp while the Cecropia DNA in pCP510 is only ~250 bp. Estimates by Northern blotting (Figure 3) showed that P4 mRNA contained ~1650 bases while P5 mRNA contained ~950 bases. It is therefore clear that pCP430 covers about half of the mRNA for P4 while pCP510 would cover about one fourth of the mRNA for P5.

The mol. wt. of the P4 cross-reacting material synthesized *in vitro* is ~50 K. Since P4 is ~48 K this is in agreement with a leader sequence of ~2 K. However, the size of P5 cross-reacting material is ~28 K while the attacins are only 20–23 K (Hultmark *et al.*, 1983). This indicates that the precursor(s) of the attacins is somewhat larger than expected from simply a leader sequence. Thus, the multiple forms of attacin which have identical N-terminal sequences could arise from processing steps.

Plasmids pCP430 and pCP510 were used to show that *de novo* synthesis of mRNA for proteins P4 and P5 was induced by either immunization with live bacteria or by an injury caused by a sham injection (Figure 4). Since in nature an infection may often start by an injury, the finding makes biological sense. It also implies that the differences between immunity and the injury reaction seen in live insects could be accounted for by regulation at the translational level or later stages such as the excretion into the hemolymph or processing from precursor forms.

Since the cDNA inserts in pCP430 and pC510 differ in size, the values in Table I can be used only for estimates of the mRNA at different times but not for a comparison of the concentrations of the two mRNA species. With this reservation, Table I indicates that, during the first 2 days, the rate of synthesis of P4 and P5 mRNA is of the same order of magnitude. During the same period the rate of appearance in the hemolymph of P5 was clearly higher than the corresponding value for P4 (Figure 5). However, P4 is known to exist in tissue and hemolymph before an infection (Rasmuson and Boman, 1979); in agreement is the fact that a weak band of P4 mRNA was visible in normal RNA (lane F in Figure 3).

Pye and Boman (1977) first purified protein P5 to apparent homogeneity on SDS-PAGE. The antisera used here were prepared against P5 and they were also used in the identification of attacins A–F (Hultmark *et al.*, 1983). We have at present no way to relate our different P5 clones or the preP5 synthesized *in vitro* to the different attacins. However, work has been initiated to solve this problem by sequencing both plasmid pCP510 and two of the attacins.

Materials and methods

Insects, bacterial strains and plasmids

Male, diapausing pupae of *H. cecropia* were obtained and immunized as described by Boman *et al.* (1981). A sham injection with an equal volume of insect saline was used to create an injury (Boman *et al.*, 1981). For labeling of P4 and P5 a pupa was injected with [^{35}S]methionine just after immunization.

As cloning vector, we used pBR322 and as its host *E. coli* K12, strain 294 (endo I$^-$, Bl$^-$, rk$^-$, mk$^+$) (Backman *et al.*, 1976). Recombinant plasmids with Cecropia cDNA were designated e.g., pCP510 where C stands for Cecropia, the next two characters indicate the corresponding immune protein (in this case P5) and the last symbols stand for the clone number.

Isolation of mRNA

When pupae are immunized, all essential RNA synthesis is completed after 5 h of incubation and the mRNA formed is stable for several days (Boman *et al.*, 1981). Unless otherwise stated, insects were immunized and incubated for 24 h at 25°C before RNA was prepared. Normal RNA was prepared from untreated pupae. The procedure for the isolation of total RNA from Cecropia pupae was as described (Boman *et al.*, 1981). If the pupae were immunized, or injured by a sham injection, the corresponding RNA is by definition referred to as 'immune RNA' or 'injury RNA'. Poly(A)-enriched RNA was isolated from the total RNA by chromatography on oligo(dT)-cellulose (Type 3, Collaborative Research, Waltham, MA) according to Aviv and Leder (1972). Binding buffer was 0.5 M NaCl, 0.01 M Tris-HCl, pH 7.5. Elution buffer was 0.01 M Tris-HCl, pH 7.5.

In vitro translation, immune precipitation and electrophoretic analysis of proteins

The rabbit reticulocyte lysate system (New England Nuclear) was used for translation of mRNA (Pelham and Jackson, 1976). Antisera against P4 and P5 were prepared as described in Hultmark *et al.* (1983). A slight modification of the method of Dobberstein *et al.* (1979) was used for immune precipitation of the cell-free translation products. As a first step, 50 μl of Nonidet P40 (NP-40) buffer (0.15 M NaCl) and 5 μl normal rabbit serum was added to 25 μl translation mixture. After incubation overnight at 4°C, 50 μl of a 20% suspension (w/v) of *Staphylococcus aureus* strain Cowan I in coupling buffer (10 mM Tris-HCl pH 7.4, 2.5 mM EDTA 0.1% NP-40, 0.15 M NaCl, 1 mg/ml bovine serum albumin) was added. After 5 min at room temperature, the cells were removed by centrifugation for 2 min in an Eppendorf centrifuge. To the supernatant was added 10 μl of antisera against either P4 or P5 and the mixture was incubated for 2 h at 4°C. Afterwards, 50 μl of 20% protein A-Sepharose (v/v) was added. After stirring for 3 min at room temperature the protein A-Sepharose beads were pelleted by centrifugation and washed as described (Dobberstein *et al.*, 1979).

In vitro translation products were separated by SDS-PAGE on 10–17% gradient gels using the conditions of Laemmli and Favre (1973). After electrophoresis the gel was treated with ^{3}H-enhancer (New England Nuclear), and autoradiography was performed at −70°C.

Construction of recombinant plasmids and transformation

The first and second strand of cDNA were synthesized on the immune mRNA using avian reverse transcriptase (a generous gift of J.W.Beard, National Institutes of Health, USA) and DNA polymerase I as described (Wickens *et al.*, 1978). The cDNA produced was purified by gel filtration on a Sephadex G-150 column. The fractions containing cDNA were pooled and the mixture was digested with S1 nuclease (Hoejmakers *et al.*, 1980). The linearized double-stranded cDNA was tailed by the addition of poly(dC) (Nelson and Brutlag, 1979) and annealed with *Pst*I-digested, deoxyguanosine-elongated pBR322 (Bolivar *et al.*, 1977). After annealing, the recombinant plasmids were used to transform *E. coli* K12, strain 294 as described (Cohen *et al.*, 1972). Competent cells were prepared according to Dogert and Ehrlich (1977). After transformation, recombinant clones were selected on nitrocellulose filters laid on LA plates (Maeda *et al.*, 1980) containing tetracycline (20 μg/ml). Tetracycline-resistant colonies were further selected for loss of ampicillin resistance using plates with 100 μg/ml.

Colony hybridization and RNA/DNA dot-blotting

Immune mRNA was labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP as described (Williams and Lloyd, 1979). For nick-translation, recombinant plasmids were digested with *PstI* and the insert DNA was purified by electroelution after electrophoresis in 2% agarose gel. Nick-translation with [α-^{32}P]CTP (Rigby *et al.*, 1977) was carried out according to the manufacturer's instructions (New England Nuclear). Colonies were grown for 8 h at 37°C on LA plates and transferred to nitrocellulose filters by laying sterile filters on the surface of the agar plates. The filters were transferred to plates containing 200 μg/ml chloroamphenicol and plasmids were amplified overnight. The hybridization and washing conditions were as described (Crampton *et al.*, 1980).

For RNA/DNA dot-blotting, nick-translated inserts from plasmids were hybridized to filter bound RNA according to Thomas (1980).

Electrophoresis of RNA and hybridization with cDNA insert

Oligo(dT)-purified RNA was separated by 1.5% agarose gel electrophoresis in a buffer system containing formaldehyde (Fellous *et al.*, 1982). Immune, injury and normal RNA (10 μg) was loaded in each slot. RNA was transferred to a nitrocellulose filter and hybridized against ^{32}P-labeled pCP510 or pCP410 (Northern blotting). RNA transfer, prehybridization, hybridization and washing conditions were according to Thomas (1980).

Identification of clones for protein P4 and P5 by mRNA selection.

mRNA selection by filter bound recombinant plasmid DNA followed by *in vitro* translation and immune precipitation was as described by Parnes *et al.* (1981), except that individual plasmids were bound to the nitrocellulose filter.

Acknowledgements

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