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Molecular analysis of α ecdysone induced 16S complexes in Drosophila Schneider's S3 cells

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The molecular organization of α ecdysone induced small heat shock proteins (small hsps) in Schneider's S3 tissue culture cells was analysed. Sucrose gradient centrifugation of cytoplasmic extracts and non-denaturing gel electrophoresis shows that hormone induced small hsps form 16S particles which differ in the relative molar ratios of the small hsps composing the 16S particles. The 16S particles possess a buoyant density in Cs_2SO_4 of ρ = 1.34 g/cm^3 which is indicative of RNP complexes with an RNA:protein ratio of 1:4. The RNA component of the 16S particles was identified by cDNA cloning using a cDNA library established from α ecdysone induced pupal 16S material. Northern hybridization using the 16S RNP specific partial cDNA clone Ec3 identifies a single α ecdysone inducible 300 nt RNA species. Our data suggest that the small hsps may unfold their so far unresolved function in form of RNP complexes.

The synthesis of the small heat shock proteins of Drosophila melanogaster , i.e. hsp27, hsp26, hsp23 and hsp22 , is induced by environmental stress, during normal development of the fly, as well as by the molting hormone α ecdysone in Schneider's S3 tissue culture cells (1,2). Their synthesis has been, independent of their mode of induction, correlated with the gain of increased thermal tolerance and phenocopy protection of the organism (3,4). Progress in the functional analysis of the small hsps has been slow and detailed analysis of their molecular organization is still lacking. In this paper we show that the small hsp are organized as 16S RNP particles and report on the characterization of the RNA associated with these RNP complexes.

MATERIALS AND METHODS

Cell culture, isolation and analysis of 16S particles

The Drosophila melanogaster tissue culture cell line Schneider S3 was used (5). Cells were grown as described by Shields and Sang (6). For α ecdysone induction cells were grown in the presence of 1μM α ecdysone (Serva) for various times. Cytoplasmic extracts (sol-80) were prepared and run on 10%-40% sucrose gradients as described for the isolation of proteasomes (7). For purification 16S fractions were applied to non-denaturing gel electrophoresis (8). SDS-PAGE was performed according to Laemmli (9). Two dimensional gel electrophoresis was performed as described by O'Farrell (10). Buoyant density of 16S complexes was determined in Cs_2SO_4 as described before (11).

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Screening of a cDNA library and RNA/DNA analysis

A cDNA library was established from RNA contained in pupal, α ecdysone induced 16S sucrose gradient fractions by random priming using the Amersham cDNA synthesis kit. The cDNAs were cloned into EcoRI 'arms' of the bacteriophage λgt10 and packaged using the Promega in vitro packaging system. Recombinant phages were propagated in E.coli host C600 HFl. About 10^4 recombinant phages were screened with 3' labelled RNA isolated from band 4 of the 16S complexes isolated from α ecdysone induced S3 culture cells. DNA sequencing and computer analysis was performed as described as before (12). RNA was recovered from non denaturing polyacrylamide gels by excising the bands and electroelution of the RNP complexes. The RNA was purified by phenol/chloroform extraction. The RNA was 3' labelled with ^{32}P pCp and T4 RNA ligase. Isolation of total RNA and northern blot analysis was performed as described by Maniatis (13).

RESULTS

αEcdysone induces the formation of 16S complexes formed by the small hsps

D. melanogaster S3 tissue culture cells were grown in the presence of 1μM α ecdysone for various times to induce the synthesis of the small hsps and the cytoplasmic supernatant (sol-80) subjected to sucrose gradient centrifugation. Treatment of S3 tissue culture cells with α ecdysone leads to a gradual increase in the amount of absorbing material sedimenting at approximately 16S until after 48h a clear optical density peak emerges. Concomitantly proteins of 27/26-kDa and 23-kDa accumlate in the 16S fraction (Fig.1a/b). As judged by their strongly increased synthesis in response to α ecdysone and their 2-D electrophoretic behaviour the 27/26-kDa and 23-kDa proteins are identical with the α ecdysone induced small hsps of early pupal stages (Fig.1c). We reproducibly found only minor amounts of the 22-kDa small hsp which is the small hsp whose synthesis is least responsive to the molting hormone.

Molecular analysis of α ecdysone induced 16S complexes

For further purification the material contained in the 16S fraction of α ecdysone induced S3 cells and uninduced control cells were separated on non-denaturing polyaqcrylamide gels. As shown in Fig.2a the 16S fraction of α ecdysone induced S3 cells can be separated into 4 different bands. Comparison with the uninduced control shows that bands 1, 3 and 4 are induced in the presence of α ecdysone. Analysis of the protein content of bands 1-4 shows that the four bands contain the small hsps at different molar ratios band 4 contains large amounts of hsp23 and only minor amounts of hsp27/26, band 3 is composed of almost equal amounts of the three hsps and band 4 consists of high amounts of hsp27/26 and only low amounts of hsp23. In addition a 45-kDa protein of unknown identity can be observed in band 1 and band 2. Band P as minor contaminant of the 16S gradient fraction contains the proteasome (data not shown) which due to its similar biochemical properties sometimes coisolates in the initial purification step. The data demonstrate that the small hsps form a heterogenous population of 16S complexes which contain the small hsps at different molar ratios. The appearance of different complexes is not due to disintegration of the 16S particles since identical results were obtained with 16S material which was fixed prior to electrophoresis (data not shown).

The 16S complexes are RNP-particles

Cs_2SO_4 density gradient analyis of 16S complexes shows that the 16S complexes formed by the small hsps possess a buoyant density characteristic of complexes with an RNA:protein ratio of approximately 1:4 (Fig.3a). An identical result was obtained when α ecdysone induced 16S particles of pupae were analysed (data not shown). To characterize the RNA component of the 16S RNPs a cDNA library of RNA

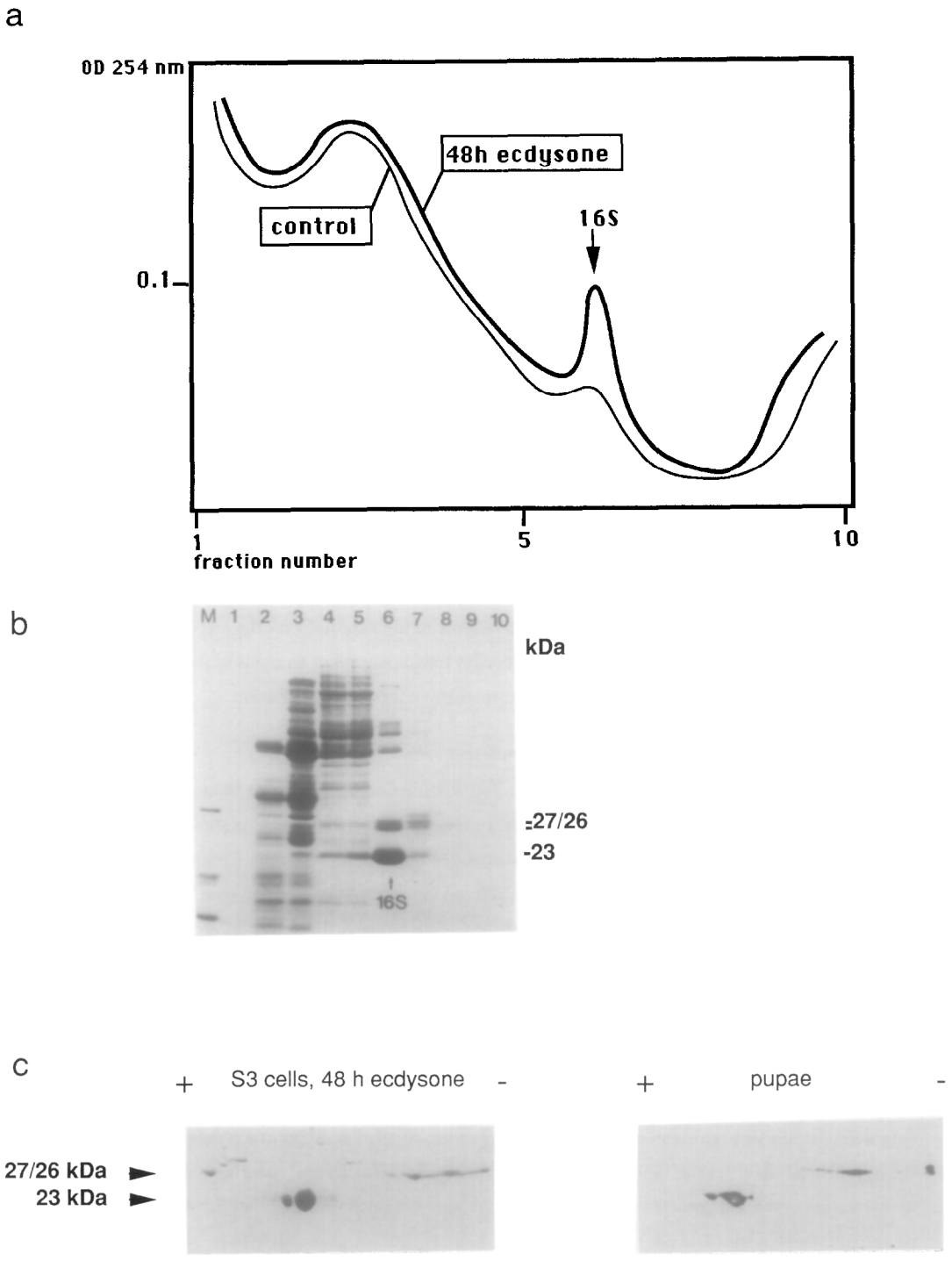

Fig.1
Analysis of the sol-80 fraction from S3 tissue culture cells grown in the presence of 1μM α ecdysone. The sol-80 fraction was analysed by centrifugation on 10%-40% sucrose gradients.
(a) Sucrose gradient profile at 254 nm of control and after 48h of hormone treatment. (b) Proteins contained in the different gradient fractions were electrophoretically analysed on 12% SDS polyacrylamide gels; M = molecular weight markers; 94kDa, 68kDa, 45kDa, 31kDa, 21.5kDa, 14.5kDa.
(c) 2-dimensional gel electrophoretic analysis of the 16S fraction from hormone treated cells and from the 16S fraction isolated from pupae.

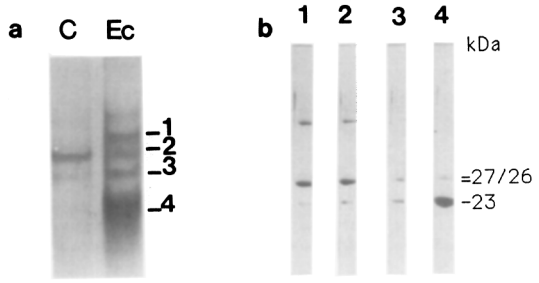

Fig.2
Analysis of the 16S material of α ecdysone induced 16S material from S3 cells on non denaturing polyacrylamide gels. (a) Analysis of the the 16S material from non induced (C) and 48h α ecdysone induced (Ec) S3 tissue culture cells on non-denaturing polyacrylamide gels. (b) Gel electrophoretic analysis of the proteins contained in bands 1-4. All gels shown were stained with coomassie blue. P= proteasome band.

isolated from pupal 16S sucrose gradient fractions was established. To identify positive recombinants this cDNA library was screened with 3' labelled RNA isolated from electrophoretically purified α ecdysone induced band 4 (Fig.2) of S3 culture cells. Of the positive clones the one with the largest cDNA insert, Ec3, was analysed in detail. To verify that the RNA encoded by Ec3 is indeed associated with the 16S particles, RNA from band 1-4 was isolated and probed by northern slot blot hybridization. As shown in Fig.3b the clone Ec3 hybridizes strongly with the RNA isolated from bands 1-4. Furthermore analysis of

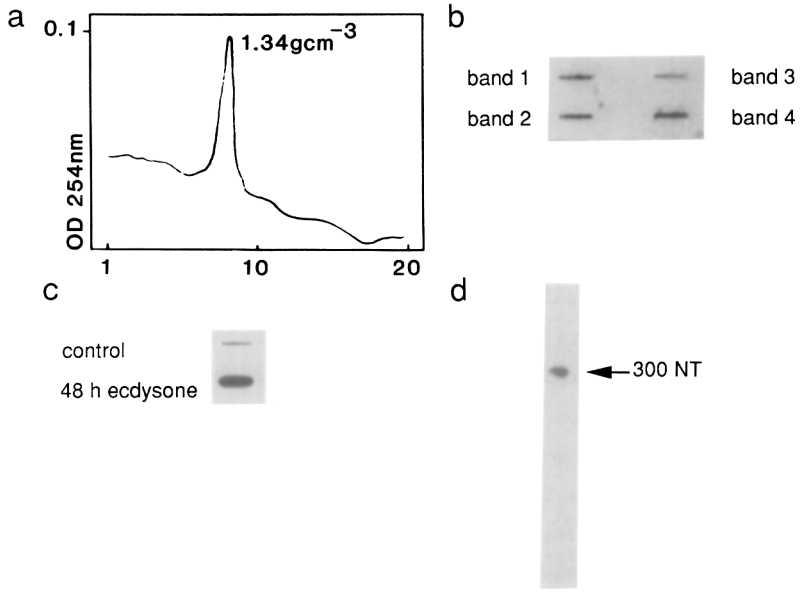

Fig.3
Characterization of the α ecdysone induced 16S particles as RNP-complexes. (a) Absorption profile of the Cs_2SO_4 density gradient containing purified 16S particles. The 16S particle bands at a density of ρ= 1.34 g/cm^3. (b) Northern slot blot analysis on RNA isolated from 16S particle bands 1, 2, 3,4 (**Fig.2b**) with the cDNA clone Ec3. (c) Northern slot blot analysis with Ec3 on total RNA from uninduced and hormone induced S3 tissue culture cells. (d) Northern hybridization on total RNA from αecdysone induced S3 tissue culture cells using Ec3 as probe.

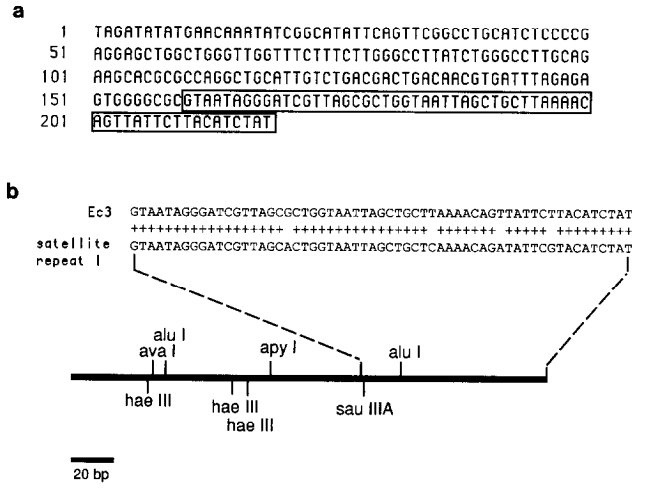

Fig.4
Sequence and partial restriction map of the cDNA clone Ec3. **(a)** Sequence of Ec3. Boxed sequence indicates the overlap with the Drosophila satellite repeat I. **(b)** Sequence of Ec3 which is almost identical to the satellite repeat I sequence and partial restriction map of Ec3.

total RNA from α ecdysone induced cells shows that the synthesis of this RNA, like the synthesis of the small hsps, is inducible by α ecdysone (Fig.3c). Northern hybridization of total RNA from α ecdysone induced S3 cells shows that Ec3 identifies a single RNA of about 300 nt (Fig.3d) and thus codes for about two thirds of the RNA molecule. Sequence analysis reveals a 93.2% identity within a 59 nt overlap with the Drosophila satellite repeat I. No homology to any known RNA transcripts were found. (Fig. 4).

DISCUSSION

In Drosophila the synthesis of the small hsps underlies at least a dual regulatory mechanism and exposure of Schneider's S3 tissue culture cells to α ecdysone strongly induces the synthesis of the small hsps(3). In this report we demonstrate that α ecdysone not only triggers the synthesis of the small hsps but also induces the concomitant formation of a populations of 16S particles which are formed by the small hsps and which exhibit all the characteristics of small RNP complexes. The 16S particles represent a population of complexes in which the small hsps are present at different molar ratios. A diversity in small hsp expression and particle population can also be observed during normal fly development (14,15,16). Therefore the heterogeneity of the 16S particles in S3 cells reflects to a large extent the physiological situation during the normal life cycle of the fly where 16S particles are formed by the small hsps in embryos as well as in pupae. The ability of the small hsps to form 16S complexes appears to be a general feature of the small hsps. The situation however differs from the one observed in Hela cells and yeast where the single small hsp forms 12S-18S complexes only after prolonged recovery from a previous stress situation (17,18). The 16S particles possess an approximate mw of 400 kDa and exhibit characteristics of RNP complexes with an RNA:protein ratio of 1:4. The isolated cDNA clone Ec3 identifies a single 300 nt RNA species within the total cellular RNA and its transcription is inducible by α ecdysone like the synthesis of the protein component of the 16S RNPs. In addition, Ec3 hybridizes to RNA isolated from different α ecdysone induced 16S RNP particle bands as well as to the RNA in

undenatured 16S particles which were transferred directly onto nitrocellulose (data not shown). In combination with the density gradient analysis these experiments demonstrate that the 16S complexes formed by the small hsps are indeed RNP particles and that this appears to be independent of the mode of their induction. This conclusion is in agreement with previous reports which showed that in Schneider's S3 cells stress induced small hsps can be uv cross linked to RNA (19). Since sequence analysis of Ec3 shows no homology to any known RNA sequence of Drosophila or other small sized RNA our data present evidence that the 16S particles represent a new type of RNP particles and that at least one organization principle of the small hsps is that of RNP complexes.

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