Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/148579

Please be advised that this information was generated on 2017-12-05 and may be subject to change.

1766

RNA METABOLISM DURING DEVELOPMENT IN DROSOPHILA HYDEI

C. ALONSO

RNA METABOLISM DURING DEVELOPMENT IN DROSOPHILA HYDEI

PROMOTOR:

PROF. DR. H.D. BERENDES

RNA METABOLISM DURING DEVELOPMENT IN DROSOPHILA HYDEI

P R O E F S C H R I F T

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF. MR. F.J.F.M. DUYNSTEE,
VOLGENS HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 14 JUNI 1973
DES NAMIDDAGS TE VIER UUR

door

CARLOS ALONSO

geboren te Mota del Marques (Valladolid, Spanje)

druk: Stichting Studentenpers, Nijmegen

Bij het verschijnen van dit proefschrift wil ik gaarne mijn dank uitspreken aan allen van de afdeling Genetika die mij bij de totstandkoming ervan hebben bijgestaan.
Mijn dank gaat in het bijzonder uit naar mijn collegae, Dr. P.J. Helmsing, Dr. H.J. Leenders, Dr. Th.K.H. Holt en Dr. C.L.M. Poels. Prof. Dr. G.T. Rudkin ben ik veel dank verschuldigd voor de vele stimulerende discussies en voor zijn bijdrage aan de kritische beoordeling van de inhoud van dit proefschrift. Mej. A. van den Berg heeft voor een belangrijk deel het typewerk verzorgd en de heer J. Gerritsen en zijn medewerkers de tekeningen.
Een deel van het proefschrift werd bewerkt op basis van een stipendium van de Juan March Foundation, terwijl ook door de Stichting Clementer et Auguste financiële steun werd verleend.

A mi madre y hermanos

Dit proefschrift is samengesteld uit overdrukken uit de volgende tijdschriften:

Developmental Biology, 28, 372-381 (1972)
Cell Differentiation, (in druk, 1973)
FEBS (Fed.Eur.Biochem.Soc.) Letters, 31, 85-88 (1973)
J. Insect Physiology, (in druk, 1973)

INHOUD

Inleiding .... 9 - 15

The influence of molting hormone on RNA synthesis in isolated polytene nuclei of Drosophila ....372 - 381

RNA synthesis in polytene salivary glands of Drosophila hydei maintained under in vitro conditions ....107 - 118

Improved conditions for in situ RNA/DNA hybridization .... 85 - 88

RNA metabolism during metamorphosis of Drosophila hydei .... 16 - 33

Samenvatting .... 34 - 36

INLEIDING

Het lijkt alleszins verantwoord om op grond van onze huidige kennis omtrent de funktie van het genoom op het niveau van de individuele cel, te veronderstellen dat de eigenschappen van iedere cel van een organisme het resultaat is van een gebruik van tenminste een deel van de informatie aanwezig in het genoom. De ontwikkeling van een meercellig organisme met een veelvoud, van op verschillende wijze naar struktuur en funktie gedifferentieerde cellen zou voor een belangrijk deel kunnen berusten op een gedifferentieerd gebruik van de voor iedere cel in principe identieke genetische informatie, tijdens de ontwikkeling.

Een dergelijke opvatting wordt onder meer gesteund door de resultaten verkregen uit het onderzoek van de aktiviteit van polytene chromosomen in verschillende weefsels van de larven van tweevleugelige insecten. Deze polytene chromosomen maken het mogelijk lokale aktiviteiten van het genoom te onderkennen en deze in bepaalde, gunstige gevallen te correleren met specifieke celfunkties (Beermann, 1961; Berendes, 1965; Baudisch en Panitz, 1968; Grossbach, 1969; Poels, 1972; Leenders en Beckers, 1972).

Eén van de belangrijkste vragen in de ontwikkelingsbiologie is nu de vraag op welke wijze de differentiële genaktiviteit wordt gereguleerd, met andere woorden, hoe wordt op een bepaald moment in een bepaalde cel een bepaald onderdeel van het genoom geaktiveerd terwijl eenzelfde aktivering in andere cellen achterwege blijft. Het is bekend dat bepaalde externe stimuli, waaronder hormonen, in staat zijn

specifieke veranderingen in het RNA metabolisme, primair in het RNA synthese patroon, tot stand te brengen. Ook dit fenomeen is duidelijk geillustreerd aan polytene chromosomen, welke na toediening van het steroid hormoon ecdyson een specifieke verandering in het patroon van RNA synthetiserende loci demonstreren (Clever en Karlson, 1960; Berendes, 1967; Ashburner, 1971; Poels, 1970). Het is echter lange tijd een strijdvraag gebleven of het steroid direkt dan wel indirekt op de reagerende genoom loci zou inwerken. Onderzoek van het werkingsmechanisme van steroid hormonen bij zoogdieren (Jensen e.a. 1968; Beaulieu e.a. 1970) en insecten (Emmerich, 1972) heeft uitgewezen dat het steroid in de cel wordt gebonden aan specifieke z.g.n. "receptor" eiwitten, die waarschijnlijk een belangrijke rol vervullen bij het intracellulaire transport van het hormoon en het binnendringen ervan in de celkern. Er is echter geen enkele aanwijzing dat het steroid daarna geassocieerd wordt met die genoom loci die geaktiveerd worden (Emmerich, 1970). Anderzijds is door Karlson's groep gepostuleerd dat een steroid hormoon wel direkt op het genoom zou aangrijpen (Karlson, 1965; Congote e.a. 1970). De resultaten van een onderzoek naar de werking van het steroid ecdyson op geisoleerde celkernen van Drosophila speekselklieren, één van de doelorganen van het hormoon, hebben uitgewezen dat het steroid niet zonder meer in staat is een specifieke genoom response op te wekken. De resultaten van dit onderzoek welke berusten op een vergelijking van door de kern gesynthetiseerd RNA in aan- en afwezigheid van het hormoon met behulp van RNA-

studie van de meest gunstige in situ hybridisatie omstandigheden waarbij de polytene chromosomen als hybridisatie basis werden benut. Een eerste belangrijke verbetering werd inmiddels gerapporteerd en is als derde onderdeel aan dit proefschrift toegevoegd. Andere verbeteringen alsmede een kritische evaluatie van de in situ RNA-DNA hybridisatie procedure, vormen tesamen met de resultaten van een aantal experimenten waarin in vivo en in vitro door de cel gesynthetiseerd RNA in hybridisatie experimenten werd gebruikt, het onderwerp van twee in voorbereiding zijnde publikaties.

Het laatste onderdeel van dit proefschrift betreft een onderzoek van het RNA metabolisme tijdens de metamorfose van Drosophila. Vanaf het laat derde larve stadium vindt geen voedselopname door de larve plaats. De ingrijpende veranderingen welke tijdens het metamorfose stadium plaats vinden, de totale afbraak van alle larvale weefsels en de opbouw van het imago, geschieden geheel in een gesloten systeem (Ring, 1972) en moeten derhalve plaats vinden op basis van de tijdens het larvale stadium opgebouwde voorraad aan energie en bouwstenen. De afbraak van larvale weefsels en een opbouw van de imaginale weefsels moet ten dele plaats vinden op basis van de in de larvale weefsels gesynthetiseerde RNA en wel via een afbraak en gebruik van de hieruit resulterende bouwstenen. Een analyse van de quantitatieve en qualitatieve veranderingen in RNA tijdens de metamorfose vormt het laatste deel van dit proefschrift.

Ashburner, M. (1971) Induction of puffs in polytene chromosomes of in vitro cultured salivary glands of Drosophila melanogaster by ecdysone and ecdysone analogues. Nature 230, 222-223.

Baudisch, W. en Panitz, R. (1968) Kontrolle eines biochemischen Merkmals in den Speicheldrüsen von Acricotopus lucidus durch einen Balbiani-Ring. Exptl. Cell Res. 49, 470-476.

Baulieu, E.E. en Raynaud, J.P. (1970) A "proportion graph" method for measuring binding systems. Eur. J. Biochem. 13, 293-304.

Beermann, W. (1961) Ein Balbiani-Ring als Locus einer Speicheldrüsenmutation. Chromosoma 12, 1-25.

Berendes, H.D. (1965) Salivary gland function and chromosomal puffing patterns in Drosophila hydei. Chromosoma 17, 35-77.

Berendes, H.D. (1967) The hormone ecdyson as effector of specific changes in the pattern of gene activities of Drosophila hydei. Chromosoma 22, 274-293.

Clever, U. en Karlson, P. (1960) Induktion von Puff-Veränderungen in den Speicheldrüsenchromosomen von Chironomus tentans durch Ecdyson. Exptl. Cell Res. 20, 623-626.

Congote, L.F., Sekeris, C.E. en Karlson, P. (1970) Alteration of the nature of RNA synthesized in isolated fat body cell nuclei as a result of ecdysone and juvenile hormone action. Z. Naturforsch. 25b, 279-284.

Emmerich, H. (1970) Ecdysonbindende Proteinfraktionen in den Speicheldrüsen von Drosophila hydei. Z. vergl. Physiol. 68, 385-402.

Emmerich, H. (1972) Ecdyson binding proteins in nuclei and chromatin from Drosophila salivary glands. Gen. comp. Endocr. 19, 543-551.

Gall, J.G. en Pardue, M.L. (1971) Nucleic Acid hybridization in cytological preparations. In: Methods of Enzymology, Vol. XXI Nucleic Acids. Ed. by L. Grossman and K. Moldave. Academic press 470-480.

Greenberg, J.R. (1969) Synthesis and properties of ribosomal RNA in Drosophila. J. mol. Biol. 46, 85-98.

Grossbach, U. (1969) Chromosomen-Aktivität und biochemische Zelldifferenzierung in den Speicheldrüsen von Camptochironomus. Chromosoma 28, 136-187.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E. Jungblut, P.W. en DeSombre, E.R. (1968) A two step mechanism for the interaction of estradiol with rat uterus. Proc. Nat. Acad. Sciences 59, 632-638.

Karlson, P. (1965) Biochemical studies of ecdysone control of chromosomal activity. J. Cell Comp. Physiol. 66, 69-76.

Leenders, H.J. en Beckers, P.A.J. (1972) The effects of changes in the respiratory metabolism upon genome activity. A correlation between induced gene activity and an increase in activity of a respiratory enzyme. J. Cell Biol. 55, 257-265.

Poels, C.L.M. (1970) Time sequence in the expression of various developmental characters induced by ecdysterone in Drosophila hydei. Dev. Biol. 23, 210-225.

Poels, C.L.M. (1972) Mucopolysaccharide secretion from Drosophila salivary glands as a consequence of hormone induced gene activity. Cell Differentiation 1, 63-78.

Ring, R.A. (1973) Changes in dry weight, protein and nucleic acid content during diapause and normal development of the blowfly Lucilia sericata. J. Insect Physiol. 19, 481-494.

Steffenson, D.M. en Wimber, D.E. (1971) Localization of tRNA genes in the salivary chromosomes of Drosophila by RNA: DNA hybridization. Genetics 69, 163-178.

Reprinted from DEVELOPMENTAL BIOLOGY Volume 28 No 2 June 1972
Copyright © 1972 by Academic Press Inc Printed in U S A

DEVELOPMENTAL BIOLOGY 28, 372-381 (1972)

The Influence of Molting Hormone on RNA Synthesis in Isolated Polytene Nuclei of *Drosophila*

C. ALONSO

Department of Genetics, University of Nijmegen, The Netherlands

Accepted February 2, 1972

Treatment of isolated polytene (salivary gland) nuclei of *Drosophila hydei* with the steroid molting hormone ecdysone results in a 1 4 times enhanced incorporation of labeled nucleotides into acid-precipitable RNA as compared with control nuclei

Gel electrophoresis demonstrated that the hormone stimulates predominantly the incorporation of precursors into low molecular weight RNA (2–6 S) and to a lesser extent two high molecular weight fractions (> 28 S)

Hybridization of RNA from control and hormone treated nuclei with DNA revealed no difference in saturation level (2 4%) between the RNA derived from the two types of nuclei Unlabeled cytoplasmic and nuclear RNA competed to the same extent with RNA from control and from hormone-treated nuclei

Cytological examination revealed that the hormone treatment failed to induce the formation of hormone-specific puffs or the initiation of RNA synthesis at these chromosome loci

It is suggested that the stimulation of RNA synthesis by *in vitro* hormone treatment of isolated nuclei is due to an increased rate of transcription of already derepressed sites rather than to a derepression of new sites

INTRODUCTION

The insect molting hormone ecdysone is known to affect genome activity of a variety of insect cell types. In the Diptera, genome responses can be identified as specific changes in the puffing pattern of the polytene chromosomes in larval tissues (Clever and Karlson, 1960; Clever, 1963; Berendes, 1967; Ashburner, 1971). These changes in the puffing pattern are generally conceded to correspond with alterations in the specifities of RNAs transcribed, since puffs are sites of active RNA synthesis (Pelling, 1964).

Because of these features, the polytene nuclei provide an excellent tool for combined cytological and biochemical studies on the mechanism of hormone action at the transcriptional level. With regard to the control of nuclear (chromosomal) RNA metabolism by steroid hormones, the evidence obtained so far in support of a *direct* control is conflicting.

By employing RNA—DNA hybridization, it was established that the pattern of RNA synthesized in isolated polytene nuclei of *Calliphora* fat body incubated with ecdysone differs significantly from that of nontreated nuclei (Congote *et al.*, 1970).

For other steroid hormones. e.g., cortisol, it has been demonstrated that *in vitro* hormone treatment of isolated nuclei and chromatin from target tissues has a definite influence on template activity (Sekeris *et al.*, 1969; Stackhouse *et al.*, 1968). It remains to be elucidated, however, whether or not this influence results from a direct interaction between the hormone and the chromatin.

In an attempt to define the effect of the steroid ecdysone at the genome level, isolated polytene nuclei of *Drosophila* were incubated with appropriate hormone concentrations. However, the nuclei, which provide the opportunity for a direct observation of a hormone-specific response (puffs), failed to reveal any indication for a response similar to that ob-

served if intact cells are supplied with the hormone (Berendes and Boyd, 1969)

The aim of the present study is to provide, on the basis of combined cytological and RNA DNA hybridization experiments, some insight into the nature of the changes in RNA metabolism of isolated nuclei provoked by treatments with the steroid hormone ecdysone

MATERIALS AND METHODS

Mid-third instar larvae of a laboratory stock of *Drosophila hydei* were used throughout this study

Isolation and Incubation of Nuclei

Isolated nuclei were prepared as described previously (Berendes and Boyd, 1969) In all experiments in which the quantity of RNA synthesis of isolated nuclei incubated with and without the molting hormone β-ecdysone) were compared, the two aliquots of nuclear suspension were carefully equilibrated with regard to the number of nuclei The equilibration was checked by a quantitative DNA measurement in a number of samples These measurements, carried out according to the method of Giles and Myers (1965), indicated that a difference in DNA quantity in two samples to be compared never exceeds 5% After isolation and repeated washing at 0°C, the nuclei were incubated for 30 min at 25°C in 0 3 ml of nuclear buffer (Berendes and Boyd, 1969), supplied with 1 μCi of each of the C^{14}-labeled nucleotides, ATP, GTP, CTP, and UTP (specific activity, mCi/mmole ATP, 550, GTP, 389, CTP, 425, UTP, 197, Radiochemical Centre, Amersham, England) The incubation medium was also supplied with 20 μg/ml cold ATP in order to reduce possible degradation of the nucleotide-triphosphates to di and monophosphates (Meisler and Tropp, 1969)

Hormone treatment was performed by supplying the standard incubation medium with 3 μg/ml β-ecdysone

Preparation of RNA

Nuclear RNA After incubation, the isolated salivary gland nuclei were suspended in an amount of 0 1 *M* Tris HCl sodium dodecyl sulfate (SDS) buffer at pH 7 2 (ice-cold) equivalent to 2 3 times the wet weight of the glands used The suspension was mixed with an equal volume of buffer-saturated phenol and shaken for 30 min at 4°C After centrifugation (10 min at 16,000 *g*) the water phase was saved and the interphase and phenol phase were reextracted with half the volume of a Tris HCl-SDS buffer, pH 8 2, at 60°C for 1 min followed by 15 min at 4°C (Georgiev *et al*, 1963) The water phase resulting from 10 min centrifugation was added to the water phase saved from the extraction at pH 7 2 This mixture was again submitted to a phenol treatment of 5 min at 4°C The RNA was precipitated from the water phase by addition of 2 vol ETOH (28°C, overnight) The RNA was purified from traces of DNA by incubation for 15 min at room temperature with RNase-free DNase (20 μg/ml DNase I, Schwarz/Mann) The DNase treatment was followed by reprecipitation of the RNA with 2 vol of ETOH (−28°C, overnight) The final RNA pellet was dissolved in 100 μl of 0 15 *M* saline–0 015 *M* citrate (SSC) and stored at 70°C

Total larval RNA Total larval RNA was labeled by feeding larvae for 48 hr with $NaP^{32}O_4$ (1 mCi/ml food medium) After this period the larvae were harvested and immediately frozen on dry ice Frozen larvae were powdered and suspended in 10 v/w Tris HCl EDTA-SDS buffer, pH 7 2, mixed with an equal volume buffer-saturated phenol and shaken for 30 min at 4°C Following centrifugation the waterphase was saved and the remaining phenol and interphase were reextracted with an equal volume of buffer The resulting waterphase and that of the first extraction were combined

and mixed with half a volume of phenol After 20 min shaking at 4°C the RNA was precipitated with 2 vol ETOH containing 0 3% NaCl The RNA was further treated as described above, however, including a phenol treatment following the DNase treatment

The purity of the RNA was determined by optical density measurements at E260/230 and E260/280 Contamination with DNA was tested by a modified diphenylamine test (Giles and Myers, 1965) The preparations were contaminated by less than 5% DNA Subsequently, the RNA was heated to 100°C and filtered through a Millipore filter to remove the remaining DNA The specific radioactivity of the RNA extracted, was 14000 cpm/μg

Cytoplasmic nonradioactive RNA Cytoplasmic RNA was extracted from whole larvae as described above b In using a Tris HCl buffer (pH 7 2) without detergents the nuclear RNA remains in the interphase (Georgiev *et al*, 1963, Cooper and Kay, 1968, Piker *et al*, 1969)

Preparation of DNA

DNA was prepared from polytenic tissues which were saved from the isolation procedure of the salivary glands These tissues, predominantly midgut and Malpghian tubules, after extensive washing were kept frozen at 70°C until use, 10 gm of tissue were mixed with 3 vol of 0 1 *M* saline EDTA, pH 8 0 containing a final concentration of 0 7% SDS The buffer was boiled for 1 min and added immediately to the tissues After 2–4 min soaking, the mixture was cooled to 0°C and extracted twice with an equal volume of chloroform–isoamyl alcohol (24 1) DNA was recovered by layering 2 vol of ETOH on top of the mixture and spooling The DNA was dissolved in 3 ml 0 1 × SSC and after it was dissolved the concentration of SSC was adjusted to 1 × SSC Contaminating RNA was digested with 200 μg/ml pancreatic RNase (DNase-free, Calbiochem) and 200 μg/ml T1 RNase (Worthington) for 3 hr at 37°C RNase and other contaminating proteins were digested with 200 μg/ml pronase (Calbiochem) for another 3 hr at 37°C After several chlorophorm-isoamyl alcohol washings until no interphase was left, 2 vol of ETOH were added and the DNA was spooled The DNA was dissolved in 0 1 × SSC and dialyzed for 8 hr against 0 1 × SSC (Melli and Bishop, 1969) After dialysis the DNA was centrifuged for 18 hr at 38,000 rpm in a Spinco preparative ultracentrifuge (5°C) The purity of the DNA was controlled by optical density measurements at $E_{260\ 280}$ and $E_{260\ 230}$, providing values of 1 80 and larger than 2 respectively Hyperchromicity of the DNA increased by 38% after 20 min at 100°C in 0 1 × SSC (cf Melli and Bishop, 1969)

RNA-DNA Hybridization Procedure

DNA was denaturated at 50 μg/ml in 0 1 × SSC at 100°C for 20 min followed by rapid cooling The DNA was immobilized on Sartorius filters (SM 11304, 0 1 μ) Each filter was loaded with 1 5 μg DNA For competition experiments the filters were loaded with 5 μg DNA In order to avoid DNA renaturation the loading was performed at 4°C The hybridization reaction was performed in 0 5 ml of 6 × SSC in closed glass scintillation vials for 16 hr at 67°C Parallel experiments were carried out with filters containing no DNA In most instances the noise level was less than 0 02% of the input radioactivity of the RNA RNA used for hybridization was previously filtered through Millipore filters (HA, 0 45 μ) (Hennig, 1968) Following annealing, the filters were soaked for 5 min in 2 × SSC, washed and treated with 20 μg/ml RNase DNase-free, Calbiochem) and washed again before counting with a Philips scintillation analyzer (50 min) Counting efficiency was 32%

All competition experiments were per

formed by using a level of labeled RNA and a range of concentrations of competing unlabeled RNA which remained within the rising portion of the DNA saturation curve up to the initial part of the saturation plateau (Jeanteur and Attardi, 1969) The competing RNA (nonlabeled) was mixed with the labeled RNA from nontreated or hormone-treated nuclei before applying it to the DNA containing filters

RNA Gel Electrophoresis

The electrophoretic mobilities of newly synthesized RNA fractions of isolated nuclei were determined on 2 4% acrylamide gels according to the method of Bishop *et al* (1967) Before use the gels were prerun without RNA at room temp for 1 hr at 5 mA per tube and 50 V The RNA samples were administered to the gels in a volume of 10 20 μl Electrophoresis was carried out for 55 min at room temp Carrier RNA was obtained from whole larvae Radioactivity of 1 mm slices was determined in Bray's solution with the Philips scintillation analyzer The vials were kept in a dark room for 24 hr before counting

RESULTS

RNA Synthesis and Electrophoresis

Nuclear RNA was isolated from equal samples of isolated nuclei following 30 min incubation with ^{14}C-labeled nucleotides together with or without ecdysone A comparison of the amount of radioactive acid-precipitable RNA in nuclei treated with and without the hormone revealed a significantly increased uptake of nucleotides if hormone is present in the incubation medium The specific activity of the RNA isolated from, hormone treated nuclei varies in the different experiments from 500-520 cpm/μg RNA The specific activity of the RNA from nontreated nuclei is 330 cpm/μg RNA

Acrylamide gel electrophoresis of RNA synthesized in isolated nuclei revealed that predominantly low molecular weight RNA (between 9 and 2 S) is produced In the hormone-treated nuclei the amount of precursor incorporation into low molecular weight RNA has increased considerably Particularly, the fractions in the 2 3 S regions have increased Only two fractions of RNA with a molecular weight > 28 S were found in increased quantities in hormone treated nuclei as compared with the controls (Fig 1) This result is consistent with the data reported on hormone treated isolated nuclei of the fat body of *Calliphora* (Congote *et al*, 1970)

Despite these changes in the RNA synthesized in isolated nuclei in the presence of hormone, cytological observations on the polytene chromosomes of these nuclei did not reveal the presence of known hormone-specific puffs (Fig 2) in agreement with earlier findings of Berendes and Boyd (1969) Furthermore, it was established by autoradiography that in addition to the absence of distinct puffs, the hormone specific loci did not incorporate ^{3}H labeled UTP

On account of these results it seemed important to determine whether or not the increase in uridine incorporation in isolated nuclei under the influence of the hormone was due to the production of new RNA species For this purpose RNA-DNA hybridization was employed

Hybridization

In the first instance, saturation experiments were performed in which filters containing 5 μg, of DNA were incubated with increasing amounts of total larval RNA (1 42 μg) (Fig 3, top) Similar experiments were carried out with nuclear RNA of nontreated nuclei and of nuclei incubated with hormone, using filters containing 1 5 μg of DNA (Fig 3, bottom) The saturation value obtained with nuclear RNA was significantly higher than that resulting from annealing with total RNA This

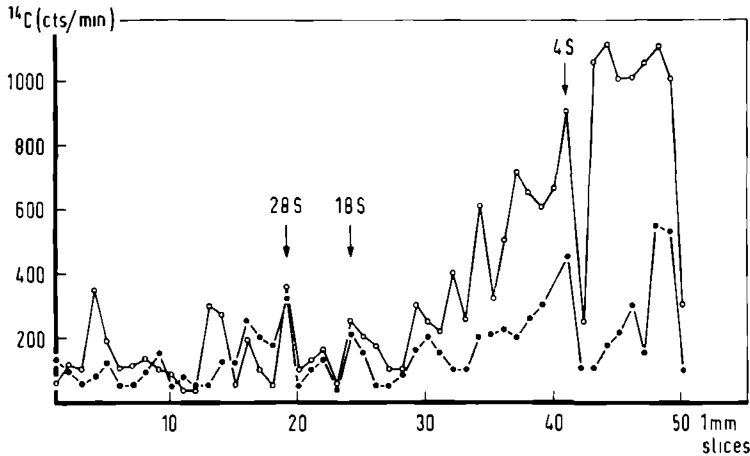

FIG. 1. Acrylamide electrophoresis of ^{14}C-RNA synthesized *in vitro* in isolated salivary gland nuclei in the absence, ●——●, and in the presence, O——O, of 3 μg/ml β-ecdysone in the incubation medium. The conditions for ^{14}C-RNA synthesis and electrophoresis are described in the text. Cytoplasmic RNA of larvae was used as a marker for the 4, 18, and 28 S RNA From nontreated nuclei radioactive RNA equivalent to 7500 cpm/100 μg of DNA was obtained whereas hormone-treated nuclei produced 12,000 cpm/100 μg of DNA The electrophoretic pattern was reproduced in at least seven experiments

appears to be a general feature (Piker *et al.*, 1969). The saturation value obtained with total larval RNA following hybridization either without formamide at 67°C, or at 24°C in the presence of 30% formamide (2 SSC) is close to the value reported by Hennig (1968) for the same species (1.3%)

In comparing the saturation values obtained with RNA from nontreated and hormone-treated nuclei, no significant difference can be found (Fig. 3, bottom). In both cases, the initial point of the saturation plateau is reached with about 8 μg of RNA per 1.5 μg of DNA. It may be concluded from reciprocal plots of the amount of DNA hybridized as a function of the amount of RNA in the reaction mixture (Fig. 4) that most of the RNA sequences from nontreated and hormone-treated nuclei have similar hybridization rates. This indicates that the composition of the fast annealing sequences does not differ significantly in the two RNA extracts (Bekhor *et al.*, 1969; Melli and Bishop, 1969).

A more sensitive test of whether or not the hormone treatment provoked the synthesis of new RNA species was made by competition hybridization experiments in which nonlabeled cytoplasmic or nuclear RNA was applied as competitor. The RNA extracts were obtained from nontreated animals. There was no detectable difference found between the competition of cytoplasmic nonlabeled RNA with RNA from either untreated or hormone-treated nuclei. The competing effect of cytoplasmic RNA as such was very poor (15% at a competition ratio of 50:1), indicating a great difference in sequence availability between the two RNA sources (cf. Piker *et al.*, 1969; McCarthy and Church, 1970). It was reported previously that nuclear RNA contains many sequences which never leave the nucleus (Shearer and McCarthy, 1967).

Nonlabeled RNA from nuclei effectively competes (85% at a competition ratio of 8:1) with labeled RNA fron nontreated as well as hormone-treated nuclei (Fig. 5). There is no significant difference in competition of the nonlabeled nuclear RNA for sequences present in the RNA of either nontreated or hormone-treated

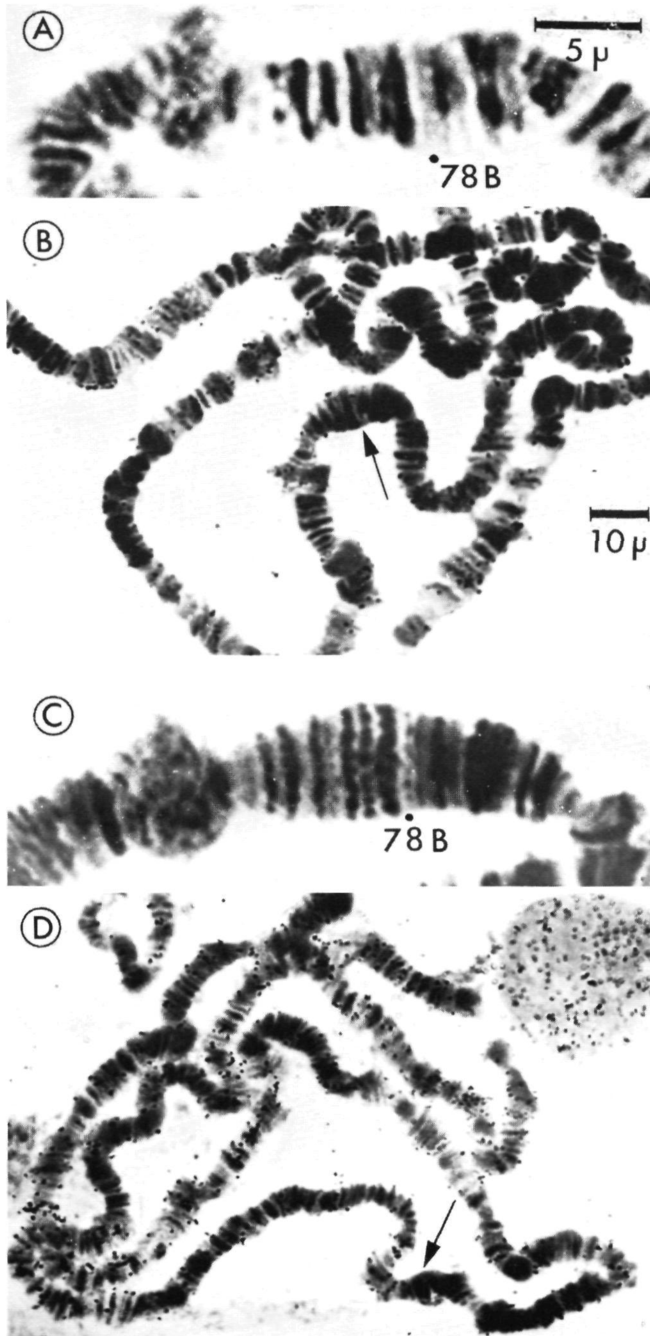

FIG. 2. (A) Region 78B in a control nucleus following incubation for 30 min in the absence of hormone. (B) Autoradiograph of ^{3}H-UTP incorporation in a nucleus incubated for 30 min in the absence of the hormone. (C and D) as for A and B after incubation for 30 min with 3 μg/ml β-ecdysone. Neither C, nor D provide indications for an effect of the hormone at genome loci responding with puff formation if hormone is applied *in vivo* (see arrows).

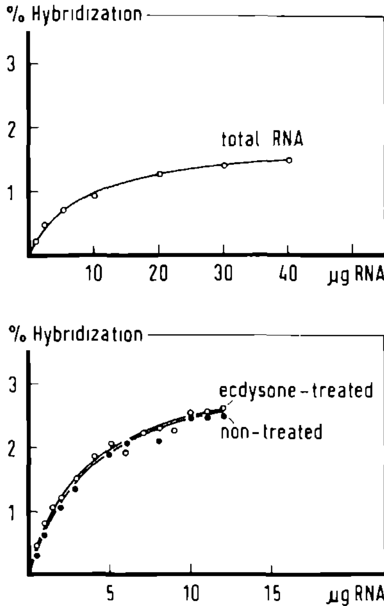

FIG. 3 *Top* Hybridization of total DNA with ^{32}P-total-RNA from late third-instar larvae The ^{32}P-RNA (14,000 cpm/µg) was incubated with the DNA filters, loaded with 5 µg DNA at 4°C, in 0 5 ml of 6 × SSC for 16 hr at 67°C *Bottom* Hybridization of total DNA with ^{14}C-labeled nuclear RNA synthesized in isolated nuclei in the absence of hormone in the medium (330 cpm/µg, ●——●) and in the presence of 3 µg/ml β eceysone in the incubation medium (520 cpm/µg; O——O) The values presented are corrected for noise, which never exceeded a level of 0 02%. The incubation of the DNA filters in the RNA solution was done in 6 × SSC at 67°C for 17 hr

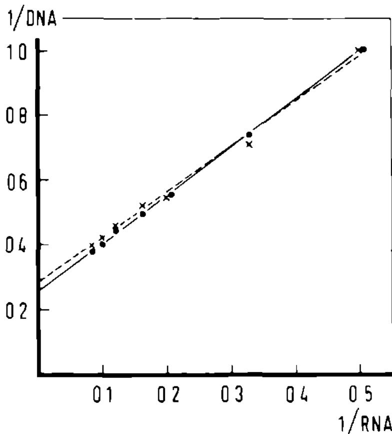

FIG 4. Reciprocal plot of the hybridization results presented in Fig 3b. ●——●, Ecdysone treated, ×——×, nontreated

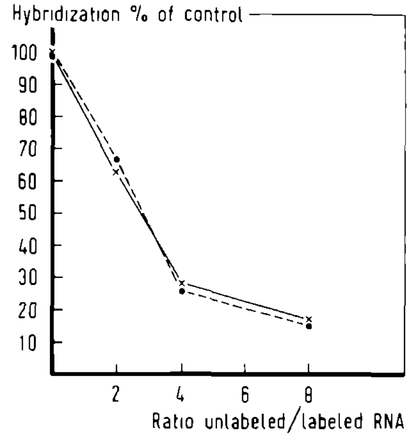

FIG. 5. Competition between nuclear unlabeled RNA and labeled RNA synthesized in nontreated nuclei (330 cpm/µg; ●——●) and in hormone-treated nuclei (510 cpm/µg, ×——×) The filters were loaded with 5 µg of DNA and annealed with 27 µg of labeled RNA and a ratio of unlabeled RNA as indicated at the ordinate. 100% hybridization represents 64 cpm for the RNA of hormone-treated nuclei and 48 cpm for the RNA extracted from untreated nuclei

nuclei. This result indicates that the composition of the bulk of the RNA sequences present in hormone-treated and nontreated nuclei are very similar. The results do not exclude the possibility that certain RNA sequences which may be synthesized in the hormone-treated nuclei in very small quantities have excaped from detection in the competition experiments. It is known that infrequent RNA transcripts may be selected against as RNA molecules react with DNA of closely related base sequence in the mammalian genome (Britten and Kohne, 1968; Church and McCarthy, 1970).

DISCUSSION

Intact larval salivary gland cells of *Drosophila* respond to the insect molting hormone ecdysone with specific changes in genome activity (Berendes, 1967). It has been suggested that this response may be brought about by a direct action of the steroid hormone at the genome level (Karlson, 1965; Congote *et al.*, 1970). In order to test this hypothesis, isolated

nuclei of *Drosophila hydei* salivary glands were incubated with the steroid ecdysone. The isolated nuclei retain upon isolation their structural integrity with regard to chromosome morphology and they are capable of RNA, DNA, and protein synthesis *in vitro* (Berendes and Boyd, 1969; Helmsing, 1970, 1971; Boyd, personal communication). Moreover, incubation of these nuclei with ecdysone results in a significant increase in the incorporation of precursors into RNA. Polyacrylamide gel electrophoresis of the RNA extracted from hormone-treated and control nuclei revealed a particularly strong increase in low molecular weight species in the RNA extracted from hormone-treated nuclei. This effect could result from an increase in template availability due to a specific derepression of particular genome sites. It cannot be excluded, however, that the permeability of the nuclear membrane for the nucleotide triphosphates has been increased by the hormone treatment. However, if this were the only cause of the increased incorporation of the labeled precursors, the increase should be evenly distributed over all RNA fractions rather than occurring predominantly in any one single class (see Fig. 1). Another mechanism would have to be invoked for the observed results. For example, the postulate that the hormone facilitates ribonuclease activity would provide a formal explanation of the altered size distribution but would demand an as yet unnecessarily complex view on the mode of action of the steroid.

With regard to the mechanism of steroid hormone action at the genome level, conflicting results have been reported. Dahmus and Bonner (1965), working on rat liver chromatin found no significant increase in template activity following cortisol treatment (cf. Drews and Bondy, 1966). In contrast to these results, Stackhouse and co-workers (1968) reported a considerable enhancement of the priming efficiency of rat liver chromatin by cortisol. A similar effect of this steroid was found with isolated liver nuclei incubated with the hormone (Dukes and Sekeris, 1965; Sekeris *et al.*, 1969).

Also isolated nuclei of *Drosophila hydei* respond to incubation with the steroid ecdysone with an increase in incorporation of RNA precursors. The uptake of radioactive RNA precursors was enhanced by a factor of 1.4 on the average. Despite this increase in incorporation, RNA-DNA hybridization studies did not support the suggestion that by the action of the steroid new sequences became available for transcription. The obvious similarity of the reciprocal plots of the quantity of DNA hybridization as a function of the amount of RNA extracted from nontreated and hormone-treated nuclei and the absence of a clear difference between RNA extracted from the two kinds of nuclei in competition hybridization experiments, contrast with the hypothesis that the hormone increases template availability in terms of new sequences. The present data do not permit, however, a conclusion with regard to differences in unique RNA sequences between hormone-treated and control nuclei. It neither can be excluded that small quantities of slowly annealing sequences which could be present in the low molecular weight fraction remained undetected by the experimental approach applied. On the other hand, it seems unlikely, that an increase in template availability if induced by the *in vitro* hormone application would result predominantly in the transcription of slowly annealing sequences. The more so, because cytological observations on hormone-treated nuclei failed to demonstrate any evidence for the induction of hormone-specific puffs or detectable RNA synthesis at hormone-specific chromosome loci.

The action of ecdysone in our system appears to be quite different from its

effect on isolated fat body nuclei of *Calliphora* (Congote *et al*, 1970) Whereas in the salivary gland nuclei of *Drosophila hydei* the incorporation of RNA precursors is enhanced and the size distribution of the product is changed by the steroid, neither of these effects was found in the nuclei of *Calliphora* There is also a discrepancy in the result of the hybridization studies.

The simplest interpretation of the results presented here is that the hormone facilitates the synthesis of RNA at sites already active in RNA synthesis, whereas only few, if any, sites become derepressed and become transcribed as a consequence of the action of the hormone The question whether the observed effects are due to a direct action of the steroid on RNA polymerase activities should be further investigated

The apparent difference between the effects of the hormone on isolated nuclei and on the same nuclei in intact cells may have a trivial explanation The isolation procedure and *in vitro* environment may so alter chromosome composition as to preclude the normal chromosomal response (the appearance of puffs and local incorporation of RNA precursors) observed after administration of the steroid to intact gland cell On the other hand, the specificity of the response of the genome in the intact cell could be attributed to a central role of cytoplasmic elements in the control of nuclear activity

The author is indebted to Dr H D Berendes for his continuous support and interest in this investigation, to Dr P J Helmsing for suggestions and to Mr van Woerkom for his help with some of the experiments

REFERENCES

ASHBURNER, M (1971) Induction of Puffs in Polytene chromosomes of in vitro cultured salivary glands of Drosophila melanogaster by ecdysone and ecdysone analogues *Nature, New Biology* **230**, 222–223

BEKHOR, I, KUNG G M and BONNER, J (1969) Sequence specific interaction of DNA and chromosomal protein *J Mol Biol* **39**, 351 364

BERENDES H D (1967) The hormone ecdysone as effector of specific changes in the pattern of gene activities of *Drosophila hydei* *Chromosome* **22**, 274–293

BERENDES, H D, and BOYD, J B (1969) Structural and functional properties of polytene nuclei isolated from salivary glands of *Drosophila hydei* *J Cell Biol* **41**, 591 599

BISHOP D H L, CLAYBROOK, J R, and SPIEGELMAN, S (1967) Electrophoretic separation of viral nucleic acids on polyacrilamide gels *J Mol Biol* **26**, 373 387

BRITTEN R J and KOHNE D E (1968) Repeated sequences in DNA *Science* *161*, 529–540

CHURCH, R B and MCCARTHY, B J (1970) Unstable nuclear RNA synthesis following estrogen stimulation *Biochim Biophys Acta* **199**, 103–114

CLEVER, U (1963) Genaktivitaten in den Riesen chromosomen von *Chironomus tentans* und ihre Beziehungen zur Entwicklung *Chromosoma* **14**, 651–675

CLEVER U and KARLSON, P (1960) Induktion von Puffänderungen in den Speicheldrusen Chromosomen von *Chironomus tentans* durch Ecdyson *Exp Cell Res* **20**, 623–627

CONGOTE L F, SEKERIS C E, and KARLSON, P (1970) Alterations of the nature of RNA synthesized in isolated fat body cell nuclei as a result of ecdysone and juvenile hormone action *Z Naturforsch* 279–284

COOPER, H L, and KAY J E (1968) Differential extraction of nuclear and cytoplasmic RNA from intact lymphocytes *Biochim Biophys Acta* **174**, 503 512

DAHMUS, M E, and BONNER, J (1965) Increased template activity of liver chromatin a result of hydrocortisone administration *Proc Nat Acad Sci U S A* **54**, 1370–1374

DREWS, J, and BONDY P K (1966) Effect of cortisol on nuclear RNA synthesis in vitro *Proc Soc Exp Biol Med* **122**, 847 850

DUKES P P and SEKERIS, C E (1965) Stimulierung des Einbaus von (2 ^{14}C) Uracil in Ribonukleinsaure von Rattenleberkernen durch Cortisol in vitro *Hoppe Seylers Z Physiol Chem* **341**, 149–151

GEORGIEV, G P, SAMARINA, O P, LERMAN, M I SMIRNOV M N, and SEVERTOV, A N (1963) Biosynthesis of messenger and ribosomal ribonucleic acids in the nucleolochromosomal apparatus of animal cells *Nature* (*London*) **200**, 1291–1294

GILES, K W, and MYERS, A (1965) An improved diphenylamine method for the estimation of deoxyribonucleic acid *Nature* (*London*) **206**, 93

HELMSING, P J (1970) Protein synthesis of polytene nuclei in vitro *Biochim Biophys Acta* **224**, 579-587

HELMSING, P J (1971) Isolation and partial characterization of the labeled product from polytene nuclei incubated in vitro with amino acids *Biochim Bophys Acta* **232**, 733-735

HENNIG W (1968) Ribonucleic acid synthesis of the Y chromosome of *Drosophila hydei* *J Mol Biol* **38**, 227 239

JEANTEUR, P, and ATTARDI, G (1969) Relationship between HeLa cell ribosomal RNA and its precursors studied by high resolution RNA DNA hybridization *J Mol Biol* **45**, 305-324

KARLSON P (1965) Biochemical studies of ecdysone control of chromosomal activity *J Cell Comp Physiol* **66**, 69 75

MCCARTHY, B J, and CHURCH, R B (1970) The specificity of molecular hybridization reactions *Annu Rev Biochem* **39**, 131-150

MEISLER, A I, and TROPP, E B (1969) Studies on ribonucleic acid synthesis in nuclei isolated from rat liver *Biochim Biophys Acta* **174**, 476-490

MELLI, M, and BISHOP, J O (1969) Hybridization between rat liver DNA and complementary RNA *J Mol Biol* **40**, 117-136

PELLING, C (1964) Ribonukleinsaure Synthese der Riesenchromosomen Autoradiographische Untersuchungen an *Chironomus tentans* *Chromosoma* **15**, 71-122

PIKER, E G, LICHTENSTEIN, A V, and SHAPOT, V S (1969) Concerning the interrelationship of nuclear and cytoplasmic RNA s from aminals cells as revealed by competition hybridization experiments *Biochim Biophys Acta* **174**, 758 760

SEKERIS, C E, HOMOKI, J, BEATO, M, GALLWITZ, D, SEIFART, K, and LUKACS, I (1969) 'In vitro action of cortisol on the nucleus of the liver cell *Advan Biosci* **2**, 222 240

SHEARER, R J, and MCCARTHY, B J (1967) Evidence for ribonucleic acid molecules restricted to the cell nucleus *Biochemistry* **6**, 283-288

STACKHOUSE, H L, CHETSANGA, C J, and TAN, C H (1968) The effect of cortisol on genetic transcription in rat liver chromatin *Biochim Biophys Acta* **155**, 159-168

Cell Differentation 2, 107–118 (1973) © North-Holland Publishing Company

RNA SYNTHESIS IN POLYTENE SALIVARY GLANDS OF *DROSOPHILA HYDEI* MAINTAINED UNDER IN VITRO CONDITIONS*

C. ALONSO and J.A. GROOTEGOED

Department of Genetics, University of Nijmegen
Nijmegen, The Netherlands

Accepted 2 March 1973

Autoradiographical analysis of uridine incorporation into in vitro maintained early-third instar salivary glands revealed over a period of up to 72 hr of incubation, a pattern of grain distribution over the nuclear components which is essentially the same as after in vivo incorporation

During a 72-hr period of incubation, involving changes of the medium every 24 hr, ^{3}H-uridine incorporation increases linearly at a rate of 0 076 pmoles UMP/hr/μg DNA At all time intervals studied the electrophoretic profiles of newly synthesized RNA displayed some high molecular weight fraction(s), 38S, 28S, 18S and 4 7S RNA

In situ hybridization experiments in which pooled 28S and 18S fractions were applied to salivary gland squashes revealed hybridization with DNA within the nucleolus as well as with the DNA fiber connecting the nucleolus with the euchromatic arm of the X-chromosome Hybridization experiments performed with total in vitro synthesized RNA revealed, in addition to labeling of the nucleolar DNA, labeling of the chromosomes

The aim of the present study was to analyse the RNA metabolism in larval salivary glands of *Drosophila hydei* maintained over various periods, up to 72 hr, under in vitro conditions (see Poels, 1972). Because it has been reported previously that *Drosophila* polytene salivary glands fail to process the 38S ribosomal precursor molecule under in vitro conditions (Greenberg, 1969), special attention was paid to the metabolism of this ribosomal precursor.

* Abbreviations rRNA, ribosomal RNA, SSC, 0 15 M NaCl, 0 015 M Na-citrate, EDTA, ethylene diamine tetra acetate, Tris, tris-(hydroxy methyl)-amino methane, SDS, sodium dodecyl sulphate, TCA, trichloroacetic acid, ETOH, 100% ethanol, MAK, methylated albumin Kieselguhr

In addition to an autoradiographical study of the chromosomal incorporation of tritiated uridine under in vitro conditions and a comparison of the incorporation pattern with that obtained after in vivo administration of the radioisotope, the migration pattern of the newly in vitro synthesized RNA in polyacrylamide gels was determined.

In vitro produced 18 and 28S RNA fractions were used in in situ hybridization experiments in order to test whether or not these fractions represent transcripts of the ribosomal cistrons which are, in *Drosophila*, clustered within the nucleolus organizer region (Ritossa et al., 1965; Pardue et al., 1970; Tartof et al., 1970; Hennig et al., 1971).

MATERIALS AND METHODS

In vitro maintenance of the salivary glands

Throughout this study early-third instar glands (100–120 hr after oviposition) of a laboratory stock of *Drosophila hydei* were used. All incubations were performed in the medium described by Poels (1972) supplied with 80 μg/ml penicilin G (Sigma) and 80 μg/ml streptomycin sulfate (Sigma). Unless otherwise indicated, the medium was replaced every 24 hr by fresh medium to avoid possible bacterial contamination.

Autoradiography

Autoradiographs were prepared from glands pulse labeled for 15 min in 10 μl of medium containing 1 μCi ^{3}H-uridine (spec. act. 27 Ci/mM) as described previously (Alonso, 1972). The pulse labeling was performed with glands following their preincubation in medium without tritiated uridine for various periods.

Extraction of RNA and gel electrophoresis

(1) Preparation of cold 28S and 18S RNA.

Cold 28S and 18S RNA was prepared from larval gut and Malpighian tubulus obtained as a byproduct from the mass isolation of larval salivary glands (Boyd et al., 1968). Portions of 5 g of these tissues were frozen and than thawed by the addition of a boiling solution of 10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$. $6H_2O$ and 1% SDS (pH 7.2) containing 300 μg/ml self digested pronase. This procedure breaks the cells and after cooling and standing for 30 min at room temp. cellular debris was pelleted by 15 min centrifugation at 12,000 rpm. Diethylpyrocarbonate (40 μl/ml) was mixed with the supernatant and after standing for 5 min at 37°C, 0.5 vol of a saturated NaCl solution was added and the solution placed in melting ice. Subsequently, the solution was centrifuged twice (15 min at 12,000 rpm) and the RNA was precipitated from the supernatant by addition of 2.5 vol ETOH. In order to remove traces of SDS from the RNA preparation, the

pellet was dissolved in 1 X SSC, stored for 20 min at 4°C and centrifuged for 10 min at 10,000 rpm.

Spectrophotometric analysis of the RNA in the supernatant revealed a ratio 260/280 of 1.85 and a ratio 260/230 of >2.3. The RNA was then incubated with electrophoretically purified DNAse I (20 μg/ml, Schwartz–Mann) for 20 min at 37°C. Following a subsequent phenol extraction (cold) (Kirby, 1965) and precipitation, the RNA, again dissolved in 1 X SSC, was heated to 90°C for 4 min and filtered through a nitrocellulose filter (BA 85, 0.45 μ). The resulting solution was made up to 12% NaCl and stored overnight at 0°C. 28S and 18S fractions were obtained by MAK column chromatography of the precipitate using an elution gradient from 0.5–1 2 M NaCl (Mandell et al., 1960). The fractions were dialyzed against 6 X SSC and stored at –70°C until use.

(2) Radioactive RNA from whole larvae.

40–50 dechorionated eggs were placed on low yeast artificial medium (Carolina Biol. Supply Cy) containing 1 mCi/ml ^{3}H-uridine (spec. act 30 Ci/nM). After 6–7 days following hatching, the larvae were collected, frozen and homogenized in Tris–HCl–SDS buffer as described RNA was extracted by using a buffer-saturated phenol solution according to the method described previously (Alonso, 1972). The specific activity of the RNA extracted was $2{,}5.10^5$ cpm/μg.

(3) Radioactive RNA from salivary glands incubated in vitro

Pulse labeled glands (see Autoradiography) (90–100 per extraction) were kept for 20 min in incubation medium deprived of the labeled nucleoside before they were homogenized and extracted three times with 2 ml of a mixture of Tris–HCl–SDS buffer and buffer-saturated phenol (1 : 1) at 60°C. 90 μg of purified *Escherichia coli* ribosomal RNA were added per 1 ml of water phase before precipitation of the RNA with 2 vol ETOH (at –28°C overnight). The precipitate was air dried, then dissolved in 10–20 μl of 0.04 M Tris HCl, 0.02 M Na-acetate, 0.001 M EDTA (pH 7.2) (E-buffer, Bishop et al., 1967).

RNA used for in situ hybridization was obtained from 90–100 gland pairs incubated for 3 days in Poels medium containing 1 μCi/μl ^{3}H-uridine (spec act. 27 Ci/mM). Every 24 hr the medium was changed. Following 3 days of incubation the glands were washed in isotope-free medium, transferred to sterile glass tubes, and homogenized in 1 ml Tris–HCl–SDS buffer containing 40 μl saturated diethylpyrocarbonate. Subsequently a cold phenol extraction was performed twice 150 μg of purified *E.coli* ribosomal RNA was added to the resulting waterphase before precipitating the RNA with 2 vol ETOH (–28°C, overnight).

(4) Gel electrophoresis

Analysis of the in vitro synthesized RNA was performed by electrophoresis for 40 min at room temp. on 2.5% acrylamide gels, which were prerun for 1 hr at 60*v*,

4 mA per gel. The gels were frozen on dry ice and sliced in 1 mm slices and counted in tolenue 2-(2-ethoxy-ethoxy) ethanol (6 : 4) (Weinberg et al., 1968). All S-values were calculated according to the method of Lewicki et al. (1970).

For in situ hybridization, 28S and 18S RNA were separated from the total newly synthesized RNA by gel electrophoresis. Regions containing 28S and 18S RNA were cut out after freezing the gels, crushed and the RNA eluted with E-buffer containing 5.10^{-3} M EDTA by continuous shaking in scintillation vials at 4°C for two subsequent periods of 12 hr. The eluted RNA was passed through a nitrocellulose filter and precipitated with 2 vol ETOH. The specific activity of the pooled 28S + 18S RNA fractions was 120,000 cpm/μg.

In situ hybridization

In situ hybridization was performed essentially according to the method of Pardue et al. (1970), using as a denaturation procedure exposure of the slides to 0.1 X SSC containing 90% formamide at 65°C for 2,5 hr (Steffenson et al., 1971).

On each slide 50–100 μl of 6 X SSC containing 1.5 μg of radioactive RNA was placed over the squashed material and the slides were incubated for 15–18 hr at 67°C. Exposure time was 4–6 months.

RESULT

The effect of in vitro maintenance upon chromosome morphology and chromosomal uridine incorporation

In all instances, prevention of mechanical damage of gland cells during dissection and transfer to the medium is essential in order to avoid rapid degeneration of the glands after the onset of in vitro incubation.

A comparison of the morphology of the glands and their nuclear contents after various periods of in vitro incubation revealed that early-third instar glands retained a virtually normal morphology up to 3 days of incubation. Glands dissected from mid- and late-third instar larvae retained a normal morphology for only 15–20 hr of incubation. Thereafter, the glands displayed clear signs of degeneration. The chromosomes either showed partial or complete disintegration or clumped together in the center of the nucleus.

Early third instar glands given a 15-min pulse of tritiated uridine display, even after 3 days of incubation, an incorporation pattern over the chromosomes which is essentially similar to that observed after an in vivo pulse (fig. 1). Older glands not only show a drastically reduced level of incorporation, but also a non-specific distribution of grains over the chromosomes.

As a result of these observations all experiments to be described have been carried out with glands dissected from early-third instar larvae.

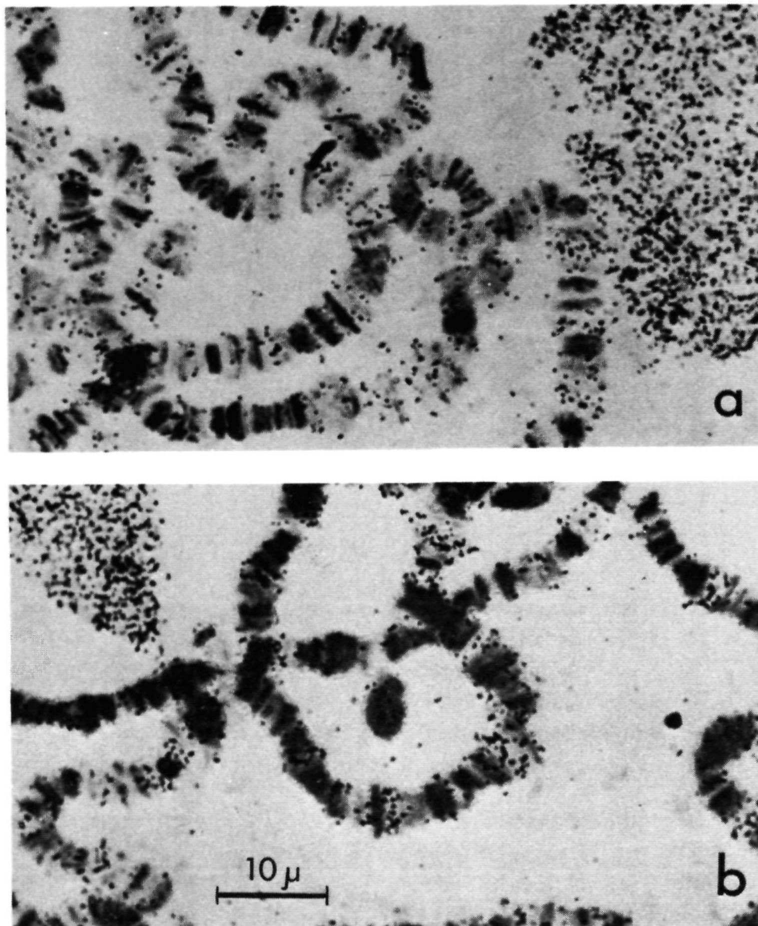

Fig. 1. Autoradiographs of polytene chromosomes from: (a) third instar salivary gland cells after a 15 min ^{3}H-uridine pulse in vivo (1 µCi/larva); (b) early-third instar gland cells after a 15 min ^{3}H-uridine pulse in vitro (0.1 µCi/µl medium) at the end of a 72-hr period of in vitro maintenance.

Kinetics of ^{3}H-uridine incorporation in vitro

The kinetics of ^{3}H-uridine incorporation into early third instar salivary glands was determined by scintillation counting of TCA precipitated material from 6 pairs of glands per experiment incubated for various periods. For comparison of the radioactivity in the different experiments, protein quantity as determined according to the method of Lowry et al. (1951), was used as a reference.

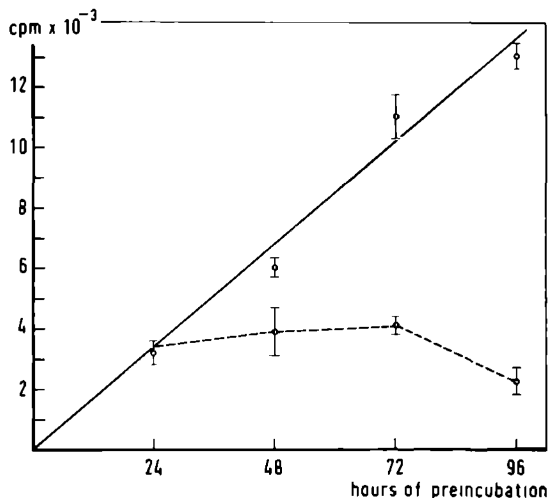

Fig 2 Kinetics of ^{3}H-uridine incorporation into early-third instar salivary gland cells The incorporation was measured by scintillation counting of TCA precipitated material of 6 gland pairs at each point of the graph Solid line Incorporation at subsequent times after onset of incubation in medium which is replaced by fresh radioactive medium every 24 hr Dotted line Similar experiment in which glands were kept in the medium without change up to 96 hr cpm/30 μg protein

Fig 2 shows that the amount of radioactivity in glands transferred every 24 hr to fresh medium containing 1 μCi ^{3}H-uridine/20 μl, increases linearly up to 96 hr of incubation at a rate of approx 135 cpm/hr Glands incubated continously in the same medium (no transfer to fresh medium) displayed a gradual reduction in the rate of incorporation from 24–72 hr as compared with the initial rate during the first 24 hr of in vitro maintenance At 96 hr the extract of the glands contained significantly less radioactivity than at 72 hr, indicating a loss of previously incorporated radioisotope Examination of the glands after 96 hr of in vitro incubation in the same medium revealed an abnormal morphology The regular banding pattern of the polytene chromosomes became indistinct Moreover, following pulse labeling of glands incubated for 96 hr without changing the medium only very low levels of incorporation into the chromosomes were found autoradiographically.

Electrophoretic profiles of newly in vitro synthesized RNA in polyacrylamide gels

As indicated previously, the sucrose gradient profile of the RNA synthesized during 12 hr of in vitro incubation, is similar to that synthesized under in vivo conditions (Poels, 1972) Electrophoretic analysis of RNA extracted from glands incubated for 72 hr revealed the presence of 4–7S, 18S, 28S, 38S and some higher molecular weight species (fig. 3). In all profiles a fraction of 13S was present (see

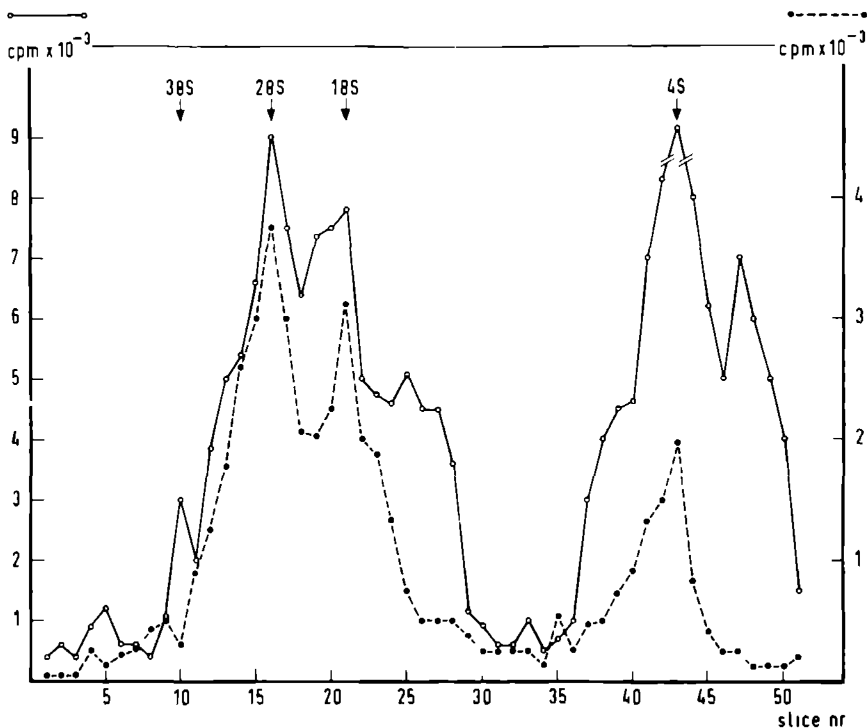

Fig 3 Electrophoretic profile of labeled RNA extracted from larvae kept for three days on food containing 250 μCi/ml ^{3}H-uridine (•——•) as compared with that of RNA synthesized in vitro in salivary glands during a 72-hr incubation period The radioactive incubation medium was replaced every 24 hr (○——○)

also fig. 4). It may be pointed out that the relative amount 4–7S RNA obtained from the incubated glands is significantly higher than that in RNA extracted from larvae which were exposed for three days to food containing 250 μCi/ml ^{3}H-uridine.

Although labeled 38S as well as 28S and 18S fractions were present after 72 hr of incubation, it remained to be established whether or not the 38S RNA is processed continuously during the entire 72-hr period. In order to investigate this question, RNA was extracted from glands which were labeled for 2 hr at the end of a period of either 12, 24, or 72 hr of preincubation in medium without the radioisotope. In all instances (see fig. 4), 38S, 28S and 4–7S peaks were observed. In some of the extracts, the 18S fraction seemed to be slightly deficient as compared with the profile of RNA synthesized under in vivo conditions. Moreover, with increasing incubation time the ratio 38S to 28S decreases. In all preparations the

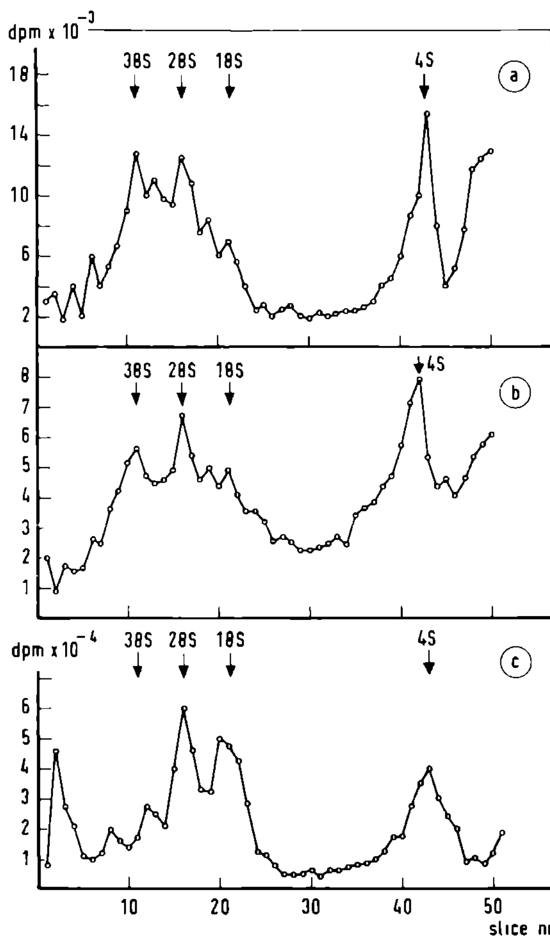

Fig. 4. Electrophoretic profiles of labeled RNA extracted from glands pulse labeled for 2 hr at the end of an incubation period in medium without ^{3}H-uridine for 12 hr (a); 24 hr (b) and 72 hr (c).

4–7S fraction was significantly increased as compared to that observed in extracts of in vivo synthesized RNA. Similar small RNA species were obtained from nuclei incubated in vitro with ^{3}H-UTP (Congote et al., 1970; Alonso, 1972).

In situ hybridization of in vitro synthesized RNA

In order to obtain evidence for a nucleolar origin of the 28S and 18S RNA fractions observed in the electrophoretic profiles, in situ hybridization was carried out with pooled 28S and 18S RNA isolated from glands after a 72-hr incubation period. Furthermore, total RNA obtained from glands incubated for 72 hr was used

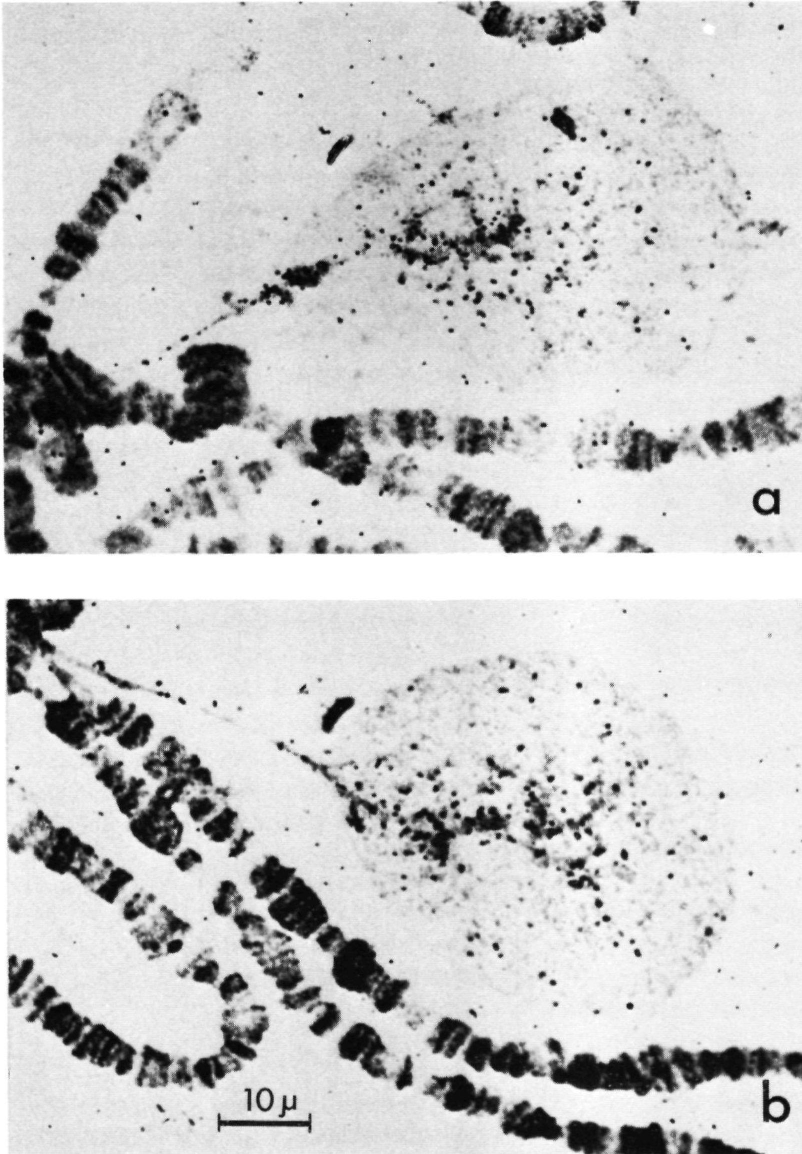

Fig. 5. Autoradiographs of salivary gland chromosomes and nucleoli following the hybridization of 28S and 18S (^{3}H)-RNA. (a) Hybridization was performed with 28S + 18S RNA extracted from whole larvae kept for 6 days on radioactive (^{3}H-uridine) food medium; (b) Hybridization of 28S + 18S fractions extracted from salivary glands incubated for 72 hr in ^{3}H-uridine containing medium (medium refreshed every 24 hr).

for in situ hybridization in order to determine the origin of RNA species other than those hybridizing with the nucleolus organizer region.

The in situ hybridization of pooled 28S and 18S RNA revealed that this RNA hybridized predominantly with DNA in the nucleolus (fig. 5b). The distribution of grains over the nucleolus indicated a specificity for DNA containing regions rather than a random location. Furthermore, grains were *always* observed over the DNA containing fiber connecting the nucleolus with, in most instances, the base of the banded X-chromosome. The same result was obtained with labeled 28S and 18S RNA extracted from whole larvae kept for 6 days on medium containing 1 mCi/ml ^{3}H-uridine (fig. 5a). In both instances, only few grains were present over the chromosomes. A further test was made by performing a competition experiment using a mixture of 200 μg cold 28S and 18S in vivo synthesized RNA and 1.5 μg labeled 28S and 18S in vitro synthesized RNA (spec. act. 120,000 cpm/μg) for in situ hybridization. No significant labeling of the nucleolar DNA was observed under these conditions. In order to test whether the low molecular weight RNA (4–7S) might be a degradation product of rRNA, these fractions were also submitted to in situ hybridization. These RNA fractions hybridized with chromosomal but not with nucleolar DNA.

In situ hybridization with total RNA revealed, in addition to a heavy labeling over the nucleolus, labeling over the entire chromosome set, suggesting the presence of RNA transcripts of various chromosome regions in the extract. As such, this result agrees with the data obtained from the autoradiographical analysis of glands pulse labeled at the end of a 72-hr period of in vitro incubation, which also indicated transcriptional activity of a variety of chromosome regions (fig. 1b).

DISCUSSION

Following the development of an artifical medium for long term in vitro maintenance of polytene tissues of *Drosophila hydei* (Poels, 1972), it seemed essential to investigate as one of the parameters indicative for the metabolic state of the cells under in vitro conditions, the synthesis and processing of RNA.

In this study the synthesis and processing of rRNA was chosen as a primary object of experimentation. It has been reported that, although the 38S precursor molecule is synthesized in salivary glands of *Drosophila* under in vitro conditions using Schneider's medium, processing of this precursor is impaired (Greenberg, 1968). On the other hand, it was found that in *Chironomus* salivary gland cells, kept in vitro, 38S RNA is synthesized, converted into 30S and 23S RNA, and subsequently into 28S and 18S final rRNA subunits (Ringborg et al., 1970).

Electrophoretic analysis of RNA synthesized in *D. hydei* salivary gland cells revealed that in addition to other RNA species, labeled 38S, 28S and 18S RNA were present after 2 hr of incubation in the presence of radioactive uridine at any

time within a 72-hr incubation period Although no distinct 30S and 23S fractions could be detected with certainty, the mere fact that 28S and 18S RNA was found, indicated processing of the newly synthesized 38S RNA By using combined 28S and 18S RNA fractions for in situ hybridization it was shown that these RNA fractions hybridized almost exclusively with DNA present within the nucleolus This result favors the idea that the in vitro labeled 28S and 18S RNA represent rDNA transcripts

Previous studies on the location of rRNA cistrons by in situ hybridization have confirmed and extended genetical and cytogenetical information indicating their location within the nucleolus organizer region(s) (Pardue et al , 1970, Gerbi, 1971) In *D hydei*, the nucleolus organizer seems to be, at least to a large extent, included within the body of the nucleolus It, thus, could be suggested that the DNA present in the body of the nucleolus of this species (Barr et al , 1966, Olvera, 1969) consists in part, or completely, of rRNA cistrons This suggestion finds support from the results of in situ hybridization in which RNA complementary to rDNA from *Xenopus* was used for hybridization with *D hydei* salivary gland cells Only the DNA located *within* the nucleolus was found to hybridize in these experiments (Pardue et al , 1970) However, in contrast to the results of Pardue and coworkers in situ hybridization performed with in vitro transcribed endogenous 28S and 18S RNA revealed that hybridization was not restricted to the DNA within the nucleolus Grains were consistently present over the Feulgen-positive fibril connecting the nucleolus, in most instances, with the base of the euchromatic arm of the X-chromosome

The results of in situ hybridization of total in vitro synthesized RNA indicate that a variety of chromosome regions is transcribed during the in vitro maintenance of the glands As such these results agree with the autoradiographical data on ^{3}H-uridine incorporation However, whereas ^{3}H uridine incorporation following pulse labeling of glands kept for 72 hr in vitro occurs to a large extent within chromosome regions displaying a puffed appearance (fig 1b), the RNA extracted from glands incubated for 72 hr with ^{3}H-uridine hybridizes to a large extent with DNA in the compact band regions (with the exception of the 18 and 28S fractions) The latter result indicates the presence of transcripts from reiterated DNA sequences among the RNA's transcribed during a 72-hr incubation period, the transcription of which is not detected by pulse labeling with uridine It could be supposed that these sequences are transcribed at a much lower rate than sequences in the puffed regions and that the transcripts are accumulated during the incubation period This assumption finds support from the observation that the chromosomes following in vitro labeling with ^{3}H uridine for one hour or more display, in addition to labeling over the puffs, labeling over the bands

The failure to detect in situ hybridization in puffed regions could result from the presence of only one or a very few identical sequences in these regions which would not be detected by the in situ hybridization procedure applied

ACKNOWLEDGEMENTS

The authors are indebted to Prof H D Berendes and Dr P J Helmsing for their suggestions and help in the course of this investigation

REFERENCES

Alonso, C Developm Biol 28, 372–381 (1972)
Barr, H J and W Plaut J Cell Biol 31, C17, 22 (1966)
Bishop, D H L, J R Claybrook and S Spiegelman J Mol Biol 26, 373 387 (1967)
Congote, L F, C E Sekeris and P Karlson Z Naturforsch 279–284 (1970)
Boyd, G B, H D Berendes and H Boyd J Cell Biol 38, 369–376 (1968)
Gerbi, S A J Mol Biol 58, 499–511 (1971)
Greenberg, J R J Mol Biol 46, 85–98 (1969)
Hennig, W and B Meer Nature 233, 70–72 (1971)
Kirby, K Biochem J 96, 266 269 (1965)
Lewicki, P P and A J Sinskey Anal Biochem 33, 273 278 (1970)
Lowry, O H, N J Rosenbrough, A I Farr and J Randall J Biol Chem 193, 265 275 (1951)
Mandell, J D and A D Hershey Anal Biochem 1, 66–77 (1960)
Olvera, R O Genetics 61 (suppl), 245–249 (1969)
Pardue, M L, S A Gerbi, R A Eckhardt and J G Gall Chromosoma 29, 268–290 (1970)
Poels, C L M Cell Diff 1, 63 78 (1972)
Ringborg, U, B Daneholt, J E Edstrom, E Fgyházi and B Lambert J Mol Biol 51, 327 248 (1970)
Ritossa F M and S Spiegelman Proc Natl Acad Sci (Wash) 53, 737 745 (1965)
Steffensen, D M and D W Wimber Genetics 69, 163–178 (1971)
Tartof, K D and R P Perry J Mol Biol 51, 171–183 (1970)
Weiberg, R A and S Penman J Mol Biol 38, 289–304 (1968)

IMPROVED CONDITIONS FOR IN SITU RNA/DNA HYBRIDIZATION

C. ALONSO
Department of Genetics, University of Nijmegen, The Netherlands

Received 4 January 1973

1. Introduction

The aim of the present investigation was the development of a method for *in situ* RNA/DNA hybridization in which the hybridization reaction can be carried out at a low temperature over long periods with small volumes of RNA solutions. Fulfillment of this aim would facilitate the localization of sequences which are not highly repetitive and of sequences with melting points well below that of the major portion of the nuclear DNA of eukaryotes.

Thus far, the hybridization reaction in *in situ* hybridization experiments have been performed at an incubation temperature of 65° with labeled RNA dissolved in 2 × SSC [1] or 6 × SSC [2]. However, it was indicated that under these conditions reassociation of the chromosomal DNA may occur within a short period of time [3–5]. This process, favoured by the proximity of complementary DNA strands in the chromosomes, should reduce the efficiency of the RNA/DNA hybrid formation considerably and in effect bias the hybrid formation in the direction of most highly repetitive sequences.

The present report describes a method for *in situ* hybridization in the presence of formamide at a temperature of 20°. Formamide was used during hybridization previously by Jones and Robertson [6]. However, they report that under their conditions it was less satisfactory than 65° in 2 × SSC.

2. Materials and methods

2.1. *RNA used for in situ hybridization*

Complementary RNA (cRNA) was prepared by *in vitro* transcription of 8 μg nuclear DNA obtained from mid-third instar salivary gland nuclei of *Drosophila hydei*. The DNA was transcribed with 5 units of *E. coli* RNA-polymerase (Sigma) in the presence of 50 μCi of each of the four tritiated nucleoside triphosphates [7]. The RNA was extracted from the transcription medium according to Perry et al. [8] and had a specific activity of 7×10^7 dpm/μg.

In vivo synthesized RNA was obtained from *Drosophila hydei* larvae grown for 7 days on a medium containing 1 mCi/ml [^{3}H]uridine and was extracted as described previously [9]. The RNA had a specific activity of 2×10^6 dpm/μg.

From the *in vivo* synthesized RNA, 18 S and 28 S RNA fractions were separated by acrylamide gel electrophoresis [10]. The pooled 18 S + 28 S fractions had a specific activity of 2.1×10^6 dpm/μg.

The purified RNA was dissolved in 1 × SSC and, by the addition of SSC and/or formamide, made to a final concentration of 6 SSC or 50% formamide and 2 × SSC before applying it to the cytological preparations.

2.2. *Cytological preparations used for in situ hybridization*

Mid-third instar larval salivary glands of *Drosophila hydei* were dissected, fixed for 10 min in ethanol–acetic acid (3:1 v/v), squashed in 45% acetic acid and, after removal of the coverslip, postfixed for 15 min in the previously used fixative, then transferred to 70% and subsequently to 100% ethanol. After rinsing in 2 × SSC the preparations were incubated for 60 min with pancreatic RNAase (100 μg/ml) in 2 × SSC, rapidly chilled and extensively rinsed with 2 × SSC [11, 12].

Denaturation was performed by a 2.5 hr incubation of the preparations in 90% formamide in

Table 1
Radioactivity in [^{3}H]cRNA solutions recovered after *in situ* hybridization to slides which were previously submitted to sham hybridization for various periods

A) Sham hybridization at 65° in 6 × SSC followed by hybridization with [^{3}H]cRNA (152,928 cpm/slide) in 50% formamide 2 × SSC at 20° for 60 min

Period of sham hybridization (min)	Radioactivity in cpm recovered per slide*	Radioactivity in cpm retained per slide*
none	125 428	27,500
10	138 030	14 898
60	147 426	5,502
120	148 485	4,443
180	147 021	5 907

B) Sham hybridization at 20° in 50% formamide, 2 × SSC followed by hybridization as in A

Period of sham hybridization (min)	Radioactivity in cpm recovered per slide*	Radioactivity in cpm retained per slide*
none	124 628	28,300
10	127 190	25,738
60	129 457	23 471
120	129 828	23,100
180	131 227	21 701

* Average of two slides per series

0 1 × SSC at 65° [13] followed by immediate transfer into ice cold 0 1 × SSC subsequently to ice cold 70% and 100% ethanol and finally drying under vacuum at low temperature

The use of a coverslip to cover the RNA solution after its application to the cytological preparation was avoided in experiments carried out at a low temperature in order to enable an almost quantitative recovery of the volume applied The incubation of the preparation with the RNA solution was performed in a sealed plastic petri dish containing filter paper wetted with the solvent of the RNA applied

Following the incubation the solution was removed, pooled with 4 washes of 50 µl 2 × SSC and the recovered radioactivity determined by scintillation counting in 15 ml of a mixture of toluene ethoxy-ethoxy ethanol (6 4, v/v) The slides were extensively rinsed and treated with 20 µg/ml RNAase for 180 min at 25°, rinsed again and subsequently coated with Kodak AR 10 stripping film [14] The slides were exposed for 6 days in those experiments in which cRNA was applied and for 2 months in the experiments using RNA synthesized *in vivo*

It should be pointed out that the denaturation procedure, the processing of the slides, and photographic procedures were identical for all slides Moreover, the preparations were made of salivary glands from larvae of essentially the same developmental stage Each preparation contained only one gland which normally contains about 130 polytene nuclei [15], or about 0 06 µg DNA

3 Results and discussion

In order to compare the relative effectiveness of the hybridization reactions performed at 65° or in the presence of formamide at 20° two series of slides, in which each slide contains essentially the same amount of DNA, were denatured and each series was submitted to different hybridization conditions in the *absence* of RNA Following application of these conditions for various periods of time (table 1), the slides were processed until the point that the autoradiographic film should be applied Instead the slides were submitted again to hybridization conditions in which each slide was incubated with 0 022 µg [^{3}H]cRNA (152,928 cpm/slide) in 50% formamide, 2 × SSC for 60 min at 20 ± 1°

In all instances the RNA solution applied was recovered as described and the radioactivity determined In order to establish the background binding of [^{3}H]cRNA slides containing non-denatured chromosomes were submitted to the same hybridization procedure The RNA solution was recovered and the reduction of the radioactivity measured The loss of radioactivity varied from 1000 to 1,300 cpm per slide The effect of the RNAase treatment following hybridization was investigated by counting the radioactivity in the washes following the treatment The radioactivity released from the slides varied from 400 to 500 cpm per slide

Since the total number of counts involved in background binding and "mismatching" is low as compared to the number involved in hybrid formation (table 1) and the variations in the numbers obtained from different slides are small the data presented in table 1 show that during incubation at 65° without

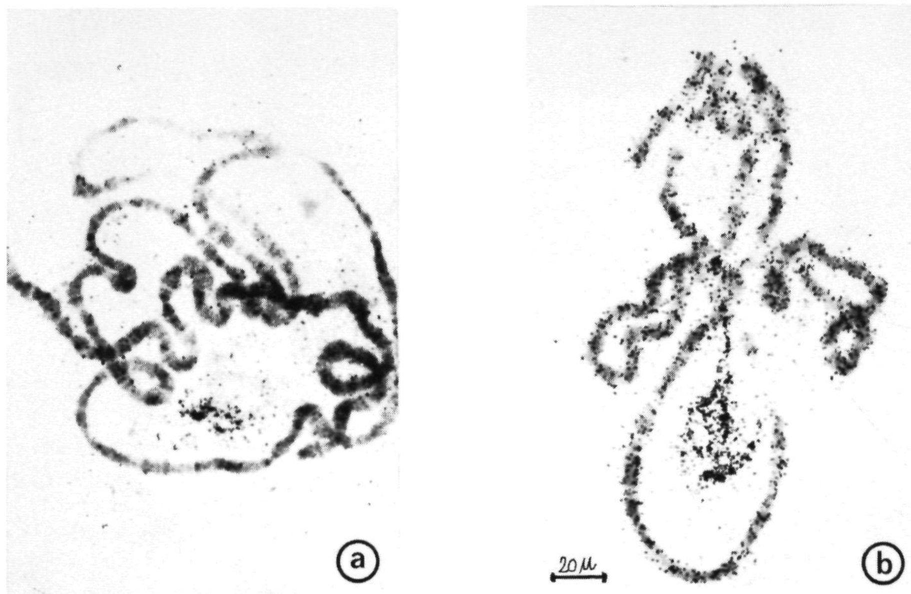

Fig. 1. Autoradiograph of *Drosophila hydei* salivary gland chromosomes after *in situ* hybridization of total RNA synthesized *in vivo* (3×10^6 dpm/slide). a) Hybridization at 65° in 6 × SSC for 12 hr. Exposure time 62 days. b) Hybridization at 20° in 50% formamide, 2 × SSC for 12 hr. Exposure time 62 days.

RNA there is a gradual reduction with time of the availability of DNA sequences which hybridize with cRNA. In slides submitted to sham hybridization conditions using formamide only a very small reduction was observed.

These results are compatible with the idea that during incubation at 65° the DNA gradually reassociates, whereas reassociation seems to be prevented to a large extent in the presence of formamide. The possibility that some DNA is lost during incubation at 65° cannot be excluded.

The higher relative effectiveness of the hybridization reaction carried out at 20° in the presence of formamide was also obvious from the autoradiographical analysis of the binding of [^{3}H]cRNA, total *in vivo* synthesized [^{3}H]RNA and pooled 18 + 28 S [^{3}H]RNA. In all instances, in which the quantity of [^{3}H]RNA applied, the hybridization time and the exposure time of the autoradiographs were identical, the hybridization reaction appeared to have occurred with much greater relative effectiveness in the presence of formamide at 20° than in its absence at 65° (fig. 1).

Hybridization of pooled 18 + 28 S RNA was restricted to the nucleolus organizer located within the nucleolus in the species used (fig. 2) [16].

It may be concluded that the hybridization procedure reported here eliminates a number of disadvantages inherent in the usual procedure.

Acknowledgements

The author is very much indebted to Dr. P.J. Helmsing for providing him with the [^{3}H]cRNA of *Drosophila hydei* DNA and to Drs. H.D. Berendes and G.T. Rudkin for discussions and critical reviewing of the manuscript.

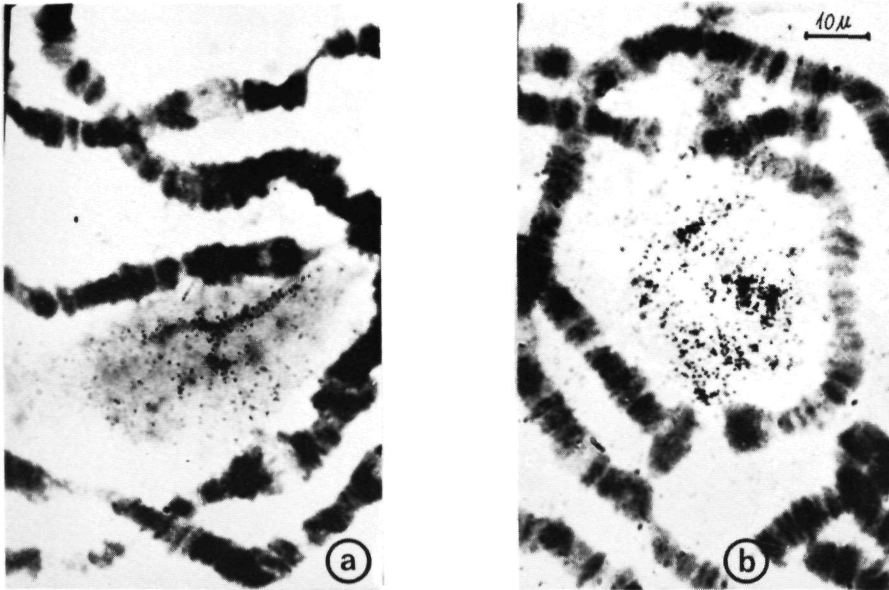

Fig. 2. Autoradiograph of *Drosophila hydei* salivary gland chromosomes after *in situ* hybridization of pooled 18 + 28 S RNA (3.6×10^6 dpm/slide). a) Hybridization at 65° in 6 × SSC for 12 hr. Exposure time 70 days. b) Hybridization at 20° in 50% formamide, 2 × SSC for 12 hr. Exposure time 70 days.

References

[1] A.H. John, M.L. Birnstiel and K.W. Jones, Nature 223 (1969) 582.

[2] M.L. Pardue and J.G. Gall, Science 168 (1970) 1356.

[3] D.E. Wimber and D.M. Steffenson, in: Nuclei acid hybridization in the study of cell differentiation, ed. H. Ursprung (Springer Verlag, 1972) 47.

[4] J.C. Stockert and J.A. Lisanti, Chromosoma 37 (1972) 117.

[5] M. Diaz, Chromosoma 37 (1972) 131.

[6] K.W. Jones and F.W. Robertson, Chromosoma 31 (1970) 331.

[7] P.J. Helmsing and O. van Eupen, Biochim. Biophys. Acta (1973) in press.

[8] R.P. Perry, J. La Torra, D.E. Kelley and J.R. Greenberg, Biochim. Biophys. Acta 262 (1972) 220.

[9] C. Alonso, Developm. Biol. 28 (1972) 372.

[10] C. Alonso, Roux Arch. Dev. Biol. (1973) in press.

[11] J.G. Gall and M.L. Pardue, Proc. Natl. Acad. Sci. U.S. 63 (1969) 378.

[12] W. Hennig, Intl. Rev. Cyt. 34 (1973) in press.

[13] D.E. Wimber and D.M. Steffenson, Science 170 (1970) 639.

[14] B. Lambert, L. Wieslander, B. Daneholt, E. Egyházi and U. Ringborg, J. Cell Biol. 53 (1972) 407.

[15] H.D. Berendes, Chromosoma 17 (1965) 35.

[16] M.L. Pardue, S.A. Gerbi, R.A. Eckhardt and J.G. Gall, Chromosoma 29 (1970) 268.

RNA metabolism during metamorphosis of *Drosophila hydei*

C. Alonso

Dept. of Genetics, University of Nijmegen, The Netherlands

ABSTRACT

Determinations of total protein and RNA quantity at subsequent times after the beginning of the pharate pupal stage of *Drosophila hydei* revealed an increase in both substances during the first 25 hours and a sudden decrease thereafter until 52 hours. From this time on the total quantity of both, protein and RNA increases slightly until emergence of the flies at 160 hours after beginning of the pharate pupal stage. A similar pattern of changes was recorded for the total radioactivity as well as the specific activity of RNA labeled with ^{3}H-uridine after the injection of the isotope immediately before the beginning of the pharate pupal stage.

The migration profile of RNA labeled with ^{3}H-uridine during larval development, revealed shortly after the onset of the pharate pupal stage an essentially normal larval pattern consisting of major fractions of 28S, 18S, 8-9S and 4-7S RNA. At 52 hours only low molecular weight RNA was present. The "normal" pattern was restored at the time of emergence of the flies indicating reutilization of degradation products of previously labeled RNA.

INTRODUCTION

Programmed degradation of the majority of larval tissues and the subsequent, or even simultaneous, morphogenesis of adult tissues during metamorphosis of holometabolous insects involves drastic changes in composition and quantity of a variety of macromolecular components. Histolysis of larval tissues, beginning during the pharate pupal stage is accompanied, if not caused, by changes in the pattern of enzyme activities (Schin and Clever, 1968; Laufer, 1968; Rodems et al., 1969; Boyd and Boyd, 1970; Henrikson and Clever, 1972; Boyd and Logan, 1972). It cannot be excluded that as a consequence of the activity of nucleases, the nucleic acid quantity as well as composition undergoes drastic changes during metamorphosis. With regard to ribonucleic acid, variations in total RNA content have been reported to occur during metamorphosis (Linzen and Wyatt, 1964; Vickers and Mitlin, 1965; Ring, 1973). However, little is known about the actual fate of RNA synthesized in larval tissues during metamorphosis.

The present study was designed to establish changes in total protein and RNA content during metamorphosis of *Drosophila hydei* as well as the fate of RNA synthesized in larval tissues, in particular in regard to the question of possible reutilization of RNA degradation products for the RNA metabolism during morphogenesis of adult tissues.

MATERIALS AND METHODS

Larvae of a laboratory stock of *Drosophila hydei* were raised under standardized conditions (Berendes, 1965). Timing of the developmental stage of pharate pupae and pupae is based on collection of pharate pupae just at the moment of immobilization and extrusion of the spiracles. Under the rearing conditions used, the beginning of the pharate pupal stage occurs at 160 $\pm$ 3 hours after oviposition. All developmental stages are expressed as hours after beginning of the pharate pupal stage (PPS).

Labeling of larval RNA for extraction and analysis during pharate pupal and pupal stages

Immediately after hatching, 100 larvae were placed on a low yeast food medium (Ritossa, 1965) containing 300 uCi/ml ^{3}H-uridine (spec.act. 27 Ci/mM; New England Nuclear). At subsequent times after the onset of the pharate pupal stage, at which time the animals were removed from the food and carefully washed, pharate pupae, pupae or freshly emerged flies were collected and frozen in a dry ice-acetone bath and stored at -70°C until extraction of the RNA.

Extraction of the RNA was performed as described previously (Alonso, 1972). Before electrophoresis on polyacrylamide gels (2.7%), the RNA was dissolved in a buffer (0.04 M Tris, 0.02 M NaAc, 0.001 M EDTA, pH 7.2) containing 0.2% sodium dodecyl sulphate (SDS) and 7% RNase-free sucrose (Schwartz/Mann).

Electrophoresis was performed at room temperature for 40 min at 60 V, 4 mA (see: Alonso, 1972). After freezing on dry ice, the gels were sliced (1 mm slices) and after addition of a toluene-2-(2-ethoxy-ethoxy) ethanol mixture (6:4; $^{v}/_{v}$), the radioactivity was determined with a Philips Scintillation counter.

Incorporation of ^{3}H-uridine during the pharate pupal stage and the fate of the labeled RNA during metamorphosis

Larvae were injected between one and two hours before the onset of the pharate pupal stage with 5 uCi ^{3}H-uridine in 0.5 ± 0.1 ul Drosophila Ringer (Leenders and Berendes, 1972). At 1, 10, 18, 25, 40, 52, 80, 110, 140 and 160 hours after onset of the pharate pupal stage, 4 pupae or emerging flies (160 hrs) were taken and frozen in a dry ice-aceton bath. The frozen animals were homogenized in 10% cold trichloroacetic acid (TCA) and the homogenate was sonicated (20 sec, Branson sonifier, step 1) before centrifuging at 8000 rpm for 10 min. The resulting pellet was washed twice with 1 ml cold 5% TCA, dried and treated for 1,5 hours with 0.5 ml 0.3 M NaOH at 37°C. After cooling to 4°C an equal volume of 1.5 N perchloric acid (PCA) was added. 20 min later, a precipitate was obtained by centrifugation for 10 min at 8000 rpm. In all instances part of the precipitate was used for determination of radioactivity. In none of the precipitates could significant radioactivity be detected.

In each experiment, aliquots of 0.2 ml of the supernatant were used for measuring radioactivity and for measurement of the quantity of RNA. Protein quantity in the pellet was determined according to Lowry et al. (1952), using different bovine albumine fraction V (Calbiochem) concentrations as a standard. RNA quantity was determined according to a modification of the Schmidt and Tannhauser (1945) procedure (Munro and Fleck, 1966). Under the conditions used, 1 ug RNA gives an absorption of 0.03 at 260 nm. The standard curve for RNA was based on total *E. coli* RNA. All values presented are the means of two series of independent measurements. The extraction procedure employed, avoids possible interference of uric acid in the RNA determinations (Linzen and Wyatt, 1964).

RESULTS

For determinations of total protein and RNA content at various stages of metamorphosis, animals were selected on a live weight basis just before the beginning of the pharate pupal stage. All animals used in the experiments, the results of which are recorded in Fig. 1, weighed 4.5 ± 0.3 mg each. Following their selection within this weight range, the larvae were injected with ^{3}H-uridine and treated as described above.

The results of the determinations of protein quantity in PCA precipitates of homogenized pharate pupae and pupae and of RNA quantity in supernatants at subsequent times after the beginning of the pharate pupal stage are recorded in Fig. 1.

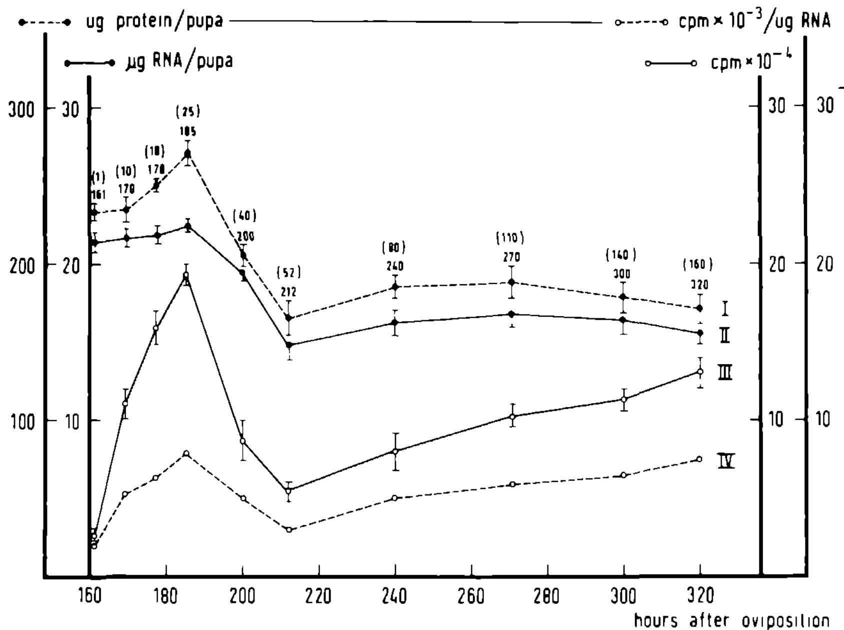

Fig. 1

Changes in total protein, total RNA, ^{3}H-uridine-labelled RNA and specific activity of ^{3}H-uridine labelled RNA (curve IV) during metamorphosis.

The values recorded for total protein (curve I) are averages of measurements of TCA precipitates after alkaline hydrolysis of four different pupae which had an identical live weight at the beginning of the pharate pupal stage. The same applies for measurements of total RNA which were performed on the hydrolysate (curve II).

The radioactivity measurements were made on TCA-precipitated RNA labelled with ^{3}H-uridine after injection of 5 uCi ^{3}H-uridine per larva one to two hours before the beginning of the pharate pupal stage (curve III).

The figures above curve I indicate the exact time of measurement in hours after oviposition. The figures between brackets indicate the time in hours after beginning of the pharate pupal stage.

Whereas the quantity of RNA changes only relatively little during the pharate pupal stage, protein quantity increases significantly shortly before pupation which occurs in *Drosophila hydei* at about 18 hours after PPS. At 25 hours after PPS, a sudden drop in protein quantity as well as in RNA quantity occurs. The lowest values for protein as well as RNA quantity were observed at 52 hours after beginning of the pharate pupal stage. From this time on, protein and RNA quantity increase slightly again.

Measurements of the radioactivity associated with TCA-precipitable RNA in the PCA supernatant following the injection of ^{3}H-uridine 1 to 2 hours before the pharate pupal stage begins, gave a curve essentially similar to that obtained for total protein. During the pharate pupal stage, the radioactivity of the RNA increased until about 25 hours after PPS, then a decrease in RNA-bound radioactivity occurred suddenly and continued until 52 hours after PPS. From that time on the radioactivity increased again up to the emergence of the flies. Calculations of the specific activity of the RNA at the various times gave, again, a curve similar to that for total radioactivity. This implies that the RNA is becoming richer in ^{3}H-uridine. By collecting the TCA washes of the precipitated RNA an attempt was made to determine the "pool" of non-precipitable radioactivity in order to test the utilization of the injected ^{3}H-uridine and eventually register the occurrence of degradation products of previously labeled RNA. The non-precipitable radioactivity de-

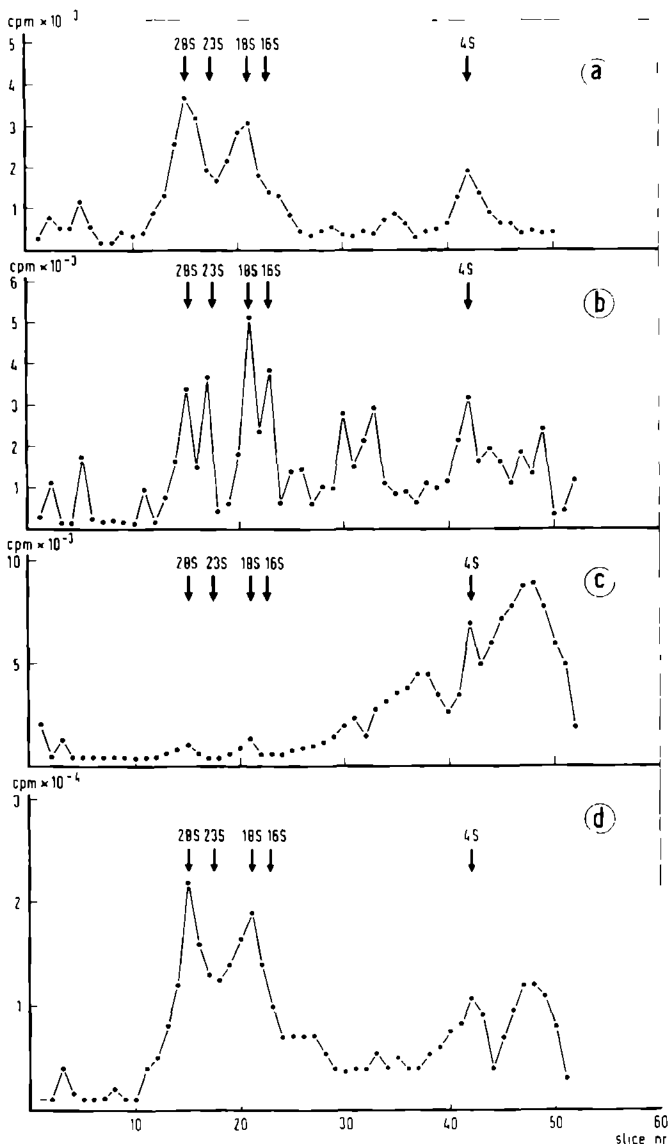

Fig. 2

Migration profiles of ^{3}H-uridine labelled RNA in polyacrylamide gels. In each experiment RNA was extracted from pupae resulting from larvae which had been fed with ^{3}H-uridine throughout larval development.

a) RNA of 1 hour old pharate pupae

b) RNA of pupae 22 hours after beginning of the pharate pupal stage

c) RNA of pupae 40 hours after beginning of the pharate pupal stage

d) RNA of freshly emerged flies

creased rapidly during the first 10 hours after the injection (until 12 hours after PPS). During the same period a strong increase in labeled RNA is observed. Subsequently, the TCA soluble radioactivity drops more slowly until about 40 hours after injection (= 40 hours after PPS) and from then on only slightly until emergence of the flies. No evidence was obtained for a significant increase in TCA-soluble radioactivity at a period during which a sharp decrease in radioactivity bound to RNA was found.

In order to investigate the nature of the sharp decrease in labeled RNA during the period from 25 till 52 hours after PPS and to obtain at the same time information as to whether or not radioactivity degradation products are reutilized for the synthesis of new RNA, RNA was extracted at 2 hours, 22 hours, 40 hours and 160 hours after the beginning of the pharate pupal stage from animals fed during the entire larval period on ^{3}H-uridine containing food. The electrophoretic profiles of the RNA extracted is shown in Fig. 2a-c.

Whereas shortly after the beginning of the pharate pupal stage peaks were observed at 28S, 18S, 8-9S and 4-7S positions, many more peaks consistently occurred in the profile of animals 25 hours later, although the 28S, 18S and 4S fractions were still present. However, another 15 hours later (40 hours after PPS) no high molecular weight fractions were found anymore. A broad peak occurs in the region 9-12S, a sharp peak at the 4S position and, again, a broad peak in the region of lower molecular weights.

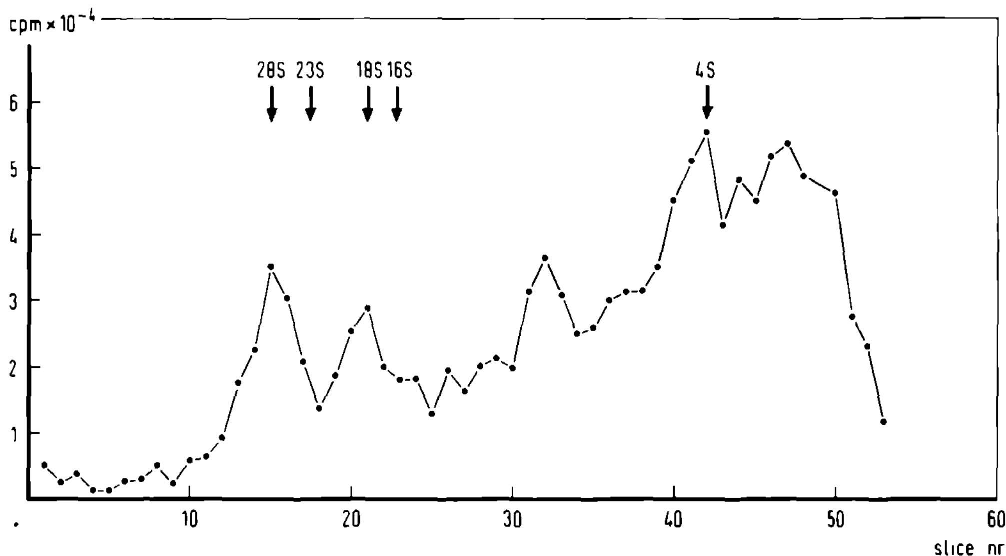

Fig. 3

Migration profile of RNA extracted from a mixture of 40 hr old pupae and freshly emerged flies. The characteristics of both the curves of fig. 2c and d can be recognized. This indicates that the absence of RNA species with S values greater than 4-7S in the extract of 40 hr old pupae (fig. 2c) reflects the actual situation rather than a result of degradation during the extraction procedure.

The profile of the RNA extracted from freshly emerged flies (160 hours after PPS) again revealed peaks at the 28S, 18S and 4S as well as a broad fraction of RNA species with lower molecular weights. It, thus, seems that following the degradation of a large part of the RNA (in particular the species with S values higher than 9-12S), new species of RNA are synthesized which probably incorporate radioactive degradation products of previously synthesized RNA.

In order to test the possibility that the profiles obtained at 40 hrs after the beginning of the pharate pupal stage are the result of degradation of the RNA during the isolation procedure, pupae of this stage and freshly emerged flies were homogenized and extracted together. The profile of the resulting RNA was a combination of the profiles obtained for 40 hour pupae and freshly emerged flies (Fig. 3); the RNA's of flies had not been degraded.

DISCUSSION

Although the RNA metabolism during larval live of Drosophila has been investigated in some detail both as regard of changes in total RNA content (Church and Robertson, 1966), and the characteristics of RNA production in particular larval and presumptive imaginal tissues (Greenberg, 1969; Tartof and Perry, 1970; Fristrom, 1972), relatively little attention has so far been paid to the RNA metabolism during metamorphosis (see also: Wyatt, 1968).

The present study indicates that during the first 25 hours after beginning of the pharate pupal stage, the total amount of TCA-precipitable RNA remains essentially unaltered. However, during the same developmental period the specific activity of the RNA increases significantly. ^{3}H-uridine is incorporated into RNA during this period. These findings suggest that after onset of the pharate pupal stage an appreciable _de novo_ synthesis of RNA occurs but that, at the same time, preëxisting RNA is degraded. It may be pointed out that the pupal moult occurs approximately 18 hours after the beginning of the pharate pupal stage.

The relatively insignificant change in RNA content during this period agrees with findings of Agrell (1964) on the metamorphosis of Calliphora. Ring (1973), on the other hand, observed a clear increase in RNA in relation to dry weight of _Lucilia cericata_ shortly after puparium formation.

The amount of TCA-precipitable RNA decreases significantly over the next 27 hours (25 to 52 hours after PPS). Because this decrease coincides with a drop in specific activity and with the appearance and increase in low molecular weight RNA in the migration profiles, it seems that the degradation of RNA occurs at a higher rate than de novo synthesis during this period. A similar decrease in total RNA in relation to dry weight occurring at a comparable time in development was reported for Lucilia cericata (Ring, 1973). In Drosophila hydei, the decrease in total RNA may reflect completion of histolysis of the larval tissues. After 52 hours after PPS the total RNA as well as its specific activity increases slowly until emergence of the flies. Since the pupa is essentially a closed system, it may be inferred that degradation products of previously synthesized RNA become utilized for de novo RNA synthesis in the proliferating and differentiating imaginal tissues. Although unlikely, it cannot be excluded that part of the increase in specific activity is a result from incorporation of free ^{3}H-uridine still present in the pool. The idea of a reutilization of degradation products of essentially larval RNA is supported by the results of electrophoresis of RNA extracted from freshly emerged flies. The profiles show again a pattern similar to those found during mid third instar (Church and Robertson, 1966) and shortly after beginning of the pharate pupal stage in which the 28S and 18S ribosomal RNA and the 4-7S RNA fractions are the most predominant.

ACKNOWLEDGEMENTS

The author is indebted to Drs. H.D. Berendes and L.M. Birt for discussion of the results, and Mr. Guillermo Gimenez for his help with some of the experiments.

REFERENCES

Agrell, I (1964) Physiological and biochemical changes during insect development. In Physiology of Insecta (Ed. by Rockstein, M.), 1, 91-149. Academic Press, New York.

Alonso, C. (1972) The influence of molting hormone on RNA synthesis in isolated polytene nuclei of Drosophila. Developm. Biol. 28, 372-382.

Berendes, H.D. (1965) Salivary gland function and chromosomal puffing patterns in _Drosophila hydei_. Chromosoma, 17, 35-77.

Boyd, J.B. and Boyd, H. (1970) Deoxyribonucleases of the organs of _Drosophila hydei_ at the onset of metamorphosis. Biochem. Genet. 4, 447-459.

Boyd, J.B. and Logan, W.R. (1972) Developmental variations of a deoxyribonuclease in the salivary glands of _Drosophila hydei_ and _Drosophila melanogaster_. Cell Diff. 1, 107-118.

Church, R.B. and Robertson, F.W. (1966) A biochemical study of the growth of _Drosophila melanogaster_. J. exp. Zool. 162, 337-352.

Fristrom, J.W. (1972) The biochemistry of imaginal disc development. In The Biology of Imaginal Discs (Ed. by Ursprung, H. and Nöthiger, R.) 109-154. Springer Verlag, Heidelberg.

Greenberg, J.R. (1969) Synthesis and properties of ribosomal RNA in Drosophila. J. mol. Biol. 46, 85-98.

Henrikson, P.A. and Clever, U. (1972) Protease activity and cell death during metamorphosis in the salivary gland of Chironomus tentans. J. Insect Physiol. 18, 1981-2004.

Laufer, H. (1968) Developmental interactions in the dipteran salivary gland. Am. Zool. 8, 257-271.

Leenders, H.J. and Berendes, H.D. (1972) The effect of changes in the respiratory metabolism upon activity in Drosophila. I. The induction of gene activity. Chromosoma (Berl.) 37, 433-444.

Linzen, B. and Wyatt, G.R. (1964) The nucleic acid content of tissues of cecropia silkmoth pupae. Relations to body size and development. Biochem. Biophys. 87, 188-198.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Munro, H.N. and Fleck, A. (1966) Recent developments in the measurement of nucleic acids in biological materials. A supplementary Review. Analyst 91, 78-88.

Ring, R.A. (1973) Changes in dry weight, protein and nucleic acid content during diapause and normal development of the blowfly, Lucilia sericata. J. Insect Physiol. 19, 481-494.

Ritossa, F.M.A. (1965) Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of Drosophila melanogaster. Proc. Natl. Acad. Sci. 53, 737-745.

Rodems, A.E., Henrikson, P.A. and Clever, U. (1969) Proteolytic enzymes in the salivary gland of Chironomus tentans. Experientia 25, 686-687.

Schin, K.S. and Clever, U. (1968) Ultrastructural and cytochemical studies of salivary gland regression in Chironomus tentans. Z. Zellforsch. 86, 262-279.

Schmidt, G. and Tannhauser, S.J. (1945) A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animals tissue. J. Biol. Chem. 161, 83-89.

Tartof, K.D. and Perry, R.P. (1970) The 5S RNA genes of Drosophila melanogaster. J. mol. Biol. 51, 171-183.

Vickers, D.H. and Mitlin, N. (1965) Changes in nucleic acid content of the boll weevil, Anthonomus grandis Boheman during its development. Physiol. Zool. 39, 70-76.

Wyatt, G.R. (1968) Biochemistry of insect metamorphosis. In: Metamorphosis (Ed. by Etkin, W. and Gilbert, L.J.) 143-184 North-Holland Publ. Cy. Amsterdam.

SAMENVATTING

Het eerste onderdeel van dit proefschrift beschrijft de veranderingen in het RNA synthese patroon, zowel kwalitatief als kwantitatief, in geisoleerde speekselklierkernen van Drosophila hydei tijdens incubatie met het steroid hormoon ecdyson. Ofschoon de incorporatie van radioaktief gemerkte uridine, een bouwsteen van het RNA, bij incubatie van de kernen met het hormoon met een factor 1.4 toeneemt in vergelijking met kernen welke over eenzelfde tijdsperiode worden geincubeerd zonder hormoon, is deze toename in incorporatie waarschijnlijk geen gevolg van synthese van andere dan in de controle kernen gesynthetiseerde RNA species. Competitieve hybridisatie experimenten wezen uit, dat onder de condities waarbij deze experimenten werden uitgevoerd, het RNA van hormoonbehandelde kernen geen transcripten bevatte van DNA sequenties welke in controle kernen niet waren afgelezen. Ook verzadigings hybridisatie experimenten met RNA uit onbehandelde en hormoon behandelde kernen gaven hetzelfde verzadigingsniveau (2.4%). Vergelijking van RNA migratie patronen in acrylamide gels wees uit dat hormoonbehandeling van geisoleerde kernen in het bijzonder het ontstaan van laag (2-6S) moleculaire RNA species stimuleerde. Het kan niet worden uitgesloten dat deze toename in laag moleculaire RNA mede een gevolg is van verhoogde degradatie van RNA onder invloed van de hormoon behandeling.

In het tweede deel van dit proefschrift wordt de synthese en verwerking van ribosomaal RNA in in vitro gecultiveerde speekselklieren beschreven. De incorporatie van ^{3}H-uridine in RNA gesynthetiseerd in deze klieren neemt over een periode van 72 uur incubatie lineair toe met 0.076 pmoles UMP/uur/ug DNA. Op verschillende tijdstippen na begin van in vitro incubatie werd het migratie profiel van nieuw gesynthetiseerd RNA in acrylamide gels onderzocht waarbij bleek dat gedurende de gehele periode 38S, 28S, 18S en 4-7S frakties gevormd werden. Teneinde een goede indicatie te verkrijgen dat de gevormde 28S en 18S frakties inderdaad ribosomale RNA species representeerden, werden deze frakties gebruikt voor in situ hybridisatie experimenten. Een exclusieve labeling van de nucleolus was het resultaat hetgeen een goede aanwijzing is dat onder de incubatie condities hier toegepast, de synthese van de ribosomale voorloper (38S) en de verwerking tot 28S en 18S ribosomale RNA voortgang vindt. Dit resultaat toont dat de incubatie condities voor geëxplanteerde speekselklieren zodanig zijn dat het RNA metabolisme in de celkern, tenminste voor wat de ribosomale RNA betreft, overeenkomst vertoont met het metabolisme onder in vivo omstandigheden.

Het derde onderdeel van dit proefschrift beschrijft een methode voor in situ RNA/DNA hybridisatie waarbij een opmerkelijke verbetering in de efficientie van hybridevorming werd bereikt door toepassing van formamide tijdens het hybridisatie proces. Bovendien kan in aanwezigheid van

formamide de hybridisatie reaktie worden uitgevoerd bij lage temperatuur (20^oC).

Het laatste onderdeel van dit proefschrift beschrijft het RNA metabolisme tijdens de metamorfose van Drosophila hydei. De totale hoeveelheid RNA per pop, de hoeveelheid ^3H-uridine gelabeld RNA en de specifieke aktiviteit van het RNA neemt tijdens het begin van de metamorfose periode toe, doch vertoont vervolgens na ongeveer 25 uur een zeer plotselinge daling. 55 uur na de pupariumvorming begint de hoeveelheid RNA weer toe te nemen, een toename welke zich voortzet tot de vliegen uitkomen. Migratie patronen van radioaktief gemerkt RNA in polyacrylamide gels op verschillende tijdstippen tijdens de metamorfose periode tonen dat tijdens de sterke daling in RNA gehalte alle hoogmoleculaire RNA afgebroken is, doch dat het normale RNA patroon hoofdzakelijk bestaande uit 28S, 18S en 4-7S RNA frakties in uitkomende vliegen wederom aanwezig is. Daar deze frakties alle radioaktief zijn, is aan te nemen dat in het gesloten systeem dat de insektenpop vormt, bouwstenen van het larvale RNA dat tijdens de metamorfose wordt afgebroken, worden gebruikt voor de opbouw van het imago. Uit deze gegevens kan men bovendien afleiden dat de afbraak (histolyse) van larvale weefsels een hoogtepunt bereikt tijdens de periode van 25-55 uur na pupariumvorming.

VI

De resultaten van circularisatie experimenten met korte DNA moleculen van Drosophila leiden tot de conclusie dat de hoeveelheid repetitieve DNA sequenties in het genoom veel hoger is dan op grond van de reassociatie kinetiek van het DNA wordt verondersteld.

Hennig, W.J. mol. Biol. 71, 419 (1972).

VII

De suggestie dat de donkerkleurende banden in zoogdier chromosomen na behandeling met de azijnzuur-Giemsa techniek de lokalisatie van gerepeteerde sequenties weerspiegelt is onjuist.

Sumner, A T , Evans, H J en Buckland, R A Nature New Biol 232, 32 (1972).

VIII

Het is waarschijnlijk dat een groot aantal nieuwe plantesoorten zijn ontstaan als gestabiliseerde fertiele hybriden van ouders waarvan de fertiliteit berust op een groot aantal onafhankelijk segregerende faktoren

Stebbins, L G. in Processes of organic evolution Prentice-Hall, Inc 122 (1966)

IX

De ethische implicaties van bepaalde moleculair biologische experimenten mogen niet uitsluitend berusten op het verantwoordelijkheidsbesef van de individuele onderzoeker.

Spiegelman, S FEBS Congress, Amsterdam (1972).

X

Verdere uitbreiding van de ambtelijke organisaties ten behoeve van de financiering van het wetenschappelijke onderwijs gaat ten koste van het rendement van dit onderwijs.

XI

Invoering van een wettelijke beperking van de verblijfsduur van buitenlandse werknemers zou neerkomen op het invoeren van een moderne versie van slavenhandel