SUMMARY

The size of the DNA replication units in salivary gland polytene chromosomes of *Chironomus* larvae at 2 developmental stages (red-head and late 4th instar) was investigated. The 2 developmental stages differ with respect to intensity of DNA replication with much lower values at the red-head stage. Also the level of polytenization is lower at the red-head stage. The size of the replication units was measured by determining the size of double-stranded DNA fragments released from active replication units during cell lysis. At both stages it is possible to release from the polytene chromosome a double-stranded DNA fragment of similar size. This indicates that there is no or very little difference in size of active replication units in the same tissue at the 2 development stages.

INTRODUCTION

During development of the dipteran *Chironomus tentans* giant polytene chromosomes are formed in certain organs such as the salivary glands (see e.g., *Results and Problems in Cell Differentiation* 4, 1972). These chromosomes offer a unique possibility to investigate whether structural features of the chromosome may determine the size of the replication units (replicons) since DNA-rich bands and DNA-poor interbands are easily distinguished in the light microscope. In *Chironomus* about 95% of the DNA is located in the DNA bands and the remaining 5% in the interbands (Daneholt & Edström, 1967). Polytene chromosomes develop in cells which grow in size but do not divide. The DNA increase in such cells occurs in a stepwise fashion where each step represents a doubling of the previous amount. Consequently the newly formed chromatids are not separated but remain arranged side by side.

It has been proposed that each band may function as a separate replication unit. This is based on early autoradiographic investigations that revealed a positive correlation between the DNA content of a band and the duration of DNA replication in the same band (Pelling, 1966). However, late autoradiographic investigations showed that in certain situations the time required for DNA synthesis in a band may be less than that predicted from the DNA content (Hägele, 1976). Thus it is possible that the sizes of the replicons in polytene chromosomes are controlled functionally.

During the development of *Chironomus* larvae, there is a regular timing of DNA replication in the salivary glands with regard to the moulting cycles implying external control of the process of initiation and/or maintenance of DNA synthesis in polytene
chromosomes. Just after the third moult of the *Chironomus* larvae there is a stage at which the frequency of replicating nuclei in salivary gland is reduced (Darrow & Clever, 1970). These animals are easily recognized morphologically by lack of black cuticle on their head (red-head stage). In this paper we have compared DNA replication in glands from red-head stage with glands obtained from late fourth instar larvae. The fourth instar larvae show a higher intensity of DNA replication. Also the level of polytenization is higher in these animals than in the red-head animals.

Fibre autoradiographic methods are most commonly used to determine the size of replication units. However, a different approach is to allow synthesis of double-stranded DNA fragments at active DNA-replicating sections, and to isolate them by lysing the cells at 25 °C in a neutral, non-denaturing buffer. The average size of this DNA population is half the size of the replication unit as determined by parallel determination with fibre autoradiography (Guy & Taylor, 1978; Taylor, 1973).

We have earlier shown that it is possible to release a double-stranded DNA fragment from *Chironomus* polytene chromosomes (Lönn, 1989). Therefore we have looked for the presence of a double-stranded DNA fragment in salivary glands from the red-head stage and compared the size of this fragment with the size of the DNA fragment obtained from late 4th-instar animals.

**MATERIALS AND METHODS**

**Biological material**

Larvae of *Chironomus tentans* were raised in the laboratory as described earlier (Lönn & Edström, 1977). The animals were cultured in deionized water containing NaCl (0.4 g/l) and cellulose tissue. Twice weekly the animals were fed fermented nettle powder. The cultures were aerated and kept at +18 °C with a 16/8 h day/night rhythm. The average time of development from eggs to pupae is about 12 weeks.

**Labelling conditions**

Red-head stage and late 4th-instar larvae (8 weeks old) were used. The red-head larvae have just completed a larval moult and have not yet formed the black cuticle pigment of the head capsule. Under our culturing conditions they are about 5 weeks old. For labelling of the DNA, the animals were injected into the bloodstream with 20 μCi of [3H]thymidine (22-25 Ci/mmol), (Amersham/Searle Corp.) dissolved in 1 μl of 0.67 % NaCl, 0.04 % KCl. The injection was done with a micropipette, held with a de Fonbrune micromanipulator, under the control of a stereomicroscope (Lönn, 1980). Pipettes are made and filled in an oil chamber arrangement as described by Edström (1964).

**Extraction of DNA**

The excised salivary glands of each animal were immediately covered with 75 μl of a solution containing 0.05 M Tris-HCl (pH 7.4), 0.002 M Na₂EDTA, 1 % sodium dodecylsulphate (SDS). After 3 h at +25 °C the sample was transferred to the trough of an agarose-flat bed gel. To extract glands at 0 °C the following solution was used: 0.05 M Tris-HCl (pH 7.4), 0.002 M Na₂EDTA, 0.5 % sarkosyl. After 3 h at 0 °C in this solution the samples were transferred to the trough of an agarose gel (Lönn, 1980).

**Agarose gel electrophoresis and CsCl gradient centrifugations**

Separation of the DNA in agarose (0.75 %) flat-bed gels was performed as described earlier (Lönn, 1980). The gland DNA to be analysed by CsCl gradient centrifugations was concen-
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treated by ethanol precipitation and then dissolved in 0.1 M Tris-HCl (pH 7.6), 0.01 M Na2EDTA. The CsCl gradient centrifugations were performed as described earlier (Lönn, 1980).

RESULTS

The red-head stage

The development of Chironomus larvae of individual cultures was followed daily using the culturing conditions described in Materials and methods. The total incorporation of [3H]thymidine into trichloroacetic acid (TCA)-soluble and -insoluble material was measured at 3 stages: younger than red-head, at red-head, and older than red-head. For this purpose the animals were injected with the same amount of [3H]thymidine into the larval blood and the salivary glands were excised 6 h later. The results show that the incorporation of radioactivity into the TCA-insoluble material is on a continuous high level with the exception of the animals which are in the red-head stage. During the red-head stage there is a drop in total incorporation of radioactivity into TCA-insoluble material (Fig. 1). On the other hand there is a con-

![Graph showing time-course of radioactivity distribution between acid-soluble and -insoluble gland extracts after injection of tritiated thymidine. At each time-point 4 animals were analysed. The pair of glands were dissolved in 0.5% SDS, 0.02 M Tris-HCl (pH 7.4). After precipitation with ice-cold 10% trichloroacetic acid for 30 min, radioactivity in the supernatant (1 ml) was measured in 4 ml of Instagel (Packard) and the radioactivity in the pellet in toluene scintillator after prior solubilization with Soluene 100. The scale to the left refers to acid-insoluble material, the scale to the right to acid-soluble material. RH stands for red-head animals. Bars give S.E.M. —O—, acid-insoluble; — ●—, acid-soluble.]
Continuous high level of radioactivity in the TCA-soluble fraction indicating that there is very little or no difference in isotope uptake during the red-head stage. Using autoradiographic measurements it has earlier been shown that at the red-head stage there is a characteristic decrease in the average frequency of replicating nuclei per gland (Darrow & Clever, 1970). The red-head salivary gland polytene chromosomes contain DNA corresponding to about 10 endomitotic duplications of the DNA. This should be compared to the late fourth-instar larvae which contain DNA corresponding to 12–13 endomitotic duplications of the DNA (Daneholt & Edström, 1967; Darrow & Clever, 1970).

Characterization of the nascent double-stranded DNA

To investigate whether labelled nascent DNA in red-head animals can be released from the polytene chromosomes in the form of double-stranded fragments the following experiments were performed. Red-head animals as well as 8-week-old animals (late 4th instar) were injected with tritiated thymidine and sacrificed 4 h later. The salivary gland DNA was extracted at 25 °C with the neutral non-denaturing buffer as described in Materials and methods, and then analysed in a CsCl-gradient (Fig. 2A). The labelled DNA of the red-head animals banded in the same position in the gradient as the labelled DNA obtained from the control 8-week-old animals. The position in the gradient is that of the double-stranded salivary gland DNA as earlier established (Lönn, 1980). Similar results were obtained with animals sacrificed 18 h after precursor injection (not shown). Thus the labelled DNA 4 h after [3H]thymidine injection exists mainly in the form of double-stranded DNA.

This DNA was analysed by agarose flat-bed gel electrophoresis. The DNA obtained from the red-head animals and DNA from late fourth-instar larvae were electrophoresed in parallel lanes of the same 0.75 % agarose gel. The results showed in both samples a heterogeneous population of labelled DNA molecules located between slices 33 and 47 (Fig. 2B). Moreover, there is a peak of radioactivity located at slices 4–6. There is no qualitative difference between the 2 stages of larval development.

Fig. 2. CsCl-centrifugation of pulse-labelled salivary gland DNA from the red-head stage and 8-week-old animals. Larvae were injected with 20 μCi [3H]thymidine 4 h before sacrifice. The salivary gland DNA was extracted and analysed by CsCl centrifugations as described in Materials and methods. The arrow to the left indicates the position of marker Micrococcus DNA (1.73 g/cm³). The labelled Chironomus DNA banded in the same position in the gradient (1.691 g/cm³, right-hand arrow) as earlier established for double-stranded salivary gland DNA (Lönn, 1980). —○—, red-head stage; —●—, 8-week-old animals. B, Electrophoretic separation of pulse-labelled salivary gland DNA from the 2 developmental stages. The salivary gland DNA was obtained from animals injected with [3H]thymidine 4 h before sacrifice and then analysed in 0.75 % agarose flat-bed gels. The 2 samples were placed in separate application troughs in the same gel and analysed in parallel. Left- and right-hand arrows indicate the position of Chironomus 28 s and 18 s ribosomal RNA, respectively, localized with the Minuvis u.v. lamp. The 2 separations are very similar with a heterogeneous material located at slices 33–47 and a peak located at slices 4–6. —○—, red-head stage; —●—, 8-week-old animals.
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![Graphs showing replication profiles](image-url)
Fig. 3. Electrophoretic separation of pulse-labelled salivary gland DNA from the red-head stage and 8-week-old animals. Larvae were injected with 20 μCi[^3H]thymidine and sacrificed 60 min (A), 18 h (B) or 48 h (C) later. The salivary gland DNA was extracted at 25 °C and then analysed in 0.75 % agarose flat-bed gels. In each gel samples from the 2 developmental stages were analysed in parallel lanes. Left- and right-hand arrows indicate the position of *Chironomus* 28 s and 18 s ribosomal RNA, respectively, localized with the Minuvist u.v. lamp. The 60-min separation is dominated by a heterogeneous material migrating to slices 33-47. The 18-h sample contains apart from this material also a peak of radioactivity located at slices 4-6. The 48-h separation is dominated by the material located at slices 4-6. There is no qualitative difference in the DNA obtained from the 2 developmental stages. —○—, red-head stage; —●—, 8-week-old animals.

However, there is a difference in the total amount of labelled material with lower values in the red-head sample.

The results with the CsCl gradient indicate that both the DNA located at slices 33-47 and at slices 4-6 exist in the form of double-stranded DNA. This is in agreement with earlier observations on the late 4th-instar larvae (Lönn, 1980).

In the late 4th-instar larvae the labelled DNA located at slices 33-47 is a replication intermediate with relatively short half-life whereas the labelled DNA located at slices 4-6 represent stable and/or steady-state labelled DNA. To investigate whether this is also true for the red-head stage experiments were performed where the animals were sacrificed after 60 min, 18 h, and 48 h (Fig. 3). Sixty minutes after precursor injection electrophoretic analysis revealed only the replication intermediates located between slices 33 and 47, with no detectable material located at slices 4-6. However,
after 18 h the electrophoretic separation has changed, mainly insofar that in addition to the material located at slices 33–47 there is also a peak of radioactivity at slices 4–6. After 48 h only the peak located at slices 4–6 is detectable. There is no qualitative difference between the red-head stage and the late 4th-instar larvae. The labelling procedure to administer the [H]thymidine has previously been shown to have the character of a pulse (Lönn, 1980). Thus the results show that also in the red-head stage the double-stranded DNA located in slices 33–47 is a replication intermediate which later is joined together to produce the high-molecular-weight DNA located at slices 4–6.

Furthermore, we wished to investigate whether it is possible to prevent the release of DNA fragments from the polytene chromosomes in animals at the red-head stage. This can be done with late fourth-instar animals by lysing the cells at 0 °C instead of at 25 °C (Lönn, 1980). Similar experiments were therefore performed using salivary glands of the red-head stage, and in these also it was thus found to be possible to prevent release of the double-stranded DNA fragments by lysing the cells at 0 °C (Fig. 4).

**DISCUSSION**

The aim of the present work was to investigate whether there is a change in size of the active replicating units in a tissue which passes through different stages during development of the animal. For this purpose the development of *Chironomus* larvae has many advantages. As shown in this paper and earlier by Darrow & Clever (1970) there is a drop in the replication activity of salivary glands of *Chironomus* during the larval red-head stage. A similar phenomenon has also been reported in the development of the salivary glands of *Drosophila hydei* (Danielli & Rodino, 1967), as well as in the last instar of *Sciara coprophila* in which virtually no replication could be detected in the salivary glands 3–4 days after the last larval ecdysis (Crouse, 1968).

The variation in replication activity in *Chironomus* salivary glands is probably under hormonal control since there is a correlation between DNA replication and metamorphic events (for references, see *Results and Problems in Cell Differentiation* 4, 1972). The S-phase in late fourth instar animals has been estimated to last between 10 and 20 h (Darrow & Clever, 1970). The length of S-phase at the red-head stage is not known. However, the fact that only a fraction of the nuclei take up autoradiographic

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**Fig. 4.** Electrophoretic separation of DNA from cells lysed at 0 °C. Larvae were injected with 20 μCi [H]thymidine and sacrificed 60 min later. One gland was extracted at 0 °C as described in Materials and methods whereas the sister control gland was extracted at 25 °C. Animals from the red-head stage (A) as well as 8-week-old animals (B) were analysed. The extracts from the 2 sister glands were analysed in parallel lanes of the same 0.75 % agarose flat-bed gel. Left-and right-hand arrows indicate the position of *Chironomus* 28 S and 18 S ribosomal RNA, respectively, located with the Minivis u.v. lamp. When the cells are lysed at 0 °C the main part of the radioactivity is located at slices 4–6 whereas in cells lysed at 25 °C the main part of the radioactivity is located at slices 33–47. —○—, cells lysed at 0 °C; —●—, cells lysed at 25 °C.
label at the red-head stage does not imply that the S-phase is prolonged in these cells. In fact, analogy with most other cells suggests that these cells are slowly cycling because of prolonged G1 phase, with an S-phase of normal duration.

Another difference between the 2 stages is the lower level of polytenization at the red-head stage. These cells have undergone about 10 endomitotic duplications as compared to 13-14 duplications at the late 4th instar. However, chromosomes of the 2 stages show identical cytological interband-band patterns with about 95% of the total DNA located in the bands.

We have in this paper examined the DNA replication in salivary glands of the red-head stage and compared the results with those obtained with late 4th-instar larvae. The results show that it is possible to label replicative intermediates in the glands of both stages. When the cells are lysed at 25 °C in a non-denaturing buffer there is release from the polytenic chromosomes of a double-stranded DNA fragment. The release is probably enzymic since it is possible to prevent it by lysing at 0 °C. The average size of this double-stranded DNA population is half the size of the replication units (Guy & Taylor, 1978).

Therefore, we determined the electrophoretic mobility of the double-stranded DNA fragments obtained from both the red-head stage and late fourth-instar larvae. Similar mobilities of the DNA fragments in agarose gel indicated similar sizes, in turn indicating that the sizes of the replication units at the 2 developmental stages are very similar. The size of the DNA fragments from the late 4th-instar animals has earlier been estimated to be 3.75-6 × 10^8 Daltons (Lönn, 1980), which corresponds to a length of 2-3 μm of double-stranded DNA, assuming that 1 μm is 2 × 10^8 Daltons. Since the average band contains about 30 μm of double-stranded DNA per chromatid (Daneholt & Edström, 1967) there is room for several replication units of this size located side by side in the band.

Thus, the size of active replicating units is similar at the 2 development stages. Consequently neither the lower level of polytenization nor the hormonal control of replication frequency in salivary glands at the third moult can have changed the size of the active replicons.

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