Qualitative changes in DNA indicating differential DNA replication during early embryogenesis of the newt *Triturus vulgaris*

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### SUMMARY

During gastrulation of the newt *Triturus vulgaris* considerable changes in the melting behaviour and in the CsCl density gradient pattern of DNA occur. The melting curves of DNAs from mid to late gastrulae (stages 11 b–12c) deviate from the regular shape. Whereas the Tm values are identical in the stages 11 a (early gastrula) and 15 (early neurula), and correspond to the standard DNA (stage 36 = tailbud), a significant rise of Tm (0.8–1.2 °C) has been recorded in the stages 12 a/b (yolk plug). The differences in melting behaviour become visible by the deviation of the curves above Tm. These deviations from the normal sigmoidal shape are caused by the fact that a portion of DNA melts at higher temperatures than usual. Therefore the thermal denaturation of DNA is completed at approximately 3–4 °C later than in standard DNA. Both the derivative curves and the plots on normal probability paper demonstrate a heterogeneity of DNA in the stages 11 b–12c which indicates the presence of an additional GC-rich satellite fraction. These findings are confirmed by CsCl density gradient studies. Thus, in the stages 12 a/b a slight shoulder on the heavy side of the gradients occurs, being absent in the other stages. From these facts we have to conclude that there is a stage-dependent multiplication and elimination of GC-rich sequences.

### INTRODUCTION

In the last decade many exceptions from the rule of DNA constancy have been found which can be classified into four major groups: gene amplification, DNA magnification, DNA under-replication and DNA elimination (Brown & Dawid, 1968; Ritossa, 1968; Kaback & Halvorson, 1977; Rudkin, 1969; Beermann, 1977; see Tobler, 1975 and Nagl, 1976 for review). The best-studied case of gene amplification involves the excessive multiplication of gene copies coding for ribosomal RNAs (rDNA) in oocytes of many vertebrates and invertebrates (Gall, 1969; Lima de Faria, Birnstiel & Jaworska, 1969). Both the fact that gene amplification is not restricted to oogenesis but also occurs within somatic cells, for example in the DNA puffs of sciarids (Pavan & Da Cunha, 1969), and the phenomenon of DNA under-replication in the course of polytenization of some insect tissues (see Laird, 1973 for review) led to the assumption that differential

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DNA replication may be a more common mechanism of differentiation in eukaryotic systems than is currently believed.

Under this aspect we studied the early phases of determination and differentiation in the development of the newt *Triturus vulgaris* with respect to quantitative and qualitative changes in the nuclear DNA. From cytophotometric measurements it became evident that the nuclear DNA content is not constant during early development, but varies with stage and region specifically (Lohmann & Vahs, 1969; Lohmann, 1972). To answer the question whether there is a stage-specific amplification of DNA sequences differing in the mean guanosine–cytosine content from bulk DNA, in the present study the DNA isolated from various developmental stages was characterized by thermal denaturation analysis and CsCl density gradient centrifugation. A short report on this subject has been presented elsewhere (Lohmann & Schubert, 1978).

**MATERIALS AND METHODS**

**Animals.** All studies were carried out with embryos of *Triturus vulgaris* from the early gastrula to the early neurula stage (Harrison stages 10–15). Late tailbud stages (stages 35, 36) have been used for reference. In order to obtain sufficient numbers of eggs the freshly collected females were treated with human gonadotropin (Predalon, Organon GmbH, München). In 24 h intervals three doses of 3 i.u. each were injected. In order to prepare homogeneous egg material, the eggs were collected within 3 h intervals and kept at 18 °C in Holtfreter solution, containing Cibazol (0-1 %, Ciba, Wehr/Baden) and penicillin (100 i.u. per ml), until the desired stage was reached. In the gastrula stages 50–60 dejellied embryos were pooled and preserved in liquid nitrogen until used.

**DNA isolation.** The isolation of nuclei was performed according to the methods of Imoh & Minamidani (1973) and Faulhaber (1972). From the nuclei fractions the DNA was prepared by the method of Marmur (1961) with some modifications. From the supernatant obtained after the removal of RNA by RNase (Ribonuclease A, 100 μg/ml) and deproteinization with an equal volume of chloroform–isoamyl alcohol (24:1, v/v) the DNA was precipitated by ethyl alcohol containing 0-2 m sodium acetate and dissolved in 0-1 SSC (0-015 M-NaCl, 0-0015 M-Na₃ citrate, pH 7-0). The remaining amounts of polysaccharides and proteins were removed by digestion with α-amylase (100 μg/ml) and pronase (500 μg/ml), followed by two treatments with chloroform–isoamyl alcohol. After dialysing the supernatant against 0-1 SSC the DNA was precipitated with ethanol and washed several times with 80 % ethanol.

**Thermal denaturation.** The melting profiles of native DNA were obtained as described by Mandel & Marmur (1968) with a Zeiss PM Q2 spectrophotometer equipped for thermal denaturation studies. In all cases the DNA was dissolved in 0-1 SSC. The samples showed a 260/280 nm absorption ratio of 1-76–1-82, and a 230/260 nm ratio of 0-42–0-47. The hyperchromicity values of different
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Fig. 1. Upper part: thermal denaturation profiles of DNA from different developmental stages (early gastrula to early neurula, and tailbuds). Ordinate: relative absorbance (hyperchromicity) (percentages). Abscissa: temperature (°C). Lower part: the appropriate derivative melting curves.

samples varied between 33 and 36 %. The mean Tm value of 28 melting curves of 'standard'-DNA (stage 36) is 69.43, with a standard error $\sigma = 0.15$. The plots on normal probability paper were carried out according to Knittel, Black, Sandine & Fraser (1968).

CsCl ultracentrifugation. Native unsheared DNA was centrifuged in a Beckman preparative ultracentrifuge in a type-65 fixed-angle rotor. Centrifugation was performed in neutral CsCl at 33000 rev./min for 65 h at 25 °C. In all cases 10 ml gradients, overlaid with paraffin oil, were loaded with equal amounts of DNA (0.6 OD unit, approx. 30 μg) from different developmental stages. The initial density of the gradients was 1.695 g/ml at 25 °C. After centrifugation the content of each tube was fractionated by the bottom harvesting technique. The u.v. gradient profiles were continuously recorded by using a flow-through micro-cuvette in a Zeiss PM Q2 spectrophotometer. Forty-six fractions of 185 μl each were collected and the density of each fraction was determined by a Zeiss refractometer.

RESULTS

1. The melting behaviour of DNA

Figure 1 illustrates the melting behaviour of DNA isolated from different developmental stages. Parallel to each embryonic DNA the standard DNA of late tailbuds (stage 36) has been measured. Standard Triturus DNA has a regular melting profile with a mean Tm value of 69.4 °C, whereby the extreme values are at 69.2 and 69.6 °C, respectively. Corresponding melting curves have been recorded in the early gastrula stages (10/11a) and early neurula (stage 15). In the stages 11b–12c, however, the melting profiles deviate from the regular
Temperature (°C)

Fig. 2. Thermal denaturation curves of DNA (stages as in Fig. 1), plotted on normal probability paper.

sigmoidal shape at temperatures above Tm being accompanied by an increase of the Tm value. Maximal values have been found in the stages 12a/b that range in different samples between 0.8 to 1.2 °C above the mean Tm of standard DNA. The deviations from the normal shape become visible by the flattening of the curves at lower hyperchromicity values (85 % H) than usual, and moreover by the fact that the thermal denaturation of DNA is completed later (about 3–4° in the stages 12a/b) than in the other stages.

If we look at the derivative curves (lower part of Fig. 1) and the plots on normal probability paper (Fig. 2), these changes in the melting profiles of DNA can be most clearly seen. The melting behaviour of DNAs from the stages 10/11a, 15 and 36 corresponds to the Gaussian distribution with respect to the mean base composition. The derivative curves of the stages 11b–12c, by contrast, show a shoulder at higher temperatures which indicates the presence of a second DNA fraction. The latter is also demonstrated by the fact that on normal probability paper the hyperchromicity values deviate from the straight line, yielding a second line at H-values above Tm. From the comparison of the temperature scales in Fig. 2 it can be easily seen that the endpoint of thermal denaturation is shifted to higher temperatures in the mid gastrula stages.

2. CsCl density gradient patterns

The characterization of DNA in neutral CsCl gradients yields corresponding curves for the early gastrula (stage 10/11a), early neurula (stage 15), and tailbuds (stage 36). Besides the main band a slight shoulder (arrow) is present on the lighter side of the gradient (Fig. 3a). In contrast, in the stages 12a/b (Fig. 4a) a little deviation from the reference curve becomes visible on the heavy side of the gradient too (arrow). In order to make this deviation more evident the
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Fig. 3. CsCl density gradient patterns of DNA from the stages 11a, 15 and 36 (Fig. 3a). The buoyant density profiles are shown in the upper part. In Fig. 3b–d the heavy fractions 10–18 (d), the middle fractions 19–27 (c), and the light fractions 28–42 (b) of three identical gradients were pooled and recentrifuged. Fractions 10–18, 19–27 and 28–42 of three identical gradients were pooled and recentrifuged in neutral CsCl. As expected, a symmetric profile has been monitored for the middle fractions (19–27, Fig. 4c); in the light fractions (28–42) the shoulder on the light side appears more prominently (Fig. 4b), and the mentioned deviation on the heavy side of Fig. 4a can now be clearly seen in the fractions 10–18 as a significant shoulder (Fig. 4d). In the same manner the DNAs from the stages 10/11a, 15 and 36 have been fractionated, whereby no deviations could be detected on the heavy side of the gradient (Fig. 3d). For the other fractions, curves have been recorded (Fig. 3b and c) corresponding with those of Fig. 4.

On the one hand the density difference of DNA which causes the heavy shoulder is obviously too small to separate itself from the main band as a satellite peak. On the other hand the portion of this fraction is so small that the formation of a separate peak is suppressed solely by the amount of main band DNA. To reduce the portion of main band DNA significantly the heavier fractions (10–18) of one
Fig. 4. CsCl density gradient profiles of DNA from the mid-gastrula stages 12a/b (a). In (b)–(d) the same fractionation and recentrifugation was carried out as in Fig. 3.

gradient with DNA from the stages 12a/b (Fig. 5, upper part) were pooled and recentrifuged. The result is presented in the lower part of Fig. 5. Now, a separate, heavy satellite peak (arrow) occurs. In order to determine the buoyant density of *Triturus* DNA, gradients loaded with equal amounts of *Triturus* and *Micrococcus luteus* marker DNA (30 μg each) were analysed. From these tracings the buoyant density of *Triturus* main band DNA was estimated with 1.702 g/ml. With respect to this value the buoyant density of the shoulder on the lighter side is 1.686 g/ml and that of the stage-specific one on the heavy side of the gradient is 1.719 g/ml. According to the formula of Schildkraut, Marmur & Doty (1962) the calculated GC contents of the main band, the light and the heavy shoulder are 43%, 27% and 60%, respectively.

**DISCUSSION**

As shown by the thermal denaturation curves, significant changes in the melting behaviour of DNA occur during gastrulation of *Triturus vulgaris*. The deviation from the normal shape and the increase of the Tm value is caused by
the fact that a portion of DNA melts at higher temperatures than usual. From the stage-specificity of the curves we have to conclude that the amount of the late melting fraction increases from the early gastrula up to the yolk-plug stage and becomes reduced in the following stages down to the normal level. There are essentially two possibilities to explain the occurrence of a late melting fraction.

First, by selective methylation of DNA. For instance Dawid, Brown & Reeder (1970) have shown that chromosomal rDNA has a higher thermal stability than amplified rDNA which contains no 5-methyl cytosine. Our results pose the old question of whether DNA modifications are generally involved in cell differentiation, as discussed by Scarano (1971) and Holliday & Pugh (1975).

Secondly, by amplification of DNA sequences, possessing a higher GC-content than bulk DNA.

In comparison with the melting curves, by ultracentrifugation only slight differences in the gradient patterns have been found between the stages studied. Since it is well known that thermal satellites do not always appear as satellite fractions in CsCl density gradients, and because we cannot decide whether and to what extent methylation of DNA participates in the melting behaviour, this point will not be discussed further. The small number of measurements at lower temperatures, when AT-rich sequences denature preferentially, accounts for the fact that the permanently present AT-rich satellite fraction, shown in the density gradient patterns, has not been visualized in the melting profiles.

The occurrence of the shoulder on the heavy side of the gradient, however, gives evidence for the existence of a stage-specific DNA fraction with a higher density than main band DNA. Then, the appearance of a heavy satellite fraction cannot be caused by methylation of DNA, because this phenomenon reduces the buoyant density of DNA. On the other hand this argument includes the
possibility that this fraction is relatively under-methylated as a result of DNA synthesis in the absence of methylation. But this effect could not account for the differences in the melting curves. Therefore we think that the presented changes in the melting behaviour together with the differences in the density gradient patterns can be plausibly explained by differential DNA replication, precisely by a stage-dependent amplification of GC-rich DNA sequences.

These results confirm our earlier studies in which we postulated that the increase of nuclear DNA content in the late gastrula is at least partly caused by amplification of GC-rich sequences, possibly rDNA (Lohmann, 1972; Lohmann & Schubert, 1977). Filter hybridization experiments with radioactive ribosomal RNA from HeLa cells (done very recently by Schubert (in preparation)), show that the stage-specific satellite fraction contains amplified rDNA to only a small extent. The function of the major component of the amplified DNA is not known, but there is much indication that the stage-dependent increase of DNA apparently serves as an enhancement of the transcription capacity in definite regions of the embryo. Then, with a phase-shift of about 2 h the DNA amplification during gastrulation and neurulation is followed by a remarkable short-term increase of the nuclear RNA and non-histone protein content (Lohmann & Jansen, 1976; Lohmann, 1979).

With respect to the relative amount of the additional DNA fraction, there is a quantitative discrepancy between the cytophotometric measurements and the presented findings. Since a correct estimation of the proportion of GC-rich sequences is not possible from the gradient profiles shown in this paper, we reproduced some of our experiments using a Gilford spectrophotometer (Type 250) equipped with a density gradient scanner. These tracings, which have been recorded without changing the absorption range, show, besides the permanently present AT-rich fraction (about 10 % of total DNA), a distinct GC-rich shoulder of about 3–4 % of total DNA. This amount is obviously low compared to the above-mentioned changes in the Feulgen–DNA content (ranging between 15 and 35 % in various regions of the embryo). If we consider that the Feulgen values reflect changes in DNA content (this point has been discussed in detail by Lohmann, 1975), then the cited discrepancy gives indirect evidence that not only GC-rich sequences are amplified during early *Triturus* development but also, and to a greater extent, DNA sequences with a mean GC-content similar to the main band DNA.

While it is generally accepted that gene amplification is a common mechanism during oogenesis, the occurrence of selective gene amplification in proliferating somatic cells is still a very disputed question (e.g. Tobler, 1975; Wintersberger, 1978). Since the amplified DNA is accumulated for a period of a few cell cycles (in neurula cells the S-phase lasts about 4 h (Callan, 1973)) there has to be a mechanism that prevents the dilution of (extrachromosomally?) amplified DNA in the following mitoses, possibly by a temporal limited integration into the genome. Evidence for the existence of such a mechanism comes also from recent
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studies on ribosomal gene amplification in mitotically dividing spermatogonia and oogonia of *Xenopus* (Bird, 1978) and on the ribosomal magnification in *Drosophila melanogaster* (Graziani, Gaizzi & Gargano, 1977). In both cases extra-chromosomal rDNA rings can be integrated into the chromosome. Furthermore, Alt, Kellems, Bertino & Schimke (1978) could show that in methotrexate-resistant lines of Sarcoma 180 murine lymphoma cells the increased synthesis of dihydrofolate reductase is associated with a proportional increased number of copies (200–250-fold) of the dihydrofolate reductase structural genes. These findings indicate that in proliferating cells the phenomenon of gene amplification is not restricted to the ribosomal RNA genes, but that the machinery required to amplify structural genes exists. In summary, we would like to interpret our results as consistent with the concept that the genetic information in the eukaryotic genome is not statically fixed, but undergoes dynamic changes in the course of embryonic differentiation.

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REFERENCES


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