STUDIES ON THE TEMPLATE ACTIVITY OF 'ISOLATED' XENOPUS ERYTHROCYTE NUCLEI

I. THE EFFECTS OF IONS

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SUMMARY
The isolation and characterization of nuclei from Xenopus erythrocytes is described. Such nuclei are freely permeable to ions and large molecules. They have an extremely low basal rate of RNA synthesis which can be increased by the addition of Escherichia coli RNA polymerase and by alteration in the ionic strength of the incubation medium within physiological limits. The results are discussed in terms of the mechanisms which control gene activity in these cells.

INTRODUCTION
The demonstration of RNA synthesis in chicken erythrocyte nuclei, following their incorporation into heterokaryons with active cells (Harris, 1967), has led to various attempts to 'reactivate' isolated erythrocyte nuclei by incubation in cytoplasmic extracts (Thompson & McCarthy, 1968; Leake, Trench & Barry, 1972). Work along these lines with chicken erythrocyte nuclei in this laboratory was abandoned in favour of nuclei from Xenopus erythrocytes since the base rate of RNA synthesis is much lower in the latter (Madgwick, Maclean & Baynes, 1972; Maclean, Hilder & Baynes, 1973). We report here on the isolation and characterization of Xenopus erythrocyte nuclei, and on the effects of various ionic solutions on their basal rate of RNA synthesis. We use the term 'isolated nuclei' for these preparations since they are essentially free from cytoplasm, but, it should be noted, the nuclei are surrounded by a damaged plasma membrane. We have studied the effect of ionic concentration of the incubation medium on the rate of RNA synthesis of these nuclei in some detail, not only because these are essential controls for subsequent attempts to 'reactivate' the nuclei, but also because the nuclear ionic concentration has been proposed as a major factor in determining specific gene activity (Kroeger, 1963; Lezzi, 1970).

MATERIALS AND METHODS
Animals
Mature Xenopus laevis were obtained from Harris' Biological Supplies (Weston-super-Mare) and maintained as previously described (Maclean & Jurd, 1971).
Chemical reagents

Tritiated reagents were obtained from the Radiochemical Centre (Amersham), other reagents from British Drug Houses Ltd (Poole) except where stated.

Preparation of nuclei

Blood was obtained by ventricular puncture as previously described (Maclean & Jurd, 1971) and washed twice in 50 vol. Rugh's amphibian Ringer (Rugh, 1962). Of the various methods of nuclear preparation tried, the most effective, in terms of yield and integrity of product, was to resuspend the erythrocytes in 100 vol. of 0.05% saponin in Nuclear Suspension Medium (NSM: 0.25 M sucrose, 50 mM Tris, 100 mM KCl, 5 mM MgCl₂, adjusted to pH 7.4 with HCl) agitate gently for 1 min, and centrifuge at 500 g for 5 min. The pellet of nuclei was washed twice in 100 vol. NSM and the final pellet resuspended in a buffered sucrose solution containing ions to the concentration required for the experiment.

In vitro ³H-UTP incorporation

Cultures of approximately 10⁸ nuclei/ml in an incubation medium containing 0.25 M sucrose, 50 mM Tris/HCl (pH 7.4), 0.125 mM each of ATP, GTP, and CTP, and 0.05 mCi/ml ³H-UTP from a stock labelled 1 Ci/mmol, and cation chlorides to the requirement of the experiment, were gently agitated at 25°C. E. coli ribonucleic acid polymerase (Sigma Chemical Corp., London), where included, was added at 5 units/ml. Samples of 0.1 ml were taken at various time intervals, applied to Whatman 3-mm filter paper disks and processed according to the method of Mans & Novelli (1961) to remove TCA-soluble counts. Radioactivity was assayed by scintillation counting of the disks as previously described (Maclean et al. 1973).

Determination of DNA, RNA and protein

Samples were extracted by the Schneider technique and assayed for DNA, RNA and protein by the diphenylamine, orcinol and Lowry reactions, respectively (Hutchison & Munro, 1961).

Flame photometry

Samples for flame photometry were briefly washed in a medium in which lithium was the only monovalent cation and completely oxidized with boiling concentrated nitric acid. The acid was evaporated off and the residue taken up in a known volume of double-distilled water. Sodium and potassium contents were measured on an Evans Electroselenium Model A flame photometer against known amounts of sodium and potassium chloride treated in the same way as the samples.

Cell volume was estimated from packed cell volume and microscopic measurement, and cell water content calculated from measurements of wet and dry weight.

RESULTS AND DISCUSSION

Nuclei prepared by the method described appear under phase-contrast to be unswollen, with condensed chromatin, and retaining their partly collapsed cell membranes (Fig. 5). They have some 24% less RNA and 95% less protein than whole cells, the DNA content remaining the same. Almost all the haemoglobin appears to be lost (Table 1); the residual absorption at 410 nm may represent some remaining intranuclear haemoglobin, as observed by Davies (1961).

Despite the presence of the ruptured plasma membrane, the nuclei are freely permeable to large molecules such as trypan blue (mol. wt. 960) and fluorescein-conjugated bovine serum albumin (mol. wt. 60000) which are excluded by the intact membranes.
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of whole cells (Figs. 6, 7). Several instances of the movement of protein into the nuclei of intact cells are discussed by Gurdon (1970).

They also appear to be freely permeable to cations, since the measured ionic concentration of the whole cell resembles that of other amphibian tissues (Table 2), whilst that of the isolated nuclei reflects that of the suspension medium (Table 3). Whilst the nuclei may not be so freely permeable in the intact cell, the observation

Table 1. Nucleic acid, protein and haemoglobin ratios of Xenopus erythrocytes and their ‘isolated’ nuclei

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
<th>A_{410 nm}</th>
<th>mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>3'33</td>
<td>0'0179</td>
<td>1'180</td>
<td>118'0</td>
<td>0'6</td>
</tr>
<tr>
<td>Nuclei</td>
<td>4'40</td>
<td>0'3012</td>
<td>0'6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values given are the means of 8 determinations.

* Haemoglobin absorption maximum.

Table 2. Sodium and potassium concentrations in whole Xenopus erythrocytes

<table>
<thead>
<tr>
<th>K, mequiv/l. cell water</th>
<th>Na, mequiv/l. cell water</th>
</tr>
</thead>
<tbody>
<tr>
<td>114 ± 6</td>
<td>20'6 ± 2'6</td>
</tr>
</tbody>
</table>

Means ± S.E. of 8 determinations are given. Water makes up 62 ± 2 % of the cell wet weight.

Table 3. Permeability of ‘isolated’ Xenopus erythrocyte nuclei to ions

<table>
<thead>
<tr>
<th>Medium</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>K Na</td>
<td>K Na</td>
</tr>
<tr>
<td>mequiv/l.</td>
<td>mequiv/unit vol.</td>
</tr>
<tr>
<td>200 0</td>
<td>3'4 &lt; 0'1</td>
</tr>
<tr>
<td>150 50</td>
<td>2'1 0'8</td>
</tr>
<tr>
<td>100 100</td>
<td>1'0 1'0</td>
</tr>
<tr>
<td>50 150</td>
<td>0'3 1'2</td>
</tr>
<tr>
<td>0 200</td>
<td>&lt; 0'1 1'7</td>
</tr>
</tbody>
</table>

Nuclei were incubated for 5 min in media containing sodium and potassium to the concentrations shown and subjected to flame photometry as described in the text.

of nucleo-cytoplasmic continuity in nucleated erythrocytes (Davies, 1961) suggests that these nuclei could not maintain large ionic concentration gradients with the cytoplasm such as have been reported for the nuclei of various other cells (e.g. Langendorf et al. 1961; Century, Fenichel & Horowitz, 1970).

Thus, the in vivo nuclear ionic concentration may be expected to be near that of the cytoplasm: incubation of the ‘isolated’ nuclei in a medium with cations to approximately this concentration results in a rate of RNA synthesis of 1'35 ± 0'25 pmol/mg DNA/h, which is maintained for at least 2 h (Fig. 1, ●). This is slightly higher than
the rate observed in whole cells (Maclean et al. 1973), the difference being probably due either to the loss of some controlling factor during the isolation of the nuclei, or to limited permeability or dilution of labelled uridine triphosphate by an internal pool in the whole cells.

![Graph](image)

**Table 4. Effect of ribonuclease treatment (incubation for 1 h at 30°C with 1 mg/ml RNase I-A (Sigma Chemical Corp.) of samples prior to application to disks, or omission of one of the nucleoside triphosphates from the incubation medium, on **3**H-UTP incorporation by `isolated' Xenopus erythrocyte nuclei**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ RNase</th>
<th>− CTP</th>
<th>− ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/10^7 nuclei</td>
<td>620</td>
<td>43</td>
<td>88</td>
<td>66</td>
</tr>
</tbody>
</table>

The rate of RNA synthesis can be considerably increased by the addition of exogenous RNA polymerase (Fig. 1, O), despite the presence of large amounts of endogenous polymerase in these cells (Tata, personal communication). A similar phenomenon has been observed in chicken erythrocyte nuclei (Madgwick, 1973). Thus, whilst the rate of synthesis in these cells does not appear to be limited by the overall level of RNA polymerase, the endogenous polymerase cannot be active on all the available template, and the major part of it must be in some way inactivated in vivo.

That the measured UTP is indeed incorporated into RNA is shown not only by the failure of repeated washing with TCA to remove the counts, but also by the virtual
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Fig. 2. Effect of monovalent cation concentration on $^3$H-UTP incorporation in 'isolated' nuclei. ▲—▲, KCl; ○—○, LiCl. Mean ± S.E. of 4 experiments done in the presence of 10 mM MgCl$_2$, 5 mM MnCl$_2$. Curves for NaCl and 1:1 NaCl:KCl lie over that of KCl.

Fig. 3. Effect of divalent cation concentration on $^3$H-UTP incorporation in 'isolated' nuclei. ●—●, MnCl$_2$; □—□, MgCl$_2$; ○—○, CaCl$_2$; ■—■, CoCl$_2$; all in the presence of 100 mM KCl.

abolition of counts following ribonuclease treatment or in cultures from which one of the nucleoside triphosphates has been omitted (Table 4).

Incubation in a medium of higher ionic strength, based on that reported to occur in rat liver nuclei, i.e. 140 mM K, 60 mM Na, 12 mM Mg, and 2 mM Ca (Langendorf et al. 1961), results in a much higher rate of RNA synthesis, both in the presence and absence of exogenous RNA polymerase (Fig. 1, ■ and □). In such a medium the
nuclei undergo a marked conformational change; their volume increases by about 65% and they become much less refractile, reflecting some decondensation of their chromatin (Figs. 8, 9). As in the chicken erythrocyte nucleus (Leake et al. 1972), a similar swelling and decondensation can be produced by media of very low ionic concentration. These changes are readily reversible by returning to the normal medium.

Fig. 4. Effect of magnesium concentration on $^3$H-UTP incorporation in 'isolated' erythrocyte nuclei, in the presence of 100 mM (●—●) and 200 mM (○—○) KCl. Mean ± s.e. of 4 experiments.

Such nuclear swelling and chromatin dispersal have been implicated in many cases of nuclear reactivation. It cannot, however, be solely responsible for the increase in activity in this case, since the conformational change can be produced by conditions of low ionic strength or by a high concentration of lithium, sodium or potassium, but the increase in RNA synthesis is observed only in the latter two cases (Fig. 2).

Of the other ions which we have studied, magnesium and manganese have the most interesting effect on RNA synthesis, both displaying clear optimal concentrations (Fig. 3). The effect of any particular ion is to some extent dependent on the level of other ionic species present. Thus, as shown in Fig. 4, the effect of high concentrations of magnesium ions is considerably influenced by the concentration of potassium.

The ionic species which most markedly affect the rate of RNA synthesis in these nuclei are precisely those which, at similar concentrations, profoundly affect the rate of the RNA polymerase reaction using purified DNA as a template. We believe, therefore, that the cation-mediated modulation of the rate of RNA synthesis takes place at the level of the efficiency with which the polymerase acts on the already available template, rather than being due to any alteration in template availability as would be required for specific gene reactivation, although this latter possibility is not conclusively excluded.
CONCLUSIONS

*Xenopus* erythrocyte nuclei can be isolated by a rapid technique which appears to leave them little changed from their condition in the cell. They are freely permeable to ions and large molecules. On incubation in the presence of RNA precursors in a medium believed to approximate in cation concentration to that of the cytoplasm which normally surrounds them, they show a very low rate of RNA synthesis. This rate can be increased by the addition of exogenous RNA polymerase and by altering the ionic concentration within physiological limits.

This alteration of the rate of synthesis does not, however, lend support to the idea that specific gene activity can be determined by the intranuclear ionic concentration. These results are more easily explained in terms of an effect on the efficiency of the RNA polymerase reaction.

The control of gene expression in eukaryotic cells is likely to be effected at various levels and it seems that the permeability of the nuclei in these cells is involved in the general rundown of synthetic activity by leading to an unfavourable environment for transcription, beyond which there are the mechanisms involved in specific switching off of genes. It is for an effect at this latter level that we are now assaying the effect of various cytoplasmic extracts on these nuclei.

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REFERENCES


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Fig. 5. 'Isolated' Xenopus erythrocyte nuclei suspended in NSM. Phase-contrast micrograph. × 1000 approx.

Fig. 6. 'Isolated' Xenopus erythrocyte nuclei suspension in the presence of 0.1% trypan blue. Bright-field micrograph. × 1000 approx.

Fig. 7. 'Isolated' Xenopus erythrocyte nuclei following 5 min incubation with 4% fluorescein isothiocyanate-labelled bovine serum albumin in NSM and a brief wash in NSM prior to fixation. Dark-field fluorescence micrograph. × 1000 approx.
Fig. 8. 'Isolated' *Xenopus* erythrocyte nuclei suspended in NSM. Phase-contrast micrograph. ×1000 approx.

Fig. 9. 'Isolated' *Xenopus* erythrocyte nuclei, from the same preparation as Fig. 8, suspended in modified NSM containing 200 mM KCl, 12 mM MgCl₂, 5 mM MnCl₂, and 2 mM CaCl₂. Phase-contrast micrograph. ×1000 approx.