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

II. THE EFFECTS OF CYTOPLASMIC EXTRACTS

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SUMMARY

The effects of exposing nuclei isolated from Xenopus erythrocytes to cytoplasmic extracts prepared from various sources on their template activity are described. The cytoplasm of rat liver cells and the immature blood cells of Xenopus contain factors which stimulate RNA synthesis in these essentially inactive nuclei, whereas the cytoplasm of many other cells does not yield such factors. The active factors in rat liver cytoplasmic extracts appear to be proteins of molecular weight about 40,000 Daltons. The implications of these results for genetic control in eukaryotes are discussed.

INTRODUCTION

Experiments with artificially formed heterokaryons (e.g. Harris, 1970) and nuclear transplantation (e.g. Gurdon & Laskey, 1970) have demonstrated the importance of factors located in the cytoplasm in the control of nuclear genetic activity. This has led to attempts to 'reactivate' the relatively inactive nuclei isolated from chicken erythrocytes by exposing them to extracts of cytoplasm from 'active' cells (Thompson & McCarthy, 1968; Leake, Trench & Barry, 1972). Whilst apparent 'reactivation' has been achieved, little information concerning the nature of the factors responsible has been gained.

We have found the endogenous activity of chicken erythrocytes and the nuclei isolated from them to be unsatisfactorily high (Madgwick, Maclean & Baynes, 1972) and consider the erythrocytes of Xenopus to be a more suitable subject for reactivation studies (Maclean, Hilder & Baynes, 1973). We have established the characteristics of the low basal level of template activity in the 'isolated nuclei' (a term used here to denote nuclei which are essentially free of cytoplasm but which retain a damaged, freely permeable, cell membrane) of Xenopus erythrocytes (Hilder & Maclean, 1974) and report here on the effects of cytoplasmic extracts from various sources on this activity.
MATERIALS AND METHODS

Animals

Adult *Xenopus laevis* (Daudin) were obtained from Harris' Biological Supplies (Weston-super-Mare) and maintained as previously described (Maclean & Jurd, 1971). Eggs and embryos were obtained from these animals using the methods of Brown (1970), and anaemia induced by injection of phenylhydrazine according to the method of Thomas & Maclean (1975).

Male Wistar rats, of approximately 100 g body weight, were bred in the University.

Preparation of erythrocyte nuclei

Blood, obtained by ventricular puncture under anaesthesia induced by MS222 (Sandoz Products Ltd, London), was washed in amphibian Ringer solution (Rugh, 1962); nuclei prepared by suspension of the erythrocytes in nuclear suspension medium (NSM - 0.25 M sucrose, 50 mM Tris, 100 mM KCl, 5 mM MgCl₂, 5 mM MnCl₂, pH 7.4) containing 0.05% saponin and washed in NSM as previously described (Hilder & Maclean, 1974).

Preparation of cytoplasmic extracts

Tissues were homogenized 1:1 w/v in NSM with up to ten strokes in a hand homogenizer (Thomas, Philadelphia). The resulting mixture was centrifuged at 500 g for 5 min to remove gross debris and the supernatant spun at 105000 g for 30 min. The soluble cytoplasmic fraction was removed from the pellet and the overlying material, and recentrifuged, if necessary, to produce a clear preparation. These operations were carried out at 0-4 °C wherever possible. The protein concentrations of the cytoplasmic extracts were usually adjusted to 10 mg/ml with NSM before use.

Liver cytoplasm was prepared from the sliced, washed liver of freshly killed rats or *Xenopus*. *Xenopus* eggs and embryos were dejellied, by gently agitating them in 0.1% papain, 2% cysteine-HCl, pH 7.8, followed by 2 washes in Ringer solution (Rugh, 1962) before homogenization.

Measurement of template activity

Unless the experiment demanded otherwise, 1 ml of a suspension containing 10⁸ nuclei in NSM was mixed with 1 ml of NSM or a cytoplasmic extract and incubated for 15 min at 25 °C. The nuclei were pelleted by centrifugation, washed twice in 100 vol. NSM, and incubated at a concentration of 10⁷/ml of NSM containing 0.125 mM each ATP, GTP, CTP, 0.05 mCi 5-^3^H-UTP from a stock labelled 2 Ci/mM (Radiochemical Centre, Amersham) and 5 units *E. coli* RNA polymerase (Sigma Chemical Corp., London). Duplicate samples of 0.1 ml were taken at zero time and after 30 min incubation applied to Whatman 3-MM filter paper disks and processed with trichloroacetic acid to remove unincorporated radioactivity prior to liquid scintillation counting as previously described (Maclean et al. 1973).

Enzyme assays

Ribonuclease was assayed for by the production of acid-soluble nucleotides from RNA by the method of Klee & Richards (1957); deoxyribonuclease by the spectrophotometric method of Kunitz (1950); protease by the production of acid-soluble Folin-positive products from denatured haemoglobin method of Anson (1938) and RNA polymerase by the incorporation of ^3^H-UTP into acid-insoluble products based on the method of Burgess (1969). In each case the buffer system used was NSM. The definition of unit activity of these respective authors was used.
Characterization of transcripts

RNA was extracted from the nuclei after incubation with labelled precursor by the cold phenol/DNase method of Knowland (1970) and analysed on polyacrylamide gels as previously described (Maclean et al. 1973).

RESULTS

A cytoplasmic extract from rat liver has been reported to induce the reactivation of isolated nuclei from chicken erythrocytes (Leake et al. 1972), results which have been successfully repeated in our own laboratory (Madgwick, 1973). Exposure of Xenopus erythrocyte nuclei to preparations from this source resulted in a significant increase in the subsequently measured incorporation of UTP (Table 1). It is on these prepara-

Table 1. Effect of exposing 'isolated' erythrocyte nuclei to cytoplasmic extracts on their rate of RNA synthesis

<table>
<thead>
<tr>
<th>Source of cytoplasm</th>
<th>Rate of UTP incorporation into nuclei relative to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Xenopus</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>O.54 1.12 2.53 1.20 0.45</td>
</tr>
<tr>
<td>Liver (anaemic frogs)</td>
<td></td>
</tr>
<tr>
<td>Liver (metamorphosing tadpoles)</td>
<td></td>
</tr>
<tr>
<td>Oocytes</td>
<td>O.52</td>
</tr>
<tr>
<td>Unfertilized eggs</td>
<td>O.45  O.52</td>
</tr>
<tr>
<td>Blastulae</td>
<td>O.65  O.68</td>
</tr>
<tr>
<td>Gastrulae</td>
<td>O.71  O.73</td>
</tr>
<tr>
<td>Neurulae</td>
<td>O.85  1.03</td>
</tr>
<tr>
<td>Erythroid cells (anaemic frogs)</td>
<td>4.70  4.85</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5.59  14.22 10.15 10.02 7.30</td>
</tr>
</tbody>
</table>

Values are the means of 2 determinations of the rate of TCA-insoluble UTP incorporation following preincubation in a cytoplasmic preparation, relative to that of nuclei preincubated in NSM. Over 95% of parallel 'control' incubations fell within the range of 0.87–1.15.

ations that most of the following results are based, but we have recently found that preparations of cytoplasm from the circulating erythroid cells of anaemic Xenopus, at a stage when they are known to be active in RNA synthesis (Thomas & Maclean, 1975), have similar reactive properties: we intend to discuss these results in a later publication after further experimentation.

Experiments using cytoplasmic extracts from Xenopus liver have also been undertaken but the effects of these preparations are rather variable. We attribute this chiefly to the difficulties involved in consistently homogenizing so tough a tissue as Xenopus liver. We have therefore concentrated on rat liver cytoplasm in our investigations.
We also examined the effects of cytoplasmic extracts prepared from cells at various points in the embryonic series of *Xenopus*, since at these times profound and partially characterized changes in gene expression take place (e.g. Bachvarova, Davidson, Allfrey & Mirsky, 1966). The effect of these preparations was consistently inhibitory, the degree of apparent inhibition declining as development proceeded. We consider that this is most readily interpreted in terms of something in the cytoplasm binding or inactivating one or more of the components of the transcription reaction other than the template. The possibility that significant amounts of ribonuclease might be carried over from the cytoplasm into the incubation was considered, but assays for RNase activity in the cytoplasm indicated that there was insufficient to account for the reduction of radioactivity in the nuclei exposed to cytoplasm compared to controls.

![Graph](image)

**Fig. 1.** $^3$H-UTP incorporation into isolated *Xenopus* erythrocyte nuclei following preincubation in a rat liver cytoplasmic extract (O) or in NSM (●).

The isolated nuclei of *Xenopus* erythrocytes do not swell in our rat liver cytoplasmic extracts, nor is there any visible decondensation of their chromatin. RNA synthesis following such exposure is linear for at least an hour, at a rate of approximately 1 nmol UTP/mg DNA/h, compared to about 0.1 nmol UTP/mg DNA/h in control nuclei (Fig. 1). The much lower rate of RNA synthesis in the absence of added polymerase (Hilder & Maclean, 1974) was also increased following exposure to rat liver cytoplasmic extracts, though only by two to three times, up to about 4 pmol UTP/mg DNA/h and maximal stimulation occurred at a much lower concentration of cytoplasm than when exogenous polymerase was added.

In order to allow a more accurate comparison of preparations and to facilitate purification of the active factor/s a standard assay for 'reactivating activity' was devised. Since the apparent activity of the preparations varies non-linearly with certain pre-
incubation conditions (Fig. 2) this assay procedure was kept constant at the conditions specified in the Methods section.

The activity of the rat liver cytoplasmic extract is retained by a dialysis membrane, as shown by extensive overnight dialysis of the cytoplasm against NSM and by separating the nuclei from the cytoplasm by a dialysis membrane during the pre-

**Fig. 2.** Effect of preincubation conditions on the enhancement of \(^3\)H-UTP incorporation into isolated *Xenopus* erythrocyte nuclei by rat liver cytoplasmic extracts. In each case the ordinate, which is a measure of the extent to which 'reactivation' occurred, represents the cpm/\(10^7\) nuclei/30 min in nuclei preincubated in cytoplasm minus the rate in nuclei preincubated in NSM. Preincubation conditions were as described in the Methods section except for the variable under investigation. A, concentration of cytoplasm; B, concentration of nuclei; C, preincubation time; D, preincubation temperature. •, freshly prepared cytoplasm; ○, cytoplasm stored at −20 °C for 8 weeks.
Fig. 3. Ammonium sulphate fractionation of rat liver cytoplasmic extracts. ---, reactivating activity; ---, protein content. Means of 4 determinations.

Fig. 4. Fractionation of rat liver cytoplasmic extract on a Sephadex G-200 column; 0.8 ml of cytoplasm was applied to a 90 x 1.5 cm column and eluted with NSM. The positions at which the marker proteins eluted are indicated: BSA, mol. wt 64 000; ovalbumin (ova), 40 000; chymotrypsinogen (chy), 25 000; and cytochrome (cyt), 12 500. ---, reactivating activity; ---, A_365 nm. The shaded portion represents the 'active fraction' on which enzyme assays were carried out.
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The activity is recoverable from the material precipitated from crude cytoplasmic extracts by ammonium sulphate in the 60-80% saturated range (Fig. 3). The macromolecular nature of the active factor(s) is confirmed by gel filtration on columns of Sephadex (Pharmacia (G.B.) Ltd, London). The reactivating activity is excluded from columns of Sephadex G-25 and eluted from columns of G-200 in the region of globular proteins of molecular weight 35-40 k Daltons (Fig. 4).

The activity is relatively thermolabile (Table 2), inactivation occurring at a lower temperature than would be expected if it were due to a hydrolytic enzyme or to DNA. It is, however, relatively stable at low temperatures, withstanding repeated rapid freezing and thawing and, indeed, increasing in activity on storage at —20 °C for 2 months (Fig. 2A).

The activity of the preparations was destroyed by treatment with a wide spectrum protease bound to an insoluble base (insoluble protease – Sigma Chemical Corp., London) (Table 3). The small decrease in activity after treatment with insolubilized protease which is seen in Table 3 is not surprising in view of the high proportion of introns in the transcripts of mammalian DNA. The reactivating activity is thus non-specific, and is not destroyed by treatment with RNase and cytoplasmic extracts.
ribonuclease may represent some contribution by RNA to the reactivating activity but is more probably due to the carriage of a small amount of the added RNase over into the incubation, since the agarose-bound RNase was much less readily pelleted than the CM-cellulose-bound protease.

We have examined the crude cytoplasmic extracts and the active fractions recovered from Sephadex G-200 columns for certain enzymic activities which might non-specifically activate the template. We cannot attribute the reactivating activity to RNase, DNase, protease or RNA polymerase activity (Table 4). The most serious concern must be that these preparations contain a low level of a specific endonuclease which might introduce a small number of nicks into the template which remain undetected in our assay. If such an activity was present, however, one might suppose that it had an important role in regulating template activity in its native cells.

Table 4. Enzymic activity of rat liver cytoplasmic extracts and the reactivating fraction recovered from Sephadex G-200 columns (Fig. 4)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (u./ml)</th>
<th>Crude cytoplasm</th>
<th>Sephadex G-200 active fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase</td>
<td>$4 \times 10^{-3}$</td>
<td>$&lt; 1 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>$&lt; 1 \times 10^{-1}$</td>
<td>$&lt; 1 \times 10^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>$30$</td>
<td>$&lt; 0.1$</td>
<td></td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>$&lt; 0.1$</td>
<td>$&lt; 0.1$</td>
<td></td>
</tr>
</tbody>
</table>

In each case the figure in the right-hand column represents the limit of sensitivity of our assay.

The nature of the RNA transcripts following reactivation was investigated by polyacrylamide gel electrophoresis of purified RNA followed by scintillation counting of gel slices. We are continuing to work on this important aspect of the system, and will publish the main findings in a separate paper. We can briefly state that there is no detectable synthesis of ribosomal RNA and that most of the transcripts are of low molecular weight, with a major peak separating at about 4 s.

**DISCUSSION**

We are now primarily concerned with the cytoplasmic preparations derived from rat livers and anaemic *Xenopus* erythroid cells, but there are one or two points concerning the earlier work which deserve comment. The failure of cytoplasmic extracts from certain sources to exert any detectable effect on the rate of RNA synthesis in isolated nuclei suggests that we are not merely observing a non-specific effect which might result from the exposure of these nuclei to any complex mixture of proteins, etc. Some cytoplasmic preparations had an opposite effect to that which we were seeking, i.e. resulted in a decreased rate of UTP incorporation in nuclei following exposure. We consider it to be most unlikely that these preparations could contain anything which would reduce the template activity of a nucleus as inert as that of the
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Xenopus erythrocyte, but rather attribute the reduction in RNA synthesis to the presence of factors which inactivate other components of the transcription reaction.

Whilst we consider the above hypothesis adequate to explain the results obtained with Xenopus embryonic cytoplasms, it is of interest to note that a specific repressor of the ribosomal cistrons has been isolated from the cytoplasm of Xenopus oocytes (Crippa, 1970), and that ribosomal RNA synthesis does not recommence until after gastrulation (Brown & Littna, 1964). The nature of the RNAs synthesized in our nuclei under a variety of conditions is currently under intensive investigation, but so far our evidence suggests that there is no significant contribution to it by the ribosomal cistrons.

The rate of RNA synthesis remains constant over a prolonged period in our system compared to many isolated nuclear preparations. This is probably due to the large amounts of exogenous RNA polymerase which we use permitting repeated initiation. The use of bacterial polymerase in eukaryotic systems has been criticized as the initiation specificities of the endogenous and exogenous enzymes, although related, do not precisely correspond (see, e.g. Reeder, 1973; Wilson, Steggles & Nieuhius, 1975). Since, however, we are simply concerned, in this study, with comparing the extent to which transcription occurs in treated and control nuclei, we consider the use of the bacterial enzyme to be justified.

The kinetics of the interaction between isolated nuclei and cytoplasmic preparations are complex and the curves presented in Fig. 2 probably reflect the interaction of a number of multicomponent systems. One of the simplest models to give a qualitative fit to these curves is based on the assumption that the cytoplasm contains both an activator, which binds to certain sites in the chromatin of the nuclei, thereby increasing the rate of transcription, and an antagonist of this activator which binds at or close to these sites, negating the effect of the activator, but also binds reversibly to a number of other sites where it has no detectable effect. The importance of non-specific binding sites for genetic regulatory elements has been discussed by Von Hippel, Revzin, Gross & Wang (1974). The antagonist is presumed to be less stable than the activator. Thus, the extent of reactivation with increasing cytoplasm concentration follows a sigmoid curve with freshly prepared cytoplasm, but after storage at —20°C this converts to a curve which is reducible to a straight line on a double-reciprocal plot and suggests a decreasing amount of a non-competitive inhibitor as storage is prolonged (Fig. 2A). The deviation of the curve in Fig. 2B from the directly proportional decline with increasing numbers of nuclei, which would be expected at a concentration of cytoplasm which appears to be limiting, would be explained by an increase in the number of sites at which non-specific binding of the antagonist could occur with a consequent decrease in the number of antagonists available to bind to the sites in terms of the progressive relocation of the antagonist from non-specific sites to sites occupied by the activators, assuming that its affinity for the latter was higher than for the former. Fig. 2D illustrates that there is no sharp transition temperature for the interaction between nuclei and cytoplasm: such a transition would indicate that ‘melting’ of the chromatin was involved (see Travers, Baillie & Pedersen, 1973).
Cytoplasm is certain to be a complex mixture and the activator/antagonist model finds some support from our preliminary attempts to fractionate the cytoplasmic extracts. When such extracts are applied to Sephadex columns they yield fractions with an apparent activity greater than the total applied to the column.

The thermolability of the activity fits well with the idea of a rapidly turning-over activator in the liver which would, therefore, need to be continuously synthesized in the cytoplasm to maintain an effect.

It is of particular interest to compare these results with those obtained by Leake et al. (1972) using a very similar cytoplasmic extract on chicken erythrocyte nuclei. The extent of the apparent reactivation is similar in the 2 systems, but they differ in a number of significant respects. The extent to which these differences are due to differences in the preparative techniques or to differences in the assay system, in particular to the greater degree of template restriction in the Xenopus erythrocyte, are unknown. No reactivation was detected in these earlier studies unless the free ionic concentration of the preincubation medium was sufficiently low to cause swelling of the chicken erythrocyte nuclei, whereas we were unable to detect any swelling of the Xenopus nuclei under conditions which led to enhanced RNA synthesis. We have shown previously that gross chromatin decondensation does not automatically imply increased template activity in this system (Hilder & Maclean, 1974) and this result illustrates that the converse is also true. It is, however, possible that highly localized decondensation of the chromatin was required for reactivation to occur.

The active factor(s) in the cytoplasmic fractions studied by Leake et al. was considerably less stable than that which we are studying, being lost on dialysis and on freezing and thawing. The relative stability of our activity at low temperatures offers considerable hope for its further purification and characterization.

CONCLUSIONS

We conclude that the cytoplasm from liver cells of young rats and from the immature blood cells of anaemic Xenopus contains factors capable of stimulating RNA synthesis in essentially inactive nuclei. The cytoplasm of many other cells does not yield such factors. We are now virtually certain that these factors are proteins and they are perhaps examples of the regulatory proteins widely believed to control eukaryotic gene expression. Since these factors are relatively stable we are currently following up 2 main lines of enquiry: (1) the further purification and characterization of these factors; and (2) analysis of the types of RNA which are transcribed during the reactivation process.

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REFERENCES


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