EFFECT OF CYTOSOL ON DNA SYNTHESIS IN ISOLATED MAMMARY GLAND NUCLEI FROM RABBITS DURING PREGNANCY AND EARLY LACTATION

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The in vitro DNA synthesis of nuclei isolated from rabbit mammary gland was stimulated by a cytosol fraction from the same tissue. Time-course of this phenomenon was followed during rabbit pregnancy at 5-day intervals. The stimulation was shown to be physiological state-dependent in that it could be detected only in the case of cytosols prepared on day-10 of pregnancy and from day-20 or 25 of pregnancy through day-5 of lactation. Moreover, only nuclei isolated on days-15 or -30 of pregnancy responded to the exogenously added cytosol.

The DNA-stimulating activity was partially characterized. It was shown to be protein-like since it was heat-labile, mostly non-dialysable and sensitive to N-ethylmaleimide and trypsin treatment. Sedimentation analysis on sucrose density gradients separated this activity into 3 - 4 peaks, distinct from the cytoplasmic DNA polymerase.

Intact isolated nuclei represent an in vitro system for DNA replication that simulates the situation in vivo. Utilizing endogenous DNA template they appear to continue DNA synthesis at chromosomal sites which were actively replicating in the living cell before isolation of the nuclei (Hershey et al., 1973; Planck & Mueller, 1977) and may under proper conditions be able to initiate synthesis of early nascent intermediates, primary DNA pieces (Benbow & Ford, 1975; Jazwinski et al., 1976; Krokan et al., 1977). Isolated nuclei are permeable to macromolecules, making possible detection and characterization of components which play a critical role in DNA replication.

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Varying claims were made about the ability of nuclei isolated from different cells to synthesize DNA, particularly regarding their ability to respond to the addition of putative cytoplasmic factors (Shimada & Terayama, 1972; Thompson & McCarthy, 1973; Hershey et al., 1973; DePamphilis & Berg, 1975; Friedman, 1975; Benbow & Ford, 1975; Jazwinski et al., 1976; Planck & Mueller, 1977; Krokan et al., 1977; Schlaeger, 1978; Burke & Pearson, 1979; Das, 1980; Gutowski & Cohen, 1983; Wawra & Wintersberger, 1983). The experiments described in this paper showed that the effect of cytosol on the rate of DNA synthesis in mammary gland nuclei isolated from rabbits during pregnancy and early lactation was physiological-state dependent. A preliminary study was devoted to the kinetics of the stimulation and the biochemical nature of the cytoplasmic factor(s) participating in DNA synthesis in the nuclei examined.

MATERIALS AND METHODS

Material. Mammary glands were obtained from nulliparous time pregnant or lactating Great Popielno-White rabbits. The glands were quickly cleared of connective tissue, clotted blood, and kept frozen at −20°C until use. The day of mating was considered as day 1 of pregnancy.

Reagents. Sucrose (RNAase-free) was from Serva (Heidelberg, F.R.G); glycerol, double distilled, from Merck (Darmstadt, F.R.G); N-ethylmaleimide (NEM) from Koch-Light (Colnbrook, Bucks., England); calf thymus DNA and pancreatic DNAase from Worthington (Freehold, N.J., U.S.A.); methyl-[^3H]dTTP (spec. act. 30 Ci/mmol, 1110 GBq/mmol) from the Radiochemical Centre (Amersham, U.K.); chromatography paper No 1 from Whatman (Springfield Mill, England) and aphydicolin was a gift from dr. A. H. Tood (Imperial Chemical Industries, England). The proteinase inhibitor phenylmethylsulphonyl fluoride (PMSF) from Sigma was dissolved in propanol to make a stock solution of 0.1 m.

Preparation of nuclei. All procedures were performed at 0 - 4°C. Samples of frozen mammary tissue from rabbits were finely minced and homogenized in 10 volumes of 0.32 m-sucrose in buffer A (10 mM-Tris/HCl, pH 7.4, 2 mM-MgCl₂, 0.5 mM-DTT, 0.1 mM-PMSF) for 2 × 15 s in a Unipan homogenizer immersed in ice/water. After filtration the homogenate was centrifuged at 800 g for 10 min. Nuclei were suspended in 1.61 m-sucrose in buffer A containing 0.05% Triton X-100 and purified by sedimentation through 1.95 m-sucrose in buffer A (27 000 rpm, 4°C, 1 h MSE SW 3 × 23 ml rotor). The nuclei were then suspended in 50% glycerol in buffer A and centrifuged (800 g, 15 min). The purity of the nuclei was checked by light microscopy.

Preparation of cytosol. The supernatant from centrifuged homogenate of mammary glands was again centrifuged at 125 000 g for 1 h at 4°C.
The resulting clear solution (cytosol) was removed taking care not to contaminate it with lipid from the surface. Cytosol was used directly or kept frozen at -20°C in small aliquots, which were thawed immediately before use. Protein concentration in the cytosol was assayed by A_{280} nm/A_{260} nm ratio according to Layne (1955).

Assay for DNA synthesis in isolated nuclei. The reaction mixtures (80 μl) contained: 30 mm-HEPES/NaOH, pH 8.0, 0.2 mm-sucrose, 6.7 mm-MgCl₂, 40 mm-KCl, 5 mm-ATP, 0.1 mm-dATP, 0.1 mm-dCTP, 0.1 mm-dGTP, 0.005 mm-dTTP, 10 μCi/ml (370 kbp/ml) of [³H]dTMP, 1 mm-DTT, freshly prepared nuclei (7-20 μg DNA equivalent) and (unless stated otherwise) about 150 μg cytosol protein (in 30 μl-aliquot of cytosol). Incubation was generally carried out at 37°C for 30 min. It was terminated by chilling the samples in ice/water and adding EDTA and SDS to 10 mm and 1%, respectively. Assays were performed in duplicate or triplicate.

[³H]dTMP incorporated into the trichloroacetic-insoluble product was measured on Whatman No 1 filter paper according to Marzluff (1978). Radioactivity was counted by liquid scintillation in toluene. The values of nonspecific background radioactivities (the zero time counts) were low (about 150 dpm) with no differences between nuclei alone, cytosol alone and nuclei plus cytosol preparations.

Sucrose gradient fractionation. Aliquot (1 ml) of the mammary cytosol was layered on a linear sucrose gradient (5-20%, 4.8 ml) containing 20 mm-HEPES/NaOH, pH 8.0, 20 mm-MgCl₂, 0.5 mm-DTT (Das, 1980) prepared on a 0.3 ml cushion of 60% sucrose. Centrifugation was carried out in a MSE SW rotor (3 × 6.5 ml) for 20 h at 2°C and 42000 r.p.m. Fractions (15 drops) were collected from each gradient from the bottom of the tube and 40 μl aliquots were assayed for DNA synthesis-stimulatory activity.

When the cytoplasmic DNA polymerase activity was assayed in sucrose gradients, activated calf thymus DNA (previously digested with DNAase I according to Schlabach et al., 1971) was used as a template-primer. Incubation was carried out under DNA synthesis assay conditions for 1 h at 37°C. The reaction was stopped by chilling in ice/water and adding EDTA and SDS to 10 mm and 1%, respectively. Aliquots (60 μl) were pipetted onto DE-81 (Whatman) filters which were washed and prepared for counting as previously described (Kleczkowska et al., 1981).

RESULTS AND DISCUSSION

Nuclei freshly isolated from rabbit mammary glands continued under appropriate conditions a replicative-like DNA synthesis; [³H]dTTP incorporation was dependent on the presence of four deoxyribonucleoside triphosphates and a high concentration of ATP (data not shown), whereas
it was strongly inhibited by the well-known specific inhibitors of DNA polymerase α, aphidicolin and N-ethylmaleimide (Table 1).

The degree to which a cell is active in DNA synthesis is correlated with its generation time and state of differentiation. The DNA synthetic activity in mammary gland nuclei is shown in Fig. 1. From the data

**Table 1**

Aphidicolin- and NEM-inhibition of replicative DNA synthesis in rabbit mammary gland nuclei

Mammary gland nuclei were isolated from rabbits on day-15 of pregnancy and their DNA-synthetic activity was measured, as described in Methods, in the presence of aphidicolin or NEM at the concentration indicated. Incubations were carried out with \(^{3}H\)dTTP at 37°C for 60 min.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Final. conc. (mM)</th>
<th>(^{3}H)dTMP incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>3104</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>0.1</td>
<td>956</td>
</tr>
<tr>
<td>NEM</td>
<td>5</td>
<td>1160</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Final. conc. (mM)</th>
<th>d.p.m</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3104</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>956</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>1160</td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Rate of DNA synthesis in isolated nuclei from mammary tissues as a function of the physiological state of the gland. Nuclei were isolated from rabbits on the indicated days of pregnancy or lactation and were incubated with \(^{3}H\)dTTP for 30 min at 37°C as described in Methods. The values are means \(\pm\) SEM

presented, confirming our earlier observations (Sokól-Misiak et al., 1984), it is apparent that mammary gland nuclei isolated from rabbits during pregnancy and early lactation display physiological-state dependent changes in their ability to synthesize DNA. Major peaks of DNA synthetic activity
were observed at specific times: on day-10 and -25 of pregnancy and on
day-1 of lactation. On those days the DNA synthetic activity was at least
1.6 times higher than in nuclei isolated 5 days later.

To study the effect of cytosol on the rate of $[^3]$HdTMP incorporation
into nuclear DNA, the nuclei were isolated from mammary glands at
5-day intervals during pregnancy and early lactation and incubated with
corresponding cytosols (obtained from mammary glands of the same animals)
or with cytosols obtained from rabbits being in a different physiological
state. Upon recombination of the two cell fractions (nuclei and cytosol)
prepared from rabbit mammary glands in the same physiological state,
only the nuclei isolated on day-30 of pregnancy responded to corresponding
cytosol with an enhanced DNA synthetic activity (stimulation about 24%,
Fig. 2b, cytosol from day-30). In nuclei derived on other days of pregnancy
or lactation we did not observe a stimulation of DNA synthesis despite
repeated attempts (data not shown). In systems reconstituted in vitro,
with nuclei incubated with heterologous cytosols (obtained from rabbits
in a different physiological state), the stimulatory effect of cytosol on $[^3]$H
dTMP incorporation was detected only in nuclei isolated on day-15 or
-30 of pregnancy (Fig. 2). Nuclei isolated from the rabbit mammary
gland at other times, in other stages of pregnancy or in early lactation
were unresponsive (data not shown). As illustrated in Fig. 2, the DNA
synthetic activity of nuclei isolated on day-15 and -30 of pregnancy was
stimulated by cytosols prepared on day-10 or from day-20 or -25 of pregnancy
through day-5 of lactation. In the case of these cytosols all the stimulatory
effects were statistically significant. In nuclei from 30-day pregnant animals
the stimulation was small but reproducible (22 - 34% over the corresponding
control values, Fig. 2b). In nuclei from 15-day pregnant animals the stimulation
was somewhat larger (at peak activity in the range 37 - 53% over the
control, Fig. 2a).

In general, in pregnancy and early lactation, at the time of the
expression of two mammary cell functions. i.e. cell proliferation and milk
protein gene expression (Toper & Freeman, 1980), the nuclei examined
were either responsive or unresponsive depending on the physiological state
of the mammary gland. It was shown that the enhancement of the
rate of DNA synthesis required both a responsive state of the nuclei and
the availability of the cytoplasmic active factor(s). The effect of cytosol
on $[^3]$HdTMP incorporation was apparent, however, only in nuclei which,
when incubated alone, were characterized by reduced DNA synthetic activity
(see Fig. 1, nuclei isolated on day-15 and -30 of pregnancy). The mechanism
whereby the nuclei acquire or lose their responsiveness to cytosol is not
known. One possible explanation is that sequential sensitivity of nuclei
to cytoplasmic DNA synthesis stimulatory factor(s) could be associated
with the sequence of hormone action during the mammary gland development.
and cell differentiation. As has been reviewed by Topper & Freeman (1980) the progression of mammary tissue from relatively dormant state in mature virgins to the lactational state is a complex process controlled by a series of interacting hormones, namely prolactin, estrogens, glucocorticoids, progesterone and insulin. Our suggestion is in agreement with the observation of Mueller et al., (1978) that dexamethasone, a steroid that regulates the expression of several genes and directs the differentiation of certain cells, can modify significantly the uptake of cytoplasmic proteins in isolated HeLa cell nuclei under conditions that support chromatin replication.

![Graph A](image.png)

**Fig. 2.** DNA synthesis-stimulating activity of cytosols prepared from rabbit mammary gland at the indicated days of pregnancy or lactation. Cytosols obtained from rabbits in different physiological states were assayed for their capacity to stimulate the $[^3]$H]dTMP incorporation into DNA of mammary gland nuclei (●) isolated on day-15 (A) or day-30 (B) of pregnancy as described in Methods. The values are means±SEM. The statistical analysis was based on the two-tailed Student's t-test. (a) NS, (b) $P<0.02$, (c) $P<0.005$, (d) $P<0.002$, (e) $P<0.001$. Control nuclei incubated in the absence of cytosol (---) shown in the same panel (A or B).

Like in our experiments, variations in the requirement for a cytoplasmic factor of nuclear DNA synthesis have also been found among other cells. HeLa cell nuclei from logarithmic cultures were not activated by a cytoplasmic
fraction from either logarithmic or S-phase cells. However, both cytosols supported DNA synthesis in nuclei from S-phase cells (Friedman & Mueller, 1968). In the latter case product analysis has demonstrated that the cytosols play a critical role in both chain elongation and the production of short DNA segments (Planck & Mueller, 1977). Shimada & Terayama (1972) have reported on stimulation of [³H]dTTP incorporation into DNA of isolated nuclei from infant rat brain by infant as well as adult rat brain cytosol. In adult brain nuclei, however, the DNA synthesis was stimulated slightly by adult rat brain cytosol but was not affected by infant brain cytosol. Thus, it has been suggested that it is the nature of nucleus that can determine the responsiveness to the cytoplasmic stimulator.

On the other hand, there are data indicating that the cytoplasmic extracts prepared from early frog embryos (Benbow & Ford, 1975), from mitogenic hormone-stimulated cells (Das, 1980), from continuously proliferating cells (Hershey et al., 1973; Thompson & McCarthy, 1973; Jazwinski et al., 1976; Gutowski & Cohen, 1983) as well as from mitogen-activated cells (Schlaeger, 1978; Gutowski & Cohen 1983) were capable of inducing DNA synthesis in isolated quiescent nuclei. The cytoplasmic state responsible for the induction of DNA synthesis appears to be characteristic of proliferating cells only, as no stimulatory activity in the cytosol of resting cells was found. It is also noteworthy that in our studies and those of other investigators utilizing other cells (Hershey et al., 1973; Jazwinski et al., 1976; Schlaeger, 1978; Burke & Pearson, 1979; Wawra & Wintersberger, 1983) some nuclear DNA-replicating systems could not be stimulated even when they were supplemented with cytoplasmic extracts of rapidly growing cells.

In our studies we also obtained some preliminary data on the kinetics of stimulation of nuclear DNA synthesis and the biochemical characterization of the stimulatory activity present in cytosol from rabbit mammary cells.

The dose dependence of the nuclear activation response is shown in Fig. 3. The rate of DNA synthesis increased gradually with the amount of cytosol protein added up to 160 μg per assay; above this value synthesis was inhibited. [³H]dTMP incorporation was linear for 60 min (Fig. 4) both in the reconstituted system (nuclei + cytosol) and in nuclei alone. This contrasts with experiments carried out with synchronized S-phase HeLa cells (Krokan et al., 1977) and Friend erythroleukemia cell nuclei (Muller et al., 1981) in which the omission of the cytoplasmic fraction from the reaction mixture led to an early decline in the rate of DNA replication which ceased entirely after 20 min. The DNA synthesis-stimulating activity of rabbit mammary cytosols was not due to possible additional DNA templates present since incubation of the cytosol alone under the assay conditions did not result in a significant incorporation of [³H]dTMP. Additional data (not shown) indicated that the rate of DNA synthesis
Fig. 3. DNA-synthetic activity of nuclei as a function of the amount of cytosol added. Isolated nuclei (16 μg DNA equivalent) from mammary gland of 15-day pregnant rabbit were incubated in the presence of cytosol prepared on day-25 of pregnancy. The incorporation is expressed as dpm of [³H]dTMP in the acid-precipitable material. Under these conditions, described in Methods, nuclei incubated in the absence of cytosol incorporated 2207 dpm of [³H]dTMP.

Fig. 4. Time course of cytosol-stimulated [³H]dTMP incorporation into nuclear DNA. Isolated mammary gland nuclei (10 μg DNA equivalent) from 15-day pregnant rabbit were incubated for the indicated times under DNA synthesis assay conditions, as described in Methods, in the absence (A) or in the presence of a given amount of cytosol (30 μl) prepared on day-25 of pregnancy (●) or on day-3 of lactation (○).

per unit time was proportional to the number of nuclei in the assay (at least up to 20 μg DNA equivalent).

To determine the nature of the active cytoplasmic component(s), the rabbit mammary cytosols were subjected to various treatments (Table 2). They lost most of their DNA synthesis-stimulating activity when heated at 57°C. Treatment of the cytosol with trypsin also abolished their effect on nuclear DNA synthesis. This was not due to soybean inhibitor used to stop the reaction of trypsin during incubation with nuclei since addition of the inhibitor concomitantly with the trypsin prevented the inactivation
of the cytosol stimulatory factor(s). Preincubation of cytosol with the thiol reagent, NEM (10 mM) reduced the DNA synthesis-stimulating activity almost completely. After dialysis for 18 h cytosol retained 70% of its original activity.

Table 2:

Characteristics of DNA synthesis-stimulating activity in cytosol
Mammary tissue from rabbits on day-25 of pregnancy was subjected to various treatments indicated below prior to assay for DNA synthesis-stimulating activity with nuclei isolated on day-15 of pregnancy. The stimulation of DNA synthesis by untreated (control) cytosol over DNA synthesis carried out in the absence of cytosol was normalized to 100% in each experiment. DNA synthesis assay conditions were as described in Methods.

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Activity (%)</th>
</tr>
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<tbody>
<tr>
<td>Untreated cytosol</td>
<td>100</td>
</tr>
<tr>
<td>Sensitivity to heat</td>
<td></td>
</tr>
<tr>
<td>Cytosol heated at 57°C, 5 min; denaturated protein removed by centrifugation</td>
<td>16</td>
</tr>
<tr>
<td>Sensitivity to trypsin</td>
<td></td>
</tr>
<tr>
<td>Cytosol treated with trypsin (25 µg/0.5 ml, 20 min, 37°C)</td>
<td>0</td>
</tr>
<tr>
<td>Suitable controls with soybean trypsin inhibitor (6.2 µg/0.5 ml)</td>
<td>100</td>
</tr>
<tr>
<td>Sensitivity to NEM</td>
<td></td>
</tr>
<tr>
<td>Cytosols treated with 10 mM-NEM (5 min, 37°C) and then dialysed overnight against buffer A</td>
<td>7</td>
</tr>
<tr>
<td>Cytosol treated with 10 mM-NEM (5 min, 37°C) and then with 12 mM-DTT, dialysis as above</td>
<td>109</td>
</tr>
<tr>
<td>Dialysis 18 h, 4°C, against buffer A</td>
<td></td>
</tr>
</tbody>
</table>

From the data presented in Table 2 it can be concluded that the stimulatory activity detected in cytosol was most probably due to a factor(s) which was protein in nature, heat-labile and required a sulphhydryl group for activity. Serum albumin, the predominant unspecific hormone binder, was incapable of stimulating nuclear DNA synthesis (data not shown).

Subsequently, it was demonstrated by the sucrose density gradient centrifugation that the cytoplastic factor responsible for stimulating DNA synthesis in isolated nuclei was heterogeneous in respect to molecular weight. Representative sucrose gradient profiles showing the stimulatory activity of cytosol fractions prepared from 10-day, 25-day pregnant and 2-day lactating rabbit mammary tissue are shown in Fig. 5A, 5B and 5C, respectively.
Fig. 5. Sucrose density gradient analysis of stimulatory factors from cytosol. Cytosols prepared from rabbit mammary gland on days-10 (A) and -25 of pregnancy (B) and on day-2 of lactation (C) were centrifuged in a linear 5-20% sucrose gradient containing 20 mm-HEPES/NaOH, pH 8.0, 20 mm-KCl, 0.5 mm-MgCl₂, 0.5 mm-DTT (42 000 rpm, 2°C, 20 h, MSE SW rotor 3×6.5 ml) and each gradient fraction was assayed for stimulation of [³H]dTMP incorporation in mammary nuclei (9 µg DNA equivalent) isolated on day-15 of pregnancy (○) and for protein content (---). Under the assay conditions (37°C, 1 h)
In the case of the cytosols tested, we found one low molecular weight stimulator at the top of the gradient and several high molecular weight stimulators, the major activity being localized in fractions sedimenting at about 4.5S (Fig. 5A, 5B, 5C), about 6.5S (Fig. 5B, 5C) and about 8.4S (Fig. 5A, 5C). When in the sucrose gradient the cytoplasmic DNA polymerase activity was assayed with activated calf thymus DNA as a template-primer (Fig. 5B), a single peak was observed which sedimented at 8.4S and could be ascribed to DNA polymerase α (Kleczkowska et al., 1981). Thus, the cytosol fractionation attempts appear to separate partially the cytoplasmic stimulators of DNA synthesis from the cytoplasmic DNA polymerase α.

Reports exist that several stimulatory activities have been revealed by the fractionation procedures in cytosols from HeLa (Friedman, 1975) and epidermal growth factor-treated 3T3 cells (3.7S, 6.6S, 12S; Das, 1980). Furthermore, a partial separation of cytoplasmic fraction and DNA polymerase activities has been obtained in the case of HeLa cells (Hershey et al., 1973; Krokant et al., 1977). It is also noteworthy that the active cytoplasmic factor, detected in the infant rat brain (Shimada & Terayama, 1972) and Taper mouse hepatoma (TLT) cells (Thompson & McCarthy, 1973) appeared to be of low molecular weight and seemed to have a peptide nature or to be reversibly associated with a high molecular weight, heat-labile cytoplasmic compound, respectively.

The exact role of cytosol in nuclear DNA synthesis has remained elusive. Attempts to demonstrate an essential role, such as DNA polymerase, DNA ligase and a RNA primer-related activity have been generally negative (DePamphilis et al., 1978; Mueller et al., 1978). There are speculations that the cytoplasmic factors from dividing cells that have been described to lead to in vitro DNA synthesis in nuclei from nondividing cells are most likely involved in the formation of an intercellular mitogenic signal (Gutowski & Cohen, 1983), i.e. in the formation of diadenosine 5',5''-P₁, P₄-tetraphosphate (Ap₄A) (Grummt, 1978). With respect to the possible role of cytosol in chromatin replication during cell differentiation it appears highly probable that the spectrum of cytoplasmic proteins that are available for the initial complexing of the newly replicated DNA can ultimately determine whether the DNA of such complexes will be in a genetically expressible state. It has been proposed by Mueller et al. (1978) that, as the spectrum of these proteins is shifted either in amounts, availability or activity by prior nutritional, hormonal or physical treatment of the

the nuclei alone incorporated 2 206 dpm of [³H]dTMP. In the case of cytosol from day-25 of pregnancy (B), each gradient fraction was assayed additionally for DNA polymerase activity with the activated calf thymus DNA as a template-primer (O). The arrows indicate the position of the sedimentation markers: rabbit mammary gland DNA polymerase α (8.4S) and beef hemoglobin (4.3S)
cells, the probability of forming certain chromatin complexes is also changed. Such changes will determine the range of genetically active chromatin complexes formed or stabilized during nuclear replication.

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