EFFECT OF CYTOSOL ON DNA SYNTHESIS IN ISOLATED HELA CELL NUCLEI

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SUMMARY

Cytosol obtained by centrifugation of cytoplasm from synchronized S-phase HeLa cells at 200 000 x g for 30 min had a stimulatory effect on the rate and extent of DNA synthesis in isolated nuclei. The cytosol preserved the ability of isolated nuclei to initiate early nascent intermediates (primary DNA pieces). The stimulatory activity was partially separated from the DNA polymerase activity present in the cytosol.

INTRODUCTION

Cytoplasmic proteins from proliferating cells have been found to stimulate DNA synthesis in isolated nuclei (1-8) and such systems may be useful in characterizing factors taking part in DNA synthesis. So far, relatively little is known about the nature of the stimulatory factors, but it has been shown that cytosol may be of importance for ligation of primary DNA pieces both in uninfected(8) and in polyoma-infected cells (6, 7). It is still a matter of controversy whether or not the cytosol polymerase activity is important for stimulation (4, 5). We report here some effects of cytosol from S-phase HeLa cells on DNA synthesis in isolated nuclei. The in vitro system has been described earlier (9-12).

MATERIALS AND METHODS

All chemicals were obtained as mentioned previously (11). HeLa S_3 cells were maintained in suspension culture, synchronized and the nuclei isolated and incubated in our standard test mixture (11) or as specified in figure legends. The incubations were stopped and the radioactivity incorporated was assayed as described earlier (9, 10). DNA polymerase activity was assayed as described by Spadari and Weissbach (13). DNA was estimated by the method of Burton (14), and protein by the Lowry method (15).

Preparation of cytosol.

Cells were harvested 3 hr after entry into S-phase, washed once in buffer A (9) by resuspension and centrifugation (750 x g for

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5 min), then resuspended in buffer A (1ml/14x10^7 cells) and broken in a Dounce homogenizer. The lysate was centrifuged at 3000 x g for 10 min at 2°C and the resulting supernatant subjected to ultracentrifugation (200 000 x g for 30 min). The final supernatant was collected, avoiding the floating lipid layer. The cytosol was then mixed with 0.5 vol of buffer D (195 mM Tris-HCl, 195 mM NH_4Cl, 1 mM EGTA, 150 mM glucose, 30 % glycerol (v/v) and 26.1 mM $MgCl_2$ with final pH 8.1) to give the same final content of other components as in the standard test mixture (11). When cytosol was fractionated in clycerol gradients, glycerol was not included in buffer D.

Glycerol gradiant analyses.

5-15 % glycerol gradients in test mixture lacking nucleotides were prepared in SW 40 nitrocellulose tubes. Cytosol (1.8 ml) was layered on top of the gradients and centrifuged for 20 hr at 200 000 x g and 2°C.
Fractions of 25 drops were collected from the top of the gradient at 4°C.

RESULTS AND DISCUSSION

The cytosol preparations contained about 15 mg protein/ml and no detectable DNA. The addition of cytosol increased the extent of DNA synthesis 2 - 2.5-fold after 75 min compared to that obtained with the standard test mixture (Fig. 1). The initial rate in the presence of cytosol was only slightly increased whereas the main effect of cytosol was to extend the time of incorporation.

The stimulatory activity of cytosol was completely lost upon heating at 50°C for 10 min, whereas storage for 2 days at 4°C did not cause significant loss. After 14 days at 4°C most of the stimulatory activity was lost.

The possibility of extranuclear DNA synthesis in the presence of cytosol was excluded by separating nuclei and the cytosol-containing test mixture by centrifugation (500 x g for 5 min at 4°C) after incubation. More than 97 % of the acid-precipitable radioactivity was intranuclear. Double labelling experiments with bromodeoxyuridine for 3 hours in vivo followed by a 1 or 5 min pulse with [^{3}H] TTP in vitro (9) showed that 80-85 % of the

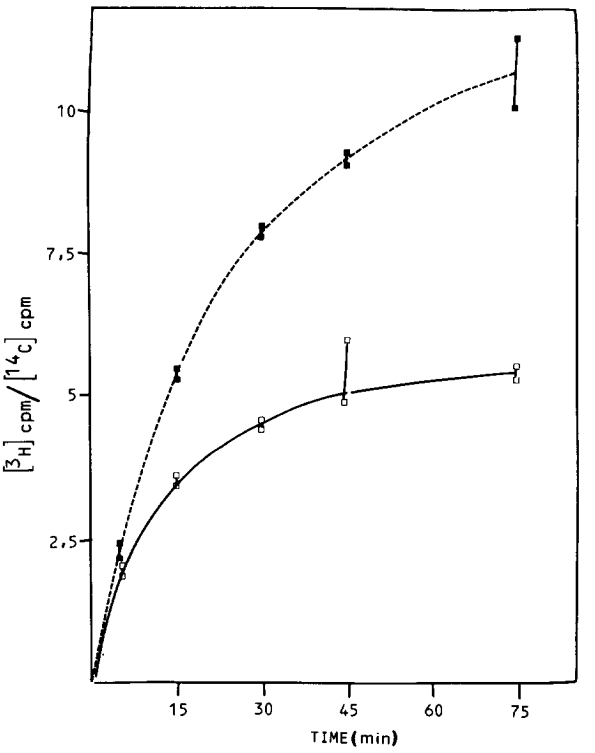

Figure 1: Effect of cytosol on [^{3}H] TMP-incorporation into DNA in isolated nuclei. Nuclei were isolated from synchronized cells 3 hours after entry into S-phase and incubated with standard test mixture (—□—) or cytosol-containing test mixture (—■—). The final [^{3}H] TTP concentration was 50 µM and the specific activity 50 mCi/mmol.
The cells were prelabelled with [^{14}C] thymidine for 48 hours (12). The total incorporation of [^{3}H] TMP after 75 min in standard test mixture was 18 850 cpm/100 µg DNA and in the cytosol containing test mixture 33 050 cpm/100 µg DNA.

DNA synthesized in the isolated nuclei in the presence of cytosol was covalently linked to the strands which contained bromodeoxyuridine. This indicates strongly that the in vitro synthesis is a continuation of the in vivo process.
When nuclei are incubated under conditions where DNA synthesis is not allowed, they rapidly lose their DNA synthesizing capacity probably by inactivation or leakage of factors essential for replication (12).

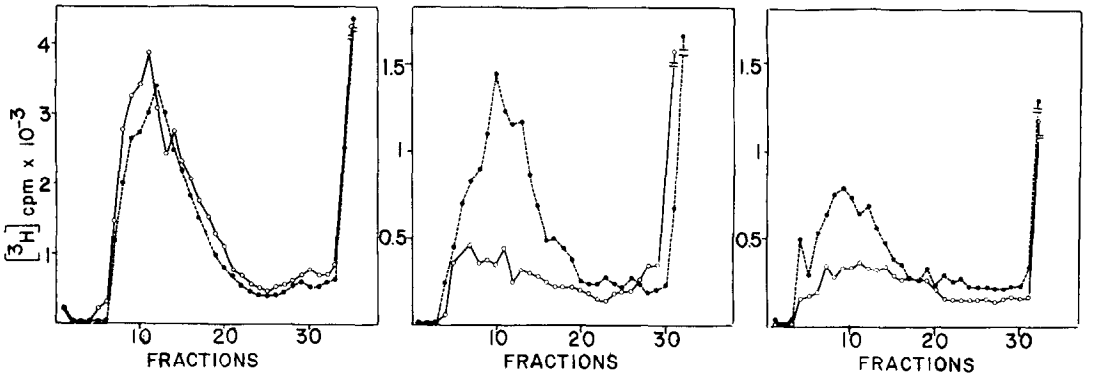

Figure 2: Effect of cytosol on the initiation of synthesis of primary DNA pieces. Nuclei were isolated from cells prelabelled with [^{14}C] thymidine for 48 hours 3 hours after entry into S-phase and incubated with 50 µl of non-radioactive test mixture with (--●--●--) or without (—o—o—) cytosol for 5 (A), 10 (B) or 20 min (C). Then 50 µl of a standard test mixture or a cytosol-containing test mixture containing 5 µM [^{3}H] TTP (specific activity 30 Ci/mmol) was added and the incubation continued for 60 sec. The pulses were terminated by adding 500 µl alkaline lysis solution (11) and the DNA was analysed in alkaline sucrose gradients (280 000 x g for 17 hr at 5°C in an SW 40-Ti rotor). The nuclear DNA content was 25 µg (A) and 12.3 µg (B and C). Total incorporation per 10 µg DNA in the absence of cytosol was (A) 32 200 cpm, (B) 11 520 cpm and (C) 11 800 cpm. In the presence of cytosol the incorporation was (A) 33 716 cpm, (B) 20 402 cpm, and (C) 17 497 cpm. All the [^{14}C] labelled DNA (bulk DNA) sedimented to the bottom of the tube in all gradients (not shown in the figure). Sedimentation is from left to right.

The kinetics of the stimulatory effect of cytosol suggested that the stimulation was caused by the supply or conservation of some function which declined during incubation in the absence of cytosol. The synthesis of the primary DNA pieces (the smallest intermediates) could possibly be an important rate limiting step. We therefore decided to investigate whether primary pieces were synthesized for a longer period in the presence of cytosol. Nuclei were incubated with cytosol-containing or standard test mixture both containing unlabelled precursors, for 5, 10 or 20 min, followed by a 1 min pulse with [^{3}H] TTP. The nuclei were lysed and centrifuged on alkaline sucrose gradients (11). The radioactivity incorporated into the peak of primary DNA pieces

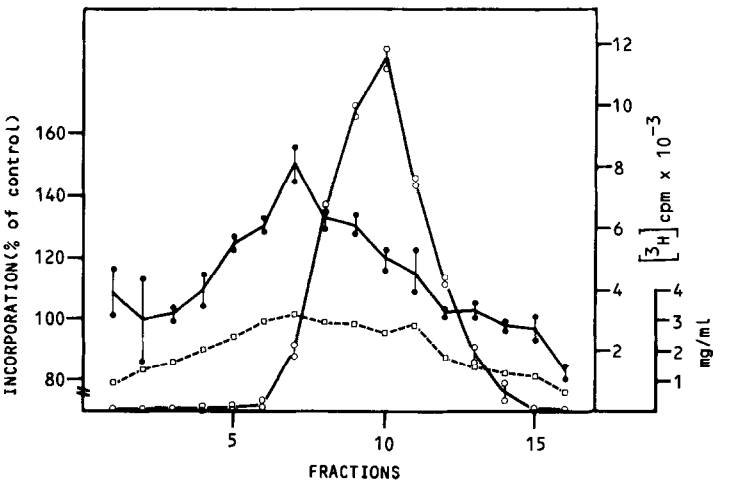

Figure 3: Fractionation of cytosol by centrifugation in a glycerol gradient. Cytosol was centrifuged in a 5 - 15 % linear glycerol gradient and each fraction analysed for DNA polymerase activity by [^{3}H] TMP-incorporation (——o——), stimulation of nuclear DNA synthesis (——●——) and protein content (mg/ml) (——□——). Sedimentation is from left to right.

declined with the time of preincubation of the nuclei, but when cytosol was present during the preincubation the synthesis of new primary DNA pieces was markedly enhanced and detectable even after 20 min (Fig. 2 C). The difference was most marked after 10 min (Fig. 2 B). We have shown earlier that the primary DNA fragments synthesized in vitro during a short pulse at the start of the incubation are attached to high molecular weight DNA within 90 sec (10,12). This ability to ligate primary DNA pieces was not inhibited by cytosol, and we did not observe any increased breakdown of nuclear DNA by cytosol (data not shown). The small fragments of DNA that appeared after a 1 min pulse (Fig. 2 A-C) must therefore have been initiated in vitro. Thus, cytosol clearly preserves the ability of isolated nuclei to initiate primary DNA pieces.

To examine whether the stimulatory cytosol factors were different from the cytosol polymerase activity, we attempted to separate these activities in 5-15 % linear glycerol gradients.

Each fraction was tested for DNA polymerase activity and for ability to stimulate DNA synthesis in isolated nuclei. The peaks of the two activities were separated, indicating that cytosol DNA polymerase activity is not the main stimulatory activity in crude cytosol (Fig. 3). Evidence for this has also been obtained by means of DEAE-Cellulose ion exchange chromatography. The polymerase activity was recovered quantitatively, but did not stimulate DNA synthesis in isolated nuclei even after concentration by ultra-filtration.
Unfortunately, the activity stimulating the isolated nuclei was not recovered in any of the other fractions.

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