ACTIVATION OF THE TEMPLATE ACTIVITY OF ISOLATED RAT LIVER NUCLEI FOR DNA SYNTHESIS AND ITS INHIBITION BY NAD

L. Burzio* and S.S. Koide+

Biomedical Division, The Population Council, The Rockefeller University, New York, New York 10021

Received June 5, 1973

SUMMARY: The template activity of isolated rat liver nuclei for DNA synthesis assayed with E. coli DNA polymerase was found to be dependent upon the presence of Ca^{2+} or Mg^{2+} in the incubation medium. DNA was prepared from isolated nuclei subjected to conditions which activated the template and centrifuged in an alkaline sucrose gradient. The distribution profile showed that smaller fragments were formed, suggesting enhancement of endonucleolytic activity. When isolated nuclei were incubated with NAD to induce poly(adenosine diphosphate ribose) formation and were subjected to the activation conditions, the template for DNA synthesis remained unchanged. The distribution profile in an alkaline sucrose gradient of DNA prepared from these nuclei and control nuclei was identical. The present findings suggest that the template-activating system for DNA synthesis was blocked when isolated nuclei were treated with NAD in vitro.

Treatment of isolated rat liver nuclei with NAD in vitro resulted in an inhibition of the amount of [^{3}H]TTP incorporated into DNA (1,2). The block was related to ADP‡-ribosylation of nuclear proteins (3,4) and was demonstrated to be due to a suppression of the template activity for DNA synthesis of nuclei or chromatin (2). In the course of these studies we found that under the assay condition currently used, the template of isolated rat liver nuclei as measured with exogenous DNA polymerase was gradually activated on incubation and that the activation process was associated with an endonucleolytic activity (5). In the present report evidence will be presented to show that the inhibition of DNA synthesis exerted by poly(ADP-Ribose) formation is associated with a block of the template activation process.

MATERIALS AND METHODS

Materials. DNA polymerase of about 5000 units per mg of protein obtained from E. coli was purchased from Biopolymers, Pine Brook, N.J.
Preparation of Nuclei. Rat liver nuclei was isolated in a medium containing 0.25 M sucrose, 2 mM $CaCl_2$ (g/5 ml) as described in a previous report (1). The crude nuclear pellet was suspended in 2.3 M sucrose, 2 mM $CaCl_2$ as described by Chauveau et al. (6). The suspension was centrifuged at 40,000 x g for 60 min. The pellet of purified nuclei was suspended in 0.25

* Present address: Instituto de Bioquimica, Universidad Austral de Chile, Casilla 567, Valdivia, Chile.
+ To whom all correspondence should be addressed.
‡ Abbreviation used: ADP, adenosine diphosphate; EGTA, ethylene glycol bis (2-aminoethylether)-N-N' tetraacetic acid.

M sucrose, 2 mM $CaCl_2$. In some preparations the pellets were washed twice with 0.34 M sucrose and suspended in the same solution.

Enzymatic Studies. Poly(ADP-Ribose) synthetase activity was assayed as described in a previous report (1). The template activity for DNA synthesis of isolated rat liver nuclei was assayed in a medium containing 75 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2-mercaptoethanol, 0.1 mM each of dATP, dCTP, dGTP, 0.5 mM [^{3}H]TTP (50 to 100 μCi/μmole), one unit of E. coli DNA polymerase and nuclei containing about 25 μg of DNA in a total volume of 0.5 ml. The reaction mixture was incubated at 25° for 15 min.

To study the effect of bivalent cations on the activation of the template, isolated nuclei were washed twice and resuspended in 0.34 M sucrose. The incubation medium contained 75 mM Tris-HCl (pH 7.4), 2 mM 2-mercaptoethanol, 150 mM sucrose, 0.25 mM EDTA, varied concentrations of bivalent cations, nuclei containing about 200 μg of DNA in a total volume of 0.5 ml. EGTA was added to chelate endogenous Ca^{2+} in the nuclei preparation. The reaction mixture was incubated at 37°. At the designated time intervals aliquots of 50 μl were removed and assayed for template activity.

To study the effect of NAD on the template activity, the isolated nuclei preparations containing 200 μg of DNA were incubated in a medium containing 75 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 4 mM NAD, in a final vol of 0.5 ml. The mixture was incubated at 25° for 30 min. The untreated-control (without NAD) and the NAD-treated nuclei samples were washed twice with a solution of 0.25 M sucrose, 2 mM $CaCl_2$. Template activity of the nuclei preparations was measured before and after being subjected to the activating process. The incorporation of [^{14}C]NAD or [^{3}H]TTP was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375. The material precipitated with 5% trichloroacetic acid was collected on a glass fiber filter (Reeve Angel, Clifton, N.J.) and processed as described in a previous report (1).

Alkaline Sucrose Gradient. Preparation of nuclei containing about 300 μg of DNA was incubated in a medium containing E. coli DNA polymerase which was used to assay for DNA synthesis. After incubation two vol of ethanol at -20° was added to the reaction mixture. The mixture was centrifuged and the pellet was washed once with cold 66% ethanol, dried under vacuum and suspended in 0.5 ml of a medium composed of 0.2 N NaOH and 5 mM EDTA. At this step the amount of radioactivity in the material precipitated with trichloroacetic acid was greater than 90%. A sample of 0.3 to 0.4 ml was layered on a 12 ml solution of 5 to 20% linear sucrose gradient containing 0.2 N NaOH, 0.8 M NaCl, 1 mM EDTA (7). The tubes were centrifuged in a Spinco Model L2-65 ultracentrifuge using a SW-40 rotor at 30,000 rpm for 17 hr at 5°. About 30 fractions of 15 drops each were collected from the bottom of the tube. Aliquots of 100 μl of each fraction were removed and the radioactivity in the trichloroacetic acid-precipitated material was measured as described by Bollum (8). DNA in the remainder of the fraction was determined by a modified method (9) of Burton (10).

Other procedures. DNA was prepared according to Marmur (11). Protein was determined as described by Lowry et al. (12).

RESULTS

In the course of these studies it was observed that the template for DNA synthesis of control nuclei preparation (without NAD) showed a gradual increase in activity with the duration of the incubation. To determine the basis for the activation of the template, a time course study of the template activity was carried out at 37° (Fig. 1). The template activity of fresh nuclei was very low. However, on incubation at 37° in a medium containing

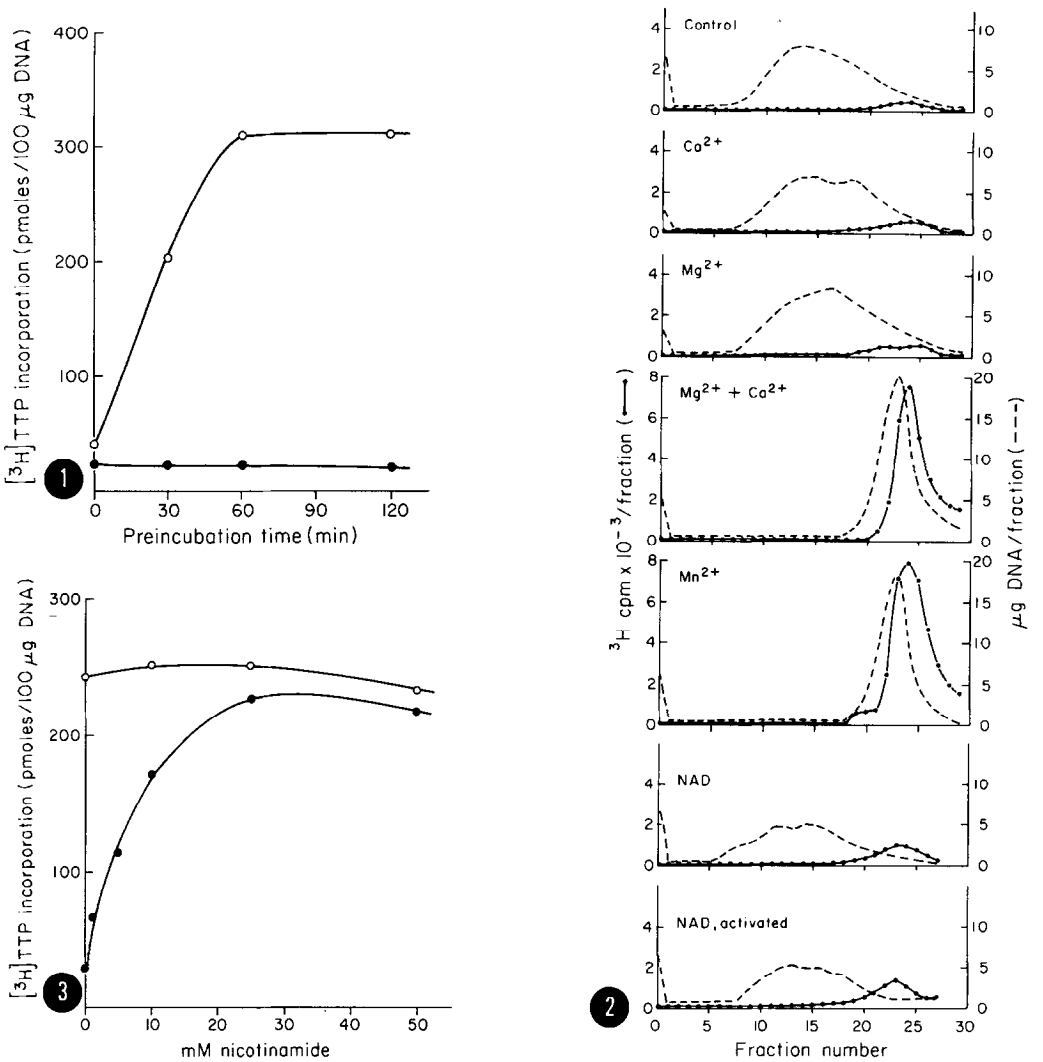

Figure 1. Activation of template for DNA synthesis of control and NAD-treated nuclei following incubation in a medium containing Mg^{2+} plus Ca^{2+}. Control and NAD-treated nuclei were incubated at 37° to activate the template as described in the text. Aliquots were removed at the designated time and assayed for template activity with E. coli DNA polymerase. (●——●) untreated-control nuclei; (○——○) NAD-treated nuclei.

Figure 2. Sedimentation profile of alkaline sucrose gradient of DNA obtained from nuclei incubated in a medium containing different cations and from NAD-treated nuclei. Nuclei preparations were incubated for 90 min at 37° with the same cation combination as shown in Table I to activate the nuclei. Control and activated nuclei were assayed for template activity with DNA polymerase. The DNA was prepared from these nuclei and analyzed on a linear alkaline sucrose gradient described in the text. About 30 fractions of 15 drops were collected from the bottom of the tube. Top of the gradient to the right.

Figure 3. Reversal of the inhibition of template-activation of nuclei treated with NAD by nicotinamide. Nuclei were incubated with or without NAD with varied concentrations of nicotinamide for 30 min at 25°. After washing, the nuclei were subjected to the activation process as described in the text and assayed for template activity with E. coli DNA polymerase. (●——●) control nuclei; (○——○) NAD-treated nuclei.

Mg^{2+} as described under methods, the template activity was stimulated.

Nuclei preparations were extracted with various solutions to establish in which fraction factors that influenced activation of the template for DNA synthesis existed. The template activity of nuclei preparations and purified chromatin was determined by measuring the increase in [^{3}H]TTP incorporation into DNA with E. coli DNA polymerase in the assay system. The template-activating system was found to be present in chromatin. It was established that the activation process required bivalent cations in the medium (Table I). In the absence of bivalent cations the template activity remained unchanged after 90 min of incubation at 37°. When either Mg^{2+} or Ca^{2+} was added to the medium, activation of the template was minimal. However, the addition of Mg^{2+} plus Ca^{2+} to the medium caused a dramatic increase in the template activity. Mn^{2+} can replace Mg^{2+} plus Ca^{2+} and was equally effective as an activator (Table I). These results suggest that the activation of the template might be due to endonuclease activity. To test this notion, DNA was prepared from isolated nuclei which were subjected to activation and its sedimentation profile in alkaline sucrose gradient was determined (Fig. 2). Fresh nuclei or nuclei incubated for 90 min at 37° with the same bivalent cations combination as shown in Table I were assayed for template activity and the DNA extracted as described under methods. The DNA was centrifuged through a linear alkaline sucrose gradient. The pattern of distribution of the DNA obtained from nuclei incubated with Mg^{2+} or Ca^{2+} alone was the same as that extracted from fresh nuclei (Fig. 2). It was noted that in the gradient the [^{3}H]TTP incorporated was associated with fractions containing smaller fragments of DNA. When the nuclei were incubated in a medium containing Mg^{2+} plus Ca^{2+} or Mn^{2+} alone, [^{3}H]TTP incorporation was markedly stimulated (Table I). Parallel with this finding a dramatic shift in the position of the nuclear DNA in the gradient was observed (Fig. 2).

In the next set of experiments we have attempted to establish a relationship between the observed inhibition of DNA synthesis induced by NAD (1,2) to the activation of the template activity of the nuclei. The template activity of nuclei preparations incubated with NAD was low (about 40%) compared to the control value (Fig. 1). When the control nuclei were activated at 37° in the presence of Mg^{2+} plus Ca^{2+}, the template activity increased to a maximum level in about 90 min. However, the template activity of NAD-treated nuclei subjected to the activation conditions remained unchanged. DNA was prepared from NAD-treated nuclei which were incubated in a medium containing Mg^{2+} plus Ca^{2+} for 90 min at 37° and from fresh nuclei which were not activated. When both DNA were analyzed by sedimentation in an alkaline sucrose gradient, their

Table I. Effect of Bivalent Cations on the Activation of Template Activity of Isolated Rat Liver Nuclei.

Cations added*	[^{3}H]TTP incorporation+ (pmoles/10 min)
Control	5
No bivalent cations	4
0.5 mM Ca^{2+}	5
5.0 mM Mg^{2+}	7
5.0 mM Mg^{2+} + 0.5 mM Ca^{2+}	72
0.5 mM Mn^{2+}	82

* All of the media used contained 0.25 mM EDTA. The nuclei were activated by incubating the system at 37° for 90 min. Aliquots containing 25 µg of DNA were removed and assayed for template activity.

\+ DNA synthesis was assayed with one unit of bacterial DNA polymerase for 10 min at 25°.

position was identical (Fig. 2). As observed with the above described experiment the incorporated [^{3}H]TTP was associated with fractions containing smaller fragments of DNA. In agreement with the above finding the template of control nuclei showed an increase in [^{3}H]TTP incorporation when subjected to the activating conditions and a parallel shift in the position of the DNA in the gradient was observed.

The present results suggest that the template activity may be dependent upon an increased formation of 3' hydroxyl primer fragments due to endonuclease activity. To substantiate this idea, the template was activated by incubating nuclei in a medium containing a small amount of pancreatic deoxyribonuclease and Mg^{2+} (Table II). In this experiment the untreated-control nuclei and NAD-treated nuclei preparations were activated by the enzyme. These results suggest that perhaps the endonuclease present in the rat liver nuclei may be a variant of deoxyribonuclease or that some other factors are operating.

Purified DNA obtained from control-activated nuclei, NAD-treated-activated nuclei and fresh nuclei were assayed for template activity with excess *E. coli* DNA polymerase. The template activity of DNA obtained from fresh and NAD-treated nuclei was practically identical. On the other hand, the template activity of DNA obtained from activated nuclei was about 20% higher, suggesting that the DNA of untreated-control nuclei underwent changes during the activation process.

Table II. Activation of Template Activity of Untreated-Control and NAD-treated Nuclei with Pancreatic Deoxyribonuclease.

Type of nuclei preparation	Treatment	[^{3}H]TTP incorporation[§] (pmoles/100 µg DNA)
Untreated-control	None	63
NAD-treated	None	21
Untreated-control	Activation[+]	207
NAD-treated	Activation[+]	17
Untreated-control	DNAase[‡]	245
NAD-treated	DNAase[‡]	263

[*]Nuclei were incubated without or with 4 mM NAD for 40 min at 25° and washed as described in the text.

[+]Template activity of the nuclei preparation was activated as described in the text.

[‡]Untreated-control and NAD-treated nuclei were incubated for 30 min at 37° in a medium containing 0.5 µg of pancreatic deoxyribonuclease, 5 mM MgCl , 50 mM Tris-HCl (pH 7.5), 0.25 mM EGTA.

[§]The nuclei preparations were assayed for template activity with E. coli DNA polymerase after the treatment as described in the text.

When ADP-ribosylation of nuclear proteins was prevented with nicotinamide (13,14), the effect of NAD was ameliorated and the nuclei were activated to the full extent (Fig. 3). In the present experiment nuclei were incubated in a medium containing 4 mM NAD, varied concentrations of nicotinamide and Mg^{2+} plus Ca^{2+}, at 37° for 90 min and the template activity was assayed. The template activity of control nuclei incubated in the presence of nicotinamide and subjected to the activation procedure was not affected (Fig. 3). Nicotinamide at a concentration of 30 mM prevented completely the inhibition exerted by 4 mM NAD. The activation proceeded at a maximal rate and the observed template activity was the same as the control value. At this concentration of nicotinamide the incorporation of [^{14}C]NAD was blocked by about 97%.

DISCUSSION

Burgoyne et al. (15-17) reported that the template capacity of isolated nuclei for DNA synthesis was dependent upon the extent of nicks in the DNA. They demonstrated that the activation process was related to Ca^{2+}-dependent endonucleolytic activity located in the nucleus. The present findings that the template activation process required Ca^{2+} and Mg^{2+} or Mn^{2+} in the medium and that the sedimentation of the DNA obtained from activated nuclei in alkaline sucrose gradient showed a shift toward smaller fragments support the con-

tention that activation of the template was due to endonucleolytic activity. The endonuclease responsible for the activation of the template activity is relatively tightly bound to the chromosomal structure. Marushige and Bonner (18) reported that purified chromatin possessed endonucleolytic activity which had the same cation requirements although the enzyme was rather labile as it existed in chromatin. The localization of the enzyme in the nucleus is in agreement with an earlier report (19). Bollum (20) showed that pancreatic deoxyribonuclease hydrolyzed poly I from the double strand copolymer, poly I·poly C, when Mg^{2+} was present in the medium. However, when both Mg^{2+} and Ca^{2+} were included in the reaction mixture, poly I as well as poly C were hydrolyzed by this enzyme.

It was demonstrated in the present study that poly(ADP-Ribose) formation by isolated rat liver nuclei resulted in an inhibition of the template activating system. The block appeared to be associated with a suppression of endonuclease activity. These results suggest that ADP-ribosylation of the enzyme might have occurred by a mechanism similar to that observed with ADP-ribosylation of transferase II by diphtheria toxin and NAD (21-23).

ACKNOWLEDGEMENTS: This work was supported in part by Grant No. 5 PO1 HD05671-02 from NICHHD. We are grateful to M. Elvira for her excellent technical assistance.

REFERENCES

1. Burzio, L., and Koide, S.S. Biochem. Biophys. Res. Commun. 40:1013 (1970).
2. Burzio, L., and Koide, S.S. Biochem. Biophys. Res. Commun. 42:1185 (1971).
3. Nishizuka, Y., Ueda, K., Honjo, T., and Hayaishi, O. J. Biol. Chem. 243: 3765 (1968).
4. Otake, H., Miwa, M., Fujimura, S., and Sugimura, T. J. Biochem. (Tokyo) 65:154 (1969).
5. Burzio, L., and Koide, S.S. Abstracts of papers, 164th National Meeting Am. Chem. Soc., New York, Aug. 27-Sept. 1, 1972, Biol., No. 73.
6. Chauveau, J., Moule, Y., and Rouiller, C. Exp. Cell Res. 11:317 (1956).
7. Olivera, B.M., and Lehman, I.R. Proc. Nat. Acad. Sci. USA 57:1426 (1967).
8. Bollum, F.J. J. Biol. Chem. 234:2733 (1959).
9. Giles, K.W., and Myers, A. Nature 206:93 (1965).
10. Burton, K. Biochem. J. 62:315 (1956).
11. Marmur, J. J. Mol. Biol. 3:208 (1961).
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.G. J. Biol. Chem. 193:265 (1951).
13. Nishizuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O. J. Biol. Chem. 242: 3164 (1967).
14. Fujimura, S., Hasegawa, S., Shimizu, Y., and Sugimura, T. Biochim. Biophys. Acta 145:247 (1967).
15. Burgoyne, L.A., Wagar, M.A., and Atkinson, M.R. Biochem. Biophys. Res. Commun. 39:254 (1970).
16. Burgoyne, L.A., Wagar, M.A., and Atkinson, M.R. Biochem. Biophys. Res. Commun. 39:918 (1970).
17. Hewish, D.R., and Burgoyne, L.A. Biochem. Biophys. Res. Commun. 52:475 (1973).

18. Marushige, K., and Bonner, J. J. Mol. Biol. 15:160 (1966).
19. Baril, E., Brown, O., and Laszlo, J. Biochem. Biophys. Res. Commun. 43:754 (1971).
20. Bollum, F.J. J. Biol. Chem. 240:2599 (1965).
21. Collier, R.J., and Pappenheimer, A.M., Jr. J. Exp. Med. 120:1019 (1964).
22. Honjo, T.Y., Nishizuka, Y., and Hayaishi, O. Cold Spring Harbor Symp. Quant. Biol. 34:603 (1969).
23. Goor, R.S., and Maxwell, E.S. Cold Spring Harbor Symp. Quant. Biol. 34:609 (1969).