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NAD^+, ADP-ribosylation and transcription in permeabilized mammalian cells

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When permeabilized hamster fibroblasts were incubated with 4mM-NAD^+, the substrate for poly(ADP-ribose) polymerase, RNA polymerase I activity was inhibited by about 85%. This inhibition was not relieved by prior incubation of cells with 3-aminobenzamide, a potent inhibitor of the poly(ADP-ribose) polymerase. Digestion of cells with pancreatic deoxyribonuclease I resulted in the inhibition of RNA polymerase I by 80% and the activation of poly(ADP-ribose) polymerase by up to 300%; prior incubation with 3-aminobenzamide did not prevent the inhibition of the RNA polymerase activity. No radioactivity was found associated with RNA polymerase I during later stages of purification of this enzyme from permeabilized cells previously incubated with [^{14}C]NAD^+. The inhibitory effect of NAD^+ on RNA polymerase I was not specific for NAD^+, as other small, negatively charged molecules with a nuclear location also inhibited the enzyme. The results do not support the concept of a role for ADP-ribosylation in transcription catalysed by RNA polymerase I.

The literature concerning a possible connection between transcription and ADP-ribosylation events is confusing and on balance seems not to support any obvious correlation between these two processes (see review by Purnell *et al.*, 1980). For example, Hilz & Kittler (1971) reported no change in poly(ADP-ribose) synthetase activity in livers from sham-operated, adrenalectomized and cortisol-treated rats. Tsopanakis *et al.* (1978) reported that poly(ADP-ribose) polymerase activity was similar both in nuclei and in the more transcriptively active nucleoli isolated from *Tetrahymena pyriformis*, and Yukioka *et al.* (1978) concluded from their work with rat liver chromatin that poly(ADP-ribose) polymerase activity is also distributed equally between transcriptively active and inactive chromatin regions. The earlier work by Mullins *et al.* (1977), reporting that poly(ADP-ribose) synthetase activity is primarily associated with transcriptively active chromatin, was criticized by Yukioka *et al.* (1978) on the grounds that the procedures used by Mullins and co-workers for shearing the chromatin (mechanical shearing by sonication) were inadequate for the subsequent separation of transcriptively active and inactive chromatin regions, and also that sonication leads to considerable alterations in chromatin structure.

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However, despite this somewhat circumstantial evidence against an involvement of ADP-ribosylation reactions in transcriptional events, a report by Müller & Zahn (1976) purported to demonstrate the modification of RNA polymerase I activity by ADP-ribose, probably by a covalent linkage, when isolated quail oviduct nuclei were incubated with NAD^+, the substrate for poly(ADP-ribose) polymerase. Our own earlier studies with nuclei isolated from baby-hamster kidney fibroblasts seemed to support this conclusion, since the activity of RNA polymerase I was inhibited when the nuclei were incubated with 4mM-NAD^+. When an $(NH_4)_2SO_4$ extract was prepared from control and NAD^+-treated nuclei, the activity of the RNA polymerase I was considerably lower in the extract from the NAD^+-treated nuclei (Furneaux & Pearson, 1980*a*).

This present paper summarizes our subsequent efforts to consolidate these earlier conclusions.

Materials and methods

Cell culture

Baby-hamster kidney cells (BHK-21/C13; Macpherson & Stoker, 1962) were used. Cells were grown routinely at 37°C in an atmosphere of O_2/CO_2 (19:1) in minimal Eagle's medium (Glasgow modification) containing 10% (w/v) tryptose phosphate broth and 10% (v/v) foetal-calf

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serum as previously described (Burke & Pearson, 1979).

Permeabilization procedure

Late-exponentially growing cells (about 2×10^7/ml) were suspended for 30min in 10ml of ice-cold permeabilization buffer, consisting of 0.01M-Tris/HCl, pH7.8 at 4°C, 0.25M-sucrose, 4mM-$MgCl_2$, 1mM-EDTA and 30mM-2-mercaptoethanol (Berger & Johnson, 1976). They were then pelleted by centrifugation (MSE bench centrifuge) and resuspended in 10ml of the permeabilization buffer ready for use in RNA polymerase or poly(ADP-ribose) polymerase assays. Cell counts and the extent of permeabilization were determined at this stage; over 85% of the cells were permeable to Trypan Blue.

Poly(ADP-ribose) polymerase assay

Cells rendered permeable as described above (about 1×10^6 cells) were added to an assay mixture (final volume 360μl) to give final concentrations of 50mM-Tris/HCl, pH8.0 at 25°C, 5mM-$MgCl_2$, 1mM-dithiothreitol and 4mM-[^{3}H]NAD^+ (7mCi/mmol, 10μCi/assay; obtained from Dr. W. J. D. Whish, University of Bath, U.K.). Incubations were at 25°C. Incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as previously described (Furneaux & Pearson, 1980*b*).

DNA-dependent RNA polymerase I assays

Assays were carried out 37°C in a final reaction volume of 360μl containing 3×10^6–6×10^6 permeabilized cells in 50mM-Tris/HCl, pH7.8 at 37°C, 40mM-$(NH_4)_2SO_4$, 2mM-$MnCl_2$, 0.5mM-dithiothreitol, 2mM-ATP, 0.1mM each of GTP and CTP, 20μM-[^{3}H]UTP (1Ci/mmol, 7μCi/assay) and 1μg of α-amanitin/ml.

Radioactivity incorporated into trichloroacetic acid-insoluble material was taken as a measure of RNA synthesis (Furneaux & Pearson, 1980*a*).

Partial purification of DNA-dependent RNA polymerase I from isolated nuclei previously incubated with [^{14}C]NAD^+

Four lots of nuclei were incubated separately for 15min (2×10^8 nuclei/3ml) in 50mM-Tris/HCl, pH8.0 at 25°C, 5mM-$MgCl_2$, 1mM-dithiothreitol and 30μM-[^{14}C]NAD^+ (100Ci/mol). They were then pelleted by centrifugation (800*g* for 10min) and resuspended (3ml each) in ice-cold 50mM-Tris/HCl, pH7.4 at 4°C, containing 1mM-dithiothreitol. Procedures used for purifying the RNA polymerase from the BHK cells are essentially those described by Cooper & Keir (1975). Briefly, the resuspended nuclei were made 0.3M with $(NH_4)_2SO_4$ and sonicated. They were diluted 3-fold by the addition of 6.45ml of glycerol buffer, consisting of 50mM-Tris/HCl, pH7.4 at 4°C, 5mM-$MgCl_2$, 0.1mM-EDTA, 0.5mM-dithiothreitol and 25% (v/v) glycerol. After centrifugation to pellet the residue, the supernatants were combined (now 34ml) and the pellets discarded. Solid $(NH_4)_2SO_4$ was added to the ice-cold supernatant over a 1h period until the final concentration was 0.42g/ml. The precipitate that formed was pelleted by centrifugation and resuspended in 5ml of 30% (v/v) glycerol buffer containing 0.05M-$(NH_4)_2SO_4$ and dialysed overnight against the glycerol buffer. The volume after dialysis was 9ml.

The dialysis residue was applied to a column (2.2cm × 23cm) of DEAE-Sephadex A25 equilibrated with the 30% (v/v) glycerol buffer containing 0.05M-$(NH_4)_2SO_4$. The column was washed with 70ml of the same buffer and then with 30% (v/v) glycerol buffer containing 0.1M-$(NH_4)_2SO_4$ until no more protein was eluted (about 100ml). RNA poly-

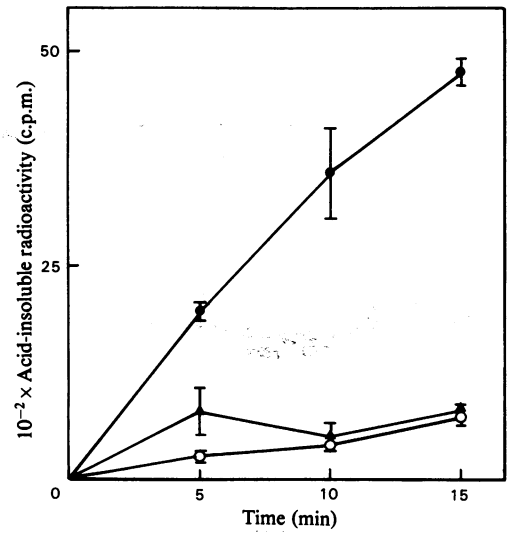

Fig. 1. *Effect of NAD^+ on DNA-dependent RNA polymerase I activity and the influence on this of 3-aminobenzamide*

Nucleotide-permeable cells (about 6×10^6) in 200μl of permeabilization buffer were incubated with or without 5.5mM-3-aminobenzamide at 37°C for 10min. Then 60μl of water, or NAD^+ solution, was added together with 100μl of an RNA assay mixture (see the Materials and methods section) to give final concentrations of 4mM-NAD^+ (or none = control) and 4mM-3-aminobenzamide. Trichloroacetic acid-insoluble radioactivity was determined at the times shown; 1000c.p.m. represents the incorporation of 1.5pmol of UMP into RNA. ●, Control; ○, with 4mM-NAD^+; ▲, prior incubation with 3-aminobenzamide before addition of NAD^+ to 4mM. Results are means ± S.E.M. ($n = 3$).

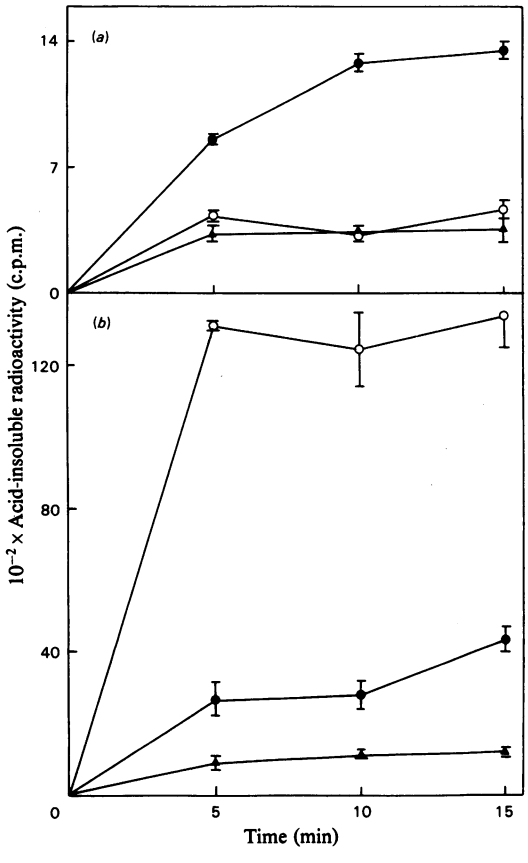

Fig. 2. *Effect of pancreatic deoxyribonuclease I (DNAase I) on DNA-dependent RNA polymerase I and poly(ADP-ribose) polymerase activities: influence of 3-aminobenzamide*

(*a*) Nucleotide-permeable cells (2×10^6) were incubated in the presence or absence of 5.5 mM-3-aminobenzamide at 37°C for 20 min. DNAase I and Triton X-100 were added to the indicated assays, followed immediately by 100 μl of an RNA polymerase assay mixture (see the Materials and methods section). The final concentrations in an assay volume of 360 μl were: 100 μg of DNAase I, 0.04% (v/v) Triton X-100 and 4 mM-3-aminobenzamide. Trichloroacetic acid-insoluble radioactivity was determined at the times shown; 1000 c.p.m. represents the incorporation of 1.5 pmol of UMP into RNA. (*b*) Nucleotide-permeable cells (2×10^6) were incubated in the presence or absence of 5.5 mM-3-aminobenzamide for 20 min at 25°C. DNAase I and Triton X-100 solutions were then added to the indicated assays, followed immediately by 100 μl of a poly(ADP-ribose) assay mixture. The final concentrations in a total assay volume of 360 μl were: 100 μg of DNAase I/assay, 0.04% Triton X-100, 4 mM-3-aminobenzamide (see the Materials and methods section for final concentrations of other components). Trichloroacetic acid-insoluble radioactivity was determined at the times shown; 1000 c.p.m. represents the incorporation of 220 pmol of ADP-ribose. ●, Control; ○, with DNAase I and Triton X-100; ▲, with DNAase I, Triton X-100 and also 4 mM-3-aminobenzamide. Results are means ± S.E.M. ($n = 3$).

merase I and II activities were eluted with a linear gradient (100 ml) of 0.1–0.4 M-$(NH_4)_2SO_4$ in 30% (v/v) glycerol buffer.

The column flow rate was 30 ml/h at 4°C and 3.9 ml fractions were collected.

Results and discussion

Permeabilized cells were used since they are thought to be more representative of the situation *in vivo* than are isolated nuclei used in our earlier studies (Berger *et al.*, 1978; Halldorsson *et al.*, 1978). Fig. 1 shows that the activity of RNA polymerase I in these BHK cells is severely inhibited (>85%) when permeabilized cells are incubated with 4 mM-NAD^+. However, the prior incubation of the cells with 3-aminobenzamide, a potent inhibitor of poly(ADP-ribose) polymerase (Purnell & Whish, 1980), did not relieve this inhibition. Control experiments showed that poly(ADP-ribose) polymerase activity was almost completely abolished (>95%) under the conditions used; RNA polymerase I is unaffected by the drug.

Since damage to DNA is known to cause a stimulation of poly(ADP-ribose) polymerase activity (see Purnell *et al.*, 1980, for references), we have examined the effect of this on RNA polymerase I activity. After treatments of permeabilized cells with pancreatic deoxyribonuclease I, the activity of RNA polymerase I was decreased about 80% (Fig. 2*a*), whereas that of poly(ADP-ribose) polymerase was increased by about 300% (Fig. 2*b*). However, there appears to be no connection between these two events, since, although the inhibitor 3-aminobenzamide almost abolished all poly(ADP-ribose) polymerase activity, it did not relieve the inhibitory effect on the RNA polymerase caused by the deoxyribonuclease.

As an alternative approach to investigating the proposed ADP-ribosylation of RNA polymerase I, we have attempted to repeat in principle one of the experiments described previously by Müller & Zahn (1976). RNA polymerase I was partially purified from nuclei previously incubated with labelled NAD^+ (at 30 μM and sp. radioactivity of 100 Ci/mol, as used by Müller & Zahn, 1976). Fig. 3 shows the elution profiles of RNA polymerase I and II activities after chromatography of an $(NH_4)_2SO_4$ extract of the cells on DEAE-Sephadex (Cooper & Keir, 1975). Our results with BHK cells (Fig. 3) contrast with those of Müller & Zahn (1976), in which quail oviduct nuclei were used, in that we did

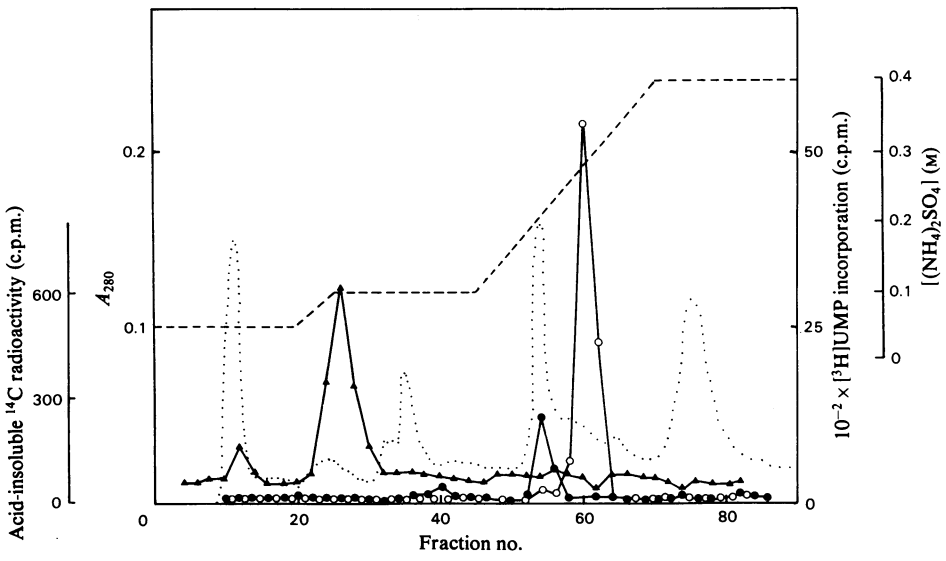

Fig. 3. *Chromatography of RNA polymerase from BHK-21/C13 cells on DEAE-Sephadex*

Nuclei from BHK cells (4×10^8) were incubated with 30μM-[^{14}C]NAD in a reaction mixture composed for poly(ADP-ribose) polymerase (see the Materials and methods section). RNA polymerase activities were solubilized from these nuclei by sonication and precipitation with $(NH_4)_2SO_4$ and applied to a column (2.2cm × 23cm) of DEAE-Sephadex, previously equilibrated with 30% glycerol buffer/0.05M-$(NH_4)_2SO_4$. After washing with the latter buffer followed by 30% glycerol buffer/0.1M-$(NH_4)_2SO_4$ until no more protein was eluted, RNA polymerase activites were eluted from the column with a linear gradient of 0.1–0.4M-$(NH_4)_2SO_4$ in 30% glycerol buffer. Fractions (3.9ml) were collected and 50μl portions assayed for RNA polymerase I or polymerase II activities. Other 0.1ml portions were spotted on to Whatman GF/C filters and trichloroacetic acid-insoluble radioactivity was determined. ●, [^{3}H]UMP incorporation, RNA polymerase I activity; ○, [^{3}H]UMP incorporation, RNA polymerase II activity; ▲, acid-insoluble ^{14}C radioactivity; · · · ·, A_{280}; – – – –, [$(NH_4)_2SO_4$].

Table 1. *Effect of various low-molecular-weight compounds on RNA synthesis catalysed by DNA-dependent RNA polymerase I in permeabilized cells*

Permeabilized cells were incubated for 15min at 37°C with [^{3}H]UTP in the presence (or absence = control) of each in turn of the compounds listed (details in the Materials and methods section). Radioactivity incorporated into acid-insoluble material was used as a measure of RNA polymerase activity. Results are the means ± S.E.M. ($n = 3$).

Compound	Activity (% of control) with added compounds at the following concn.: 1mM	4mM
None	100	100
Nicotinamide	99 ± 17	84 ± 1
NMN	85 ± 6	88 ± 5
β-NAD$^+$	51 ± 6	20 ± 6
α-NAD$^+$	84 ± 8	49 ± 8
NADP$^+$	51 ± 4	51 ± 10
ADP-ribose	63 ± 3	23 ± 12
5-Phosphoribosyl 1-pyrophosphate	92 ± 6	73 ± 14
Thymidine	81 ± 4	85 ± 2
dTMP	72 ± 4	80 ± 13
dTDP	45 ± 2	46 ± 7
dTTP	36 ± 4	44 ± 5
Pyruvate	98 ± 4	90 ± 8
Succinate	81 ± 8	80 ± 4
Citrate	96 ± 2	100 ± 11

not observe a major peak of radioactivity associated with the fractions containing the RNA polymerase I. Most of the radioactivity was eluted with the proteins in the flow-through fractions at 0.05M- and 0.1M-$(NH_4)_2SO_4$. (Similar results were obtained when we used mouse L cells in this type of experiment; result not shown.) We have purified the enzyme further by glycerol-density-gradient analysis, but the radioactivity in the subsequent gradient fractions was too low to measure accurately. There was no indication that the low amount of radioactivity present was preferentially associated with the RNA polymerase.

Does NAD+ have a direct effect on RNA polymerase I activity?

In view of the experimental results described above, it seemed unlikely that ADP-ribosylation was involved in the inhibitory effect of NAD^+ on the RNA polymerase. We then considered whether NAD^+ could inhibit the polymerase directly and specifically, or whether the inhibitory effect that we had observed could be produced by any small, similarly charged molecule. Table 1 summarizes the results obtained. It is apparent that inhibition of RNA polymerase is only caused by those molecules expected to have a nuclear location, such as NAD^+ and related compounds and the thymidine derivatives, and that only those compounds with phosphate groups cause any appreciable inhibition. Small negatively charged molecules not expected to be found in the nucleus, such as phosphoribosyl pyrophosphate and the carboxylic acids, had little or no effect on the enzyme.

In conclusion, our results do not support the concept of a role for ADP-ribosylation in the control of RNA polymerase I activity, contrary to our earlier tentative conclusions based on preliminary data (Furneaux & Pearson, 1980*a*). NAD^+ has been shown to have a direct inhibitory effect on the RNA polymerase in permeabilized cells (true also for the partially purified enzyme eluted from DEAE-Sephadex), but this is not specific for NAD^+. It is possible, however, that NAD^+ (and perhaps related compounds) does exert a similar effect *in vivo*, since it is present in cells at millimolar concentration (see Furneaux & Pearson, 1980*a*), although the fluctuations in nuclear concentration are unknown.

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