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A Novel Function of Poly(ADP-ribosyl)ation: Silencing of RNA Polymerase II-Dependent Transcription†

Shiao Li Oei,* Joachim Griesenbeck, Mathias Ziegler, and Manfred Schweiger

Institut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany

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ABSTRACT: Poly(ADP-ribosyl) transferase (ADPRT) is a nuclear enzyme that catalyzes the synthesis of ADP-ribose polymers from NAD^+ as well as the transfer of these polymers onto acceptor proteins. The predominant acceptor of the poly(ADP-ribose) chains appears to be the enzyme itself. The function of ADPRT is thought to be related to a number of nuclear processes, including DNA repair and transcription. In this study, it was found that polymerase II-dependent transcription in nuclear HeLa extracts was repressed in the presence of NAD^+ at concentrations as low as 1 μM. This repression was strictly dependent on the activity of ADPRT and correlated with the auto(ADP-ribosyl)ation of the enzyme. Subsequent degradation of the ADP-ribose polymers by enzymatic activities present in the nuclear extracts restored transcriptional activity. It would appear from these results that poly(ADP-ribosyl)ation represents the key event of the mechanism underlying NAD^+-dependent silencing of transcription. Importantly, ADPRT- and NAD^+-dependent silencing was observed only if poly(ADP-ribosyl)ation had taken place before formation of the transcription complex was completed. That is, if the nuclear extract was preincubated for more than 15 min in the presence of template DNA, transcription was rendered entirely insensitive to NAD^+. These results suggest that poly(ADP-ribosyl)ation may prevent polymerase II-dependent transcription, but does not interfere with ongoing transcription. Taking into account the known function of ADPRT, this enzyme may facilitate recovery from DNA damage by stimulating DNA repair and silencing transcription.

The nuclear enzyme poly(ADP-ribosyl) transferase (ADPRT,[1] EC 2.4.2.30) modifies proteins by forming and attaching to them poly(ADP-ribose) chains. It is a highly conserved and abundant protein occurring ubiquitously in eukaryotic cells, except yeast. The posttranslational transfer of ADP-ribose moieties from NAD^+ to proteins is thought to be involved in the regulation of processes such as DNA repair, differentiation, proliferation, and neoplastic transformation (reviewed in ref *1*). The major cellular pathway of NAD^+ catabolism has been ascribed to ADPRT activity (*2*, *3*). The enzymatic activity of ADPRT is strongly enhanced after genotoxic treatment of mammalian cells and correlates with the depletion of the intracellular NAD^+ pool (*4*). DNA repair of γ-irradiated plasmids is stimulated by ADPRT in an NAD^+-dependent manner (*5*), and a positive correlation between ADPRT activity and repair of DNA damage induced

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* Author to whom correspondence should be addressed. Telephone: +49 30 8382910. Fax: +49 30 8386509. E-mail: Lity@chemie.fu-berlin.de.

[1] Abbreviations: ADPRT, poly(ADP-ribosyl) transferase; 3-ABA, 3-amionobenzamide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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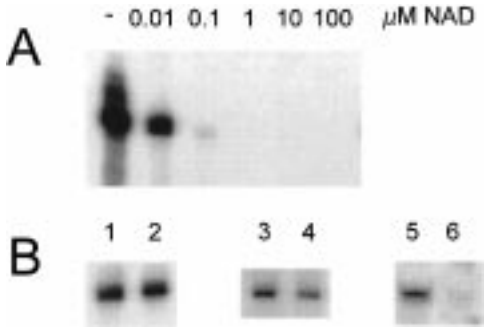

FIGURE 1: NAD^+ affects Pol II-dependent in vitro transcription. Following a preincubation for 30 min as outlined below, transcription reactions were started by the addition of NTPs and incubation was continued for 1 h. Transcripts were separated by PAGE and subjected to autoradiography. (A) HeLa nuclear extract and template pML(C_2AT) were incubated in the absence (−) or presence of NAD^+ as indicated. (B) HeLa nuclear extract and template pML(C_2-AT) were incubated in the presence of 0.6 mM 3-ABA in the absence (lane 1) or presence (lane 2) of 100 μM NAD^+. HeLa nuclear extracts depleted of ADPRT by immunoprecipitation and template pML(C_2AT) were incubated in the absence (lane 3) or presence (lane 4) of 100 μM NAD^+. HeLa nuclear extract, the template pML(C_2AT), and an equal amount (200 ng) of the promotor-less plasmid p(C_2AT) were incubated with 100 μM NAD^+ in the absence (lane 5) or presence (lane 6) of 100 ng of ADPRT.

by alkylating agents has been documented (*6*). Elimination of ADPRT in knocked-out mice rendered cells more susceptible to γ-irradiation as detected by impaired proliferation (*7*). These and other investigations have established ADPRT as a regulator of important nuclear processes. Nevertheless, the actual biological function of ADPRT and its catalytic activity have still largely remained obscure.

Eukaryotic transcription is a complex and tightly controlled process. Regulators of class II gene transcription include the general factors, and other effectors (reviewed in ref *8*). The requirement for transcription factors has been demonstrated (*9*), and fractionation studies led to the characterization of multiple RNA polymerase II-dependent (Pol II) transcription factors (*10*). Among them, the transcription factor II C was isolated from cell extracts (*10*), the active component of which was subsequently identified as the enzyme ADPRT (*11*). However, it was demonstrated to be non-essential for basal transcription (*11*). Studies with a chimeric protein of ADPRT fused to the glucocorticoid receptor revealed that ADPRT activity can be targeted to specific DNA sequences and repress gene expression (*12*). Meisterernst et al. (*13*) reported that unmodified ADPRT possesses the intrinsic capacity to enhance activator-dependent transcription in vitro. The observed ADPRT coactivator function on supercoiled templates did not depend on damaged DNA, but was reversed by NAD^+ (*13*).

In this study, Pol II-dependent transcription was analyzed in HeLa nuclear extracts with regard to the possible role of ADPRT and its catalytic function. When poly(ADP-ribosyl)-ation was induced before a stable transcription complex had been formed, transcription was virtually completely silenced. On the other hand, when the transcription complex had been allowed to form, poly(ADP-ribosyl)ation was without any effect on transcription. These results suggest that ADPRT, when activated (e.g., by DNA strand breaks), may prevent transcription.

EXPERIMENTAL PROCEDURES

Materials

Reagents were purchased from Sigma unless otherwise noted. Nucleotides and HeLa nuclear extracts were obtained from Promega. [α-^{32}P]NAD^+ and [α-^{32}P]CTP were obtained from NEN, DuPont. tRNA and RNase T1 were purchased from Boehringer Mannheim. Polyclonal antibodies raised against human ADPRT, developed in goat, were used as described before (*14*). The recombinant ADPRT-automodification domain and full-length enzyme were overexpressed as His-tagged fusion proteins and purified as described previously (*15*).

Methods

Standard in Vitro Transcription Assays. Standard transcription reactions were conducted using a supercoiled plasmid pML(C_2AT) as a template (*16*). HeLa nuclear extracts containing 18 μg of protein per reaction mixture were incubated with 200 ng of pML(C_2AT) for 30 min at ambient temperature in transcription buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.25 mM DTT, 10% glycerol, and 7 mM $MgCl_2$]. Transcription was started by adding NTPs [0.6 mM ATP, 0.6 mM UTP, 25 μM [α-^{32}P]CTP (10 000 cpm/pmol), and 0.1 mM 3′-*O*-methyl-GTP]. The final volume was 15 μL. Incubation was then continued for 1 h at 30 °C. For some experiments, the transcription assays contained further additions, which were added at the times and concentrations indicated in the figure legends. The transcription reactions were stopped, and after phenol/chloroform extraction, RNA was precipitated with ethanol, resuspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and incubated with RNase T1 (1 unit per reaction) for 15 min at 37 °C. Then, samples were subjected to 5% PAGE, containing 7 M urea in TBE buffer, and autoradiography.

Preparation of Antibodies and Immunodepletion of Nuclear HeLa Extracts. Polyclonal antibodies developed in rabbit and raised against either the recombinant DNA-binding domain (amino acids 1−232) or the automodification domain (amino acids 337−573) were affinity-purified according to a published method (*17*). These antibodies were highly specific and did not exhibit cross-reactivity with any other nuclear proteins. Either of the purified antibodies (0.3 μg) was coupled to 350 μg of protein A Sepharose (*18*) and incubated with 100 μg of HeLa nuclear extracts in transcription buffer for 1 h at 4 °C. After centrifugation, the supernatant was used for the transcription assays.

ADP Ribosylation and Chain-Length Determination of Protein-Bound ADP-Ribose Polymers. HeLa nuclear extracts containing 18 μg protein per reaction mixture were incubated with 200 ng of pML(C_2AT) in the presence of 1 μM [α-^{32}P]-NAD^+ (3 μCi per reaction) in transcription buffer in a total volume of 15 μL at 30 °C. Incubation was stopped by adding 15 μL of SDS sample buffer. Proteins were separated by 12% SDS−PAGE and subjected to autoradiography. Size analysis of ADP-ribose polymers was performed as described by Panzeter and Althaus (*19*).

RESULTS

NAD^+-Dependent Silencing of Transcription Is Mediated by ADPRT Activity. Standard in vitro Pol II transcription

assays were carried out with cell-free HeLa nuclear extracts (*20*) in the presence of various amounts of NAD^+. The plasmid pML(C_2AT) (*16*), carrying the adenovirus major late promoter, was used as the template (Figure 1A). The presence of only 10 nM NAD^+ was sufficient to significantly repress transcription. Moreover, at concentrations higher than 100 nM NAD^+, basal transcription was apparently completely silenced (Figure 1A).

As ADPRT was the major candidate mediating the effect of NAD^+, this possibility was explored in detail. First, 3-aminobenzamide (3-ABA), a potent inhibitor of ADPRT activity (*21*), was tested to interfere with NAD^+-dependent transcriptional silencing. When in vitro transcription was carried out in the presence of 0.6 mM 3-ABA, transcription was slightly reduced (Figure 1B, lane 1), but the addition of 100 μM NAD^+ did not affect transcription (Figure 1B, lane 2). In a further experiment, HeLa nuclear extracts were depleted of ADPRT by immunoprecipitation. ADPRT-free nuclear extracts exhibited transcriptional activity, whether NAD^+ was present in the assay (Figure 1B, lanes 3 and 4). Some residual ADPRT activity ($\ll$10%) in the nuclear extracts after immunodepletion may account for the slight suppression of transcription in the presence of NAD^+ (Figure 1B, lane 4). Significant transcriptional activity remained in the presence of 100 μM NAD^+ when the transcription reaction was carried out in the presence of 200 ng of pML(C_2AT) template and 200 ng of the promotor-less plasmid p(C_2AT) (Figure 1B, lanes 5 and 6). The amount of endogenous ADPRT in the nuclear extracts (about 20 ng of full-length enzyme per transcription reaction) could have been limiting for efficient silencing of transcription when 400 ng of DNA was present; that is, ADPRT may have bound to the additional DNA without being part of a transcription complex. Such a possibility exists owing to the high affinity of this enzyme for DNA (*22*). Importantly, under these conditions, addition of 100 ng of purified recombinant ADPRT restored NAD^+-dependent silencing of transcription (Figure 1B, lane 6). In the absence of NAD^+, the addition of purified recombinant ADPRT had no detectable effect on transcription (not shown). These findings indicate that, not the absolute amounts, but the ratio of DNA to ADPRT may be critical for the NAD^+-dependent silencing.

ADPRT- and NAD^+-Dependent Silencing Occurs during Transcription Initiation. Preincubation of nuclear extract with 100 μM NAD^+ for 30 min before the addition of the pML(C_2AT) template led to silencing of transcription (Figure 2A, left lane). In contrast, when additions were made in the opposite order [preincubation of pML(C_2AT) template with nuclear extract, followed by the addition of 100 μM NAD^+ after 30 min], transcription was not repressed (Figure 2A, right lane). This result suggested that the silencing effect of NAD^+ should take place during transcription initiation, presumably during the formation of the transcription complex. From Figure 2B, it is obvious that repression of transcription occurred only if NAD^+ was added within the first minutes following the addition of template DNA to the nuclear extract. Addition of NAD^+ to activated ADPRT (i.e., in the presence of DNA) leads to the synthesis of large ADP-ribose polymers within a few minutes (*23*). Therefore, NAD^+-dependent silencing of transcription may depend on the rate of the ADP ribosylation reaction.

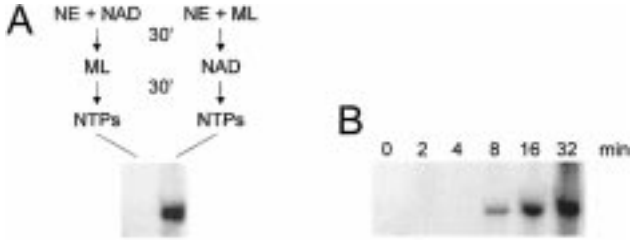

FIGURE 2: ADPRT- and NAD^+-dependent silencing occurs only within the first minutes of transcription initiation. (A) HeLa nuclear extracts (NE), template pML(C_2AT) (ML), 100 μM NAD^+, and nucleotides (NTPs) were added to the transcription assays in the order and at the times indicated. After addition of NTPs, the transcription reaction was allowed to proceed for 1 h. (B) HeLa nuclear extracts and the template pML(C_2AT) were preincubated for the times indicated followed by the addition of 100 μM NAD^+ and NTPs. Incubation was then continued for 1 h.

To address this point, NAD^+ analogues substituted in the purine base were examined in their ability to silence Pol II transcription. These NAD^+ analogues are suitable as substrates for the transfer and the polymerization reaction catalyzed by ADPRT, but the velocity of the automodification reaction is substantially reduced (*24*). Within the time frame of the experiment (1 h), nicotinamide guanine dinucleotide and nicotinamide hypoxanthine dinucleotide did not affect transcription (not shown). Another NAD^+ analogue, 3-acetylpyridine adenine dinucleotide, which exhibited a reaction rate similar to that of NAD^+ (*24*), silenced transcription as efficiently as NAD^+ (not shown). These observations indicate that the rate of polymer fomation by ADPRT may be crucial for NAD^+-dependent silencing of transcription.

Poly(ADP-ribosyl)ation Correlates with Silencing of Pol II Transcription. Since ADPRT activity appeared to be important within the first minutes of transcription initiation, this time period was analyzed with regard to ADP ribosylation (Figure 3). Nuclear extract and template DNA were incubated in the presence of 1 μM [^{32}P]NAD^+. Incubation was stopped at the times indicated, and proteins were then subjected to gel electrophoresis and autoradiography. The main target for modification with poly(ADP-ribose) was ADPRT itself as determined by Western blotting and immunodetection with anti-ADPRT antibodies (Figure 3A). Besides ADPRT (M_r = 116 000), another significantly labeled protein migrating with an apparent molecular weight of about 85 000 was identified as a C-terminal ADPRT fragment present in the nuclear extract. Other weakly radiolabeled bands detected in the autoradiogram were presumably the result of heteromodification of nuclear acceptor proteins (for example, labeled bands with M_rs of about 70 000, 50 000, and 35 000). In parallel experiments, the labeled polymers were detached from the proteins and their sizes analyzed. The occurrence of long ADP-ribose polymers with more than 30 ADP-ribose residues per chain was observed already within the first minutes of the incubation (Figure 3B). The size of the polymers was stable for about 15 min, while after 60 min, only short polymers with less than 7 ADP-ribose residues per chain remained (Figure 3B, last lane). This disappearance of poly(ADP-ribose) can be attributed to the activity of catabolic enzymes [e.g., poly(ADP-ribose) glycohydrolase or phosphodiesterases] present in the nuclear extracts (*25*). If the transcription

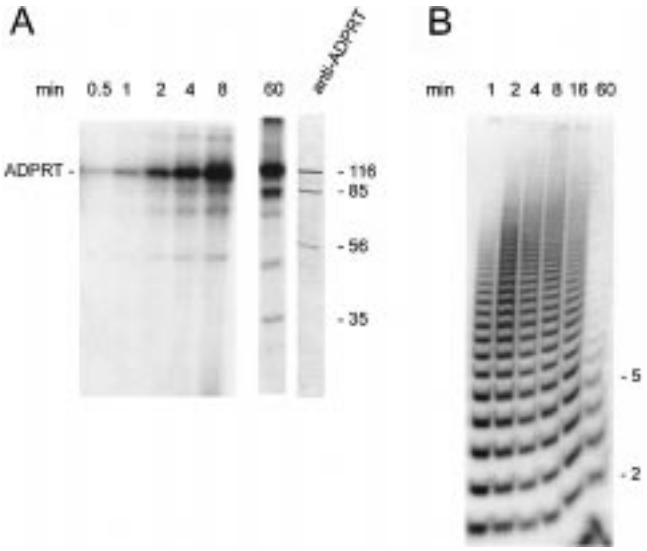

FIGURE 3: Time course of poly(ADP-ribosyl)ation during silencing of transcription. HeLa nuclear extracts and the template pML(C_2-AT) were incubated in the presence of 1 μM ^{32}P-labeled NAD$^+$ for the times indicated. (A) Incubation was stopped by the addition of SDS−PAGE sample buffer. Proteins were separated by 12% SDS−PAGE and subjected to autoradiography (first six lanes). For the rightmost lane, after SDS−PAGE, proteins were transferred onto nitrocellulose and immunostained using anti-ADPRT antibodies. The position of ADPRT and relative molecular sizes are indicated ($M_r \times 10^{-3}$). (B) Incubation was stopped by TCA precipitation, and poly(ADP-ribose) was detached from proteins by alkaline treatment. ^{32}P-labeled polymers (20 000 cpm per lane) were separated by 20% PAGE and subjected to autoradiography. Numbers of ADP-ribose units are indicated on the right.

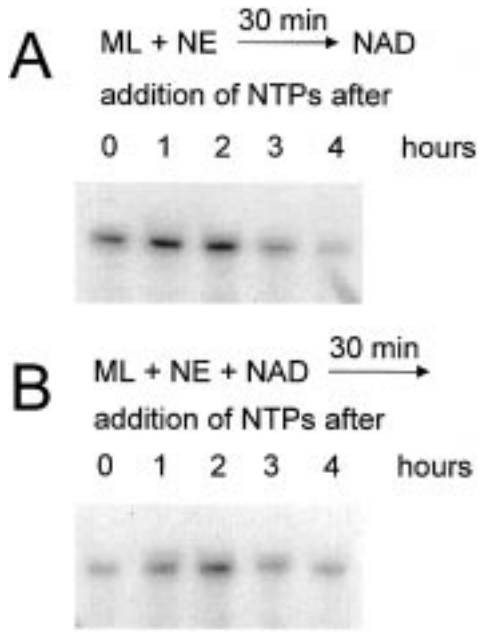

FIGURE 4: ADPRT-dependent silencing of transcription is reversible. Following a preincubation for 30 min as outlined below, incubation was continued for the time indicated. Transcription reactions were then started by the addition of NTPs, and incubation was continued for 1 h. (A) HeLa nuclear extracts and the plasmid pML(C_2AT) were incubated for 30 min, and then 1 μM NAD$^+$ was added. These samples served as a control, because addition of NAD$^+$ at this stage had no effect on transcription (see Figure 2A). (B) HeLa nuclear extracts and the plasmid pML(C_2AT) were incubated for 30 min in the presence of 1 μM NAD$^+$.

reaction was carried out in the presence of 100 ng of the isolated recombinant automodification domain of ADPRT (including amino acids 337−573), the NAD$^+$-dependent silencing effect was absent even in the presence of 100 μM NAD$^+$. The automodification domain serves as an acceptor for poly(ADP-ribose) and, therefore, drastically reduced the extent of automodification of the endogenous full-length ADPRT (not shown).

ADPRT-Dependent Silencing of Transcription Is Reversible. As shown in Figure 3B, addition of NAD$^+$ to the nuclear extracts led to transient occurrence of poly(ADP-ribose) polymers. This observation indicated the possibility that the silencing of transcription could be temporary as well. The data reported in Figure 4B support this suggestion. Addition to the nuclear extracts of NAD$^+$ together with the DNA template prevented transcription only if the nucleotides were added within 30 min (see above). After 1 h, the effect of NAD$^+$ was already much less pronounced. When transcription was started by nucleotides 2 h after adding NAD$^+$ and template DNA to the nuclear extracts, the transcription reached almost control levels (compare panels A and B of Figure 4). Incubation with longer durations led again to a decrease in transcriptional activity. However, this decrease can be attributed to the deterioration of the nuclear extracts, since it occurred similarly in the control samples (compare the last two lanes of Figure 4A,B). It should be pointed out that the recovery of transcriptional activity coincided with the disappearance of protein-bound poly(ADP-ribose) polymers (Figure 3B), lending further support for the role of poly(ADP-ribosyl)ation in the observed silencing of transcription.

DISCUSSION

This study demonstrates that transcription by Pol II can be silenced in an NAD$^+$-dependent manner mediated by the nuclear enzyme ADPRT. The catalytic activity of ADPRT, that is, the synthesis of protein-bound ADP-ribose polymers from NAD$^+$, is essential for this repression of transcription and correlates with the extent of poly(ADP-ribosyl)ation. When activated by binding to DNA, ADPRT is able to synthesize large polymers from NAD$^+$ within a time frame (1−2 min) that precedes the completed assembly of the transcription complex. It would appear, therefore, that ADPRT exerts its regulatory function during the initiation of transcription, presumably by interfering with the formation of the preinitiation complex. This view is supported by the observation that activation of ADPRT (by adding NAD$^+$) has no effect on transcription when the preinitiation complex has been allowed to form (Figure 2A).

Transcription is controlled by a variety of mechanisms. One principal way includes the covalent modification of proteins that participate in transcription or its regulation. For example, reversible phosphorylation of the transcriptional machinery represses transcription at mitosis (reviewed in ref *26*). Furthermore, histone deacetylation has been reported in several recent studies to exert a regulatory effect on transcription (reviewed in refs *27* and *28*). As a result of this study, poly(ADP-ribosyl)ation should also be regarded as a covalent modification regulating transcription. Although the crucial acceptor(s) of the ADP-ribose polymers responsible for the silencing of transcription has not been identified, there are indications that it may be the automodification of ADPRT itself. ADPRT was by far the most prominently

modified protein in the nuclear extracts. Moreover, detectable heteromodification occurred only several minutes after the ADPRT-dependent silencing was already maximal (Figure 3A). Nevertheless, the possibility of poly(ADP-ribosyl)ation of other transcription factors has been demonstrated (*28*) and may be essential for the observed silencing. It is relevant to this point that the degradation of ADP-ribose polymers and the reversal of ADPRT-dependent silencing of transcription occurred concomitantly. This observation may indicate a role for poly(ADP-ribose) glycohydrolase in the regulation of transcription.

Besides DNA repair, cell cycle regulation, and differentiation, ADPRT function has already been implicated in the regulation of transcription in previous studies. For example, ADPRT was found to be a component of the transcription complex (*10*, *11*), and a stimulating effect of ADPRT on activator-dependent transcription which was completely reversed by NAD^+ has been reported (*13*). However, the role of ADPRT as a negative regulator of transcription with the requirement for its fast catalysis adds an unexpected new aspect concerning the biological function of this enzyme. In this regard, it is important to note that ADPRT stimulated excision DNA repair in the presence of NAD^+ (*5*, *9*). Combined with the results presented herein, a model that places ADPRT as a switch between DNA repair and transcription may be proposed. Occurrence of DNA damage would be detected by ADPRT, thereby stimulating its enzymatic activity. As a result, transcription would be silenced and DNA repair enhanced. Therefore, one of the functions of ADPRT could be providing resources for efficient DNA repair by liberating some of the factors required. In addition, recent evidence suggests that the ADP-ribose polymers may be used to generate ATP in DNA repair sites by the concerted action of poly(ADP-ribose) glycohydrolase and ADP-ribose pyrophosphorylase (*31*). A regulatory role is further supported by a number of studies demonstrating the requirement of ADPRT during the recovery from DNA damage (*6*, *32*−*36*). The proposed model is also in line with the finding that ADPRT knocked-out mice do not lack any fundamental cellular function (*7*). Importantly, as demonstrated here, the activation of ADPRT would not interfere with ongoing transcription, but only prevent the formation of new transcription complexes. Such regulation appears to be plausible, because it would focus nuclear processes on the repair of damaged DNA without interrupting vital processes.

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