Activation of the Human DNA Polymerase \( \beta \) Promoter by a DNA-alkylating Agent through Induced Phosphorylation of cAMP Response Element-binding Protein-1*

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Satya Narayan, Feng He, and Samuel H. Wilson†

From the Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555

Treatment of cells with the DNA-alkylating agent N-methyl-\( N \)-nitro-\( N \)-nitrosoguanidine (MNNG) induces expression of the endogenous mammalian DNA polymerase \( \beta \) (\( \beta \)-pol) gene and of the cloned promoter in transient expression studies. The lone CAMP response element (CRE) in the core promoter, along with functional protein kinase A, is critical for the MNNG-induced up-regulation. Recently, we described a kinetic mechanism for transcriptional regulation of the \( \beta \)-pol promoter in vitro and found that CRE-binding protein (CREB) from MNNG-treated cells differentially up-regulates the promoter by stimulating formation of closed preinitiation complex (RP\( \gamma \)). Here, using a CRE-dependent chimeric \( \beta \)-pol promoter, we purified the RP\( \gamma \) assembled with nuclear extract from MNNG-treated and control HeLa cells. Comparison of proteins in the purified RP\( \gamma \) samples revealed that the MNNG induction is associated with a strong increase in the Ser\( ^{133} \)-phosphorylated form of recombinant CREB (CREB-1). CREB depletion of the nuclear extracts diminished transcriptional activity, and addition of purified Ser\( ^{133} \)-phosphorylated CREB-1 restored activity, whereas unphosphorylated CREB-1 did not. Addition of phosphorylated CREB-1 to the control cell extract mimicked the MNNG-induced up-regulation of transcriptional activity. These results indicate that phosphorylation of CREB-1 is the probable mechanism of activation of the \( \beta \)-pol promoter after treatment of cells with the DNA-alkylating agent MNNG.

DNA polymerase \( \beta \) (\( \beta \)-pol)\(^1\) is a eukaryotic cellular polymerase involved in "gap-filling" synthesis during base excision repair (for review, see Ref. 1). Accordingly, \( \beta \)-pol has been found to promote repair of a G:U base pair (2), a monofunctional DNA adduct (3, 4), and an abasic site in DNA (5). Embryonic mouse fibroblasts with a homozygous \( \beta \)-pol gene deletion are deficient in base excision repair activity and are hypersensitive to monofunctional DNA-alkylating agents (6). The core promoters of the human, bovine, and rodent \( \beta \)-pol genes have been described (7–9). These cloned \( \beta \)-pol promoters lack typical TATA and CCAAT elements, are G-C-rich, and have distinct binding elements for Sp1 and activating transcription factor (ATF)/cAMP response element-binding protein, referred to as CREB throughout. The CRE site in the \( \beta \)-pol promoters is required for full promoter activity (7), and a purified CREB from bovine testis binds specifically to the conserved CRE site of the \( \beta \)-pol promoters and stimulates promoter activity in vitro (7, 10). The human and bovine \( \beta \)-pol promoters also are known to have a functional binding element for the YY1 family of initiation site binding proteins (11).

\( \beta \)-Pol gene expression is induced after exposure of cells to the DNA-alkylating agent MNNG. This induction required transcription (12), and use of a transfected \( \beta \)-pol core promoter fusion gene revealed transcriptional up-regulation of the \( \beta \)-pol promoter after MNNG treatment. This response is mediated through the CRE of the \( \beta \)-pol promoter (13), and up-regulation of promoter activity is dependent upon the protein kinase A (PKA) signal transduction pathway (14, 15).

Using an in vitro transcription assay system, we found that the rate of transcript formation from a chimeric human \( \beta \)-pol promoter was much higher with nuclear extract (NE) from MNNG-treated HeLa cells than with extract from control HeLa cells. Further results indicated that this up-regulation was dependent upon CREB. The role of CREB from normal and MNNG-treated cells on the transcription initiation process has been described; CREB from MNNG-treated cells supports recruitment of more RP\( \gamma \) than CREB from control cells (16). The present investigation was conducted to isolate RP\( \gamma \) assembled from control and MNNG-treated HeLa cells and to characterize the CREB family member(s) present and its possible modification as a function of cellular MNNG treatment.

**EXPERIMENTAL PROCEDURES**

Treatment of Cells with MNNG and Preparation of NE—HeLa cells (S3, from ATCC) were grown as a monolayer in 150-mm culture dishes in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum. After cells were grown to 80% confluence, the medium was replaced with fresh medium containing 30 \( \mu \)M MNNG. The MNNG stock solution was prepared in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the medium was 0.1% (v/v). For \( ^{32} \)P metabolic labeling experiments, cells were treated simultaneously with 0.2 mCi/ml \( ^{32} \)P orthophosphate. After 5 h of MNNG treatment, cells were scraped and washed twice with cold phosphate-buffered saline (pH 7.4). Normal cells received 0.1% (v/v) dimethyl sulfoxide solution and were treated in the same way without MNNG. NEs from normal and MNNG-treated cells were prepared by the procedure of Shapiro et al. (17) as described previously (10, 16). CREB-depleted NE (NEd) was prepared as described previously (10) and showed no detectable CRE binding activity in electrophoretic mobility shift assays (10).

Preparation of Biotinylated pSH15 Template—The pSH15 template, a derivative of the human \( \beta \)-pol promoter (16), was restricted with EcoRI, and overhanging ends were filled with Klenow fragment, replacing GTTP with biotin-11-dUTP. The standard reaction mixture for biotinylation (in a final volume of 100 \( \mu \)l) contained 100 \( \mu \)g of pSH15

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‡To whom correspondence should be addressed: Sealy Center for Molecular Science, Medical Research Bldg., J-68, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1068. Tel.: 409-772-3367; Fax: 409-772-6334.

\(^{1}\)The abbreviations used are: \( \beta \)-pol, DNA polymerase \( \beta \); ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; MNNG, N-methyl-\( N \)-nitro-\( N \)-nitrosoguanidine; PKA, protein kinase A; NE, HeLa nuclear extract; NEd, CREB-depleted HeLa nuclear extract; RP\( \gamma \), closed preinitiation complex; PAGE, polyacrylamide gel electrophoresis; phospho-CREB-1, protein kinase A-phosphorylated recombinant CREB-1; CBP, CREB-binding protein.
template (EcoRI-restricted); a 500 μm concentration each of dATP, dCTP, dGTP; 40 μm biotin-11-dUTP; 1× TMD buffer (50 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 0.2 mM dithiothreitol); and 10 units of Klenow fragment. The reaction mixture was incubated at 25°C for 15 min. DNA was extracted with phenol-chloroform, precipitated with ethanol, and finally purified on a Sephadex G-50 column to remove unincorporated biotin-dUTP.

Purification of Preinitiation Complex—Transcriptionally active RPc was prepared from control and MNNG-treated cells by magnetic Streptavidin-agarose bead selection. One μg of biotinylated pSH15 template (pSH15 (biotin)) was incubated for 30 min at 22°C with 30 μg of NE from control or MNNG-treated cells in 25 μl of transcription buffer containing 20 mM Hapes (pH 7.9), 6.5 mM MgCl₂, 65 mM KCl, 2 mM dithiothreitol, and 10% (v/v) glycerol. Then 2 μl of magnetic Streptavidin-agarose beads (10 mg/ml Dyna beads M-280 from Dynal Inc.) was added to the above mixture, and the incubation was continued for an additional 30 min on a rotary mixer. The RPc, assembled on pSH15 (biotin) template was separated from the rest of the NE proteins utilizing a Dynal MPC E/E-1 magnet (Dynal Inc.), and used directly for transcription assays. For electrophoretic mobility shift assay and Southwestern and Western blot analysis, the RPc purification was scaled up 25-fold. Transcription factors assembled in RPc were released from the pSH15 (biotin) template in 100 μl of transcription buffer containing 20 mM Hapes, (pH 7.9), 6.5 mM MgCl₂, 65 mM KCl, 2 mM dithiothreitol, and 10% (v/v) glycerol. Then 2 μl of magnetic Streptavidin-agarose beads was extracted with phenol-chloroform, precipitated with ethanol, and resolved on a 6% polyacrylamide, 8M urea gel. The run-off transcript formation was measured (Fig. 1A, left panel). To compare the rate of RPc assembly for the two NEs, the preincubation mixture was taken at different times, and the amount of RPc, assembled was measured by single-cycle run-off transcription formation. The data are plotted in Fig. 1A (right panel). The computer-derived curves fitting the data indicated that the rate constants of RPc formation with control cell NE and MNNG-treated cell NE were similar, yet the amount of RPc formed with the MNNG-treated cell NE was much greater than that formed with the control cell NE. From these results it appeared that the amount, rather than the rate constant, of RPc assembly was stimulated by MNNG treatment of cells.

Purification of Transcriptionally Active RPc—In our previous studies, a NE was used for RPc assembly. Under these conditions, nuclear factors or other nuclear proteins not involved in RPc assembly are also present in the incubation mixture, potentially complicating analysis of factors assembled on the promoter. Since RPc dissociation is very slow under the conditions described (10), we applied a technique with immobilized promoter DNA to purify preassembled RPc (19, 20). A similar approach was used by Zawel et al. (21), among others, to describe the fate of transcription factors during RNA polymerase II transcription initiation and elongation. Biotinylated pSH15 was incubated with NE from control cells and MNNG-treated cells to assemble RPc, which was then purified by use of magnetic streptavidin-agarose beads as described in "Experimental Procedures." In vitro transcription with this purified RPc revealed that the correctly initiated single cycle run-off transcript (284 nucleotides) was formed with RPc, assembled with NE from both control and MNNG-treated cells (Fig. 1C). The amount of transcript formed by purified RPc, from MNNG-treated cells was greater than that with RPc from control cells. Since transcription was initiated with purified RPc, and was limited to a single cycle, these results indicated that the amount of RPc assembled was stimulated by MNNG treatment. The results, therefore, corroborated the findings in Fig. 1A.

CREB-1 in RPc—Previously, we reported that pSH15 promoter activity is dependent upon CRE and that the enhanced transcriptional activity of the MNNG-treated cell NE is mediated through CREB (16). Thus, CREB purified from MNNG-treated cells was transcriptionally more active than CREB-1, was eluted with 0.5 M KCl and purified on a CRE oligonucleotide affinity column. The purified CREB-1 was phosphorylated in a 100-μl reaction mixture containing 5 μg of CREB-1 (100 pmol), 25 mg Tris-HCl (pH 7.5), 20 mg MgCl₂, 1 mM ATP, 6 mg/ml dithiothreitol, 10% (v/v) glycerol, and 100 units of the catalytic subunit of PKA (Sigma). The sample was incubated at 37°C for 30 min. To prepare phosphorylated CREB-1, PKA-phosphorylated CREB-1 (2 pmol) was incubated with 2 units of (Boehringer Mannheim) at 37°C for 1 h. Phosphorylated and dephosphorylated CREB-1 was purified on an ATP/CREB oligonucleotide affinity column, dialyzed, and stored at −70°C for further analysis. To dephosphorylate CREB from control and MNNG-treated cells, 50 μg of NE was incubated with 20 units of calf intestinal phosphatase at 37°C for 1 h.

RESULTS

Recruitment of Closed Preinitiation Complex Is Stimulated by MNNG Treatment—In previous experiments, we used a fixed saturating concentration of promoter DNA (12 nm) to examine transcription by NE from control or MNNG-treated HeLa cells (16). We found an ~10-fold greater overall rate of run-off transcript formation with NE from MNNG-treated cells than with NE from control cells (16). A similar result is illustrated in Fig. 1A where the pSH15 concentration dependence of run-off transcript formation was examined. The RPc was assembled with NE from control or MNNG-treated cells, and after a preincubation to allow RPc assembly, single-cycle run-off transcript formation was measured (Fig. 1A, left panel). To compare the rate of RPc assembly for the two NEs, the preincubation was performed for different periods with a limiting amount of promoter. Aliquots from the preincubation mixture were taken at different times, and the amount of RPc, assembled was measured by single-cycle run-off transcription formation. The data are plotted in Fig. 1A (right panel). The computer-derived curves fitting the data indicated that the rate constants of RPc formation with control cell NE and MNNG-treated cell NE were similar, yet the amount of RPc formed with the MNNG-treated cell NE was much greater than that formed with the control cell NE. From these results it appeared that the amount, rather than the rate constant, of RPc assembly was stimulated by MNNG treatment of cells.
from control cells (16). However, it was unclear which member of the CREB superfamily is the target of MNNG treatment. In the present study, using purified RPc assembled from NE of control and MNNG-treated cells, experiments were performed to identify and characterize the RPc-associated CREB. DNA binding analysis of proteins in purified RPc used Southwestern blotting with a 32P end-labeled CRE oligonucleotide as a probe. The same amount of NE was used in the preparation of RPc from either control or MNNG-treated cells. Results from these experiments are presented in Fig. 2A; lanes 1 and 2 contained proteins from control and MNNG-treated cells, respectively. In lanes 3 and 4 recombinant CREB-1 and ATF-1, respectively, were run as controls. A 43-kDa polypeptide, similar in size to CREB-1, was the major CRE DNA-binding species detected. Also, the amount of 32P CRE oligonucleotide binding activity was similar with purified RPc proteins from control and MNNG-treated cell NE (Fig. 2A). Protein-DNA interactions were also studied by incubation of proteins isolated from purified RPc assembled from NE of control and MNNG-treated cells, with a 32P end-labeled oligonucleotide probe and subsequent resolution of the protein-DNA complexes by electrophoretic mobility shift assay (data not shown). The CRE oligonucleotide binding was specific, as an excess of unlabeled CRE oligonucleotide competed binding, whereas a nonspecific oligonucleotide did not. The amount of CRE oligonucleotide binding activity with protein isolated from each RPc was similar by this gel shift assay. The presence of 43-kDa CREB-1 in RPc was confirmed further by Western blot analysis. CREB-1 specific antibody detected a 43-kDa protein in both the NE (lanes 1 and 2) and in purified RPc (lanes 3 and 4) assembled from control or MNNG-treated cells (Fig. 2B). It appeared from these results that CREB-1 was present in a similar amount in purified RPc assembled from each NE.

MNNG-induced phosphorylation of 43-kDa CREB-1—CREB purified from MNNG-treated cells is known to have greater transcriptional activity than that from control cells (16). It is also known that the MNNG-induced transcriptional up-regulation is mediated through the PKA signal transduction pathway and that an intact CRE in the β-pol promoter is required for the response (15; for review, see Ref. 22). CREB is known to be phosphorylated by PKA in vivo, and this phosphorylation is critical for transcriptional activation of some CREB-dependent genes (23). Phosphorylation of CREB does not affect its affinity for strong CRE sites (for review, see Ref. 24) such as the site in the β-pol promoter. We examined phosphorylation of CREB in
response to MNNG treatment of cells metabolically labeled with \(^{32}\text{P}\)orthophosphate with or without simultaneous treatment with MNNG. RP, was eventually purified from NE from \(^{32}\text{P}\)-labeled control and MNNG-treated cells, and RP, DNA-binding proteins were purified further on a CRE oligonucleotide affinity column. The affinity column-bound proteins were resolved by SDS-PAGE (Fig. 3). The results indicated that phosphorylation of the 43-kDa CREB-1 was much higher in RP, from MNNG-treated cells (duplicate lanes 3 and 4) than in RP, from control cells (duplicate lanes 1 and 2).

Previous studies have shown that the PKA signal transduction pathway is required for MNNG-induced \(\beta\)-pol promoter up-regulation (14, 15) and that PKA phosphorylates CREB-1 at Ser\(^{133}\) (23). In the present study, we examined whether the increased CREB-1 phosphorylation after MNNG treatment (Fig. 3) involved Ser\(^{133}\). We used an anti-phospho-CREB polyclonal antibody (25), which is specific for recognition of Ser\(^{133}\) (23). In the present study, we examined whether the phosphorylation of the 43-kDa CREB-1 was much higher in RP, from MNNG-treated cells (duplicate lanes 3 and 4) than in RP, from control cells (duplicate lanes 1 and 2).

To examine further the MNNG-induced phosphorylation of CREB-1 and to confirm the specificity of the anti-phospho-CREB polyclonal antibody, NEs were treated with calf intestinal phosphatase and probed with the polyclonal antibody. The results showed a strongly diminished signal in the NE from MNNG-treated cells after calf intestinal phosphatase treatment (Fig. 4, lane 4; compare with lane 2). These results indicate that MNNG treatment induces CREB-1 phosphorylation at Ser\(^{133}\). To confirm further that the protein recognized by the anti-phospho-CREB polyclonal antibody was CREB-1, NE was prepared and used to perform the Western blot analysis. That antibody did not show a phosphorylated CREB-1 signal with NE (Fig. 4, lanes 5 and 6) suggested that all of the phosphorylated CREB-1 was removed from the NE by CREB oligonucleotide affinity column depletion. The depletion of CREB from the NE was confirmed by electrophoretic mobility shift assay (data not shown). With NE from control cells (Fig. 4, lane 1) the very low or undetectable signal observed with this antibody further suggests that phospho-CREB-1 was present in a limiting amount in control cells.

Recombinant CREB-1 Phosphorylated at Ser\(^{133}\) Mimics the MNNG Effect and Restores Transcriptional Activity in NEd—Recombinant CREB-1 was purified and phosphorylated with PKA as described under “Experimental Procedures.” PKA phosphorylation of CREB-1 at Ser\(^{133}\) was measured by Western blot analysis with anti-phospho-CREB polyclonal antibody (Fig. 5A, lane 5). Unphosphorylated CREB-1 (recombinant or phosphatase-treated) is not cross-reactive with this antibody (Fig. 5A, lanes 4 and 6). After observing that phosphorylation of RP, associated CREB-1 is induced by MNNG treatment, we examined the effect of CREB-1 on transcriptional activity of pSH15 (Fig. 5B). A dose-dependent increase in transcript formation was observed. The NE from control cells supplemented with 10 ng of phosphorylated CREB-1 had transcriptional activity similar to that of NE from MNNG-treated cells (striped bar). On the other hand, the same NE supplemented with 10 ng of unphosphorylated CREB-1 showed promoter activity (open bar) that was the same as control NE alone. These results are consistent with the idea that phosphorylated CREB-1 is limiting in the control cell NE and that phosphorylation of CREB-1 is stimulated by MNNG treatment, in turn resulting in up-regulation of the \(\beta\)-pol promoter activity. To test this idea further, we first purified NEd and then assembled RP, with either pSH15 or the wild type \(\beta\)-pol promoter (pP8) in the presence or absence of unphosphorylated or Ser\(^{133}\)-phosphorylated CREB-1. pP8 was included in this experiment for comparison. The results from these experiments are shown in Fig. 6, and run-off transcripts generated with pSH15 (180 nucleotides) and pP8 (210 nucleotides) are indicated by arrows. Run-off transcript formation with control NE (lane 1) was higher than with NEd (lane 2) with both templates (panels A and B). This observation was consistent with previous findings that NEd exhibits only a low basal level of transcriptional activity (16). Addition of unphosphorylated CREB-1 to NEd (lane 3, panels A and B) did not restore promoter activity. However, addition of PKA-phosphorylated CREB-1 restored transcriptional activity of both promoters. These results demonstrate that phosphorylated CREB-1, but not unphosphorylated CREB-1, is required for \(\beta\)-pol promoter transcriptional activity.

**DISCUSSION**

The present study describes characterization of a CREB family member that is involved in \(\beta\)-pol gene regulation, as well as a mechanism for transcriptional activation of \(\beta\)-pol gene expression by an alkylating agent in vivo. Previous work, with the cloned human \(\beta\)-pol promoter, indicated that the PKA signal transduction pathway plays a required role in transcriptional activation after exposure of cells to MNNG (12, 13, 15) and that the CRE site of the \(\beta\)-pol promoter is required for the MNNG response (15). Recently, we extended these observa-
instead of the rate of assembly, was increased after MNNG treatment. The apparent rate of RP assembly was not altered by MNNG treatment. Immobilized promoter DNA was used to purify RP. A larger amount of single cycle run-off transcript was formed by purified RP from MNNG-treated cells than from the same number of control cells, indicating that more RP was formed from MNNG-treated cells. Purified RP was used as starting material to characterize the CREB family members present. RP assembled from both NEs had a 43-kDa protein, similar to CREB-1 in mass. The identity of this protein was confirmed by DNA binding analysis and Western blotting (Fig. 2). We found that the amount of CREB in RP from MNNG-treated cells was similar to that in RP from control cells. These results are consistent with previous findings (15, 16) and suggested that a physical modification of CREB secondary to MNNG treatment may account for the transcriptional up-regulation.

Phosphorylation of CREB-1 by PKA may be indispensable for CREB transcriptional activation (23, 26, 27). The phosphorylation of CREB-1 by PKA does not affect the protein’s dimerization or DNA binding affinity for strong CRE sites (26). However, in some cases PKA phosphorylation enhances binding of CREB-1 to asymmetrical CREs, such as that in the tyrosine aminotransferase gene (TGACGCA) (28; for reviews, see Refs. 29 and 30). Since the MNNG transcriptional response is mediated through the PKA pathway (15), and CREB purified from MNNG-treated cells is transcriptionally activated (16), it was reasonable to propose that the transcriptional activation is due to increased phosphorylation. We examined this possibility by metabolically labeling cells with [32P]orthophosphate and simultaneously treating them with MNNG. [32P]-Labeled CREB was purified from RP, and resolved by SDS-PAGE. A 43-kDa protein identical in mass to CREB-1 was much more strongly phosphorylated after exposure of cells to MNNG than in control cells. Using an anti-pha-pho-CREB polyclonal antibody that is specific for the Ser133-phosphorylated form of CREB-1, we further confirmed that phosphorylation of CREB-1 at Ser133 was strongly increased by MNNG treatment. To test the functional activity of CREB and to attempt to mimic the MNNG response, we used a mixed activator approach in which PKA-phosphorylated recombinant CREB-1 was added to the NE from control cells. A very small amount of added phosphorylated CREB-1 (10 ng) (but not unphosphorylated CREB-1) was able to stimulate promoter activity. The role of phosphorylated CREB-1 in transcriptional up-regulation of the β-pol promoter was examined further with NEd. Phosphorylated CREB-1 could restore transcriptional activity of NEd, but unphosphorylated CREB-1 could not. The results indicate
that exposure of cells to the alkylating agent MNNG induces phosphorylation of CREB-1, which in turn recruits more 
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