DNA damage response of cloned DNA β -polymerase promoter is blocked in mutant cell lines deficient in protein kinase A

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ABSTRACT

DNA β -polymerase (β -pol), one of the recognized DNA polymerizing enzymes in vertebrates, has a role in 'very short patch' gap-filling synthesis during nucleotide excision DNA repair. In human and mouse, the enzyme is encoded by a single-copy gene located on the short arm of chromosome 8 near the centromere. In a series of studies, we have found that the cloned human β -pol promoter is regulated by signals acting through the single ATF/CRE palindrome in the core promoter. These signals include transactivation by: adenovirus E1a/E1b proteins; activated p21ras; and in CHO cells, treatment with the DNA damaging agent MNNG. Hence, several types of stimulatory signals are mediated through the single ATF/CRE site, including DNA damage induction. To understand the mechanism of β -pol promoter activation by MNNG in CHO cells, we asked whether induction of the cAMP/protein kinase A pathway can increase transcription of the cloned promoter in this system. Agents that increase cellular cAMP levels (8-Br-cAMP; forskolin and IBMx) activated the β -pol promoter fusion gene in transient expression experiments, and a mutation in the ATF/CRE palindrome blocked this response. Thus, the ATF/CRE site appears to be cAMP responsive in the CHO cell system. We found that the activation of the cloned β -pol promoter by MNNG does not occur with two mutant CHO cell lines that are deficient in protein kinase A activity. Further, simultaneous treatment of wild-type CHO cells, with MNNG and to elevate cAMP, failed to result in an additive effect for activation of the β -pol promoter. Thus, these effectors may act through a common pathway. These results suggest that the activation of the cloned β -pol promoter in CHO cells following MNNG treatment is mediated through the cAMP/protein kinase A signal transduction pathway.

INTRODUCTION

DNA polymerase β is a vertebrate DNA repair enzyme considered to be involved in 'gap-filling' DNA synthesis during nucleotide excision DNA repair.^{1, 3-15} The purified enzyme

conducts gap-filling synthesis on single-stranded (ss) DNA templates *in vitro*,^{2, 16} and this reaction is consistent with 'very short patch' synthesis during DNA repair.⁴⁻⁷ Inhibitor studies had implicated β -pol in some pathways of mammalian cell DNA repair,⁸⁻¹² and other recent studies also have implicated β -pol in gap-filling synthesis involved in mismatch repair by a HeLa cell nuclear extract,¹³ in repair of UV-damaged DNA¹⁴ and abasic lesions¹⁵ by *Xenopus l.* oocyte extract, and in correction of damaged residues following exposure of human cells to monofunctional DNA damaging agents, such as MNNG or MMS.¹²

In cultured CHO cells, β -pol mRNA level is induced several-fold after exposure to MNNG or MMS.¹⁷ In addition, a transfected β -pol promoter-CAT fusion gene responded to MNNG treatment of CHO cells by exhibiting a strong transcriptional activation (c.f., Figure 3, ref. 18). Analysis of this response indicated that it is mediated by the single palindromic ATF/CRE-binding element in the core promoter of the β -pol gene;¹⁸ the 10 nucleotide element GTGACGTCAC is at -40 to -49 in the core promoter.³⁸

The ATF/CRE-binding element is implicated in cAMP-induced modulation of gene expression for a number of promoters (for reviews, see refs. 19 and 20), and it has been well documented that the ATF/CRE-binding family of nuclear proteins recognizes this element. Several of these proteins carry consensus substrate recognition sites for protein kinase A²¹⁻²³ and it appears that the phosphorylation status of these proteins influences the transcriptional activity of their target promoters.²⁴⁻²⁷ Biochemical analysis of the bovine testes ATF/CRE-binding family protein²⁸, which binds in a highly specific manner to the β -pol promoter ATF/CRE site and has the capacity to regulate transcription of the promoter *in vitro* revealed that this protein is phosphorylated *in vivo*.²⁹ Since DNA binding *in vitro* by the purified protein was altered by phosphorylation,²⁹ the results suggested the possibility of involvement of phosphorylation in transcriptional control of the β -pol promoter via this ATF/CRE-binding protein.

In HeLa cells it has been shown that the ATF/CRE site mediates a response of the cloned β -promoter to elevated levels of cAMP, and a phosphoprotein that binds with high specificity to the β -pol promoter ATF/CRE site was identified as a member of the

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Figure 1. The cloned β -pol promoter is activated by elevated level of cAMP in the wild-type (10001) CHO cell line. A photograph of an autoradiogram illustrating CAT activities of duplicate samples 40 h after transfection is shown. Exposure was to 1 mM 8-bromo cAMP (8-Br cAMP) or to 20 μ M forskolin (F) plus 1 mM IBMx (I). The time (hours) of exposure prior to harvesting is indicated at the top for each set of duplicates. Percent conversion of substrate to acetylated product is shown at the bottom and was measured by cutting the chromatogram and counting.

Figure 2. The effect of MNNG treatment on the activity of the β -pol promoter-CAT fusion gene in wild-type and mutant CHO cells. A photograph of an autoradiogram is shown illustrating CAT activities of duplicate samples 40 h after transfection. Cells were exposed as indicated for 14 h prior to harvesting to 30 μ M MNNG.¹⁸ 150 μ g of cellular proteins were used for measuring CAT activity. The results shown are representative of three independent experiments.

HeLa CRE-binding protein family.³⁰ Protein binding to the cloned β -pol promoter has been studied also with nuclear proteins from CHO cells. It was shown that the ATF/CRE forms the center of a strong binding site for CHO nuclear proteins.³¹ Further, Kedar et al¹⁸ used mutants in the ATF/CRE site to demonstrate that the activation of the β -pol promoter by MNNG correlated with binding to the ATF/CRE site by nuclear proteins from CHO cells. Thus, ATF/CRE site mutants that fail to bind protein also fail to exhibit the MNNG activation.

In the current study, we further analyzed the mechanism of the β -pol promoter response to MNNG treatment using wild-type CHO cells (10001), as well as two protein kinase A deficient CHO cell mutants: The 10248 cell line carrying a defective gene for the RI subunit^{32, 33}; and the 10260 cell line with diminished protein kinase A activity.³⁴ In addition, transfected cells were treated with MNNG plus agents to increase cellular cAMP and then tested for activation of the cloned β -pol promoter. Results from both approaches point to the protein kinase A signal transduction pathway as mediating the MNNG response.

MATERIALS AND METHODS

Cell culture and transfection

Chinese hamster ovary cell lines were maintained as a monolayer in α -modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine and 50u/ml penicillin, 50 μ g/ml streptomycin. The 10248 and 10260 mutant cell lines were selected following mutagenesis of the 10001 wild-type cell line for their ability to grow under conditions of elevated cellular cAMP.^{35, 36} Cells were seeded 24 h before transfection at 5×10^5 cells/10 cm tissue culture dish and transfected by the calcium phosphate co-precipitation technique. A total of 20 μ g DNA was added to each dish: 15 μ g of β -pol reporter plasmid and 5 μ g of carrier plasmid (pTZ18). Cells were exposed to the precipitate for 4 h, glycerol shocked, washed three times with medium and refed. In DNA damaging experiments, cells were exposed to the monofunctional alkylating agent MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) at 30 μ M for 14 h prior to harvesting. For cAMP induction experiments, the treatments were

with 1mM 8-bromo-cAMP or 20 μ M forskolin and 1mM IBMx for the indicated periods of time prior to harvesting at 40 h after transfection. CAT activity was determined according to Gorman et al.³⁷

Plasmids

The wild-type and mutant β -pol promoter-CAT fusion constructs spanning the 100 bps of the human β -pol gene promoter region immediately flanking the transcription start site were described.^{18, 38} The 180 bp DNA fragment used in band shift assays was derived from the wild type β -pol promoter-CAT construct.

Gel mobility shift assays

Each binding reaction in a final volume of 20 μ l was assembled with 10 μ g of nuclear extract protein, 20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM DTT, 12% glycerol, 1.5 μ g sonicated salmon sperm DNA, 0.5 μ g poly(dI)·poly(dC), and 10 fmole of ³²P-labeled 180-bp promoter fragment DNA. Catalytic subunit of protein kinase A (Sigma) and 0.5 mM ATP was added directly to binding mixtures, and the incubation was at 29°C for 20 min; the samples were then cooled on ice prior to addition of the labeled probe. Following addition of the probe, the mixtures were incubated at 25°C,³¹ and the complexes were resolved in native 4% polyacrylamide gels in 0.5×TBE.

RESULTS

The transfected β -pol promoter-CAT fusion gene is activated by MNNG treatment in wild-type CHO cells.¹⁸ Mutated β -pol promoter-CAT fusion genes lacking the intact ATF/CRE element failed to respond to MNNG treatment of cells, indicating that the activation is mediated through the GTGACGTCAC element at position -40 to -49 in the 'TATA-less' core promoter.¹⁸ Further, the mutated constructs carrying the modified ATF/CRE site were not active in binding CHO nuclear proteins, indicating that protein binding to the ATF/CRE site is required for the MNNG-induced activation of the β -pol promoter.¹⁸ These results pointed to involvement of the protein kinase A pathway in the MNNG induction, since an intact DNA element (cAMP response element) was required. In the present study, we used cultured CHO cells, wild-type (10001) as well as protein kinase A deficient mutant cell lines, to further examine the question of a role of protein kinase A activity in the MNNG-induced transcriptional activation of β -pol promoter-CAT fusion gene. The cell lines mutated in protein kinase A were as follows: the 10248 mutant carrying an altered type I regulatory subunit (RI) of the cAMP dependent protein kinase. This defective subunit competes for binding of the catalytic subunit resulting in decreased kinase activity^{32, 33}; the 10260 mutant, which is defective in one of the catalytic subunits and has ~80% reduction of cAMP dependent protein kinase activity compared with wild-type cells.³⁴

First, to ascertain whether the β -pol promoter fusion gene is cAMP responsive in wild-type CHO cells, we examined the activation of the β -pol promoter-CAT fusion gene as a function of cellular level of cAMP. This fusion gene had been shown previously, in transient expression assays with two transformed human cell lines,^{29, 30} to be responsive to cAMP induction. However, the fusion gene is not cAMP responsive in all cell types (Widen and Wilson, unpublished observation). Wild-type CHO cells were transfected with the cloned β -pol promoter fusion gene

Figure 3. The effects of MNNG and forskolin plus IBMx on the β -pol promoter CAT fusion gene are not additive in the wild-type CHO cell line. A photograph of an autoradiogram illustrating CAT activities of duplicate samples 40 h after transfection is shown. Cells were exposed for 14 h prior to harvesting as indicated to 30 μ M MNNG and 20 μ M forskolin (F) + 1mM IBMx (I). The results shown are representative of three independent experiments.

Figure 4. Protein binding to the β -pol promoter ATF/CRE site as revealed by gel mobility shift analysis. A photograph of an autoradiogram of protein-DNA complexes resolved in a 4% polyacrylamide gel is shown. Binding mixtures were assembled with nuclear extracts from wild-type (10001) cells, untreated or treated with 30 μ M MNNG for 14 h prior to harvesting, and from the mutant cells (10248 and 10260), as indicated. The extracts were incubated with purified protein kinase A in the presence of 0.5 mM ATP, as indicated. Labeled 180-bp DNA probe spanning the core β -pol promoter³¹ was added for incubation in the binding mixture as described in Materials and Methods. The observed complexes were completely eliminated by including a 20-fold excess of the 24 bps oligonucleotide carrying the sequence corresponding to the footprint over the ATF/CRE site.³¹ The free probe and top of the gel are indicated; the right-hand lane was loaded with a binding mixture incubated without nuclear extract protein.

and then treated to elevate the cellular cAMP level with either 1mM 8-BrcAMP or simultaneously with 20 μ M forskolin and 1mM IBMx. The exposure was for varying periods of time prior to harvesting the cells at 40 h after transfection. CAT activity in the cell extract then was measured. As shown in Figure 1, we observed an activation of the promoter following this regimen of induction of cAMP. The activation could be detected after 4 to 6 hr. of exposure (Fig. 1). These results indicate that wild-type CHO cells are able to confer cAMP responsiveness to the cloned β -pol promoter.

Next, we observed that the cloned β -pol promoter-CAT fusion gene is active at a basal level in the two mutant protein kinase A-deficient CHO cell lines (Fig 2). In wild-type CHO cells, the

promoter was transcriptionally activated, as expected, by exposure to 30 μ M MNNG. However, the promoter was not activated in either of the protein kinase A-deficient cell lines (Fig 2). These results indicate that the MNNG response requires fully active protein kinase A.

We examined the possibility of a synergistic effect between the transcriptional response of the β -pol promoter-CAT fusion gene to the two types of effectors studied here, MNNG and elevators of cellular cAMP. Wild-type CHO cells were transfected and then exposed to simultaneous treatment with MNNG and agents to elevate cellular levels of cAMP (forskolin plus IBMx). The simultaneous exposure was for 14 h. time. The effect of the combined exposure on the activity of the β -pol promoter was no greater than the effect of each treatment alone (Figure 3 and Figure 1). These results suggest that both effectors act on a common target or pathway, rather than on separate targets capable of incremental stimulation, and are consistent with the possibility that protein kinase A is the target.

Finally, we have found that the ATF/CRE site in the β -pol core promoter was an active binding site for nuclear extract proteins from wild-type, as well as the two mutant CHO cell lines (Fig 4). The band-shift pattern was similar for nuclear proteins from all the indicated sources and was not changed by protein kinase A treatment of the nuclear extracts *in vitro* prior to incubation with the labeled probe. These results indicate that the failure to observe activation of the promoter by MNNG in the mutant cell lines did not appear to be due to an intrinsic deficiency in the amount of ATF/CRE-binding protein(s).

DISCUSSION

Several signal transduction pathways have been implicated in DNA damage triggered responses in mammalian cells, including protein kinase mediated mechanisms. DNA damage inducible genes controlled via protein kinase C pathways have been identified: Herrlich and coworkers found that DNA damage activates transcription factors such as AP-1 which stimulates the collagenase gene,³⁹ serum response factor which modulates the c-fos gene,⁴⁰ and the NF kappa B transcription factor which regulates the HIV-1 LTR control region.⁴¹ Devary et al.⁴² have shown a protein kinase C mediated activation of c-jun during the mammalian cell UV response, and casein kinase II has been implicated in conferring partial UV resistance to XP cells.⁴³

We presented here further evidence for the involvement of the protein kinase A pathway in the β -pol promoter/CHO cell response to DNA damage by MNNG. This is the first example to our knowledge of a DNA damage response mediated through the protein kinase A pathway. Earlier, it was shown that the bovine testes phosphoprotein purified by virtue of its high specificity binding to the β -pol ATF/CRE²⁹ did not recognize oligonucleotides carrying the AP-1 element,²⁸ and the cloned β -pol promoter was not responsive to TPA treatment of cells, supporting the idea that regulation of the cloned β -pol core promoter involves a pathway distinct from that mediated by protein kinase C. Next, the integrity of the ATF/CRE element in the human β -pol promoter was found to be critical for the MNNG induced response in CHO cells¹⁸, pointing to an involvement of the protein kinase A signal transduction pathway in the β -pol promoter DNA damage response. Here, we have shown that wild-type CHO cells are able to support a cAMP induced response by the cloned β -pol promoter. This response reached the same level of induction as that observed after MNNG

treatment. However, the magnitude of stimulation of the β -pol promoter by these two activators was not additive; both agents gave the same level of activation as either agent alone. Therefore, both agents could have a common target in the transcriptional activation process. We used mutant CHO cell lines that harbor a lower basal protein kinase A activity and are unable to increase protein kinase A activity to levels comparable to that of normal cells in response to inducing agents. These mutant cells also were unable to support the positive transcriptional response of the β -pol promoter to DNA damage. Taken together, these observations indicate a correlation between the DNA damage response mechanism and the presence of normal protein kinase A activity in CHO cells. We failed to detect any correlation between protein binding to the β -pol ATF/CRE site by wild-type and mutant cell nuclear extracts and the absence of MNNG activation. Overall, we can conclude that the MNNG activation of the promoter requires protein binding to the ATF/CRE,¹⁸ but this is not sufficient to cause activation. An intact protein kinase A pathway appears to be required also.

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