The ATF/CREB transcription factor-binding site in the polymerase β promoter mediates the positive effect of N-methyl-N'-nitro-N-nitrosoguanidine on transcription

PADMINI S. KEDAR†, STEVEN G. WIDEN‡, ELLA W. ENGLANDER†, ALBERT J. FORNACE, JR.,‡, AND SAMUEL H. WILSON†

Laboratories of †Biochemistry and ‡Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT DNA polymerase β (pol β) is a constitutively expressed DNA repair enzyme in vertebrate cells. Yet, it had been shown previously that the pol β mRNA level increases in Chinese hamster ovary (CHO) cells within 4 h after treatment with several multifunctional DNA damaging agents, notably, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Herein we report that a transfected pol β promoter fusion gene is activated by MNNG treatment of CHO cells; mRNA from the transfected gene is ≈ 10-fold higher in treated cells than in untreated cells 16 h after treatment. This activation is mediated through the decanucleotide palindromic element GTGACGTCA at positions −49 to −40 in the “TATA-less” core promoter. This element, which is similar to the ATF/CREB transcription factor-binding site in a number of mammalian genes, forms the center of a strong protein-binding site for CHO cell nuclear extract proteins. Mutated pol β promoter fusion genes lacking the element fail to bind protein at this site and fail to respond to MNNG treatment of cells.

DNA polymerase β (pol β) is a vertebrate DNA repair enzyme considered to be involved in “very short-patch” DNA synthesis during both mismatch repair (1) and correction of damaged residues after exposure of cells to multifunctional DNA damaging agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or methyl methanesulfonate (2–7). In several cultured cell systems, pol β protein and its mRNA are constitutively expressed as a function of the cell cycle and of growth stage (6–8). Yet, pol β mRNA level is rapidly induced ≈ 4- to 5-fold in Chinese hamster ovary (CHO) cells after exposure to MNNG or methyl methanesulfonate (9). This mRNA induction was not associated with an increase in pol β protein for reasons that have not yet been investigated. The increase in mRNA level, however, is likely the result of transcriptional activation, at least in part, since transcription is required for the induction as revealed by actinomycin D experiments (9).

The model pol β promoter that we used was cloned from the single-copy gene on human chromosome 8. By transient expression assays, full promoter activity of the 5′ flanking region was localized within the first ≈ 100 nucleotides, termed the core promoter (10). This sequence does not have “TATA” or “CAAT” elements but does have GC boxes and the decanucleotide palindromic sequence GTGACGTCA at positions −49 to −40. This palindrom is similar to a regulatory element (enhancer) in several adenovirus promoters and to the cAMP-responsive element (CRE) in many cellular genes for which transcription is regulated by changes in the cellular level of cAMP. The adenovirus enhancer element and CRE are binding sites for the DNA binding proteins activating transcription factor (ATF) of HeLa cells and CRE binding protein (CREB) of rat brain, respectively (11–16). These proteins are generally believed to be members of a large family of sequence-specific DNA binding proteins recognizing sites containing the TGACGTCA palindrome in a number of individual genes. This palindromic element in the pol β promoter is required for efficient promoter activity, when assayed in 293 or HeLa cells (10), and is the binding site (17) for a purified DNA binding protein that is a positive regulator of in vitro transcription from the pol β mRNA major start site (18).

In the present study, we further examined the subject of transcriptional activation of the pol β promoter after MNNG treatment of CHO cells. We found that a transfected pol β promoter–chloramphenicol acetyltransferase (CAT) fusion gene responds to MNNG treatment of cells by exhibiting a strong transcriptional activation. Analysis of this response indicated that it is mediated by the ATF/CREB site. Thus, the results suggest that a ATF/CREB element can function in transcriptional regulation of a DNA repair gene, in response to treatment of cells with a DNA damaging agent. Possible regulatory pathways leading to this response are discussed.

MATERIALS AND METHODS

Plasmids. pBP8 and its derivatives were the pol β–CAT fusion plasmids as described (10). The plasmid for the herpes simplex virus thymidylate kinase (TK) promoter–CAT fusion construct was pBLCAT2 (19); the chimeric plasmid construct with one copy of the pol β palindromic element oligonucleotide in the TK promoter construct was as indicated in Fig. 4B.

Cell Cultures and Transfections. CHO cells were grown in Ham’s F12 medium with 10% (vol/vol) heat-inactivated fetal calf serum and gentamycin (100 μg/ml). Approximately 1 × 10⁶ cells were seeded into 100-mm culture dishes 24 h before the transfection. The cells were refed with 15 ml of fresh medium and transfected by the calcium phosphate technique (10). A total amount of 20 μg of DNA in 1 ml was added to each dish: 15 μg of pol β reporter plasmid, 1 μg of reference plasmid (pCH110), and 4 μg of pTZ18. Cells were treated with 15% (vol/vol) glycerol 4 h after transfection, rinsed with phosphate-buffered saline, and refed. At 24 h after transfection, the cells were exposed to 30 μM MNNG for 4, 6, 8, 10, 12, 14, or 16 h. Control and damaged cells were harvested at the same time and CAT activity was determined as described (10). Typically, 20% of the cell extract was incubated for 1 h at 37°C with 0.2 μCi of [14C]chloramphenicol (DuPont/New England Nuclear; 1 Ci = 37 GBq). β-Galactosidase assays were performed as described (10) and used to normalize DNA synthesis.

Abbreviations: pol β, polymerase β; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; HN2, mechlorethamine hydrochloride; cis-Pt, cis-platinum(II) diammine dichloride; PMA, phorbol 12-myristate 13-acetate; CRE, cAMP-responsive element.
variations due to differences in transfection efficiencies. To obtain the CAT level values for the experiment in Fig. 1, actual CAT values were multiplied by β-galactosidase level correction factors for control and MNNG-treated cells, respectively, as follows: 4 h, 3 and 3; 6 h, 1.6 and 2.9; 8 h, 2.9 and 1.8; 10 h, 2.8 and 1.7; 12 h, 1.8 and 1.3; 14 h, 1.3 and 1.9; 16 h, 1.7 and 1.5. Treatment with other DNA damaging agents also was conducted. Beginning 24 h after transfection, control dishes were maintained in culture while other dishes were treated with mechlorethamine hydrochloride (HN2) at 40 μM or cis-platinum(II) diammine dichloride (cis-Pt) at 45 μg/ml. These agents were removed after 4 h by changing the medium. Another set of dishes was treated with x-rays (1200 rads; 1 rad = 0.01 Gy) for 11 min and then refed with medium. Additional dishes were treated with 400 μM H2O2 for 1 h, heat-shocked for 9 min at 45.5°C, or treated with phorbol 12-myristate 13-acetate (PMA; 30 ng/ml) for 2 h. After treatment with each of these agents, the transfected cells were allowed to grow for 16 h before harvesting.

**DNase I and S1 Nuclease Protection Assays.** Wild-type and mutated pol β promoter fragments were assayed for DNase I digestion patterns (17). DNA fragments from the promoter region were derived from the relevant constructs described in Table 1, and the DNA damaging treatment of CHO cells with MNNG was as described above. Preparation of nuclear extracts and DNase I footprinting assays were performed as described (17). Total RNA from cells was prepared and analyzed by S1 nuclease protection as described (10).

**RESULTS**

To examine the effect of MNNG treatment on pol β promoter activity, CHO cells were transfected with a human pol β promoter–CAT fusion gene construct (pβP8) and held in culture for 20 h. Half of the cultures then were treated with 30 μM MNNG and the other cultures were held as controls; this level of MNNG has been shown to cause a 4-fold increase in pol β mRNA level in these cells. CAT levels in the MNNG-treated cells were higher than in control cells (Fig. 1) and reached the highest level about 12 h after treatment. CAT levels in these experiments were measured relative to β-galactosidase levels in the same extracts; this latter enzyme was expressed from a reference plasmid containing the simian virus 40 promoter linked to the *Escherichia coli* β-galactosidase gene, and co-transfection with this plasmid was conducted as a precaution to eliminate any differences due to transfection efficiency.

Analysis of the effect of MNNG treatment on actual pol β–CAT fusion gene mRNA level and start site(s) is shown in Fig. 2. For treated and untreated CHO cells, the transcript from the pol β–CAT construct had been initiated at the correct start sites, based upon earlier experiments with the pol β promoter in human cells (10). The level of transcript is much higher (~10-fold) in MNNG-treated cells than in untreated cells, as expected from the results of CAT level measurements shown in Fig. 1.

The specificity of pol β promoter activation was examined in the experiment shown in Fig. 3. The experiment was conducted with the perspective that treatment with several well-known DNA damaging agents, including x-ray and nitrogen mustard (HN2), does not lead to an increase in pol β mRNA level in these cells (9). We found a similar pattern for activity of the transfected promoter (Fig. 3). Thus, treatment with HN2, cis-Pt, and x-ray failed to activate the promoter, whereas H2O2 treatment gave an activity intermediate between that found with control cells and MNNG-treated cells. Stress induction by heat shock or PMA treatment failed to activate the promoter.

The human pol β promoter does not have an obvious TATA element, and a key feature required for the activity of the cloned pol β promoter is the decanucleotide palindromic element GTGACGTCAC at positions −49 to −40 (10). This element is the center of a strong protein binding site for nuclear extract proteins of mammalian cells (17, 18) and is the recognition element for a sequence-specific DNA binding protein purified from bovine tissue (18). This decanucleotide element is very similar to an element, TGACGTCAC, that is part of the ATF protein binding site in several promoter systems and that confers cAMP responsiveness to several promoters (10). We investigated the question of whether this element in the context of the pol β promoter could be the target for mediation of the MNNG activation. Mutated pol β promoter–CAT constructs (10) were examined. Each contained an alteration in the palindromic element but was otherwise identical with pβP8 (Table 1). These two constructs showed low basal promoter activity in cells not treated with MNNG and failed to show any stimulation upon MNNG treatment. Truncation of the core promoter in pβP8 to remove about one-half the residues at the 5' end (positions...
nucleotide corresponding to the pol β palindromic element linked to the herpes simplex virus TK promoter in the CAT fusion construct pBLCAT2 (20). Thus, an oligonucleotide with the decanucleotide palindromic element and seven residues on each side from the pol β promoter was attached to an Xba I linker at one end and a BamHI linker at the other end and then inserted into an Xba I and BamHI site at the 5' end of the TK promoter. As shown in Fig. 4, transfection of CHO cells with 1–15 μg of the wild-type TK–CAT construct DNA (pBLCAT2) revealed a slight, but reproducible, stimulation by MNNG treatment (1.5-fold). For the TK–CAT construct containing the pol β palindrome, stimulation by MNNG treatment was higher, equal to ≈3-fold. Thus, it appears that the pol β palindromic element and surrounding nucleotides can impart additional responsiveness to the TK promoter. The wild-type TK promoter is relatively strong in transfection experiments and has both CAAT and TATA sequence motifs, and neither of these properties is shared by the pol β promoter (10). These facts may be relevant to the question of why palindromic element responsiveness in the chimeric TK promoter is not as high as it is in the pol β promoter.

We have shown that the ATF/CRE palindromic motif GTGACGTCAC in the pol β core promoter is a DNA-

![Fig. 3. Effect of various DNA damaging and stress agents on activity of transfected pol β promoter in CHO cells. Twenty-four hours after transfection, the plates were treated with various damaging agents or stress agents as indicated. Amounts of these agents are as follows: HN2, 40 μM; cis-Pt, 45 μg/ml; x-ray, 40 Gy; H2O2, 400 μM; MNNG, 30 μM; heat shock, 46°C for 9 min; PMA, 30 ng/ml. Relative pol β mRNA levels in identically treated cells shown at the top are from ref. 9.](image-url)

![Fig. 4. Effect of MNNG treatment on promoter activity of a wild-type TK promoter construct (A) and a chimeric plasmid construct with one copy of the pol β palindromic element oligonucleotide–TK promoter construct (B) as indicated. Experiments were conducted as described in Fig. 1, 16 h after treatment with 30 μM MNNG (●) or with untreated cells (○).](image-url)

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**Table 1. Comparison of pol β promoter activity for wild-type and mutated fusion gene constructs in CHO cells treated with MNNG**

<table>
<thead>
<tr>
<th>Promoter fusion gene</th>
<th>CAT activity</th>
<th>Fold induction</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>− MNNG</td>
<td>+ MNNG</td>
</tr>
<tr>
<td>pβP8 (pol β)</td>
<td>15.7 (0.85)</td>
<td>46.9 (0.2)</td>
</tr>
<tr>
<td>pβP8*</td>
<td>4.3 (0.6)</td>
<td>4 (0.6)</td>
</tr>
<tr>
<td>pβP8*A</td>
<td>1.7 (0.3)</td>
<td>1.7 (0.3)</td>
</tr>
<tr>
<td>pβP9</td>
<td>3.5 (0.75)</td>
<td>10.3 (1.3)</td>
</tr>
<tr>
<td>pβP10</td>
<td>1.4 (0.3)</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>β-actin</td>
<td>3.4 (0.6)</td>
<td>4.5 (0.6)</td>
</tr>
</tbody>
</table>

pol β promoter has the palindromic element GTGACGTCAC at positions −49 to −40. The construct with this element in the wild-type core pol β promoter (positions −114 to +62) linked to CAT is termed pβP8 (10). The mutated plasmids pβP8* and pβP8*A, respectively, have an altered palindrome element sequence as underlined GTGACGTCACCCAC and GTGGACGTCAC in the otherwise wild-type promoter sequence (10). In pβP9 the palindrome motif is intact, but two contiguous Sp1 sites on the 5' side of the palindrome are removed by virtue of 5' truncation to position −52; in pβP10 sequence to position −42 is removed by 5' truncation (10), destroying the palindrome motif. Cells carrying the promoter fusion genes (15 μg, except for β-actin at 3 μg) as indicated were either treated or not treated with MNNG and CAT activity was measured. Values shown are the mean. SD is shown in parentheses.
damage-responsive element in the sense that MNNG activation of a cloned pol β promoter in CHO cells requires the motif. To examine the mechanism of this promoter activation, we conducted DNase I footprinting studies with nuclear extracts from both untreated and MNNG-treated CHO cells. It is clear that the ATF/CREB palindrome forms the center of a strong protein binding site in the promoter (Fig. 5A) (17). This DNase I footprint was not observed with the activation-negative mutated promoter of pβ8* A (Fig. 5B) but was seen with a truncation mutant pβ9 that retains the palindrome and the ability to respond to MNNG activation (Table 1).

Thus, the MNNG response correlates with and probably is mediated through proteins binding to the palindrome and surrounding residues. However, binding to this site with the wild-type pol β promoter is qualitatively similar for extracts for untreated and treated cells (Fig. 5A and C). Therefore, some alteration in properties of the palindrome binding protein(s) may occur after MNNG treatment, rather than a change in the amount of the DNA binding protein(s). Alternatively, an individual member of the ATF/CREB protein family (11–16, 21, 22) may be induced to bind to the palindromic element in place of other constitutive palindrome binding protein(s).

**DISCUSSION**

In the present study, we used the transient expression approach with the CHO cell system to identify an element in the core pol β promoter that mediates a positive response to certain types of DNA damaging agents. This element is the ATF/CREB site located near the major transcription start site. This is the first example, to our knowledge, of the involvement of the ATF/CREB site in a DNA-damage response pathway and is of particular interest because regulation and action of proteins binding to this site are subjects of broad investigation (11–16, 17, 18, 21, 22) and also because at least two other human DNA metabolism genes contain ATF/CREB sites in their core promoters, proliferating-cell nuclear antigen and DNA polymerase α (23, 24). It is conceivable that low levels of certain types of DNA damage can be repaired by constitutively expressed pol β and that cells can overexpress the pol β gene, and perhaps other genes, in response to higher levels of DNA damage (25).

A positive transcriptional response after DNA damage in mammalian cells has been documented for several genes (26–32). UV damage, for example, is followed by induction of c-fos and collagenase genes, which are responsive to activation of protein kinase C. These genes have regulatory sequences that are believed to mediate the response to agents that induce protein kinase C activity. Thus, the AP-1 transcription factor binding element, TGACTCA, has been found to mediate response of the collagenase gene to protein kinase C activation after UV damage of cells (for review, see ref. 29). This response pathway probably involves a set of DNA binding proteins different from those binding to the pol β ATTF/CREB site. The latter proteins in crude nuclear extracts (17) and in purified form (18) do not recognize the AP-1 element, and we have shown here that the pol β promoter transfected in CHO cells does not respond to PMA treatment of the cells. Therefore, the ATF/CREB-site-mediated response to MNNG probably reflects a DNA binding protein–promoter element pathway distinct from those of the AP-1 and protein kinase C pathways discussed above.

Our results have shown that an intact ATF/CREB site is required for the positive transcriptional response to MNNG, but the detailed mechanism of the response beyond protein binding to the site is unknown. The ATF/CREB site, characterized by the central 8-base-pair sequence TGACGTCA, is present in many mammalian promoters and has been found in several cases to be important in regulation of transcription from cloned promoters and in specific protein binding to the promoter. It is now clear that ATF/CREB sites are recognized by a family of nuclear DNA binding proteins (11–16, 21, 22), including the well-characterized examples of ATF, CREB, and ΔCREB. These proteins share an overall domain structure including a DNA binding domain with a degree of sequence similarity, a leucine-zipper domain, and possible phosphorylation sites for protein kinases. Several members of the protein family have been found to exhibit binding discrimination between various closely related DNA sequences containing the 8-base-pair ATTF/CREB palindrome. For the pol β promoter, the ATF/CREB palindrome forms the center of a strong protein binding site, and the precise sequence of the palindrome is important for transcriptional activity of the cloned promoter. A bovine testes protein purified on the basis of binding to this sequence exhibited the ability to discriminate in its sequence-specific binding between the pol β ATTF/CREB site compared with the ATF/CREB sites of the somatostatin, chorionic gonadotropin, or glucagon promoters (18). Therefore, the pol β ATTF/CREB site may be recognized by a subset of the family of ATF/CREBs. Since the sequence similarity among the various ATF/CREB palindrome sites in different promoters is limited to the central 8-residue palindrome of the 18–22 residues of the protein-occluded site generally found for each promoter, it is reasonable to expect that residues flanking the palindrome provide for protein binding specificity by individual members of the ATF/CREB family (for discussion, see ref. 22). As this appears to be the case for the protein binding to the pol β promoter ATF/CREB site, modification of this particular protein, secondary to MNNG treatment of cells, could be involved in the transcriptional response reported here. Although other possibilities cannot be ruled out, this modification could involve phosphorylation of the ATTF/CREB site binding protein. The purified protein from bovine testes is a phosphoprotein whose sequence-specific DNA binding is modulated by phosphorylation (E.W.E., S.G.W., and S.H.W., unpublished results). This protein is phosphorylated in vivo at several sites including a site for protein kinase A. This multiplicity of phosphorylation sites indicates.

**Fig. 5.** CHO cell nuclear extract protein binding to the pol β promoter as determined by DNase I footprinting. Autoradiograms are shown of the portion of gels corresponding to residues –84 to +30 of the 32P-labeled pol β promoter (14). The probe was a 5'-labeled pol β insert in pβ8 (A), pβ8*A (B), or pβ9 (C). Experiments were conducted as described (14). Lanes: 1, bovine serum albumin at 100 µg; 2, CHO nuclear extract at 100 µg; 3, MNNG-treated CHO cell nuclear extract (100 µg). Sequence elements in the core promoter (10, 14) are noted to the left of each panel, and the approximate region protected from DNase I (17) is noted to the right in A and C. Palindrome refers to the ATTF/CREB palindromic sequence GTGACGTCA, and GC box refers to GCCCGCCCCC.
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that several second messenger pathways may converge to regulate the promoter through this protein.

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