DNA Damage-Induced Transcriptional Activation of a Human DNA Polymerase β Chimeric Promoter: Recruitment of Preinitiation Complex *in Vitro* by ATF/CREB†

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ABSTRACT: Treatment of hamster cells in culture with the DNA alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induces DNA polymerase β (β-pol) gene expression and cellular levels of the enzyme. Transcriptional activity of a cloned β-pol promoter in transient expression assays is also stimulated. Among the requirements for these responses are methylation damage to genomic DNA, cellular cAMP-dependent protein kinase, and the ATF/CREB site of the cloned β-pol promoter. In the present study, HeLa cell nuclear extract from MNNG-treated cells was much more active in an *in vitro* transcription assay than nuclear extract from normal cells. By using an oligonucleotide affinity column to deplete the nuclear extract of ATF/CREB, we showed that the difference was due to ATF/CREB activator. Purified ATF/CREB activator from MNNG-treated cells was ~10-fold more active than ATF/CREB purified from normal cells as a transcriptional activator for the depleted nuclear extract. ATF/CREB in the extract from normal cells is known to activate *in vitro* transcription by increasing the rate of promoter clearance [Narayan, S., Widen, S. G., Beard, W. A., & Wilson, S. H. (1994) *J. Biol. Chem. 269,* 12755−12763]. With ATF/CREB from MNNG-treated cells, the amount of preinitiation complex formed was much greater than with ATF/CREB from normal cells, and the kinetics of both the closed to open preinitiation complex isomerization and promoter clearance were altered. These results indicate that the mechanism of transcriptional activation secondary to DNA alkylation damage is recruitment of more preinitiation complex and alteration of the kinetic scheme of transcription initiation.

DNA polymerase β (β-pol)[1] is a cellular DNA polymerizing enzyme, long believed to be involved in DNA repair. Recent studies have further implicated β-pol in gap-filling synthesis involved in the following systems: base excision repair (Matsumoto & Bogenhagen, 1989; Dianov et al., 1992; Wiebauer & Jiricny, 1990; Singhal & Wilson, 1993; Singhal et al., 1994), replication of M13 DNA in *Xenopus laevis* oocyte extract (Jenkins et al., 1993), and correction of damaged residues following exposure of human cells to DNA alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or methyl methanesulfonate (Hammond et al., 1990). Expression of β-pol in cultured cells has been described as constitutive (Chang, 1976; Fry, 1983; Zmudzka et al., 1986), and β-pol is expressed in a tissue-specific manner in rodents (Hirose et al., 1989; Nowak et al., 1989). The promoters for the human and rodent β-pol genes have been cloned and extensively characterized (Widen et al., 1988; Yamaguchi et al., 1994). In the human gene, the sequence of the core promoter lacks typical TATA and CCAAT elements, is G+C-rich, and has three Sp1 binding sites and many potential methylation sites. A palindromic sequence with perfect dyad symmetry, GTGACGTCAC, is located at position −40 to −49 and is surrounded by the Sp1-binding elements (Widen et al., 1988). The central 8 bp of this palindrome are identical to the consensus binding site for the activating transcription factor (ATF)/cAMP responsive element-binding (CREB) transcriptional activator proteins that are implicated in cAMP-induced modulation for a number of mammalian promoters [for a review, see Montminy et al. (1990)]. It was shown that the human β-pol promoter can be regulated through the cAMP-induced protein kinase signal transduction pathway and that the palindromic sequence at −40 to −49 both is a functional protein binding site and is required for promoter activity of a cloned promoter (Englander & Wilson, 1992a).

The β-pol mRNA is induced in Chinese hamster ovary (CHO) cells after exposure of cells to the DNA-damaging agent MNNG, and this induction is blocked by treatment of cells with actinomycin D (Fornace et al., 1989), suggesting that transcriptional activation is required. Further, a transfected β-pol promoter fusion gene is strongly activated in CHO cells by MNNG treatment, and this transcriptional activation was shown to be mediated by the ATF/CREB site in the β-pol core promoter (Kedar et al., 1991). Two CHO cell lines deficient in cAMP-dependent protein kinase activity failed to support the MNNG-induced promoter stimulation (Englander & Wilson, 1992b), corroborating the hypothesis that stimulation of the cloned β-pol promoter following MNNG treatment is mediated through the cAMP-dependent protein kinase signal transduction pathway (Kedar et al., 1991). Finally, a CHO cell line transformed to overexpress

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[1] Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ATF, activating transcription factor; CRE, cAMP response element; β-pol, DNA β-polymerase; NE, HeLa cell nuclear extract; NEd, ATF/CREB-depleted HeLa cell nuclear extract; (Normal) ATF/CREB, ATF/CREB purified from normal HeLa cells; (MNNG) ATF/CREB, ATF/CREB purified from MNNG-treated HeLa cells; nt, nucleotide; R, RNA polymerase; RNA pol II, RNA polymerase II; P, promoter; RP_c, closed preinitiation complex; RP_o, open preinitiation complex; RP_i, intermediate preinitiation complex; DTT, dithiothreitol; CHO, Chinese hamster ovary.

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O^6-methylguanine DNA methyltransferase does not show β-pol induction after MNNG treatment, suggesting that DNA methylation, presumably in trans to the β-pol promoter, is required for the transcriptional activation (Srivastava et al., 1994).

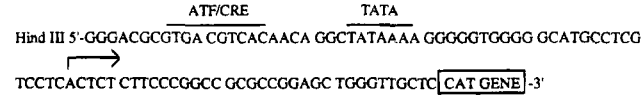
Induction of β-pol was reported in human cell lines from tumors resistant to cisplatin chemotherapy [for a review, see Scanlon et al. (1989)]. β-pol induction also was found during proliferation of mitogen-stimulated human lymphocytes (Suzuki et al., 1991) and after treatment of CHO cells with the protein kinase C modulator TPA (Srivastava et al., 1994). In other studies, it was shown that expression of the cloned β-pol promoter is activated by the transforming oncoprotein $p21^{ras}$ [Kedar et al., 1990; for a review, see Englander and Wilson (1992c)]. A strong activation of the β-pol promoter also was observed in human kidney 293 cells constitutively expressing the oncoproteins E1A/E1B (Widen et al., 1988). As expected, these latter trans-activating effects were found to be dependent on the integrity of the ATF/CREB site (Widen et al., 1988; Yamaguchi et al., 1992). Taken together, the results point to the ATF/CREB transcriptional activator as a key regulatory protein in the activity of the β-pol promoter. To understand the mechanism of β-pol promoter activation by MNNG treatment, we chose an *in vitro* transcription system with a chimeric β-pol promoter that exhibits a dependence on ATF/CREB. Additionally, this ATF/CREB-dependent transcriptional activation has been kinetically characterized (Narayan et al., 1994). In the present work, ATF/CREB was purified from MNNG-treated cells and studied to determine the kinetic mechanism of its transcriptional regulation. This protein is different from ATF/CREB isolated from normal cells, in that it has the ability to stimulate recruitment of more transcriptional preinitiation complex.

MATERIALS AND METHODS

Treatment of HeLa Cells. HeLa cells (S3, from ATTC) were grown as a monolayer in 150-mm culture dishes in DMEM with 10% FCS. After cell growth reached 80% confluence, the medium was replaced with fresh medium containing 30 μM MNNG. MNNG stock solutions were prepared in DMSO, and the final concentration of DMSO in the medium was 0.1% (v/v). After 5 h of MNNG treatment, cells were scraped and washed twice with cold phosphate-buffered saline, pH 7.4. The normal cells were treated in the same way without MNNG.

Purification of ATF/CREB from MNNG-Treated HeLa Nuclear Extract. HeLa nuclear extract (NE) was prepared by the procedure of Shapiro et al. (1988) with the following modifications. The hypotonic, sucrose restore, and nuclear resuspension buffers were supplemented with 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 100 μM sodium vanadate. The dialysis and nuclear resuspension buffers were supplemented with 1 μM sodium vanadate, 1 μg/mL leupeptin, and 1 mM PMSF. The ATF/CREBs from the NE of normal and MNNG-treated cells were purified on an ATF/CRE sequence specific DNA affinity column. A 24-bp double-stranded ATF/CRE oligonucleotide comprising residues -32 to -55 of the human β-pol promoter was used to prepare the column as described previously (Widen & Wilson, 1991). The oligonucleotide affinity column (1 mL bed volume) was washed thoroughly with NE buffer (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 2 mM DTT). NE (1 mL, 8.5 mg of protein) was loaded on the column, and the flow-through was collected. To ensure that all the ATF/CREB was bound to the column, the procedure was repeated four times. The flow-through (designated NEd, ATF/CREB-depleted NE) was collected in a separate tube, aliquoted, and stored at -70 °C. The column was washed with 5 ml of NE buffer to remove unbound NE proteins. The ATF/CREB was eluted from the column with elution buffer (1 M KCl, 20 mM Hepes, pH 7.9, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, and 0.1% Nonidet P-40) (Widen & Wilson, 1991). The eluted sample was diluted 10-fold with the same buffer without KCl and was concentrated using a Centricon-10 microconcentrator (Amicon). Purified ATF/CREB was aliquoted and stored at -70 °C. The proteins from normal and MNNG-treated cells are designated (Normal) ATF/CREB and (MNNG) ATF/CREB, respectively. Gel-mobility shift assay was done to determine the ATF/CREB activity of the NE, NEd, and purified (Normal) ATF/CREB or (MNNG) ATF/CREB (Widen & Wilson, 1991). The binding reaction mixture was assembled with 10 μg of NE or with 10 ng of purified ATF/CREB in a final volume of 20 μL.

In Vitro Run-Off Transcription Assay. A derivative of the human β-pol promoter, pSH15, was used in this study. The detailed structure of the pSH15 promoter has been described (Narayan et al., 1994):



The *in vitro* transcription reaction mixture (25 μL final volume) contained 20 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 2 mM DTT, 65 mM KCl, 10% glycerol, 20 units of RNasin (Promega), and 1−2 μg of pSH15 plasmid linearized with *Pvu*II. Forty-five micrograms of NE, NEd, or NEd supplemented with 100 ng of purified (Normal) ATF/CREB or (MNNG) ATF/CREB was used in each reaction as described in the figure legends. The reaction mixture was incubated for 30 min at 30 °C to form closed preinitiation complex (RP_c). ATP, GTP, and UTP (500 μM each) and 25 μM [α^{32}P]CTP were added, and incubation was continued. The reaction was stopped at different times as indicated. The product RNA was extracted with chloroform/phenol, precipitated with ethanol, and electrophoresed on 6% polyacrylamide/8 M urea gels. The dried gels were exposed to X-ray films, and the 180-nt transcript was quantified by laser scanning densitometry (LKB Ultro-gel Scan XL) and analyzed using GSXL software or quantified with a PhosphorImager (Molecular Dynamics). Background corrections were made in all analyses.

In Vitro $KMnO_4$ Footprinting of Open Preinitiation Complex (RP_o) Formation. The *in vitro* run-off transcription assay with pSH15 plasmid, NEd, or NEd supplemented with purified (Normal) ATF/CREB or (MNNG) ATF/CREB was set up as described above. The $KMnO_4$ footprinting assay for RP_o formation was done as described by Wang et al. (1992a). After a 30-min preincubation at 30 °C to form closed preinitiation complex (RP_c), 400 μM dATP was added and the preincubation was continued for different periods. The reaction mixture was then treated with 6 mM $KMnO_4$ for 4 min. The DNA was purified, and the $KMnO_4$-modified T residue at the +1 position on the "transcribed strand" of

the pSH15 promoter was analyzed by primer extension using Pol I (Klenow fragment). The selection of the primer and the characterization of the $KMnO_4$ footprinting assay has been described earlier (Narayan et al., 1994).

Kinetic Analysis. A minimal model for transcription initiation has been described by McClure (1985) and discussed in detail by Narayan et al. (1994) for the ATF/CREB-dependent transcriptional regulation of the pSH15 promoter. Three general steps are shown in the following scheme:

$$R + P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RP_c \xrightarrow{k_c} RP_0 \xrightarrow[NTPS]{k_3} \text{transcript} \qquad (1)$$

K_B
step I step II step III

where R = RNA pol II, P = promoter, RP_c = closed preinitiation complex, RP_o = open preinitiation complex, K_B = equilibrium association constant, k_1 = association rate constant, k_{-1} = dissociation rate constant, k_2 = isomerization rate constant, and k_3 = rate constant for promoter clearance.

A quantitative analysis of the various rate constants of transcription initiation with respect to the ATF/CREB and the pSH15 promoter was described earlier (Narayan et al., 1994). In order to define the effect of (MNNG) ATF/CREB on the kinetics of transcriptional activation of pSH15, time courses of run-off transcript formation were followed. The NE and other reaction components, as described for the run-off transcription assay, were assembled in one microcentrifuge tube, and the pSH15 plasmid and NTPs were put in another microcentrifuge tube. The reaction mixtures were incubated separately for 10 min at 30 °C and then mixed at zero time. Aliquots of 25 μL were removed at different time intervals. The run-off product was purified and separated on 6% polyacrylamide/8 M urea gels.

The 180-nt transcript bands were densitometrically analyzed, and the time courses were fitted to eq 2 by nonlinear least squares methods:

$$N = V_{ss}t - V_{ss}\tau_{obs}(1 - e^{-t/\tau_{obs}}) \qquad (2)$$

where N = the amount of product formed at time t, V_{ss} = the final steady-state velocity (run-off transcript/min = $[RP_o]k_3$), and τ_{obs} = the observed lag of transcript formation, i.e., the average time for the transition of a closed preinitation complex (RP_c) to an open preinitiation complex (RP_o).

RESULTS

Kinetics of Transcriptional Activity of Nuclear Extract as a Function of MNNG Treatment. In vitro transcription was conducted with the pSH15 promoter (Narayan et al., 1994) and NE prepared from normal or MNNG-treated HeLa cells. Although the experiments to be described were with HeLa cells, similar results also were obtained with CHO cells; HeLa cells were chosen for detailed study because this system was found to be a more active NE source for *in vitro* transcription than CHO cells, and more ATF/CREB could be purified from HeLa NE than from CHO NE (data not shown). To examine transcriptional regulation, we first obtained time courses of *in vitro* transcription with NEs prepared from normal and MNNG-treated HeLa cells (designated as MNNG-treated NE). Reaction mixtures were assembled and incubated for various periods, and accumulation of a 180-nt run-off transcript, representing correct initiation, was quantified (Figure 1). After a brief lag, the amount of transcript formed by MNNG-treated NE was ~10-fold greater than that formed by normal NE, throughout the time course. The data were fitted to eq 2 (Figure 1B) and were consistent with a three-step model for transcriptional initiation as outlined in eq 1 (McClure, 1985; Narayan et al., 1994). The lag in the time course represents isomerization (k_2) of closed to open preinitiation complex (Narayan et al., 1994). Because a similar lag was observed with normal NE and MNNG-treated NE, the respective rate constants for isomerization (k_2) were not very different. The observation of a lag in the time course is consistent with a kinetic model where promoter clearance (k_3) is rate-limiting; this was the case with each NE, even though the linear rate of transcript formation was higher with the MNNG-treated NE. Failure to observe a lag in the time course with the MNNG-treated NE would have suggested a faster rate of promoter clearance with this NE, thus making an early step in the ordered sequential process rate-limiting. The linear rate of transcript formation is defined by just two terms; in eq 2, the steady-state velocity of product formation is equal to $[RP_o]k_3$. In the following experiments, we evaluated

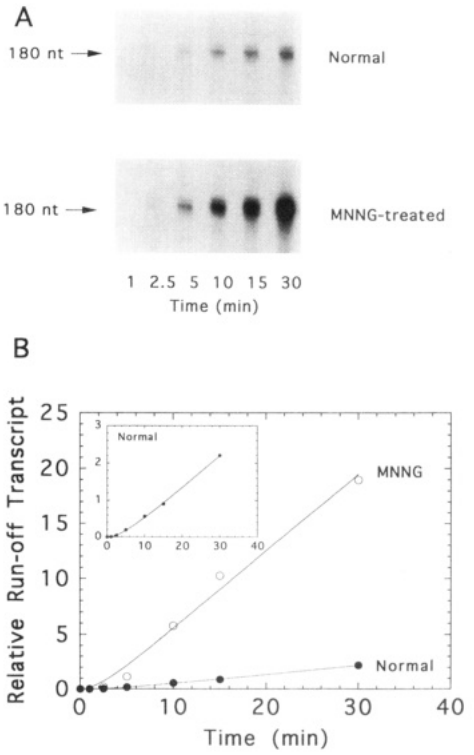


FIGURE 1: Time course analysis of transcript formation with pSH15 and NE prepared from normal and MNNG-treated HeLa cells. The *in vitro* run-off transcription assay was assembled with pSH15 plasmid and NE prepared from normal or MNNG-treated cells. The HeLa cells were treated with 30 μM MNNG for 5 h, and then the NE was prepared. Normal cells received the same amount of vehicle solution [DMSO, 0.1% (v/v) final concentration]. To determine the time required for the transition of RP_c to RP_o and the effect of MNNG treatment on the rate of transcript formation, the reaction mixtures were assembled and initiated with NTPs at zero time. Panel A is an autoradiogram showing the time course of appearance of 180-nt run-off transcript. Densitometric analysis of the data from panel A is shown in panel B with the ordinate corresponding to arbitrary absorbance units of run-off transcript. The solid lines represent computer-derived theoretical curves based upon the best-fit of the data to eq 2 to determine the kinetic parameters, lag (τ_{obs}, relaxation time required for RP_c to RP_o transition), and V_{ss} (steady-state velocity = run-off transcript/min). The data from the normal NE is replotted (inset) to illustrate the lag that is not apparent in panel B due to the range of the ordinate scale.

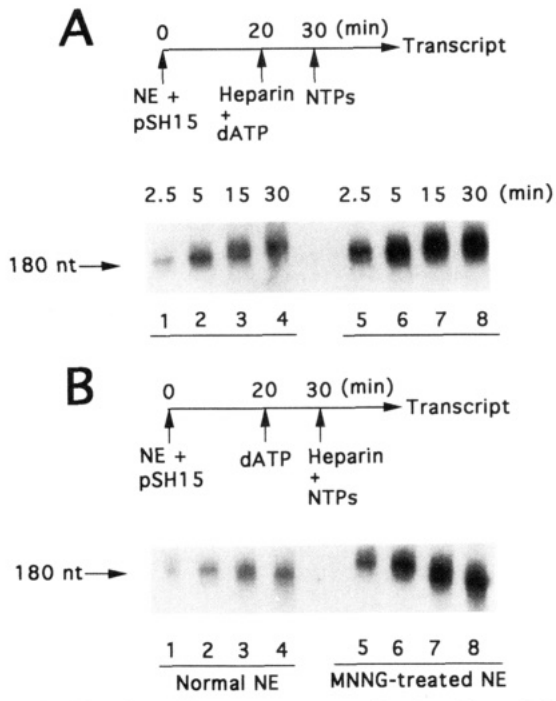


FIGURE 2: Heparin challenge assay to examine the effect of MNNG treatment on the assembly of preinitiation complex on pSH15 promoter. The *in vitro* run-off transcription assay was assembled with pSH15 plasmid and NE prepared from normal and MNNG-treated cells. Heparin (25 μg/mL), dATP (400 μM), and NTPs were added at the indicated times in the protocol at the top of each panel. Each autoradiogram is representative of three independent experiments.

alteration of these two terms as a function of MNNG treatment in order to explain the greater velocity of product formation seen in Figure 1 with MNNG treatment. However, we did not expect to find much change in k_3, because k_2 and k_3 with the normal NE are 0.26 and 0.03 min^{-1}, respectively, and a 10-fold increase in k_3, to account for the results in Figure 1, would have nearly eliminated the lag in the time course.

The mechanism of the faster rate of transcript formation with MNNG-treated NE was first examined by preincubating the NE with promoter in the presence of dATP to accumulate RP_o in the reaction mixture. The amount of RP_o formed was then measured by adding NTPs in the presence of heparin, so as to limit synthesis to one round of promoter clearance and elongation. Heparin prevents assembly of RP_c, but does not affect RP_c once formed (Reinberg & Roeder, 1987; Conaway & Conaway, 1990; Narayan et al., 1994). In the experiment in Figure 2A, RP_c was assembled with normal NE or MNNG-treated NE using a 20-min preincubation. Heparin and dATP were added, and the incubation was continued for 10 min, to convert RP_c into RP_o (isomerization half-time = 2.6 min). NTPs were then added to initiate transcription, and product formation was followed (Figure 2A). More transcript was formed by the MNNG-treated NE than by normal NE, suggesting that the greater rate of product formation with MNNG-treated NE was due to an increase in RP_o. As a control for this experiment, we altered the protocol by adding heparin after the formation of RP_o (Figure 2B). This tested the stability of RP_o and also the unlikely possibility that RP_o could accumulate by cycling of the RNA pol II preinitiation complex. The results were similar to those in Figure 2A, suggesting that RP_o was stable during the period used and that cycling to accumulate RP_o did not occur, as predicted in eq 1. Taken together, these results indicate that the increase in linear rate of transcript formation observed with NE from MNNG-treated cells (Figure 1) is due to recruitment of a larger amount of RP_o.

Purified ATF/CREB and Transcription from the pSH15 Promoter. After establishing that NE from MNNG-treated cells had stronger transcriptional activity than normal NE, we next examined the question of whether this effect is mediated by the ATF/CREB activator, since it is possible that MNNG treatment influenced transcription proteins other than the ATF/CREB activator. To address this point, ATF/CREB was removed from normal or MNNG-treated NE by passing the extract through an oligonucleotide affinity column. Virtually no residual ATF/CREB could be detected after this procedure by band-shift assay, indicating that the ATF/CREB protein had been quantitatively removed from the depleted NE preparations (designated NEd). The depleted nuclear extracts were then assayed for transcriptional activity. The depleted extracts had similar, relatively low transcriptional activities (Figure 3A, lanes 1 and 3), suggesting that a factor(s) required for the difference between the two original NEs had been removed. Next, to restore ATF/CREB activation to the depleted NE system, a reference purified ATF/CREB from bovine testis (Widen & Wilson, 1991) was added. The results showed that the depleted NEs from normal and MNNG-treated cells were able to support similar amounts of transcription activity in the presence of this reference activator protein (Figure 3A, compare lanes 2 and 4).

To further examine the role of the ATF/CREB activator, the protein was highly purified from normal and MNNG-treated NEs. We note that the amounts of ATF/CREB activator in the two extracts were similar, as was observed earlier with MNNG-treated CHO cell NE (Englander & Wilson, 1992b,c). In these experiments, similar recovery of the activator proteins was obtained from each NE after purification. The two preparations of purified ATF/CREB (100 ng), with equal DNA-binding activity, were compared in an *in vitro* transcription assay assembled with depleted nuclear extract from normal cells (Figure 3B). ATF/CREB from MNNG-treated cells activated transcription more (~10-fold) than ATF/CREB from normal cells. We conclude from these results that MNNG treatment leads to a modification in ATF/CREB, which increases transcriptional activity of the NE. The purified activators from normal and MNNG-treated cells are designated (Normal) ATF/CREB and (MNNG) ATF/CREB, respectively.

MNNG Treatment and Promoter Clearance. To rule out the possibility of an effect of (MNNG) ATF/CREB on the promoter clearance step (see above), time courses of transcript formation were obtained starting from preformed RP_o. RP_c was first assembled with NEd, and dATP was added to form RP_o (Narayan et al., 1994). This was followed by addition of (Normal) ATF/CREB or (MNNG) ATF/CREB and NTPs to initiate transcription (Figure 4B). Since ATF/CREB was added after RP_o had formed, any differences in the rate of transcription would be insensitive to changes in k_2, but instead must be a function of k_3 (i.e., the rate of product formation is equal to $[RP_o]k_3$). The rate of transcript formation was similarly increased by each purified ATF/CREB (4−5-fold), as found earlier with (Normal) ATF/CREB (Narayan et al., 1994). These results indicate that (MNNG) ATF/CREB must be present prior to formation of RP_o in order for it to show differential activation and that

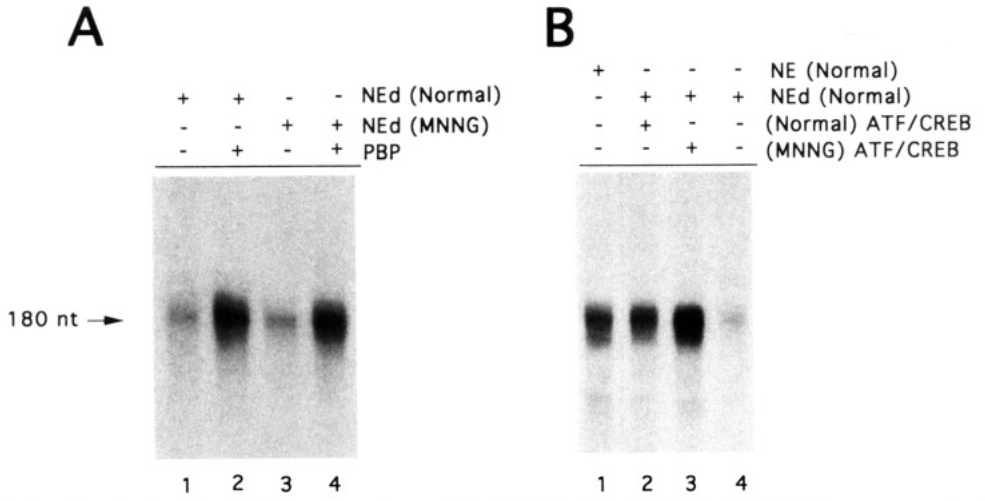


FIGURE 3: Transcriptional activation of pSH15 promoter by purified (MNNG) ATF/CREB. Panel A demonstrates that MNNG treatment did not modify transcription factors other than the ATF/CREB that is required for the transcriptional activation of the pSH15 promoter. The preinitiation complexes were assembled with NEd prepared form normal (lanes 1 and 2) or MNNG-treated cells (lanes 3 and 4), and 100 ng of ATF/CREB (palindrome binding protein, PBP, purified from bovine testis) was added to the mixtures in lanes 2 and 4. The transcription conditions were as in Panel B. Panel B demonstrates the effect of (MNNG) ATF/CREB on the activation of transcription of the pSH15 promoter. The *in vitro* transcription assay was assembled with pSH15 plasmid and ATF/CREB-depleted nuclear extract (NEd) supplemented with purified (Normal) ATF/CREB or (MNNG) ATF/CREB. The preinitiation complex was assembled by incubation for 30 min at 30 °C, and then NTPs were added to initiate transcription. Transcription was stopped after 1 h. Lanes 1 and 4 show run-off transcript with complete NE and NEd, respectively. Lanes 2 and 3 show run-off transcript with NEd supplemented with (Normal) ATF/CREB and (MNNG) ATF/CREB, respectively.

once RP_o is formed, the rate of promoter clearance (k_3) is not influenced by (MNNG) ATF/CREB.

Effect of Purified ATF/CREB on RP_c and RP_o Formation. To evaluate RP_o formation as a function of MNNG treatment, we used permanganate ($KMnO_4$) footprinting (Wang et al., 1992a; Narayan et al., 1994). $KMnO_4$ reacts selectively with thymines in single-stranded DNA (Wang et al., 1992a,b), and in our system, the transcribed strand is probed to measure hypersensitivity of the +1T residue as RP_o is formed (Narayan et al., 1994). We conducted reactions where RP_c was preformed. Then dATP was added, and the incubation was continued for a period long enough to convert most of the RP_c to RP_o (10 min). There was no difference in the amount of RP_o formed, i.e., there was no difference in hypersensitivity at the +1T residue, with the NEd and the NEd supplemented with (Normal) ATF/CREB (Figure 5). However, with NEd supplemented with (MNNG) ATF/CREB, RP_o formation was much greater. The increase in the amount of RP_o must have been due to an increase in the amount of RP_c. The isomerization rate constants, k_2, determined from kinetic studies as in Figure 1 with NEd, NEd supplemented with (Normal) ATF/CREB, and (MNNG) ATF/CREB were similar: the values were 0.28, 0.26, and 0.22 min^{-1} with NEd, NEd supplemented with (Normal) ATF/CREB, and (MNNG) ATF/CREB, respectively. It was apparent, therefore, that the effect of (MNNG) ATF/CREB was to increase the amount of RP_c formed.

Kinetics of RP_o Formation. We measured the time course of RP_o formation starting from preformed RP_c with NEd alone and with NEd supplemented with (MNNG) ATF/CREB (Figure 6). The results with NEd were as expected, where the time course was exponential and consistent with an isomerization rate constant (k_2) of 0.28 min^{-1}. However, the time course of RP_o formation with NEd supplemented with (MNNG) ATF/CREB was unexpected (Figure 6); the data were clearly nonexponential and indicated that the rate of RP_o formation was initially slow and followed by a more rapid rate. The multiphasic nature of RP_o formation indicates that at least two steps are involved:

$$RP_c \xrightarrow{k_{2a}} RP_i \xrightarrow{k_{2b}} RP_o \qquad (3)$$

The lag in open-complex formation is consistent with a "slow" accumulation of an intermediate complex designated RP_i. The simulations in Figure 7 (upper panel) illustrate the expected time courses for one- and two-step models for open complex formation, and these curves qualitatively resemble the data in Figure 6.

Additionally, the slow rate of formation of RP_o would be expected to influence the observed lag for transcript formation, as noted in Figure 1. Since the apparent lag in RP_o formation for (MNNG) ATF/CREB (Figure 6) is approximately equivalent to the lag observed for transcript formation (Figure 1), the rate-limiting step would be expected to be the same, i.e., the rate of open complex formation. This implies that the rate of promoter clearance (k_3) is much faster with (MNNG) ATF/CREB than with (Normal) ATF/CREB, where the rate constant has been determined to be slow and the lag for transcript formation would therefore be expected to be much greater than the lag observed for open complex formation. This is illustrated by the simulated time courses in Figure 7 (bottom panel). Thus, our results indicate that eq 1 is not adequate to explain the results with (MNNG) ATF/CREB and needs to be amended to reflect the existence of an intermediate preinitiation complex (RP_i):

$$R + P \xrightarrow{k_1} RP_c \xrightarrow{k_{2a}} RP_i \xrightarrow{k_{2b}} RP_o \xrightarrow{k_3} \text{transcript} \qquad (4)$$

In the presence of (Normal) ATF/CREB, closed to open complex formation can be described by a simple one-step model, suggesting that either RP_i formation or conversion to RP_o in eq 4 is rapid, so there is not accumulation of RP_c or RP_i, respectively. However, RP_o accumulates due to the slow rate of promoter clearance (k_3). In contrast, in the presence of (MNNG) ATF/CREB, the slow rate of open complex formation becomes significant, but RP_o does not accumulate due to the faster rate of promoter clearance. The lag observed in open complex formation, as well as transcript

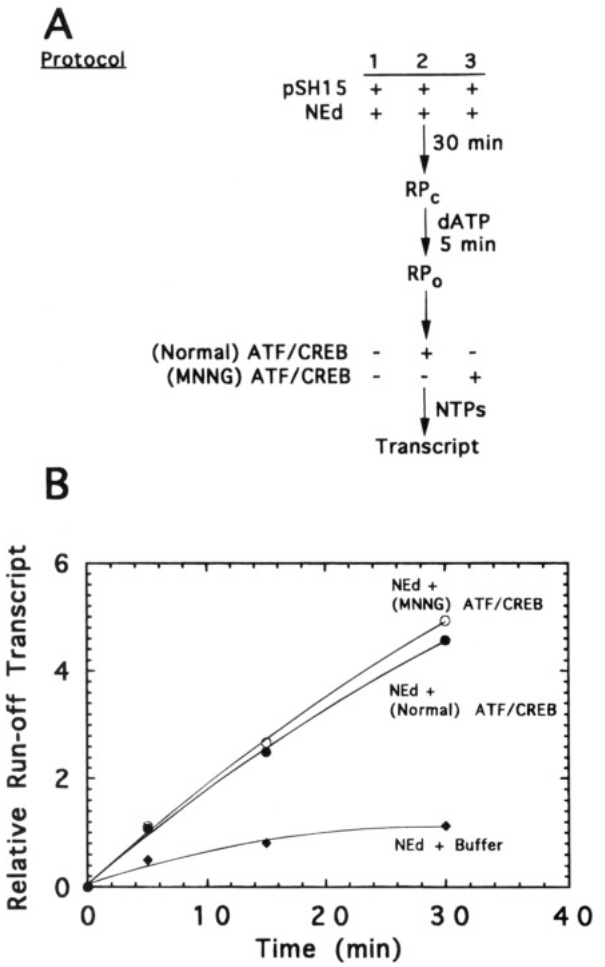


FIGURE 4: Role of ATF/CREB in the rate of promoter clearance (k_3). The run-off transcription assay was done with NEd, purified (Normal) ATF/CREB or (MNNG) ATF/CREB, and pSH15 plasmid. The reaction protocol is indicated in panel A. Preinitiation complex was assembled by 30 min of preincubation of pSH15 and NEd. Four hundred micromolar dATP was added to allow RP_c conversion to RP_o, and after 5 min, 100 ng of (Normal) ATF/CREB (protocol 2) or (MNNG) ATF/CREB (protocol 3) was added. Protocol 1 received a similar volume of NE buffer, and NTPs were then added immediately to initiate transcription. Panel B shows the rate of transcript formation determined from densitometric scanning of autoradiograms.

formation, indicates that closed to open complex formation requires at least two steps, as depicted by k_{2a} and k_{2b} in eq 4.

DISCUSSION

Signals for responding to DNA damage in mammalian cells generally are transmitted through intracellular signal transduction pathways, eliciting programs of gene expression and other alterations of protein activity. One such signal transduction pathway activates protein kinase C, leading to phosphorylation of proteins which elevate the expression of "DNA damage inducible" genes (Buscher et al., 1988; Devary et al., 1991). Previous work from this laboratory, with the cloned human β-pol gene, indicated that a second well-known signal transduction pathway, mediated by cAMP-dependent protein kinase, plays a role in the transcriptional activation of the β-pol promoter after treatment of CHO cells with the DNA-damaging agent MNNG (Fornace et al., 1989; Kedar et al., 1991; Englander & Wilson, 1992a,b). Studies indicated that the ATF/CRE site of the β-pol core promoter is required for the alkylation agent response (Englander &

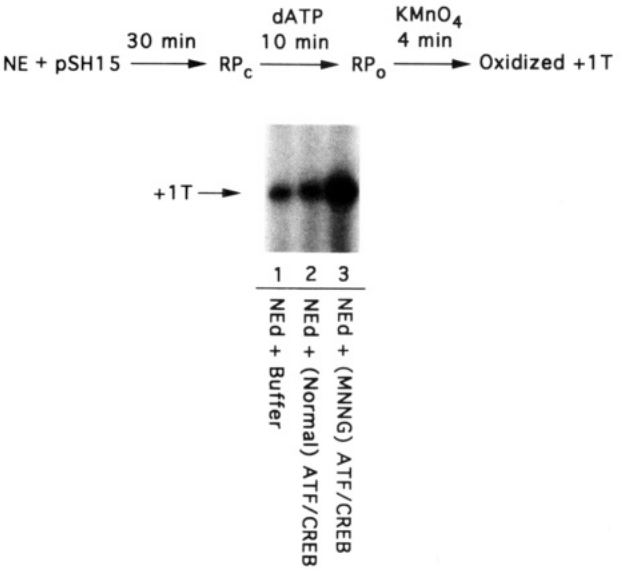


FIGURE 5: $KMnO_4$ footprinting to determine RP_o formation. RP_c was assembled by preincubation for 30 min at 30 °C with pSH15 plasmid, NEd, and NEd supplemented with a saturating amount (100 ng) of (Normal) ATF/CREB or (MNNG) ATF/CREB. dATP (400 μM) was added, and incubation was continued for an additional 10 min. The reaction mixture was then treated with 6 mM $KMnO_4$ for 4 min. DNA was purified, and the hypersensitive T residue at the +1 position of the transcribed strand, indicated by an arrow, was identified by primer extension analysis. Lane 1, NEd alone; lane 2, NEd supplemented with (Normal) ATF/CREB; lane 3, NEd supplemented with (MNNG) ATF/CREB. The autoradiogram shown is representative of multiple independent experiments.

Wilson, 1992b,c). Thus, the transcriptional activator ATF/CREB was viewed as a likely mediator of the response to MNNG treatment by the β-pol promoter. The present studies were designed to examine this possibility and to understand the mechanism of its transcriptional activation.

We recently applied an *in vitro* transcription system with HeLa nuclear extract (Narayan et al., 1994) and a chimeric β-pol promoter (pSH15) that exhibits dependence on the activator protein ATF/CREB. Individual steps in transcription initiation (eq 1) were quantified, and the kinetic effect of ATF/CREB was assigned (Narayan et al., 1994). In general, a transcriptional activator such as ATF/CREB can stimulate a promoter at any one or all of the three main steps in the transcriptional initiation model, as depicted in eq 1: initial binding between preinitiation complex and promoter to form a closed preinitiation complex (RP_c); isomerization (k_2) of the RP_c to form an open complex (RP_o), and the rate constant for promoter clearance (k_3). We found earlier that the ATF/CREB activator stimulated our HeLa extract *in vitro* system at the promoter clearance step, and we noted that transcript elongation is relatively fast and is not a rate-limiting step accounting for changes in the rate of run-off transcript formation. In prokaryotic transcription systems, activator or repressor proteins have been shown to regulate *in vitro* transcription by modifying either the equilibrium association constant for step I or k_2, or both. For example, the λ repressor activates a bacteriophage λ promoter by increasing k_2 (Hawley & McClure, 1982; Hershberger & deHasetts, 1991), and regulation at the *Escherichia coli* galactose operon by the catabolite activator protein occurs at both the equilibrium constant (K_B) and k_2 steps (Goodrich & McClure, 1992). In these studies, kinetic analysis generally were used to define the role of the regulatory protein.

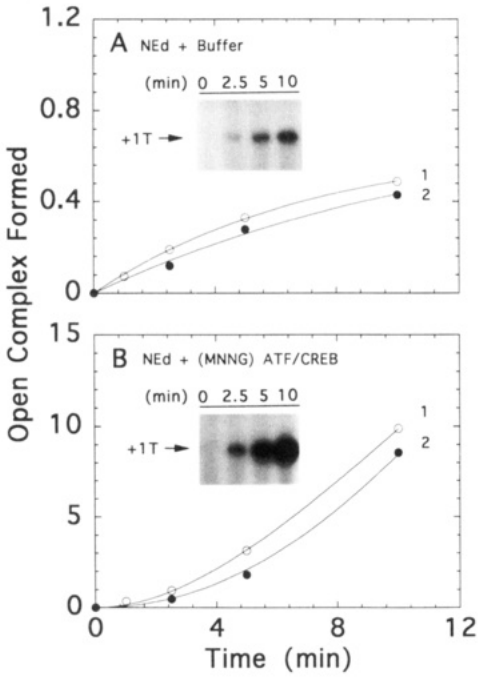


FIGURE 6: Kinetics of RP_o formation as measured by $KMnO_4$ footprinting. $KMnO_4$ footprinting conditions were as described in Figure 5. To examine the rate of RP_o formation, RP_c was assembled as before (Figure 5). The reaction mixture was treated with dATP for 0, 2.5, 5, or 10 min, as indicated (inset autoradiograms in both panels). The hypersensitive T residue at the +1 position is shown by the arrow. The autoradiograms shown are representative of multiple independent experiments. Panel A shows the results from NEd alone. The curve was fitted to an exponential equation ($k_{obs} = 0.14\ min^{-1}$). Panel B shows the results from NEd supplemented with (MNNG) ATF/CREB. The curve was fitted to lag eq 2, $\tau_{obs} \sim 5$ min. Curves 1 (○) and 2 (●) in both panels are from two independent experiments. The data for curve 2 in each panel was obtained from the inset autoradiogram shown in that panel.

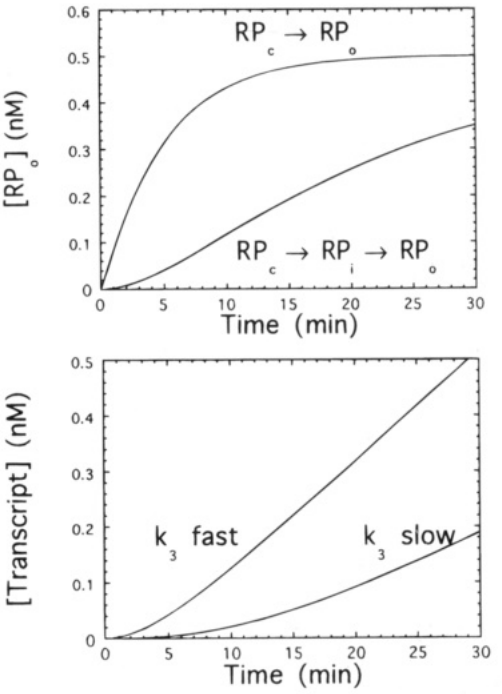


FIGURE 7: Influence of intermediate preinitiation complex (RP_i) formation on simulated time courses. Simulated time courses were generated from the models illustrated in eqs 1 and 4. The RNA polymerase and promoter concentrations were 0.5 and 15 nM, respectively. The upper panel represents simulated time courses for RP_o formation as observed with $KMnO_4$ footprinting. With this experimental protocol, RP_c is preformed, and RP_o formation is initiated by the addition of dATP and measured by $KMnO_4$ probing as described in Materials and Methods. Since NTPs are not included, the rate of promoter clearance (k_3) is zero. For a single-step model for RP_o formation (eq 1), a simple exponential time course governed by the isomerization rate constant (k_2) is observed. In contrast, if RP_o formation proceeds through a precursor intermediate complex (eq 4), an apparent lag in RP_o formation is observed. The time courses illustrate that a multiphasic time course indicates the accumulation of an intermediate complex. For these simulations, the rate of promoter clearance was zero, and the rate of RP_c formation was rapid. The isomerization rate constants were 0.2 s^{-1} for the single-step model and 0.2 and 0.05 s^{-1} for the two-step model for RP_o formation. The same time courses are generated whether k_{2a} or k_{2b} is larger. The concentration of RP_i would need to be monitored to distinguish between these alternatives. Simulated time courses of transcript formation for eq 4 are illustrated in the lower panel. These time courses were generated with the rate constants used in the upper panel, except that k_3 was either 0.05 (slow) or 1000 s^{-1} (fast). If the rate of promoter clearance (k_3) is slow, then the lag in transcript formation is greater than the apparent lag for RP_o formation. However, when k_3 is fast, the lags for RP_o and transcript formation are similar.

In the present study, with MNNG-treated HeLa cells we found that *in vitro* transcription with the nuclear extract was much stronger than with the corresponding extract from normal cells (Figure 1). To confirm the idea that ATF/CREB activator mediated the effect of MNNG treatment, we depleted ATF/CREB from the NE of normal or MNNG-treated cells and then assembled *in vitro* transcription assays with these depleted extracts. The depleted extracts had similar ("basal") activities. We confirmed that ATF/CREB was responsible for the MNNG-mediated transcriptional activation with purified protein from either normal or MNNG-treated cells. Thus, ATF/CREB from MNNG-treated cells was found to be transcriptionally more active than ATF/CREB from normal cells.

We found that ATF/CREB from MNNG-treated cells, designated (MNNG) ATF/CREB, stimulates formation of RP_c, such that much more RP_c is formed than with ATF/CREB from normal cells. Surprisingly, the rate of RP_o formation from RP_c was altered and the rate of promoter clearance was stimulated >10-fold, such that it was not rate-limiting, as in the case with normal NE or ATF/CREB from normal cells. This differential effect of (MNNG) ATF/CREB on promoter clearance seems inconsistent with the results in Figure 4, where both ATF/CREB proteins stimulated promoter clearance to the same extent (6-fold). However, for (MNNG) ATF/CREB to exert its differential effect, it must be present during the assembly of RP_c. When this is the case, the pathway shown in eq 4 is followed, where both promoter clearance (k_3) and closed to open preinitiation complex formation accumulation are different from those in eq 1. Thus, the transcriptional activation by ATF/CREB from MNNG-treated cells involves a combination of effects: formation of more RP_c, a different $RP_c \rightarrow RP_o$ isomerization pathway, and a greater k_3. In earlier studies by Wang et al. (1992a), the activator protein GAL4-VP16 was shown to increase RP_c formation, and to do so, the presence of the activator protein was necessary at the beginning of the promoter and extract preincubation. Hence, this result is similar to our present results with ATF/CREB, since the differential stimulation with MNNG treatment is not observed unless ATF/CREB is present during the initial preinitiation complex formation (i.e., RP_c).

We observed that MNNG treatment did not result in an increased ATF/CREB DNA-binding activity in the extract, suggesting that simple occupancy of the ATF/CREB site does

not explain promoter activation secondary to MNNG treatment. Instead, a physical modification of ATF/CREB may lead to the differential transcriptional activation. A post-translational modification, such as phosphorylation status, of the (MNNG) ATF/CREB could conceivably facilitate assembly of transcription proteins at the β-pol promoter initiation site. This idea is similar to that proposed for enhancement of the transcriptional potential of c-Jun via phorbol ester-induced phosphorylation of the transactivation domain (Franklin et al., 1992). Furthermore, the phosphorylation of rat brain CREB by protein kinase A both *in vitro* and *in vivo* has been shown to increase its transcriptional activity (Yamamoto et al., 1988; Gonzalez & Montminy, 1989; Hagiwara et al., 1992). ATF/CREB activators, as a group, are regulated by phosphorylation.

In summary, ATF/CREB transcriptional activator from normal HeLa cells stimulates the rate of promoter clearance (k_3) in our *in vitro* system (Narayan et al., 1994); ATF/CREB from MNNG-treated cells is altered such that it is a much stronger transcriptional activator. The ATF/CREB from MNNG-treated cells alters preinitiation complex assembly, resulting in recruitment of more preinitiation complex at the initiation site. This preinitiation complex follows a kinetic pathway through an intermediate preinitiation complex not observed with normal ATF/CREB. The rate of promoter clearance is dramatically increased in the presence of (MNNG) ATF/CREB so that isomerization of the closed preinitiation complex is rate-limiting during steady-state transcript formation.

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