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Functional role of the ultraviolet light responsive element (URE; TGACAACA) in the transcription and replication of polyoma DNA

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ABSTRACT

We have previously identified a novel 8 bp sequence (UV-responsive element, URE: TGACAACA) present in the regulatory region of polyoma DNA that interacts with protein factors induced in rat fibroblast cells by exposure to UV light. In the present study, we demonstrate through competitive binding assays that this sequence is distinct from the partially homologous AP1 and CRE target sequences. The proteins that bind to the URE appear to have transcriptional activity in UV-exposed rat fibroblasts. In addition, the URE appears to play a role in promoting the replication of polyoma DNA as determined through two different experimental approaches. Together, these findings suggest that the URE is a novel DNA binding element that interacts with proteins involved in the transcription and replication of polyoma sequences.

INTRODUCTION

DNA tumor viruses provide a useful tool for studying inducible responses to DNA damage in mammalian cells. It has been demonstrated that papovaviruses (i.e. SV40 and polyoma) and parvo-associated helper viruses are induced to undergo asynchronous replication, independent of the host mammalian cell cycle, by both physical and chemical carcinogens known to cause DNA damage (Reviewed in 1). Similarly, various genotoxic agents have been shown to induce selective amplification of cellular DNA (2–4), and increasing evidence suggests that common regulatory sequences and inducible protein factors play an important role in the regulation of these processes (5–10). The replication of DNA tumor viruses is dependent upon both the production of viral proteins (such as the large T antigen of SV40 and polyoma viruses) and on a set of species specific accessory regulatory factors that must be provided by the host cell. Therefore, viral genomes serve as sensitive and useful markers for the identification of the regulatory elements and cellular factors involved in replication and transcriptional events.

The polyoma enhancer is divided into 2 regions, designated A and B (11). The transcription of polyoma sequences is regulated through sites located on the two enhancers (reviewed in 12) and together with the viral origin, these regions comprise the critical regulatory region of polyoma DNA (13). While both the transcription and replication of polyoma sequences require the participation of the viral large T antigen, the functional activities of the polyoma regulatory region are mediated through the array of proteins provided by the permissive cell that interact with the regulatory domain.

Previously we have shown that *trans*-acting factors, purified from UV-treated normal rat fibroblasts, are capable of inducing polyoma DNA replication in a rat fibroblast cell line that contains an integrated copy of polyoma DNA (5,6). In an attempt to identify the factors that are responsible for this activation, DNase I footprinting assays were performed using nuclear proteins from sham and UV-irradiated normal rat fibroblasts reacted with a 257bp DNA fragment encompassing the polyoma regulatory region (7). This analysis yielded 2 regions that were protected in UV treated cells, the ubiquitous CAAT motif, and a novel 8 bp sequence, TGACAACA, designated the URE (UV-responsive element). As the CAAT sequence has already been shown to play a role in both transcription and replication (14), subsequent studies have been aimed toward investigating the functional role of the URE and identifying the proteins that bind this sequence.

The URE (nt 5256–5263 of the polyoma A2 strain) is located at the 3′ end of the B enhancer adjacent to a series of AT rich base pairs that define the late boundary of the polyoma origin of replication (15). This region of polyoma DNA, including the URE sequence, represents part of the binding site of the large T antigen, however, the URE lies in a region of low affinity T antigen binding (16). The URE shares homology with two other common regulatory elements, the cyclic AMP (CRE) responsive element (TGACGTAA, 17) and the AP1 target sequence ($TGA^{C}/_{G}TCA$, 18) and may represent the target binding site for an as yet unidentified member of the ATF or AP1 family of transcription factors. The URE is found on the 5′ regulatory domain of several cellular genes (19), including alpha amylase and alpha feto-protein and is found with one basepair modification on the c-*jun* regulatory domain (20). The latter sequence (TGACATCA) has been shown to interact with a factor distinct from AP1 and to increase the transcriptional activity of the c-*jun*

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regulatory region upon UV-irradiation (20). Using antibodies raised against URE-binding proteins we have recently demonstrated that the expression of a 40 kDa protein is induced by UV-irradiation in rat fibroblast and human keratinocyte cells and that the expression pattern of the 40 kDa protein was found to be distinct from that of c-*jun* and CREB proteins (21). Together, these observations suggest that the URE is part of the complex response of mammalian cells to DNA damage.

The purpose of the present study was to identify whether the URE plays a role in the transcription and/or replication of polyoma DNA and to determine the specificity by which proteins bind to this sequence when compared with CRE and AP1 target sequences. We demonstrate here that the URE is important in both of these events and that the complex of proteins that binds the URE is distinct, in part, from that which binds the CRE and AP1 target sequences.

MATERIALS AND METHODS

Cells

Rat 6 fibroblast cells and WOP mouse fibroblast cells were both maintained in DMEM (Gibco) supplemented with 10% Calf serum (Hyclone) at 37°C in the presence of 5% CO_2. The WOP cells (22), kindly provided by C.Basilico, were routinely maintained at 37°C and transferred to 33°C upon transfection with DNA. In all cases, cells were UV irradiated as described previously (7) using UV-C (254nm) at a dose of 20 J/m^2.

DNA sequences

The polyoma construct used here contains the entire 5.2 Kb polyoma genome (A2 strain) cloned into the Bam H1 site of the pBR322 plasmid. The region encoding the polyoma large T antigen remains intact in this plasmid, and similar polyoma constructs have been demonstrated to be able to replicate in mammalian cells without excision and re-circularization (23). In addition, polyoma mutated at the URE site (*dl*1031 mutant, 24) was kindly provided by G. Magnusson (University of Uppsala). A 257 base pair region encompassing the polyoma regulatory region (7) was cloned upstream of the CAT gene (pCAT257) in the pCAT enhancer plasmid (purchased from Promega). The pCAT control vector (Promega) in which the expression of the CAT gene is driven by SV40 flanking sequences is used in this study as well.

Oligonucleotide sequences were synthesized in-house using a Cyclone DNA synthesizer (Milligen) and phosphoramidite reagents, and both purified and annealed employing standard procedures. The upper strands of these sequences written 5′ to 3′ in orientation are as follows:

URE tetramer:
CTAGAGGCGCCTGACAACAGCTATGACAACAGCTA-
TGACAACAGCTATGACAACAT
URE dimer:
ACTATGACAACAGCTATGACAACAGT
CRE dimer:
ACCATGACGTCAGCTATGACGTCAGT
AP1 dimer:
CTGACTCATCCGTGAGTAACT

The URE tetramer was cloned into the Xba I site of the pGEM7 vector yielding the pURE. The correct orientation of this sequence upon cloning was confirmed through the use of an internal restriction site (Ban I).

All other oligonucleotide sequences were annealed to complementary strands and used in EMSA as described. Note that the AP1 dimer contains two related AP1 sequences, one that is found on the polyoma regulatory region (TGACTAA, nt 5114−5121) and one that represents the consensus cellular AP1 binding site (TGA$^{C}/_{G}$TCA).

Transfection assays

Calcium Phosphate Precipitation. Twenty-four hours before transfection, cells were plated at a density of 5×10^5 cells per 100mm dish. The medium was changed four hours before the addition of precipitates which were incubated with the cells for a period of 4 hours. At this time, cells were washed once with PBS prior to sham irradiation or exposure to UV light and treated with 10% glycerol for 1.5 minutes to enhance DNA uptake. The plates were then washed extensively with PBS to remove DNA adherent to the plates. A total of 25 μg of DNA (5−15 μg of pURE and 10 μg of pCAT257 vector see Results for details) were precipitated onto the cells, the differences in amounts of pURE were compensated by the addition of the parent pGEM7 plasmid (Promega) such that the amount of pURE and pGEM7 represented a total of 15 μg in each transfection mixture.

Electroporation. WOP cells (5×10^6 cells/ml) in the log phase of growth were electroporated (using a GENEZAPPER from IBI) at 4°C with 255 volts and 1000 μF in 25mM HEPES pH 7.1, 0.25mM $NaHPO_4$ and 140mM NaCl.

Transcriptional assays

Protein extracts were prepared from the cells 40 hours after calcium phosphate transfection and UV exposure. Prior to harvest, the cells were washed twice with PBS. Protein extracts were prepared according to the method of Gorman et al. (25). 50 μg of protein was reacted with ^{14}C-chloramphenicol in the presence of 4 mM acetyl CoA for 2 hours prior to separation by thin layer chromatography. Resulting amounts of acetylated chloramphenicol were quantitated using a radioimaging blot analyzer (AMBIS) and visualized through autoradiography. To monitor DNA uptake, the amount of pCAT257 in the transfected cells was quantitated through Southern blot hybridization using the parent pCAT vector as ^{32}P-labeled probe.

Preparation of DNA for replication assays

Twenty-four hours after transfection, extrachromosomal DNA was prepared according to the method of Hirt (26), extracted with phenol and ethanol precipitated. Equal amounts of DNA (5 μg), as determined through A_{260} absorption, were fractionated on a 0.9% agarose gel. Levels of polyoma replication were determined through Southern blotting using ^{32}P labelled polyoma DNA (the entire 5.2 Kb genome purified away from the vector) as probe. The blots were analyzed through autoradiography and quantitated through the use of a computerized radio-imaging system (AMBIS).

Electrophoretic mobility shift assays (EMSA)

Nuclear proteins were prepared from rat 6 cells according to the method of Dignam et al. (27). DNA oligonucleotide fragments were ^{32}P-labelled (Amersham) utilizing T4 polynucleotide kinase (Boehringer Mannheim) according to standard procedures. EMSA assays were performed by reacting 7.2 or 14.4 μg of nuclear proteins with 0.2 ng of ^{32}P-labelled target DNA (specific activity 4×10^{10} cpm/μg) as specified in Results in the presence

of 10mM HEPES, 50mM KCl, 0.1mM EDTA, 0.5mM DTT, 4mM $MgCl_2$, 10% glycerol and 2 μg poly dI-dC (Boehringer Mannheim) for 20 minutes before separating on 7.5% PAGE. Competition assays were performed in the presence of 500, 2500 and 5000 fold amounts of cold DNA relative to labeled probe added to the reaction 10 minutes prior to the addition of ^{32}P-labeled DNA as specified in the Results.

RESULTS

Specificity of proteins that bind the URE sequence

We have shown in previous studies that quantitative differences and qualitative increases in the binding of proteins to the URE occur upon UV-irradiation of mouse and rat fibroblasts and human keratinocytes (21). Here, we wished to determine the specificity of the proteins that bind the URE in comparison with those that interact with the CRE and AP1 target sequences. To this end, the URE, CRE and AP1 sequences were used both as a ^{32}P-labeled target sequence and as competitor sequences for the binding of proteins to the URE. When each of these DNA elements was used as a ^{32}P-labeled target sequence, the pattern of complexes formed with nuclear proteins prepared from UV-exposed rat 6 cells suggested that different proteins may interact with each sequence (Figure 1A). While the binding of proteins to the URE generated multiple complexes, the binding to the AP1 target sequence yielded fewer complexes and the binding to the CRE was found to be the weakest, although, this sequence shares the greatest similarity with the URE. It should be noted, however, that the DNA binding activity of the CREB protein is normally low in unstimulated rat fibroblasts (28). In contrast, it has been demonstrated that the AP1 transcription factor is inducible by UV-irradiation (20, 29). That a complex of similar size was formed with each of these sequences may suggest that the proteins that bind these target sites are of a similar molecular weight.

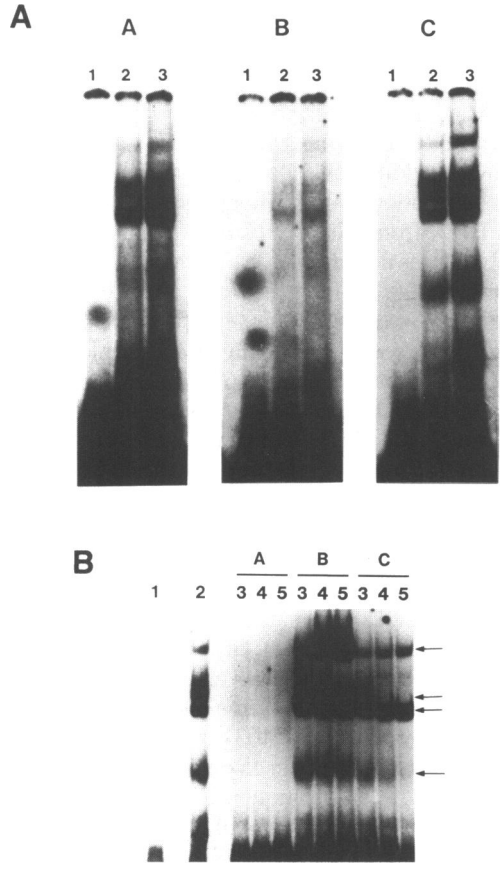

Figure 1. (A) The binding of proteins to the AP1, CRE and URE target sequences. ^{32}P-labelled oligomers representing the AP1 (panel A), CRE (panel B) and URE (panel C) target sequences were reacted with 7.2 μg (lanes 2) and 14.4 μg (lanes 3) of nuclear proteins from UV-exposed rat 6 cells. The migration of the probes alone is shown in the lanes marked 1. The autoradiogram shown in panels A and B were exposed for 24 h while that in C was exposed for 72 h. (B). Specificity of proteins that bind the URE. Nuclear proteins (7.2 μg) extracted 24 hrs after irradiation of rat 6 cells were reacted with 500×, 2500× and 5000× fold amounts (lanes 3–5 respectively) of unlabeled URE (panel A), CRE (panel B) and AP1 (panel C) dimer sequences for 10 minutes prior to the addition of 0.2 ng ^{32}P-labeled URE. This mixture was reacted for 20 minutes prior to fractionation on a 7.5% polyacrylamide gel. The binding pattern of proteins with the URE dimer as target sequence is shown in lane 2, the profile of the URE probe alone is shown in lane 1.

To further determine the specificity of protein binding to the URE in comparison with the AP1 and CRE target sites, each of these sequences were used as non-labelled competitor sequences in EMSA with the URE as a ^{32}P-labelled probe. This assay revealed that competition with cold URE completely inhibited binding to the ^{32}P-labeled URE (Figure 1B). In addition, the AP1 dimer was capable of partially inhibiting 2 of the 4 DNA-protein complexes when used in 2500-fold excess (Figure 1B), suggesting that some of the proteins that bind to the URE also participate either directly or indirectly in binding to the AP1 target sequence. This is supported by the similar pattern of complexes formed with each of their sequences (Figure 1A) and further supports our previous observation that c-*fos* (which together with the c-*jun* protein forms the AP1 transcription factor) is one of the four proteins that bind to the URE (7). Interestingly, when the CRE was used as a competitor sequence, no decrease in the binding of proteins to the URE was noted, but a slight increase in the intensity of the URE-protein complexes was observed. This may be due to the large excess of DNA sequences used in the reaction, or alternatively, to an indirect repressive role CRE may play in the formation of complexes with the URE. These findings demonstrate not only specific binding to the URE sequence, but that the proteins that bind the URE are, in part, distinct from those that bind the CRE and AP1 target sequences. Furthermore, these observations indicate that the URE may represent a novel DNA binding element, although the proteins that bind this sequence may be closely related to those that bind the AP1 target site.

The URE has a positive role in transcription

In order to determine whether the URE plays a role in the transcription of polyoma sequences, the pURE was co-transfected with the pCAT257 vector into sham and UV-irradiated rat fibroblast cells. In this experimental design, the pURE can compete for the binding of proteins that would otherwise bind to the URE sequence present on the polyoma regulatory region thereby removing them from the transcriptional assay. The pCAT257 vector demonstrated greater transcriptional activity in these cells after UV exposure (Figure 2A lanes 3 and 4) indicating the activation of transcription factors by UV irradiation. Interestingly, in the presence of increasing amounts of pURE, a concentration-dependent decrease in transcriptional activity of pCAT257 was seen in the UV-exposed cells that was less significant after sham exposure (Figure 2A, lanes 5–8). To insure that equal amounts of DNA were contained in the transfection mixture and to ensure that this assay demonstrates

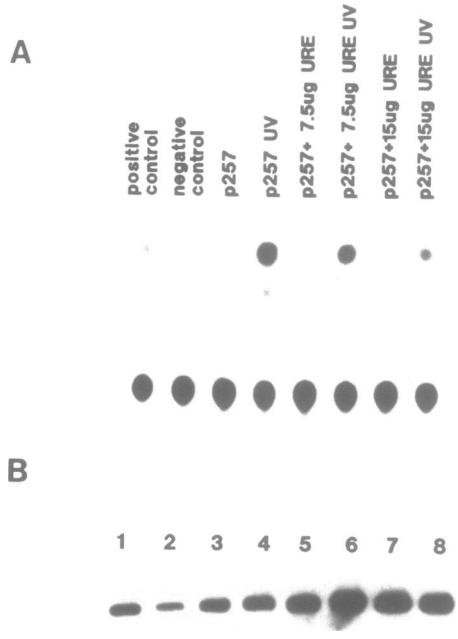

Figure 2. The role of the URE in transcription. (A) A 257 base pair fragment of polyoma DNA encompassing the regulatory region cloned upstream of the CAT gene was analyzed for transcriptional activity in sham and UV-irradiated rat fibroblast cells in the presence of 7.5 μg or 15 μg of pURE as indicated in the figure. In all cases pGEM 7 DNA was added to generate a total of 25 μg of transfected DNA. In the positive control vector, the 257 base pair region is replaced by the SV40 promoter and the negative control represents the activity of the CAT vector in the absence of any promoter sequences. (B) To control for transfection efficiency, the amount of pCAT vector taken up by the cells was determined through Southern blot hybridization of extrachromosomal DNA isolated from the transfected cells and linearized with Hpa I. (C) Quantitation of the assay represented in A generated by a radioimaging blot analyzer (Ambis).

a specific effect of the URE, inverse amounts of the parent pGEM7 plasmid were co-transfected with the pURE to a total of 15 μg test plasmid in all cases. These results indicate that the URE-bound proteins may become transcriptionally active upon UV exposure, suggesting their possible role as UV-inducible regulatory factors. No difference in CAT activity was noted when the parent pCAT vector (controlled by SV40 sequences) was transfected into sham and UV-treated cells (data not shown). Southern blot analysis of extrachromosomal DNA prepared from the transfected cells was performed to verify similar uptake of DNA between each test group using the pCAT control vector as ^{32}P-labeled probe (Figure 2A). The quantitation of the CAT assay shown in Figure 2A, obtained from a radioimaging blot analyzer (AMBIS), is shown in Figure 2B.

The URE is involved in polyoma replication

We have determined that the URE plays a role in polyoma replication through two different experimental approaches. Initially, the pURE was co-transfected with a plasmid construct of polyoma DNA into mouse fibroblast cells (WOP cell line) that are permissive for polyoma replication and express polyoma large T antigen at 33°C (22). Levels of replicated polyoma DNA are determined through Southern blotting of Dpn I digested extrachromosomal DNA. Dpn I digestion distinguishes between replicated and non-replicated DNA sequences based on methylation patterns rendering replicated DNA resistant to digestion. The Dpn I digested polyoma DNA that migrates to the lower part of the gel serves as a control for transfection efficiency.

When either the pURE or a dimer oligonucleotide of the URE sequence was co-transfected together with polyoma DNA into WOP cells, a concentration-dependent decrease in replication was seen as shown in Figure 3A (I), indicating that the pURE was able to compete for proteins important in promoting the replication of polyoma DNA. In the presence of approximately 45-fold excess URE sequence (provided by co-transfection of the pURE), a 40% decrease in replication was seen relative to co-transfection of polyoma DNA with the parent pGEM7 plasmid that does not carry the URE sequence. The amount of radioactivity measured in both of the replicated bands (form I and form II) was determined by a radioimaging blot analyzer (AMBIS, Figure 3A, II). The ability of the pURE to modulate polyoma replication was observed in both sham and UV-irradiated WOP cells (not shown). That the URE affected the replication of polyoma in non-irradiated WOP cells suggests that the proteins that bind the URE may be part of the permissive factors provided by mouse cells that are induced by UV-irradiation in the semi-permissive rat fibroblast cells. We have previously demonstrated that proteins that bind the URE are present in mouse cells prior to UV-irradiation, however, these proteins demonstrate enhanced binding capacity following UV-exposure (21).

To provide additional support for the role of the URE in polyoma replication, we have employed a mutant form of polyoma DNA from which a 17 base pair region that includes the 8 bp URE was deleted (*dl*1031 mutant, 24). When originally characterized, the dl1031 mutant was found to exhibit substantially lower replication and transformation capacities (24). Interestingly, however, this mutant was unable to replicate in WOP cells (Figure 3B). The *dl*1031 mutant is cloned into the pBR322 plasmid at the EcoR1 site whereas our wild type polyoma DNA is cloned at the BamH1 restriction site. Digesting polyoma at the EcoR1 site for cloning interrupts the polyoma large T coding region. Thus, it is critical that large T be provided by an external source for such a plasmid to replicate. By performing these experiments at the permissive temperature for large T expression in WOP cells, this requirement is met. A similar polyoma construct, the B1 mutant (30, mutated at the AP1 target site on the polyoma enhancer), also cloned at the EcoR1 site, was capable of replicating in WOP cells maintained at 33°C (data not shown). This indicates that large T is expressed by these cells and that the inability of the *dl*1031 mutant to replicate in WOP cells is not due to differences in cloning site, but may be attributed to the lack of the URE sequence. We cannot exclude the possibility that the inability of the dl1031 mutant to replicate can be attributed to the fact that the URE sequences also provides a low affinity binding site for polyoma large T and may therefore interfere with large T binding.

DISCUSSION

In this study, we have demonstrated a functional role of the URE sequence in the transcription and replication of polyoma DNA. Furthermore, the specificity of the proteins that bind the URE was demonstrated through EMSA in which neither the CRE nor AP1 sequences were able to compete for the binding of proteins to the ^{32}P-labeled URE to the degree obtained with excess URE sequence. However, the finding that the AP1 sequence, at the highest concentration, inhibited 2 of the 4 complexes formed with

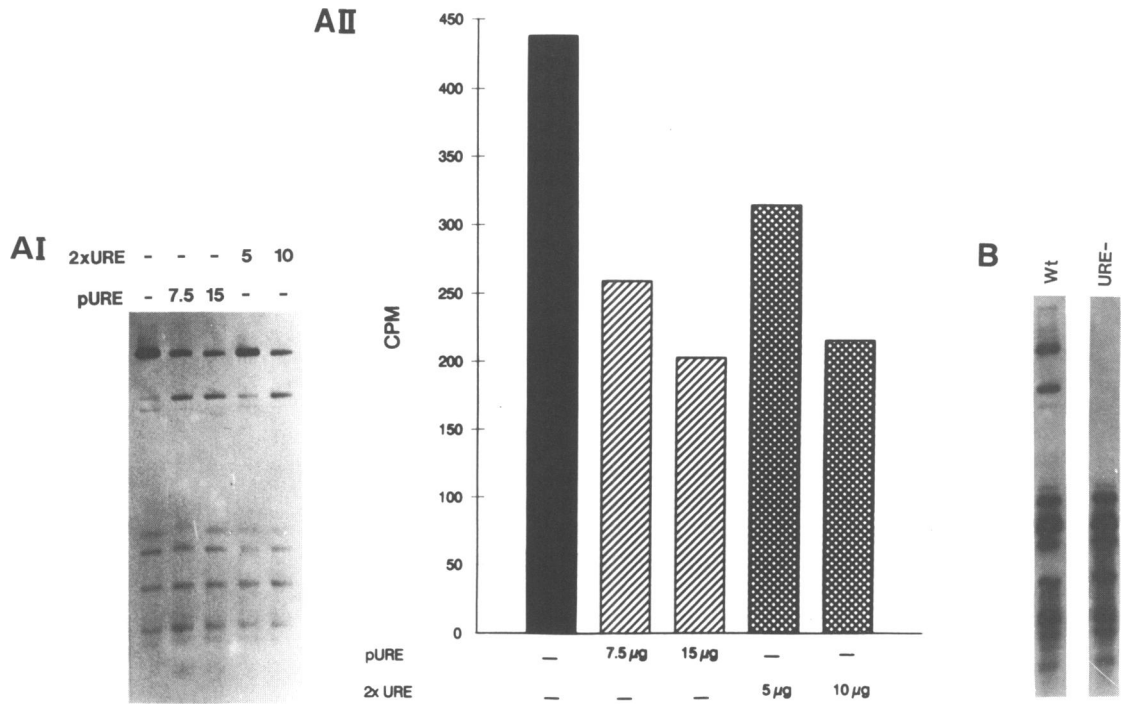

Figure 3. The role of the URE in the replication of polyoma DNA. **(A) I.** WOP cells were transfected through electroporation with 1 µg of a plasmid construct containing the polyoma genome co-transfected with 15 µg of the parent pGEM7 plasmid and with 7.5 µg of pURE together with 7.5 µg of pGEM7 or with 15 µg of pURE alone as indicated. In addition, 5 or 10 µg of a dimer of the URE sequence was co-transfected with polyoma DNA in the presence of 10 µg of pGEM7 as indicated. In all cases, extrachromosomal DNA was isolated, digested with Dpn I and analyzed through Southern blot hybridization using ^{32}P-labelled polyoma DNA as probe. Two bands are seen representing the random distribution of nicked (upper band) or supercoiled (lower band) replicative forms. The Dpn I digested material at the lower part of the gel provides a control for transfection efficiency. **II.** Quantitation of the blot generated by a radioimaging blot analyzer (Ambis) is shown to the right. **(B)** Polyoma lacking the URE sequence does not replicate. WOP cells were transfected through electroporation with wild type (wt) polyoma DNA and a mutant form of polyoma lacking the URE sequence (URE$^-$; *dl*1031 mutant, 26). The Dpn I digested polyoma DNA that migrates to the lower part of the gel provides a control for transfection efficiency.

the URE suggests that similarities exist between the proteins that participate directly or indirectly in the binding to these sequences. Moreover, the URE-bound proteins may represent as yet unidentified members of the AP1 or ATF families of transcription factors, an hypothesis supported by the finding that c-*fos* has been identified as one of four proteins that bind to the URE.

Decreased transcriptional activity of the polyoma enhancer was seen upon co-transfection of the pCAT257 vector with excess URE sequences (provided by the pURE), suggesting that the proteins that bind the URE play a positive role in the transcription of polyoma sequences through interactions within the B enhancer element. That the effect of the URE on transcription was observed predominantly in UV treated cells is in accordance with our finding that the URE was protected in DNase I footprinting assay by proteins prepared from rat 6 cells following UV-irradiation (7). A similar approach demonstrated that the URE is also involved in the replication of polyoma DNA as co-transfection of the pURE with polyoma DNA yielded a dose-dependent decrease in replication. In both of these experiments, the effect observed was specific to the URE sequence as inverse amounts of the parent pGEM7 plasmid were co-transfected with the pURE in each transfection mixture. Support for the role of the URE in polyoma replication is derived by the inability of a mutant form of polyoma (*dl*1031 mutant, lacking both the URE and 3 A/T basepairs at the origin, 24) to replicate. The position of the URE, adjacent to a tract of 8 AT-rich base pairs that defines the polyoma origin of replication (15), may indicate a possible function of this sequence in replication through interactions with cellular proteins that initiate the replication of this virus. The URE is present within low-affinity binding sites of polyoma large T antigen as well (16), and the protein that binds this sequence may play a role in complexing with, or guiding large T to, the appropriate binding site. Indeed, the activities of polyoma large T-antigen appear to be mediated by cooperation with several cellular protein factors (31), one of which may bind to the URE. At this point we can not rule out the possibility that the decrease in polyoma replication seen with co-transfection of the pURE may relate to the proximity of this sequence to the large T antigen binding site. Recently, Ustav et al. (32) have identified a minimal origin region required for the papilloma virus E1 and E2 protein-dependent replication of bovine papilloma virus (BPV) that contains a sequence similar to the URE (TGTTCAACA). This sequence is highly conserved among papilloma viruses (32) further suggesting its importance in the replication process.

Taken together, these findings suggest that the URE-bound protein(s) may be required both for transcription and replication of polyoma sequences, similar to the dual activities defined for the API and CTF transcription factors in this and other viral systems (30, 33–35). Alternatively, the dual role of the URE in transcription and replication may be due to interactions of this sequence with different regulatory proteins required for transcription and replication. In characterizing proteins that bind the URE with antibodies prepared against affinity purified URE-bound proteins, we have detected a 40 KDa protein factor that

is induced by UV-irradiation as well as by other stress-inducing agents. The expression of this factor is greatest during the S phase of the cell cycle (21), supporting the possible role of the URE in the replication of DNA sequences. As DNA tumor viruses provide a model system of events occurring within the cell, it is likely that the URE may affect the response of cellular genes to certain types of damage through the changes in the expression or activity of the URE bound proteins. The expression of various cellular genes that carry the URE on their 5′ regulatory region has been shown to modulate following UV-irradiation (2, 19, 20). Studies are currently in progress to determine the role of the URE in the response of these genes to DNA damage.

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