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Received 13 March 2003; accepted 7 May 2003.

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Aberrant cryptic responsiveness of the pCAT[®]3- and pGL3-promoter reporter vectors

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BioTechniques 35:332-339 (August 2003)

Transfection analyses are an informative method to assess the activity of specific promoter or enhancer elements in mammalian cells. Commercially available reporter vectors can be extremely useful investigative tools for such studies. This study reports that the pCAT[®]3- and pGL3-promoter vectors display cryptic responsiveness to androgens when they contain a DNA insert, while the empty vector, a commonly used negative control, is nonresponsive. Our studies initially aimed to characterize novel androgen-responsive DNA sequences in human genomic DNA through transactivational analyses. An isolated DNA fragment, designated ARC-3, contained three putative androgen response element "half-sites" and was androgen-responsive when cloned into the pCAT3-promoter vector. While we originally believed this to be a novel enhancer element, subsequent analyses of this clone revealed that this vector displays cryptic activity in the presence of an androgen. This was confirmed by cloning several unrelated DNA fragments that did not contain any known classic response elements into the pCAT3-promoter vector; all of which were found to be responsive. The empty vector (negative control) was again nonresponsive. The ARC-3 DNA fragment was also weakly responsive to stimulation when cloned into the pGL3-promoter vector; which is identical to the pCAT3-promoter vector; with the exception of an intron located 5' of the chloramphenicol acetyltransferase gene, and the reporter genes. This work demonstrates that both the pCAT3- and pGL3-promoter vectors are inappropriate to assess androgen-responsive enhancers and emphasizes the importance of the careful selection of reporter vectors and controls when conducting transactivational analysis.

INTRODUCTION

Transfection analyses in mammalian cells are a standard method to assess the transactivational abilities of gene promoters and enhancer elements. There are several commercially available reporter vectors for analyzing the activity of DNA fragments. Such activity is measured through the expression of downstream reporter genes such as chloramphenicol acetyltransferase (*cat*) or luciferase (*luc*). Our studies are focused on the analysis of promoters and enhancers regulated by androgens. Transcriptional regulation mediated by the androgen receptor (AR) requires the binding of the ligand-bound receptor complex to DNA sequences, known as hormone response elements (HREs) or androgen response elements (AREs), which are present in or near the promoter regions of responsive

genes. Consensus AREs, through which the AR can modulate gene transcription, consist of two inverted non-identical hexamers that are separated by a three-nucleotide spacer (1,2). Nonconsensus binding sites may consist of different arrangements of these hexamers or "half-sites." We aimed to identify novel AREs using the technique of whole-genome PCR, a method by which genomic DNA sequences are isolated through the binding of a transcription factor (3), in our case, the AR. One of the isolated clones, which was highly responsive to androgens in initial transactivation analysis, was designated ARC-3. Further analysis demonstrated the presence of three putative consensus ARE half-sites (4,5) that we initially believed were responsible for the androgen-responsiveness of ARC-3. The original aim of this investigation was to identify which regions of ARC-

3 were important for the induction of downstream gene expression in the presence of androgens. Transactivation studies were conducted using the pCAT[®]3-promoter vector (GenBank[®] accession no. U57027), and the pGL3-promoter vector (GenBank accession no. U47298) (both from Promega, Madison, WI, USA), which were designed for the analysis of enhancer elements through the expression of the downstream *cat* or *luc* reporter genes, respectively (6). These data revealed that the pCAT3- and pGL3-promoter vectors display aberrant cryptic activity in response to androgens when a range of unrelated DNA fragments are inserted upstream of the promoter-*cat* or -*luc* transcriptional unit. This finding emphasizes the critical importance of multiple and appropriate controls in transactivation studies and a thorough characterization of the reporter system chosen for such analyses to avoid the generation of false-positive data. This study describes the experiments that led us to this conclusion.

MATERIALS AND METHODS

Cell Culture

CV-1 cells, African green monkey kidney cells (provided by Dr. David Findlay, St. Vincent's Institute of Medical Research, Victoria, Australia), were cultured in Dulbecco's modified Eagle's medium (DMEM) (MultiCell[™], Trace Biosciences Pty Ltd., Castle Hill, Australia), containing 10% fetal calf serum (FCS) (CSL Ltd., Victoria, Australia), 100 IU/mL penicillin, and 100 µg/mL streptomycin (ICN Biomedicals, Costa Mesa, CA, USA) and were maintained at 37°C and 5% CO₂.

Reporters and Other Expression Constructs

The backbones of the pCAT3- and pGL3-promoter vectors are approximately 95% homologous, with the difference of the distinct reporter genes and a chimeric intron situated downstream of the simian virus 40 (SV40) promoter sequence in the pCAT3-promoter vector. These vectors are designed for the analysis of enhancer ele-

Figure 1. Responsiveness of the ARC-3 DNA fragment to R1881 in the pCAT3-promoter vector. (A) Sequence of the ARC-3 DNA fragment with androgen response elements (AREs) half-sites highlighted and Oct-1 binding site underlined. (B) Representation of the ARC-3 DNA fragment, including the three ARE half-sites, cloned upstream of the chloramphenicol acetyltransferase (*cat*) gene in the pCAT3-promoter vector. Coordinates above the representation refer to the ARC-3 DNA sequence, and coordinates below are for the pCAT3-promoter vector. (C and D) Influence of R1881 stimulation on CV-1 cells co-transfected with the AR expression vector, CMV-AR, and the pCAT3-promoter vector alone or containing ARC-3 or various deletion fragments of ARC-3. Diagrams down the left-hand side of the figure depict the structures of the deletion constructs. (E) Influence of R1881 on ARC-3 when each of the ARE half-sites is mutated either alone or in combination. A diagram demonstrating the mutated AREs (MUT) is shown on the left-hand side of the figure. Each graph is representative of at least three independent experiments. Fold induction of *cat* activity by R1881 over unstimulated levels is represented as the mean ($\bar{x} \pm \text{SD}$) of triplicate samples from one experiment.

ments (6). DNA fragments were subcloned into the pCAT3-promoter vector via blunt-end ligation into the *Sma*I restriction site in the multi-cloning region. The TA Cloning[®] Kit containing the pCR[™] II vector (Invitrogen, Carlsbad, CA, USA) was used for subcloning PCR products. The AR expression plasmid, pCMV-AR (kindly provided by Drs. Frank French, Dennis Lubahn, and Elizabeth Wilson, University of North Carolina, Chapel Hill, NC, USA), contains the full-length human AR cDNA, with expression driven by the cytomegalovirus (CMV) promoter (7). The androgen-responsive vector, ARR₃-TK, contains three copies of the ARE derived from the probasin promoter and drives the expression of *luc* through the thymidine kinase (TK) promoter (kindly provided by R. Matusik, Vanderbilt University, Nashville, TN, USA) (8). Plasmid DNA was isolated using the JETSTAR Maxi Prep

Kit (Genomed, Research Triangle Park, NC, USA), following the manufacturer's protocol, and stored at -20°C.

DNA Insert Details

Putative androgen-responsive sequences. Whole-genome PCR methodology was utilized to isolate putative genomic DNA sequences that bind transcription factors (3), which, in this case, is the AR. Of several DNA fragments isolated, a 155-bp fragment, designated ARC-3, was further characterized. The ARC-3 DNA fragment contained three consensus ARE half-sites (4,5) separated by 34 and 26 bp, respectively (Figure 1A). Subclones of the ARC-3 DNA fragment were derived via annealing of self-complementary oligonucleotides for smaller DNA fragments or via PCR, using standard methodologies. Site-directed mutagenesis (9) was used to mutate the putative ARE half-sites, 5'-

AGAAGA-3' and 5'-TGTACA-3', to the nonfunctional sequence, 5'-CAGC-TG-3', to investigate the significance of each in responsiveness to androgens (5) (Figure 1D). All ARC-3 subclones were verified by sequencing.

Other DNA fragments. "Neg 1" is a 104-bp DNA fragment that was isolated from the pPCR-Script[™] Amp SK(+) plasmid (Stratagene, La Jolla, CA, USA) using the restriction sites for *Kpn*I and *Sac*I (657–759 bp). The "Neg 2" (236 bp) and "Neg 3" (208 bp) DNA fragments were amplified from human genomic DNA by PCR, using standard methodology. The following oligonucleotides pairs were used: Neg 2 sense, 5'-GAGAGTGATGCAGGGAATGCTC-3'/Neg 2 antisense, 5'-GATCCTCTTTTCAGCCTGCTG-3'; and Neg 3 sense, 5'-CAGGCCAAGAAATTTGGA-3'/Neg 3 antisense, 5'-CTCCCTCTTAAAATAAAT-3'. None of the "Neg" DNA fragments contained known an-

drogen-responsive sequences, as determined by standard transcription factor database searches (TFSearch[®], version 1.3, by Yutaka Akiyama; <http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>) (10). All DNA fragments were verified by sequencing.

Transfection, *cat*, and *luc* Assays

CV-1 cells were transfected by the calcium-phosphate precipitation method using the ProFection[®] Mammalian Transfection System (Promega), following the manufacturer's protocol. Briefly, CV-1 cells were seeded at a density of 0.3×10^5 cells/well (12-well plates) 24 h before transfection. The cells were transfected with 2 μ g DNA (1 μ g reporter and 1 μ g AR expression vector) per well. The pPCR-Script Amp SK⁽⁺⁾ plasmid was used in transfections to correct for molar differences between different plasmids. For steroidal stimulation, media were replaced the following day with DMEM (+10% FCS, depleted of steroids by treatment with dextran-coated charcoal) containing either the synthetic androgen, methyltrienolone (R1881) (10^{-9} M), or ethanol (24 h). The cells were harvested in 100 μ L Reporter Lysis Buffer (Promega), and the lysates were maintained at -80°C prior to the assay.

The measurement of the *cat* enzyme in cell lysates was performed using a modification of the standard *cat* assay whereby [¹⁴C]acetyl coenzyme A (acetyl CoA) (Amersham Biosciences Pty Ltd., Castle Hill, Australia) is substituted for [¹⁴C]chloramphenicol (11). The assay mixture contained 60 μ L cell extract, 1.6 mM chloramphenicol, 0.09 mM acetyl CoA (Roche Diagnostics, Sydney, Australia), and 2 μ L [¹⁴C]acetyl CoA (0.1 μ Ci/ μ L) in a final volume of 100 μ L. The reaction was incubated at 37°C (4 h), and then the [¹⁴C]acetyl CoA-labeled derivatives of chloramphenicol were separated from residual [¹⁴C]acetyl CoA through a series of extractions with ethyl acetate. The organic phases were counted on a Wallac 1410 liquid scintillation counter (Perkin Elmer Life Sciences, Gaithersburg, MD, USA). For the determination of *luc* activity, 20 μ L cell lysate were mixed with *luc* reagent

(Promega), and relative light units (RLUs) were measured in a TD-20/20 luminometer (Turner Designs; Promega). Each experiment was per-

formed in triplicate. Data are expressed as the mean ($\bar{x} \pm \text{SD}$) fold induction over unstimulated levels from a single experiment.

Figure 2. Responsiveness of ARC-3 and unrelated DNA fragments to R1881. (A) ARC-3 and DNA fragments Neg 1–3 in the pCAT3-promoter vector, and (B) the effect of different amounts of co-transfected AR expression vector on the transactivation of ARC-3 and the unrelated DNA fragment, Neg 1. Each graph is representative of at least three independent experiments. Fold induction by R1881 over unstimulated levels is represented as the mean ($\bar{x} \pm \text{SD}$) of triplicate samples from one experiment. (C) Sequences of the Neg 1–3 DNA fragments with ARE half-sites are boxed, and ARE-like half-sites are underlined.

RESULTS AND DISCUSSION

The initial objective of this study was to characterize a putative androgen-responsive DNA fragment (ARC-3). The 155-bp DNA fragment contained three putative ARE half-sites, suggesting that it may be a transcriptional target for the activated AR (Figure 1A). Figure 1C reveals the androgen-responsive activity with the full-length ARC-3 DNA fragment and three deletion constructs derived from ARC-3 when each was cloned separately into pCAT3-promoter vector. As indicated, the full-length ARC-3 DNA fragment induced approximately a 10-fold increase in *cat* activity when compared to the empty vector (no insert). Furthermore, each different ARC-3 deletion construct, which still included all three putative ARE half-sites, revealed androgen-responsive activity that was similar to the full-length ARC-3 DNA fragment (Figure 1C). Similar experiments were performed with two other cell lines, the human neuroblastoma cell line, SK-N-SH, and the rat pheochromocytoma cell line,

PC12, confirming the responsiveness of ARC-3 and its subclones (data not shown). Furthermore, the responsiveness observed was AR-dependent, as similar transactivational analyses using co-transfected glucocorticoid or mineralocorticoid receptor and their cognate ligands failed to induce reporter activity (data not shown). The nonspecific steroid-responsive mouse mammary tumor virus (MMTV) promoter was used as a positive control in these experiments. These experiments indicate that the cryptic activity in the pCAT3-promoter vector is androgen-specific. This was confirmed in analyses where the AR expression vector was omitted, and activity in the presence and absence of androgens was investigated. In all cases, no induction of reporter activity was observed (data not shown). We chose to conduct transactivation analyses in the cells that were devoid of endogenous AR expression so that activity in the absence of the expression vector was not expected.

To more thoroughly define the androgen-responsive sequence(s) in ARC-3, a second series of deletion con-

structs were generated using the pCAT3-promoter vector. Specifically, these ARC-3 deletion constructs were designed to identify which, if any, of the ARE half-sites were responsible for the androgen-responsive activity observed in Figure 1C. As indicated in Figure 1D, the deletion constructs contained either one or two ARE half-sites in a range of configurations. All ARC-3 deletion constructs exhibited similar androgen-responsiveness when compared to the full-length ARC-3, with at least a 10-fold induction of reporter gene expression when compared to the empty control vector (no insert). This series of experiments suggested that androgen-responsive activity was not dependent on one specific region of the ARC-3 DNA fragment and was not consistent with previous studies defining other AREs, in which a deletion or mutation of putative binding sites resulted in a reduction of transactivation (12–15). These data also indicated that androgen-responsive activity was possible when only one ARE half-site was

Figure 3. Responsiveness of ARC-3 and the subclone ARC-3a to R1881 in the pGL3-promoter vector. (A) Schematic representation of the ARC-3 DNA fragment, including the three ARE half-sites, cloned upstream of the luciferase gene (*luc*) in the pGL3-promoter vector. (B) Induction of luciferase expression of ARC-3 and ARC-3a (refer to Figure 1C for the structure) in comparison to the empty pGL3-promoter vector (no insert) and the androgen-responsive vector, ARR₃-TK. Each graph is representative of at least three independent experiments. Fold induction of *luc* by R1881 over unstimulated levels is represented as the mean ($\bar{x} \pm \text{SD}$) of triplicate samples from one experiment. SV40, simian virus 40.

present in the construct (ARC-3/d4 and ARC-3/d6). While this was unexpected, the possibility of a single ARE half-site being functional could not be discounted. To examine the specific role of each ARE half-site in androgen-responsiveness, they were mutated either alone or in combination. Once again, all the ARC-3 fragments were responsive to R1881, even when all three of the sites were mutated (Figure 1E). Based on these results, it remained possible that either novel androgen-responsive DNA sequences were present that were yet to be defined or that the responsiveness observed was cryptic activity. Because of the combinations of sequences that were tested, we have excluded the possibility that this androgen effect was sequence-specific. Cryptic activity was the most likely explanation, based on the fact that each of the deletion constructs contains diverse sequences.

To investigate the possibility that the responsiveness of the ARC-3 DNA fragments reflected cryptic activity rather than the presence of specific regulatory sequences, several unrelated DNA fragments (Neg 1–3) were subcloned into the pCAT3-promoter vector and assessed for responsiveness in transfection experiments. None of the DNA fragments contained AR-binding consensus sites, as determined by standard transcription factor database searches (TFSearch; version 1.3) (10). However, the Neg 2 DNA fragment contained a single half-site (5'-TGTTCT-3'), and both the Neg 2 and Neg 3 each contained an ARE-like half-site sequence (5'-CAGAG-3'; only five nucleotides are homologous) (Figure 2C). Note that a single half-site is nonfunctional unless it is in close proximity to another half-site because the ligand-bound dimerized receptor complex is unable to bind. The Neg 1 DNA fragment did not contain any ARE half-sites. As demonstrated in Figure 2A, all of the Neg DNA fragments were responsive to androgen stimulation. These results indicate that androgen-responsiveness is not dependent on the DNA sequence of the insert. Furthermore, while no AREs were identified in the pCAT3-promoter vector, two half-sites were identified, 5'-AGTCCC-3' and 5'-AGAACA-3' at positions 4175 and 4200, respectively, which are located

upstream of the polylinker. Whether these contribute to the responsiveness of the vector remains to be determined.

A recent report showed that overexpression of the AR interfered with transactivation, which creates a squelching or interference effect in the reporter expression (16). To eliminate this possibility, transactivation experiments were performed by altering the amount of AR expression vector that was co-transfected into the cells. Figure 2B reveals that a reduction in the amount of co-transfected AR expression vector (1–0.05 µg) did not consistently or significantly influence reporter transactivation. Thus, the potential squelching or interference effect from excess AR did not play a role in modulating the transactivation activity in the presence of R1881.

To confirm that the ARC-3 DNA fragment did not contain any androgen-responsive sequences, ARC-3 was subcloned into the pGL3-promoter vector. This vector differs from the pCAT3-promoter vector only in the reporter gene used, *luc*, and the presence of a chimeric intron situated downstream of the SV40 promoter sequence in the pCAT3-promoter vector. It is of importance to note that, similar to the pCAT3-promoter vector, the pGL3-promoter vector contains two ARE half-sites proximal to the polylinker. While this experiment was primarily conducted to confirm that the androgen-responsiveness of ARC-3 in the pCAT3-promoter vector was cryptic, given the high degree of homology between the pCAT3- and pGL3-promoter vectors, we wanted to establish whether the pGL3-promoter vector was also responsive to androgens. Figure 3 demonstrates that the empty vector had a 2-fold induction of *luc* expression in response to androgens, the pGL3-ARC-3 vector had a nonsignificant 2- to 3-fold induction, while the positive control, ARR₃-TK, induced *luc* expression 250-fold. While the same degree of responsiveness of the pGL3-promoter vector was not observed when compared to the pCAT3-promoter vector, it is apparent that the pGL3-promoter vector is also unsuitable for examining androgen-dependent transactivation, especially given the intrinsic responsiveness of the empty vector to androgen treatment.

Transcription factor binding site

database searches (TFSearch) were conducted to examine the presence of other known AR coactivators in the DNA fragments examined. These searches revealed the presence of a consensus octamer motif for the POU-domain protein Oct-1 within the ARC-3 DNA fragment, positioned between the first and second ARE half-sites (Figure 1A). This was considered to be potentially significant because the Oct-1 transcription factor has been demonstrated to play a synergistic role with the AR in the nonspecific MMTV promoter (17). However, results from our mapping analyses indicate that the deletion of this consensus binding site (in constructs ARC-3/d3 and ARC-3/d6) does not alter transactivational ability in the presence of androgens (Figure 1D). We did not identify any other sites that may have contributed to responsiveness. The diversity of the sequences that responded to androgen stimulation would tend to indicate that there aren't any other transcription factor binding sites within the ARC-3 DNA fragment that interact with the activated AR to enhance reporter expression. Similarly, we did not identify any other potential transcription factor binding sites in the Neg 1–3 DNA sequences (Figure 2C).

The observation of cryptic responsiveness in reporter vectors is not without precedent, particularly for the pGL3 *luc* reporter vector (18–20). The detailed study by Bert and colleagues (18) provided particularly useful insights into the likely mechanisms underlying the cryptic activity of the pGL3 vectors. According to this study, short DNA fragments were able to induce significant cryptic responsiveness when inserted into pGL3. Furthermore, this aberrant activity could be eliminated by the inclusion of an efficient SV40 poly(A) signal upstream of the multiple cloning site (MCS). This poly(A) signal was more efficient at blocking read-through transcription than the synthetic poly(A) signal in the pGL3 vector (18). It therefore seems most likely that aberrant activities seen with both the pGL3 and its *cat* equivalent, pCAT3, arise from read-through transcription due to an inefficient poly(A) signal.

The MCS in the pCAT3- and pGL3-promoter vectors each contain TAA or TAG translation stop codons in all three

reading frames. This would presumably terminate any read-through translation that initiates in the vector to create a fusion protein containing *luc* or *cat*. This may account for the low activity of the basic vector. Furthermore, the insertion of DNA fragments could put at least one of these out-of-frame, thereby enabling translation to proceed through this region to produce functional *luc* or *cat* protein. While beyond the scope of this study, it would be of interest to employ primer extension to establish the underlying mechanism.

It is important to note that while true strong promoters and enhancers have much higher activities than the cryptic pCAT3 and pGL3 elements, weak activities such as those observed in this study are often described by researchers scanning the genome for promoter elements.

In summary, these results indicate that the pCAT3- and pGL3-promoter vectors are not appropriate for the study of androgen-responsive sequences. It is evident that the use of another unrelated fragment as a control was advantageous to verify nonspecific activity induced through the reporter vector. These results caution against the use of empty vectors as negative controls in transactivation analysis, despite that it has been used as the sole negative control in multiple studies (12,21–24). This work emphasizes the care with which researchers need to approach such studies and that careful consideration should be given to the appropriate controls used to examine *cis*-acting sequences that are believed to play a regulatory role in downstream gene expression.

ACKNOWLEDGMENTS

This work was supported by funds from the National Health & Medical Research Council of Australia and the Royal Children's Hospital Research Foundation. G.G. was supported by a Ph.D. scholarship from the University of Melbourne. Support from the Centre of Hormone Research is gratefully acknowledged.

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Received 31 March 2003; accepted 15 May 2003.

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