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The promoter competition assay (PCA): a new approach to identify motifs involved in the transcriptional activity of reporter genes

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1. ABSTRACT

Identifying particular motifs responsible for promoter activity is a crucial step toward the development of new gene-based preventive and therapeutic strategies. However, to date, experimental methods to study promoter activity remain limited. We present in this report a promoter competition assay designed to identify, within a given promoter region, motifs critical for its activity. This assay consists in co-transfecting the promoter to be analyzed and double-stranded oligonucleotides which will compete for the binding of transcription factors. Using the recently characterized SBEM promoter as model, we first delineated the feasibility of the method and optimized the experimental conditions. We then identified, within an 87-bp region responsible for a strong expression of the reporter gene, an octamer-binding site essential for its transcriptional regulation. The importance of this motif has been confirmed by site-directed mutagenesis. The promoter competition assay appears to be a fast and efficient approach to identify, within a given promoter sequence, sites critical for its activity.

2. INTRODUCTION

Gene regulation, primarily achieved at the level of gene transcription, represents an attractive target for therapeutic drugs. Indeed, such drugs can act either by stimulating the transcription of specific genes required for a desired beneficial effect or by inhibiting the transcription of genes involved in an undesirable event. It is therefore not surprising that more than 10% of the 50 FDA-approved best selling drugs, including salicylate or tamoxifen (1,2), are known to target gene transcription. Increasing further our knowledge of transcriptional gene regulation is essential to develop novel drug targets. Identifying particular motifs responsible for promoter activity is a crucial step toward the development of new preventive and therapeutic strategies.

Currently, the experimental method of choice to study promoter activity is the transfection of the 5´-flanking promoter sequence driving a reporter gene. Importance of particular regions and motifs are further established using deleted constructs containing different lengths of the

promoter region, and by site-directed mutagenesis (3,4). To a lesser extend, knock down and co-transfection of specific transcription factors are also used to test their ability to modify gene activation (5,6). Such techniques have however several limitations. Indeed, mutagenesis necessitates additional handworks (new transformation, DNA preps and sequencing), which are rather costly and time consuming, whereas knock down of transcription factor genes require new constructs and specific skills.

In 2002, Sun *et al.* have described a novel method, the promoter competition assay designed to investigate the role of cis-acting DNA regulatory elements *in vitro* (7). The assay was based on the passive transfer into cells of short double-stranded DNA fragments, which corresponded to a known transcription factor binding site. These DNA fragments were expected to compete for the binding of the endogenous transcription factor, thus resulting in an altered expression of the endogenous target genes. This assay was successfully used to assess the role of NF-kappa-B transcription factor in the activation of matrix metalloproteinase genes. The transfer of a NF-kappa-B competitor motif indeed resulted in the down-regulation of the endogenous MMP-1 and MMP-13 (7).

Herein, we described a competition assay (PCA) which we have modified to identify, within a given promoter region, motifs critical for its activity.

3. MATERIALS AND METHODS

3.1. Plasmids

P1 and P2 plasmids were constructed by subcloning the small breast epithelial mucin (SBEM) promoter fragments (-357/-51 and -270/-51 from the ATG, respectively) in the promoterless, enhancerless expression vector pGL3-Basic (Promega, Madison, WI, USA) as previously described (8); Hube *et al.*, submitted 2005). P3 corresponds to the P1 plasmid except that the octamer-binding site (-282/-274, AGCATATTT) has been mutated (TCTAATGTA) using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Sequences of all promoter constructs were confirmed by dideoxynucleotide chain-termination sequencing (DNA Sequencing and Genetic Analysis Laboratory, University of Calgary, Canada).

3.2. Cell Culture and transfections

BT-20 cells were cultured in complete media (DMEM, 5% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 15 mM sodium bicarbonate and 2 mM glucose, all from Life Technologies, Inc., Burlington, ON, Canada). Confluent cells were detached by 0.05% trypsin-0.02% EDTA (Life Technologies), seeded in 24-well plates (Corning Inc., NY, USA) and cultured in complete media until use.

SBEM/luciferase reporter constructs were transiently transfected in BT-20 cells cultured in a 24-well plate, using LipofectAMINE Plus® Reagent (Life Technologies) and following the manufacturer'sinstructions. Briefly, 1.33 nM of appropriate plasmid was combine with 8 micrograms of LipofectAMINE and 4 microliters of Plus® Reagent in a final volume of 200 microliters of complete media without FBS. The renilla luciferase reporter vector (0.11 nM) was co-transfected each time to normalize transfection efficiency. A positive control containing CMV promoter sequence (pGL3-Control) and a negative control (pGL3-Basic) were used for each experiment. Firefly luciferase and renilla luciferase activities were measured 24h after transfection using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and the Lmax Luminometer (Molecular Devices, Sunnyvale, CA, USA). Luciferase activities were acquired as RLU (relative luciferase unit), corrected by renilla luciferase activity, adjusted to pGL3-Control activity, and expressed in fold of pGL3-Basic activity +/- standard error of the mean (SEM).

3.3. Identification of transcription factors binding sites and patterns in the SBEM promoter

In order to identify motifs potentially important for the SBEM promoter activity, database search was performed using the 87-bp ENH region as template. Identification of transcription factor binding sites was done with TESS 1.2.2 and MatInspector v2.2 programs based on TransFac database (8-10), Oligorep was used to detect repeats, and purine-rich motifs were identified using a personal algorithm.

The different softwares and databases used are available at the following addresses: TESS, www.cbil.upenn.edu/tess; MatInspector, www.genomatix.de; TransFac, www.gene-regulation.com; Oligorep, wwwmgs.bionet.nsc.ru/mgs.

3.4. Promoter competition assay (PCA)

Promoter competition assay (PCA) was performed under the conditions stated above and as previously published (7), with some modifications. Briefly, double-stranded competitors consisting either in PCR products or in double-stranded oligonucleotides were co-transfected in BT-20 cells with the human SBEM/luciferase P1 construct.

In a first series of experiments, the competitor (ENH) consisted of the 106-bp PCR fragment (-363/-257) encompassing the full length 87-bp enhancer region (-357/-270) present in the SBEM promoter (Hube *et al.*, submitted 2005). An exogenous PCR fragment (amplification of the GAPDH cDNA region from +868 to +1045) was used as negative control (EXO).

In a second series of experiments, double-stranded oligonucleotides were used as competitors (Table 1). Equal amount of complementary single-stranded oligonucleotides (purchased from Life Technology), were mixed in annealing buffer (100 mM NaCl, 50 mM HEPES pH 7.4), incubated at 90°C for 10 min, and slowly cooled to room temperature (70°C for 10 min, 55°C for 10 min, then 37°C for 10 min).

Overall, 6 different competitors were used (Table 1). The WT (wild-type) oligonucleotide competitor consisted of the native promoter region from -319 to -267.

Table 1. Oligonucleotides used in this study

Name	Sequence (5'-3')
WT	TACATGTAAGAGGATGCCTGGAAGAGAAGTTGCCTGGAGCATATTTAACATGA ATGTACATTCTCCTACGGACCTTCTCTTCAACGGACCTCGTATAAATTGTACT PR1 PR2 Oct DR DR
PR1	-------AT-CAT---------- -------TA-GTA----------
PR2	----------T-CATGC---------- ----------A-GTACG----------
DR	----------ATGCAT----------G-TG--C---------- ----------TACGTA----------C-AC--G----------
OM	----------TCT-ATG-A------- ----------TCG-ATA-A-------
NC	AGACGA--GT-C-TA-TAC-T-GT-CGTAG-A-G-A-TA-G--G-ACGATA-- TCTGCT--CA-G-AT-ATG-A-CA-GCATC-T-C-T-AT-C--C-TGCTAT--

'-' indicates nucleotides that are similar to the wild-type sequence. WT, wild-type SBEM promoter sequence from -319 to –267 upstream of the ATG; PR1, wild-type sequence mutated in the first purine-rich motif; PR2, wild-type sequence mutated in the second purine-rich motif; DR, wild-type sequence mutated in the direct repeat motif; OM, wild-type sequence mutated in the octamer-binding motif; NC, negative control with an unrelated SBEM nucleotide sequence.

PR1 and PR2 (purine-rich 1 and 2) competitors corresponded to the WT sequence without the first (PR1) or without the second (PR2) purine-rich motifs identified in -312/-307 and -298/-291, respectively (i.e. AAGAGGA and AAGAGAAG were replaced by ATGCATG and ATGCATGC). The DR oligonucleotide consisted of the WT sequence in which the direct repeat (-311/-304 and -295/288) was changed from TGCCTGG-N_9-TGCCTGG to ATGCATG-N_9-GGTGTGC. The octamer-binding site (-282/-274, AGCATATTT) was replaced in OM oligonucleotide by TCTAATGTA. An exogenous oligonucleotide (NC), with identical nucleotide composition but randomly determined, was used as negative control. The size (53 bp) was the same for each double-stranded oligonucleotide competitor. In addition, oligonucleotides were carefully designed to avoid the creation of any new transcription factor binding sites (as assessed by TESS and MatInspector programs), any new repeats (as evaluated by Oligorep), and any new purine-rich motifs (using a personal algorithm).

PCA was performed in the presence of 1.33 nM of P1 vector and molar excess of competitors (ENH and EXO, 1x, 10x, 25x, and 100x molar excess; WT, NC, PR1, PR2, DR, and OM, 100x molar excess).

4. RESULTS

4.1. Principle of the Promoter Competition Assay (PCA)

The assay presented here relies on the principle that if particular motifs are needed for the activity of a reporter gene, the co-transfection of double-stranded short DNA fragments corresponding to these motifs will modify the promoter activity measured. The promoter competition assay (PCA) therefore consists in co-transfecting the promoter construct (reporter gene driven by the promoter region to test), together with a molar excess of a double-stranded oligonucleotide competitor corresponding to all or a part of the promoter sequence. The principle of the PCA is illustrated Figure 1. In the given example, the binding of 3 transcription factors controls the activity of the studied promoter. In basal conditions (Figure 1A), cells are transfected with the promoter construct to be studied and a given amount of control unrelated double-stranded oligonucleotide (NC, negative control). Indeed, it is critical to consistently transfect cells with the same molar amounts of DNA (plasmid + oligonucleotide) in order to control for any non-specific effect due to DNA rather than the sequence used. In these standard conditions, positive and negative factors controlling the promoter activity will bind to their corresponding DNA motifs in the promoter region and lead to the reference luciferase activity. In contrast, when the promoter construct is co-transfected with an excess of a competitor corresponding to a native promoter sequence (WT), regulating factors will preferentially bind to the competitor and the luciferase activity measured will be decreased (Figure 1B).

Double-stranded competitors can be easily mutated, experimentally by PCR or during their design if oligonucleotides are ordered from a supplier. If mutations are performed in motifs needed for the binding of factors controlling the activity of the promoter, the effect of the competitor will be modified. Three possible situations could be observed. In the first one, the positive factor 1, unable to bind on its mutated site within the competitor (Comp 1), will be available to bind on the promoter (Figure 1C). The resulting luciferase activity will be increased as compared to the one seen with a full competitor. In contrast, if the negative regulator 2 cannot bind to the mutated competitor (Comp 2), its binding on the promoter will further down-regulate the activity of the reporter gene (Figure 1D). If the mutation does not affect the binding of a factor participating to the regulation of the promoter (Comp X, Figure 1D), the efficiency of the competitor will remain unchanged.

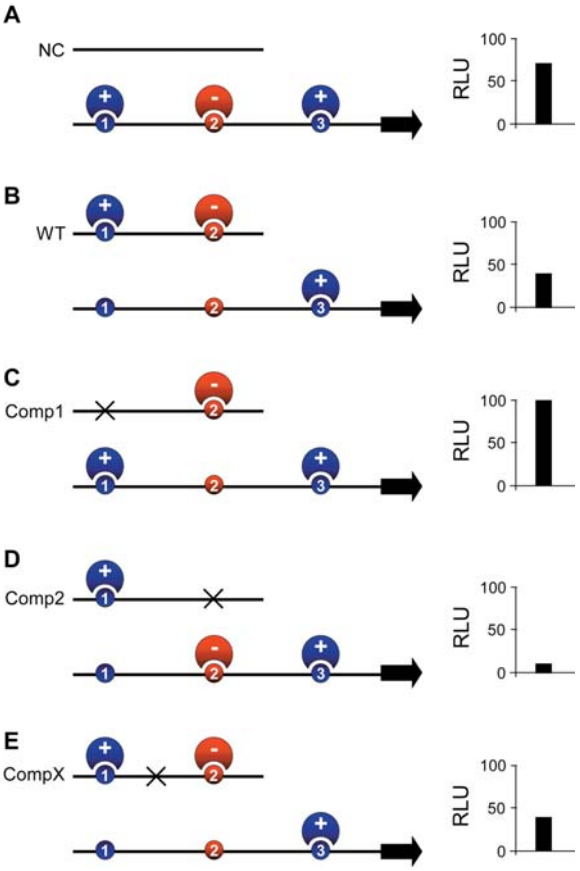

Figure 1. Principle of the promoter competition assay (PCA). PCA consists in co-transfecting the promoter construct (reporter gene driven by the promoter region to test), together with a molar excess of a double-stranded oligonucleotide competitor corresponding to all or a part of the promoter sequence. Blue spheres represent positive transcription factors and red correspond to negative factors. Small spheres in the schematic promoter and competitor sequences (black lines) represent intact DNA binding motifs and a cross indicates mutation in the competitor sequence. RLU, relative luciferase unit.

4.2. Feasibility of the promoter competition assay in our model

PCA was tested to identify critical motif(s) within a region of 87 bp found in the SBEM promoter and recently described to be sufficient for driving a strong luciferase activity in breast cancer cell lines (Hube *et al.*, submitted 2005). Two promoter fragments fused to the coding region of the luciferase gene (Figure 2A) were constructed as described in the "Material and Methods" section, transfected into the BT-20 cells, and the resulting luciferase activities were measured. As seen in Figure 2B, the promoter construct P1, containing region from -357 to -51 upstream of the ATG, led to a strong luciferase activity of 95±16 fold over the empty vector (the baseline control), whilst the removal of this region drastically reduced the activity of P2 to 36±4 fold over the empty vector. This suggested the presence within this region of important regulatory motifs.

We first determined the amount of competitor to be co-transfected with P1 to observe an efficient promoter competition. Competitors corresponding to double-stranded DNA (PCR product), either similar to the whole 87-bp region (ENH competitor, Figure 3A) or unrelated to the SBEM promoter sequence (negative control, GAPDH PCR product NC), were rapidly generated by PCR, as described in the Material and Methods section. Increasing amounts of ENH competitors were then co-transfected with the P1 promoter construct (Figure 3B). Co-transfection with 100x molar excess of the negative control (NC competitor) did not significantly modify the P1 promoter activity (104±12 fold compared to 95±16 fold without the NC oligonucleotide). In contrast, increasing amount of ENH competitor gradually inhibited the P1 luciferase activity to reach 30±1 fold at 100x molar excess of ENH oligonucleotide competitor (Figure 3B). The reduction in the promoter activity was about 70% compared with those observed with P1 alone or P1 co-transfected with NC only. As one hundred molar excess of competitor provided the strongest promoter competition, this concentration was selected for subsequent experiments.

4.3. Computational identification of putative important motifs in the 87-bp region

Using several online softwares (see Material and Methods), different motifs potentially important for the SBEM promoter activity were identified within the 87-bp region. One octamer-binding transcription factor motif was identified in -282/-274 (grey box in Figure 4A, AGCATATTT), 2 purine-rich motifs were located in -312/-306 and -300/-291 (white boxes in Figure 4A, PR1, AAGAGGA; PR2, GGAAGAGAAG) and a direct repeat was found in -305/-298 and -289/-282 (black boxes in Figure 4A, DR, TGCCTGG-N_9-TGCCTGG).

4.4. Identification of the octamer-binding site as a critical motif in the SBEM promoter

In order to establish whether any of these motifs were involved in the strong SBEM promoter activity, small oligonucleotide competitors (53 bp) were used to perform promoter competition assay. As described in the Material and Methods section and Table 1, oligonucleotide competitors were designed (Figure 4A) to fully overlap the SBEM promoter region from -319 to -267 upstream of the translation start site. WT competitor corresponded to the native SBEM promoter sequence from -319 to –267. PR1 and PR2 competitors were overlapping the same region but contained mutations in the purine-rich motifs, whereas DR and OM were mutated in the direct repeat and the octamer-binding sites, respectively. In addition, a competitor (NC) corresponding to an oligonucleotide unrelated to the SBEM promoter sequence (but with identical nucleotide composition randomly distributed) was used as a negative control. As shown in Figure 4B, when co-transfected with P1 promoter construct, the control competitor did not significantly modify the promoter activity (86±7 fold compared to 95±16 fold without oligonucleotide competitor). In contrast, co-transfection with the WT competitor led to a reduction of 80% of the P1 activity. A similar inhibition was observed when P1 was co-transfected either with PR1, PR2 or DR competitors. This

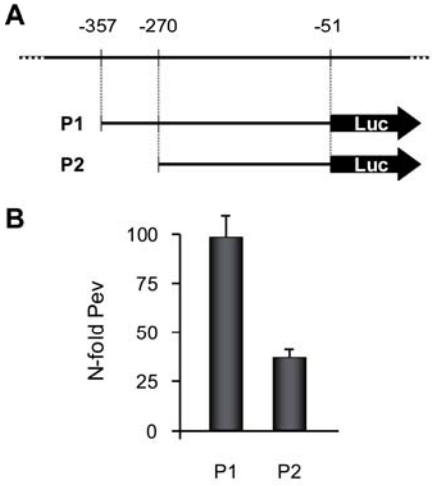

Figure 2. Activity of P1 and P2 constructs used in luciferase assay. (A) P1 contains the SBEM promoter region from -357/-51 from the translation start site; P2 contains the region from –270/-51. (B) P1 and P2 constructs were transiently transfected in BT-20 cells, and luciferase was measured 24h post-transfection as described in the Material and Methods. Luciferase activity is shown as n-fold value compared to cells transfected by the promoterless pGL3-basic vector. Data represent the means of at least three independent transfection experiments.

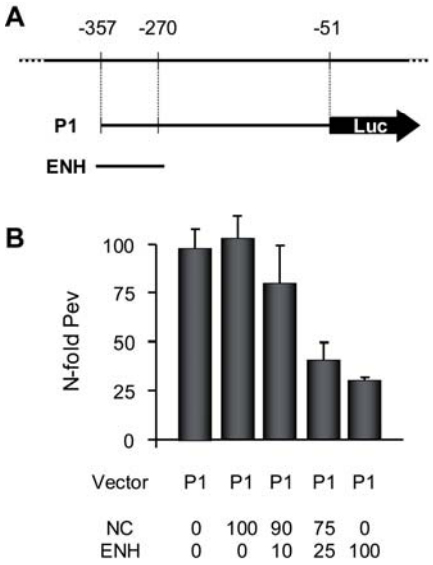

Figure 3. Promoter competition assay using PCR products as competitors. (A) P1 contains the SBEM promoter region from -357/-51 from the translation start site; ENH, 106-bp PCR product corresponding to the SBEM promoter region from –363 to –257 upstream of the ATG. (B) P1 plasmid and competitors were co-transfected in BT-20 cells, using increasing amount of ENH competitors. Twenty-four hours post-transfection, cells were lysed and luciferase activity was measured, as described in Material and Methods. Luciferase activity is shown as n-fold value compared to cells transfected by the promoterless pGL3-basic vector. Data represent the means of at least three independent transfection experiments.

suggests that the presence of these mutated motifs does not alter the competition observed. In other words, factors needed for the promoter activity are still captured by the mutated double-stranded oligonucleotides. In contrast, when the competitor was mutated within the octamer-binding site (OM), its inhibitory effect on the promoter was abolished (Figure 4B). It appears that by suppressing the binding site for Oct factors on the competitor, we restored the possibility for these corresponding factors to participate to the activity of promoter. Similar results were obtained when using two other cancer cell lines (data not shown), demonstrating that the results of this promoter competition assay are not cell line-dependent. Altogether, our data suggested that the octamer-binding transcription factor motif was crucial for the strong activity of the SBEM promoter.

4.5. Confirmation that the octamer-binding site is an important motif in the SBEM promoter

To check whether the octamer-binding transcription factor motif identified using the promoter competition assay was indeed critical for the promoter activity, this motif was mutated by site-directed mutagenesis as described in the Material and Methods section. P1 (-357/-51, Figure 5A) and P3 (corresponding to the mutated P1 promoter construct, Figure 5A) were then transfected in BT-20 cells and the luciferase activity measured as described above. As shown in Figure 5B, the P1 plasmid resulted in a luciferase activity of 95±16 fold whereas mutation of the octamer-binding site in P3 totally abolished the luciferase activity.

5. DISCUSSION

In this report, we present a modified promoter competition assay, based on the principle elaborated by Sun *et al.* in 2002 (7), and designed to identify, within a given promoter region, motifs needed for the reporter gene activity.

The promoter competition assay is based on the hypothesis that competitors, corresponding to short double-stranded oligonucleotides containing specific DNA motif, will penetrate into the cells and trap the corresponding transcription factors. Whether the competitors indeed enter the cells, and are recognized *in vivo* by transcription factors remain to be demonstrated. Introducing short DNA fragment into cells has been successfully done in the past, mainly for transferring DNA fragments acting as antisense oligonucleotides (11,12). We are therefore confident that the competition effect observed results from an intracellular effect of the competitors. Similarly, it is well established that transcription factors have the ability to bind *in vitro* short double-stranded DNA fragments, as shown in other type of experiments, such as EMSA or footprinting (13,14). More recently, Moxley *et al.* have even used an 'oligonucleotide trapping system' to purify transcription factors (15,16). In these experiments, short double-stranded DNA fragments were incubated with crude protein extracts, allowing the formation of a DNA-protein complex. These complexes were then purified on columns by DNA-affinity chromatography (16), confirming the aptitude of

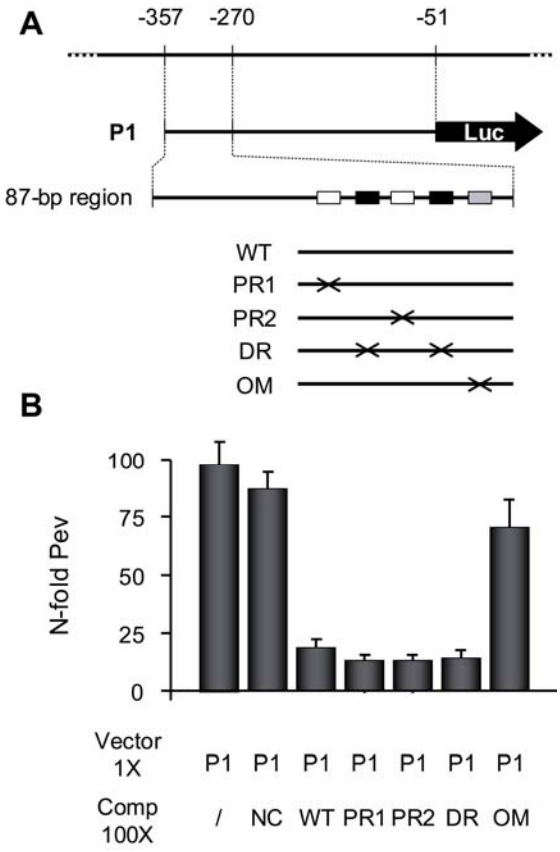

Figure 4. Promoter competition assay using double-stranded oligonucleotide competitors. (A) P1 contains the SBEM promoter region from -357/-51 from the translation start site. In the 87-bp region, 1 octamer-binding transcription factor motif was identified in -282/-274 (grey box, AGCATATTT), 2 purine-rich motifs were located in -312/-306 and -300/-291 (white boxes, PR1, AAGAGGA; PR2, GGAAGAGAAG) and a direct repeat was found in -305/-298 and -289/-282 (black boxes, DR, TGCCTGG-N_9-TGCCTGG). WT, wild-type oligonucleotide competitor consisting of the native promoter region form -319 to -267; PR1 and PR2, WT sequence mutated for the first (PR1) and the second (PR2) purine-rich motifs, respectively; DR, WT sequence mutated for the direct repeat; OM, WT sequence mutated for octamer-binding site. (B) P1 and competitors were co-transfected in BT-20 cells, using 100x molar excess of double-stranded oligonucleotide competitors. Twenty-four hours post-transfection, cells were lysed and luciferase activity measured as described in Material and Methods. Luciferase activity is shown as n-fold value compared to cells transfected by the promoterless pGL3-basic vector. Data represent the means of at least three independent transfection experiments.

transcription factors to bind to short oligonucleotides in these experimental conditions. The experimental observation that indeed the co-transfection of double-stranded oligonucleotides suppresses the promoter activity, as opposed to randomly designed or mutated sequences, corroborates the model presented Figure 1. The exact mechanism of action of such competitors *in vivo* is however still unknown.

Although the principle of the promoter competition assay presented here (trapping of transcription factors using short oligonucleotide competitors) relies on a previously published method (7), the protocol itself and the potential information gathered using both techniques are different. Herein, we co-transfected the competitor oligonucleotides with the reporter gene construct using cationic lipids whereas Sun *et al.* passively transferred their competitors. Moreover, in the approach presented here the competitor modulate the activity of an exogenous promoter as opposed to the activity of an endogenous promoter. We choose to actively co-transfect because our oligonucleotides were larger (53 bp) than the ones used by Sun *et al.* (18 bp). Indeed, transfection using liposomes is much more efficient than passive DNA fragment transfer (17,18), and was recently used successfully to investigate the role of Sp1 transcription factor in the PTH promoter activity (19). Another important difference lies in the fact that our competitor corresponded to the exact sequence of the promoter region to test, whilst a consensus sequence for transcription factor binding site was used in the original method (7). Such specificity in the competitor sequence also likely participates to an efficient competition with only 0.133 microM of competitors (100x molar excess) as opposed to 5 microM (7).

Our system was designed to assess the importance of several motifs within one exogenous promoter, while the previous method aimed to analyze the effect of sequestrating transcription factors on the activity of several endogenous promoters (7).

Computational identification of transcription factor motifs in promoter regions usually leads to a profusion of data, not easily exploitable. For example, using online softwares, numerous motifs were identified in the model used in this report, i.e the 87-bp region of the SBEM promoter. Amongst these motifs, two purine-rich motifs, a direct repeat and an octamer-binding motif, had the potential to be involved in the SBEM promoter activity. Indeed two such purine-rich motifs were previously shown to participate to the transcriptional regulation of another breast-specific gene, mammaglobin (20). Similarly, direct repeats are often recognized by hormonal receptors actively involved in the biology of breast tissues (21-23). Finally, octamer-binding transcription factor are known to participate in the transcriptional regulation of numerous breast-specific genes (24-27). The promoter competition assay, allowed us to quickly establish the important role played by the octamer-binding factor motif, as opposed to other sites, in the SBEM promoter activity.

Several applications for the promoter competition assay can be foreseen. It could be used to test the significance of promoter polymorphisms, i.e. to examine whether or not a particular mutation within a given promoter region participate to the regulation of its activity. It could also allow the identification of new binding sites

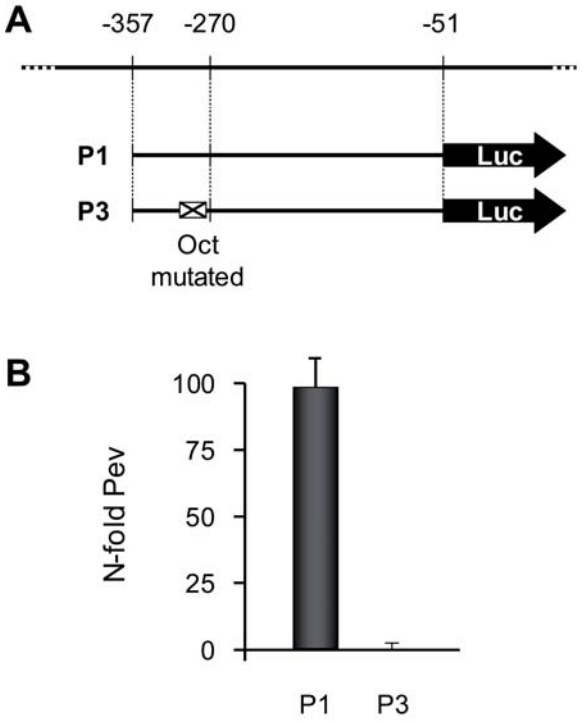

Figure 5. Activity of P1 and P3 constructs used in luciferase assay. (A) P1 contains the SBEM promoter region from -357/-51 from the translation start site; P3 contains the region from -357/-51 but in which the octamer-binding site was mutated. (B) P1 and P3 promoter activities transiently transfected in BT-20 cells and luciferase was measured 24h post-transfection as described in the Material and Methods. Luciferase activity is shown as n-fold value compared to cells transfected by the promoterless pGL3-basic vector. Data represent the means of at least three independent transfection experiments.

for known or unknown transcription factors, by designing competitors bearing serial mutations.

We strongly believe that this fast and easy promoter competition assay will, in the near future, become a method of choice to complement already well-established techniques aiming to analyze transcription factors mechanism of actions.

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Abbreviations: ENH, enhancer region; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Oct, octamer-binding transcription factor; RLU, relative luciferase unit; SBEM, small breast epithelial mucin

Key Words; Promoter Competition Assay, Transcription Factor Binding Site, Small Breast Epithelial Mucin, Luciferase Reporter Gene

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