Design of a chimeric promoter induced by pro-inflammatory mediators in articular chondrocytes

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Abstract We have designed a chimeric promoter that can be stimulated by various pro-inflammatory mediators and so drive the expression of therapeutic genes under inflammatory conditions. The promoter has two parts, the [−247/+20] fragment of the human type IIA secreted phospholipase A2 gene promoter, which is stimulated by the pro-inflammatory cytokine interleukin-1β (IL-1β), and a double peroxisome proliferator-activated receptor response element that is activated by some eicosanoids and by non-steroidal anti-inflammatory drugs (NSAIDs). Transfection experiments using rabbit articular chondrocytes in primary culture showed that this chimeric promoter produced a low basal activity and was induced by NSAIDs, WY-14643, IL-1β, and 15-deoxy $\Delta^{12,14}$ prostaglandin J2. The latter two compounds stimulated the promoter synergistically.

Key words: Type IIA secreted phospholipase A2; Peroxisome proliferator-activated receptor; Interleukin-1β; Transcription; Chondrocyte

1. Introduction

Several studies have shown that $sPLA_2$-IIA (type IIA secreted phospholipase A2), also known as 'synovial PLA_2', is involved in inflammatory diseases such as atherosclerosis and rheumatoid arthritis. The gene coding for $sPLA_2$-IIA has been cloned from patients suffering from rheumatoid arthritis [1]. This enzyme releases arachidonic acid from cell membrane phospholipids, thus providing the substrate for prostaglandin synthesis. The gene has no detectable basal level of transcription in a number of cell lines and tissues. However, the amount of its mRNA increases markedly in cells exposed to pro-inflammatory cytokines, in particular interleukin-1β (IL-1β) [2]. The human sPLA2 gene promoter has been extensively studied in our laboratory [3–5]; we have shown that stimulation by IL-1β triggers the binding of the transcription factors C/EBP β and δ (CAAT/enhancer binding protein). This promoter is also stimulated (two-fold) by glucocorticoids through a half-GRE.

PPARs (peroxisome proliferator-activated receptor) are ligand-induced transcription factors belonging to the nuclear receptor family. PPARs form heterodimers with the retinoic X receptor (RXR), which then bind to a specific element, the PPRE (PPAR response element), in the promoter region of the target gene. Three PPAR isotypes have been identified: PPARα (mainly in liver), PPARβ/δ (quite ubiquitous) and PPARγ (mainly in adipose tissue) [6], and two isoforms, α and γ, were found in chondrocytes [7]. They were first believed to mediate the effects of synthetic compounds, peroxisome proliferators, on transcription. But, PPARs are also activated by several natural and synthetic molecules, including hypolipidemic fibrate drugs, anti-diabetic thiazolidinediones, acyl CoA, some eicosanoids and fatty acids like arachidonic acid. PPARα is especially sensitive to leukotriene B4 [8], while PPARγ is strongly activated by 15-deoxy $\Delta^{12,14}$ prostaglandin J2 (PGJ2) [9]. Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and indomethacin, bind and activate both isoforms of PPAR [10].

We have constructed a chimeric promoter that can be induced in an inflammatory context and may thus be used for the gene therapy of diseases involving inflammation. This promoter consists of two transcription units: the proximal sequence is part of the $sPLA_2$-IIA gene promoter and the distal part contains two PPRE. This report describes the responses of these two parts to inflammatory stimuli, IL-1β for the sPLA2-IIA part and certain eicosanoids for the PPRE, in primary cultures of chondrocytes. The results demonstrate that the short human $sPLA_2$ gene promoter triggers a strong response to IL-1β in the vicinity of PPRE. Moreover, this synthetic hybrid promoter is not only stimulated by all these compounds (IL-1β, WY-14643, PGJ2), but also by indomethacin and ibuprofen. Lastly, these activators can stimulate greater transcription when they act together, probably due to cross-talking between the transcription factors.

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Abbreviations: sPLA2-IIA, type IIA secreted phospholipase A2; PPAR, peroxisome proliferator-activated receptor; IL-1β, interleukin-1β; PGJ2, 15-deoxy $\Delta^{12,14}$ prostaglandin J2; NSAID, non-steroidal anti-inflammatory drug; DR1, direct repeat 1

2. Materials and methods

2.1. Plasmid constructs

The [−247/+20] fragment of the human $sPLA_2$-IIA promoter was inserted into a PUC-SH-CAT plasmid. The various units – direct repeat 1 (DR1), (DR1)2-18, (DR1)2-21 and (DR1)2-30 – were inserted upstream into *Asp*718/*Xba*I restriction sites (Fig. 1A).

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The PPARα, PPARγ and RXR expression vectors were kindly donated by Dr W. Wahli (Lausanne, Switzerland).

2.2. Chondrocyte culture and transfection

Chondrocytes were isolated from 3-week-old female 'Fauve de Bourgogne' rabbits and transfected as previously described [2]. β-Galactosidase activity was measured to correct for the transfection efficiency. [3]

2.3. Preparation of COS-1 whole cell extracts

COS-1 cells were transfected as described [11] with 20 μg PPARα, PPARγ or RXR expression vectors.

2.4. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described [3] and the retarded complexes were quantified using a phosphorimager (Imagequant software).

The equations used to calculate the cooperativity ratio were adapted from Tsai et al. [12] for the (DR)2-*x* units (*x* is the number of bp separating the two DR1 centers); it corresponds to the ratio between the K_m for the formation of the double dimer and the K_m for the formation of the single dimer.

3. Results

3.1. Design of an optimal PPRE

We designed four sequences containing different arrangements of PPRE (Fig. 1A) to produce a readily induced eicosanoid response element. The classical PPRE sequence, or DR1, consists of a direct repeat of AGGTCA separated by 1 bp. We increased the affinity of the PPARs for DR1 by adding a CAAAACT sequence upstream of the first AGGTCA. (DR1)2-*x* consisted of two DR1, separated by 18, 21 or 30 bp, center to center. In the case of (DR1)2-18, the AGGTCA sequence of the first PPRE overlapped the CAAAACT 5′-sequence of the second PPRE by 2 bp. The two PPREs of (DR1)2-21 and (DR1)2-30 are separated by two or three DNA turns, respectively (21 and 30 bp).

3.1.1. PPAR binding to the PPRE constructs. We then tested the binding of PPARα and -γ isoforms to these synthetic response elements. We performed EMSA with increasing amounts of COS-1 whole cell extracts enriched with either PPARα (Fig. 1B) or PPARγ (Fig. 1C), and in both cases we added whole cell extracts enriched with RXR, as PPAR need to heterodimerize with RXR to bind the PPRE. The PPAR/RXR ratio was 3/1 to avoid the formation of the RXR/RXR homodimer. PPARα formed a single complex, corresponding to a PPAR/RXR heterodimer, with the DR1. (DR1)2-18, (DR1)2-21 and (DR1)2-30 formed two complexes. The first co-migrated with the dimeric complex (complex II) and corresponded to a PPAR/RXR dimer. Another, slowly migrating complex, was also produced by these constructs. This high-molecular-weight complex probably corresponded to a PPAR/RXR double heterodimer (complex IV). The amounts and proportions of complexes II and IV produced in the presence of each (DR1)2-*x* were different. For PPARα, the cooperativity ratio was the same for all the (DR1)2-*x* constructs (about 10), which suggests that the PPARα/RXR double heterodimer bound cooperatively to these sequences, regardless of the distance between the two DR1.

The experiments with PPARγ showed differences in the proportions of complexes II and IV depending on the PPRE structure (Fig. 1C). Complex II contained 35–40% of the total radioactivity of the probe in the presence of (DR1)2-18 and (DR1)2-30 units. In contrast, complex II never contained more than 20% of the total radioactivity in the presence of

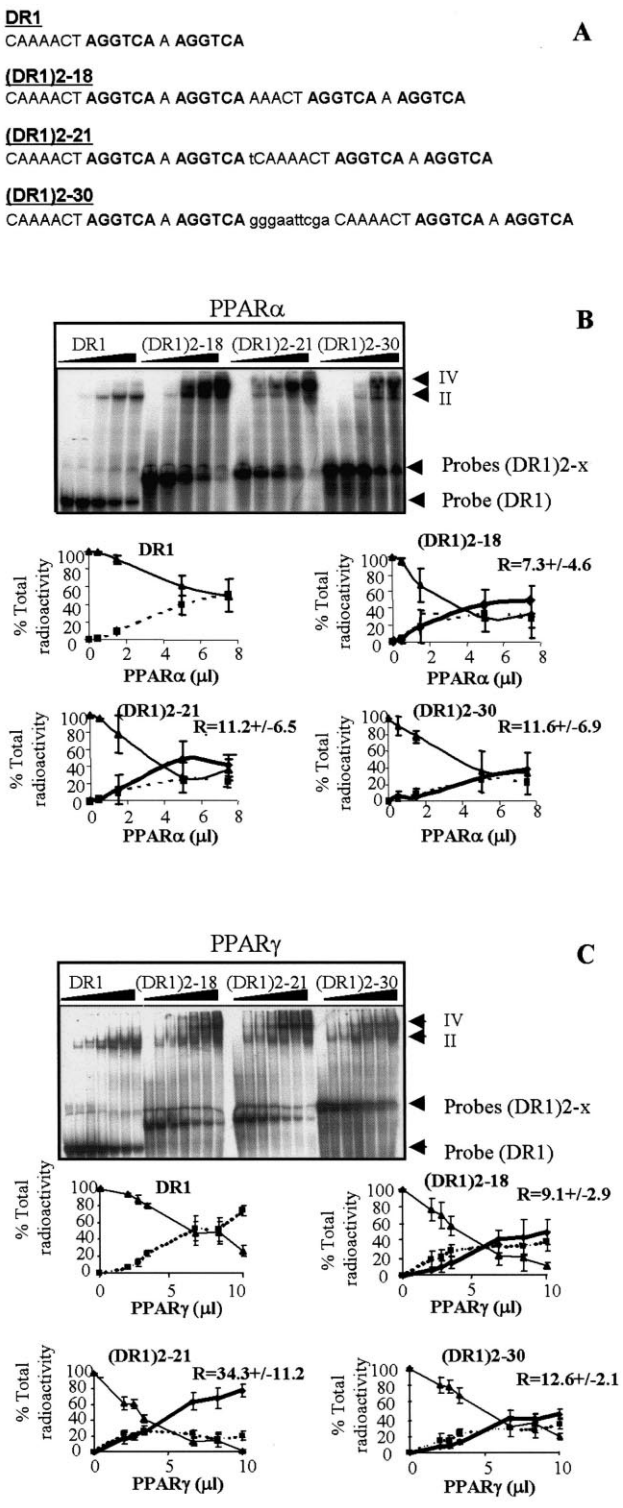

Fig. 1. Cooperative binding of the PPAR/RXR to the PPRE sequences. A: Sequences of the various PPRE arrangements. DR1 half-sites are in bold type. Radiolabeled DR1, (DR1)2-18, (DR1)2-21 or (DR1)2-30 were incubated with increasing amounts (in μl) of COS-1 whole cell extract enriched with PPARγ (B) or PPARα (C). Arrowheads indicate the free probe (Probe), complex II (II) or complex IV (IV) on each autoradiogram. Results are expressed as the percentage of the total radioactivity in each band. Squares represent complex II, diamonds represent complex IV and triangles represent free probe. (R) is the cooperativity ratio. The curves are the means of three different EMSA.

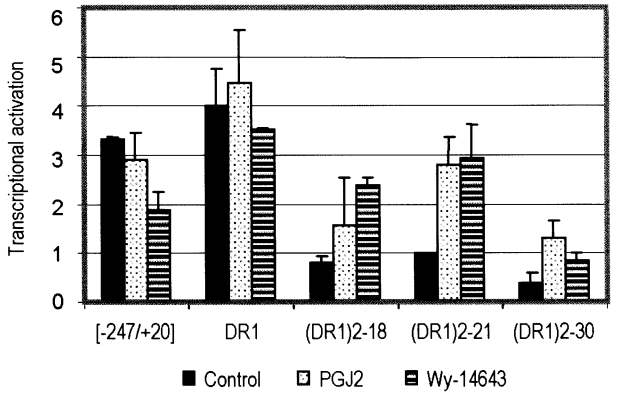

Fig. 2. Functionality of the PPREs. Primary cultures of chondrocytes were transfected with one of these constructs: pUC-SH-[−247/+20]-CAT, pUC-SH-DR1-[−247/+20]-CAT, pUC-SH-(DR1)2-18-[−247/+20]-CAT, pUC-SH-(DR1)2-21-[−247/+20]-CAT or pUC-SH-(DR1)2-30-[−247/+20]-CAT and stimulated with PGJ2 (5.10^{-6} M) or WY-14643 (10^{-4} M). Each point is the mean ± S.E.M. of four independent experiments performed in duplicate. Data are expressed as stimulated transcription relative to the pUC-SH-(DR1)2-21-[−247/+20]-CAT basal activity.

the (DR1)2-21 unit, while complex IV contained 80%. The cooperativity ratios for (DR1)2-18 and (DR1)2-30 were very similar (about 10), whereas that of (DR1)2-21 was higher (34 ± 11). These findings show that the three units cooperatively bind two PPARγ/RXR dimers and that (DR1)2-21 elicits greater cooperative binding.

3.1.2. Functionality of the different PPRE constructs. We tested the capacity of the various constructs to stimulate transcription. Primary cultures of chondrocytes were transiently transfected with either [−247/+20]-CAT, (DR1)-[−247/+20]-CAT, (DR1)2-18-[−247/+20]-CAT, (DR1)2-21-[−247/+20]-CAT, or (DR1)2-30-[−247/+20]-CAT (Fig. 2) and stimulated with PGJ2, a potent PPARγ activator, or WY-14643, a potent PPARα activator. The basal activities of the five constructs tested were very different. Those of [−247/+20] and DR1-[−247/+20] were three- to four-fold higher than that of (DR1)2-*x* constructs. Neither PGJ2 nor WY-14643 activated the transcription of [−247/+20] or DR1-[−247/+20] constructs, but PGJ2 and WY-14643 both stimulated the transcriptions of (DR1)2-18, -21 and -30 nearly three-fold. These three constructs also had very low basal activities that could prevent the 'leakage' of the promoter. The EMSA and transfection experiments led us to select (DR1)2-21-[−247/+20] as the most efficient inducible promoter. This sequence bound PPARα/RXR and PPARγ/RXR most cooperatively, and promoted transcription most efficiently under stimulation.

3.2. Effects of IL-1β, eicosanoids and glucocorticoids on the promoter activity

We investigated the transcription elicited by the promoter in response to different stimulations. The [−247/+20] $sPLA_2$ part of the promoter contains a C/EBP site at [−200/−191] that mediates activation by IL-1β and a half-GRE site at [−229/−224] [3]. The PPRE should allow the promoter to respond to PGJ2, WY-14643 or any other PPAR ligand. IL-1β produced a moderate (two-fold) increase in transcription, and PGJ2 stimulated transcription less than three-fold (Fig. 3). Incubation with a combination of these drugs resulted in a 10-fold increase in transcription. The PPARα activator WY-

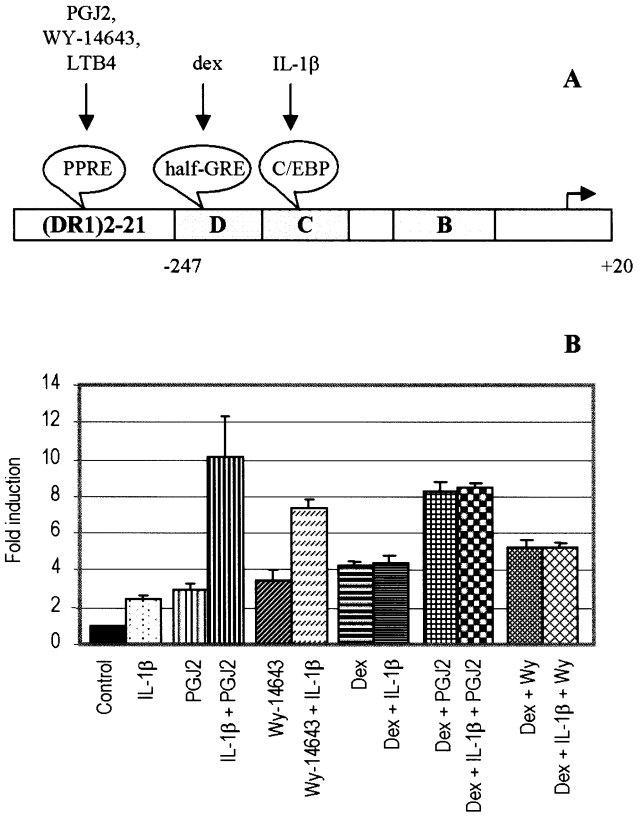

Fig. 3. A: Diagram showing the chimeric promoter, transcription factor binding sites and related putative inducers. B: Effects of IL-1β, PGJ2, WY-14643 and Dex on chimeric promoter activity. Primary cultures of chondrocytes were transfected with pUC-SH-(DR1)2-21-[−247/20]-CAT and cultured with IL-1β (10 ng/ml), PGJ2 (5.10^{-6} M), WY-14643 (10^{-4} M), Dex (10^{-7} M) or combinations of these inducers. Each point is the mean ± S.E.M. of six independent experiments performed in duplicate. Data are expressed as fold induction compared to unstimulated cells. The combination of WY-14643 and PGJ2 was highly toxic to the chondrocytes, although the vehicle did not affect them.

14643 produced 3.5-fold more transcription than the basal activity and IL-1β and WY-14643 acted additively (7.5-fold stimulation). The synthetic glucocorticoid, dexamethasone (Dex), activated the $sPLA_2$-IIA promoter two-fold [5], but it produced a four-fold increase in transcription with the synthetic promoter. PGJ2 plus Dex acted additively (eight-fold increase in transcription), whereas WY-14643 plus Dex had a less marked action (five-fold stimulation). IL-1β and Dex did not act additively, as described by Massaad et al. on the $sPLA_2$-IIA promoter [3] and combinations of Dex+IL-1β+PGJ2 or Dex+IL-1β+WY-14643 did not increase the stimulation produced by Dex+PGJ2 or Dex+WY-14643.

We compared the transcription elicited by the synthetic promoter to that of strong virus promoters commonly used in gene therapy. The basal activity of the (DR1)2-21 promoter was weaker (2 and 5% respectively) than those of SV-40 (Simian Virus 40) or RSV (Rous Sarcoma Virus). The various ligands all significantly stimulated the synthetic eukaryote promoter: 30% of SV-40, and 40% of RSV activities (data not shown).

3.3. Effects of NSAIDs on the promoter activity

Patients suffering from inflammatory diseases are usually

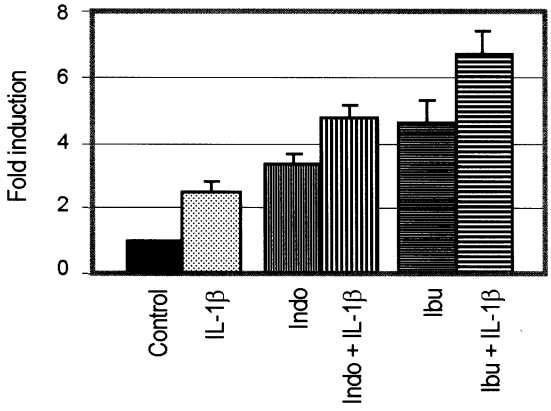

Fig. 4. Effects of NSAIDs on the chimeric promoter activity. Primary cultures of chondrocytes were transiently transfected with pUC-SH-(DR1)2-21-[−247/+20]-CAT and stimulated with IL-1β (10 ng/ml), indomethacin (10^{-4} M), ibuprofen (10^{-4} M) or a combination of IL-1β plus a NSAID. Each point is the mean ± S.E.M. of three independent experiments performed in duplicate. Data are expressed as fold induction compared to unstimulated cells.

treated with NSAIDs. These drugs not only inhibit cyclooxygenase activity, they also bind to and activate PPAR-α and -γ isoforms [10]. We therefore tested the effects of indomethacin and ibuprofen on the transcription triggered by the synthetic promoter (Fig. 4). Indomethacin activated the construct 3.5-fold and had an additive effect with IL-1β (five-fold activation). Ibuprofen increased transcription five-fold and acted additively with IL-1β (seven-fold activation). The combination of either indomethacin or ibuprofen with Dex had no additive effect (data not shown).

4. Discussion

We have constructed a promoter that can be modulated by the various pro-inflammatory mediators present in tissues in inflammatory diseases like Crohn's disease [13], arthritis [14], atherosclerosis [15] and acute respiratory distress syndrome [16]. We wanted it to have a low basal activity under non-inflammatory conditions but to be very active in the presence of inflammation. This promoter is a potentially useful clinical tool. It could be used to develop new protocols of gene therapy, in which the synthesis of an anti-inflammatory or anti-destructive protein is regulated in response to the patient's endogenous signals. Hence, the amount of the curative protein produced would vary with the intensity and duration of the inflammatory condition. We therefore used a short promoter that acts well in chondrocytes fused to DNA elements that respond to a wide range of inducers found in inflamed tissues. The promoter we have adopted consists of the [−247/+20] fragment from the human $sPLA_2$-IIA gene promoter, which is induced by the pro-inflammatory cytokine IL-1β through a C/EBP site located at [−200/−191] and by the synthetic glucocorticoid Dex through a half-GRE located at [−229/−224], plus a double PPRE that is stimulated by certain eicosanoids and prostaglandins. Therefore, the high concentrations of IL-1β, leukotrienes and prostaglandins produced during inflammation should stimulate the promoter.

We first constructed the most efficient transcription unit responding to PPAR activators. The (DR1)2-21 PPRE construct was chosen because of its low basal activity and responsiveness to PGJ2 and WY-14643. IL-1β, Dex, PGJ2, WY-14643 or NSAIDs such as ibuprofen and indomethacin stimulated the (DR1)2-21-sPLA2-IIA chimeric promoter to varying degrees; some combinations of stimulators had additive effects. Of particular interest was the transcription elicited in response to a combination of IL-1β and PGJ2. This resulted in the greatest promoter activity.

PPARs are involved in the regulation of many crucial physiological and developmental processes [6,17,18]. Although PPARα and -γ seem to be antagonistic partners in lipid homeostasis [19], both have been implicated in the anti-inflammatory process [8,20–22]. The eicosanoid PGJ2 is the most potent natural ligand of PPARγ described so far and the chemotactic leukotriene B4 has been implicated in the control of the inflammatory response through binding to PPARα. And, some cyclooxygenase inhibitors belonging to the NSAIDs family have been identified as PPAR ligands [10].

Transcription is initiated by cross-talking between inducible transcription factors and general transcription factors bound to the promoter. For example, transcription of the rat CYP2DS P450 is mediated by cooperative interaction between C/EBPβ and SP1 [23]. The $sPLA_2$-IIA gene promoter contains binding sites for both C/EBP and SP1 [3,4]. The concentrations of C/EBPβ and -δ increase in most tissues after inflammatory stimuli, suggesting that these factors are important in inflammation [24]. But, few studies have described the interaction between PPAR and C/EBP factors.

Clearly, the human $sPLA_2$-IIA promoter contains several binding sites for ubiquitous and inducible activation factors lying a short distance (up to −200 bp) from the initiation site. This promoter organization is also reminiscent of that of the COX-2 promoter studied in our laboratory [25]. This particular organization provides a good context for cross-talk between transcription factors, especially through docking to common co-activator proteins like PGC-1, CBP/p300 and SRC-1 [26–28]. These interactions between transcription factors and co-activators, and between co-activators [29], may cause conformational changes facilitating the recruitment of the pre-initiation complex and chromatin remodeling.

Therefore, the $sPLA_2$-IIA gene promoter provides an interesting model for driving the expression of a gene that can reduce inflammation. The addition of PPAR binding sites may provide a new tool for coordinating transcription in response to selected stimuli. Our findings show that the PPRE-$sPLA_2$-IIA chimeric promoter contains enough information to direct the expression of a curative gene. We also find that IL-1β and PGJ2 act synergistically through C/EBP and PPARγ respectively, in chondrocytes. The results obtained by mutation of the C/EBP binding site confirm the central role of C/EBP factors in stimulating the inflammatory response gene promoters of $sPLA_2$-IIA and COX-2 [3,25,30].

Studies with transgenic animals would give us access to the way the promoter responds to inducers like eicosanoids and NSAIDs and to screen the effect of new drugs on inflammatory responsive promoters.

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The promoter described above has been patented by the Université Pierre et Marie Curie (Patent Number: 00 03262)

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