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and the character of the deleted region is inferred. Here we provide an alternative approach for detailed promoter analysis, which is especially useful for studying relatively weak promoters. Promoter constructs with internal deletions are generated by means of inverse polymerase chain reaction (IPCR). In this way each putative regulatory region is deleted individually, while the rest of the sequence is preserved. Promoter constructs with internal deletions generally provide higher activities whereby the contribution of each deleted regulatory region can be seen directly. “Classical” exonuclease-based protocols (12) are not suitable for dissecting relatively small elements with one nucleotide accuracy. PCR protocols are the methods of choice (5). IPCR (4) is especially noteworthy because it minimizes downstream steps by amplifying the recombinant circular template using a pair of primers that has the region to be deleted between the 5′ ends of the primers. Linear products of such IPCRs are recircularized and mutants can be identified in a relatively low background of parental clones.

The [-172, +31] region of the gene coding for the tumor-associated MN/CA 9 protein (8) contains five PRs as identified by in vitro DNase I footprinting with the following distribution: PR1 [-45, -24]; PR2 [-71, -56]; PR3 [-101, -85]; PR4 [-134, -110]; PR5 [-163, -145]. Previously, we found that reporter constructs containing the MN/CA 9 gene 5′-upstream region and its fragments generated low reporter activity in all cell lines tested (not shown). In an attempt to maintain the highest available reporter activity, internally deleted constructs generated by IPCR were used for detailed analysis of the [-172, +31] region of the MN/CA 9 gene. We expected that by retaining as many regulatory regions as possible, fragments should preserve relatively higher transcriptional activity compared with progressively deleted promoter constructs. To characterize the role of PRs in transcriptional regulation, a set of promoter mutants was prepared in which each PR (putative cis element) and its flanking region was deleted while the rest of the promoter was preserved. A chloramphenicol acetyltransferase (cat) reporter gene construct driven by the MN/CA 9 [-172, +31] fragment was directly amplified with pairs of promoter-specific primers as shown in Figure 1. From reporter activities generated by these mutants and the control, the contribution of the deleted PR to the activity of the MN/CA

| Table 1. Sequences of Primer Pairs Used for IPCR and Screening of Clones |
|-----------------|-----------------|-----------------|
| **IPCR primers** | **Screening primers** |
| **(-PR1)** | GCTCTCGTTTTCCAATGCACG [-24, -4; s] |
| | AGCAGGCTGACTCACAGAG [-54, -72; a] |
| **(-PR2)** | AGGCTTTGCTCCTCCCCACCCAG [-46, -24; s] |
| | TGCAGAGATGGAGCCAAAGTCTCA [-81, -104; a] |
| **(-PR3)** | CGCTCTGTAGTGAGCCTG [-74, -56; s] |
| | AGTCTTGTGCGCTTTAGCGCCTCTCC [-106, -133; a] |
| **(-PR4)** | ACCTGTGAGACTTTGGCTCCATCTC [-109, -85; s] |
| | AGCTAGGATGGGGGATTGAGTCA [-143, -166; a] |
| **(-PR5)** | GGGGGAGAGGGCGAGCCAGACAAAC [-137, -110; s] |
| | AGCTAGGATGGGGGATTGAGTCA [-143, -166; a] |

**Note:** Primers are written in the 5′-3′ direction, s and a stand for sense and antisense orientations, respectively.
9 promoter was deduced.

Enzymes, kits and reagents were used according to the manufacturers’ recommendations. Plasmid pBMN5 was constructed by subcloning the [-172, +31] fragment into the XbaI and BglII sites of the pBLCAT6 vector (2). Primer pairs used for IPCR are given in Table 1. PCRs were performed in 25 µL containing a pair of phosphorylated primers at 20 µM each, 0.2 mM each of the four dNTPs and 1 U of Pwo DNA Polymerase in 1× Reaction Buffer (Boehringer Mannheim, Indianapolis, IN, USA), supplemented with 2 mM MgSO4. The pBMN5 plasmid (20 ng) was used as a template and amplified in a Model 480 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) for 30 cycles: 94°C for 30 s, 56°C for 30 s (-PR1 and -PR3 constructs) or 68°C for 30 s (-PR2, -PR4 and -PR5 constructs) and 72°C for 3 min followed by a final extension at 72°C for 5 min. PCR products (ca. 30 ng), after purification on a 1% agarose gel and QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA, USA), were recircularized with T4 DNA Ligase (Life Technologies, Gaithersburg, MD, USA) and transformed into competent INVαF′ bacteria (Invitrogen, Carlsbad, CA, USA). The pBMN5(-PR5) product was first cut with XbaI to remove the antisense primer, blunt-ended and recircularized. Transformants were screened for appropriate deletions by PCR (-PR2, -PR3 and -PR4 constructs) with primers given in Table 1 or restriction analysis (-PR1 and -PR5 constructs). Inserts in deleted constructs were sequenced with a T7 Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to check for the exact extent of deletion and random mutations. Plasmid DNA for transfection experiments was purified with a QIAfilter Plasmid Midi Kit (Qiagen).

MaTu cells (9) were grown in Dulbecco’s modified Eagle medium (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies) and 0.16 mg/mL gentamicin (Sigma, St. Louis, MO, USA). Cells at 50%-80% confluence on 3.5-cm plates were exposed to a mixture of plasmid DNA (1.5 µg) and 6 µL of LIPOFECTAMINE Reagent (Life Technologies) for 16 h. Transfected cells were harvested 72 h post-transfection, and CAT activities were assayed with a CAT ELISA Kit (Boehringer Mannheim). Protein concentrations were determined with BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL, USA).

Under conditions described, IPCR generated sufficient quantities of specific products with little background (not shown) despite some imbalance in the primer pairs. These primers were originally synthesized for electrophoresis mobility shift assay (EMSA) of individual PRs in the MN/CA 9 promoter region and some may have not been optimal for PCR. The yield of deleted constructs was within 70%-85%. In all sequenced clones, the expected deletions were confirmed and only one point mutation in one clone was detected.

The CAT activity generated from the parental construct pBMN5 and its deletion mutants are shown in Figure 2. From the markedly decreased CAT assay results obtained from pBMN5 (-PR1) and (-PR2) constructs, it can be concluded that at least in MaTu cells PR1 and PR2 bind strongly activating transcription factors. Analysis with the Signal Scan program (10) revealed presence of a consensus binding site for the AP2 transcription factor (CC-MNSSS) in the PR1 sequence and a binding site for the AP1 transcription factor (5′-TGAGTCAG-3′) in the PR2 sequence.
sequence. Decreased CAT expression from pBMN5(-PR1) and (-PR2) is thus in good agreement with the expected activating role of AP2 (13) and AP1 (1). Decreased CAT expression from pBMN5(-PR3) and (-PR5) constructs suggests that PR3 and PR5 also bind trans-acting factors, positively contributing to the transcriptional activity of the MN/CA 9 promoter in MaTu cells, albeit to a lesser extent. Finally, since deletion of PR4 yielded CAT assay results almost 3× higher than the control, at least in the context of the MN/CA 9 promoter, PR4 functions as a silencer element. The conclusion that this region binds as yet uncharacterized repressor is further supported by an absence of a PR4-specific complex in the MN/CA 9 positive cell line vs. presence of this complex in negative cell lines (not shown). Although further experiments are required to confirm the identity of the trans-acting factors that bind to the PRs, IPCR made possible the rapid characterization of putative cis elements within the MN/CA 9 promoter.

All primers used for generating deletion mutants were from the previous EMSA analysis, except for the [-24, -4; s] primer, not corresponding to any PR, which had to be synthesized to preserve the context of the transcription initiation site and immediate upstream region. In the case of the (-PR5) construct, the antisense primer in fact corresponded to PR5 and was removed by restriction digestion. Thus the approach described in this paper requires minimal extra costs and makes efficient use of previously made primers.

In this paper, the alternative type of detailed functional promoter analysis that we propose is based on IPCR, which uses information from a DNase I footprinting assay and primers from EMSA. Although serving essentially similar purposes, constructs internally deleted by IPCR could have some advantage over progressively deleted ones in certain situations. Interactions of certain combinations of transacting factors with RNA polymerase complex could have a synergistic rather than an additive effect on transcription. Internally deleted promoter constructs should generally produce higher reporter activities as a result of retaining more activating cis elements and at least some of the possible interactions between transcription factors. Especially in the case of relatively weak promoters, this increased activity could allow more accurate measurement. As with the case of the pBMN5(-PR4) construct, by internal deletion of negatively acting cis elements it should be possible to “optimize” the activity of weak promoters, thus generating templates that could be more suitable for trans-repression studies. In addition, recent evidence confirms that the combination of a cis element and the influence of a protein that is bound nearby can determine the conformation and subsequently the function of a transcription factor (6). Finally, progressive deletions create conditions that gradually depart from “normal” conditions of the control. Constructs with internal deletions generated by IPCR, having at least to some extent preserved the original context, could be more comparable to the full-length construct.

Application of IPCR-generated internally deleted constructs to promoter analysis has the following advantages: (i) it generates internal deletions in one step, (ii) individual PRs are precisely deleted with their flanking regions while the rest of the promoter is preserved, (iii) the effect of deleted PR on transcriptional activity can be seen directly in a more natural context and (iv) internally deleted constructs should offer higher reporter activities.

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Benchmarks

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Immunofluorescence Localization of Glycoproteins Using Tissue Printing: Detection of Pistil Extensin-Like Proteins in Tobacco

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Immunocytochemical methods often encounter difficulties with both soluble antigens and with processing large numbers of samples efficiently. As a complement to in situ hybridization (5), western blotting and ultrastructural immunolocalization (3), we searched for a suitable method to gain information on the overall subcellular localization of highly soluble extensin-like glycoproteins in Nicotiana tabacum (tobacco) pistils at various stages of development. Whereas the cryofixation technique that is used in conjunction with embedding, sectioning and immunofluorescence labeling (1) limits the risks of translocation or loss of epitopes during the preparation procedure, this technique is time-consuming and not appropriate for autofluorescent organs like tobacco pistils. The tissue print-alkaline phosphatase (AP) detection method (see References 2, 10, 13 and 14 for general techniques and References 6, 7, 9 and 11 for applications to styles and stigmas) is much more rapid; however, it can be biased by endogenous enzymes known to be active in the pistil (i.e., peroxidase, esterase and phosphatase; References 6, 7 and 15). As an alternative, we introduced a new and sensitive variant of the tissue print technique by applying fluorescent antibody labeling and using low- and high-magnification fluorescence microscopy for detection.

Unpollinated tobacco pistils at various stages of development (i.e., stages 2-4, 6, 8, 10 and 11, and mature +0, 24 or 48 h; References 4 and 15) and pollinated pistils (mature +0, 24 or 48 h) were collected in the greenhouse of the University of Nijmegen. Using a razor blade, longitudinal hand-sections were made through the pistils, which consist of a stigma, a style that contains a transmitting tract and an ovary. The exposed pistillar tissue was printed by finger pressure for 30 s on pieces of reinforced Optițran® BA-S85 Nitrocellulose Membrane (pore size 45 μm; Schleicher & Schuell GmbH, Dassel, Germany). Since most pistil material was quickly removed with forceps after printing, the level of autofluorescence due to chlorophyll, lignin compounds and other biological substances was limited. Each piece of printed membrane was immediately soaked in Blocking Buffer (100 mM potassium phosphate buffer, pH 6.4, 5 mg/mL bovine serum albumin [BSA] fraction V [Sigma, St. Louis, MO, USA]) and incubated for at least 30 min at room temperature (RT). In some series, prints were incubated in 20% goat serum or fetal calf serum. Prints were transferred to a fresh solution of Blocking Buffer containing a noncommercial polyclonal rabbit antisemur (I-C3P; diluted 1:200) (5) against the C-terminal domain of extensin-like glycoproteins (3.5). In control experiments, incubation with the primary antibody was omitted and replaced either by preimmune serum (PI-C3P; Reference 5) or by Blocking Buffer. After an incubation overnight at RT on a Model RS 500 LaboTech® rotating plate (50 rpm; Breda, The Netherlands), prints were quickly rinsed 3× in Blocking Buffer and transferred to the secondary antibody, a fluorescein isothiocyanate (FITC) goat-anti-rabbit antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) that was diluted 1:200 in Blocking Buffer. This incubation was done in the dark for 2–3 h, at RT on a rotating plate set at 50 rpm. After 3 brief final washes in Blocking Buffer without BSA, tissue prints were mounted on microscope slides and placed either in a mixture of an 80% glycerol, 20% phosphate buffer (pH 6.4 or 7.0) or in SlowFadeLight® dissolved in buffer and glycerol (ca. pH 10.5; Molecular Probes, Eugene, OR, USA). For comparison, an enzymatic detection reaction was carried out on approximately 30 tissue prints. For these experiments, the secondary antibody was a horseradish peroxidase (HRP) goat anti-rabbit serum (1:200; Pierce Chemical, Rockford, IL, USA). The substrate solution contained 1 mg/mL 2,2′ azinodi-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma), 0.003% H2O2, 28 mM cit-