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A Nuclear Factor Recognizing a Positive Regulatory Upstream Element of the *Antirrhinum majus* Chalcone Synthase Promoter

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ABSTRACT

A positive regulatory element directing maximal expression of the *Antirrhinum majus* chalcone synthase promoter was characterized by protein-DNA-interaction studies and *cis* deletion analysis. The positive regulatory element consists of a 47 base pair direct repeat between positions −564 and −670 and provides three binding sites for nuclear protein factors from *Nicotiana tabacum* and *Antirrhinum majus*. Oligonucleotide competition assays revealed that the same factor(s) interact(s) with all three binding sites. Transient expression of chimeric chalcone synthase-neomycin phosphotransferase II genes in parsley protoplasts demonstrated that both halves of the 47 base pair repeat element are required for its *in vivo* function. A possible role of redundant binding sites for the positive regulatory function of the 47 base pair repeat element is discussed.

Flavonoids represent a class of secondary metabolites which serve a variety of functions such as flower and fruit pigmentation. Flavonoids accumulating in leaf epidermal cells serve as UV protective compounds with characteristic light absorption in the range of 230 to 380 nm. Chalcone synthase is the first enzyme of the flavonoid-specific branch of the phenylpropanoid pathway (7). It catalyzes the formation of the basic structure of the flavonoids, naringenin chalcone, through condensation of one molecule 4-coumaryl-CoA with three molecules of malonyl-CoA. Transcriptional activation of *chs*[3] has been used as a model for studying both developmental regulation of gene expression and molecular responses to environmental stimuli, such as UV light, wounding or pathogen infection, in a variety of plants (9, 10, 19, 21, 22, 24).

In the promoter of the *Antirrhinum majus chs* gene (23) sequence elements located downstream from position −357 mediate the UV light regulated *chs* gene expression in transgenic tobacco seedlings (9; K Fritze, DS JS, and D Wing, unpublished observation). Light-induced transient expression of *chs-npt*II gene fusions in parsley protoplasts delineated the light-responsive *cis* element to sequences located between positions −39 and −197 (12). A nuclear factor, CG-1, has been identified which interacts with a CACGTG motif located between positions −129 and −134 within the light responsive element (25). Related sequence motifs within the upstream region of the maize alcohol dehydrogenase gene, tomato ribulose-bisphosphate carboxylase small subunit gene and parsley chalcone synthase gene were also reported to bind nuclear factors (3, 4, 21).

A fragment of the *Antirrhinum majus chs* upstream region spanning positions −357 to −1100 was shown to increase the basal expression level both in transgenic tobacco (9; K Fritze, DS, JS, and D Wing, unpublished observation) and in parsley protoplasts (12). In order to determine which DNA sequence motifs are involved in quantitative regulation of *chs* gene expression, the interaction of nuclear factors with promoter upstream elements was studied by protein-DNA binding studies. Here we describe a nuclear factor that specifically binds to a positive regulatory element located between positions −564 and −670 of the *chs* promoter. The positive regulatory element contains a direct repeat of a 47 base pair DNA sequence. Deletions removing the entire 47 bp direct repeat element or either half of the 47 bp repeat element decrease the expression level of chimeric *chs-npt*II genes in parsley protoplasts.

MATERIALS AND METHODS

Preparation of Nuclear Extracts

Nuclei were prepared from 4-week-old seedlings of *Nicotiana tabacum* (var. W38) and *Antirrhinum majus* (commercial hybrid line Tip top Karmesin) as described (25). All steps were performed at 4°C. The seedlings were homogenized by Waring blendor in NiB (25 mM Mes-KOH [pH 6], 0.25 M sucrose, 5 mM EDTA, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) supplemented with 0.5% Triton X-100 and 0.5 mM spermidine. The homogenate was filtered through 80 μm and 20 μm nylon mesh and the nuclei were pelleted at 2,000 *g*. The crude nuclear pellet was washed twice with NiB and resuspended in 3 volumes of NEB (25 mM Hepes-KOH [pH 7.8], 5% (v/v) glycerol, 0.1 mM EGTA, 420 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 μM leupeptin, 1 μM pepstatin). Nuclear proteins were extracted by stirring on ice for 45 min and nuclear debris was pelleted at 100,000 *g* for 30 min. The supernatant was dialyzed against 25 mM Hepes-KOH [pH

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[3] Abbreviations: *chs*, chalcone synthase; *npt*II, neomycin phosphotransferase II.

7.8], 20% (v/v) glycerol, 0.1 mM EDTA, 50 mM KCl, 14 mM β-mercaptoethanol, quick frozen in liquid nitrogen and stored at −70°C.

DNA Probes

The RsaI-HpaII fragment of the *A. majus chs* promoter (23) spanning positions −564 to −670 was isolated from a pUC19 subclone by digestion with *Eco*RI and *Sal*I and labeled with DNA polymerase I (Klenow fragment) using [^{32}P] dCTP and [^{32}P]dATP. A cauliflower mosaic virus 35 S promoter fragment spanning from +1 to −135 (15) was isolated by *Eco*RI-*Hind*III digestion of a pUC19 subclone. Oligonucleotides were synthesized on an Applied Biosystems model 380 A DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis. The annealed oligonucleotides were labeled with DNA polymerase I (Klenow fragment) and purified from a polyacrylamide gel.

Gel Retardation Assay

Labeled DNA probes (0.5 ng) were incubated in a total volume of 20 μL of 12.5 mM Hepes-KOH (pH 7.8), 5 mM $MgCl_2$, 100 mM KCl with nuclear extract and *Hpa*II-cut pUC19 DNA, as indicated, for 10 min at room temperature. Polyacrylamide gels (5%) were run in 20 mM Tris base, 8 mM sodium acetate, 15 mM glacial acetic acid, 1 mM EDTA at 4°C, dried, and autoradiographed.

Construction of Chimeric *A. majus chs-npt*II Genes

DNA manipulations were carried out according to (13). A 850 bp *Hpa*II fragment containing the *chs* promoter region from −564 to −1100 and 310 bp of pUC9 sequence upstream of the *Eco*RI site was isolated from the plasmid pUC9-1.2 (9). After limited *Bal*31 digestion the ends were repaired with Klenow fragment of DNA polymerase I and the fragments were recut with *Eco*RI. Fragments between 440 and 540 base pairs in size were subcloned into *Eco*RI-*Sma*I cut pUC19 vector and the endpoints of the deletions determined by DNA sequencing (14). A clone with a deletion to position −674 (pBAL29) and a clone with a deletion to position −615 (pBAL53) were chosen for further manipulation. To obtain construct Delta 107 pUC lacking the entire 47 base pair repeat element (−564 to −674), the 208 base pair *Hpa*II-*Hinc*II fragment spanning positions −564 to −357 of the *chs* upstream region was inserted into the *Hinc*II site of pBAL29 after filling in the *Hpa*II site. For construction of Delta 3′ r pUC, lacking the 3′ half of the 47 base pair repeat element (−564 to −615) the 208 bp *Hpa*II-*Hinc*II-fragment (positions −564 to −357) was inserted into the *Hinc*II site of pBAL53 after filling in the *Hpa*II site. For Delta 5′ r pUC lacking the 5′ half of the 47 base pair repeat (−609 to −674) the 252 base pair *Hinc*II-*Mae*III fragment (positions −357 to −609) was inserted into the *Hinc*II site of pBAL29 after filling in the *Mae*III site. The wild-type *chs* upstream region linked to a *npt*II reporter gene and to the parsley *chs* 3′ end (17) in the plasmid 714 pUC (9) was replaced by the mutated *Eco*RI-*Sna*BI fragments spanning positions −1100 to −400 to yield the constructs Delta 107 pUC, Delta 3′r pUC, and Delta 5′r pUC, respectively (*cf.* Fig. 5).

Transient Expression in Parsley Protoplasts

Parsley suspension culture (*Petroselinum crispum*) was a kind gift of Dr. D. Scheel, MPI Köln. Protoplast preparation and DNA transformation were carried out using the $Ca(NO_3)_2$-PEG method as described previously (12). Following DNA uptake, the protoplast samples were divided. One-half was irradiated continuously with UV-containing white light (referred to as light) for 15 h (Phillips TL 20W18 lamps) while the other half was kept in dark. *Npt*II enzyme activity was assayed using established procedures (18, 20).

RESULTS

Interaction of Nuclear Factors with a 47 Base Pair Repeat Element of the *Antirrhinum majus* Chalcone Synthase Promoter

Deletion of the 5′ upstream region from position −1100 to position −357 decreased the expression level of reporter genes linked to the *chs* promoter in transgenic tobacco (9; K Fritze, DS, JS, and D Wing, unpublished observation) and in parsley protoplasts (12). Inspection of deleted 5′ upstream sequences (23) revealed a direct repeat of a 47 base pair sequence separated by a viral enhancer core sequence between positions −564 and −661. To explore a possible functional significance of this promoter element protein-DNA interactions within this 47 base pair repeat element were mapped using gel retardation assays.

Nuclear extract prepared from tobacco seedlings was incubated with an endlabeled *chs* promoter fragment spanning

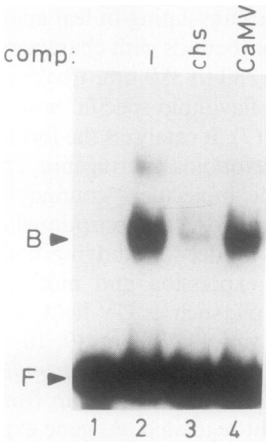

Figure 1. Binding of tobacco nuclear factors to the 47 base pair repeat element of the *Antirrhinum majus chs* promoter. Five-tenths of labeled fragment spanning positions −564 to −670 was incubated with tobacco nuclear extract in the presence of 5 μg *Hpa*II-cut pUC19 DNA (lane 2). For competition, 50 ng of the unlabeled homologous fragment (lane 3) or a fragment spanning positions +1 to −135 of the CaMV 35 S RNA promoter (15) (lane 4) were added. This corresponds to a 100-fold molar excess over the labeled fragment. Lane 1: Free fragment (no extract). B, Retarded complex; F, free probe.

positions −564 to −670 (Fig. 1). All reactions included 5 μg *Hpa*II-cut pUC19 DNA to eliminate nonspecific protein-DNA interactions. Binding of a nuclear factor was identified on the basis of the lower electrophoretic mobility of the protein-DNA-complex in polyacrylamide gels compared to the unbound input DNA (Fig. 1, lane 2). The binding was specifically inhibited by a 100-fold molar excess of unlabeled homologous fragment (lane 3) but not by a 100-fold molar excess of a fragment of the cauliflower mosaic virus 35 S promoter carrying a known viral enhancer (15) (lane 4). The additional faint complex seen in lane 2 is considered to be nonspecific because it is also competed by the heterologous cauliflower mosaic virus promoter fragment (lane 4). Complex formation was sensitive to proteinase K treatment showing that the retarded complex contains an essential protein component (data not shown).

The binding site was further characterized by using synthetic oligonucleotides as probes in gel-shift assays. A putative binding site within the 3′ half of the repeat which was recognized as a weakly protected region in DNAseI footprints (D. Staiger, unpublished observation) was synthesized (oligonucleotide 3′r I; positions −573 to −593; Fig. 2, A and B). Upon incubation of the endlabeled oligonucleotide with nuclear extracts in the presence of 1 μg *Hpa*II-cut pUC19 DNA a retarded complex was observed (Fig. 2C, lane 2). A 40-fold molar excess of the unlabeled homologous oligonucleotide greatly reduced the amount of retarded complex (Fig. 2C, lane 3). In contrast, a 120-fold molar excess of an oligonucleotide which derived from the 5′ half of the repeat and which contained beside four base pair substitutions an identical sequence (oligonucleotide 5′r I; positions −623 to −643; Fig. 2, A and B) competed only very weakly with protein binding to oligonucleotide 3′r I (Fig. 2C, lane 6). This indicates that the sequence spanning positions −573 to −593 within the repeat serves as a binding site for a sequence-specific tobacco nuclear factor and that four base pair mismatches as they occur in oligonucleotide 5′r I prevent binding almost completely.

The 47 Base Pair Repeat Contains Three Binding Sites for the Same Nuclear Factor(s)

To test whether additional sites of the 47 base pair repeat interact with nuclear factors, two additional oligonucleotides, 3′r II (positions −594 to −622) and 5′r II (positions −643 to −663) were synthesized (Fig. 2, A and B). Retarded complexes obtained by incubation of oligonucleotides 3′r II and 5′r II with tobacco nuclear extract migrated with R_F values similar to the complex formed with oligonucleotide 3′r I (Fig. 2D, lanes 3, 4). Oligonucleotide 5′r I did not show a significant interaction with nuclear factors present in the extract (Fig. 2D, lane 2). Competition experiments using labeled oligonucleotide 3′r I as probe demonstrated that oligonucleotides 3′r II and 5′r II competed well with oligonucleotide 3′r I for binding of the nuclear factor (Fig. 3A): A 40- and 120-fold molar excess of oligonucleotide 3′r II (lanes 5, 6) and oligonucleotide 5′r II (lanes 7, 8) significantly reduced complex formation. A 120-fold molar excess of an 18mer oligonucleotide containing the binding site for tobacco nuclear factor CG-1 (25) failed to compete (Fig. 3A, lane 9). Similarly, when the entire 47 base pair repeat element was used as labeled probe, binding of the nuclear factor was inhibited by oligonucleotide 3′r I (Fig. 3B, lanes 2, 3), oligonucleotide 3′r II

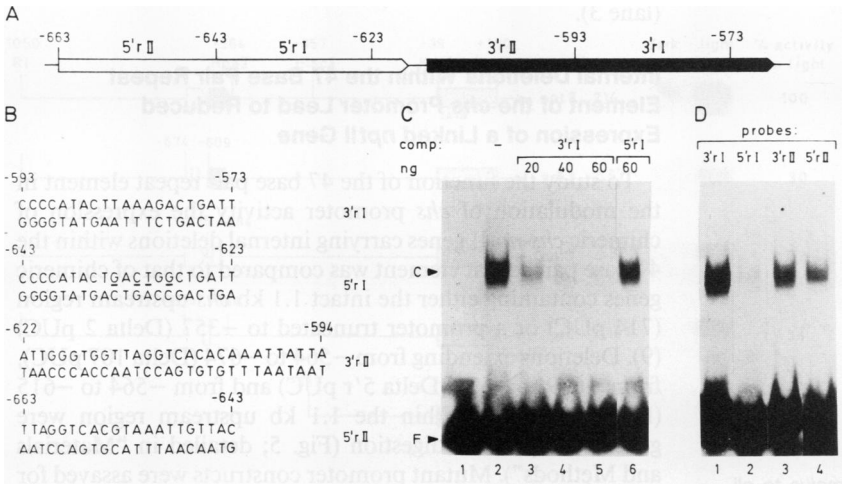

Figure 2. Binding of nuclear factors to subfragments of the 47 base pair repeat element. A, Position of oligonucleotides used for gel-shift assays. The unfilled arrow represents the distal (5′) repeat, the filled arrow the proximal (3′) repeat. B, Sequence of the oligonucleotides. Underlined are the four mismatches between oligonucleotides 3′r I and 5′r I. The oligonucleotides contained in addition flanking restriction sites for labeling. C, Gel retardation competition assay with oligonucleotide 3′r I as probe. Five tenths ng of labeled oligonucleotide 3′r I were incubated with 12 μg of tobacco nuclear extract in the presence of 1 μg *Hpa*II-cut pUC19 (lane 2). For competition, 20, 40, or 60 ng of the homologous oligonucleotide 3′r I corresponding to a 40-, 80-, or 120-fold molar excess, respectively (lanes 3, 4, 5), or 60 ng of oligonucleotide 5′r I corresponding to a 120-fold molar excess (lane 6) were added. Lane 1: Free oligonucleotide (no extract). D, Gel-shift assay with oligonucleotides 3′r I (lane 1), 5′r I (lane 2), 3′r II (lane 3), and 5′r II (lane 4) as probe. Binding assays contained 0.5 ng of the respective labeled oligonucleotide, 12 μg of tobacco nuclear extract, and 1 μg of *Hpa*II-cut pUC19. C, Retarded complex; F, free probe.

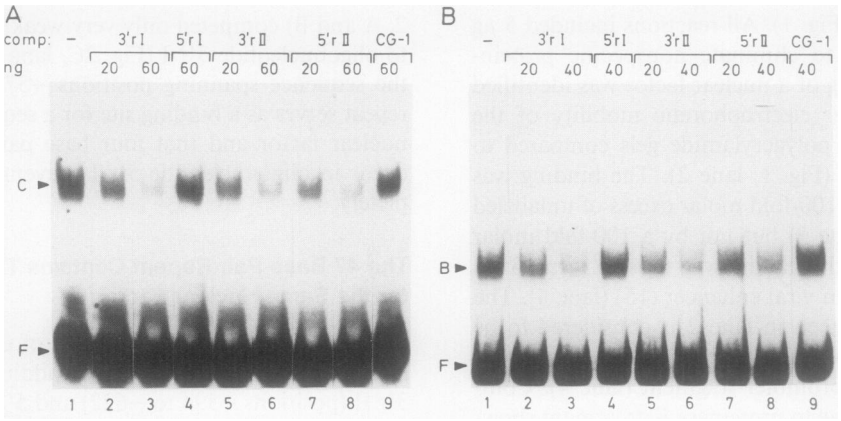

Figure 3. A, Gel retardation competition assay using oligonucleotide 3′r I as probe. Five-tenths ng of labeled oligonucleotide 3′r I were incubated with 12 μg of tobacco nuclear extract in the presence of 1 μg *Hpa*II-cut pUC19 (lane 1). Specific competitor DNAs were 20 and 60 ng of unlabeled oligonucleotide 3′r I (lanes 2, 3), 60 ng of unlabeled oligonucleotide 5′r I (lane 4), 20 and 60 ng of unlabeled oligonucleotide 3′r II (lane 5, 6), 20 and 60 ng of unlabeled oligonucleotide 5′r II (lanes 7, 8), and 60 ng of an unrelated oligonucleotide containing the binding site for nuclear factor CG-1 CATGTCACGTGCCAACTG (25) (lane 9). C, Retarded complex; F, free probe. B, Gel retardation competition assay using the entire 47 base pair repeat element (positions −564 to −670) as probe. One ng of labeled fragment was incubated with 12 μg of tobacco nuclear extract in the presence of 2 μg *Hpa*II-cut pUC19 (lane 1). Specific competitor DNAs were 20 and 40 ng of oligonucleotide 3′r I (lanes 2, 3), 40 ng of oligonucleotide 5′r I (lane 4), 20 and 40 ng of oligonucleotide 3′r II (lanes 5, 6), 20 and 40 ng of oligonucleotide 5′r II (lanes 7, 8), or 40 ng of the oligonucleotide containing the CG-1 binding site (25) (lane 9). B, Retarded complex; F, free probe.

(lanes 5, 6) and oligonucleotide 5′r II (lanes 7, 8) but not by oligonucleotide 5′r I (lane 4) or by the oligonucleotide containing the CG-1 binding site (lane 9). The ability of the oligonucleotides to cross-compete indicates that the same factor is able to recognize three binding sites within the 47 base pair repeat element.

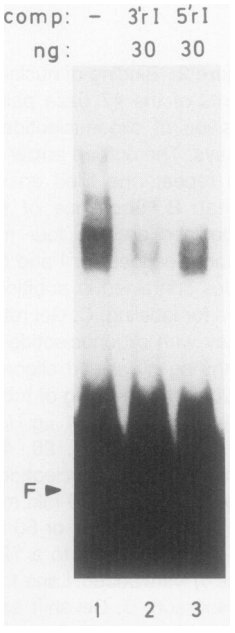

Figure 4. Binding of nuclear factors from *Antirrhinum majus* to oligonucleotide 3′r I; 0.5 ng of labeled oligonucleotide 3′r I was incubated with 7 μg of *A. majus* nuclear extract in the presence of 1 μg *Hpa*II-cut pUC19 (lane 1) and 30 ng of competitor oligonucleotide 3′r I (lane 2), or oligonucleotide 5′r I (lane 3). F, free probe.

A Nuclear Factor Exhibiting the Same Binding Specificity is Also Present in *Antirrhinum majus*

Since most data on *cis*-regulatory elements of the *Antirrhinum majus chs* promoter were derived from studies done in heterologous tobacco and parsley systems (9, 12) we analyzed whether a nuclear factor with the same binding specificity was also present in *Antirrhinum majus*. Upon incubation of labeled oligonucleotide 3′r I with nuclear extracts prepared from *Antirrhinum majus* seedlings in the presence of 1 μg *Hpa*II-cut pUC19 DNA retarded complexes were observed (Fig. 4, lane 1) which were specifically competed by a 60-fold molar excess of the unlabeled oligonucleotide 3′r I (lane 2) but not by a 60-fold molar excess of oligonucleotide 5′r I (lane 3).

Internal Deletions Within the 47 Base Pair Repeat Element of the *chs* Promoter Lead to Reduced Expression of a Linked *npt*II Gene

To study the function of the 47 base pair repeat element in the modulation of *chs* promoter activity the expression of chimeric *chs-npt*II genes carrying internal deletions within the 47 base pair repeat element was compared to that of chimeric genes containing either the intact 1.1 kb *chs* upstream region (714 pUC) or a promoter truncated to −357 (Delta 2 pUC) (9). Deletions extending from −564 to −674 (Delta 107 pUC), from −609 to −674 (Delta 5′r pUC) and from −564 to −615 (Delta 3′r pUC) within the 1.1 kb upstream region were generated by Bal31 digestion (Fig. 5; detailed in "Materials and Methods"). Mutant promoter constructs were assayed for light-inducible transient expression in parsley protoplasts (2, 12, 21, 22). Following transformation, half of the protoplasts were kept in the dark whereas the other half was induced by light irradiation for 15 h. In comparison to the wild-type

promoter construct 714 pUC, the deletion of the entire 47 base pair repeat in Delta 107 pUC led to an approximately threefold reduction of *npt*II expression to the level of the truncated promoter construct Delta 2 pUC in light induced protoplasts (Fig. 5). The expression level in uninduced protoplasts was affected as well. This indicates that the 47 base pair repeat element is a positive regulatory element. Interestingly, the other deletion constructs Delta 3′ r pUC and Delta 5′ r pUC displayed a similar reduction in expression as Delta 107 pUC. This suggests that both halves of the 47 base pair repeat which contain factor binding sites are required for maintaining the enhancing function of the 47 base pair repeat element.

DISCUSSION

We describe a nuclear protein factor from *Nicotiana tabacum* and *Antirrhinum majus* binding to a positive regulatory element in the *Antirrhinum majus* chalcone synthase promoter. This element is comprised of two direct repeats of 47 base pairs, having 85% homology to each other. Gel retardation assays with synthetic oligonucleotides representing subfragments of either the 3′ half or the 5′ half of the repeat element revealed three nuclear factor binding sites between positions −573 and −593, −594 and −622, and −644 and −663, respectively. Cross-competition experiments indicated that these sites are recognized by the same nuclear factor(s). To define a common motif present in oligonucleotides 3′r I, 3′r II, and 5′r II which serve as recognition sites for the factor, we searched for base exchanges in comparison to the inactive oligonucleotide 5′r I. Oligonucleotides 3′r II and 5′r II occupy equivalent positions within the 47 base pair direct repeat and thus exhibit considerable sequence homology, whereas oligonucleotide 3′r I shows no obvious sequence homology to neither oligonucleotide 3′r II nor oligonucleotide 5′r II. One potential consensus sequence derived by sequence comparison of the three oligonucleotides is ACNYAAA. However, a synthetic oligonucleotide comprising three copies of the sequence CACTTAAA does not compete for binding of the nuclear factor to the 47 base pair repeat element (our unpublished observation). Therefore, it is possible that the factor is able to recognize at least two sequence elements bearing no obvious sequence similarity. Albeit unusual, such properties are not unprecedented: The yeast activator protein HAP 1 has been shown to bind to apparently unrelated sequences within the upstream region of the CYC1 and CYC7 genes (16). The HeLa cell octamer-binding protein recognizes the conserved octamer motif ATGCAAAT found in numerous promoters but is also capable of binding a motif within the herpes simplex virus immediately early promoter which bears as little as 4 matches over a 14-base pair sequence (1). The DNA-binding protein C/EBP, present in rat liver nuclei, binds selectively to the CCAAT homology of several viral promoters as well as to the core homology of many viral enhancers (8). A functional role for the 47 base pair repeat element in the regulation of *chs* gene expression had been revealed previously using the light-responsive parsley protoplast transient expression system (12). It was reported that the 47 base pair repeat element increases the level of *chs* gene expression irrespective of UV-induction when fused to the minimal UV light responsive element (positions −39 to −197) of the *A. majus chs* promoter. Here we demonstrate that deletions within the 47 base pair repeat element reduce the activity of the full length

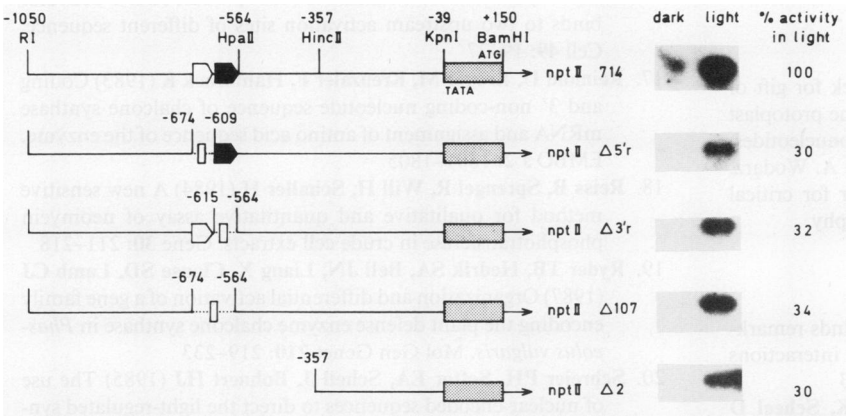

Figure 5. Identification of a positive regulatory element within the *A. majus chs* upstream region by transient expression in parsley protoplasts. In the map of the *chs-npt*II constructs, the dotted region marks the *chs* TATA box and untranslated leader sequence. The unfilled arrow represents the distal (5′) repeat; the filled arrow the proximal (3′) repeat. The deletion endpoints are indicated. At the junction between sequences upstream and downstream of the 47 base pair repeat the internal deletion constructs have residual 21 base pairs of pUC19 polylinker indicated by the vertical bar (5′ GGGGATCGATCCTCTAGAGTC 3′). Construct Delta 3′r pUC has a change in spacing of 43 base pairs, Delta 5′r pUC of 28 base pairs, and Delta 107 pUC of 87 base pairs. For the *npt*II activity test, protoplasts (3×10^6) were transformed with 30 μg DNA of plasmids 714 pUC, Delta 5′r pUC, Delta 3′r pUC, and Delta 107 pUC or 26 μg Delta 2 pUC. Half of the protoplasts were induced by light-irradiation for 15 h and the other half was kept in the dark. *NPT*II activity was quantitated by liquid scintillation counting and is presented as percentage of activity of construct 714 pUC. For dark samples the absolute expression level was found to be too low to be quantitated reliably.

chs promoter to the level of the truncated Delta 2 promoter. This suggests that sequences located between positions −564 and −674 represent a major positive regulatory element within the *chs* promoter upstream region. A similar reduced expression level of construct Delta 107 in comparison to construct 714 has also been observed in transgenic tobacco seedlings (K Fritze, DS, JS, and D Wing, unpublished data). Our data also show that both halves of the 47 base pair direct repeat element are required for the positive regulatory function in parsley protoplasts since deletion of either repeat reduced the level of *chs* gene expression. It is unlikely that the reduced expression of Delta 3′r pUC is due to a change in spacing between the distal (5′) 47 base pair sequence and the TATA proximal promoter region because the 47 base pair repeat element was shown to function in a position-independent manner (12). Our data indicate that two binding sites spanning the proximal (3′) half of the 47 base pair repeat element as well as a small part of the distal (5′) half of the 47 base pair repeat element and one binding site within the distal (5′) element are recognized by the same nuclear factor(s). It is therefore conceivable that at least one binding site is required in each half of the repeat for the enhancer function and redundant binding is actually essential for proper *in vivo* function. This contrasts with the functional redundancy of regulatory elements that has been reported for a light-responsive pea small subunit gene (11). In that case, reiterated binding sites for a nuclear factor, GT-1 (5, 6), are located upstream and downstream of position −170, respectively, and a mutation or deletion of the TATA proximal GT-1 binding sites downstream from position −170 can be compensated by the distal binding sites. Interestingly, this redundancy is dependent on the developmental stage of the plant (11). *In vivo*, the observed degeneracy in binding sites within the 47 base pair repeat element of the *Antirrhinum majus chs* promoter may create a variation in binding affinities which could be relevant for modulating the positive regulatory activity of the 47 base pair repeat element. Unraveling the precise function of the three binding sites has to await the analysis of *in vivo* binding properties of the protein factor(s).

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