

Full Length Research Paper

Analysis of *AVR4* promoter by sequential response-element deletion

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Several reports have associated the variability in physico-chemical properties of avidin protein to dynamism inherent in the consensus regulatory networks within the promoter region of avidin genes. An *Avr4* promoter region ligated to chloramphenicol acetyltransferase plasmid vector (pBLCAT2) to produce recombinant plasmid *Avr4pBLCAT2* was sequentially deleted to produce five distinct mutants: *Avr4pBLCAT2*₉₀₇₋₁₇₆, *Avr4pBLCAT2*₈₀₉₋₁₇₆, *Avr4pBLCAT2*₇₈₉₋₁₇₆, *Avr4pBLCAT2*₄₂₉₋₁₇₆ and *Avr4pBLCAT2*₃₀₂₋₁₇₆. The transformants elicited different chloramphenicol acetyl transferase (CAT) activities.

Key words: Avidin, *AVR4* promoter, chloramphenicol acetyl transferase.

INTRODUCTION

Avidin gene when expressed in oviductal tissues of oviparous animals and in response to inflammation results in the production of a tetrameric protein (Green, 1975; Zerega et al., 2001). The avidin protein is heterogeneous in function and shows variation in its several properties, including biotin binding, thermostability, antigenicity and immunomodulation (Bayer and Wilchek, 1990; Houen and Hansen, 1997; Chinol et al., 1998). These variations seem to provide indications that avidin gene display diversity in structure even within the 5' flanking promoter region containing a network of regulatory cis elements such as hormone response elements (HRE), heat shock response element (HSE) and acute phase protein (APRE) (Yamamoto, 1985; Bienz and Phelam, 1986). These consensus sequences have been found to be highly conserved in several organisms and provide domains for co-operativity via DNA-DNA interactions and serve as enhancer sequences for transcriptional factor mediation of avidin

gene expression (Wallen et al., 1995; Ahlroth et al., 2001a). However, the patterns of interactions, number and type of response elements involved in the regulation of avidin gene expression are inadequately understood, and supportive experimental studies to improve understanding are few. Hybrid avidin mutants have been expressed in baculovirus infected insects to enhance biotin binding for improved application as a detection tool in immunology, histopathology and molecular biology (Airenne et al., 1997). Furthermore, Marttila et al. (2000) engineered the biotin- binding pocket in avidin protein mutants to alter glycosylation and increase stability under high temperatures. By virtue of high conserved sequence and exons homology, avidin related genes (AVRs) have been found as perfect analogues of avidin gene for genetic studies (Laitinen et al., 2002). This is supported by the recovery of *AVR1*, *AVR2* and *AVR3* mRNAs from chicken during inflammatory conditions but no clues as to whether these transcripts express proteins were given (Kunnas et al., 1993). *AVR4* and *AVR5* are the other human avidin related genes that have been extensively studied (Keinanen et al., 1994).

Gene deletion experiments are exceedingly useful in the understanding of gene functions and regulation. In this study, the avidin *AVR4* promoter subcloned into

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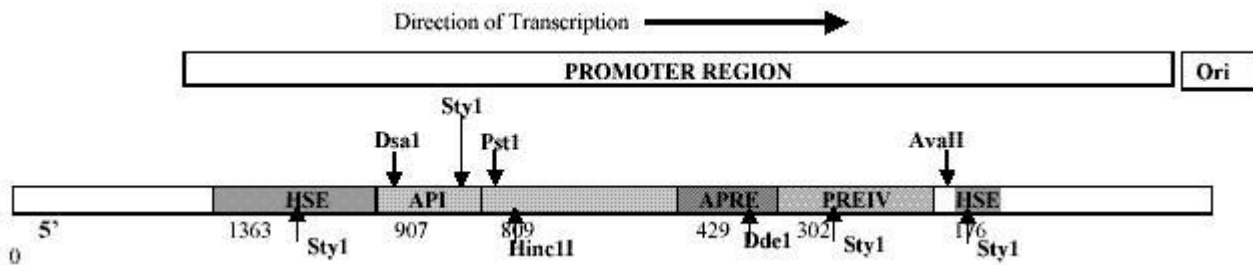


Figure 1. Avidin related gene 4 (*Avr4*) promoter region showing the map positions of consensus response elements from transcription start site and restriction enzyme cleavage sites.

pBLCAT2 expressing chloramphenicol acetyltransferase (CAT) was subjected to timed deletions. The influence of promoter activity was investigated by CAT activity.

MATERIALS AND METHODS

Recombinant plasmid and vectors

Chloramphenicol acetyltransferase plasmid vector (pBLCAT2) and *AVR4* promoter region ligated to pBLCAT2 to produce a recombinant plasmid *Avr4pBLCAT2* was obtained from the Dept of Molecular Medicine University of Jyvaskyla, Finland. Stock culture of *E. coli* HB101 harbouring pBR322 plasmid was obtained from our laboratory.

The nucleotide sequence *AVR4* promoter region investigated was obtained from EMBL database search with accession number Z22883 and used to map the cleavage sites of the restriction endonucleases used (Figure 1).

Creation of 5' and 3' overhanged *Avr4pBLCAT2* plasmids

A 5' overhang of *Avr4pBLCAT2* susceptible to Exonuclease III deletion and 3' overhang resistant to the enzyme activity were obtained by *Sall* and *SphI* digestion, respectively. The linear plasmids were subjected to Exonuclease III digestion at 25°C according to manufacturer's instructions. The assay mixtures were incubated at different time intervals to allow sequential deletion of response elements of *Avr4* promoter within each recombinant plasmid in 5' - 3' direction. The incubation period ranged from 29 - 59 min and was terminated by the addition of 2 l 0.5M EDTA at 69°C for 10min. This was followed by agarose gel electrophoresis and the desired bands were excised and eluted by using the QIAEX II (R) method (QIAGEN Inc, Canada), and re-ligated. *E. coli* DH5 cells were made competent prior to transformation with the plasmids according to the protocol of Hanahan et al. (1983).

Each *AVR4pBLCAT2* mutant was digested with a pair of restriction enzymes to confirm the deletion of an upstream response element and the presence of the expected terminal response element as indicated in Figure 1. For instance a pair of *DsaI* and *StyI* was used to confirm the absence of HSE and the presence API as the terminal response element in *AVR4CAT2* mutant 1 (API-API?-APRE-PREIV-HSE-pLCAT2).

Chloramphenicol acetyl transferase (CAT) assay

CAT activity *in vitro*, as a measure of chloramphenicol degradation in broth was determined by modifying the protocol of Charles and

Alan (1999). Each *Avr4 E.coli* transformant (10^7 cells) was grown aerobically with shaking at 30°C in LB medium containing 20 g/ml chloramphenicol (Sigma, USA). At 2 hourly intervals cells were pelleted by centrifugation (3000 rpm for 5 min) and aliquots of supernatants were extracted with ethylacetate (equal volume). The resulting organic phase was dried under reduced pressure and heat and the extract reconstituted with 20 l ethylacetate. The concentration of chloramphenicol in the reconstituted extract was determined by extrapolation from chloramphenicol standard curve as previously described (Shaw, 1975) CAT activity was measured as the micrograms of chloramphenicol degraded per hour under the assay conditions.

RESULTS AND DISCUSSION

In vitro Exonuclease III deletion of the 5' and 3' overhanged *Avr4pBLCAT2* generated five distinct mutants at different incubation periods (Table 1). The CAT activities of the constructs are recorded in Figure 2.

The present study is aimed at understanding the importance of the upstream response elements in the regulation of avidin gene expression. The obliteration of *DsaI* cleavage site but the presence of *StyI* site confirmed API? as the 5'terminal response elements in mutant 1. That mutant 2 has API as its 5'terminal cis element was established by the absence of *PstI* site within the API? locus of mutant 1. Meanwhile, *HincII* cleavage within the API terminal of mutant 2 was the cause of the 786bp, 1521bp and 2811bp bands observed in this study. Similarly, *DdeI* produced different fragment length in mutants 3 and 4 to confirm APRE and PREIV as their terminal cis elements. Finally, the obliteration of *AvaII* site in mutant 5 confirmed PREIV deletion from the precursor mutant. While *StyI* established HSE as the terminal regulatory motif in this mutant. Similar sequential deletion studies have been conducted elsewhere (Ahlroth et al., 2001b).

The observed increased CAT activity among the transformants implies decreased promoter activity with decreased promoter length, indicating the importance of the different promoter elements.

In conclusion, the successful transformation of *E. coli* DH5 with all the generated mutants as observed in this study coupled with altered gene expression suggests a

Table 1. AVR4 promoter constructs generated at different time intervals.

Mutant	Promoter element(s)	Generation time (min)
Avr4pBLCAT2 ₉₀₇₋₁₇₆	API-API?-APRE-PREIV-HSE	32
Avr4pBLCAT2 ₈₀₉₋₁₇₆	API?-APRE-PREIV-HSE	32
Avr4pBLCAT2 ₇₈₉₋₁₇₆	APRE-PREIV-HSE	36
Avr4pBLCAT2 ₄₂₉₋₁₇₆	PREIV-HSE	41
Avr4pBLCAT2 ₃₀₂₋₁₇₆	HSE	45

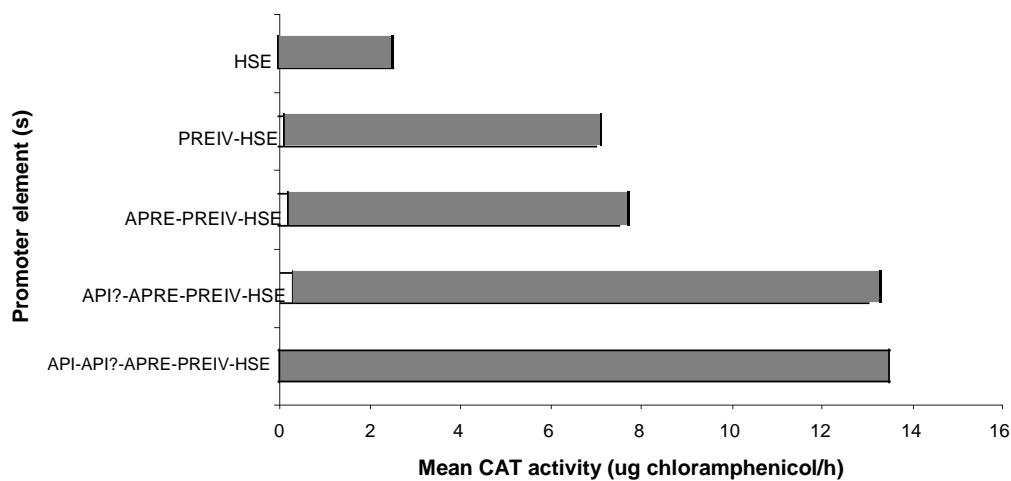


Figure 2. Mean CAT activity of the Avr4 promoter deletion constructs.

possible means of studying avidin gene expression. There is also the need to ascertain the stability of these mutants for long-term storage and use towards elucidating the mechanisms of avidin gene expression as well as applicability in future avidin-biotin technological challenges.

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