

Isolation and Characterization of the Promoter and Partial Enhancer Region of the Porcine Inter- α -Trypsin Inhibitor Heavy Chain 4 Gene

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A porcine genomic library was screened for clones containing the promoter of the major acute-phase protein in pigs, inter- α -trypsin heavy chain 4 (ITIH4). Following isolation of the promoter, a functional analysis was performed with Hep3B cells. The promoter was induced by interleukin-6 (IL-6) but not by IL-1 β . However, IL-1 β was shown to inhibit the IL-6-induced activation of the porcine ITIH4 promoter.

Outbreaks of infectious disease in livestock can prove costly, and persistent infections can result in economic losses that run into billions of euros (15, 17, 20). The current control methods (vaccination, antibiotics, culling) are rarely successful in controlling infectious diseases. Given the costs involved in controlling outbreaks of infectious disease in livestock with conventional methods, there is clearly a need for alternative approaches to improving disease resistance in livestock.

Transgenic technology offers a new approach to increasing disease resistance in animals (for a review, see reference 14). Of particular interest to us is the use of cytokine gene-encoding constructs. However, when such constructs are used, it is essential that the expression of the cytokine gene be controlled; otherwise, lethal pathological effects are sometimes seen (13, 21). To address this problem, we recently described an inducible expression system based on the human C-reactive protein gene (4) which would allow induction of cytokine gene expression when it is needed, i.e., during an infection. The aim of this study was to investigate whether it is possible to modify the system for use in animals of economic importance. Here we describe the isolation and characterization of the region approximately 1.6 kb upstream of the major acute-phase protein in pigs, inter- α -trypsin inhibitor heavy chain 4 (ITIH4).

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The PigE BAC library (1) was screened by using the porcine ITIH4 cDNA clone described by Buchman et al. (3) as a probe. The library screen resulted in the isolation of eight putative positive clones (Table 1). Two separate PCRs with primers designed from published sequence data (3, 11) were performed to identify true-positive clones. This analysis confirmed that the clones were positive for BAC1, BAC2, and BAC7 (data not shown). To check if each clone contained the ITIH4 promoter, a PCR was performed with a 5' primer from the human ITIH4 sequence (18) and a primer from the cDNA sequence of the

porcine ITIH4 gene, designed so that the PCR product would span a reported 1.4-kb intron. These experiments revealed that only BAC1 and BAC7 contained the porcine ITIH4 promoter and partial enhancer region (Fig. 1). The isolated sequence from BAC1 was subsequently cloned and sequenced and logged in the GenBank database. Surprisingly, although the sequence mapped to the segment of human chromosome 3 containing the human ITIH4 gene, homology between the two promoters was limited (about 65%). Although this finding was unexpected, this may explain why in pigs ITIH4 is the major acute-phase protein (12), whereas in humans it is only a minor one (for a review, see reference 5). Indeed, it has previously been proposed that the species specificity of the acute-phase response is due to the absence of specific transcription factors or changes in the DNA sequence of the promoter which affect binding of the transcription factors and, consequently, gene expression (6). Sequence analysis of the promoters for putative transcription factor binding sites revealed that the same binding sites were present in both promoters but that their positions and frequencies differed. For example, a putative binding site for an interleukin-6 (IL-6)-responsive element (22) was found at positions 1427 to 1432 of the submitted sequence (GenBank accession number AY737719), and a putative nuclear factor IL-6 binding site (7) was found at positions 1010 to 1019. Of particular interest was the finding that there were three LF-A1 binding sites (10) in the region 1 kb upstream of the start codon of the ITIH4 gene (positions 1225 to 1230, 1315 to 1320, and 1343 to 1348). Hardon et al. (10) proposed

TABLE 1. Putative positive BAC clones

PigE BAC library clone identification	Clone renamed as:
PigE BAC 037a02	BAC1
PigE BAC 044h21	BAC2
PigE BAC 049g22	BAC3
PigE BAC 096n06	BAC4
PigE BAC 203b11	BAC5
PigE BAC 231i07	BAC6 ^a
PigE BAC 246i06	BAC7
PigE BAC 258c15	BAC8

^a Did not grow and was excluded from further analysis.

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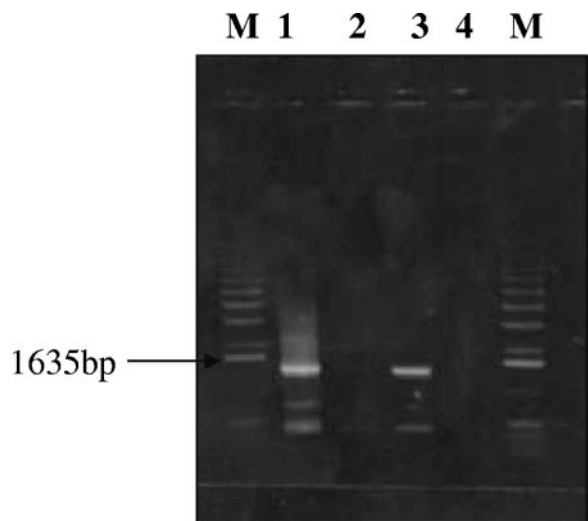


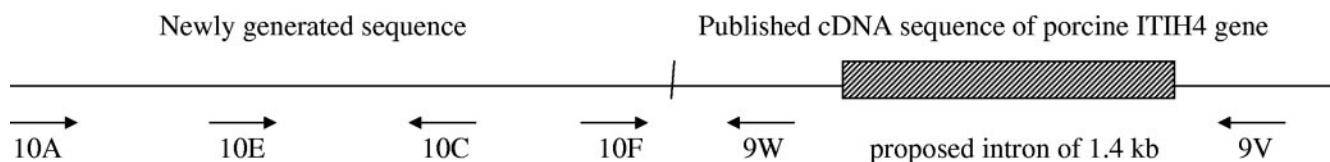
FIG. 1. Confirmation by PCR that the proposed intron of 1.4 kb and the promoter region of the porcine ITIH4 gene are present in BAC1. This region is also present in BAC7 but absent in BAC2. Lanes: M, 1-kb DNA ladder (Invitrogen); 1, BAC 1; 2, BAC2; and 3, BAC7. The 1.6-kb band of the DNA ladder is indicated with an arrow.

that the affinity of the LF-A1 transcription factor for its binding site in the promoter region of the haptoglobin gene as well as the number of binding sites to which LF-A1 could strongly bind influenced the basal levels of expression of the gene. Significantly, in the corresponding region upstream of the human ITIH4 gene there is only one LF-A1 site.

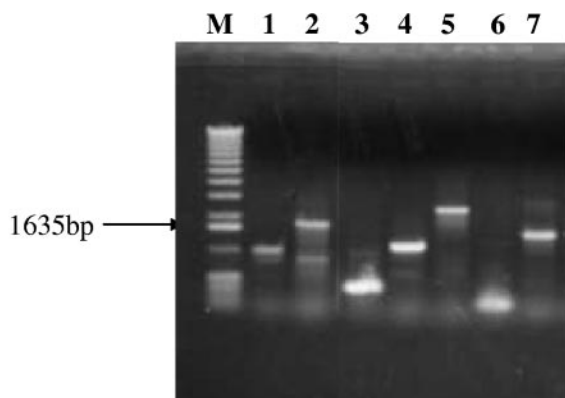
In order to confirm that the porcine ITIH4 promoter had been isolated, a series of PCRs were performed with the newly generated porcine ITIH4 promoter sequence and the published porcine ITIH4 gene sequence. As shown in Fig. 2, all the reactions gave PCR products of the expected size, strongly suggesting that the porcine ITIH4 promoter had indeed been isolated.

To confirm that the ITIH4 promoter was functional and responded to an inflammatory stimulus, a number of expression studies were performed with the chloramphenicol acetyltransferase (CAT) reporter system. The porcine ITIH4 promoter and partial enhancer region was cloned in the pCAT3-Basic vector (Promega) and transfected in the human hepatoma cell line Hep3B, which has been used extensively in studies of the acute-phase response. Although

(A)



(B)



(C)

Lane	Primer pairs	Expected size	Actual size
1	10A + 10C	~ 1 kb	1 kb
2	10A + 9W	~ 1.7 kb	1.7 kb
3	10E + 10C	~ 500 bp	~ 400 bp
4	10E + 9W	~ 1 kb	1.1 kb
5	10E + 9V	~ 2.8 kb	2.5 kb
6	10F + 9W	~ 170 bp	170 bp
7	10F + 9V	~ 1.6 kb	1.6 kb

FIG. 2. PCR to confirm that the promoter isolated is that of the porcine ITIH4 gene. (A) Schematic diagram (not to scale) of the promoter region and the 5' end of the porcine ITIH4 gene, showing the location of the primers used for the PCR; (B) electrophoretic analysis of the PCR products; (C) table showing the primer combinations as well as the expected and the actual sizes of the PCR products. As seen in the table, all PCRs gave products of the expected size. The exception was the PCR product in lane 3, which gave a smear. This was because we were unable to generate a defined band using this primer pair.

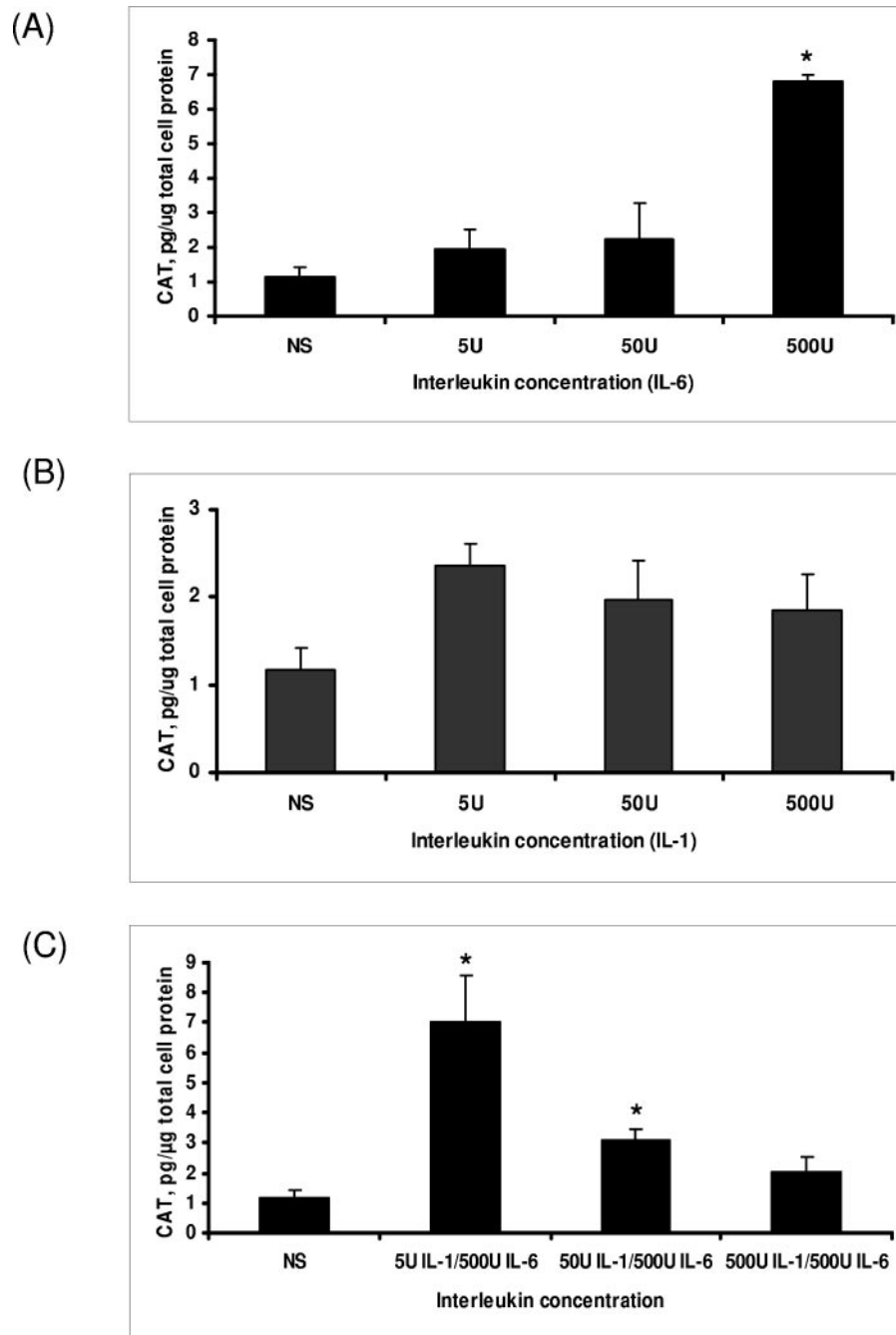


FIG. 3. In vitro analysis of the inducibility of the porcine ITIH4 promoter in response to various concentrations of IL-6 (A), IL-1 β (B), and a combination of IL-6 and IL-1 β (C). Hep3B cells were seeded in six-well plates 24 h prior to transfection. Cells were transfected with 2 μ g DNA by using the GenePorter transfection reagent (GeneTherapy Systems). After 24 h the growth medium was removed and replaced with medium containing the appropriate concentration of interleukins. After 48 h the cells were harvested. The amount of CAT produced was measured by enzyme-linked immunosorbent assay (Roche), and the total protein concentration was determined by the method described by Bradford (2). The data shown are representative data from one experiment performed in triplicate. All experiments were performed at least twice independently, with similar results. *, $P < 0.05$ (Mann-Whitney U test).

it is desirable to perform these studies in a porcine cell line, such a cell line was not commercially available.

Previous studies have shown that ITIH4 is a class II acute-phase protein that is induced only by IL-6 (9, 16). From our studies we could also show that the isolated promoter and partial enhancer region was induced only by human recombi-

nant IL-6, with a significant sixfold increase in expression seen following treatment with 500 U IL-6 ($P < 0.05$, Mann-Whitney U test) (Fig. 3A). Stimulation with human recombinant IL-1 β did not significantly affect expression of the reporter gene (Fig. 3B). Therefore, our findings are in good agreement with previously published data. However, previously published stud-

ies on the expression of the ITIH4 gene have not addressed the effect of a combination of cytokines on gene expression. As expression of other acute-phase genes (8, 23) has been shown to be affected by a combination of cytokines, we therefore investigated the effect of a combination of 500 U IL-6 together with different combinations of IL-1 β . Unexpectedly, we found that increasing concentrations of IL-1 β resulted in inhibition of the IL-6-induced expression (Fig. 3C). A combination of 500 U IL-6 and 5 U IL-1 β resulted in a sixfold increase in expression, which was similar to that seen in cells stimulated with 500 U IL-6 alone. However, a combination of 500 U IL-6 with either 50 U IL-1 β or 500 U IL-1 β resulted in a significant ($P < 0.05$) decrease in the expression of the CAT gene. A combination of 500 U IL-6 with 50 U IL-1 β resulted in a 2.5-fold increase in CAT expression, although this is still significantly higher ($P < 0.05$) than that by cells receiving no stimulation. However, a combination of 500 U IL-6 and 500 U IL-1 β abolished the inducibility of the construct seen when the cells were stimulated with IL-6 alone. The level of expression of the CAT gene is similar to that of cells receiving no stimulation. A number of other studies have also noted this phenomenon, whereby IL-1 β inhibits the activity of IL-6 and the inducibility of the gene. Zuraw and Lotz (24) showed that stimulation of HepG2 cells with IL-6 or gamma interferon results in an increase in C1 inhibitor secretion, whereas IL-1 β antagonizes the effect of IL-6 on the C1 inhibitor. In the case of the fibrinogen gene, which is also a class II acute-phase gene, IL-1 β is believed to act by inhibiting the activation of STAT-1 by IL-6 (19).

In conclusion, we have isolated the porcine ITIH4 promoter and confirmed that it is functional in vitro. Sequence analysis of the promoter and comparison of the sequence with that of the human ITIH4 promoter may give insight to the species specificity of the acute-phase response. Additional studies with porcine hepatic cells will allow assessment of the suitability of the porcine ITIH4 promoter for use in an acute-phase expression system and further studies on the downregulation of the IL-6-induced activation of the ITIH4 gene by IL-1 β .

Nucleotide sequence accession number. The sequence isolated from BAC1 has been logged in the GenBank database under accession number AY737719.

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