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

Niamh Harraghy* and Timothy J. Mitchell
Institute of Biomedical and Life Sciences, Division of Infection and Immunity, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Received 17 June 2005/Returned for modification 28 July 2005/Accepted 18 August 2005

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-1β. However, IL-1β 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).

(This research was conducted by N.H. in partial fulfillment of the requirements for a Ph.D. from the Division of Infection and Immunity, University of Glasgow.)

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 AF737719), 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 approximately 1.6 kb upstream of the major acute-phase protein in pigs, inter-α-trypsin inhibitor heavy chain 4 (ITIH4).

The PigE BAC library

| 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 231107                     | BAC6*           |
| PigE BAC 246h06                     | BAC7            |
| PigE BAC 288c15                     | BAC8            |

* Corresponding author. Present address: Institute of Medical Microbiology and Hygiene, University of Saarland Hospital, Building 43, D-66421 Homburg/Saar, Germany. Phone: 49 6841 1623900. Fax: 49 6841 1623985. E-mail: bhnhar@uniklinik-saarland.de.
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, 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

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.

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.
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 recombinant 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-1β 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-1β. Unexpectedly, we found that increasing concentrations of IL-1β resulted in inhibition of the IL-6–induced expression (Fig. 3C). A combination of 500 U IL-6 and 5 U IL-1β 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-1β or 500 U IL-1β 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-1β or 500 U IL-1β 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-1β 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-1β 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-1β 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-1β 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-1β.

**Nucleotide sequence accession number.** The sequence isolated from BAC1 has been logged in the GenBank database under accession numberAY737719.

This work was funded by a BBSRC CASE studentship with assistance from PIC (a subsidiary of Sygen International).

We thank Gary Evans at Sygen International for helpful discussions and critical reading of the manuscript. We are also grateful to Susan Anderson and Alan Archibald at the Roslin Institute, United Kingdom, for their assistance in the screening of the porcine BAC genomic library. The sequence isolated from BAC1 has been logged in the GenBank database under accession numberAY737719.

### REFERENCES

1. Anderson, S. I., N. L. Lopez-Corrales, B. Gorick, and A. L. Archibald. 2000. A large-fragment porcine genomic library resource in a BAC vector. Mammalian Genome 11:811–814.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248–254.
3. Buchman, T. G., D. E. Cabin, S. Vickers, C. S. Deutschman, E. Delgado, M. M. Sussman, and G. G. Bulkley. 1990. Molecular biology of circulating shock. Part II. Expression of four groups of hepatic genes is enhanced after resuscitation from cardiogenic shock. Surgery 108:559–566.
4. Burke, B., A. Pridmore, N. Harraghy, A. Collick, J. Brown, and T. Mitchell. 2004. Transgenic mice showing inflammation-inducible overexpression of granulocyte macrophage colony-stimulating factor. Clin. Diagn. Lab. Immunol. 11:588–596.
5. Choi-Miura, N. H. 2004. Novel human plasma proteins, IHRP (acute phase protein) and PHBP (serine protease), which bind to glycosaminoglycans. Curr. Med. Chem. Cardiovasc. Hematol. Agents 2:239–248.
6. Ciliberto, G., R. Arcone, E. P. Wagner, and U. Ruther. 1987. Inducible and tissue-specific expression of human C-reactive protein in transgenic mice. EMBO J. 6:4017–4022.
7. Fainst, S., and S. Meyer. 1992. Compilation of vertebrate-encoded transcription factors. Nucleic Acids Res. 20:23–26.
8. Gantner, U., A. Roche, C. Toniatti, G. Moronne, and G. Ciliberto. 1989. Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. EMBO J. 8:3773–3779.
9. Gonzalez-Ramon, N., K. Hoebe, M. A. Alava, L. Leengoed, M. Pineiro, S. Carmona, M. Iturralde, P. Lampreave, and A. Pineiro. 2000. Pig MAP/ITIH4 and hapto globulin are interleukin-6–dependent acute-phase plasma proteins in porcine primary cultured hepatocytes. Eur. J. Biochem. 267:1878–1885.
10. Muller, E. M., M. Frain, G. Paonessa, and R. Cortese. 1988. Two distinct factors interact with the promoter regions of several liver-specific genes. EMBO J. 7:1711–1719.
11. Hashimoto, K., T. Tobe, J. Sumiya, Y. Sano, N.-H. Choi-Miura, A. Ozawa, H. Yasue, and M. Tomita. 1996. Primary structure of the pig homologue of human IHRP: inter-alpha-trypsin inhibitor family heavy chain-related protein. J. Biol. Chem. 269:557–564.
12. Lampreave, F., N. Gonzalez-Ramon, S. Martinez-Ayensa, M.-A. Hernandez, H.-K. Lorenzo, A. Garcia-Gil, and A. Pineiro. 1994. Characterization of the acute phase serum protein response in pigs. Electrophoresis 15:672–676.
13. Lang, R. A., D. Metcalf, R. A. Cuthbertson, I. Lyons, E. Stanley, A. Kelso, G. Kannourakis, D. J. Williamson, G. K. Klintworth, T. J. Gonda, and A. R. Dunn. 1987. Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. Cell 51:675–686.
14. Muller, M. G., and B. Proctor. 1996. Intracellular, genetic or congenital immunisation—transgenic approaches to increase disease resistance of farm animals. J. Biotechnol. 44:233–242.
15. Ngategie, P. K., T. Beckel, and G. Tilahun. 1993. Financial losses caused by ovine fascioliasis in the Ethiopian highlands. Trop. Anim. Health Prod. 25:155–161.
16. Pineiro, M., M. A. Alava, N. Gonzalez-Ramon, J. Osada, P. Lasierra, L. Larrad, A. Pineiro, and F. Lampreave. 1999. ITIH4 serum concentration increases during acute phase processes in human patients and is up-regulated by interleukin-6 in hepatocarcinoma HepG2 cells. Biochem. Biophys. Res. Commun. 263:224–229.
17. Satakkamp, H. W., P. B. M. Berentsen, and H. S. Horst. 2000. Economic aspects of the control of classical swine fever outbreaks in the European Union. Vet. Microbiol. 78:221–237.
18. Saguchi, K., T. Tobe, K. Hashimoto, Y. Sano, Y. Nakano, N.-H. Miura, and M. Tomita. 1995. Cloning and characterization of cDNA for inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP), a novel human plasma glycoprotein. J. Biol. Chem. 270:14–18.
19. Shen, X., Z. Tian, M. J. Holtzman, and B. Gao. 2000. Cross-talk between interleukin-1β (IL-1β) and IL-6 signalling pathways: IL-1β selectively inhibits IL-6–activated signal transducer and activator of transcription factor 1 (STAT1) by a proteosome–dependent mechanism. Biochem. J. 352:913–919.
20. Stegeman, A., A. Elbers, H. De Smit, H. Moser, J. Smak, and F. Phuimers. 2000. The 1997–1998 epidemic of classical swine fever in The Netherlands. Vet. Microbiol. 73:183–196.
21. Taverne, J. 1993. Transgenic mice in the study of cytokine function. Int. J. Exp. Pathol. 74:525–546.
22. Wegenka, U. M., J. Buschmann, C. Lutticken, P. C. Heinrich, and F. Horn. 1993. Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level. Mol. Cell. Biol. 13:276–288.
23. Zhang, D., S. L. Jiang, D. Rzewnicki, D. Samols, and I. Kushner. 1995. Characterization of the 117:143–148.
24. Zuraw, B. L., and M. Lota. 1990. Regulation of the hepatic synthesis of C1 inhibitor by the hepatocyte stimulating factors interleukin-6 and interleukin-1b. J. Biol. Chem. 265:12666–126670.