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Mechanisms of Regulation of Polymeric Immunoglobulin Receptor Expression: Cytokine Induction and Tissue Specificity.

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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January, 1996
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GRADUATE STUDIES

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November 13, 1995

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Mechanisms of Regulation of Polymeric Immunoglobulin Receptor Expression:
Cytokine Induction and Tissue Specificity.

Abstract

by

KENNETH ROBERT YOUNGMAN

Secretory immunoglobulins form the first line of antigen-specific defense at mucosal surfaces. In the human intestine polymeric immunoglobulins (dimeric IgA and pentameric IgM) are produced by plasma cells in the lamina propria and secreted via polymeric immunoglobulin receptor (pIgR) mediated transcytosis across the intestinal epithelium. In the human colon carcinoma cell line HT-29, pIgR expression is up-regulated by the recombinant cytokines IFN-γ, TNF-α, and IL-4.

We have observed synergistic stimulation of pIgR expression by human rIFN-γ, rTNF-α, and rIL-4. We have further demonstrated that activation of freshly isolated human intestinal lamina propria mononuclear cells induces production of natural IFN-γ, TNF-α, and IL-4, which act synergistically as potent stimulators of pIgR expression in HT-29.74 cells. In our studies, IFN-γ was the central regulator of pIgR expression.

We have cloned the pIgR gene and determined the site at which transcription of mRNA is initiated. A fragment of the pIgR gene (-563 bp to +29 bp, relative to the start site of transcription) confers both basal
transcriptional activity and IFN-γ inducible transcription upon chimeric prompter-reporter constructs in transiently transfected HT-29.74 cells.

Our studies have also revealed a unique cis-element in the in the pIgR gene, which we have named the intestinal epithelial specificity element or IESE. Intestinal epithelial cell nuclear proteins bind the IESE, and levels binding activity correlate with levels of pIgR expression in human intestinal cell lines which express pIgR, and in primary human intestinal epithelial cells. The transcription factor interferon regulatory factor-1 was induced in HT-29.74 cells by IFN-γ and binds an interferon stimulus response element located in the first exon of the pIgR gene. The interactions of these two cis-elements, and their with trans-acting factors found in intestinal epithelial cells provide the first insights into the mechanisms by which expression of pIgR, a vital component of mucosal immune responses, is regulated.
This dissertation is dedicated to the memory of my grandmother, Josefine Reissman. She would have shared it, as she did everything she had, with the rest of our family; my mother, my father, my sister Eva, my nephews Brian and Sam, and my nieces Wendy and Christine. My grandmother lived through The Holocaust and still taught me to seek the beauty inherent to our world.
I would like to acknowledge the people who contributed directly to the work presented in this dissertation. I am grateful to Dr. Kote Rao Chintalacharuvu for his work in subcloning the HT-29.74 cell line and in developing the secretory component ELISA. I thank John France for his efforts in cloning the plgR gene and developing the RNase protection assay. Carol Tamer and May Hsieh Blanchard also helped with the cloning of the plgR gene. Kim Phillips, in Dr. Kaetzel's lab at the University of Kentucky, was instrumental in subcloning plgR gene fragments into luciferase expression vectors and executing transient transfection experiments. I thank Paula Hempen for her help with EMSA. I am especially grateful to Dr. Janet Piskurich for her persistence in cloning the plgR gene, which laid the ground work for much of this dissertation. I thank Dr. Claudio Fiocchi for his collaboration in the study of intestinal mononuclear cell cytokine induction of plgR expression, and Dr. Alan Levine for providing the IL-4 used in these studies. I thank Dr. Michael Lamm for his support, and my advisor Dr. Charlotte Kaetzel, without whom, none of this would have been accomplished.

My committee members, Drs. David Kaplan, Ganes Sen, and Dennis Templeton have helped me become a better scientist. Their constructive criticism and insightful guidance are much appreciated. My interactions with them were an ideal type of education; like sitting under a tree with my teachers, learning fascinating things.
It took me a long time to get to graduate school, so there are many people who helped me who helped me along the way. First and foremost I thank my parents Albert and Ann Youngman who have pushed me to pursue my education from my earliest memories. Perhaps this is because they were themselves denied these opportunities, but they are the smartest, hardest working people I know. They have supported my education from nursery school through Ph.D. candidacy.

In grade school, my classmates identified me as a science nerd. Back then it was the solar system and dinosaurs, but it was the beginning. Biology became the focus of my scientific curiosity at Stuyvesant High School. There, the faculty and students taught one another, and I discovered that the more you learn the more you are. In particular, Ralph Ferraro, my biology teacher, and Frank McCort, my English teacher for three semesters, taught me to love to learn.

I thank Dr. Jim Zull who gave me my first job in science. This was a collaboration with Dr. Arnold Caplan, and for the first time I had a real laboratory to play in. I learned a great deal from both of them.

In 1980 it was my great fortune to meet Dr. Claudio Fiocchi. He gave a job in immunology, and the opportunity to see far I could take it. From him, I learned the art of research. In him, I found my best friend. We have maintained a productive working relationship for 15 years, and become better friends every year. When circumstances lead my graduate advisor to leave Case Western Reserve University, Claudio helped fill that very large void. I do not where I would be without him.
In 1986 Gail West came to work with Claudio and me. She brought a vibrant spirit to the lab, and the three of made a good team. Gail helped me see the world in a different way. I will always look to her for enlightenment.

Mike McKee helped me decide what to do when I grew up. The fact that this occurred when I was thirty didn’t make it any easier. He helped me find confidence I did not know I had.

Dr. Alan Levine introduced me to the field of molecular biology in 1987, and has been an invaluable source of scientific guidance, and good friend, ever since. It is rare and rewarding to make an instant connection with someone and have it grow into the kind of genuine friendship we share. I am grateful he arrived in Cleveland before my departure, giving us a chance to discover this.

When the decision to go back to school was made, Dr. Alan Tartakoff was the first contact I made. He gave me sound advice, even if his answers to my questions were not the ones I wanted to hear. He was my first teacher, the second time around, and perhaps the best one I could have had. His intense curiosity and keen intellect inspire me to this day. Alan is perhaps the most gifted teacher I have encountered and it has been a privilege to be his student.

Among my new classmates, I have made great friends. Lief Stordal, Milita Panin, Nywanna Sizemore, John Biesterfeld, Carol Tamer, and Sue Burden, and I studied together for core molecular biology and they made it fun for me. Once again, the students learned from each other, and not just molecular biology. Kathy O’Conner and Lynn Horton studied separately, but
were part of a very close group of friends. These are a very special group of people who made me feel like a kid again.

I found a new home in Charlotte Kaetzel's lab. I thank Janet Robinson for taking care of me in the lab, and for her sensitive and warm friendship. Tom Blanchard always had a kind word and a patient ear. John France, John Nedrud, Norma Sigmund, May Hsieh Blanchard, Dave Fletcher, Janet Petara, Chris Brown, Dennis Huang, Steve Czinn, and Michael Lamm all helped make it a comfortable place to work. Carol Tamer and Melita Panin, my classmates as well as labmates always made things interesting. And then there is Janet Piskurich. What can I say about JP that she hasn't said already! JP and I worked very closely together for three years and did good work together. In many ways we knew each other too well. We exasperated each other on an almost daily basis, and perhaps because of that became fast friends. I miss you JP.

I also had two neighbors who took me in. The Templeton Lab was my refuge when I needed to get away and do "real molecular biology". Dennis also provided brewing tips, sound scientific advice, and kept the computers running. He is a true Renaissance Man, and if the money runs is one scientist who will be able to carry on in his garage with spare parts from where ever he can find them. Lynn Horton has been my best friend in graduate school. I have fond memories of The Cleveland Orchestra, sushi, and much more. Malathy Mahalingham brings warmth and good humor everywhere she goes. Thanks for letting me whine and always making me feel better. Margaret Lewis brought cheer and Indians' World Series tickets! Joe Deak and Minhong Yan were always there to help with technical problems, and discuss
the big issues from Chinese history to the sports page. In 1993 Claudio Fiocchi’s Lab moved to CWRU, and my home at the Cleveland Clinic was now five floors down. Having Claudio and Gail so close made coming to work fun again. Mike Dobrea let me “steal” anything I needed and helped me get back in the groove many times. Teaching others is rewarding to me because it repays the debt owed to those who have taught me. David Binion, Alessandro Musso, and Kenji Ina gave me an opportunity to share some of what I’ve learned the past five years, and I gained many insights into clinical aspects of research from them.

There is an axiom, "If you’re going to work hard, you have to play hard." I have always found this to be the case. I guess it’s lucky that I’ve had some good playmates during my time in graduate school. Thanks Dad, for ski trips that helped keep me going. The Yeastie Boys were lots of fun, and we brewed some tasty stuff. Thanks to Dave Singleton, Joel Kreps, and Mike Hoffman for sharing nights around the brew pot. "Warp 6" and "The Army of Darkness" will strike fear in the hearts of Cleveland Heights volleyball teams for years to come. Bob Botti, Marion Korosec, Jason Tasch, Tracy Mourton, and Daynna Wolff have been my teammates and good friends. Sailing with the members of Highlander Fleet 14 at Edgewater, and the other fleets at National’s, Pipers’, and even Atwood were a joy. Frank and Mary Anne Gerry, Rick, Chris, and Bill Meyers, Jim Mounts, Roger Walko, and the rest provided stiff competition, but the crews of Highlander 747 were the best. Dave Singleton and Rick Pepler helped me blow off steam when I needed it most. But thanks most of all to you Cap’n Vinicky. We make a great team
and I thank you, and the Admiral, for your generosity and friendship. May you always have “FREETIME AIR!”.

I have a few special friends for whom I have special thanks. Chris Block helped me through some difficult times when I went back to school. We are now both Yankee fans who came to love the Tribe. Joel Kreps was a brewing buddy who became my close friend. I will never forget our discussions of life and science. Joel introduced me to Michael Hoffman, and I discovered that architects can be both as interesting and boring as biologists. Daynna Wolff’s warmth and intensity are a rare combination, and make her an ideal teammate and wonderful friend. She is an exemplar scientist and person. Bob Botti has been my friend for seventeen years and has always been like a sharpening stone for me. I thank you marrying Marion, I would not have gotten to know her otherwise. I thank Tom and Sue Piccorelli for their friendship through the years, and for making me feel like a part of their family. Chick Carroll and Lois Geist are kind of friends that make time and distance irrelevant. And then there is Steve Krug, who has been my friend forever. His coming back to Cleveland was a gift I cherished, especially because it gave me the chance to get to know Lori. I miss you both, and hope the future brings us together again.

Lisa Hall came into my life at a stressful time for both of us. You helped me through the graduate school end game and eased the transition from my realm in Cleveland to yours in the Bay Area. I know how hard this was, considering your love of your home for more than a decade, and our feelings for each other. Thank you for your love and friendship.
I am very grateful to Dr. Michael Laimin for establishing the department I have been a part of for the past five years. His example defines the term mentor. The members of the faculty of the Department of Pathology have been a priceless resource for Case Western Reserve University, especially the departments’ students.

Finally, I must thank my advisor, Dr. Charlotte Kaetzel. I chose to work with Dr. Kaetzel because she is the most dedicated teacher I know, and I have learned so much from her. She is proof that a warm heart and a sharp mind are not mutually exclusive.
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ABBREVIATIONS

bp base pair
dIgA dimeric IgA
Ca+2 ionophore calcium ionophore A-23187
CAT chloramphenicol acetyltransferase
DEPC diethyl pyrocarbonate
DTT dithiothrietol
fSC free secretory component
GAF interferon-γ activated factor
GAS interferon-γ activated sequence
h hours
HBSS Hank's balanced salt solution
hPTEC human primary intestinal epithelial cells
Ig immunoglobulin
kb kilobases
IESE intestinal epithelial specificity element
IFN interferon
IL interleukin
IRF interferon regulatory factor
ISGF interferon stimulated gene factor
ISRE interferon-stimulated response element
EMSA electrophoretic mobility shift assays
Jak Janus kinase family
LPMC intestinal lamina propria mononuclear cells
<table>
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<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>plg</td>
<td>polymeric immunoglobulins, dimeric IgA and pentameric IgM</td>
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<tr>
<td>plgR</td>
<td>polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td>PIPES</td>
<td>1,4-piperazine diethanesulfonic acid</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light units</td>
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<tr>
<td>SC</td>
<td>secretory component</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
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<tr>
<td>slg</td>
<td>secretory immunoglobulins</td>
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<td>slgA</td>
<td>secretory IgA</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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CHAPTER 1

INTRODUCTION
The polymeric immunoglobulin receptor and its role in secretory immunity

Among the earliest observations in the discipline of immunology were those of host defense functions in the body's secretions, including the presence of specific antibodies in the feces of dysentery patients (1). Several groups studying the mechanisms of these defense functions discovered differences between systemic and local immune responses (1).

The immunoglobulins, originally referred to as γ-globulins, were initially discovered as a class of serum proteins with electrophoretic mobility different than albumin, α-globulins, and β-globulin (2). They have also been found to be a diverse population of antigen binding molecules, generated by the recombination of multiple gene segments, and the antigen specific component of humoral immunity. The basic unit of immunoglobulin structure was characterized as two identical light chains and two identical heavy chains linked by disulfide bonds (2). Five subclasses of human immunoglobulins have been designated IgA, IgD, IgE, IgG, and IgM (2). IgA and IgM were found to be polymers of the basic unit (dimers and pentamers, respectively) associated with an additional polypeptide chain, referred to as the J- (joining) chain (2).

Chodirker and Tomasi presented evidence of differences in the ratios of IgG : IgA found in human serum as compared with ratios found in several body fluids and secretions, including small intestinal fluid and bile (3). In a subsequent study it was demonstrated that IgA in saliva and colostrum differed from IgA in serum both immunochemically and in susceptibility to disulfide bond reduction in the absence of urea (4).
By the 1970's IgA was recognized as the predominant antibody in the secretions of man and several other species (1, 5). Secretory IgA (sIgA) was shown to differ from other immunoglobulins (including serum IgA) in that it contained a specific, usually covalently linked polypeptide (5). This secretory component (SC), also found in human milk unassociated with Ig (free SC or fSC), was shown to be capable of binding IgA \textit{in vitro} producing complexes closely resembling sIgA (6). Immunohistochemistry revealed both SC and IgA associated with mucosal epithelial cells and provided evidence of differential sub cellular localization (6). Both free and IgA complexed SC were found at the cell membranes and apical cytoplasm, while only SC was detected in the Golgi region. These observations lead Brandtzaeg to propose that SC functions as a specific epithelial receptor for IgA and in the secretion of the resulting complex into lumenal spaces (6). A subsequent study of isolated colonic epithelial cells showed both free SC and SC coupled to polymeric immunoglobulins (pIg) on the surface of these cells (7). These data demonstrated that colonic epithelial cells express SC on their cell surface where it can bind pIg.

In 1978 Crago, et al, reported that a human colon carcinoma cell line (HT-29) was capable of intracellular and cell surface binding of pIg (IgA and IgM) (8). This binding was demonstrated to be SC dependent by the specific blocking of binding by anti-SC antisera. In the title of their report the authors defined SC as, "...a surface receptor for polymeric immunoglobulins." Subsequently, several groups began to refer to the membrane bound pIg binding form of SC as the polymeric immunoglobulin receptor (pIgR). The term SC was originally based on association with secreted antibody, and as
described above, plgR is synthesized and inserted into the basolateral plasma membrane prior to this association.

The maintenance of a physical barrier against harmful agents in the environment is of paramount importance to an organism's survival. While protecting the host, such barriers might also prevent various immune defense mechanisms from attacking environmental pathogens and toxins. The mucosal immune system has evolved separate mechanisms to detect and eliminate pathogens (see Fig. 1.1). The afferent response begins in lymphoid follicles such as the Peyer's patch, where specialized epithelial cells called M-cells absorb potential antigens from the lumen and pass them on to antigen presenting cells in the dome region. Putative antigens are presented to naive lymphocytes, which upon recognition, begin the process differentiation and maturation into mature effector cells, such as plasma cells. Lymphocytes activated in mucosal sites home back to these tissues where they participate in the efferent response, in the case of plasma cells, by producing antibodies to antigens detected during the afferent phase. In the specific transport of plg across mucosal epithelial barriers plgR plays a crucial role in immune defense.

**plgR protein structure**

Human plgR is an integral membrane glycoprotein. The primary sequence of human fSC was initially determined by protein sequencing (9). Later, using cDNA probes derived from rabbit plgR clones (10), a partial human plgR cDNA was cloned (11), and finally cDNA's containing the entire coding sequence were cloned (12, 13). The deduced amino acid sequence had
Figure 1.1 The afferent and efferent arms of the mucosal immune response and the function of pIgR in facilitating effector functions of sIgA.
an overall length of 764 amino acids, including an 18 amino acid signal peptide and was 99% homologous with the sequence derived directly from fSC protein (11). The above data revealed 5 Ig like domains in the 80 kD extra-cellular domain, a 23 amino acid hydrophobic transmembrane domain, an 11 kD cytoplasmic domain, and 56% and 64% homology to the amino acid sequences of rat and rabbit pLgR, respectively (13).

Transcytosis

pLgR is synthesized on the rough endoplasmic reticulum, trafficked through the Golgi complex and inserted in the basolateral plasma membrane of polarized epithelial cells (14). The intracellular trafficking of pLgR is regulated, at least in part, by its cytoplasmic domain. In canine kidney epithelial cells (MDCK) transfected with a rabbit pLgR cDNA, a 14 amino acid sequence in the 103 amino acid cytoplasmic domain of rabbit pLgR targets the receptor to the basolateral compartment (14). Deletion of this sequence results in sorting of the mutant protein to the apical plasma membrane, and its insertion into an apically targeted protein misdirects it to the basolateral domain (14). Once at the basolateral surface pLgR is endocytosed and translocates to the apical surface. Receptor bound plg as well as unoccupied receptor are endocytosed. Efficient transcytosis of rabbit pLgR in MDCK cells is associated with phosphorylation of a serine residue its cytoplasmic domain (15). Serine 644 is phosphorylated in this system and replacement of this amino acid with alanine (a neutral amino acid) results in decreased transcytosis and increased recirculation to the basolateral domain.
Replacement of serine 644 with asparagine (a negatively charged amino acid) results in increased transcytosis and decreased recirculation (15).

Membrane bound pIgR is proteolytically cleaved releasing fSC or sIg at the apical plasma membrane of polarized epithelium or the bile canalicular membrane (15, 16). The precise location of cleavage (intracellular or cell surface) has not been conclusively determined in intestinal epithelial cells, but has been localized to bile canalicular membranes in rat hepatocytes (16).

pIgR gene structure

The structure of the human pIgR gene including the intron-exon organization were published in 1992 by Krajci, et al (17). The sequences coding for the first, fourth, and fifth Ig like domains are reported to be contained on separate exons, domains 2 and 3 on a single exon, and the cytoplasmic tail encoded by four exons. The human gene structure is similar to that found in rabbits, where alternative splicing results in the synthesis of 2 mRNA species, one containing all 5 Ig like domains, the other lacking domains 2 and 3 (18).

The human pIgR gene has been mapped to human chromosome region 1q31→q41 by Southern blot analysis of a panel of human-rodent somatic cell hybrids and in situ chromosomal hybridization (13, 19).

Cytokine regulation of pIgR expression

Cytokines are soluble proteins that mediate interactions between cells. They are multi-functional, and form regulatory networks coordinating complex intercellular interactions including immunity and inflammation (20,
In human colonic epithelial cells, plgR expression is regulated by IFN-γ, TNF-α, and IL-4 (22-24).

Kvale et al., reported additive induction of total cellular SC, cell surface SC, and active secretion of SC by IFN-γ and TNF-α in HT-29 colon carcinoma cells (23). These effects were dose dependent for both cytokines with IFN-γ more potent than TNF-α. Time courses for both cytokines, alone or in combination showed small induction in total cellular SC at 12 hours and steadily increasing levels up to 50 hours. Induction of major histocompatibility complex (MHC) class II was also additively induced by IFN-γ and TNF-α, however TNF-α alone had no effect in the absence of IFN-γ.

In studies by Phillips et al., induction of plgR expression (in the same colon carcinoma cell line) by IFN-γ and IL-4 was synergistic (24). PlgR expression was measured as binding of 125I-labeled plgA. Binding was blocked by unlabeled plgA, anti-SC rabbit antisera and unaffected by IgG, monomeric IgA, or plgA Fab fragments. Induction by both cytokines was dose dependent. IL-1α, IL-2, IL-3, IL-5, and IL-6 had no effect on plgR expression alone and IL-2 and IL-6 had no effect on induction by IL-4 and IFN-γ, alone or in combination.

We have observed synergistic stimulation by human recombinant IFN-γ and TNF-α, IFN-γ and IL-4, TNF-α and IL-4, and the three cytokines combined in human colonic epithelial cells (HT-29.74) (see Chapter 2). We have also obtained cytokine containing culture supernatants from stimulated human intestinal lamina propria mononuclear cells (see Chapter 2). These supernatants contain IFN-γ, TNF-α, and IL-4, and are potent inducers of plgR protein expression in colonic epithelial cells. Antibody-mediated
neutralization of IL-4 (which is present in very low concentrations) had no effect on induction. TNF-α neutralization reduces induction significantly. IFN-γ neutralization abolishes 80 to 90% of induction and had the same effect as simultaneous neutralization of all 3 cytokines, demonstrating that induction by TNF-α is through synergy with IFN-γ. These experiments demonstrate that cytokines produced by intestinal mononuclear cells have the capacity to regulate pIgR expression in intestinal epithelial cells. IFN-γ, TNF-α, IL[4, and its mechanism of action are briefly described below.

Human IFN-γ, a 146 amino acid glycoprotein, also known as type II and immune interferon, is produced by T-lymphocytes and NK cells (25). Examples of IFN-γ responsive cells are lymphocytes, macrophages, astrocytes, and epithelial cells. Recently, there have been major advances in our understanding of the mechanisms of IFN-γ signal transduction and transcriptional activation (26-28). The IFN-γ receptor, composed of α and β subunits, associates with two tyrosine kinases, JAK1 and JAK2, upon ligand binding, resulting in the phosphorylation of the 91 kD latent transcription factor STAT 1 (26-28). Phosphorylated STAT 1 forms dimers (sometimes referred to as gamma activating factor or GAF), translocates to the nucleus, and binds DNA elements referred to as gamma activation sites (GAS) (26-28). GAS elements have been found in the regulatory sequences of several IFN-γ immediate activation genes, including guanylate binding protein (GBP), FcγR1, Ly-6E, monokine induced by IFN-γ (MIG), interferon consensus sequence binding protein (ICSBP), interferon regulatory factor family, and keratin (26, 27, 29). Some of these immediate activation genes are themselves transcriptional regulatory factors, and in turn activate the transcription of a
second set of IFN-\(\gamma\) inducible proteins, including MHC class I and class II antigens, \(\beta_2\)-microglobulin, invariant chain, \((2'\cdot\cdot\cdot5')(\Lambda_h)\) synthetase, RNase L, dsRNA dependent protein kinase (12, 23, 30).

Human TNF-\(\alpha\) is a 157 amino acid glycoprotein produced mainly by monocytes and macrophages and to a lesser extent by T-cells, B-cells, NK cells, neutrophils, astrocytes, endothelial cells, and smooth muscle cells (31). The majority of human cells bind TNF-\(\alpha\) with high affinity stimulating numerous signal transduction pathways including activation of PKC, protein kinase A, and EGF tyrosine kinase (32). TNF-\(\alpha\) also stimulates the intracellular release of diacylglycerol and prostaglandins (31). The transcription factor NF-\(\kappa\)B is activated by TNF-\(\alpha\), as is de novo synthesis of the transcription factors AP-1 (c-fos and c-jun), IRF-1, IRF-2, NF-IL-6, and NF-GMa (33).

Human IL-4 is a 143 amino acid glycoprotein produced by T-cells (34). Cell types responsive to IL-4 include B-cells, T-cells, macrophages, mast cell lines, and megakaryocyte, granulocyte, and erythroid precursors (34). IL-4 stimulates MHC class II, low affinity \(\text{Fc}_{\varepsilon}\) receptor expression, and Ig class switching in B-cells and modulates T-cell cytotoxicity (35). In human B-cells, IL-4 stimulates rapid and transient increases in inositol 1,4,5-triphosphate and intracellular calcium and a delayed sustained increase in cAMP (36). These events are not associated with increased protein phosphorylation. In murine B-cells IL-4 induction of MHC class II expression is blocked by inhibition of intracellular calcium release (37). IL-4 induces MHC class II mRNA expression in mouse pre-B cell lines in 1 hour independent of protein synthesis (38). In murine B-lymphoma cells, reporter constructs containing
MHC class II promoter elements are IL-4 inducible and levels of a constitutive class II promoter specific DNA binding protein are increased by IL-4 exposure (39).

**Regulation of eukaryotic mRNA transcription**

As previously mentioned, plgR expression in human colonic epithelial cells is regulated by IFN-γ, TNF-α, and IL-4 (22-24). The response to IFN-γ is preceded by a proportional increase in steady state plgR mRNA (12, 40). Increased mRNA was not observed until 24 hours after IFN-γ exposure while protein expression did not appear until 48 hours. Cycloheximide treatment completely blocked increased mRNA levels, demonstrating dependence of induction on *de novo* protein synthesis. Therefore, mechanisms of regulation of transcriptional initiation may be relevant to the regulation of plgR expression.

The first step in the flow of genetic information from DNA sequence to protein expression is the transcription of RNA. For this to occur an RNA polymerase must bind DNA in a specific region and transcribe the appropriate sequence. In the case of most eukaryotic genes mRNA is transcribed by RNA polymerase II (RNA pol II) (41, 42). This enzyme does not bind directly to DNA, rather it binds to a variety of DNA-protein complexes. The DNA elements to which these proteins bind are referred to as cis-acting elements. Such elements only effect the transcription of sequences to which they are directly linked. Proteins which bind cis-acting elements are referred to as trans-acting factors, reflecting the notion that such factors can influence transcription of sequences not linked to their own coding sequences. Trans-acting factors produced in response to various
Developmental and environmental cues can regulate the transcriptional activity of various genes by binding to cis-regulatory elements of those genes and increasing the probability of RNA pol II initiating transcription. In this way the basal or constitutive activity of a promoter can by enhanced or repressed in response to specific stimuli such as hormones, growth factors, or cytokines. Thus, the transcriptional activity of a gene can be programmed by the arrangement of regulatory elements, and modulated by the activation or production of various trans-acting factors in the cell (41, 42).

Conclusion

In this dissertation I will present evidence of the physiologic significance of cytokine regulation plgR expression. This will consist of experiments demonstrating that mononuclear cells isolated from an anatomic site in close association with the intestinal epithelium produce cytokines which up-regulate plgR expression. I will then present data relevant to molecular mechanisms of regulation of plgR expression. These data will describe cis-elements in the plgR promoter proximal region which: 1) bind intestinal epithelial cell specific nuclear proteins, and 2) bind the IFN-γ inducible transcription factor IRF-1.
CHAPTER 2

Cytokines produced by human intestinal mononuclear cells
up-regulate polymeric immunoglobulin receptor expression
in intestinal epithelial cells
ABSTRACT

The polymeric immunoglobulin receptor (pIgR) mediates transcytosis of polymeric IgA across mucosal epithelia. Expression of this receptor in HT-29.74 human colon carcinoma cells is up-regulated by the recombinant cytokines IFN-γ, TNF-α, and IL-4. Here we demonstrate that activation of freshly isolated human intestinal lamina propria mononuclear cells (LPMC) induces production of natural cytokines, and these act synergistically as potent stimulators of pIgR expression in HT-29.74 cells. LPMC from normal colonic mucosa were stimulated with PMA and calcium ionophore A-23187. The resulting supernatants consistently induced dose-dependent increases in pIgR expression by HT-29.74 cells, up to 130-fold. Analysis of 4 separate LPMC supernatants revealed mean concentrations of 8260 pg/ml for IFN-γ, 420 pg/ml for TNF-α, and 15 pg/ml for IL-4. Antibody-mediated neutralization of these cytokines suggested that the central regulator of pIgR expression in these supernatants was IFN-γ. IL-4 neutralization had no effect on induction and TNF-α neutralization slightly reduced induction. In contrast, IFN-γ neutralization abolished up to 93% of pIgR induction and had essentially the same effect as simultaneous neutralization of all 3 cytokines. In conclusion, our data demonstrate that natural cytokines, predominantly IFN-γ, produced by stimulated human intestinal lymphocytes and macrophages have the capacity to dramatically up-regulate pIgR expression in an intestinal epithelial cell line, strongly suggesting that their action in vivo leads to enhancement of local defense functions mediated by IgA.
SECRETORY Ig provide the first line of antigen-specific defense at mucosal surfaces (43-46). In the human intestine, pIg (dimeric IgA and pentameric IgM) are produced by plasma cells in the lamina propria and are secreted via receptor-mediated transcytosis across intestinal epithelial cells (47). Transcytosis is mediated by the polymeric Ig receptor (pIgR) and regulated by intracellular targeting signals located in its intracellular domain (14, 15, 48, 49). First, pIgR is inserted in the basolateral plasma membrane of polarized epithelial cells where it can bind pIg in the extracellular fluid. Both pIgR-pIg complexes and unoccupied receptors are then endocytosed and translocated to the apical surface, where pIgR is proteolyticly cleaved between the extracellular domain and transmembrane domain. This cleavage event causes the release of either slg (pIg and the associated extracellular domain of pIgR, also referred to as SC) or unoccupied extracellular domain of pIgR (also known as SC) into the luminal space (50). Thus, pIgR facilitates the traditional function of slg in host defense (neutralization of pathogens and toxins before they enter the body proper) as well as newly postulated defense functions including intra-epithelial cell viral neutralization and the elimination of pIg containing immune complexes (47, 51-53).

Regulation of immune responses involves complex interactions among multiple cell types. Communication between LPMC and intestinal epithelial cells is mediated, in part, by soluble factors referred to as cytokines, which form intricate networks regulating and coordinating immunological responses (20, 21, 54). In the human colonic adenocarcinoma cell line HT-29, pIgR expression is up-regulated by at least three cytokines: IFN-γ, TNF-α, and
IL-4 (12, 22-24, 40). Solin et al. reported dose-dependent increases in pIgR expression by HT-29 cells in response to IFN-γ exposure (22). Kvale et al. reported additive induction of total cellular and cell surface pIgR, and secreted SC by IFN-γ and TNF-α in the same cells (23). These effects were dose-dependent for both cytokines, IFN-γ being more potent than TNF-α. In subsequent studies by Phillips et al., IFN-γ and IL-4 acted synergistically in the induction of pIgR expression in HT-29 cells (24). IL-1α, IL-2, IL-3, IL-5, and IL-6 alone had no effect on pIgR expression, and addition of IL-2 or IL-6 had no effect on induction by IL-4 and IFN-γ. IFN-γ has been shown to up-regulate pIgR mRNA levels in HT-29 cells by a protein synthesis dependent mechanism (12, 40). Furthermore, Wira et al. reported that IFN-γ enhanced pIgR expression in the rat uterus in vivo (55). Another cytokine, TGF-β1 has been reported to enhance pIgR expression in a rat epithelial cell line (56).

The intestinal lamina propria is richly populated by mononuclear cells with the capacity to produce these cytokines, thereby providing a potential source for local regulation of pIgR mediated responses (57-63). LPMC differ from cells isolated from the periphery in their state of activation and capacity to produce cytokines (64-68). Given the close anatomic association of the intestinal lamina propria and epithelial cells expressing pIgR, mononuclear cells residing in this compartment are likely to provide important regulatory signals coordinating epithelial pIgR expression with other effector mechanisms, such as increased pIg production.

Here we report the synergistic stimulation of pIgR expression by human rIFN-γ, rTNF-α, and rIL-4. More importantly, we demonstrate that stimulated LPMC produce natural IFN-γ, TNF-α, and IL-4, and that culture
supernatants derived from these cells are potent inducers of epithelial cell plgR expression. Induction is dose-dependent and is specifically inhibited by IFN-γ and TNF-α neutralizing antibodies, with IFN-γ neutralization abrogating the inductive capacity of these supernatants.

MATERIALS AND METHODS

Cell Culture

The derivation of the HT-29.74 cell line, selected for high expression of plgR, has previously been described (69). Cells were maintained in culture medium (RPMI-1640 medium, 10% FCS, 100 mM sodium pyruvate, 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and 20 mM HEPES, pH 7.3) at 37°C in a humidified 5% CO2 atmosphere. Cells from passage numbers 8-12 were used in these experiments. Cells were plated at 1.5x10⁵/cm²; 48 h later culture medium was removed and replaced with culture medium containing recombinant cytokines, LPMC supernatants, or culture medium alone (controls). At 72 h the media were replaced with identical fresh media, and at 96 h the culture supernatants were collected and stored at -20°C. Cells were detached with trypsin and EDTA, counted using a hemacytometer, and viability determined by trypan blue exclusion. All reagents for cell culture were obtained from Life Technologies (Grand Island, NY).

Recombinant Cytokines

Human rIFN-γ (>98% pure, 1x10⁷ U/mg protein) and human rTNF-α
(>95% pure, 1x10⁷ U/mg protein) were obtained from R&D Systems (Minneapolis, MN). Human rIL-4 (>98% pure, 2x10⁷ U/mg protein) was generously provided by Dr. Alan D. Levine (Searle Research and Development, St. Louis, MO).

Measurement of pIgR Protein Expression

Concentrations of SC in HT-29.74 cell culture supernatants were determined by ELISA as previously described (69). Briefly, microtiter plates were coated with guinea pig anti-human SC serum and then incubated with either purified human SC (diluted in culture medium) or experimental samples. Bound SC was detected by incubation with rabbit anti-human SC serum, followed by alkaline phosphatase conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) and phosphatase substrate (Sigma, St. Louis, MO). Data are expressed as ng SC/10⁵ cells. We previously demonstrated that induction of pIgR mRNA and protein by IFN-γ is paralleled by increased SC secretion (12); therefore, measurement of SC in culture supernatants gives an index of SC production as well as secretion.

Northern Analysis of pIgR mRNA Expression

Total cellular RNA was isolated from HT-29.74 cells by a modification of the technique of Chomcynski and Sacchi (70). Briefly, cells were cultured as described above in 35 mm culture dishes. After 6, 12, or 24 h in culture, with either control or cytokine containing media, cells were rinsed twice with warm PBS and lysed in Trizol (Life Technologies, Grand Island, NY). One tenth volume of chloroform was added, the mixture shaken, and incubated at
room temperature for 5 min. After centrifugation (30 min, 12,000 X g, 4°C) the aqueous phase was collected and RNA precipitated by addition of an equal volume of isopropanol and centrifugation as above. RNA was washed in cold 70% ethanol and dissolved in DEPC treated H2O. Twenty micrograms of each RNA was denatured with glyoxal and DMSO, size fractionated by electrophoresis in 1% agarose, and transferred to Genescreen (DuPont NEN Research Products, Boston, MA) by electro-blotting. The filter was baked at 80°C for 2 h and prehybridized for 3 h in 50% formamide, 5X SSPE, 5X Denhart’s solution, 5% (w/v) dextran sulfate and 100 mg boiled single stranded salmon sperm DNA. Hybridization was performed under the same conditions in the presence 10^6 cpm/ml human pIgR specific probe and 10^6 cpm/ml human α-tubulin specific probe.

The pIgR probe spanning nt -8 to +462 of the human pIgR cDNA sequence (12) was generated by PCR (Perkin-Elmer Cetus, Norwalk, CT) in the presence of [^32P]-dCTP without the addition of unlabeled dCTP (71). Human pIgR cDNA was used as template with primers corresponding to nt -8 to +17 and the reverse complement to nt +439 to +462 (12). The final concentration of reactants in 100 μl reaction mixtures were 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001% (w/v) gelatin, 20 μM each dATP, dGTP, dTTP, 0.5 μM[^32P]-dCTP (3,000 Ci/mmol; DuPont, NEN Research Products), 0.1 μM of each primer, 10 pg template DNA/μl, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus). Amplification was performed using a DNA thermal cycler (Perkin-Elmer Cetus) for 40 cycles of 1 min at 94°C, 2.5 min at 55°C, and 4 min at 72°C, followed by a final elongation for 10 min at 72°C. The α-tubulin probe, corresponding to nt 22-204 (relative to the start
Preparation of LPMC Supernatants and Measurement of Cytokine Concentrations

Surgical specimens were obtained from four patients undergoing resection for adenocarcinoma of the colon. The tissue used was at least 10 cm distal from the tumor, and was macroscopically and histologically normal. LPMC were isolated as described (73). In brief, the dissected intestinal mucosa was freed of mucus with dithiothreitol and of epithelial cells with EDTA. The tissue was then digested with collagenase and DNase (both from Worthington Biochemical Corp., Freehold NJ). The resulting crude cell suspension was purified using a Ficoll-Hypaque gradient and resuspended in RPMI-1640 (Whittaker Bioproducts, Walkersville, MD). The composition of the final cell suspension has previously been defined in many reports (65, 68, 74). Cells prepared using this technique from comparable tissues contain approximately 60% T cells (CD2/3+; 45% CD4+, 15% CD8+); 2% NK cells, 15% macrophages, and 20% B cells. The cells were cultured at 5x10^6 cells/ml in RPMI-1640, stimulated for 3h with 40 ng/ml PMA and 0.5 μM Ca^{2+} ionophore (both from Sigma Chemical Co., St. Louis, MO), washed 3 times, resuspended in the original volume of RPMI-1640 with 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and 20 mM HEPES, pH 7.3), and cultured for 2 days. The cultures were centrifuged, the supernatants
filtered (0.22 μm), and stored at -70°C. Parallel control cultures were also prepared without stimulation.

IFN-γ, TNF-α, and IL-4 concentrations in LPMC supernatants were determined by ELISA, using commercially available reagents (Endogen, Inc., Boston, MA, for IFN-γ and TNF-α, and R&D Systems, Minneapolis, MN, for IL-4). The sensitivity of these assays were 5 pg/ml, 10 pg/ml, and 3 pg/ml, respectively.

**Antibody Mediated-Cytokine Neutralization**

Natural cytokines contained in LPMC supernatants were neutralized with the following antibodies: monoclonal mouse anti-human IFN-γ (The Green Cross Corporation, Osaka, Japan), monoclonal mouse anti-human TNF-α (Clone F12, Olympus Immunochemicals, Lake Success, NY), and polyclonal goat anti-human IL-4 (R&D Systems, Minneapolis, MN). The neutralizing dose for each antibody was determined by titration against recombinant human cytokine induced pIgR expression in HT-29.74 cells. Culture media containing 210 pg rIFN-γ/ml, 3,160 pg rTNF-α/ml, or 100 ng rIL-4/ml were incubated with serial dilutions of the appropriate neutralizing antibody overnight at 4°C. HT-29.74 cells were then cultured with these media for 48 h (see Cell Culture, above). Antibody concentrations of 10 neutralizing units/ml, 1 μg/ml, and 1 μg/ml were required to neutralize the above concentrations of rIFN-γ, rTNF-α, and rIL-4, respectively. Antibody concentrations sufficient to neutralize at least twice the cytokine level present in LPMC supernatants (as measured by ELISA) were used in all experiments. Neutralizing antibodies were added to LPMC supernatants diluted in culture.
medium and incubated at 4°C for 24 h prior to addition to HT-29.74 cell cultures.

**Statistical Analyses**

Differences in pIgR expression among various groups were tested by one way analysis of variance and pairwise multiple comparisons made using the Students-Newman Keuls method. Analysis was performed using the SigmaStat program (Jandel Scientific, San Rafael, CA).

**RESULTS**

**Induction of pIgR expression by recombinant cytokines**

Treatment of HT-29.74 cells with IFN-γ, TNF-α, and IL-4 revealed dose-dependent induction of pIgR expression by each cytokine, as determined by measurement of SC in culture supernatants (Fig. 2.1). Half maximal responses were observed at approximately 600, 300, and 100 pg/ml for IFN-γ, TNF-α, and IL-4 respectively. Maximal responses were 16, 8, and 7 fold above control levels for IFN-γ, TNF-α, and IL-4 respectively.

We observed a marked synergistic effect on induction of pIgR expression by these three cytokines when combined at maximally stimulatory doses (2,100 pg/ml IFN-γ, 8,700 pg/ml TNF-α, and 5,000 pg/ml IL-4), minimally stimulatory doses (21 pg/ml IFN-γ, 87 pg/ml TNF-α, and 50 pg/ml IL-4), and intermediate doses (210 pg/ml IFN-γ, 870 pg/ml TNF-α,
Figure 2.1 Dose dependent induction of pIgR expression in HT-29.74 cells by recombinant cytokines.

Cells were cultured for 2 days with the indicated concentrations of rIFN-γ, rTNF-α, or rIL-4, and concentrations of SC in culture supernatants were determined by ELISA. Data are expressed as ng SC/10^5 cells (mean ± SD, n=4)
and 500 pg/ml IL-4), (Fig. 2.2 A). These responses were respectively, 3, 2, and 5 times the additive responses observed when cells were cultured with each cytokine separately. Synergistic induction of pIgR expression was also observed when we exposed HT-29.74 cells to any combination of two of these recombinant cytokines (Fig 2.2 B).

Northern blot analysis of HT-29.74 cell RNA failed to detect pIgR mRNA in control cells (Fig. 2.3). However, exposure to IFN-γ, TNF-α, and IL-4 resulted in weak but detectable levels at 6 h, stronger induction at 12 h and the greatest response at 24 h (Fig. 2.3). The three cytokines combined at 1/10 and 1/100 of the concentrations employed separately appeared to demonstrate cooperative induction (Fig. 2.3), however, since basal levels of pIgR expression were undetectable by this technique, it was impossible to determine if this induction was synergistic or additive. We have previously detected pIgR mRNA by RNase protection analysis (12).

**Analysis of cytokine concentrations in LPMC supernatants**

In order to determine if mononuclear cells isolated from an anatomic site in close association with intestinal epithelial cells produce cytokines which regulate pIgR expression, we prepared culture supernatants from activated human LPMC. IFN-γ, TNF-α, and IL-4 concentrations were determined by ELISA (Table I). The concentrations of IFN-γ and TNF-α fluctuated according to the expected variability encountered with human samples. In all 4 supernatants, IFN-γ concentrations were by far the highest, with intermediate TNF-α concentrations and relatively low IL-4.
A. Synergistic induction of pIgR expression in HT-29.74 cells by recombinant cytokines.

Cells were cultured for 2 days with the following concentrations of rIFN-γ, rTNF-α, and rIL-4: Low dose, 0.021 ng/ml IFN-γ, 0.087 ng/ml TNF-α, and 0.05 ng/ml IL-4; Medium dose, 0.21 ng/ml IFN-γ, 0.87 ng/ml TNF-α, and 0.5 ng/ml IL-4; High dose, 2.1 ng/ml IFN-γ, 8.7 ng/ml TNF-α, 5 ng/ml IL-4. Control cultures were treated with culture medium alone. Concentrations of SC in culture supernatants were determined by ELISA. Data are expressed as ng SC/10^5 cells (average of 2 cultures).

B. Synergistic induction of pIgR expression in HT-29.74 cells by recombinant cytokines.

Cells were cultured for 2 days with the following concentrations of rIFN-γ, rTNF-α, and rIL-4 alone or in the indicated combinations: 2.1 ng/ml IFN-γ, 8.7 ng/ml TNF-α, 5 ng/ml IL-4. Control cultures were treated with culture medium alone. Concentrations of SC in culture supernatants were determined by ELISA. Data are expressed as ng SC/10^5 cells (average of 2 cultures).
Figure 2.5: Time dependence of induction of pIgR mRNA expression in HT-29.74 cells by recombinant cytokines.

Northern blot analysis was performed using 20 μg total cellular RNA from cells cultured for the indicated times with the following concentrations of rIFN-γ, rTNF-α, and rIL-4: 10 ng/ml IFN-γ, 10 ng/ml TNF-α, 5 ng/ml IL-4. In the panel labeled "Combined 1/10", cells were treated with 1 ng/ml IFN-γ, 1 ng/ml TNF-α, 0.5 ng/ml IL-4, and in "Combined 1/100" with 0.1 ng/ml IFN-γ, 0.1 ng/ml TNF-α, 0.05 ng/ml IL-4. Control cultures were treated with culture medium alone. Bands hybridizing to probes specific for pIgR and α-tubulin mRNA are indicated.
<table>
<thead>
<tr>
<th>LPMC Supernatant</th>
<th>[IFN-γ] (pg/ml)</th>
<th>[TNF-α] (pg/ml)</th>
<th>[IL-4] (pg/ml)</th>
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<tr>
<td>#1</td>
<td>9,520</td>
<td>250</td>
<td>9.0</td>
</tr>
<tr>
<td>#2</td>
<td>13,600</td>
<td>208</td>
<td>17</td>
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<td>#3</td>
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<td>16</td>
</tr>
<tr>
<td>#4</td>
<td>1,070</td>
<td>153</td>
<td>18</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>8,260±2,270</td>
<td>420±188</td>
<td>15±1.8</td>
</tr>
</tbody>
</table>

* Concentrations determined by ELISA*
Induction of pIgR expression by human intestinal lamina propria mononuclear cell culture supernatants

LPMC supernatants induced striking dose-dependent increases in pIgR expression by HT-29.74 cells, up to 130-fold at 10% (v/v) dilution (Fig. 2.4). Supernatant concentrations above 10% were not used as they compromised HT-29.74 cell viability and recovery (data not shown). This effect was also observed with combined rIFN-γ and rTNF-α at concentrations present in LPMC supernatants at dilutions above 10%. Supernatants obtained from cultured unstimulated LPMC did not affect pIgR expression (data not shown). Supernatants derived from Raji cells (a human B-cell line) treated with PMA and Ca++ ionophore, or direct exposure to PMA plus Ca++ ionophore (at the concentrations used to stimulate LPMC) failed to affect HT-29.74 cell pIgR expression, demonstrating that residual concentrations of these reagents, if present, were unlikely to have contributed to stimulation of pIgR expression by LPMC supernatants.

We prepared culture medium containing rIFN-γ, rTNF-α, and rIL-4 at concentrations 10% of those measured in each LPMC supernatant (derived from Table I). All 4 of these mixtures stimulated pIgR expression significantly above control levels (p.<0.05, Fig. 2.5). However, each LPMC supernatant induced significantly higher levels of pIgR expression than the corresponding mixtures of recombinant cytokines (p.<0.05, Fig. 2.5).
Figure 2.4  Induction of pIgR expression in HT-29.74 cells by culture supernatants from activated lamina propria mononuclear cells (LPMC).

HT-29.74 cells were cultured for 2 days with the indicated dilutions of LPMC supernatant. The level of pIgR expression in control cultures treated with culture medium alone is indicated by the dashed line. Concentrations of SC in culture supernatants were determined by ELISA. Data are expressed as ng SC/10^5 cells (average of 2 cultures).
Figure 2.9. Comparison of the induction of pigK expression in HT-29.74 cells by LPMC supernatants and recombinant cytokines (rIFN-γ, rTNF-α, and rIL-4) at levels present in each supernatant. HT-29.74 cells were cultured for 2 days with culture medium containing 10% of the indicated LPMC supernatant (Sup.) or culture medium containing recombinant cytokines at 10% of the concentrations shown in Table I. Control cultures were treated with culture medium alone. Concentrations of SC in culture supernatants were determined by ELISA. Data are expressed as ng SC/10^5 cells (mean ± S.D., n=4)
IFN-γ is the central regulator of pIgR induction by LPMC supernatants

Antibody-mediated cytokine neutralization was employed in order to determine the relative contributions of the cytokines found in LPMC supernatants to induction of pIgR expression (Fig. 2.6). Neutralization of IL-4 did not significantly affect induction of pIgR expression by any of the LPMC supernatants (p>0.05). Neutralization of TNF-α inhibited induction of pIgR expression by 16-30%, and was statistically significant for LPMC supernatants 2, 3, and 4 (p<0.05). Interestingly, the inhibition of pIgR induction by anti-TNF-α was no greater for supernatant 3 than for supernatants 2 and 4, even though the concentration of TNF-α was 5 times greater in supernatant 3 (Table I). By far the most potent inhibition of pIgR induction (80-93%) was achieved by neutralization of IFN-γ. This inhibition was significant for all for all 4 supernatants (p<0.05) and reduced pIgR expression to levels that were not significantly different from control cultures (p>0.05). No additional inhibition was observed when anti-TNF-α and anti-IL-4 antibodies were combined with anti-IFN-γ antibody. We examined the effect of simultaneous neutralization of TNF-α and IL-4 in supernatant 3 and found those results to be the same as neutralization of TNF-α alone (data not shown).

DISCUSSION

The regulation of pIgR expression in intestinal epithelial cells by cytokines produced by mononuclear cells derived from the intestinal lamina propria provides evidence for an important mechanism of communication between LPMC and epithelial cells in regulating transcytosis of IgA (12, 22-24, 40). We report here that rIFN-γ, rTNF-α, and rIL-4 are capable of marked
Figure 2.6 Antibody mediated neutralization of cytokines in LPMC supernatants.

HT-29.74 cells were cultured for 2 days with culture medium containing 10% of the indicated LPMC supernatant (Sup.) in the presence or absence of the indicated neutralizing Ab(s). Control cultures were treated with medium alone. Concentrations of SC in culture supernatants were determined by ELISA. Data are expressed as ng SC/10^5 cells (mean ± S.D., n=4)
synergistic regulation of pIgR expression in HT-29.74 cells. Previous reports have been limited to the investigation of the effects of combinations of two of these cytokines. In one study the cooperative induction of pIgR expression by rIFN-γ and rTNF-α was simply additive (23). In contrast, we observed a synergistic response, that is, the response to simultaneous exposure to rIFN-γ and rTNF-α was 2-3 times greater than the sum of individual responses. In another study, the combination of IFN-γ and IL-4 resulted in a synergistic effect approximately equal to twice that of the sum of the individual responses (24), similar to the results we observed. Using previously untested combinations of recombinant cytokines we found that TNF-α and IL-4 induced pIgR expression synergistically (2 fold the sum of the additive responses) and the three cytokines, when combined, induce levels of pIgR expression far greater than those attainable by exposure to individual cytokines, even at high concentrations. Our data clearly demonstrate that maximal induction of pIgR expression in this system requires the presence of multiple cytokines.

However, treatment of epithelial cells in vitro with arbitrarily chosen cytokine concentrations does not address the issue of the relative contributions of these various cytokines in vivo. To do so, we isolated intestinal LPMC and stimulated them under conditions designed to potentiate but not maximally stimulate cytokine production (61, 64, 75). Stimulation with reagents such as PHA or Con A maximally stimulate most lymphocyte populations to produce uniformly high levels of cytokines. Observations supporting this approach include differences in IL-2, IFN-γ, and IL-4 production by cells isolated from the intestines of patients with
different diseases and differences in cytokine production of LPMC as compared with PBMC. Treatment of mononuclear cells with PMA and Ca\(^{+2}\) ionophore, while not physiologic, stimulates many cell types via activation of protein kinase C and mobilization of intracellular calcium, and these signaling pathways are involved in responses to a variety of physiologic stimuli, including engagement of the TCR/CD3 complex and cytokine exposure (76). Induction of pIgR expression by supernatants derived from activated LPMC was dominated by IFN-\(\gamma\), with TNF-\(\alpha\) exerting an effect only through synergy with IFN-\(\gamma\), and IL-4 exerting no significant effect (Fig. 2.6). The dose-responses for each supernatant were remarkably similar in light of the considerable variation of IFN-\(\gamma\) and TNF-\(\alpha\) concentrations (Fig. 2.4 and Table 2.1).

We have noted that the strong induction of pIgR expression by LPMC supernatants could not be entirely reconstituted by rIFN-\(\gamma\), rTNF-\(\alpha\), and rIL-4 at the concentrations measured by ELISA in these supernatants (Fig. 2.5). This may be due to differences in the specific activity of the recombinant cytokines vs. the natural cytokines present in the LPMC supernatants. It is also possible that the presence of other cytokines in the LPMC supernatants may contribute to induction of pIgR expression. While TGF-\(\beta1\) has been reported to increase surface expression of pIgR in rat intestinal epithelial cell line (56), we found that human rTGF-\(\beta1\) produced only a small increase in pIgR expression in HT-29.74 cells and only at concentrations greater than 30 ng/ml, which are unlikely to be physiologically relevant. Furthermore, TGF-\(\beta1\) neutralizing antibody, at concentrations sufficient to neutralize 100 ng/ml TGF-\(\beta1\), did not affect induction of pIgR expression by LPMC supernatants,
demonstrating that this cytokine is unlikely to act synergistically. Phillips et al, have shown that rIL-1α, rIL-2, rIL-3, rIL-5, and rIL-6 do not affect pIgR expression in HT-29 cells, and that IL-2 and IL-6 do not act in synergy with IL-4 and IFN-γ (24). However, we cannot rule out a possible role for these or other cytokines in interacting with other factor(s) in LPMC supernatants. Since neutralization of IFN-γ in LPMC supernatants abrogated their induction of pIgR expression, it is likely that any other cytokines in these supernatants that may contribute to pIgR expression do so through synergy with IFN-γ.

Cooperative induction by multiple cytokines would provide a powerful and flexible mechanism for the regulation of an important immune-effector molecule such as pIgR. Epithelial transcytosis of IgA via the pIgR is an important prophylactic defense mechanism and is critical for mediating both intracellular and extracellular defenses (47, 53), and it is therefore not surprising that pIgR is found at high levels throughout the normal intestine (7, 77). However, in the face of immunological stimuli concentrations of one or more of the cytokines regulating pIgR expression may rise, resulting in focused increases in pIgR expression at sites of infection or inflammation. There is convincing evidence for the presence of IFN-γ and TNF-α producing cells in the human intestine (57, 58, 60, 78), and infection with several pathogenic organisms enhances production of these cytokines in intestinal tissues: *Salmonella typhimurium* infection has been observed to stimulate both IFN-γ and TNF-α production in murine gut-associated lymphoid tissue (79-81); *Listeria monocytogenes* infection induces IFN-γ production by murine intestinal intraepithelial cells (82); cholera vaccination results in increased numbers of IFN-γ producing cells in the human intestinal mucosa (83); and
cytomegalovirus infection correlates with increased TNF-α production by intestinal macrophages in AIDS patients (84). In addition, IFN-γ injected into the lumen of the rat uterus increased the concentration of SC present in uterine secretions, providing evidence for cytokine regulation of pIgR expression in vivo (55, 85).

The immune system responds to a wide range of challenges to the host by detecting antigens and pathogens and then mobilizing various effector functions to neutralize them. Communication between these arms of the immune response is mediated, in part, by cytokines including IFN-γ, TNF-α, and IL-4 (21, 54). These responses may involve detection by different cell types, leading to cytokine production as a means of coordinating effector functions, such as production IgA by plasma cells in the intestinal lamina propria, epithelial cell expression of pIgR, and secretion of sIg (86, 87). It is interesting that the cytokines which enhance epithelial cell pIgR expression are the products of different subsets of mononuclear cells. IFN-γ is produced predominantly by Th1 cells and NK cells, IL-4 by Th2 cells, and TNF-α by macrophages (21, 54, 88). Different immune stimuli may result in the activation of one or more cell types (89), and the ability of intestinal epithelial cells to increase pIgR expression in response to cytokines produced by each of them would enhance the local sIg response in the face of a variety challenges to the host. In addition to LPMC, intraepithelial lymphocytes might also regulate pIgR expression by virtue of cytokine production and their proximity to intestinal epithelial cells (59). This interplay provides great flexibility in the ability of epithelial cells to respond to immunological stress by increasing production of pIgR, thus maximizing secretion of IgA.
CHAPTER 3

Characterization of

the Human Polymeric Immunoglobulin Receptor

Promoter Proximal Region
ABSTRACT

The polymeric immunoglobulin receptor (pIgR) mediates secretion of immunoglobulins across mucosal epithelia, providing the first line of antigen specific defense at these sites. Expression of the pIgR in humans is tissue specific, being restricted to mucosal epithelial cells, and has been found to be cytokine inducible in a colonic epithelial cell line.

In order to study the mechanisms by which pIgR expression is regulated, we have cloned the promoter proximal region of the human pIgR gene and determined the site at which transcription of pIgR mRNA is initiated. We have identified potential cis-acting regulatory elements in the pIgR promoter proximal region, constructed chimeric reporter plasmids, and demonstrated that a human genomic DNA fragment spanning nt -280 to +29 relative to the start site of pIgR mRNA transcription, confers both basal transcriptional activity and IFN-γ inducibility in transiently transfected colonic epithelial cells.

INTRODUCTION

Cytokine regulation of pIgR expression in the colonic epithelial cell line HT-29 has been described in Chapter 2 of this dissertation and numerous scholarly publications (12, 22, 24, 40, 90). The physiological relevance of these observations has been emphasized by demonstration that mononuclear cells isolated from an anatomic site in close proximity to mucosal epithelium produce cytokines which up-regulate pIgR expression in these cells (Chapter 2 and (91)).
Expression of plgR in humans is restricted to the mucosal epithelium in the airways, intestines, salivary glands, gall bladder, lacrimal glands, and mammary glands (92). In both the small intestine and colon, epithelial stem cells give rise to daughter cells, divide, and subsequently differentiate into mature epithelial cell types as they migrate to the villus tips (in the small intestine) or to the luminal surface (in the colon). In the small intestine plgR expression is greatest in the relatively immature cells within the crypts, with decreased expression as the cells mature and migrate up the villi. Similarly, in the colon expression is greatest in the lower two thirds of the crypts and submucoasl glands, decreasing as the cells migrate to the luminal surface (92).

IFN-γ induction of increased plgR expression in HT-29 cells has been correlated to steady state mRNA levels of plgR mRNA, which in turn has been shown to be dependent on de novo protein synthesis (12, 40). This observation suggests that IFN-γ may induce expression of transcription factor(s) which stimulate plgR expression through interaction with cis-elements in the plgR promoter, however, the mechanisms by which plgR tissue specificity and cytokine inducibility is regulated have not been uncovered. Krajci, et al. have isolated plgR genomic clones including the promoter proximal region and determined its intron-exon structure (17). While this report included conjecture as to possible promoter elements, it did not include identification of the start site plgR mRNA transcription.

We have independently cloned the plgR promoter proximal region and mapped the start site of plgR mRNA transcription. A putative "TATA"-box was found 28 bp upstream the transcriptional start and several other
possible cis-regulatory elements, located from 167 nt upstream to 22 nt downstream, identified. Chimeric reporter plasmids were constructed to identify elements in the pLgR promoter involved in the regulation of pLgR tissue specificity and IFN-γ inducibility.

MATERIALS AND METHODS

Isolation of the human pLgR Gene

A human genomic DNA library (Stratagene, La Jolla, CA) was screened using 32P-labeled PCR probes corresponding to pLgR DNA sequences +25 to +123 (Fig. 3.2). Three overlapping clones were obtained, and pLgR promoter-proximal regions identified (Fig. 3.1) by restriction enzyme digestion and Southern blot analysis using 32P-labeled oligodeoxynucleotide probes corresponding to nt +25 to +49, Fig. 3.2 (93).

DNA sequencing

A 6 kb EcoRI / BamHI genomic DNA fragment of one of the clones described above (see Fig. 3.1) hybridized to an oligodeoxynucleotide probe corresponding to nt +25 to +49 of the pLgR (see Fig. 3.2). This fragment was subcloned into pBluescript II (Stratagene) creating pLgR-pBS. Approximately 700 bp of the 3' end of the pLgR gene fragment were sequenced by the dideoxy chain termination method (94), using the Sequenase version 2.0 sequencing kit (United States Biochemical Co., Cleveland OH) (95). Homology searches of the GenBank, EMBL DNA, and Transcription Factor
Isolation and purification of primary human intestinal epithelial cells

Isolation and purification of primary human intestinal epithelial cells (pHIEC) was according to the technique of Youngman, et al. (68). Briefly, intestinal mucosal strips were treated at room temperature with DTT, 1.5 mg/ml in Hank's Balance Salt Solution (HBSS) to remove mucous. Five to thirty grams of tissue were placed in a 50 ml tube containing 30 ml RPMI-1640 (Life Technologies) with 3 mg/ml Dispase (Boehringer Mannheim Corp., Indianapolis, IN) and incubated at 37°C for 15 min with gently vortexing every 5 min. The medium containing dislodged epithelial cells was removed and the incubation repeated as above. Epithelial cell containing medium was pooled, the cells collected by centrifugation, resuspended in 15 ml Dispase-RPMI plus 1 mg/ml DNase I (Sigma, St. Louis, MO), and incubated as above to produce a single cell suspension. Purification of epithelial cells was achieved by layering the cell suspension over a 40% Percoll gradient (Pharmacia, Piskataway, NJ), centrifuging for 20 min, 1,500 RPM at room temperature, and collecting cells equilibrating at the medium-Percoll interface. Contaminating mononuclear cells were less than 2% (68).

Ribonuclease protection assay

Ribonuclease protection analysis was performed as previously described (12, 96). The pIgR-pBS plasmid was linearized with Hinc II and a 610 nt 32P-labeled RNA probe, including the antisense pIgR sequence
corresponding to nt +156 to -374 (Fig. 3.2) generated using T3 RNA polymerase and the T3 promoter of the pBS plasmid (RNA Transcription Kit, Stratagene). Reaction conditions were as follows: 1 µg linearized pIgR-pBS DNA, 10 U T3 polymerase, 10 mM each of ATP, CTP, and GTP, 50 µCi α[32P]-UTP (800 Ci/mmol; DuPont NEN Research Products, Boston, MA) in a final volume of 26 µl. The labeled probe was separated from unincorporated nucleotides by electrophoresis on a 6% polyacrylamide gel containing 7M urea (Gelmix-6, Life Technologies, Gaithersburg, MD), eluted with a solution of 2 M ammonium acetate, 1% SDS, and 25 µg yeast tRNA, and ethanol precipitated.

Varying amounts (1.5, 0.50, and 0.25 µg) of total cellular RNA from pHIEC (see Chapter 2, Materials and methods for RNA isolation technique) were precipitated and air dried. The RNA precipitates were resuspended in 30 µl hybridization buffer (40 mM PIPES, pH 6.4, 40 mM EDTA, 80% (v/v) formamide) containing 5 x 10^5 cpm of the 32P-labeled pIgR antisense RNA probe. The RNA was denatured by incubation at 85°C for 10 min followed by overnight incubation at 45°C to allow annealing of complementary sequences.

Three hundred microliters RNase digestion mixture (10 mM Tris-HCl, pH 7.4, 300 NaCl, 5 mM EDTA, 2 µg/ml RNase T1, and 40 µg/ml RNase A, United States Biochemical Corporation) was added to each hybridization mixture and incubated at 30°C for 60 min to digest single stranded RNA. The reaction was stopped by addition of 20 µl 10% (w/v) SDS and 10 µl 10 mg/ml proteinase K (Boehringer Mannheim Corp.). RNA was extracted with phenol : chloroform (1:1), ethanol precipitated and air dried. The RNA precipitates were resuspended in 10 µl of 80% (v/v) formamide, 10 mM EDTA, size
fractionated by electrophoresis as described above, and protected $^{32}$P-labeled probe visualized by autoradiography. The size of the protected fragment was determined by comparison to $^{32}$P-labeled DNA molecular size standards ($\Phi$X174 RF DNA/Hae III fragments, 100 and 123 bp ladders, Life Technologies) and to the undigested 610 nt pIgR probe.

**Primer extension analysis**

Primer extension analysis was performed essentially as described by Sambrook, et al. (96). An antisense oligodeoxynucleotide primer complementary to nt +94 to +123 (Fig 3.2) was end labeled with T4 polynucleotide kinase (Life Technologies) and $\gamma^{32}$P]-ATP (DuPont NEN Research Products). The labeled primers were incubated with 10 $\mu$g RNA from pHIEC (see above) in a solutions of 80% (v/v) formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA at 80°C for 10 min to denature the RNA followed by annealing of complementary sequences by incubation in the same solution for 18 h at 30°C. RNA : primer hybrids were ethanol precipitated and the primers extended using a modified Moloney murine leukemia virus reverse transcriptase, Superscript (Life Technologies) for 1 h at 42°C. The reaction was stopped and the RNA degraded by the addition of EDTA. pH 8.0 to a final concentration of 20 mM and boiled RNase A (Boehringer Mannheim Corp.), final concentration 40 $\mu$g/ml, and incubation at 37°C for 30 min. The extended primers were precipitated, heated in denaturing buffer and analyzed by 7 M urea 6% PAGE (Gelmix-6, Life Technologies). The length of the extended primers was determined by
Construction of chimeric reporter plasmids

Human plgR gene fragments containing putative promoter sequences were generated by PCR (Perkin-Elmer Cetus, Norwalk, CT). Three 5\'-primers were used corresponding to nt -93 to -64, -280 to -251, and -563 to -534 (Fig 3.2) in combination with two 3\'-primers corresponding to the reverse complements of nt +1 to +29 and -24 to +6 (Fig 3.2). Each primer had 12 nt 5\'-extensions generating Hind III sites following PCR. PCR was performed using standard reaction conditions (Perkin-Elmer Cetus) with plgR-pBS as template. Amplification was performed using a DNA thermal cycler (Perkin-Elmer Cetus) for 40 cycles of 1 min at 94°C, 2.5 min at 55°C, and 4 min at 72°C, followed by a final elongation for 10 min at 72°C. Human plgR gene fragments were extracted with phenol : chloroform (1:1), ethanol precipitated, digested with Hind III (Life Technologies), and size fractionated using 2% low melting point agarose (Life Technologies) with 1X TAE buffer. The Hind III digested plgR gene fragments were purified using Wizard PCR Prep columns (Promega, Madison, WI), and subcloned into the luciferase expression plasmid pGL2-Basic (Promega), which had been Hind III digested (Life Technologies) and treated with calf intestinal alkaline phosphatase (Life Technologies). Insert/vector complexes were closed with T4 DNA ligase (Life Technologies) and used to transform DH5α subcloning efficiency competent cells (Life Technologies). Colonies expressing ampicillin resistance were picked, plasmid DNA isolated, and insert size, number, and orientation
confirmed by restriction enzyme digestion with enzymes cutting outside the insert and asymmetrically within the insert. Transformed cells carrying pIGR promoter-pGL2 plasmids were cultured overnight at 37°C in 5 ml LB medium with 50 µg/ml ampicillin. The culture was then used to inoculate 500 ml LB medium with 50 µg/ml ampicillin, which was incubated overnight at 37°C. DNA for transfection was prepared using QIAGEN-tip 500 columns (QIAGEN Inc., Chatsworth, CA).

The pCMV IE CAT plasmid, a chloramphenicol acetyltransferase (CAT) expression plasmid with CAT expression driven by the cytomegalovirus immediate early promoter-enhancer, was provided by Dr. E. Fodor, University of California, San Francisco (97), and was using to control parallel cultures for transfection efficiency (see below).

Transient transfection of HT-29.74 cells

The HT-29.74 cell line has been previously described (Chapter 2) (69). Cells were maintained in RPMI-1640 medium, 10% FBS, 100 mM sodium pyruvate, 20 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and 20 mM HEPES, pH 7.3) at 37°C in a humidified 5% CO2 atmosphere. Four days prior to transfection cells were induced to differentiate switching to Leibovitz's L-15 medium, 0.2% sodium bicarbonate, 10% dialyzed FBS, 100 mM sodium pyruvate, 20 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and 20 mM HEPES, pH 7.3 (L-15) (69). One day prior to transfection the cells were detached with trypsin/EDTA and plated in 6 well tissue culture plates at 5 X 10^5 cells per well with 2 ml L-15/well.
HT-29.74 cells were transiently transfected by the Lipofectin technique, using the appropriate promoter-pGL2-Basic plasmid (2 μg/well), the pCMV IE/CAT plasmid (1 μg/well, and the Lipofectin reagent (20 μl/well) (Life Technologies). For each promoter construct to be tested, the appropriate amount of DNA (2 μg/well times the number of wells/treatment) was ethanol precipitated and resuspended in H2O at a final concentration of 0.5 μg/μl. The pCMV IE/CAT plasmid was precipitated in a separate tube and resuspended in H2O at a final concentration of 0.25 μg/μl. In a third tube, the Lipofectin Reagent (1 μg/μl) was mixed with L-15 medium (without serum and antibiotics) at a ration of 1:10 (v/v) Lipofectin : L-15, and incubated for 30 min at room temperature to allow complete dispersion of the Lipofectin in the culture medium. Separate transfection solutions were then prepared for each treatment group by mixing the three solutions at a ratio of 4 μl : 4 μl : 220 μl promoter-pGL2-Basic : pCMV IE/CAT : Lipofectin, times the total number of wells/treatment group. The transfection solutions were incubated for 20 min at room temperature to allow formation of DNA-liposome complexes.

While the transfection solutions were incubating, culture medium was removed from the HT-29.74 cells and all cultures were washed once with PBS and once with serum free, antibiotic-free L-15 medium. Transfection was initiated by adding 228 μl of the appropriate transfection solution to each well. Cultures were incubated with the DNA-liposome complexes at 37°C for 24 h in a humidified 10% CO2 atmosphere. The transfection solutions were then removed, cultures were washed with once with L-15, and 2 ml of the same medium was added to each well. After 24 h the medium was replaced with fresh L-15 with or without 100 U/ml recombinant IFN-γ (R&D Systems,
Preparation of cell lysates for luciferase and CAT assays

HT-29.74 cells were harvested for enzyme assays by washing each culture twice with PBS, and adding 300 μl Reporter Lysis Buffer (Promega) and incubating for 20 min at room temperature. Lysed cells were collected by scraping, transferred to microcentrifuge tubes, and vortexed for 10 sec. Aliquots were of each lysate (80 μl) were removed for CAT assays and treated as described below. The remaining lysates were microcentrifuged for 2 min at 14,000 rpm, and the supernatants removed to fresh microcentrifuge tube and assayed immediately for luciferase activity. The aliquots for CAT assays were incubated at 60°C for 10 min to inactivate endogenous deacetylases, microcentrifuged for 2 min at 14,000 rpm, the supernatants removed to fresh microcentrifuge tubes, and stored at -80°C.

Luciferase assay

Luciferase activity in transiently transfected HT-29.74 cells was assayed using the Luciferase Assay System (Promega). Fifty μl aliquots of cell lysates were prepared in duplicate in 12x75 mm culture tubes, the tubes placed in a LB 9501 luminometer (Wallac Inc., Gaithersburg, MD). 100 μl Luciferase Assay Substrate (Promega) injected, and relative light units (RLU) measured for 15 seconds following a 3 second pause. Luciferase activity was converted to RLU/min/ml lysate by averaging duplicate, subtracting
Chloramphenicol acetyltransferase assay

CAT activity in lysates of transiently transfected HT-29.74 cells was assayed using a modified version of the technique of Seed and Sheen, (98). Briefly, 50 µl of each cell extract, heat treated to inactivate endogenous deacetylase activity (see above), was added to a microcentrifuge tube containing 5 µl of 5 mg/ml n-butyryl CoA (Sigma) in 250 mM Tris, pH 8.0 (Sigma), 3 µl of 0.05 mCi/ml [14C]-chloramphenicol (DuPont NEN Research Products), and 67 µl H2O. The tubes were gently vortexed, pulsed in a microcentrifuge, incubated at 37°C for 3 h, and the reaction stopped by adding 300 µl mixed xylene (Aldrich Chemical Company, Inc., WI). The tubes were vortexed for 30 seconds, microcentrifuged for 3 min at 14,000 rpm and the upper xylene phase (containing butyrylated-[14C]-chloramphenicol) transferred to fresh microcentrifuge tubes. The organic phase was back-extracted twice with 100 µl of 250 mM Tris, pH 8.0, a 100 µl aliquot was removed for liquid scintillation counting. Background CAT activity was determined by assaying lysates from untransfected HT-29.74 cells.

CAT activity was expressed as cpm butyrylated [14C]-chloramphenicol produced/3h, calculated as [(cpm/100 µl - background) x 3]. CAT activity was converted to cpm/ml lysate by multiplying by 20.
Luciferase activity /ml lysate was divided by CAT Activity /ml lysate to determine normalized luciferase activity (Luciferase activity/CAT Activity) for each independent culture. The significance of differences in luciferase expression among various groups were tested by one way analysis of variance and pairwise multiple comparisons made using the Students-Newman Keuls method. Analysis was performed using the SigmaStat program (Jandel Scientific, San Rafael, CA).

RESULTS

Identification of genomic clones containing the pIgR promoter proximal region.

The structure of the pIgR gene has been described by Krajci, et al (17). They found pIgR 5'-UTR sequences on 2 exons separated by a 6.1 kb intron. The start site of pIgR mRNA transcription was not identified in this report. We have independently isolated genomic clones containing the 5'-end of the pIgR gene (93). We hypothesized that a 6 kb EcoR I / BamH I genomic DNA fragment, containing the 5'-most pIgR exon and extending 6 kb upstream of this region, would contain the pIgR transcription start site (Figure 3.1). Approximately 700 bp of the 3'-end of this fragment was sequenced (Figure 3.2).
Figure 3.1. Restriction map of the pigK gene including the promoter proximal region.

The transcription start site was determined by primer extension and ribonuclease protection (Fig. 3.3). The approximate location of exons are boxed and hatched. Restriction enzyme sites used in mapping are indicated by the following letters: A=Apa I, B=Bam H I, N=Not I, R=EcoR I.
Figure 3.2: Sequence of the pIgR promoter proximal region.

Nucleotides of the pIgR gene coding strand are numbered relative to the transcription start site (†) as determined in Fig. 3.3. Putative cis-regulatory elements are boxed. Restriction enzyme sites, intron/exon boundaries, and the ends of used in chimeric reporter plasmids are labeled and marked with arrows.
Mapping the start site of pIgR mRNA transcription

In order to determine the start site of pIgR mRNA transcription an RNase protection assay and primer extension analysis were employed. RNase protection assay revealed a protected pIgR antisense fragment of approximately 132 nt (Fig 3.3 B). Note that the probe used in this experiment contained 80 nt of pBluescript II and 24 nt of pIgR intron sequence which would not be protected by pIgR mRNA. Using this datum, an antisense primer corresponding to nt +94 to +123 was chosen for use in primer extension analysis, and a single prominent band of 123 nt observed (Fig 3.3 A).

Primer extension and RNase protection analysis indicate a single transcriptional start site which we have designated as position +1 on Fig. 3.2. Both techniques confirm the same site, making extremely unlikely the possibility of the site identified by RNase protection being an intron/exon boundary or the site identified by primer extension the result of premature termination of the reverse transcriptase reaction.

Sequence analysis of the pIgR promoter-proximal region

Sequence analysis of the pIgR gene upstream of the transcriptional start site revealed a "TTTAA" motif at position -28 (Fig 3.2). This represents a close approximation of the TATA box consensus sequence of "TATAA" (99, 100). Our analysis failed to identify potential binding sites for the transcription factors CREB, AP-1, AP-2, CTF, Sp1, OCT-2, or CAAT-binding proteins (41, 99).
Figure 3.3. Determination of the start site of plgR mRNA transcription.

A. Primer extension analysis.

A $^{32}$P-labeled oligodeoxynucleotide complementary to nt +94 to +123 of the plgR sequence (Fig 3.2) was hybridized to 10 $\mu$g primary human intestinal epithelial cell RNA or yeast tRNA (negative control) and extended by reverse transcriptase. Dideoxy chain termination sequencing reactions using the same primer are shown in adjacent lanes and were used to determine the size of extended primers. The sequence around the deduced transcriptional start site (← +1) is shown to the right.

B. Ribonuclease protection assay.

A 610 nt $^{32}$P-labeled antisense plgR probe was prepared by in vitro transcription and hybridized to 1.5, 0.5, and 0.25 $\mu$g pHIEC RNA (lanes 1,2,3). The hybridization mixture was treated with RNase to digest single stranded regions and the protected fragments resolved by PAGE. The size of the protected fragment was deduced by comparison to $^{32}$P-labeled DNA size standards (not shown). The band at 132 nt is the length of the probe protected, and represents the length of the 5'-most plgR exon. (Note: the full length probe includes 80 nt of pBluescript II and 24 nt of plgR intron 1 sequence which would not be protected by plgR mRNA.)
The GenBank, EMBL DNA, and Transcription Factor databases were searched for motifs homologous to those found in the pIgR promoter proximal region. Four elements with partial homology from other epithelial specific or IFN-γ inducible genes were identified (Fig 3.2).

An element at nt -167 to -146 in the pIgR gene was found to be 70% homologous to the X-box of MHC class II genes(101) and MHC class II associated invariant chain (102). Based on subsequent analysis demonstrating that this pIgR element binds nuclear proteins distinct from those binding the MHC class II X-box and present only in intestinal epithelial cells, we have named this unique element the intestinal epithelial specificity element or IEE (see Chapter 4).

A pair of inverted repeats at nt-121 to -112 and -62 to -53 was 80% homologous to the MHC class II Y-box, but lack the core "CAAT" associated with Y-box elements (101). Two copies of a 13 nt motif at positions -137 to -125 and -98 to -86 were homologous to sequences in the promoter regions of eleven cytokeratins expressed in epidermal and or gastrointestinal epithelial cells (103-110).

Finally, the pIgR sequence from nt +7 to +20 matched 12 of 14 nt of the consensus interferon-stimulated response element (ISRE) found in many IFN inducible genes (111). ISRE's are found in many IFN inducible genes (30, 112). Although most often associated with immediate responses to type I IFN, via interaction with the trans-acting factor interferon-stimulated gene factor-3 (ISGF-3), the ISRE also plays a role in late IFN-γ responses, mediated by members of the interferon regulatory factor (IRF) family (28).
Transcriptional activity and IFN-γ inducibility of the pIgR promoter in transiently transfected HT-29.74 cells

To test for functional promoter/enhancer activity, HT-29.74 cells were transiently transfected with chimeric reporter vectors with fragments of the pIgR gene inserted upstream of the luciferase gene (Fig. 3.4). A fragment of the pIgR gene spanning nt -280 to +29, which includes all the putative cis-regulatory elements described above, conferred both basal transcriptional activity (-280/+29, -IFN-γ vs. None, -IFN-γ, p<0.05) and IFN-γ inducibility (-280/+29, +IFN-γ, vs. -280/+29, -IFN-γ, 4.2 fold, p<0.05) on luciferase expression in transfected cells. Three prime truncation of the fragment to nt +6 (which deletes the ISRE) did not significantly effect basal transcription (-280/+29, -IFN-γ, vs. -280/+6, -IFN-γ, p<0.05), and transcription was still IFN-γ inducible (-280/+6, +IFN-γ, vs. -280/+6, -IFN-γ, 5.73 fold, p<0.05).

A fragment of the pIgR gene spanning pIgR nt -563 to +29 conferred basal transcriptional activity (-563/+29, -IFN-γ, vs. None, -IFN-γ, p<0.05) and was induced by IFN-γ (-563/+29, +IFN-γ, vs. -563/+29, -IFN-γ, 3.41 fold, p<0.05). However, while -563 to +6 fragment conferred basal transcription (-563/+6, -IFN-γ, vs. None, -IFN-γ, p<0.05), IFN-γ inducibility was lost (-563/+6, +IFN-γ, vs. -563/+6, -IFN-γ, p>0.05).

A fragment of the pIgR gene spanning nt -93 to +29 (Fig 3.2) conferred basal transcriptional activity in HT-29.74 cells (-93/+29, -IFN-γ, vs. None, -IFN-γ, p<0.05), however transcription was no longer IFN-γ inducible (-93/+29, +IFN-γ, vs. -93/+29, -IFN-γ, p>0.05). Plasmids with pIgR nt
Figure 3.4. Transcriptional activity and IFN-γ inducibility of the human plgR promoter in transiently transfected HT-29.74 cells.

HT-29.74 cells were transfected with chimeric reporter plasmids with the indicated fragments of the plgR gene subcloned upstream of the luciferase gene, and pCMV IE/CAT to control for transfection efficiency. Bars labeled "None" represent cells transfected with the pGL2-Basic plasmid with no plgR sequence. Data are expressed as Normalized luciferase activity (mean ± SEM, n=12). * denotes statistically significant differences (p<0.05) between + IFN-γ and -IFN-γ treated groups.
-93 to +6 inserts conferred also conferred basal transcription (-93/+6, -IFN-γ, vs. None, -IFN-γ, p<0.05) without IFN-γ inducibility (-93/+6, +IFN-γ, vs. -93/+6, -IFN-γ, p>0.05).

DISCUSSION

Expression of the plgR in humans is tissue specific, being restricted to mucosal epithelium (92), and cytokine inducible in the HT-29 colonic epithelial cell line (12, 22, 24, 40, 90). Krajci, et al. have isolated plgR genomic clones including the promoter proximal region and determined its intron-exon structure (17). However, the gene's transcriptional start site was not identified, nor any conclusive evidence of cis-regulatory elements involved in the regulation of plgR expression offered.

We have independently cloned the plgR gene and mapped the site plgR mRNA transcriptional initiation (Figs 3.1 and 3.3). A "TATA"- box 28 bp upstream of the transcriptional start site was identified (Fig. 3.2). The plgR sequence at this site, TTTAA, varies from the consensus "TATA" sequence (TATAA) at a single base pair, and the substitution of T for A at this site occurs in 10% of the genes surveyed by Bucher (113). We propose that the TTTAA element in the plgR promoter is likely to interact with TATA-binding proteins in order to regulate initiate mRNA transcription (100).

A fragment of the plgR gene spanning nt -280 to +29 confers both basal transcriptional activity and IFN-γ inducibility to reporter gene expression in transfected HT-29.74 cells (Fig 3.4). Truncation of the fragment at the 3'-end to nt +6 (which deletes the ISRE) does not significantly effect basal transcription or IFN-γ induced transcription. The -280 to -93 region of the
plgR gene is clearly required for IFN-γ inducibility, as neither the -93 to +29 nor -93 to +6 fragment are IFN-γ inducible. The presence of upstream plgR sequences down-regulates transcriptional activity. An insert including plgR nt -563 to +281 (spanning nt -563 to +6) is not IFN-γ inducible. These data could indicate the presence of repressor elements between plgR nt -563 and -280. No such elements were found in searches of the GenBank or EMBL DNA, or Transcription Factor databases, however, novel inhibitory cis-regulatory elements could be present. Repressors could be identified by producing another set of nested deletions between -563 and -280, or sequential mutation of sequences within this region using techniques such as scanner-linker mutagenesis.

Basal transcription driven by plgR promoter fragments in transiently transfected HT-29.74 is low compared to that of two other eukaryotic promoters tested (IRF-1, Table 4.3, and β-actin, data not shown). This is not surprising given the low levels of basal plgR expression in these cells (12, 91). It is possible that HT-29.74 cells represent a stage of epithelial cell development where plgR expression is low, such as epithelial stem cells (92). This hypothesis is supported by the pluripotent nature of HT-29 cells, which have been shown to differentiate into either mucinous or absorptive epithelial cells when grown in the absence of glucose (114-116). Other intestinal epithelial cell lines with different patterns of plgR expression, such as Caco-2, T-84, and Vaco-235 cells (Table 4.3), transfected with plgR promoter/reporter plasmids may help identify the cis-elements required for robust basal plgR expression. The Vaco-235 colonic adenoma cell line, in which basal expression of plgR is high (Table 4.3), should be especially useful in these
studies. It is possible that inhibitory trans-acting factors binding repressor elements in the pIgR gene keep basal pIgR expression in HT-29.74 cells low, and that stimulatory trans-acting factors, not present in epithelial cells at this stage of development, are required for robust pIgR expression. Local production of cytokines, such as IFN-γ, at sites of infection could also contribute to increased epithelial cell pIgR expression (79-84).

In HT-29.74 cells, IFN-γ up-regulates pIgR expression (12, 22). Our data clearly demonstrates that pIgR sequences -280 to -93 are required for IFN-γ inducibility. In the presence of pIgR sequences -563 to -280, the region spanning pIgR nt +6 to +29, which contains the pIgR ISRE is also necessary for IFN-γ induction. IFN-γ induction might involve a cooperative mechanism requiring the ISRE and cis-element(s) between pIgR nt -280 to -93. Enhancer complexes composed of multiple cis-elements binding multiple proteins, which activate transcription by altering DNA conformation and combining trans-activatiing domains, are found in the TCRα and IFN-β genes (42). The precise location of these elements can be determined by constructing reporter plasmids with mutations of potential cis-elements between -280 and -93, such as the pIgR IES.

Our experiments have not been informative regarding the multiple cytokine inducibility of the pIgR promoter (Chapter 2). We have cultured HT-29.74 cells transiently transfected with the various pIgR promoter/reporter plasmids with TNF-α, IL-4, and combinations of IFN-γ, TNF-α, and IL-4, and cooperative induction is not observed(data not shown). We are currently constructing a chimeric reporter plasmid including -1800 to
+130 bp of the pIgR gene to determine if cis-regulatory elements involved in multiple cytokine induction are present in this larger.

This is the first report of molecular mechanisms involved in the regulation of pIgR expression. We have identified a cis-regulatory element, the pIgR ISRE involved IFN-γ induction of pIgR expression and demonstrated that nt -280 to -93 of the pIgR promoter are required for IFN-γ inducibility. The roles of cis-regulatory elements within this region of the pIgR promoter in tissue specific and IFN-γ inducible pIgR expression are currently under investigation.
CHAPTER 4
Interactions of Nuclear Proteins
with Elements of
the Human Polymeric Immunoglobulin Receptor Promoter
ABSTRACT

The polymeric immunoglobulin receptor (pIgR) mediates secretion of immunoglobulins across mucosal epithelia, providing the first line of antigen specific defense at these sites. Expression of pIgR in humans is tissue specific, being restricted to mucosal epithelial cells, and cytokine inducible in the HT-29 colonic epithelial cell line.

We have demonstrated that a fragment of the pIgR gene, nt -563 to +29 relative to the transcription start site, confers basal transcription activity and IFN-γ inducibility upon a reporter gene when transiently transfected into the colonic epithelial cell line HT-29.74. Several potential cis-regulatory elements were found within this region of the pIgR gene.

Our studies reveal two elements which bind intestinal epithelial cell nuclear proteins. First, a unique cis-element, which we have named the intestinal epithelial specificity element (IESE) binds nuclear proteins found only in intestinal epithelial cells, and levels of binding activity correlate with levels of pIgR expression in human intestinal cells which express pIgR. Second, the transcription factor IRF-1 is induced in HT-29.74 cells by IFN-γ and binds an interferon-stimulated response element (ISRE) located in the first exon of the pIgR gene. The presence of these two cis-elements, and their interactions with trans-acting factors found in intestinal epithelial cells provide the first insights into the mechanisms by which tissue specific and IFN-γ induced expression of pIgR, a vital component of mucosal immune responses, is regulated.
INTRODUCTION

Regulation of the expression of eukaryotic genes during development, or in response to external signals, involves the interaction of trans-acting factors with cis-regulatory elements (41, 42). In humans, expression of the plgR gene is tissue specific, developmentally regulated (92), and cytokine responsive in the colonic epithelial cell line HT-29.74 (Chapter 2). In Chapter 3, cis-regulatory elements involved in the regulation of plgR expression were identified. In order to better understand the mechanisms involved in regulation of plgR expression we have carried out experiments designed to determine if nuclear proteins from cells expressing plgR bind these elements, which would indicate their potential to function as trans-acting factors. Using in vitro electrophoretic mobility shift assays (EMSA) we have shown that nuclear proteins bind two cis-regulatory elements in the plgR gene, providing evidence of interplay between cis-regulatory elements and trans-acting factors in the regulation of plgR expression.

MATERIALS AND METHODS

Cell culture

HT-29.74 human colon carcinoma cells were grown as described in Chapter 3. IFN-γ treated HT-29.74 cells were cultured with 100 U recombinant human IFN-γ/ml (R&D Systems (Minneapolis, MN) for specified time courses. The T-84 human colon carcinoma (117), Caco-2 human colon carcinoma (118), HeLa cervical carcinoma (119), and Hep-G2 hepatoma (120) cell lines were obtained from the American Type Culture
Collection, Rockville, MD. T-84 and Caco-2 cells were grown in RPMI-1640 medium, 10% FBS, 100 mM sodium pyruvate, 20 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and 20 mM HEPES, pH 7.3. HeLa cells were grown in DMEM with high glucose, 5% FBS, 20 mM HEPES, 0.1 mM MEM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. Hep-G2 cells were grown in DMEM F12 with L-glutamine, 10% FBS, 25 mM HEPES, pH 7.3, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. The Vaco-235 human rectal villous adenoma cell line (121) was generously provided by Dr. James Wilson (Department of Medicine, Division of Hematology and Oncology, School of Medicine, Case Western Reserve University) and grown in MEM with 10% FBS, 20 mM HEPES, pH 7.3, 20 mM L-glutamine, 0.02% (v/v) GMS-A Supplement, 20 ng/ml hydrocortisone, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. All tissue culture reagents were purchased from Life Technologies, Gaithersburg, MD.

Isolation and purification of primary human intestinal epithelial cells

Isolation and purification of primary human intestinal epithelial cells (pHIEC) was according to the technique of Youngman, et al. (see Chapter 3 for protocol) (68).

Preparation of nuclear proteins

A modification of the method of Dignam, et al. was used of the preparation of nuclear proteins (122). Approximately 2x10^8 cells, cultured as
described above, were detached by trypsinization, washed in medium with 10\% FBS to inactivate trypsin, pelleted by centrifugation at 700 x g for 10 min, the volume of the pellet recorded, resuspended in cold PBS and counted using a hemacytometer. Freshly isolated pHIEC were extracted immediately after isolation. The cells were then pelleted by centrifugation at 700 x g, 10 min, 4\(^\circ\)C, and resuspended in 5 times the pellet volume of 10 mM (pH 7.9), 1.5 mM MgCl\(_2\), 10 mM KCl, 0.5 mM DTT (Dignam Buffer A) and incubated at 4\(^\circ\)C for 10 min. The cells were centrifuged as above, supernatant removed, the pellet resuspended in twice original pellet volume of Dignam Buffer A, and lysed with a Dounce homogenizer. Lysis was confirmed by microscopic observation. The lysate was transferred to a round bottom high speed centrifuge tube, spun as above, the supernatant removed, and the pellet centrifuged at 25,000 x g, 4\(^\circ\)C, for 10 min. The supernatant was removed, the pellet resuspended in 1.5 ml/10\(^8\) cells (from cell count above) of 20 mM (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, homogenized using a Dounce homogenizer, incubated for 30 min at 4\(^\circ\)C with occasional vortexing, and centrifuged at 25,000 x g, 4\(^\circ\)C, for 30 min. The supernatant was collected and dialyzed overnight at 4\(^\circ\)C against 1 liter 20 mM (pH 7.9), 20\% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT (Dignam Buffer D). The dialysate was centrifuged at 25,000 x g, 4\(^\circ\)C, 20 min, the supernatant collected, 50 \(\mu\)l aliquots prepared, and stored at -70\(^\circ\)C. Protein concentration was determined by the technique of Bradford (123, 124)(BioRad, Hercules, CA)
Electrophoretic mobility shift assays

The method used for electrophoretic mobility shift assays (EMSA) was a modification of the technique of Chodosh (125). Probes were double stranded oligodeoxynucleotides, end labeled with T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD) and γ-[32P]-ATP (DuPont NEN Research Products, Boston, MA), and purified by PAGE in 20% acrylamide : bisacrylamide (19:1), 1X TBE, and 5% glycerol. Labeled double stranded probe was visualized by autoradiography, the gel slice pulverized, and the probe eluted in 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.6 (NET).

Nuclear protein/DNA complexes were formed by preparing 10⁻¹⁴ mol 32P-labeled probe, 2 μl poly d(I-C) (10 OD₂₆₀ U/ml in NET) (Boehringer Mannheim, Indianapolis, IN) in a total volume of 20 μl NET, with competitor oligodeoxynucleotides added where indicated. Nuclear extracts were diluted to 0.5 μg/μl in Dignam Buffer D (see above). The ratio of probe mixture (in 1X NET) to nuclear extract (in Dignam Buffer D) was always 1:1. Probe mixture and diluted nuclear extract were combined, mixed by gentle pipetting and incubated at room temperature for 15 min. For antibody-supershift EMSA, 2 μl anti-IRF-1, anti-IRF-2, or anti-STAT-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were then added and the mixture incubated for 60 min at 5°C.

DNA/nuclear protein complexes and unbound DNA probe were resolved by PAGE in 6% acrylamide : bisacrylamide (49:1) with 5% glycerol and either 25 mM Tris, 190 mM glycine, pH 7.9 (high ionic strength), or 0.25X TBE (low ionic strength). Gels were dried and complexes visualized by
Northern blot analysis of pIgR mRNA Expression

Total cellular RNA from pHIEC (isolation described in Chapter 3) and IFN-γ treated HT-29.74 cells (cultured as described in Chapter 2) was purified using techniques described in Chapter 2. Northern blot analysis of pIgR mRNA expression also was carried out as described in Chapter 2.

Measurement of pIgR Protein Expression

Concentrations of SC in HT-29.74 and Vaco-235 cell culture supernatants were determined by ELISA as described in Chapter 2 (69).

Determination of IFN-γ inducibility of the IRF-1 promoter in transiently transfected HT-29.74 cells

The IRF-1 promoter was generously provided by Dr. A. B. Deisseroth, Department of Hematology, The University of Texas M. D. Anderson Cancer Center, Texas (29). A 1.3 kb Sst I fragment of 5'Δ-1312 LUC containing the IRF-1 promoter was subcloned into the Sac I site of the luciferase expression vector pGL2-Basic (Promega, Madison, WI). HT-29.74 cell culture, transient transfection, and luciferase assay was performed as described in Chapter 3.
Identification of cis-elements in the pIgR promoter which bind intestinal epithelial cell nuclear proteins

Four elements in the pIgR promoter-proximal region with partial homology from other epithelial specific and IFN-γ inducible genes have been previously described (Chapter 3). Using EMSA, we tested all four elements for in vitro binding to nuclear proteins isolated from HT-29.74 with and without IFN-γ treatment. We did not detect specific binding to double-stranded oligodeoxynucleotides representing individual copies of the pIgR cytokeratin element or the inverted repeat (data not shown). We did observe specific binding to probes including the intestinal epithelial specificity element (IESE) in nuclear extracts from both IFN-γ treated and control cells (Figs. 4.1, 4.2, 4.3, 4.4, and Tables 4.1 and 4.3) and the pIgR interferon-stimulated response element (ISRE) in extracts from IFN-γ treated cells (Figs. 4.6, 4.7, and Table 4.3). These observations will be described and discussed in detail in the remainder of this chapter.

A pIgR promoter element binds nuclear proteins distinct from those which bind MHC class II X-box and GAS

Because the IESE was identified as a consequence of its homology to the MHC class II X-box, it was necessary to determine if these elements bound similar or distinct nuclear proteins. We also observed a sequence homologous to interferon-gamma activating sequence (GAS) overlapping the 5'-end of the IESE, and therefore wished to determine if this motif was
capable of binding interferon-gamma activated factor (GAF, also referred to as STAT 1) (26, 27). We performed EMSA using nuclear extracts from IFN-γ treated HT-29.74 cells, since STAT 1 is known to be activated and translocated to the nucleus only after IFN-γ exposure (26, 27). We have not observed any difference in the levels of nuclear protein binding to the IESE in IFN-γ and untreated cells (data not shown). Probes used were double stranded, 32P-labeled, oligodeoxynucleotides representing the IESE (pIgR bp -67 to -146, Chapter 3, Fig. 3.2), an MHC class II X-box (bp -112 to -91 of the HLA DRA gene (101)), and an IFN-γ activating sequence or GAS (bp -152 to -121 of the FcγRI gene (27, 126)). These probes were used alone or in combination with IESE, MHC class II X-box, or FcγRI GAS oligodeoxynucleotides as competitors (Fig. 4.1). Each probe bound a distinct set of nuclear proteins and competition for binding was only observed when homologous competitor was used. The IESE probe formed 5 complexes (see arrows) with HT-29.74 cell nuclear proteins, and this binding was inhibited by homologous competitor demonstrating sequence specificity of binding. In the case of all three probes, nuclear protein binding was only inhibited by homologous competitor demonstrating that the proteins bound by the IESE are distinct from those binding the MHC class II X-box and FcγRI GAS. An anti-STAT-1 antibody which blocked the formation STAT-1/GAS complexes had no effect on complex formation with the pIgR IESE, suggesting that STAT-1 does not participate in any of the IESE-protein complexes.
Figure 4.1: IESE binding nuclear proteins are distinct from MHC II X-box binding proteins and STAT-1.

High ionic strength EMSA were performed using the following double stranded, $^{32}$P-labeled, oligodeoxynucleotide probes: IESE, plgR bp -67 to -146, Chapter 3, Fig. 3.2; MHC II X-box, bp -112 to -91 of the HLA DRA gene (101); and FcγRI GAS, bp -152 to -121 of the FcγRI gene (27, 126). Unlabeled competitor oligodeoxynucleotides were used at 100X probe concentration as designated. Nuclear extract were from HT-29.74 cells cultured with IFN-γ (100 U/ml) for 24 h. Complexes where binding was sequence specific (defined by displacement by homologous competitor oligodeoxynucleotides) are labeled with arrows at left.
Cell type specificity of nuclear proteins binding a novel plgR promoter element

In order to determine the tissue specificity of nuclear proteins binding the IESE, EMSA was carried out using nuclear extracts from several intestinal epithelial epithelial cell lines, non-intestinal epithelial cell lines, and primary human intestinal epithelial cells (pHIEC) and IESE probe (Fig. 4.2). Complexes 1 and 2 were only observed in lanes with nuclear extracts from intestinal epithelial cells and formation of these complexes was greatest with pHIEC extracts. These observations, and those demonstrating that the IESE and MHC class II X-box bind different nuclear proteins, formed the basis for our naming this unique element the intestinal epithelial specificity element or IESE.

Sequence specificity of IESE binding nuclear proteins

In order to determine which nucleotides within the IESE are critical to nuclear protein binding, we tested a set of overlapping or mutated plgR sequence derived oligodeoxynucleotides for their ability to compete with IESE probe: nuclear protein binding in EMSA (Fig. 4.3 and Table 4.1). The plgR ISRE, which did not displace IESE binding proteins, was used as a negative control. Oligodeoxynucleotides overlapping the 5' or 3' end of the IESE did not affect the formation of complexes 1 and 2 suggesting that binding sites for nuclear proteins are broadly distributed along the IESE. Two mutant IESE sequences, designated 5M GAS X box and 3M GAS X box, which completely destroy the GAS concensus (see Table 4.1 for mutations), were tested. The 3M GAS X box oligodeoxynucleotide did not compete with the
Figure 4.2. Cell type specificity of IESE binding nuclear proteins.

High ionic strength EMSA were performed using a double stranded, $^{32}$P-labeled, oligodeoxynucleotide IESE probe corresponding to pIgR bp -67 to -146 (Chapter 3, Fig 3.2). Nuclear extracts were from the following cell types HT-29.74, Caco-2, T-84, Vaco-235, pHIEC, Hep-G2, and HeLa. Homologous competitor oligodeoxynucleotide at 100X probe concentration was used where indicated. Complexes specific for intestinal cells designated by arrow at left.
High ionic strength EMSA were performed using a double stranded, $^{32}$P-labeled, oligodeoxynucleotide IESE probe corresponding to pIgR bp -67 to -146 (Chapter 3, Fig 3.2). Nuclear extracts were from HT-29.74 cells. Competitor oligodeoxynucleotides (See Table 4.1) were used at 100X probe concentration.
Table 4.1. Summary of sequence specificity of IESE-binding proteins

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Complex 1/2</th>
<th>Complex 3</th>
<th>Complex 4/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt IESE</td>
<td>ATTCGTAAGTACAGAGCTC1C</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5' IESE-1</td>
<td>AACGATGAGG-............</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5' IESE-2</td>
<td>ATGGAGG-..............-CCAAGG1</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>3' IESE</td>
<td>-CC-..................</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>5MGAS IESE</td>
<td>-GG-..................</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3MGAS IESE</td>
<td>-GG-..................</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcγRI GAS</td>
<td>GA-AIT-TCACAAG-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MHC II Xbox</td>
<td>T-C-T-C-T-G-G-T-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Competition with wt IESE for binding to nuclear proteins*"
IESE for binding of nuclear proteins suggesting that the mutated residues (pIgR nt -157 and -156) are crucial for nuclear protein binding, while the mutation in the 5M GAS X box oligodeoxynucleotide (pIgR nt -166 and -165) only slightly decreased its ability to compete with the IESE for binding to nuclear proteins, suggesting a less important role.

**Correlation of IESE binding activity with pIgR expression**

Binding of nuclear proteins from HT-29.74 cells and pHIEC to radiolabeled IESE probes was further tested in EMSA by competition with serial concentrations of homologous unlabeled oligodeoxynucleotide (Fig 4.4). Much higher levels of IESE binding were observed with pHIEC nuclear extracts than HT-29.74 extracts (consistent with previous experiments, Fig. 4.1) and at least 10 times higher concentration of competitor required to completely displace probe binding.

It was of interest to note a direct correlation between the levels of IESE binding nuclear proteins and levels of pIgR expression in intestinal epithelial cells expressing pIgR. This relationship was observed between HT-29.74 cells and pHIEC by comparison of their levels IESE binding nuclear proteins (Figs. 4.4 and 4.1) and pIgR mRNA expression relative to α-tubulin expression (Fig 4.5), and in HT-29.74 versus Vaco-235 cells by comparing levels of IESE binding (Fig 4.1) and pIgR protein expression (Table 4.2).
Figure 4.4. Displacement of nuclear proteins binding the IESE probe by serial concentrations of homologous oligodeoxynucleotide competitor.

High ionic strength EMSA were performed using a double stranded, $^{32}$P-labeled, oligodeoxynucleotide IESE probe corresponding to plgR bp -67 to -146 (Chapter 3, Fig 3.2). Homologous competitor oligodeoxynucleotide was used at the indicated competitor : probe concentration ratios.

A: Nuclear extracts from HT-29.74 cells.

B: Nuclear extracts from pHIEC
A. Nuclear Extract from HT-29.74 Cells

B. Nuclear Extract from pHIEC
Figure 4.5. Comparison of pIgR mRNA expression in primary human intestinal epithelial cells and IFN-γ treated HT-29.74 cells.

Northern blot analysis was performed using 20 μg total cellular RNA per lane from HT-29.74 cell cultures with and without IFN-γ and from primary human intestinal epithelial cells isolated from normal intestinal mucosa from 2 surgical specimens. Bands hybridizing to probes specific for pIgR and α-tubulin mRNA are indicated.
A

IFN-γ Treated

plgR

α-tubulin

6h 12h 24h

Control

6h 12h 24h

B

plgR

α-tubulin

Specimen 1

Specimen 2
Table 4.2. Cell-type specificity of pIgR expression

<table>
<thead>
<tr>
<th>Cell-line</th>
<th>-IFN-γ</th>
<th>+IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29.74 well-differentiated colon adenocarcinoma</td>
<td>5.1 ± 0.78</td>
<td>24.4 ± 2.46</td>
</tr>
<tr>
<td>VACO 235 rectal villous adenoma</td>
<td>410 ± 59.6</td>
<td>426 ± 54.8</td>
</tr>
<tr>
<td>Caco-2 colon adenocarcinoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T84 colon adenocarcinoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HepG2 hepatoma</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Cells were cultured for 2 days in the presence and absence of 100 U/ml recombinant human IFN-γ. Expression of pIgR was determined by measuring release of SC (the cleaved extracellular domain of pIgR) in culture supernatants by ELISA. Data are expressed as mean ± S.E.M. (n=6).
Time course of IFN-γ induction of ISRE binding proteins in HT-29.74 cell nuclear extracts

In order to determine if the ISRE in first exon of the plgR gene could bind IFN-γ induced nuclear proteins EMSA was performed using nuclear extracts prepared from HT-29.74 cells cultured with 100 U/ml IFN-γ for 6, 12, and 24 h and from cells cultured without IFN-γ and 32P-labeled double stranded oligodeoxynucleotide probes spanning plgR nt +3 to +22 (Fig. 4.6). Two specific bands (arrows at right) were observed in lanes with nuclear extracts from IFN-γ treated cells, with peak ISRE binding activity observed at 12 h IFN-γ exposure. These bands were not present in lanes with nuclear extracts from cells not treated with IFN-γ, suggesting that binding activity was induced by IFN-γ.

The transcription factor IRF-1 is induced by IFN-γ in HT-29.74 cells and binds the plgR ISRE

The identity of the nuclear protein(s) binding the plgR ISRE was determined by EMSA, performed using nuclear extracts from HT-29.74 cells cultured with and without IFN-γ for 12 h, plgR ISRE probe, and antibodies with specificity for IRF-1, IRF-2, and STAT-1 (Fig. 4.7). The anti-IRF-1 antibody caused a "supershift" of both specific bands from IFN-γ stimulated nuclear extracts, resulting from antibody binding the nuclear protein/probe complexes and further decreasing their electrophoretic mobility. This binding was IRF-1 specific, as demonstrated by the lack "supershift" effects by either anti-IRF-2 of anti-STAT-1 antibodies.
Figure 4.6 Time course of IFN-γ induction of plgR ISRE binding nuclear proteins in HT-29.74 cells.

Low ionic strength EMSA were performed using a double stranded, $^{32}$P-labeled, oligodeoxynucleotide IESS probe corresponding to plgR nt +3 to +22 (Fig. 3.2). Nuclear extracts were prepared from HT-29.74 cells cultured with 100 U/ml IFN-γ for 6, 12, and 24 h and from cells cultured without IFN-γ. Homologous competitor oligodeoxynucleotide at 100X probe concentration was used where indicated. Complexes where binding was sequence specific (defined by displacement by homologous competitor oligodeoxynucleotides) are labeled with arrows.
Figure 4.7 The transcription factor IRF-1 is induced by IFN-γ in HT-29.74 cells and binds the pIgR ISRE.

Low ionic strength EMSA were performed using a double stranded, 32P-labeled, oligodeoxynucleotide IESE probe corresponding to pIgR nt +3 to +22 (Fig. 3.2). Nuclear extracts were prepared from HT-29.74 cells cultured with 100 U/ml IFN-γ 12 h and from cells cultured without IFN-γ. Antibodies specific for IRF-1, IRF-2, and STAT-1 were added as indicated. Complexes where binding was sequence specific are labeled with arrows. DNA/nuclear protein complexes appear below the prominent non-specific band. Complexes "supershifted" by antibody binding appear above this band. The sequences of the upper strand of double stranded oligodeoxynucleotide competitors used at 100X probe concentration were as follows:

ISRE: AGCAGAGTTTCAGTTTTGGCAG
Mut ISRE: AGCAGAGTGTCACTGTGTTGCAG
IESE: ATCCCAAGTAACAGAGTCTCC
We also tested the IFN-\(\gamma\) inducibility of the IRF-1 promoter in HT-29.74 cells (Table 4.3). IRF-1 promoter driven luciferase expression in transiently transfected cells was induced 13.6 and 9.67 fold at 8h and 24 h respectively. The time course of IFN-\(\gamma\) inducibility of the IRF-1 promoter in HT-29.74 cells correlates with time course IFN-\(\gamma\) induced ISRE binding nuclear proteins (Fig 4.6) subsequently identified as IRF-1 (Fig. 4.7).

**DISCUSSION**

Polymeric immunoglobulin receptor expression in humans is restricted to mucosal epithelial cells, developmentally regulated, and IFN-\(\gamma\) inducible in the colonic epithelial cell line HT-29.74 (12, 22, 92). In Chapter 3, we demonstrate that a fragment of the pIgR gene confers basal and IFN-\(\gamma\) inducible reporter gene expression in transfected colonic epithelial cells. Evidence of the involvement of the pIgR ISRE in IFN-\(\gamma\) inducible expression and the requirement for another unspecified cis-element(s) within the pIgR promoter for IFN-\(\gamma\) induction and tissue specific basal transcription is also presented.

We now present evidence of nuclear proteins which bind cis-regulatory elements in the pIgR promoter. Expression of the trans-acting factor IRF-1 is induced in HT-29.74 cells by IFN-\(\gamma\) (Table 4.3) and IRF-1 binds the pIgR ISRE (Fig 4.7). Additionally, intestinal epithelial cell nuclear proteins, distinct from MHC class II gene X box binding proteins, and GAF bind the pIgR IESE (Fig. 4.1), and levels of IESE binding correlate with pIgR expression in intestinal epithelial cells expressing pIgR (Figs. 4.2, 4.4, 4.5, and Table 4.2).
Table 4.3. Transcriptional activity and IFN-γ inducibility of the IRF-1 promoter in transiently transfected HT-29.74 cells

<table>
<thead>
<tr>
<th>Transfection and Treatment</th>
<th>Time in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8h</td>
</tr>
<tr>
<td>pGL2-IRF-1</td>
<td>6.87±0.173*</td>
</tr>
<tr>
<td>pGL2-IRF-1 + IFN-γ</td>
<td>93.3±16.4*</td>
</tr>
<tr>
<td>pRSVluc#</td>
<td>343±24.9*</td>
</tr>
</tbody>
</table>

* Data are expressed as:
(Experimental RLU/min/well - Background RLU/min/well) x 10^{-3}, Mean±SEM, n=3. Background RLU/min/well were measured in HT-29.74 cells transfected with pGL2Basic and were 5.16±0.366 and 6.21±0.0141 at 8 h and 24 h, respectively.

# Luciferase expression vector with expression regulated by the Rouse sarcoma virus promoter enhancer
Single copies of the pIgR inverted repeats and cytokeratin elements do not bind HT-29.74 cell nuclear proteins (data not shown). It is important to note that while these limited negative findings rule out simple specific interactions between HT-29.74 cell nuclear proteins and individual copies of these elements, they do not preclude functions requiring two linked copies or interaction other pIgR cis-elements.

ISRE's are found in many IFN inducible genes (30, 112). Although most often associated with type I IFN induction, via interaction with the trans-acting factor ISGF-3, the ISRE also plays a role in IFN-γ inducibility of guanylate-binding protein (111) and IRF-1 is required for induction (127). Nuclear proteins from IFN-γ treated HT-29.74 cells bind the pIgR ISRE (Figs. 4.6). Two sequence specific protein/DNA complexes are observed, and both are "supershifted" by anti-IRF-1 antibodies but not by antibodies to IRF-2, or STAT-1 (Fig. 4.7). This confirms that IRF-1 is part of both complexes, IRF-2 or STAT-1 is not part of either. This does not rule out the presence of proteins, other than IRF-2 or STAT-1, in these complexes.

Expression of IRF-1 is induced by IFN-α and IFN-γ (28, 29, 128). We find that reporter gene expression driven by the IRF-1 promoter is IFN-γ inducible in transiently transfected HT-29.74 cells, suggesting that IRF-1 expression may be induced by IFN-γ in these cells (Table 4.3). ISRE EMSA confirm this suggestion (see above). These observations support the finding that IFN-γ induction of pIgR mRNA expression in HT-29 cells is dependent on de novo protein expression (12, 40). The kinetics of IFN-γ induction of pIgR mRNA and protein expression, which reach maximum levels at 24 and 72 h, respectively (12), support the hypothesis that this is a delayed response
requiring *de novo* synthesis of trans-activating proteins in response to IFN-γ (25).

The kinetics of IFN-γ induction of the IRF-1 promoter and the appearance of ISRE binding nuclear proteins in HT-29.74 cells are concordant. Induction of the IRF-1 promoter is higher at 8 than 24 h, but clearly induced at both times (Table 4.3). ISRE binding peaks at 12 h, but is still high at 24 h (Fig. 4.7).

IRF-1 is the only trans-acting factor thus far confirmed to bind the pIgR ISRE. This does not rule out important roles for other ISRE binding factors in the regulation of pIgR expression. IRF-2, a trans-acting factor related to but with sometimes opposite actions of IRF-1, also binds ISRE’s (28). While IRF-1 usually functions as a transcriptional activator, IRF-2 is most often a transcriptional repressor. IRF-2 could play a role in silencing pIgR expression. At this point, no information on the expression of IRF-2 in tissues expressing pIgR is available, but given their common DNA binding specificity and opposite regulatory functions, their relative levels of expression could be important in the regulation of pIgR expression.

The pIgR IESE sequence is unique from all known cis-acting elements in the Transcription Factor database. It does resemble the MHC class II X box (Table 4.1), which is the basis for its initial identification (Chapter 3). However, the variance in the pattern of DNA/protein complexes observed in EMSA using IESE and X box probes (Fig. 4.1) and the lack of cross competition between these two elements supports the hypothesis that they bind distinct nuclear proteins. Two of the complexes formed by nuclear proteins and the IESE are specific for intestinal epithelial cells (Fig 4.2) and
levels of complex formation correlate with levels of plgR expression in cells expressing plgR (Fig. 4.4, 4.5, and Table 4.2).

Two intestinal epithelial cell lines tested do not express plgR protein; Caco-2 and T-84 (Table 4.2). Both of these cell types express intestinal cell specific IESE binding proteins (Fig. 4.2) and IFN-γ treated T-84 express IRF-1 which binds the plgR ISRE (data not shown). The reasons why these cells line do not express plgR, either constitutively or in response to IFN-γ cannot be accounted for by the observations presented here, but may be due to idiosyncrasies of the cell lines beyond the scope of this study including, but not limited to, chromosomal translocations or mutations involving the plgR gene.

It is possible that IESE and X box binding proteins are related. Three MHC class II X box binding proteins; hXBP, R-FX, and NF-X have been identified (129). It is not known what, if any, relationship IESE binding portions have to the X box binding proteins, however, they do not have the same DNA sequence binding specificity (Fig. 4.1). That does not rule out other structural or functional similarities.

The plgR and MHC class II antigens are members of the immunoglobulin superfamily (130). Key structural components of these genes are conserved through evolution. In the case of the plgR and MHC class II genes, regulatory elements of the genes have also been conserved. Not only are the IESE and X box similar (Table 4.1), but the inverted repeats of the plgR gene are similar to MHC class II Y box (Chapter 3). This could, in part, account for the distinct but related patterns of expression of the plgR and MHC class II genes (92, 131).
CHAPTER 5
Conclusion and Considerations for Future Studies of
Polymeric Immunoglobulin Receptor Expression
In this dissertation investigations detailing pIgR protein structure and function are summarized (Chapter 1). This body of work confirms the importance of the pIgR in host defense, and provides the rationale for studying mechanisms regulating its expression.

We have demonstrated synergy in the induction of pIgR expression in HT-29 cells by IFN-γ, TNF-α, and IL-4 (Chapter 2 and (91)). We have further demonstrated that mononuclear cells isolated from an anatomic site in close proximity to the intestinal secretory epithelium produce these cytokines, and that culture supernatants derived from these mononuclear cells strongly induce pIgR expression (91)). This observation provided indirect evidence of the importance of cytokine regulation of epithelial cell pIgR expression in the enhancement of local defense functions mediated by IgA.

Our laboratory initiated studies aimed at elucidating the molecular mechanisms regulating tissue specific and IFN-γ induced pIgR expression by cloning the pIgR gene and identifying the promoter proximal region (93). We mapped the start site of pIgR mRNA transcription, and demonstrated that the gene segment corresponding to nt -280 to +29, relative to the transcription start site, confers transcriptional activity and IFN-γ inducibility in the colonic epithelial cell line HT-29.74 (Chapter 3, Fig. 3.4). This pIgR gene segment contains two cis-elements bound by intestinal cell nuclear proteins with sequence specificity. The intestinal epithelial cell specificity element (IESE) binds nuclear proteins unique to intestinal epithelial cells, and levels of IESE binding proteins correlate with levels of pIgR expression (Chapter 4, Figs. 4.2, 4.4, 4.5, and Table 4.2). The pIgR ISRE is involved in IFN-γ inducibility of the pIgR promoter (Chapter 3, Fig. 3.4), and binds the trans-activating factor IRF-
1, expression of which is induced in HT-29.74 cells by IFN-γ (Chapter 4, Fig. 4.7). These observations constitute the first reports detailing molecular mechanisms involved in the regulation of pIgR expression.

Many interesting questions regarding mechanisms of regulation of polymeric immunoglobulin receptor expression remain unanswered. In the remainder of this chapter several will be posed and discussed. One point that must be made immediately is that the scope of the studies undertaken here is limited to mechanisms of regulation of transcriptional initiation. Expression of pIgR protein could be regulated at many other points, including transcriptional elongation, RNA processing, RNA stability, and protein synthesis (132). The presence of long 5' and 3'-untranslated regions in pIgR mRNA provide potential targets for regulation of translation initiation and elongation (133, 134).

The sequence TTTCAGTTTTT is found in pIgR exon 1, spanning nt +10 to +19. Sequences containing runs of T's separated three nucleotides have been associated with transcriptional pausing (Dr. Caroline Kane, University of California, Berkeley, personal communication, and (135, 136)). This sequence is within the pIgR ISRE, and raises the possibility of IRF-1 binding to this site not only inducing pIgR expression by transcriptional transactivation, but also by overcoming a pause signal. Current techniques used to study such mechanisms, such as nuclear run off transcription would be complicated by the proximity of this element to the pIgR transcription start site. The putative pause signal could be mutated to TTTCAGTTTCC, without disrupting the ISRE consensus, and the effects of the mutation in the context of pIgR promoter fragments on transcriptional activity and IFN-γ inducibility
tested. Verification of IRF-1 binding to this new element would also be necessary.

The location of the pIgR ISRE within the gene's first exon is a unique finding, further complicated by its overlapping a possible pause signal. In the limited context of the -280 to +29 pIgR gene fragment, the ISRE is not required for IFN-γ inducibility (Chapter 3, Fig 3.4), however, the ISRE domain is required in the presence of pIgR nt -563 to -281. The IFN-γ response of a construct with the ISRE moved to a location upstream of the transcription start site would provide information of how the unique location of the pIgR ISRE influences it function.

The presence of the ISRE in the pIgR gene raises another possibility. Could IFN-α or IFN-β participate in the regulation of pIgR expression? Reports of cytokine inducibility of pIgR expression by three independent groups (including our own) do not include data relevant to this issue (12, 22-24, 40, 90, 91).

The kinetics of IFN-γ induction of pIgR mRNA and protein expression, which reach maximum levels at 24 and 72 h, respectively (12), support the hypothesis that this is a delayed response requiring *de novo* synthesis of trans-activating proteins in response to IFN-γ (25). However, there is also evidence of an immediate response to IFN-γ stimulation in HT-29.74 cells. First, pIgR mRNA expression is slightly but clearly increased after only 6 h IFN-γ exposure (Fig 4.5 and (12)). Secondly, the kinetics of IFN-γ induced pIgR mRNA expression is non-linear. The rate of increase from 0 to 12 h is less than that from 12 to 24 h (12). These observations indicate that IFN-γ induction of
plgR expression may involve both activation of latent transcription factors, such as GAF, and induction of secondary trans-acting factors, such as IRF-1.

There are apparently cis-elements other than the plgR ISRE involved in IFN-γ induction of plgR expression in HT-29.74 cells (Chapter 3, Fig. 3.4). The MHC class II gene X box and Y box have been linked to IFN-γ inducibility of these genes (101, 129). There are similarities between these elements and the plgR IESE and inverted repeats, respectively (Chapter 3). Mutation of these elements within the context of the plgR promoter would provide insight into their possible roles in IFN-γ induced plgR expression.

Since IESE is a novel cis-element, the nuclear proteins which interact with it may be novel trans-acting factors. It would be of interest to know if these factors are unique or related to other proteins such as the MHC class II X-box binding proteins (129) and if the expression of IESE binding proteins correlates with plgR expression in intestinal epithelial cells as they mature and differentiate in vivo. Tools for addressing these questions could be obtained by cloning IESE binding proteins. At least two strategies could be employed.

An expression library derived from primary human intestinal epithelial cell cDNA could be screened with an IESE oligonucleotide probe (either single copies or IESE multimers). Replicate lifts could be screened with the same probe in the presence of excess unlabeled DNA to verify sequence specificity of binding, or MHC class II X-box probe.

Alternatively, IESE binding proteins from primary human intestinal epithelial cell nuclear extracts could be isolated by affinity chromatography using immobilized IESE oligonucleotides and purified by preparative SDS
PAGE. Their amino acid sequence could then be used to design probes to screen a primary human intestinal epithelial cell cDNA library. IESE binding protein cDNA sequences could then be compared with those of known transcription factors or used to design probes for in situ hybridization to determine tissue distribution.

One of the most interesting and potentially important aspects of pIgR expression is cooperative multiple cytokine induction (Chapter 2 and (24, 90, 91)). We have not found the molecular basis for any cytokine inducibility beyond IFN-γ. As mentioned in the discussion of Chapter 4, a chimeric reporter construct including bp -1800 to +130 of the pIgR gene is being engineered and may provide information on cis-regulatory elements involved in TNF-α, IL-4, and cooperative multiple cytokine induction of pIgR expression.

As discussed above and in previous chapters, expression of the pIgR is cytokine inducible and developmentally regulated in intestinal epithelial cells (12, 22, 24, 40, 90, 91). It is possible that these two aspects of pIgR expression may be related. Do cytokines provide cues for differentiation and maturation of intestinal epithelial cells, or is cytokine responsiveness a consequence of maturation of intestinal epithelial cells? These questions could be addressed by determining whether pIgR expression precedes expression of receptors for cytokines, such as IFN-γ, or vice versa.

In vitro electrophoretic mobility shift assays are convenient relatively simple tools which can be used to demonstrate nuclear protein binding to potential cis-regulatory elements. Probe size is a limiting factor, banding patterns with probes with multiple cis-elements can be confusing, and these
assays do not provide information as to whether sites are bound in vivo. In vitro DNA footprinting allows analysis of longer stretches of DNA for binding of nuclear proteins and can identify contact nucleotides involved in nuclear protein binding (137, 138). In vivo genomic footprinting allows the determination of sequences protected from methylation in living cells, and would facilitate determination of pIgR cis-regulatory elements occupied in various cell lines in the presence and absence of IFN-γ (139, 140).


