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Quantification of gene transcripts in psoriatic skin by using reverse transcription coupled to the polymerase chain reaction

by

Kenneth K.S. Ng

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

MASTERS OF SCIENCE DEGREE

UNIVERSITY BIOCHEMISTRY GROUP

CALGARY, ALBERTA

August, 1992

ISBN 0-315-79073-3

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Quantification of gene transcripts in psoriatic skin by using reverse transcription coupled to the polymerase chain reaction" submitted by Kenneth K.S. Ng in partial fulfillment of the requirements for the degree of Master of Science.

A. T. Garber

Supervisor, Dr. Anthony T. Garber
Departments of Pediatrics and Medical Biochemistry

Dylan Edwards

Dr. Dylan Edwards
Department of Pharmacology and Therapeutics

Lashitew Gedamu

Dr. Lashitew Gedamu
Department of Biological Sciences

Gilbert A. Schultz

Dr. Gilbert A. Schultz
Department of Medical Biochemistry

August 24, 1992

Date

ABSTRACT

The fundamental pathogenesis of psoriasis is poorly defined, because it has yet to be subjected to detailed molecular analysis. The molecular analysis of perturbations in the abundance of specific mRNA's can help to identify the molecular mechanisms causing psoriasis and related diseases. The analysis of gene expression in psoriatic skin requires a very sensitive assay, however, because only small biopsies from human volunteers are available for analysis. The most sensitive assay for gene transcripts uses the polymerase chain reaction coupled to reverse transcription, the RT-PCR assay. Because the RT-PCR assay has not been optimized and thoroughly studied, however, it is potentially more variable and more prone to artefacts than conventional quantitative assays.

An RT-PCR assay for precisely measuring gene transcript levels in small skin biopsy samples was thus developed, tested, and applied. The assay was characterized for accuracy and precision by comparing its results against known amounts of *in vitro* synthesized RNA transcripts. Measurements of some abundant transcripts obtained from Northern blot, slot-blot, and *in situ* hybridization techniques by our lab and others were also consistent with the results obtained from the RT-PCR assay.

Once the assay's validity had been established, the RT-PCR assay was used to investigate the expression of the insulin-like growth factors. The levels of transcripts for the insulin-like growth factors, receptors, and binding proteins in unaffected and affected skin were measured. The assay's sensitivity was indispensable in precisely defining the low levels of expression of most of these species. Measurements of the transcript levels of insulin-like growth factor binding protein-3 confirmed and elaborated upon earlier observations suggesting that this transcript was increased in both affected psoriatic skin fibroblasts and skin. The high absolute levels and significantly increased levels of IGFBP-3 transcript in psoriatic affected skin, and the quantitative profile of transcript levels for the members of the IGF family in skin have many implications on skin biology and the pathogenesis of psoriasis.

ACKNOWLEDGMENTS

Many people helped me during the course of my work. I owe the most to Dr. Tony Garber, who introduced me to molecular biology five long years ago, and who initiated the molecular biological studies into psoriasis which led to my work. His sound and expert guidance has helped shape the best of the work presented here. I also owe the other members of Dr. Garber's laboratory, especially Mr. Manfred Herfort, for valuable discussions over experimental design and the analysis of results. Special thanks must be given to Dr. Tom Enta and his nurses, who took time from their busy schedule to provide us with skin biopsy samples. Thanks must also be given to the volunteers who gave up little plugs of skin for the advancement of science.

Many people in the excellent laboratories of the Departments of Medical Biochemistry and Pediatrics have also helped me by providing access to their equipment and by providing sound counsel in their areas of expertise, and I am very grateful to all of them. Special thanks are extended to Dr. Gil Schultz, who has generously provided several of the oligonucleotide primers used in my studies, since we have a common interest in quantitative PCR and the insulin-like growth factors. Finally, I want to thank the members of my supervising and examining committees, Drs. Dylan Edwards, Tony Garber, Lash Gedamu, and Gil Schultz, for providing me with constructive criticism and thoughtful discussion on this thesis.

This work was supported by the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

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ABBREVIATIONS

bp	basepair
cDNA	complementary DNA
cpm	counts per minute
EGF-R	epidermal growth factor-receptor
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGF-1R	type 1 IGF receptor
IGF-2R	type 2 IGF receptor
IL	interleukin
kb	kilobase
K_d	dissociation constant
MoMLV-RT	Moloney Murine Leukemia Virus reverse transcriptase
MOPS	3-[N-morpholino]propanesulfonic acid
PCR	polymerase chain reaction
RT-PCR	reverse transcription coupled to the PCR
TGF-α	transforming growth factor alpha
TGF-ß	transforming growth factor beta

Metric units were used throughout.

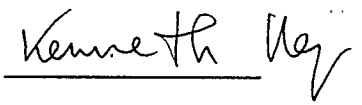
m	milli	10^{-3}
μ	micro	10^{-6}
n	nano	10^{-9}
p	pico	10^{-12}

g	gram
L	litre
m	metre

CLAIM TO ORIGINALITY

The work presented in this thesis was performed by Mr. Kenneth Ng, with the exception of the following.

1. The data presented in figure 3. was generated by Dr. A.T. Garber.
2. The pBSpolyA vector was constructed by Dr. A.T. Garber.



Kenneth Ng
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INTRODUCTION

Psoriasis is a chronic disease affecting roughly 2% of Caucasians and varying proportions of other ethnic groups (Farber and Nall 1985). Those afflicted with psoriasis suffer from recurring skin lesions causing itching, pain, and disfigurement. The current therapies prescribed for these skin lesions are either ineffective, expensive, or toxic over prolonged periods of treatment. Because it is a chronic, incurable disease with only palliative therapies available, the economic cost of managing the disease is high: in 1989, the annual cost of psoriasis in the United States was estimated to be $2.5 billion (National Institutes of Arthritis, Musculoskeletal, and Skin Disease, USA). Moreover, the disease can impair physical activity and cause loss of productivity, as well as inflict psychological trauma due to disfigurement. Current research into psoriasis is aimed at understanding the genetic and biochemical events involved in the development and the resolution of psoriatic skin lesions. This information, it is hoped, will suggest more directed, and thus more effective and less toxic therapies for psoriasis in the future.

An old but important observation is that psoriasis tends to run in families. Many epidemiological studies suggest that one or more genes contribute to the risk of developing psoriasis, but the exact mode of inheritance is still not known. Sibship analysis and twin studies are consistent with either an autosomal dominant mode of inheritance with decreased penetrance and variable expressivity or a multifactorial mode of inheritance with roughly 60 to 90% heritability in different populations (Farber and Nall 1985, Iselius and Williams 1984). A reasonable current hypothesis is that defective alleles at several gene loci, including one particularly important locus, may act in conjunction to predispose the development of psoriasis, while one or more environmental factors also act to trigger the onset of the disease. According to this hypothesis, several genes and several environmental factors would have to be identified and interrelated to explain the development of psoriasis.

One approach to identifying and elaborating the factors, especially the genes, involved in causing psoriasis is to analyze the molecular phenotype of psoriatic skin. The genetic factors contributing to the onset of psoriasis most likely involve defects in the immune response and in the control of epidermal growth and differentiation, since these are the most obvious changes in the diseased skin. Thus, one would predict that psoriatic skin contains changes in either the specific activities or levels of expression of the proteins that control the immune response, growth, and differentiation of skin.

A first step in cataloguing the physiological changes in psoriatic skin is to determine whether specific proteins and other factors that have the potential to control skin growth, differentiation, or immune response are in fact altered in activity. For example, are there increased levels of known mitogenic cytokines or accessory proteins, such as receptors and binding proteins, stimulating epidermal hyperproliferation? Alternatively, are certain inhibitory cytokines downregulated? With regards to the perturbed immune response, are there defects in the proteins involved in antigen presentation and response? Are there changes in the expression of specific cytokines and accessory proteins controlling the response of immune cells?

The answers to these questions would provide the foundations for uncovering the underlying causes of psoriasis as well as potential avenues for more effective treatments. After identifying the molecular changes responsible for the psoriatic phenotype, it may also be possible to trace the pathways leading to these molecular changes back to their ultimate source. Determining the ultimate causes of psoriasis as well as the incipient pathways of pathogenesis would allow pharmacological and other treatments to be more effectively directed towards interrupting the epidermal hyperproliferation and inflammation of psoriatic skin.

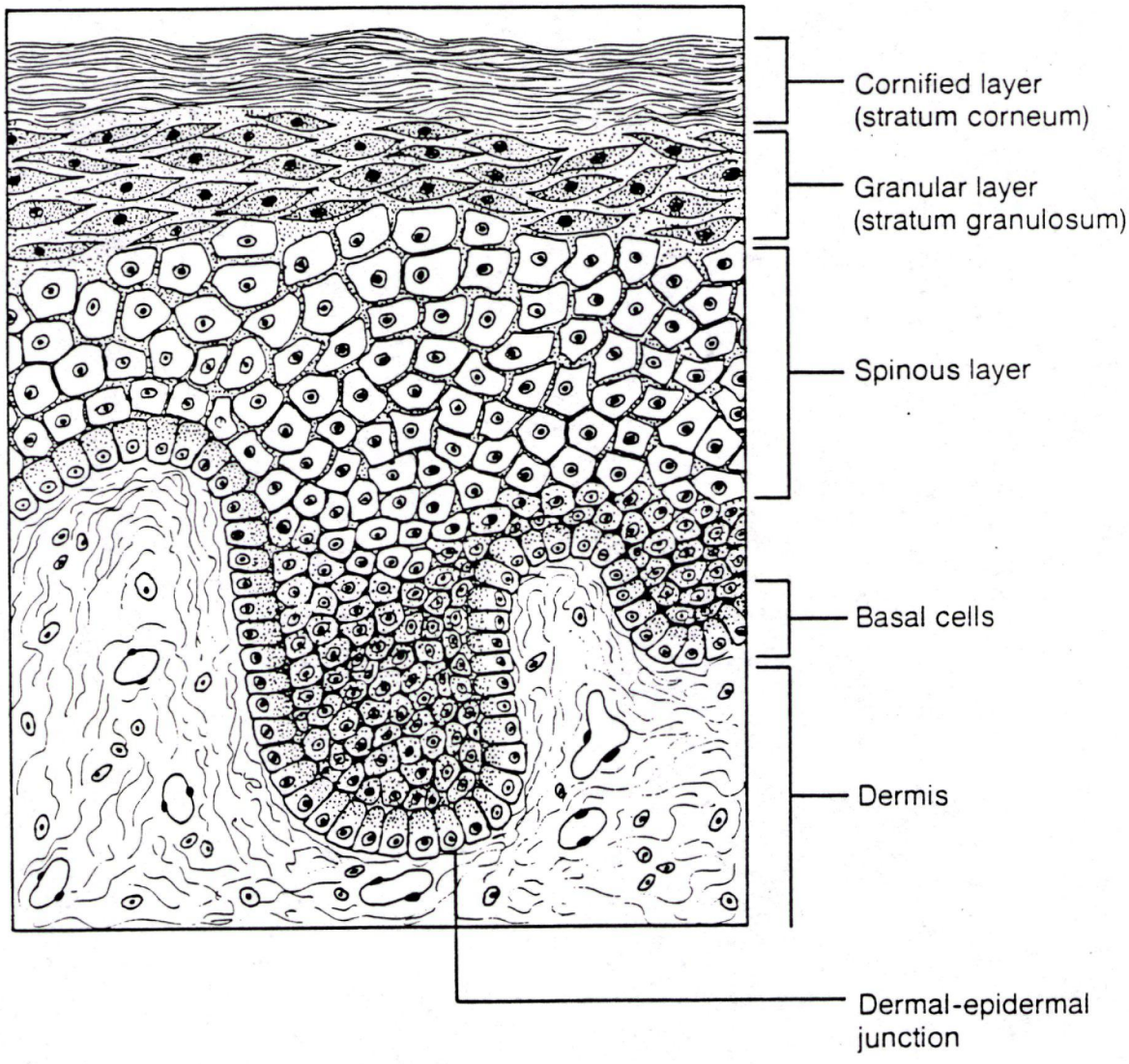
Besides these primary goals, characterizing the molecular phenotype of psoriatic skin will provide information helpful for understanding other physiological conditions and diseases. Understanding the mechanisms giving rise to psoriasis has in the past and will inevitably continue to shed light on basic skin biology, including the physiology of the wound response and allergic reactions. Advances in the understanding of psoriasis may also give insight to a large number of related inflammatory diseases like rheumatoid arthritis, ankylosing spondylitis, and multiple sclerosis, which have similarities to psoriasis in many respects.

Cell Biology of Skin and Psoriasis

The normal skin consists of several distinct layers of cells combined with intercellular matrix (reviewed in Eckert 1989, Odland 1983) (Figure 1). The deepest compartment is the hypodermis, a layer of fat. Above this lies the dermis, which mostly consists of a complex network of collagen fibrils, some blood vessels, and scattered cells. The main dermal cell is the skin fibroblast, a cell derived from the embryonic mesoderm. Among the fibroblast's many functions is the maintenance of the dermal matrix and the secretion of proteins for communicating with cells in the epidermis and blood. Situated above the dermis and just below the epidermis is the epidermal-dermal junction. This layer consists of basement membrane and blood capillaries which regulate the interchange of factors and cells among the dermis, epidermis, and blood.

The epidermis is a mostly cellular compartment composed of several different types of cells, including melanocytes, Langerhans cells, and keratinocytes. The keratinocytes comprise over 90% of the cellular mass of the epidermis (Eckert 1989). Keratinocytes are derived from the embryonic ectoderm and form a continuously growing and differentiating population that produces and organizes most of the protective structural components of the skin. Keratinocytes in the basal layer of the normal epidermis divide throughout life with an average cell cycle time of roughly 13 days, depending upon the site on the body (Weinstein et al 1984). New cells

Figure 1. Schematic diagram of the structure of normal skin (from Orkin et al 1991).


Cornified layer
(stratum corneum)
Granular layer
(stratum granulosum)
Spinous layer
Basal cells
Dermis
Dermal-epidermal
junction

occupy the bottom layer and push old cells upwards. As keratinocytes age and migrate upwards, they progressively differentiate, expressing a strictly defined series of enzymes and structural proteins during their maturation. The spinous layer lies above the basal layer, and contains more flattened cells with many desmosomal interconnections. Among the many important changes occurring in the cells progressing through this layer is the production of new forms of the key structural intermediate filament proteins, the keratins. The overlying granular layer of cells expresses involucrin, profilaggrin, and transglutaminase, which are the structural and enzymatic components of the topmost cornified layer, and stores these components in distinctive keratohyalin granules. The keratinocytes at the end of the differentiation pathway, the corneocytes, have lost their nuclei and release the keratohyalin granules, leaving a complex web of tough structural proteins and lipid that protect the body from external insults. The average lifetime for a normal keratinocyte, from birth to being sloughed off from the body, is roughly 39 days (Weinstein et al 1984).

Psoriatic skin lesions display two prominent changes in skin physiology which are mostly confined to the epidermis. First, immune cells and inflammatory molecules accumulate and become activated in the epidermis. Second, keratinocytes hyperproliferate and undergo abnormal patterns of differentiation. The two changes are likely related, but the exact relationships are still not clear. Indeed, the primary defect or defects in psoriasis may potentially reside in many loci. Neither the temporal nor the causal order of changes in psoriatic skin have been determined, and it is unclear whether the specific alterations in immune cell, keratinocyte, fibroblast, and endothelial cell physiology are primary or secondary events in the pathogenesis of psoriasis. The best that can be done at present is to describe the overt changes evident in psoriatic skin and guess at the causal relationships between the perturbations. Hopefully, as observations accumulate, the primary and secondary effects can be sorted out.

Immune cell infiltration and an inflammatory response are characteristic of psoriasis, and give rise to many of the symptoms of the disease (Baker and Fry 1992). Psoriatic lesions contain increased numbers of and increased activity in CD4+ T cells, neutrophils, and monocytes. Though far from being understood fully, these cells have many known functions, including the ability to stimulate and be stimulated by other cells through complex cytokine, eicosanoid, and chemotactic peptide pathways. Of importance to psoriasis and other inflammatory diseases, most of these cells release and respond to the cytokines interleukins -1, 6, and 8 (IL-1, IL-6, and IL-8), and tumor necrosis factor-α (TNF-α); and eicosanoids like leukotriene B4 and 12-hydroxyeicosatetraenoic acid (12-HETE); all of which are increased or perturbed in other ways in the psoriatic epidermis when compared to normal (Nickoloff 1991). Moreover, most of these cytokines and eicosanoids, as well as cell-surface trafficking molecules like intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial cell adhesion molecule-1 (ELAM-1), are also produced in significant amounts by keratinocytes and endothelial cells, and many specific perturbations in the expression of these factors have already been observed in psoriatic lesional skin. In response to these and other inflammatory signals, capillaries in the epidermal-dermal junction increase both in size and in tortuosity (Mordovtsev and Albanova 1989) and the blood flow in the growing edge of psoriatic lesions is increased over 3-fold when compared to normal skin (Hull et al 1989). Moreover, plasminogen activator is increased nearly 10-fold in activity in psoriatic lesions (Jensen et al 1988) and leads to the generation of chemotactic peptides. Some neurogenic peptides, like the vasoactive intestinal peptide (VIP), are also increased in psoriatic lesions (Anand et al 1991). Altogether, these many inflammatory changes are believed to contribute to the redness, edema, and pain associated with psoriatic lesions.

The initiating cause of the inflammatory response in psoriasis is not well defined, but the following scenario is likely, given our current knowledge of inflammation in skin and other tissues. Similar to the processes occurring in a wound response or allergic reaction, an environmental insult may induce keratinocytes and endothelial cells to produce chemotactic peptides, eicosanoids, cytokines, and cell adhesion molecules which attract immune cells to the skin and then activate them. The activated immune cells then release inflammatory mediators which induce additional changes in other epidermal cells, resulting in a full-scale inflammatory response (Cooper and Baadsgaard 1991). An observation that supports the primary role of immune cells in the development of psoriasis is that relatively low doses of the drugs cyclosporine and FK506, which are potent immunosuppressors, help to resolve psoriatic lesions (Khandke et al 1991). These drugs have many other known effects, however, and their role in resolving psoriatic lesions remains unclear (Cooper et al 1990).

The other characteristic perturbation in psoriatic skin, which consists of dramatic changes in the growth and differentiation of the epidermal keratinocytes, may also be related to inflammatory processes. The most undifferentiated epidermal cells, the basal keratinocytes, enter mitosis more frequently and complete cell divison more quickly in psoriatic lesions than do the basal keratinocytes from normal skin. Roughly three times as many keratinocytes are actively dividing, and the cell cycle time is shortened to nearly one-tenth, resulting in a nearly thirty-fold increase in the total production of cells in the skin (Weinstein et al 1985). In addition, the rate at which basal cells migrate through the epidermis and mature increases roughly 7-fold (Weinstein et al 1985). As expected, the maturation of these much more quickly migrating keratinocytes is also severely perturbed (Bernard et al 1988).

These changes in keratinocyte growth and maturation are unique to psoriasis and result in the characteristic flaky skin phenotype of psoriatic lesions. During the normal maturation of keratinocytes, the patterns of protein synthesis and hence cellular morphology change according to a well-defined sequence. Consequently, normal skin has a defined series of layers, each of which serves specific structural functions. The most important protective layer in the skin lies at the end of the maturation pathway at the skin surface. Normally, terminally differentiated anucleate corneocytes degenerate into a tough layer of highly cross-linked proteins combined with lipid to form the skin's main protective barrier. In the psoriatic epidermis, however, many structural proteins and enzymes are either not expressed, expressed in an aberrant sequence, or expressed at altered levels (Bernard et al 1988). Remarkably, the total transit time of a psoriatic keratinocyte from birth to desquamation is also reduced from the normal 39 days to roughly 5 days due to the increased rates of growth and migration, and the decreased mechanical integrity of the mature cells (Weinstein et al 1984). Because of the improper maturation of psoriatic keratinocytes, the corneal layer of the epidermis is structurally weak and flakes off more easily and profusely than normal.

The dramatic changes in both the keratinocytes and inflammatory pathways of psoriatic skin likely give rise to most of the characteristic symptoms of the psoriatic lesion. Thus, understanding the causes of these changes in psoriatic skin would help identify the causes of psoriasis and suggest more effective means of treating the disease. Many molecular defects could potentially lead to the altered physiology observed in psoriatic skin, however. The problem, then, is to identify the most likely targets for the primary defects in psoriasis, and then to efficiently screen for perturbations in those molecules which can be consistently linked to the development of psoriasis.

Cytokines and Psoriasis

Many molecules are known to play key roles in regulating and effecting the functions of the immune system and epidermal growth and maturation systems in the skin. Among these, the cytokines, their signal-transducing receptors, and their signal-modulating binding proteins are perhaps the most attractive candidates for analysis in psoriasis. Cytokines are defined as soluble polypeptides which carry signals between separate cells by binding to specific receptors on target cells at picomolar to nanomolar concentrations (Nathan and Sporn 1991). Several cytokine signalling systems are already known to control the normal growth and development of skin, and the abnormalities in psoriatic skin must arise from the subversion of at least some of these normal control mechanisms (reviewed in Nickoloff 1991).

Two competing hypotheses can be forwarded to involve cytokines in the development of psoriasis. First, a defect in a specific cytokine signalling pathway could act as the primary cause of psoriasis. The other characteristics of the disease, like immune cell activation and epidermal hyperproliferation, could be driven by a primary defect in a cytokine pathway. Alternatively, the basic genetic defect in psoriasis may lie outside of a cytokine signalling pathway, possibly in a component of the immune system for example, and this primary defect may trigger normal cytokine pathways into hyperactivity to create the psoriatic skin phenotype. If the first scenario is true, detecting hyperactivity in specific cytokine pathways may directly identify the basic defect in psoriasis. If the second possibility is true, screening for defects in cytokine pathways may help us indirectly trace the pathogenetic mechanisms in the disease towards the ultimate cause. Regardless of which hypothesis is true, an understanding of those cytokine pathways which control the activity and development of skin cells will inevitably advance our understanding and treatment of psoriasis.

Several cytokine pathways have already been investigated in the skin, all of which have relevance to psoriasis. Transforming growth factor-α, the transforming growth factor-ß's, basic fibroblast growth factor, keratinocyte growth factor, and the insulin-like growth factors have all been identified as functional growth regulators of cultured keratinocytes (reviewed in Aaronson et al 1990). Moreover, increasingly detailed studies are beginning to show how different cytokines interact with each other and how the signals from each cytokine affect the target cell. To date, however, there have not been appropriate methods for quantitatively assessing the true physiological roles and importance of specific cytokines in living skin. Indeed, systematic and comprehensive screens for the physiologically important cytokines are not yet feasible, and the current set of known cytokines may be far from complete.

Approaches to identifying the molecular perturbations in psoriatic skin

The ultimate goal of my project has been to identify proteins which may be responsible for the development of the psoriatic skin phenotype. There are several approaches which theoretically could be taken, several of which are now being tried by our lab and others. One approach exploits the genetic basis of psoriasis. Many genetic studies of psoriasis suggest a major heritable component in the development of the disease. Thus, one approach is to use linkage analysis to map the loci conferring susceptibility to psoriasis and then attempt to identify the "psoriasis gene" by positional cloning.

A clearly different approach is to describe the biochemical and physiological characteristics of psoriatic skin and to identify the molecular perturbations in the diseased skin. If we can identify the most important perturbations and then interconnect them by physiological mechanisms, then we would hope to be able to understand how the skin lesions develop and what basic defects cause the cascade of changes in psoriatic skin. As a result, pharmacological or other types of therapy could be directed in an informed and thus more effective manner to stop

the progress of the disease. My project focuses on the latter approach, although the results of my work could have implications for genetic linkage studies as well. That is, by identifying genes whose patterns of expression are altered, a candidate gene may be traced down and tested for linkage to psoriasis.

Psoriasis is particularly amenable to this second approach, because the high frequency of the disease and the accessibility of skin have facilitated an extensive description of psoriasis at the clinical and histological levels, in particular, but more recently at the cell physiological and biochemical levels, too. Consequently, as was described earlier, two fundamental changes in psoriatic skin account for nearly all of the characteristic symptoms, and the molecular basis of these changes should clearly be the focus of further study. Moreover, it is clear that many known molecules, especially the cytokines, their receptors, and their binding proteins, can now be screened for perturbed functions in psoriasis.

The functions of specific proteins may be changed in many different ways to induce the psoriatic skin phenotype. First, a specific protein may be altered in activity due to a mutation in the coding sequence of the gene. The coding sequence is a primary determinant of the protein's final conformation, which in turn is a primary determinant of the protein's function. Changes in sequence and conformation may lead to changes in enzymatic activity, binding affinity, and the regulation of those activities. An example of a coding sequence change leading to a disease is the $\triangle$F508 mutation in the chloride channel protein that has now been linked to the development of cystic fibrosis (Kerem et al 1989).

A second mechanism for altering a protein's function is to change its level of expression. Changes in the rates of transcription, transcript processing, translation, and post-translational processing could all arise from mutations in the regulatory sequences of the gene encoding a particular protein, or from changes in the regulation of these processes due to the alteration in expression of a protein higher in the regulatory hierarchy. Changes in the levels of expression

of normal proteins can also have serious effects on normal cell physiology. For example, a two-fold increase in cyclin A can completely disrupt the course of the cell cycle in yeast and other eukaryotes (Forsburg and Nurse 1991).

For most proteins it is much easier to detect altered levels of normal proteins or their transcripts than alterations in the specific activity of mutant regulatory proteins, because for most proteins, measurements of specific activity usually require technically inconvenient assays and large amounts of material. Fortunately, however, screening for changes in the levels of expression of specific genes will often reveal the presence of changes in the activities of proteins higher in the regulatory hierarchy. That is, the identification of a gene that is expressed at altered levels indicates that the regulation of its expression is perturbed and thus an analysis of its regulation should reveal the cause of its altered levels of expression. Changes in regulatory sequences or in the alterations of the activities of regulatory proteins can be identified and characterized. If necessary, this analysis could be repeated indefinitely until the ultimate cause of psoriasis is identified.

Ideally, candidate proteins should first be evaluated at the level of protein activity, and if that is found to be altered in psoriatic skin, then the exact nature of the change (i.e., whether it arises at the level of transcription, translation, etc.) should be identified. For most proteins, including the cytokines, cytokine receptors, and cytokine binding proteins, however, specific and sensitive assays for protein activity are either difficult to develop or completely unavailable.

A practical alternative to evaluating levels of gene expression is to measure the steady-state levels of specific gene transcripts. While neglecting the translational and post-translational levels of control on gene expression, measurements of steady-state transcript levels are by far the most specific, sensitive, and efficient way to screen a large number of candidate molecules for perturbations in their levels of expression. After first identifying the species of mRNA whose steady-state levels are altered in psoriatic skin, subsequent studies can pinpoint and elaborate the

exact nature of the perturbation. Since these measurements comprise only a primary screen for transcripts with perturbed levels of expression, subsequent analyses of the regulation of that gene's expression should reveal perturbations in the activity of upstream regulators, if any. Hence, screening for perturbations by looking only at steady-state mRNA levels should be reasonably comprehensive without being excessively tedious.

Attesting to the effectiveness of this approach are several early successes, including the identification of transforming growth factor-alpha (TGF-α) (Elder at al 1989), interleukin-6 (IL-6) (Grossman et al 1989), and interleukin-8 (IL-8) (Nickoloff et al 1991) as upregulated transcripts encoding key cytokines in the psoriatic skin. Subsequent to these initial observations, intensive study into these proteins has begun to reveal their central roles in skin physiology and their specific roles in the pathogenesis of psoriasis. By looking at the regulation of the expression of these and other factors whose levels are perturbed in psoriatic skin, there is hope of tracing down the fundamental causes of psoriasis. In addition to the analytical approach, the effects of these factors on different aspects of skin physiology can also be tested directly in transgenic animals and *in vitro* skin-equivalent models to elaborate how inflammation and epidermal hyperproliferation develop at the molecular level in psoriatic skin.

Methods for quantifying gene transcripts in psoriatic skin

While providing some successes, previous molecular screens for perturbations of transcript levels in skin have been limited by the sensitivity and accuracy of the methodology for quantifying specific species of RNA. The lack of reliable animal or *in vitro* cultured tissue models for psoriasis requires the direct analysis of psoriatic skin in humans. Since the skin samples provided by volunteers are usually very small, however, hybridization assays like Northern and slot-blot hybridization are necessarily limited to examining only abundant transcripts. Solution hybridization coupled to nuclease protection can quantify lower levels of

transcripts, but is rather cumbersome. An efficient and highly sensitive assay exploiting reverse transcription and the polymerase chain reaction was thus developed during my thesis work. This thesis will mainly address the development, testing, and application of this method for quantifying transcript levels from small skin samples.

There are two approaches currently used for quantifying small amounts of specific RNA molecules. One approach exploits the ability of cloned nucleic acid sequences to hybridize specifically with native transcripts. Cloned sequences are labeled to high specific activity with either a radioactive or non-radioactive tag and allowed to hybridize with target molecules either in solution or immobilized on a membrane. Specific hybrids are selected by nuclease digestion or high-stringency washing, and the amounts of target in the sample can be quantified by measuring the amount of label present in the hybrids. The most popular of these techniques include Northern blot hybridization, slot-blot hybridization, and solution hybridization coupled to nuclease protection.

There are several problems with these assays for quantifying transcripts in psoriatic and normal skin. Most importantly, most common hybridization assays lack the sensitivity needed to measure even moderately abundant transcripts in the small skin samples obtainable from volunteers. The sensitivity of a hybridization assay is limited by the specific activity of the probe and the efficiency of separating non-specifically hybridized probe from specifically hybridized probe. Simple calculations show that a typical single-stranded, 500 bp, ^{32}P-labeled probe with a specific activity of 10^8 cpm/μg has an equivalent specific activity of 1 cpm / 40,000 molecules. Assuming that the hybridization of all target molecules can proceed to near completion, and that the background can be reduced to 50 cpm, the lowest signal that could be reproducibly quantified would be 70 cpm, which is equivalent to $3x10^6$ molecules, or 2 pg of a 1 kb RNA transcript. This is comparable to the levels of sensitivity reported for conventional solution hybridization assays coupled to RNase protection (Durnam and Palmiter 1983) and is less than or equal to one-

fifth of the lowest number of transcripts reported to be measurable by Northern and slot-blot hybridization.

This level of sensitivity may preclude the accurate measurement of a number of physiologically relevant species from small samples. For example, if the hybridization assay consumed 500 ng of total RNA in an assay (one-quarter of the typical yield from a 3-mm punch biopsy from normal skin), this corresponds to the RNA extracted from roughly $2.5x10^4$ cells (assuming 20 pg RNA/cell) (Lee and Costlow 1987). If one-tenth of the cells (say, the basal epidermal cells) were expressing a moderately active gene at the level of 40 steady-state transcripts per cell, we would expect 10^5 transcripts to be present in the sample to be analysed. Expression at this level would not be quantifiable by even the best solution hybridization assay. A moderately abundant transcript being expressed by all cells would be marginally measurable but transcripts of low abundance or even moderately abundant transcripts expressed only in a subset of cells would lie beyond the scope of quantification methods relying on hybridization. Moreover, such assays consume a large amount of sample and would prevent the repeated analysis of a number of low-abundance transcripts from the same sample. A method of higher sensitivity would be very valuable, even for quantifying abundant transcripts, because more sensitive methods consume less of each very valuable sample.

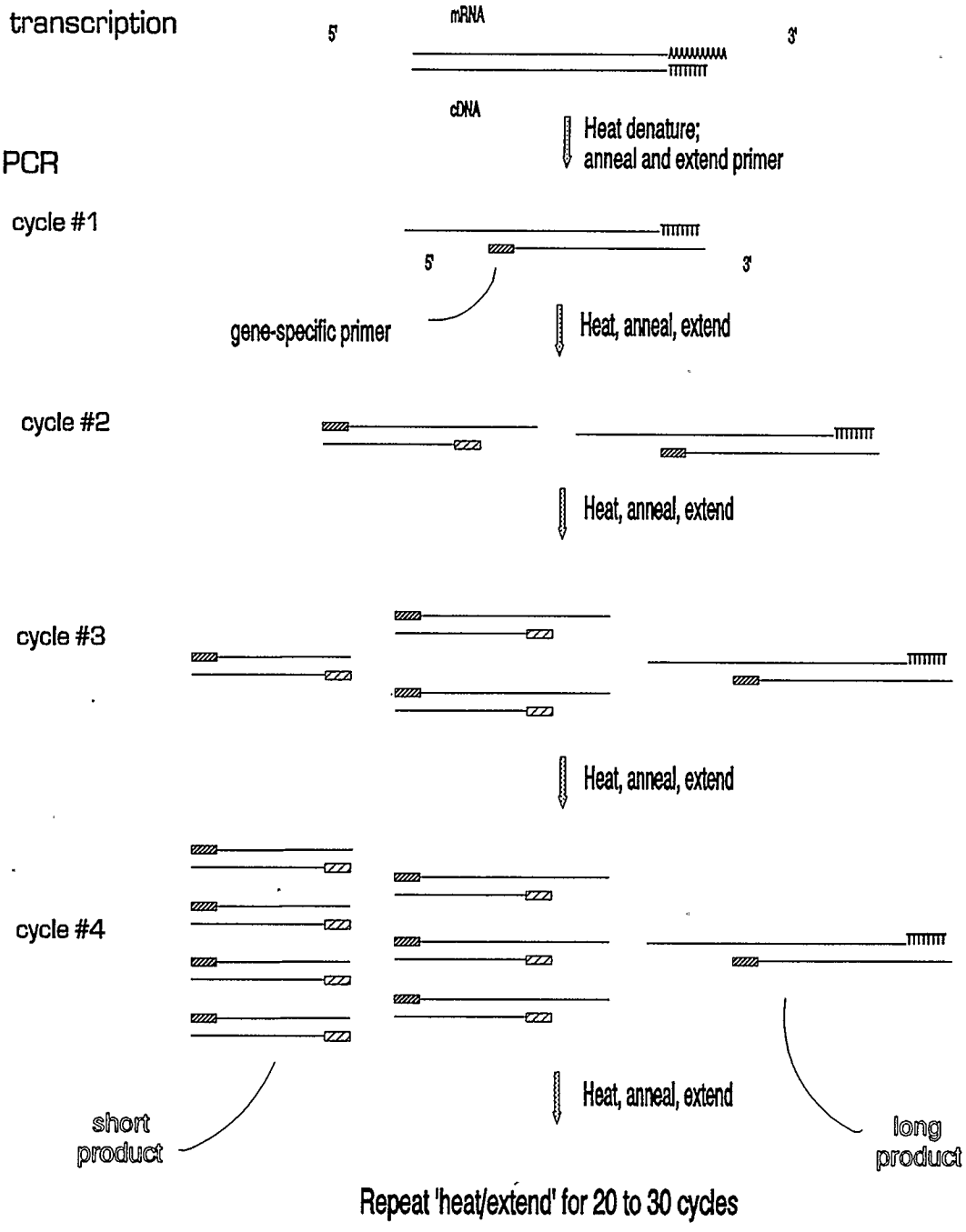
Reverse transcription coupled to the polymerase chain reaction (RT-PCR)

A second approach to quantifying gene transcripts exploits the enzymatic reactions of reverse transcription and the polymerase chain reaction (PCR). The PCR is now used to qualitatively detect the presence of specific gene transcripts for many experimental and forensic studies (reviewed by Bej et al 1991). The sensitivity of the PCR is much higher than hybridization methods and even single gene copies can be detected.

The PCR uses two oligonucleotide primers to initiate roughly twenty to forty cycles of enzymatically catalyzed reactions which synthesize DNA strands complementary to a short stretch of target DNA called the amplicon (Figure 2). The primers are designed so that each anneals to a different strand of the amplicon and each initiates the synthesis of a complementary DNA strand that contains the amplicon.

In the first cycle of the PCR, a template consisting of either genomic DNA or cDNA (reverse transcribed from RNA) is denatured and each DNA strand binds a complementary PCR primer. The latter are in vast excess over the amplicon sequences and drive the annealing reaction to completion in a short time (Syvanen et al 1988). After each primer has annealed, a DNA polymerase molecule catalyzes the synthesis of DNA strands starting from each annealed primer and extending across the amplicon. These newly synthesized DNA strands are called the long products of the reaction and are the only products of the first cycle of the PCR. The second cycle of the PCR involves the denaturation of all previously formed duplexes; after the first cycle, only hybrids containing the long products and the original templates are present. Primers then anneal to all available sequences on the original templates and long products and new DNA strands are synthesized beginning at the annealed primers. The products of the second cycle and all subsequent cycles include long products formed from the synthesis of DNA complementary to the original templates, as well as short products. Short products arise from the synthesis of DNA from a primer annealing to either a long product or another short product. Clearly, short product begins to accumulate exponentially after the second cycle, so long as primers anneal to all possible complementary binding sites at the ends of previously formed short products. In contrast, long products only increase in a linear fashion, and do not significantly contribute to the extent of amplification after the first cycle.

Figure 2. Schematic diagram of the RT-PCR process


Reverse
transcription
mRNA
5'
3'
cDNA
Heat denature;
anneal and extend primer
PCR
cycle #1
5'
3'
gene-specific primer
Heat, anneal, extend
cycle #2
Heat, anneal, extend
cycle #3
Heat, anneal, extend
cycle #4
Heat, anneal, extend
short
product
long
product
Repeat 'heat/extend' for 20 to 30 cycles

The actual yield from a PCR obviously depends upon the actual efficiency of the synthesis of new amplicons from existing copies. If the efficiency is defined as the fraction of existing amplicons which are copied during each cycle of the PCR, then the product yield after n cycles from a reaction starting with A_0 copies of cDNA can be predicted roughly as follows:

$$\text{Yield}_n = A_0 * (1 + \text{efficiency})^{n-1}$$

Clearly, product yield can only be directly related to the amount of input cDNA if the efficiency of the reaction can be controlled to reproducible levels.

Many variables can potentially affect the efficiency of short product synthesis in a PCR. Incomplete product synthesis during any cycles of the PCR will lower the product yield from the reaction and obscure the proportionality between product yield and the amount of transcript in the sample. Two general strategies for controlling variations in the efficiencies of separate reactions have been proposed: 1) product yields from a PCR are normalized to those from internal standards; or 2) PCR conditions are optimized and the amplification reaction is terminated before the reaction reaches plateau, so that product yields from different reactions can be compared.

In the first approach, an internal standard template is constructed and added to the sample to be assayed (Becker-Andre and Hahlbrock 1989, Wang et al 1989, Gilliland et al 1990). The internal standard contains sequences hybridizing to the same primers as those specific for the desired target; however, an alteration is made inside the amplicon sequence such that the internal standard amplicon can be distinguished from the native target. This alteration usually causes the loss of a restriction site or a slight increase in the size of the amplicon, either of which can be detected after amplification. This internal standard template will be amplified at roughly the equivalent efficiency to that of the native transcript in the PCR, if the internal standard transcript

is present at amounts comparable to the native transcript, say within 3-fold. Hence, sample to sample variations are controlled for, and the quantification of a particular sample is carried out by comparing the yield of product from the amplification of the native transcript versus the yield of product from the amplification of the internal standard.

While the use of internal standards provides the best theoretical controls on sample variability, significant additional work must be performed during each assay to quantify the signal from the internal standard as well as the signal from the sample to be measured. The inclusion of internal standards in the PCR is especially laborious, because valid comparisons between the yields of product from the internal standard and the native transcript can only be made if the amount of internal standard transcript added to a particular sample is within 3-fold of the actual amount of native transcript in the sample (Becker-Andre and Hahlbrock 1989, Gilliland et al 1990). Product yields differing by more than three-fold are related in a non-linear fashion which is very hard to predict. Thus, the amount of internal standard added to each sample must first be guessed at, and then empirically determined from the results of trial assays. For a set of samples with variable amounts, a number of trials must be performed to determine the appropriate amount of internal standard to add to an assay, and then a number of additional measurements must be made to ensure that the amount of standard added brackets the amount of target to be measured.

An additional complication of the internal standard method is that the internal standard transcript must be specially constructed in such a manner that it can be distinguished from the native transcript. Most often, this is done by deleting a restriction enzyme recognition site in the amplicon by site-directed mutagenesis, cloning the mutant, and preparing transcripts from the clone *in vitro* .(Becker-Andre and Hahlbrock 1989, Gilliland 1990). Then, following amplification, the internal standard product must be distinguished from the native product by quantitative restriction enzyme digestion and high resolution gel electrophoresis. Variations from

this approach can be imagined, but each involves a comparable amount of extra work. Temperature gradient gel electrophoresis, for example, may be used to distinguish between native and standard PCR products differing by a point mutation (Henco and Heibey 1990).

The advantage of including internal standards in assays is to provide a control for the efficiencies of reverse transcription and amplification in different reactions. These reactions are inherently sensitive to a number of variables, including sample quality, the amounts of reagents added, the times and temperatures allowed for the reactions, and especially in the case of the PCR, the effect of the amount of product on the efficiency of the amplification. The last variable is particularly important, because of the exponential nature of the PCR.

The most important causes of variations in amplification efficiencies between different PCR reactions have been briefly investigated (Syvanen et al 1988, Murphy et al 1990). They include

1) the accumulation of short product to levels where primers, polymerase, or deoxynucleotides are insufficient to drive the complete synthesis of one new strand for each strand carried over from the previous cycle,

2) programming times and temperatures of incubation which are insufficient to drive the complete synthesis of one new strand for each strand carried over from the previous cycle,

3) variations in the incubation temperatures generated by the thermal cycler (Linz 1990, Hoelzel 1990), and

4) slight variations in pipetting.

These factors have been systematically studied by only a few groups. Early studies suggested that the accumulation of product to high levels was the only significant problem to compromising reproducible efficiencies between different reactions (Syvanen et al 1988). Primer annealing, duplex denaturation, and primer extension were clearly shown to occur to completion during all cycles of the PCR except where product yields reached high levels. Without limiting the number of PCR cycles executed, it was obvious that PCRs without internal standards would fail to yield amounts of short product that were proportional to the amount of input template.

Several later studies showed that reactions at high efficiencies produced predictable amounts of product so long as product accumulation was monitored and the PCR was terminated before the reaction entered plateau (Singer-Sam et al 1990, Murphy et al 1990, Robinson and Simon 1991). Many quantitative assays developed from these principles, including one which is very similar to the one described in this thesis (Robinson and Simon 1991). Variability has not been fully addressed, but the work of many labs shows that under some conditions, variability can be rigorously controlled without resorting to the tedious application of internal standards.

Identification of abnormal gene expression by subtractive hybridization screening

Subtractive hybridization screening permits the identification of transcripts which are specifically overexpressed in one sample compared to another (Sargent 1987). Transcripts that are specifically overexpressed in affected psoriatic skin as opposed to unaffected psoriatic or normal skin may indicate a perturbation specific to and important in the development of the psoriatic phenotype. Thus, complex cDNA libraries have been constructed from small amounts of RNA from psoriatic skin cell lines, and these libraries have been screened by subtractive hybridization techniques (Herfort and Garber 1991).

Repeated cycles of subtractive hybridization were found to result in a nearly 10 000 fold enrichment of sequences which are more abundant in one library than the other. The enriched population of cDNA clones can be analyzed by a number of approaches. A sophisticated approach includes transfection into normal fibroblasts and selection for clones whose behavior appears to be altered to the psoriatic phenotype, though this approach could not be expected to succeed in a disease like psoriasis where more than one disease gene is likely involved. More simply, a large number of clones representing overexpressed transcripts can potentially be identifed by partial sequencing and comparing those sequences against the known databases of nucleic acid and protein sequences.

Each species of RNA identified by subtractive hybridization screening must be further characterized before subsequent studies can be initiated. Most importantly, the patterns of expression should be quantified in psoriatic skin itself to establish the actual degree of overexpression. Limited to small samples of skin tissue, evaluating the degree of overexpression of each transcript usually demands a highly sensitive yet accurate quantitative assay. Hence, the RT-PCR assay would be ideal for screening clones identified by subtractive hybridization screening. Once these levels are measured and are shown to be altered in psoriatic skin, more intensive efforts could then be directed to characterize the transcript's patterns of expression, regulation, and function in the skin. Ultimately, the significance of the putatively overexpressed transcript could be tested as a candidate for genetic linkage to psoriasis.

IGFBP-3 and Psoriasis

Subtractive hybridization screening studies have identified several dozen cDNAs that were preferentially found in affected psoriatic skin fibroblasts as opposed to unaffected psoriatic skin fibroblasts (Herfort and Garber 1991). Several of these clones were partially sequenced and two were identified by alignments to the nucleic acid sequences in the Genbank database. One of

these clones was identical to part of the insulin-like growth factor binding protein-3 coding sequence. This clone was used as a hybridization probe to estimate its prevalence in (a) cDNA libraries from psoriatic affected skin fibroblasts and psoriatic unaffected skin fibroblasts; (b) RNA from psoriatic affected and unaffected skin fibroblasts; and (c) RNA from psoriatic affected and unaffected skin (Figure 3). In all three comparisons, material from affected psoriatic skin fibroblasts and skin contained more IGFBP-3 clones and transcripts than material from unaffected psoriatic skin fibroblasts and skin. These findings implicate IGFBP-3 and the insulin-like growth factors in the pathogenesis of psoriasis.

The insulin-like growth factors

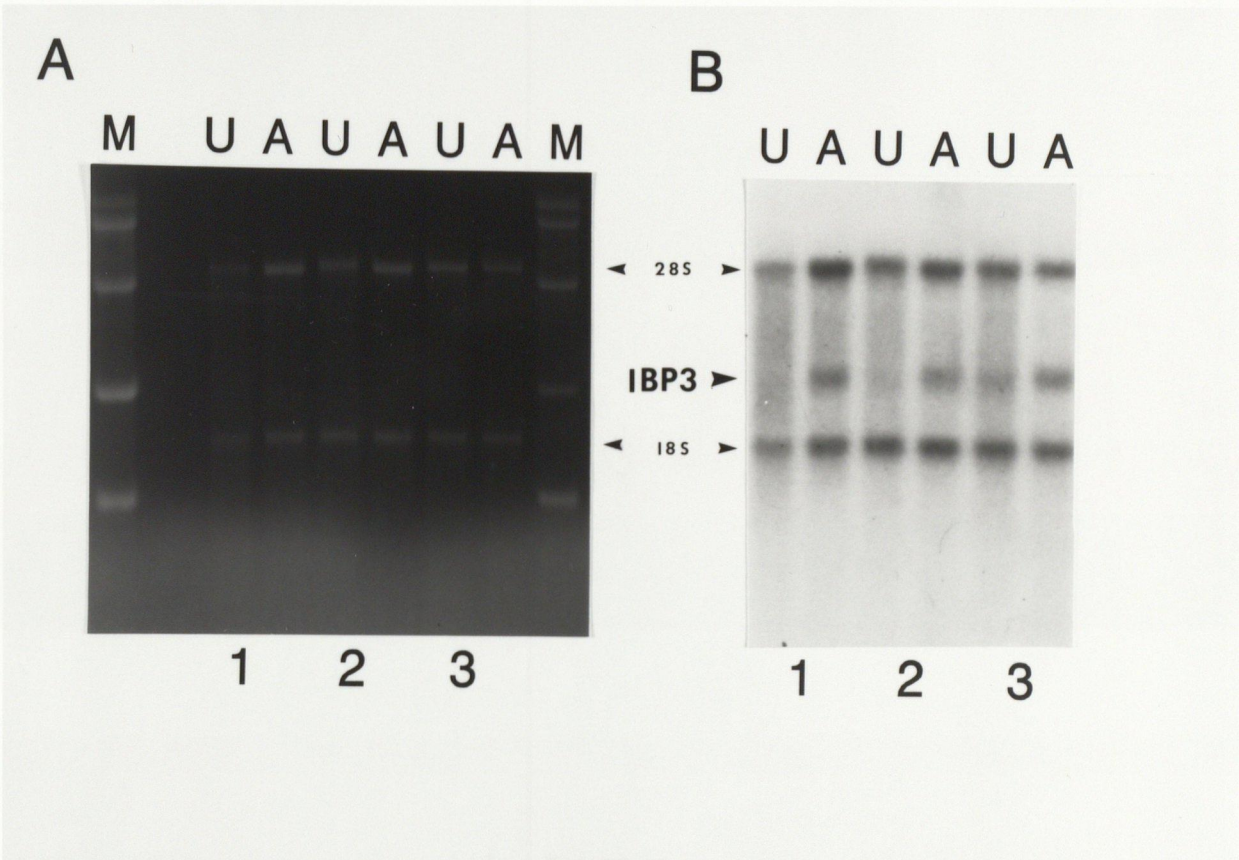
The insulin-like growth factors are a family of related polypeptides involved in signalling between many cell types (reviewed in Herington 1991, Schmid and Ernst 1992). Two genes encode the known kinds of IGF's, IGF-I and IGF-II. Primary transcripts undergo alternate splicing and processing, resulting in a number of different steady-state transcripts differing mainly in the non-coding regions. There is some suggestion of tissue-specific processing, but the functional differences of different transcripts is not known. The translated polypeptides also undergo variable modifications, including glycosylation. The amino acid sequence of the known IGF-I forms are nearly identical, except for slight deletions and point mutations that have been attributed to alternate splicing and proteolytic processing. The sequences of the known IGF-II forms are also the same, so at present there is evidence for only two genes encoding the two insulin-like growth factors (Herington 1991).

The structures of IGF-I and IGF-II are very similar to each other, and together with insulin, they form a closely related family of cytokines. IGF-I and IGF-II are the most related to each other, sharing 63% sequence identity (45 out of 70 residues) in an optimal alignment (Sara and Hall 1990). IGF-I and IGF-II also have 43% and 41%, respectively, sequence identity

Figure 3.A. Denaturing formaldehyde agarose gel electrophoresis of total RNA from psoriatic skin biopsies. One μg RNA loaded per lane. Gel stained with 0.5 μg/mL ethidium bromide and photographed under ultraviolet light. M = RNA molecular weight marker (BRL): sizes of transcripts = 9.5, 7.5, 4.4, 2.4, and 1.4 kb. U = unaffected skin, A = affected (lesional) skin. Pairs of A and U samples were from patients 1 to 3.

Figure 3.B. Northern blot hybridization analysis of the gel from Fig. 3.A. with IGFBP-3 riboprobe. 28S and 18S ribosomal RNA bands are denoted by the small arrowheads. IGFBP-3 transcript (2.5 kb) is denoted by the large arrowhead. U = unaffected skin, A = affected skin. Samples are paired from patients 1 to 3.

(Northern blot and denaturing agarose gel electrophoresis were performed by Dr. A.T. Garber)


A
M U A U A U A M
1 2 3
B
U A U A U A
28S
IBP3
18S
1 2 3

with insulin. Computer modeling and preliminary NMR structural studies suggest that the overall folds of these three proteins are similar (Blundell et al 1983, Cooke et al 1991). The structural differences are functionally significant with respect to binding and signalling specificities for the IGF receptors and binding proteins, however.

IGF-I and IGF-II are differentially regulated and expressed in many different tissues during early development and throughout life. Both IGF-I and IGF-II are expressed in many human fetal tissues (16-20 weeks), as well as the placenta (Han et al 1987a,b; Han et al 1988; Hill et al 1988; Moller et al 1991). IGF-II transcripts are particularly abundant in the liver, adrenals and skin, while IGF-I is expressed at several hundred-fold lower levels in these tissues. Of particular interest, IGF-II transcripts are only expressed deep in the dermis of developing fetal human skin and not in the epidermis. IGF-I, at 200-fold lower levels, has a similar pattern of expression. Because IGFBP-1 protein localizes to the basal epidermis as well as the superficial dermis, however, it seems likely that the IGF's being made in the skin are acting in a paracrine fashion on the epidermis (Han et al 1987a,b; Hill et al 1988).

In the adult, patterns of tissue-specific expression are not as well characterized. In the blood, IGF-I and IGF-II are present at 30 and 90 nM respectively throughout adulthood (Luna et al 1983); for comparison, these levels are roughly 20-fold higher than post-prandial serum levels of insulin. The production of IGF-I is stimulated by growth hormone, so its concentration rises to 90 nM during puberty before declining to adulthood levels. The levels of IGF-II in circulation are steady throughout life. In the adult, the main source of both peptides appears to be the liver, which expresses IGF-I at 3-4 copies per cell and IGF-II at 15-20 copies per cell (Moller et al 1991). In the adult, IGF-II is the most abundant in the choroid plexus of the brain, though its abundance in the fetal brain is relatively low (Stylianopoulou et al 1988). Both the IGF-I and IGF-II proteins have been immunologically localized to neonatal foreskin and adult skin, but their levels appear to be low in the epidermis (Ahmed et al 1992). These results

conflict with the findings from Gray and coworkers (1987), who detected large amounts of IGF-II transcripts in adult skin. A possible explanation is that IGF-II is only expressed in the dermis and exerts paracrine effects on the epidermis, as in the fetus.

IGF ligands produced in the liver and possibly other tissues may have endocrine effects on many other tissues. The ligands appear to have short half-lives in circulation unless they are bound by IGF-binding proteins (IGFBPs). The main IGFBP in circulation, IGFBP-3, is produced in large amounts by the liver and kidney. In combination with an acid-labile α-subunit, the acid-stable IGFBP-3-ß subunit binds to and protects IGF-I and IGF-II from proteases in circulation. Active IGF-I can be released from the IGFBP-3 complex by specific proteases, including one whose activity is increased during pregnancy or severe illness.

The transport of IGF ligands into various tissues has been studied by measuring the redistribution of radiolabeled IGF-1 tracer injected into a rat's circulation (Ballard et al 1991). After 15 minutes at rest, large amounts are found in kidney, liver, lungs, and adrenals, while moderate amounts accumulate in the heart, stomach, and intestines, and trace amounts can be found in the skin, muscle, and brain. After two hours, most tissues have lost from 60 to 80% of the IGF label, except for the skin, which retains nearly all of the original label.

At present, it is not known how IGF's are transported to various tissues from circulation. However, there are at least six known binding proteins and two signal-transducing receptors which could compete with circulating carrier IGFBPs for the IGF ligands (reviewed in Schmid and Ernst 1992). Potentially, specific tissues may selectively release IGF ligands from binding protein complexes by secreting specific proteases. Both an unidentified serum protease whose levels in circulation rise during pregnancy and illness (Davies et al 1991) and plasminogen activator acting with plasminogen in a cultured fibroblast cell line (Campbell et al 1992) can release active IGF ligands from (IGF-I)-(IGFBP-3) complexes by selectively cleaving IGFBP-3. The physiological relevance of these proteases to the transport of IGF-I *in vivo* is unclear, but a

tissue may potentially regulate the amount of IGF-ligand available to it by controlling the release of specific proteases.

Although IGFBP-3 accounts for over 90% of the IGFBP activity in adult human serum, five other IGFBPs are known to be expressed in the adult (reviewed in Schmid and Ernst 1992). All six binding proteins are evolutionarily related, each sharing from 50 to 60% amino acid sequence homology with each other (Shimasaki et al 1991). IGFBP-3 binds each ligand in a one-to-one ratio, but the specific interactions between the binding protein and the ligands are not known (Martin and Baxter 1986). The binding affinities of IGF-I and IGF-II for IGFBP-1 are similar (K_d = 0.21 and 0.33 nM respectively) (Martin and Baxter 1986). These affinities are roughly ten times higher than the binding affinity of IGF-I for the IGF-1R (K_d = 5.6 nM, Clemmons et al 1990; K_d = 1.5 nM, Steele-Perkins et al 1988). Clemmons and coworkers (1990) also show that mutants of IGF-I which bind IGFBP-1 and IGFBP-3 with lower affinity, while retaining high binding affinities for IGF-1R, exert stronger mitogenic effects on aortic smooth muscle cells than normal in the absence of exogenous IGFBP-1, but exert weaker mitogenic effects in the presence of exogenous IGFBP-1. These latter observations and others suggest that the exact binding affinities of IGF ligands to cell-secreted IGFBPs, IGFBPs in circulation, and IGF receptors may be very important in controlling the activities of the IGF ligands.

Two unrelated signal-transducing receptors are known to bind the IGF ligands with high affinity (reviewed in Czech 1989). The type I IGF-receptor binds both IGF-I (K_d = 1.5 nM) and IGF-II (K_d = 3 nM) with high affinity (Steele-Perkins et al 1988). Although details of the binding interactions are not known, IGF-I and IGF-II likely bind differently to the type I IGF-receptor and may induce different conformational changes that result in ligand-specific signals. Some aspects of the signalling from the type I receptor are known. The type I receptor belongs to the class of receptor tyrosine kinases including the well-studied EGF, PDGF, and insulin receptors (reviewed in Cadena and Gill 1992). Like those other receptors, the binding of a

ligand, either IGF-I or IGF-II, induces autophosphorylation at a tyrosine residue. Downstream of this, a nuclear phospholipase that leads to specific changes in nuclear but not cytoplasmic phosphoinositide levels is somehow activated by the binding of IGF-I to type I receptors on Swiss 3T3 cells (Diveche et al 1991). Other steps in the IGF-1R signal transduction pathway are still obscure.

The type II IGF-receptor also has high affinity for IGF-II (K_d = 2 nM) (Roth et al 1991). There are also conflicting reports about its affinity for IGF-I (Sara and Hall 1990). This receptor has no homology with the type I receptor or other tyrosine kinases, and its signalling mechanisms are unclear. However, it clearly does transduce signals from IGF-II binding in a number of cell types that completely lack type I receptor (for example Tally and Hall 1990). This receptor also has another function as the cation independent mannose-6-phosphate receptor involved in lysosomal protein targeting (MacDonald 1991).

Although the biochemical pathways connecting ligand binding to changes in cell physiology are mostly unknown, the binding of IGF-I and IGF-II to the signal-transducing receptors clearly lead to important changes in growth and differentiation in many cell types. IGF-I, either alone or in combination with a large number of other cytokines, including EGF, FGF, and PDGF, can stimulate entry into or progression through the cell cycle in osteoblasts, dermal fibroblasts, and keratinocytes (reviewed in Humbel 1990). In BALB/c 3T3 fibroblasts from mice, IGF-I clearly can act as a progression factor that signals the cell to enter S phase after G1; thus, IGF-I can stimulate cell proliferation only with the cooperation of a competence factor like PDGF, which can signal a cell to first exit Go (reviewed in Goldring and Goldring 1991). In several different lines of fibroblasts transfected with plasmids encoding type 1 IGF receptors and IGF-I, the type I IGF receptor and IGF-I can form an autocrine loop to sustain growth without any serum dependence (Pietrzkowski et al 1992). NIH-3T3 fibroblasts overexpressing the type 1 IGF receptor can also be stimulated into mitogenesis with only IGF-I stimulation through the

type 1 IGF receptor (Lammers et al 1989). In keratinocytes, several studies show that IGF-I alone only stimulates growth moderately, but if either EGF, PDGF, or basic FGF are also applied, IGF-I exerts an additional synergistic effect on mitogenesis (Krane et al 1991).

In addition to stimulating growth, IGF-I has many effects on the differentiation of cells. IGF-I can induce differentiation in myoblasts, osteoblasts, adipocytes, and oligodendrocytes through mechanisms independent of direct effects on growth (Sara and Hall 1990). The effects of the IGF's on differentiation have been best studied in primary osteoblast cultures and established cell lines, where IGF-I induces alkaline phosphatase activity, an increase in collagen mRNA, and a decrease in collagen degradation, all markers of terminal differentiation (Schmid and Ernst 1992). The effects of IGF-I on normal keratinocyte differentiation have not yet been reported, but in a keratinocyte cell line transformed with SV40, IGF-I induces a pattern of keratinization and differentiation similar to the normal differentiation pathway of non-transformed keratinocytes. That is, IGF-I appears to induce differentiation in keratinocyte cells that are undergoing active mitogenesis due to SV40 infection (Kamalati et al 1989).

Specific differences in the physiological activity of IGF-I and IGF-II have not been well characterized, but the many structural differences between the two proteins may result in significant differences in binding affinities, specificities, and physiological effects. Many differences in each factor's affinities for the different binding proteins and receptors have also begun to be tabulated. It is clear that the potential number of signals carried by these important cytokines, receptors, and binding proteins is enormous.

GOALS OF THE PROJECT

The purpose of my studies has been to develop, test, and apply a sensitive yet accurate RT-PCR assay for measuring specific gene transcripts in psoriatic skin. First, the assay was thoroughly optimized and tested to characterize and validate the assay. Then, once the assay's validity had been established, the expression of a number of cytokines and housekeeping transcripts were evaluated. In particular, the patterns of expression for the IGF ligands, receptors, and binding proteins in psoriatic skin were investigated, because of suggestive subtractive hybridization studies carried out in the lab. Measurements of the levels of IGF ligands, receptors, and binding proteins in psoriatic skin implicate the IGF's in the pathogenesis of psoriasis and warrant further descriptive and functional studies on the roles of the IGF's in the development of psoriasis.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized at the Calgary Regional DNA Synthesis Facility (Faculty of Medicine, University of Calgary). The DNA sequences of each PCR primer pair are given in Table 1. Primer design was facilitated by commercial nucleic acid analysis software (Primer Designer 2.0, Scientific and Educational Software). *Thermus aquaticus* DNA polymerase was purchased from Promega. MoMLV-RT was obtained from Bethesda Research Laboratories. All other enzymes, deoxynucleotides, and poly(I) were purchased from Pharmacia. High specific activity α-^{32}P dCTP (>3000 Ci/mmol) was purchased from ICN. Oligo-(dT) cellulose was purchased from Collaborative Research. All PCR reactions were carried out in a Coy Model 60 tempcycler.

Routine molecular biology procedures

Unless otherwise specified, routine molecular biology procedures like organic extraction and precipitation of nucleic acids, restriction enzyme digestion, blunting sticky ends, ligation, plasmid preparation, and dideoxynucleotide chain-termination sequencing were performed according to Sambrook et al (1989).

pBSpolyA cloning vector and cloning of PCR products

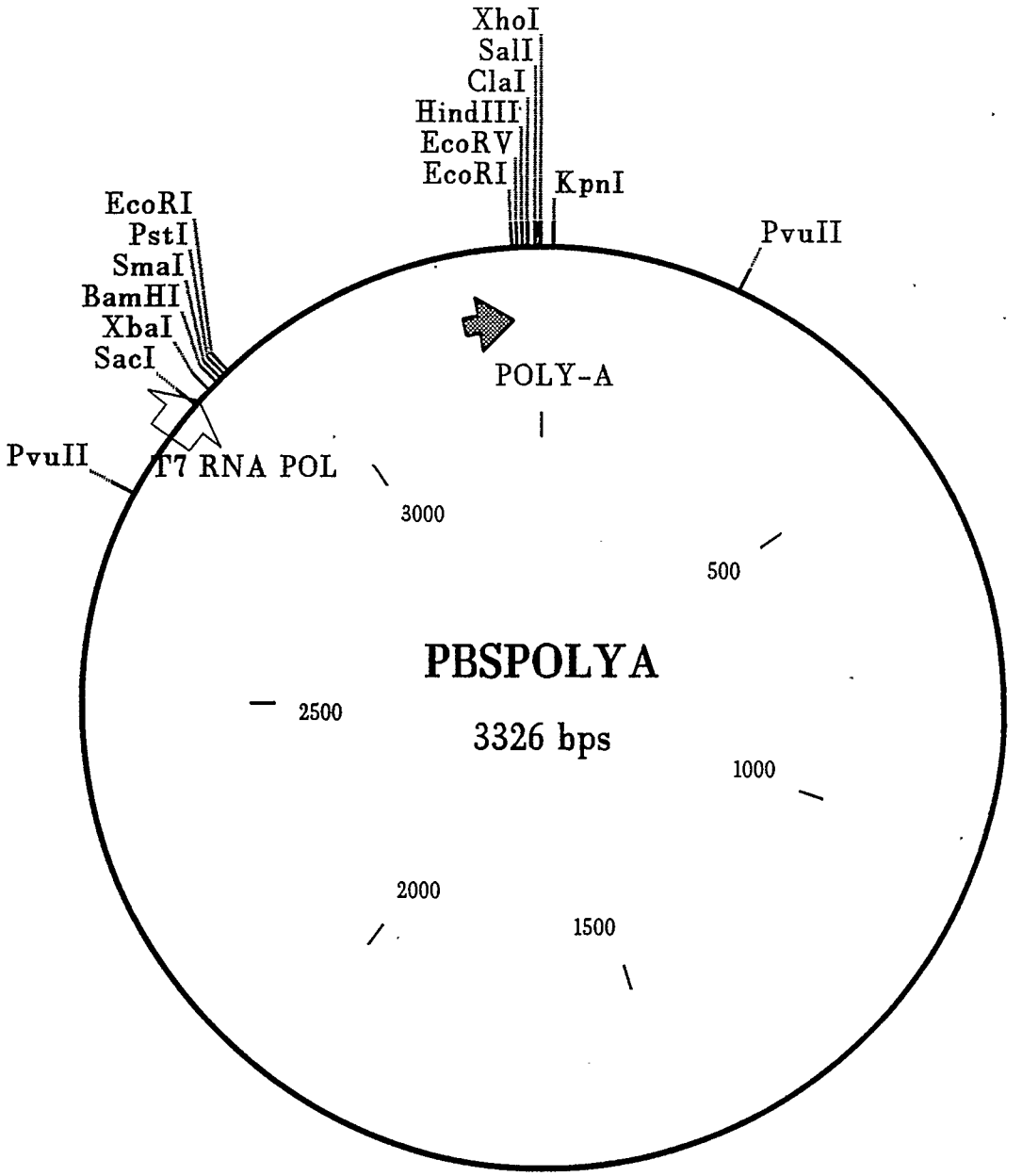
The pBSpolyA vector was constructed by ligating a 540 bp EcoRI fragment containing the 3' end and 70 residues of the poly(A) tail of a testis-specific trout creatine kinase cDNA (Garber et al 1991) into a EcoRI site of pBluescript(KS+) (Stratagene) (Figure 4).

Table 1 – PCR primer sequences

Gene		Sequence	Position
cyclophilin A	5'	TTGGGCCGCGTCTCCTTTGA	63-82
	3'	ATGCCAGGACCCGTATGCTTTA	304-283
Haendler, B. et al (1988) EMBO J. 6:947-950.			
ß-actin	5'	CGTGGG*C*CGCCC*T*AGGCACCA	1258-1278
	3'	TTGGCCTT*A*GGGTTCAGGGGGG	1634-1613
Ng, S.-Y., et al (1985) Mol. Cell. Biol. 5:2720-2732.			
TGF-α	5'	ACCTGCAGGTTTTTGGTGCAGGAGG	209-233
	3'	GCAGATGAGGGCCCGGCACCACT	448-426
Jakowlew, S.B. et al (1988) Mol. Endocrinol. 2:1056-1063.			
EGF-R	5'	AAGTGTGTAACGGAAT*TC*GTATTGGTGAA	1189-1218
	3'	CCATCACTTATCTCCT*C*GAGGGAGCG	1562-1537
Merlino, G.T. et al (1985) Mol. Cell. Biol. 5:1722-1734.			
IGF-I	5'	CACACCATGTCCTCCTCGCA	255-274
	3'	GCACGGACAGAGCGAGCTGA	550-531
Rotwein, P. (1986) PNAS 83:77-81.			
IGF-II	5'	CGCCCCAG*C*GAGAC*T*CTGTGC	329-349
	3'	GCCCACGGGGTATCTGGGGAA	583-563
Bell, G.I. et al (1984) Nature 310:775-777.			
IGF-1R	5'	ATGCTGTTTGAACTG*CA*GCGCATGTGCTGG	3750-3779
	3'	CCGCTC*GAG*CTTGCGGCCCCCGTTCAT	4110-4084
Ullrich, A. et al (1986) EMBO J. 5:2503-2512.			
IGF-2R	5'	AAGAGGCTGAGATACGTGGA	4882-4901
	3'	ACA*GAT*G*T*TGATGTAGAA*GA*CAGG	5232-5209
Oshima, A. et al (1988) J. Biol. Chem. 263:2553-2562.			
IGFBP-2	5'	ACTGTGACAAGCATGGCCTGT	905-925
	3'	CCTCCTGCTGCTCATTGTAGA	1072-1052
Binkert, C. et al (1989) EMBO J. 8:2497-2502.			
IGFBP-3	5'	CCAGAACTTCTCCTCCGAGTC	700-720
	3'	TATCCACACACCAGCAGAAGC	908-888
Wood, W.I. et al (1988) Mol. Endocrinol. 2:1176-1185.			
IGFBP-4	5'	GTGCATGGAGCTGGCGGAGA	322-341
	3'	TAGAGGTCCTCGTGGGTGCG	615-596
Latour, D. et al (1990) Mol. Endocrinol. 4:1806-1814.			

- All sequences read 5' to 3'.
- Italicized letters indicate mismatches with the human sequences, since mouse sequences were used for the design of primers obtained from Dr. G. Schultz.

Figure 4. Diagram of the pBSpolyA phagemid vector, showing the positions of unique restriction sites, the T7 RNA polymerase promoter and the poly(A) sequence.


XhoI
SalI
ClaI
HindIII
EcoRV
EcoRI
KpnI
PvuII
POLY-A
EcoRI
PstI
SmaI
BamHI
XbaI
SacI
PvuII
T7 RNA POL
3000
500
PBSPOLYA
3326 bps
2500
1000
2000
1500

Reverse transcription of total skin fibroblast RNA was carried out as described below. cDNA was amplified by the PCR for 25 to 30 cycles of amplification under standard conditions until a product could be detected. The PCR product was purified by electroelution after agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 0.2 mM EDTA) and subsequently made blunt-ended with the Klenow fragment of E.coli DNA polymerase I. Blunt-end fragments were ligated into the SmaI site of pBSpolyA using T4 DNA ligase. The ligated DNA was purified by organic extraction and ethanol precipitation before being electroporated into the bacterial host, JM109 (Yanisch-Perron et al 1985). Transformants were screened for plasmids containing inserts of the correct sequence and in the correct orientation. Screening consisted of diagnostic restriction enzyme digestions and dideoxynucleotide chain-termination sequencing.

Denaturing agarose gel electrophoresis

Purified RNA samples were denatured in 50% formamide, 6% formaldehyde, and MOPS buffer (20 mM MOPS, 5 mM Na-acetate, 1 mM EDTA, pH 7) at 65°C for 7 minutes and cooled on ice. Samples were electrophoresed for one hour in the presence or absence of ethidium bromide at 100 V through a 1% agarose gel containing 2% formaldehyde and MOPS buffer.

Synthesis and purification of standard RNA transcripts

Plasmid DNA templates were prepared by standard methods, including a final purification step of cesium chloride equilibrium centrifugation (Sambrook et al 1989). Purified plasmid containing the desired PCR product in the pBSpolyA vector was linearized by HindIII digestion downstream of the poly(A) sequence and the DNA was purified by organic extraction. These linearized templates were subjected to run-off transcription using T7 RNA polymerase and transcripts were purified by oligo-(dT) cellulose chromatography (Sambrook et al 1989). Purified fractions were

concentrated by ethanol precipitation and quantified by spectrophotometry at 260 nm. Two hundred nanogram samples of purified cRNA were tested for integrity by electrophoresis on denaturing formaldehyde-agarose gels and staining with ethidium bromide. Standard cRNA transcripts were diluted into 2mM Tris-Cl (pH 8.0) and stored in siliconized tubes at -70°C. Final dilutions contained 30 ng/μL poly(I).

Purification of RNA from skin biopsies

Total RNA was purified by the acid-guanidinium-phenol-chloroform (AGPC) method of Chomczynski and Sacchi (1987). Three or 4 mm skin punch biopsies were obtained by a standard protocol and placed immediately in the denaturing solution containing 4M guanidinium thiocyanate. The solution was vortexed vigorously and the insoluble residue was pelleted by centrifugation. The supernatant was collected and processed as described previously with the following modifications. First, the RNA was purified by an additional final step of phenol-chloroform extraction and ethanol precipitation and, second, the RNA was dissolved in 100 μL DEPC-treated water and stored at -70°C for up to six months. The integrity of RNA preparations was tested by loading 1 μg on a denaturing formaldehyde agarose gel. Total RNA was quantified by two complementary methods. First, spectrophotometry at 260 and 280 nm was performed. Second, a slot-blot hybridization analysis was performed on the RNA samples using a radiolabelled cRNA probe containing part of the human 28S rRNA gene (Gonzalez et al 1985). After overnight hybridization at 65°C and stringent washing, slots were located by autoradiography and cut out for liquid scintillation counting. Known amounts of dermal fibroblast total RNAs were hybridized in parallel to generate a calibration curve against which unknown biopsy sample RNAs were compared. Typical yields of total RNA by this method were 5 μg for both normal skin and unaffected psoriatic skin, and 30 μg for affected psoriatic skin.

Controlled hydrolysis of RNA

To test for the effects of degradation of RNA samples on the yield of PCR short product, samples of mouse liver RNA were degraded by controlled alkaline hydrolysis. Immediately before use, 15 mM sodium hydroxide, 2 mM Tris (orig. pH = 8.0) (solution A) was prepared and kept on ice, and acetic acid was diluted to 7.5 mM (solution B). 19μL of mouse liver RNA (1 μg/μL) was added to 19 μL of chilled solution A and mixed. 6μL of this mixture was aliquoted to each of twelve microcentrifuge tubes chilled on ice. Tubes were incubated for 0, 40, 80, 120, 160, and 200 seconds at 60°C before alkaline hydrolysis reactions were terminated by the addition of 6μL of chilled solution B and transfer of the reaction to ice. Samples were diluted in 2 mM Tris, pH 8.0 and analyzed immediately by the RT-PCR assay and by denaturing agarose gel electrophoresis, Northern blotting, hybridization, and densitometry.

Quantitative RT-PCR assay

Samples of 120 ng of total skin RNA or dilutions of synthetic RNA in 120 ng polyinosine were denatured along with 200 ng of random hexamer primers at 70°C for 5 minutes and cooled on ice. The 5x reaction buffer (Bethesda Research Laboratories, final conc.: 50 mM Tris-Cl, pH 8.3 (at 22°C), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$), 0.5 mM of each dNTP, 100 units of MoMLV-RT, and 10 units of RNasin were added from a master mix and incubated at 37°C for 90 minutes; the final reaction volume was 10 μL. An equal volume of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) (10 μL) was added to chelate free magnesium ion and distilled water (30 μL) was added to bring the volume to 50 μL. The reaction was heated at 95°C for 5 minutes and cooled on ice. These diluted cDNAs were stored at -20°C for up to three months.

A 4 μL aliquot of the diluted cDNA solution was added to a master mix containing PCR reaction buffer (final conc.: 10 mM Tris-Cl, pH 8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% NP-40), 7.5 pmol of each of the appropriate PCR primer pair, 0.2 mM of each dNTP, 1

μCi α-^{32}P-dCTP (3000 Ci/mmol), and 0.4 units of Taq DNA polymerase. The reaction (10 μL) was overlaid with mineral oil (20 μL) and rapidly heated to 90°C in a hot water bath. The tubes were quickly transferred to the tempcycler and incubated at 95°C for 30 seconds after which the PCR was executed using a temperature cycling program specific for each primer-template combination.

Denaturation was always set for 95°C for 1 minute. Extension was always set for 72°C for 1 minute. Annealing times and temperatures, and the total number of PCR cycles executed were set differently for different primers. Optimized annealing conditions were determined by comparing product yields and the specificity of amplification under different conditions. Optimized annealing conditions for each primer are listed in Table 2. Additional programs differing in annealing temperature or cycle number were also used to evaluate condition-dependent tempcycler block heterogeneity and to determine amplification efficiencies.

Analysis of PCR products

A 9 μL aliquot from each PCR reaction was mixed with 9 μL sucrose loading buffer. 5 μL of this was analyzed for total input radioactivity and 10 μL was electrophoresed on non-denaturing polyacrylamide gels containing TBE buffer, 7% acrylamide, and 0.2% bis-acrylamide. Gels were pre-run at 100 V for 1 hour. After sample loading, gels were run at 160 V for 5 minutes and 100 V for 60 minutes. Gels were stained in distilled water containing 1 μg/mL ethidium bromide for 10 minutes. Gels were photographed under ultra-violet illumination and fluorescent bands were cut out by scalpel. Cerenkov radiation from gel pieces was quantified by a Beckman LS8100 liquid scintillation counter.

Table 2

Optimized annealing conditions and number of amplification cycles executed for primers used in this study

target gene	optimized annealing temperature (°C)	number of cycles of amplification
ß-actin	60	22,23
cyclophilin	60	21,22
TGF-α	68	24,25
EGF-receptor	57	25
IGF-I	66	30
IGF-II*	63	29
IGF-1R	70	27
IGF-2R	59	31
IGFBP-2	65	26
IGFBP-3	65	23,25
IGFBP-4	67	26

Note that the conditions prescribed here are specific for the Coy Tempcycler 60, and transfer of conditions to other designs of thermal cyclers requires adjustments for rates of temperature equilibration within the block and between the block and the reaction vessels. Also note that changes in the number of amplification cycles executed may be required for samples with significantly different amounts of transcripts.

Temperature ramping at the fastest possible rate was found to optimal.

The thermal cycler was programmed to hold the heating block at the indicated annealing temperatures for 1 minute.

* This set of primers required the addition of 3% formamide to the PCR to eliminate non-specific amplification.

Northern blotting

RNA was fractionated on denaturing formaldehyde agarose gels. The gel was rinsed in 10xSSC (1.5 M NaCl, 0.15 M sodium citrate) with gentle agitation for 15 minutes. Using a standard capillary blot configuration, with 10xSSC as the transfer buffer, RNA was blotted overnight onto Hybond-N+ nylon membrane (Amersham, Inc.). After blotting, the RNA was fixed onto the membrane by laying the membrane, with RNA facing upwards, onto a pad of filter paper soaked in 50 mM NaOH. After five minutes, the membrane was rinsed for 1 minute in 2xSSC and blotted dry on filter papers. Filters were subsequently pre-hybridized immediately or after one or two days storage at 4°C.

Slot blotting

Purified RNA was denatured in 50% formamide, 6% formaldehyde, and MOPS buffer at 65°C for 7 minutes and cooled on ice. The sample was diluted with an equal volume of ice-cold 20xSSC (3 M NaCl, 0.3 M sodium citrate) and applied to Hybond-N+ nylon membrane by vacuum filtration in a manifold (BioDot-SF) according to the directions of the manufacturer (BioRad). After washing with 10xSSC and 2xSSC, the RNA on the membrane was fixed with 50 mM NaOH like the Northern blot.

Hybridizations, Autoradiography, and Densitometry

Membranes containing RNA were prehybridized in 5xSSPE (0.9 M NaCl, 50 mM sodium phosphate, 5x Denhardt's solution [0.2% each of bovine serum albumin, Ficoll 400, and polyvinylpyrrolidone], 1 mg/mL yeast tRNA, and 5% dextran sulfate, final pH 7) at 65°C for 2 hours. A probe, either α-^{32}P-UTP labeled single-stranded RNA or heat-denatured α-^{32}P-dCTP labeled double-stranded DNA, was added at $2x10^6$ cpm/mL and incubated overnight at 65°C. The filters were washed in 1 L of 2xSSC, 0.1% SDS at 60°C for 30 min, and then in 1 L of 0.1xSSC, 0.1% SDS at 60 to 65°C for 30 min. The damp filter was wrapped in a thin sheet of polyvinylchloride and exposed to pre-flashed Kodak XAR-5 X-ray film next to an intensifying screen at -70°C for 24 to 72 hours. The exposed X-ray films were developed and analyzed by a Model DNA 35 densitometer and "Quantity one" densitometry analysis software (PDI, Inc.).

Statistical analysis

Differences of the means were tested by Student's T-test for normal populations. Differences of the medians was tested by the Mann-Whitney U-test. Linear regression analysis and correlation coefficients were calculated by standard formulae (Campbell 1989).

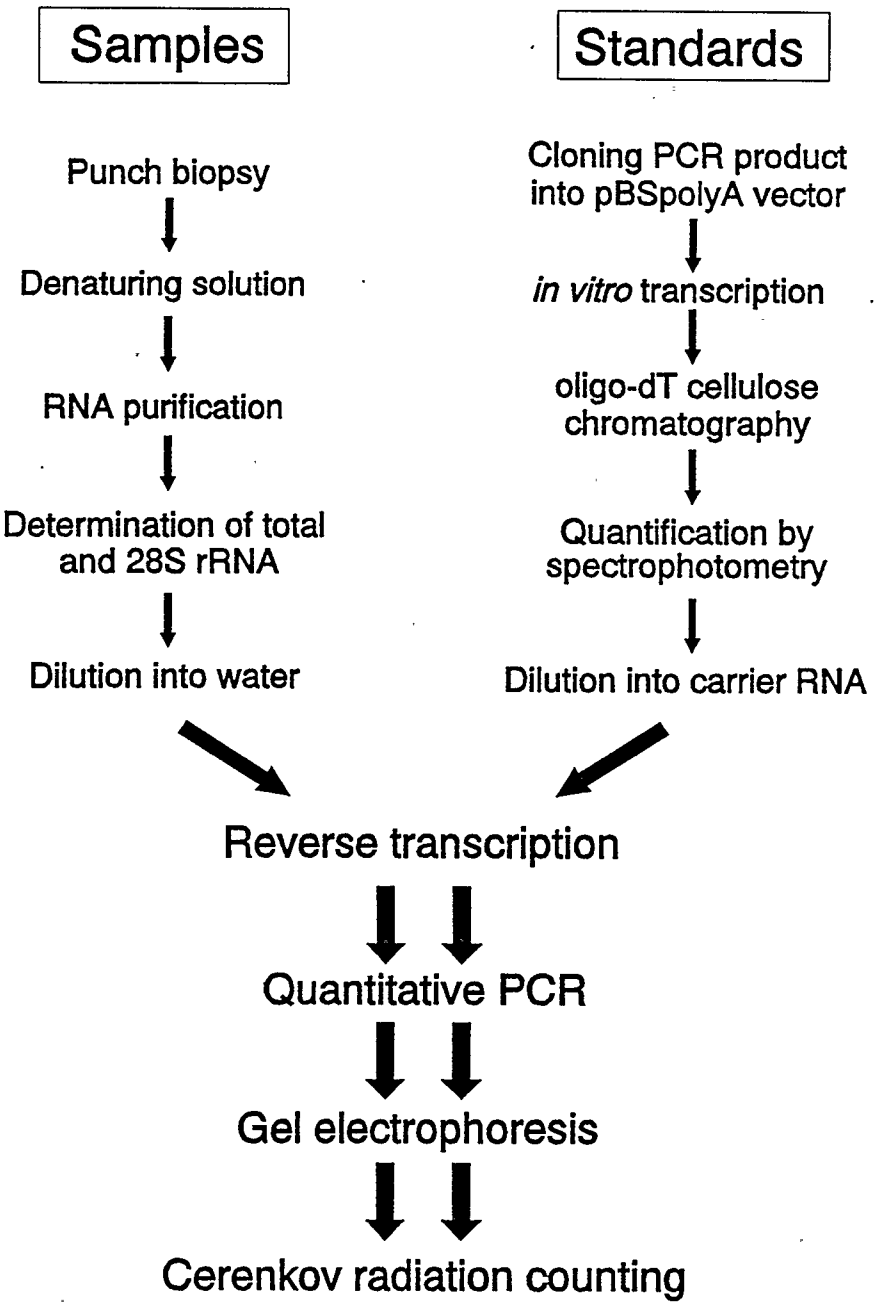
RESULTS

A sensitive assay for gene transcripts was developed, tested, and applied to measure the levels of expression of several different genes. The assay uses enzymatic reactions to amplify a specific target sequence over one million-fold before quantification. The conditions of the assay were optimized and experiments were performed to test the reproducibility of the enzymatic synthesis steps and to test the correlation between the product yields from these reactions and the amounts of input transcript. Once the assay's validity had been verified, measurements from this assay were compared with measurements made by more conventional assays for gene transcripts. Results from the RT-PCR assay were comparable to results from the hybridization assays. Finally, the RT-PCR assay was used to measure the transcript levels of a large number of genes encoding the ligands, binding proteins, and signal-transducing receptors for the insulin-like growth factors, because subtractive hybridization screening studies suggested that one of these genes is overexpressed in psoriatic lesional skin.

The RT-PCR assay for gene transcripts

The RT-PCR assay involves five main steps, RNA preparation, normalization of the RNA concentrations in each sample, reverse transcription, the polymerase chain reaction (PCR), and the measurement of the yield of PCR short product (Figure 5). First, small skin samples are obtained by punch biopsy, the skin cells are instantly lysed in a powerful denaturant, and total RNA is extracted into solution. RNA is then purified, the concentration of each sample is measured by spectrophotometry and 28S ribosomal RNA hybridization, and each sample is diluted to a concentration of 30 ng/μL. Standard transcripts are synthesized by *in vitro* transcription from cloned cDNAs containing the entire PCR amplicon and are purified by oligo-dT cellulose chromatography. Then the concentration of each standard is measured by spectrophotometry, and each sample is diluted into poly(I) at a concentration of 30 ng/μL. Skin

Figure 5. Flow diagram for the quantitative PCR process


Samples
Standards
Punch biopsy
Denaturing solution
RNA purification
Determination of total and 28S rRNA
Dilution into water
Cloning PCR product into pBSpolyA vector
in vitro transcription
oligo-dT cellulose chromatography
Quantification by spectrophotometry
Dilution into carrier RNA
Reverse transcription
Quantitative PCR
Gel electrophoresis
Cerenkov radiation counting

RNA samples and standards are then processed in parallel through two enzymatically catalyzed steps. First, the gene transcripts are reverse transcribed by the Moloney Murine Leukemia virus reverse transcriptase, using random hexanucleotides to prime complementary DNA synthesis. The products of this reaction, double-stranded cDNA/RNA hybrids, are denatured by boiling and then subjected to twenty to thirty cycles of the polymerase chain reaction, the number of amplification cycles being determined in advance through pilot experiments to ensure that reactions do not reach plateau. After an appropriate number of amplification cycles, the yield of short product accumulates to ng or fmol levels and remains proportional to the number of transcripts present in the original sample. The short product is then purified by polyacrylamide gel electrophoresis and the Cerenkov radiation from purified bands is used to compare yields between the reactions containing standards and those containing sample RNA. If the product yields of the standards are proportional to the amounts of standard transcript added, a standard curve is calculated by linear regression analysis of the appropriate standards. Finally, the short product yields from skin biopsy RNA samples are compared to the standard curve to estimate the amount of template in each biopsy sample.

During the development of the assay, the steps involving product quantification, the PCR, reverse transcription, and RNA preparation were optimized and tested for quantitative reliability. The order in which I present the results of these tests is the order in which the experiments had to be carried out, though it is opposite to the order in which the steps arise in the assay. This order was required, because the reliability of the product quantification step had to be verified before the quantitative characteristics of the PCR step could be assessed, and the quantitative validity of the PCR had to be proven before the reverse transcription step could be assessed.

Quantification of product yield

A method for quantifying the product yield from a PCR was first developed and tested. A desirable method would be convenient, precise, and moderately sensitive. The incorporation of trace amounts of radioactive deoxynucleotides into PCR products provides a simple and accurate method of comparing product yields from different PCR reactions. A small amount of radioactive nucleotide, 10^5 cpm per μL of PCR reaction, is diluted into a master mix containing all of the reagents needed for the polymerase chain reaction, and this mix is added to the sample to be analyzed. The simplicity of this method minimizes the variation in the distribution of total tracer radioactivity. By measuring the variation in total radioactivity distributed to different reactions, the absolute variation contributed by the aliquoting of reagents was measured: over thirty samples, the standard deviation was less than 4% of the mean.

Besides precision, the method of quantifying product yields must be sufficiently sensitive. 10^6 cpm of α-^{32}P-labeled dCTP in a standard 10 μL reaction containing 2 nmol of each deoxynucleotide will yield 500 cpm in 1 pmol of incorporated dCMP. 1 pmol of dCTP is found in 20 fmol or 1.3 ng of a 200 bp PCR product with a base composition of 50% (G+C). A range in PCR product yields over the range of 0.2 to 4 ng/μL, which was shown to be the desirable range for analysis, provides 1 to 20 ng of product for analysis, which is equivalent to a range in radioactivity of 400 to 8000 cpm. These amounts of radioactivity yield an amount of Cerenkov radiation that is quickly and accurately measured using a conventional liquid scintillation counter.

The other important requirement for quantifying short product yields from the PCR assay was to separate the incorporated nucleotides from the vast excess of unincorporated, and to ensure that the amplification specifically generated only the desired short product. A polyacrylamide mini-gel system provides excellent resolution and convenience for isolating the short product from the PCR (for example, Figure 6). In particular, polyacrylamide provides superior resolution of PCR products from 100 to 1000 bp in length when compared with agarose gels, retains very low

Figure 6.A. TGF-α short product: standards. Polyacrylamide gel electrophoresis of PCR short products (denoted by arrowhead) from reactions containing TGF-α oligonucleotide primers and cDNA reverse transcribed from a dilution series of standard RNA transcripts synthesized *in vitro*.

Lane	Number of transcripts added to RT-PCR assay
1,7:	160000
2,8:	96000
3,9:	51200
4,10:	25600
5,6,11:	6400
12:	0

Figure 6.B. TGF-α short product: patient samples. Polyacrylamide gel electrophoresis of PCR short products (denoted by arrowhead) from reactions containing TGF-α oligonucleotide primers and cDNA reverse transcribed from psoriatic skin biopsy total RNA. Lanes 1-6 = reactions from the unaffected skin of patients A-F respectively. Lanes 7-12 = reactions from the affected skin of patients A-F respectively. M = DNA molecular weight marker (pBR322 digested with HinfI): 1.60, 0.51, 0.40, 0.30, 0.22, 0.15 kb.

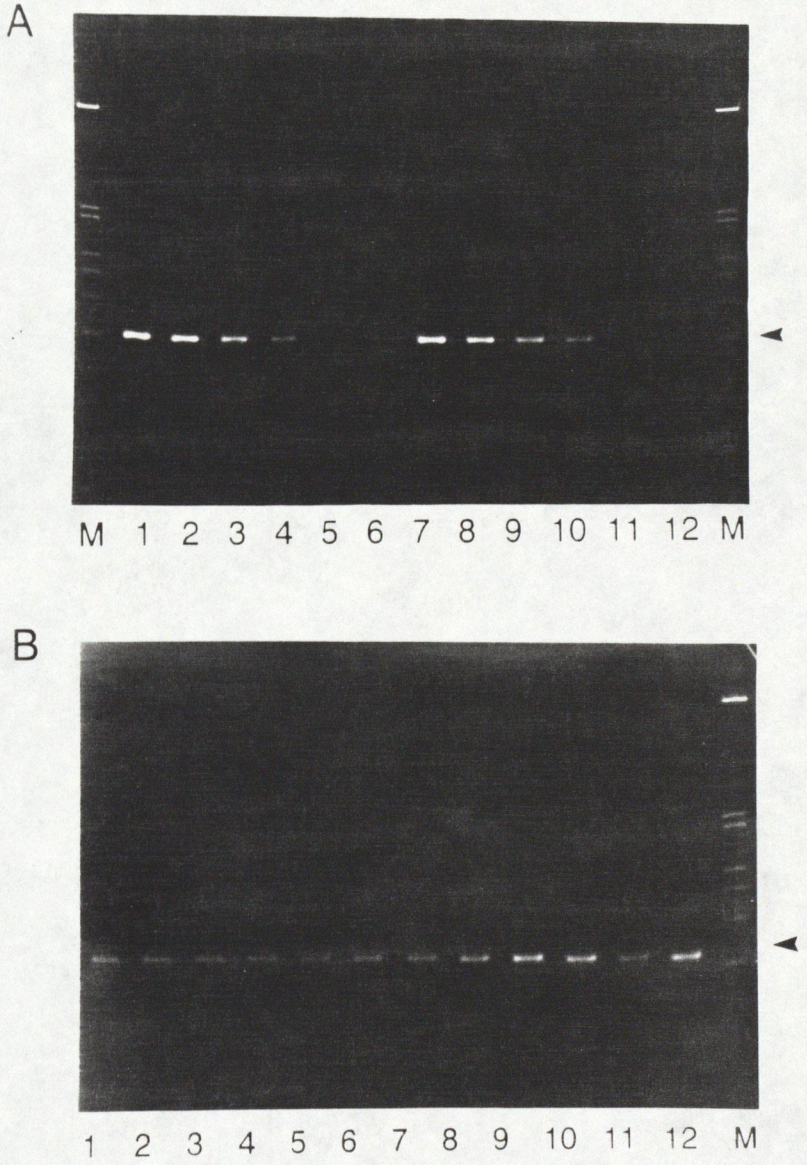
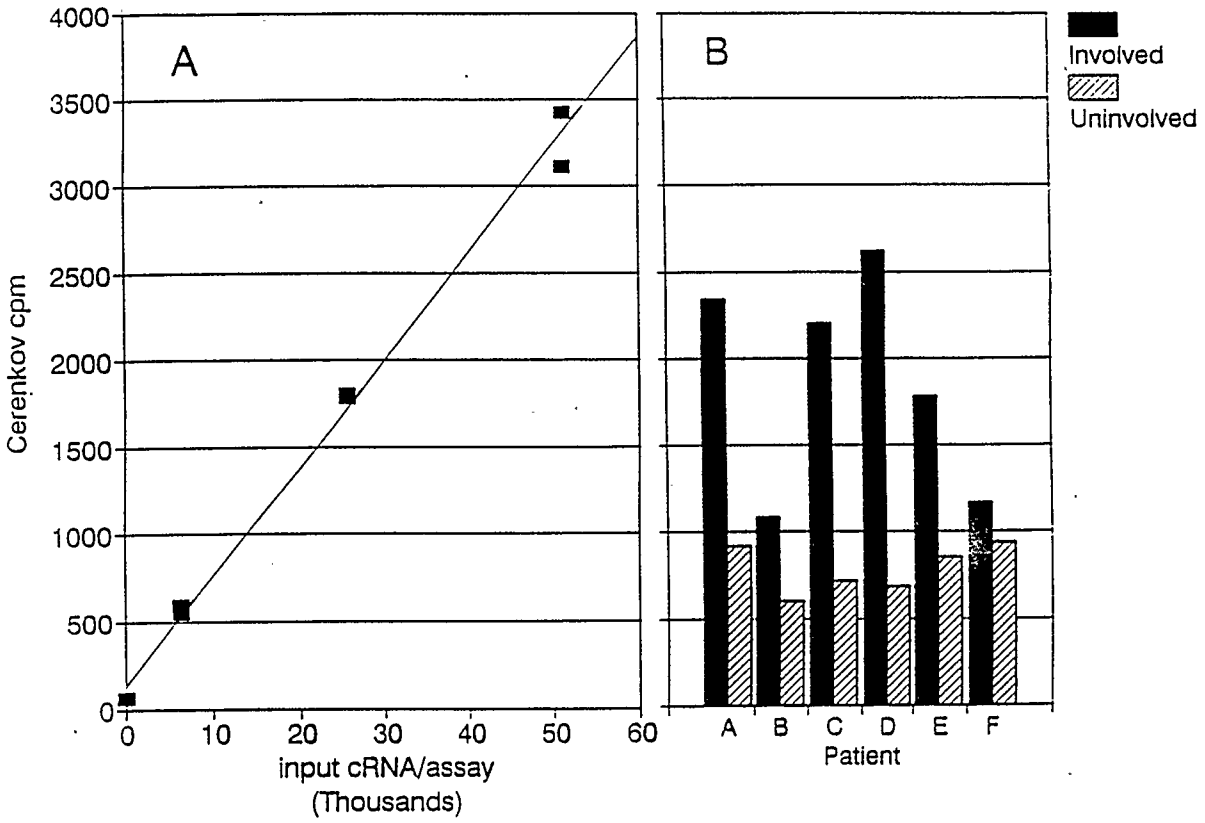

A
M 1 2 3 4 5 6 7 8 9 10 11 12 M
B
1 2 3 4 5 6 7 8 9 10 11 12 M

Figure 7.A. Standard curve of RT-PCR short product yields from a dilution series of RNA transcripts. Tracer radioactivity in the bands in Fig. 6.A. was evaluated by measuring Cerenkov radiation. For this experiment, 100 cpm = 1.2 ng of DNA. The correlation coefficient of the data over the range shown = 0.99.

Figure 7.B. Graph of RT-PCR short product yields from psoriatic skin RNA samples. Tracer radioactivity in the bands in Fig.6.B. was evaluated by measuring Cerenkov radiation.


A
B
Involved
Uninvolved
Cerenkov cpm
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
input cRNA/assay
(Thousands)
A
B
C
D
E
F
Patient

background counts from unincorporated nucleotides, and is very quick and economical to run. Background counts from pieces of gel equivalent in size and position to those containing bands of PCR product typically yielded less than 100 cpm per 10^6 total cpm loaded.

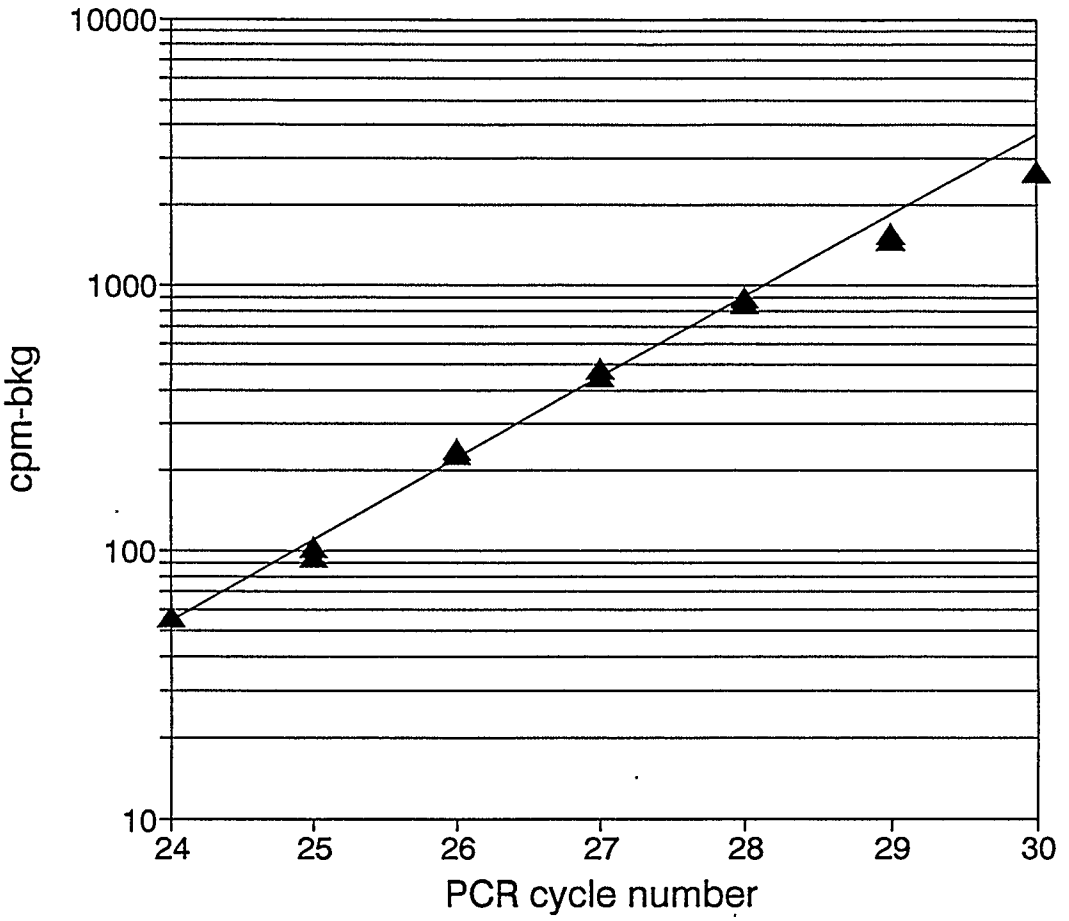
Quantitative PCR

Once a method for quantifying the PCR product had been established, the quantitative characteristics of the PCR step could be analyzed and optimized. To be useful in a routine assay, the PCR must typically synthesize an amount of short product that is between one million and one billion times the amount present in the initial sample. Since this degree of amplification has the potential to contribute a large amount of variation to the product yields of different reactions, the correlation between product yields and the amount of initial template added to separate PCRs was rigorously tested in several experiments.

The first experiment measured the efficiencies of PCRs during their exponential phases. The efficiency of a PCR is defined as the ratio of the amount of product synthesized during a cycle of the PCR to the amount of target available. An efficiency of 100% is achieved when all targets in the reaction are successfully used as templates for the synthesis of new product. Clearly, a consistent correlation between product yield and the amount of input template will only arise if the efficiencies of all reactions are equal. That is, variations in the efficiencies of different PCRs destroy the correlation between product yield and the amount of input template.

Under optimized conditions, PCRs using five different combinations of primers and templates underwent exponential amplification with 95 to 100% efficiency, so long as the product in each reaction did not accumulate to very high levels (for example, Figure 8). By comparing the pre-plateau product yields from reactions using all other primer/template pairs, it was estimated that most other carefully designed primer pairs also yielded amplification efficiencies between 90 and 100%. Under conditions where incubation temperatures were too high or too

Figure 8. Evaluation of the efficiency of the PCR. Graph of the yields of short product at cycles 24 to 30 of a PCR containing IGFBP-2 oligonucleotide primers and 600 copies of denatured plasmid containing the IGFBP-2 amplicon. Product yields were compared by comparing the incorporation of α-^{32}P-dCTP from a master mix of reagents into the short product. The yields of short product from two duplicate reactions were measured after 24 to 30 cycles of amplification. The efficiency was calculated by linear regression analysis over cycles 24 to 28 inclusive. Efficiency = 102%. Correlation coefficient = 0.99.


10000
1000
100
10
cpm-bkg
24
25
26
27
28
29
30
PCR cycle number

low, or where the soak times for certain steps were too long or too short, efficiencies dropped significantly.

Experiments measuring the efficiencies of different reactions also revealed that when the level of short product accumulated to higher levels, each reaction's efficiency decreased to variable levels. This phenomenon has been observed by many others, including Syvanen et al (1988), who characterized some of its causes. Their results and my own both suggest that a decrease in amplification efficiency occurs when product yields accumulate to levels high enough to prevent the primers from saturating all of the potential binding sites on short products or to prevent the limited amounts of polymerase from extending all annealed primers. A PCR enters plateau phase under a standard set of conditions only after a threshold amount of short product has accumulated in the reaction. Under the conditions routinely used for my assay, product yields in the easily quantifiable range of 0.2 to 4 ng/μL were proportional to the amount of starting template (for example, Figures 6 and 7). Once product yields rose above 4 ng/μL, the reaction entered plateau.

Once it was shown that the efficiency of optimized PCRs can reach the theoretical optimum so long as short product levels remain moderate, it was reasonable to assume that the same maximum efficiency could be reproduced in separate reaction tubes. As a result, if the efficiencies in separate tubes are the same, the yield of short product in each tube should then be proportional to the amount of initial template. To test this, separate reactions containing a range of template amounts were subjected to the PCR under optimized conditions. When the reactions were terminated before the short product yield reached 4 ng/μL, the amount of short product synthesized was proportional to the amount of input template (for example, Figure 6 and 7). The correlation coefficient for all standard curves was over 0.95 over this range of short product yields. Moreover, the product yields from 30 separate reactions containing the same amount of starting template were shown to have a standard deviation of less than 5% of the mean for five

different primer combinations under optimized conditions. These results are comparable with those of several others who optimize the PCR and terminate reactions before product yields reach plateau levels (Murphy et al 1990, Robinson and Simon, 1991).

Reverse transcription

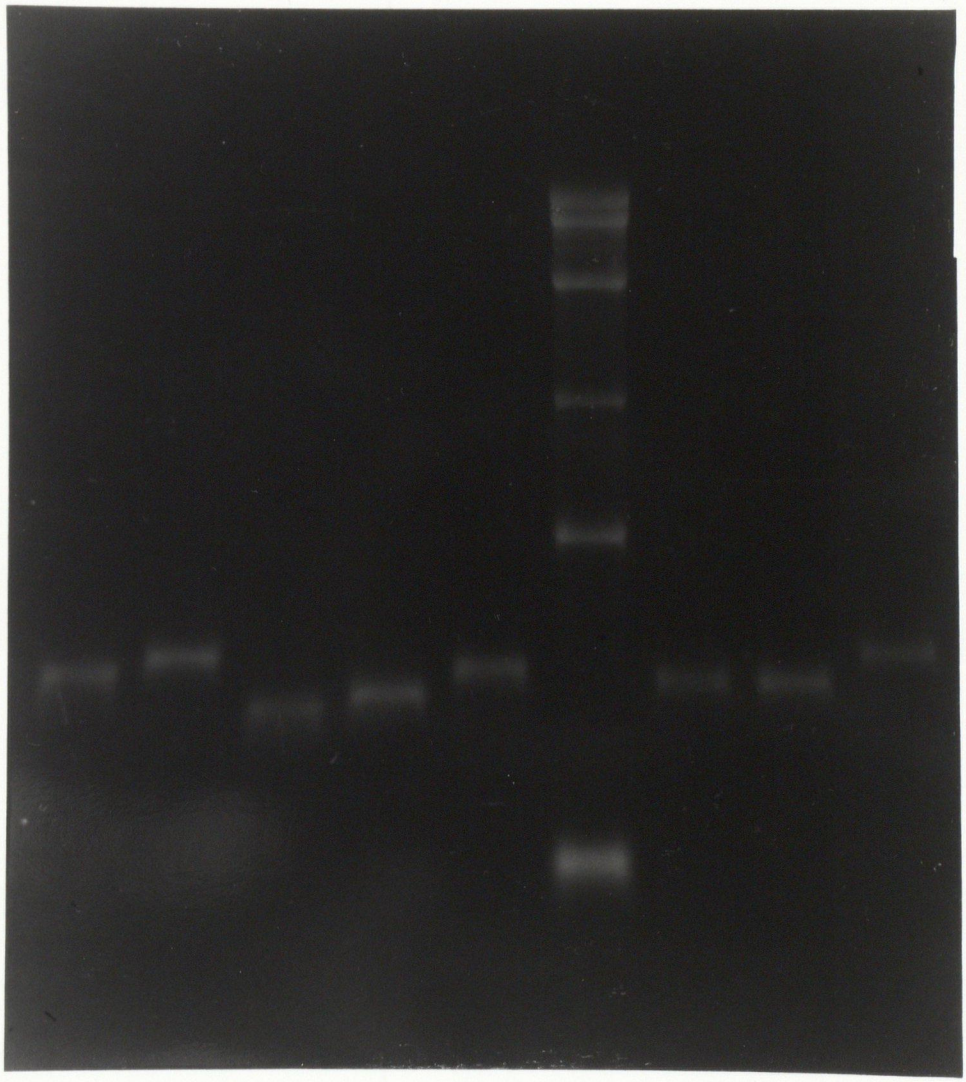
Once the product yields from the PCR were shown to be proportional to the amount of starting template under controlled conditions, the quantitative nature of the reverse transcription step could then be tested. The PCR is used to quantify the yields from reverse transcription reactions, because only small amounts of each transcript are present in each skin sample.

To test the correlation between transcript number and the amount of cDNA synthesized by reverse transcription, a coliphage T7 *in vitro* transcription system was used to make transcripts containing the entire PCR amplicon linked to a stretch of adenosine residues. The polyadenosine sequence allows the purification of full-length transcripts by oligo(dT) cellulose chromatography (Figure 9). The purified RNA consists of a homogeneous population of full-length transcripts of known sequence composition which can be accurately quantified in terms of copy number by spectrophotometric absorption at 260 nm. A range of amounts of transcripts were added to a constant concentation of poly(I), and each standard was reverse transcribed in parallel. The product yields from these reverse transcription reactions were measured by the quantitative PCR protocol described above, and the yield of short product was compared to the amount of RNA transcript added to each standard.

The product yields from the PCR are proportional to the amounts of RNA template added to a reverse transcription reaction over a very wide range, so long as the same master mix of reverse transcription reagents is used to carry out all RT reactions. The correlation coefficient of a set of standards over a 20-fold range is typically over 0.95. A typical standard curve is shown in Figure 7.A. Moreover, when different reverse transcription reactions are carried out

Figure 9. Denaturing formaldehyde agarose gel electrophoresis of standard transcripts synthesized *in vitro*. 300 ng of RNA were loaded in each lane. Samples stained with ethidium bromide (0.1 $\mu g/\mu L$) prior to electrophoresis. M = RNA molecular weight marker (BRL): sizes of transcripts = 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb.

Lane 1:	IGF-I
2:	IGF-II
3:	IGF-1R
4:	IGF-2R
5:	IGFBP-3
6:	ß-actin
7:	TGF-α
8:	EGF-R



1 2 3 4 5 M 6 7 8

with the same master mix of reagents, the standard deviation of PCR yields from eight separate reactions is less than 10% of the mean for four different transcripts. Several features of the reverse transcription reaction were optimized to ensure maximum efficiency and reproducibility. The amounts of random primers and reverse transcriptase added to each reverse transcription reaction are intentionally in vast excess to the small amounts of template present in the reaction, RNA samples are heated at 70°C prior to reverse transcription, and the incubation time for the reaction is two hours. These conditions intend to maximize the probability that all targets in the reaction are used for cDNA synthesis. The efficiencies of the reverse transcription reactions were measured by comparing the amounts of cDNA synthesized from known amounts of transcripts versus known amounts of denatured plasmid containing the same PCR amplicon. Reverse transcription efficiencies were typically between 60 and 80% for five different transcripts tested. Notably, a small variability in efficiencies was noticed when reverse transcription reactions were carried out with different master mixes, supposedly due to variations in the aliquoting of enzyme, primers, and nucleotide. When using separate reaction mixes, the standard deviation of product yields from eight separate reactions was measured to be close to 20% of the mean. This is significantly higher than the 10% standard deviation measured for reactions using the same reagent mix, and suggests that reactions carried out in parallel with the same reverse transcription reagent mix yield more reliable results. The low variability and high correlation between product yield and input transcript number for reverse transcription reactions carried out in parallel from the same reagent mix suggest that the product yields from separate reverse transcription reactions containing RNA from different samples and standards can be compared to infer the amounts of transcript present in the original samples.

Total RNA purification and normalization

The measurements for individual transcripts had to be normalized to the amount of RNA in the sample, because yields of RNA are not equivalent from different biopsy samples. Total RNA was estimated by spectrophotometry and by measuring the hybridization of a 28S ribosomal RNA probe to RNA samples slot-blotted onto nylon membrane. Both methods gave comparable measurements, but the 28S ribosomal RNA measurements provided a higher degree of precision, since replicates were more easily performed and the signal to noise ratio was higher than for the spectrophotometric determinations. Normalization of RNA concentrations was also important in that it allowed the same amount of RNA to be added to each reverse transcription reaction, which minimizes differences in reverse transcription efficiency due to varying the amount of RNA template present in different reactions.

A particular problem in the use of RNA from skin biopsies was that some samples were slightly degraded due to the method of sampling. Skin has a high level of ribonuclease (Elder et al 1990a), and although cells are instantly lysed in a powerful denaturant, guanidinium thiocyanate, the length of time the sample is held before it can be processed can lead to some degradation of the RNA. The storage of RNA over time also leads to some variable degrees of degradation. The extent of degradation was thus tested by electrophoresing aliquots of total RNA prepared from biopsies and staining with ethidium bromide. The RNA samples were mostly of high quality, since the 28S and 18S ribosomal RNA bands appear mostly intact and in the correct proportion to each other (Figure 3.A.). However, probing with a 28S ribosomal RNA probe revealed a smear which suggested a mild amount of degradation. The sensitivity of the RT-PCR assay to a mild degree of RNA degradation was thus also tested. A series of RNA samples degraded by limited alkaline hydrolysis was analyzed by Northern blot hybridization to 28S ribosomal RNA and densitometry, and the RT-PCR assay. It was found that even after more than 80% of the 28S ribosomal RNA, a 5.5 kb transcript, had been cut by one or more hydrolytic

cuts, the product yield from a small 210 bp PCR amplicon remained at 80% of unhydrolysed levels (Figures 10 and 11). The small target quantified by the assay apparently minimizes the effects of limited degradation on the measurement of the original amounts of transcripts present before degradation.

Tests of the PCR on cytokine and housekeeping gene transcripts in skin samples

The previous experiments show that synthetic transcripts in carefully controlled experimental systems can be measured precisely and accurately by the RT-PCR assay, and that sample to sample variations, even moderate amounts of degradation, should not drastically affect the yields of PCR product from skin biopsy RNA samples. As a final test of the assay's validity, the assay's measurements of transcripts in skin biopsies was compared to the results obtained by more established assays. Measurements from the RT-PCR assay were thus compared with measurements obtained from hybridization assays using radioactive probes.

Since the hybridization techniques are less sensitive than the PCR technique, comparisons were limited to the more abundant transcripts. The levels of transcripts for cyclophilin were measured by Northern blot hybridization and densitometry. The absolute and relative quantification results were correlated with the RT-PCR results. A correlation coefficient of 0.89 was calculated when comparing duplicate measurements of cyclophilin transcript from five different RNA samples analysed by the RT-PCR assay and by Northern blotting and densitometry.

Figure 10. Northern blot analysis of hydrolysed mouse liver RNA hybridized to human 28S ribosomal RNA riboprobe. 0.5 μg total RNA loaded per lane.

Time of hydrolysis (seconds)

Lane	1,7:	0
	2,8:	40
	3,9:	80
	4,10:	120
	5,11:	160
	6,12:	200

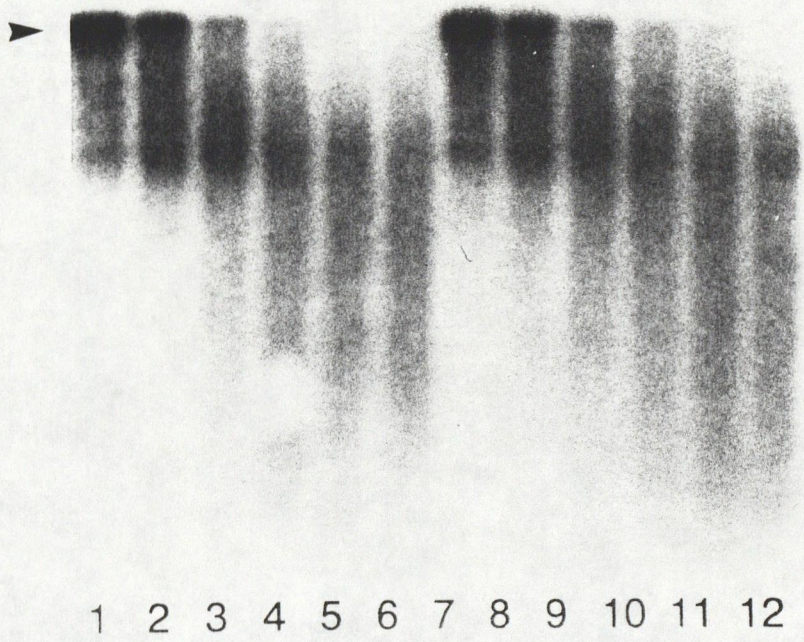
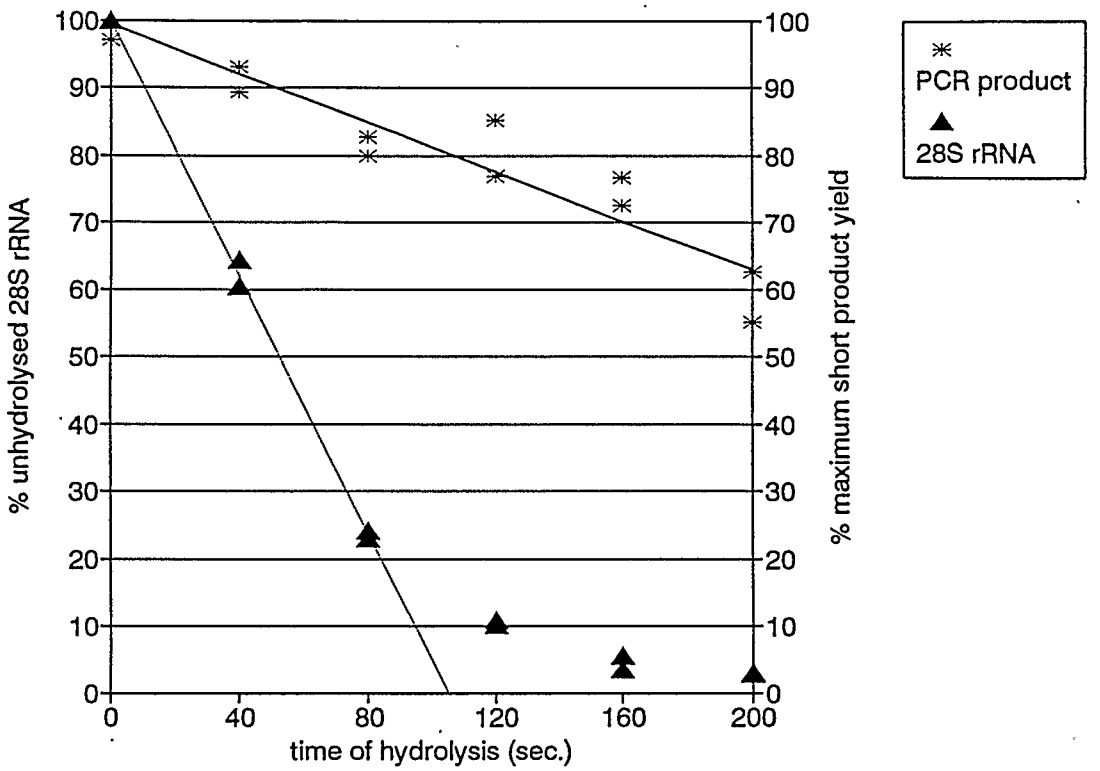

1 2 3 4 5 6 7 8 9 10 11 12

Figure 11. Graph of (a) the percent of residual integrated density in the 28S ribosomal RNA band of the autoradiogram in Fig.10, and (b) the percent of maximal RT-PCR product yields from the corresponding hydrolysed mouse liver RNA samples. Regression lines were calculated to fit the densitometric signals (correlation coeff. = 0.99 for first three time points) and the PCR product yields (correlation coeff. = 0.89).


100
90
80
70
60
50
40
30
20
10
0
% unhydrolysed 28S rRNA
% maximum short product yield
0
40
80
120
160
200
time of hydrolysis (sec.)
PCR product
28S rRNA

Measurements of cyclophilin, ß-actin, TGF-α, and EGF-R transcripts from affected and unaffected skin using the RT-PCR assay were also compared with the results published by several other groups (Table 3, Figures 12 and 13). The results from the RT-PCR assay are comparable to those derived by Northern blot and *in situ* hybridization by other groups. The close agreement between my results and previously reported results partially validate the RT-PCR assay as well as the method of sampling and the population of psoriatic lesions examined in my studies. Unfortunately, only the relative levels between affected and unaffected psoriatic or normal skin could be compared with the results from the RT-PCR assay, because most other groups do not report absolute measurements of transcript number.

Measurements of the IGF family of ligands, receptors, and binding proteins

Using subtractive hybridization methods to screen cDNA libraries from the fibroblasts of affected and unaffected psoriatic skin, Herfort and Garber (1991) identified several dozen different transcripts as being putatively overexpressed in affected psoriatic skin when compared to unaffected skin. One of these transcripts encoded insulin-like growth factor binding protein-3 (IGFBP-3). Northern blot hybridization confirmed that IGFBP-3 is increased in the psoriatic affected skin of three patients (Figure 3; Garber, unpublished).

The RT-PCR assay was used to measure the absolute levels of steady-state IGFBP-3 transcripts in both affected and unaffected psoriatic skin. Most strikingly, the levels of IGFBP-3 mRNA are roughly three-fold higher in affected psoriatic skin when compared to unaffected psoriatic skin after normalization to total RNA or 28S ribosomal RNA; paired samples from the same patient always showed a significant increase in the affected skin versus the unaffected skin. Moreover, the absolute amounts of the IGFBP-3 transcript present in normal and psoriatic skin are moderately high, being roughly 1000 to 4000 transcripts per ng total RNA or roughly 20 to 60 copies averaged over all cells, assuming 20 pg RNA per cell.

Table 3

Comparisons of previously published measurements of transcript levels with RT-PCR measurements

Transcript	RT-PCR ratio (A/U)	published	nA/nU	method	reference
cyclophilin	2.6	2.3*	9/9	NB/D	Elder et al 1990a
ß-actin	1.9	1.1*	3/5	NB/D	Kobayashi et al 1990
TGF-α	2.6	3.0	10/10	NB/D	Elder et al 1989
		3.1	5/5	ISH	Turbitt et al 1990
EGF-R	0.93	0.81*	3/5	NB/D	Kobayashi et al 1990

Notes:

Sample sizes for the RT-PCR results were 9/9 for all transcripts except for ß-actin, which was 6/6 (nA/nU).

* Published ratios marked with an asterisk denote a ratio determined between affected and normal skin rather than affected skin and unaffected skin.

abbreviations:

A: affected psoriatic skin
U: unaffected psoriatic skin
nA: number of affected samples in published study
nU: number of unaffected or normal samples in published study
NB/D: Northern blot hybridization/densitometry
ISH: *in situ* hybridization

Figure 12. Graph of cyclophilin and ß-actin transcript levels in psoriatic affected and unaffected skin. (n=9 for cyclophilin, n=6 for ß-actin)

Bars denote the mean of each sample. Error bars denote the sample standard deviation of the sample. Dashed lines denote the median of each sample.

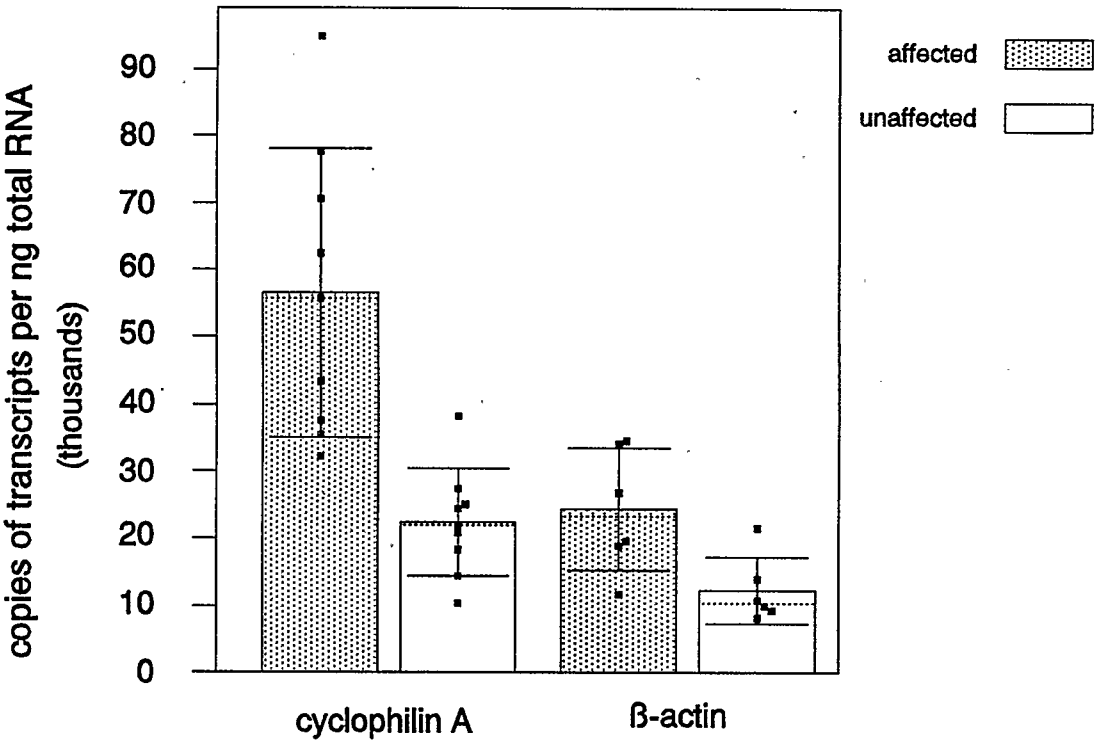
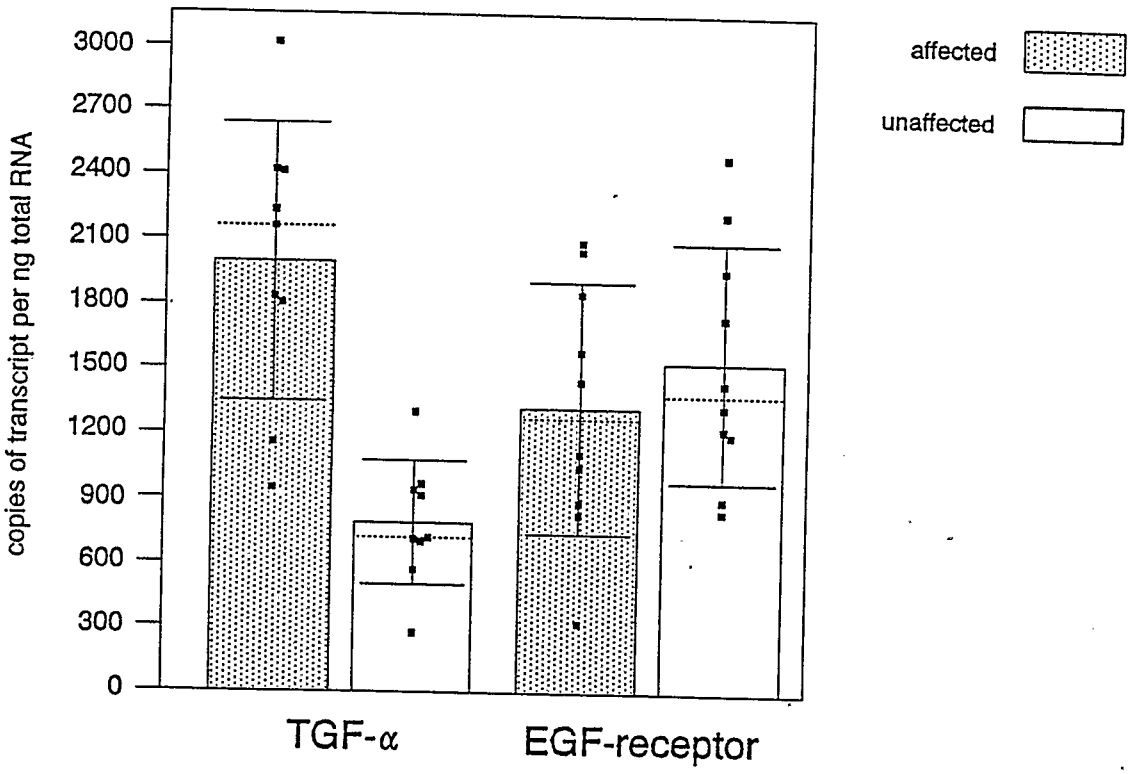

copies of transcripts per ng total RNA
(thousands)
90
80
70
60
50
40
30
20
10
0
cyclophilin A
ß-actin
affected
unaffected

Figure 13. Graph of TGF-α and EGF-R transcript levels in psoriatic affected and unaffected skin. (n=9)

Bars denote the mean of each sample. Error bars denote the sample standard deviation of the sample. Dashed lines denote the median of each sample.


copies of transcript per ng total RNA
3000
2700
2400
2100
1800
1500
1200
900
600
300
0
TGF-α
EGF-receptor
affected
unaffected

Since IGFBP-3 appeared to be expressed at moderately high levels in skin and was being overexpressed in psoriatic lesional skin, it was hypothesized that this factor might be a major component regulating IGF activity in the skin, especially psoriatic skin. Thus, it was asked whether other members of the IGF-family of ligands, receptors, and binding proteins were also being expressed in the skin, and whether any of these factors were also being aberrantly expressed in psoriatic skin. Perhaps the expression of the ligands and receptors were specifically perturbed as either a reaction to or as a cause of the altered IGFBP-3 levels.

The sensitivity, accuracy, and convenience of the RT-PCR assay allowed the levels of the two known ligands, the two known signal-transducing receptors, and three of the six known binding proteins to be measured quickly in many samples (Figures 14 and 15, Table 4). In summary, considering all of the transcripts encoding members of the IGF cytokine family, the transcript encoding IGFBP-3 is expressed at the highest level. The ligands are both expressed at low levels: there are roughly 40 and 20 copies of IGF-I and IGF-II respectively per ng total RNA, which correspond to less than 1 copy per cell, assuming 20 pg total RNA per cell. The levels of expression of one of the receptors, the IGF-IR, are higher, being roughly 200 copies per ng total RNA, which corresponds to 4 copies per cell. This result is consistent with physiological, ligand-binding, and immunocytochemical studies of skin and keratinocytes, which have previously found moderate levels of IGF-IR expression in a small compartment of normal skin (Krane et al 1991). The relatively low overall level of expression is consistent with the finding that in normal skin, expression is limited to the basal cells, while in psoriatic skin, expression extends suprabasally, but may not increase significantly when averaged over each cell in the skin. Notably, the levels of expression are only low if averaged over all cells in the skin, but in that subset of cells which actually expresses the receptor, levels are significant.

Table 4 -- Transcript Quantification by RT-PCR

Transcript	Affected Psoriatic Skin (copies/ng total RNA) (mean ±SEM)	Unaffected Psoriatic Skin (copies/ng total RNA) (mean ±SEM)	Ratio (A/U)	#Obs.	Probability (that means are the same)
High abundance					
cyclophilin A	57000 ±7000	22000 ±2700	2.5	9	**0.0012**
ß-actin	24000 ±4000	12000 ±2000	1.9	6	0.023
Medium abundance					
TGF-α	**2000 ±220**	**790 ±100**	**2.59**	**9**	**0.0003**
EGF-R	1230 ±160	1320 ±180	0.93	10	0.706
IGFBP-3	**3920 ±770**	**1090 ±230**	**3.59**	**9**	**0.0063**
Low abundance					
IGF-I	10 ±3	12 ±4	0.83	6	0.698
IGF-II	26 ±10	25 ±9	1.04	9	0.939
IGF-IR	185 ±32	205 ±26	0.90	9	0.635
IGF-IIR	17 ±3	17 ±2	0.97	9	0.976
IGFBP-2	109 ±15	147 ±10	0.74	9	0.059
IGFBP-4	388 ±86	237 ±56	1.64	9	0.164

The probability that the means of involved and uninvolved populations are the same was calculated from Student's T-distribution in each population.

Underlined (cyclophilin A, ß-actin, TGF-α, IGFBP-3) >95% confidence of unequal means.

Bold (cyclophilin A, TGF-α, IGFBP-3) >99% confidence of unequal means.

Abbreviations: SEM = standard error of the mean
A = affected psoriatic skin
U = unaffected psoriatic skin.

Figure 14. Graph of IGF-I, IGF-II, IGF-1R, IGF-2R, IGFBP-2, and IGFBP-4 transcript levels in psoriatic affected and unaffected skin. (n=9)

Bars denote the mean of each sample. Error bars denote the sample standard deviation of the sample. Dashed lines denote the median of each sample.

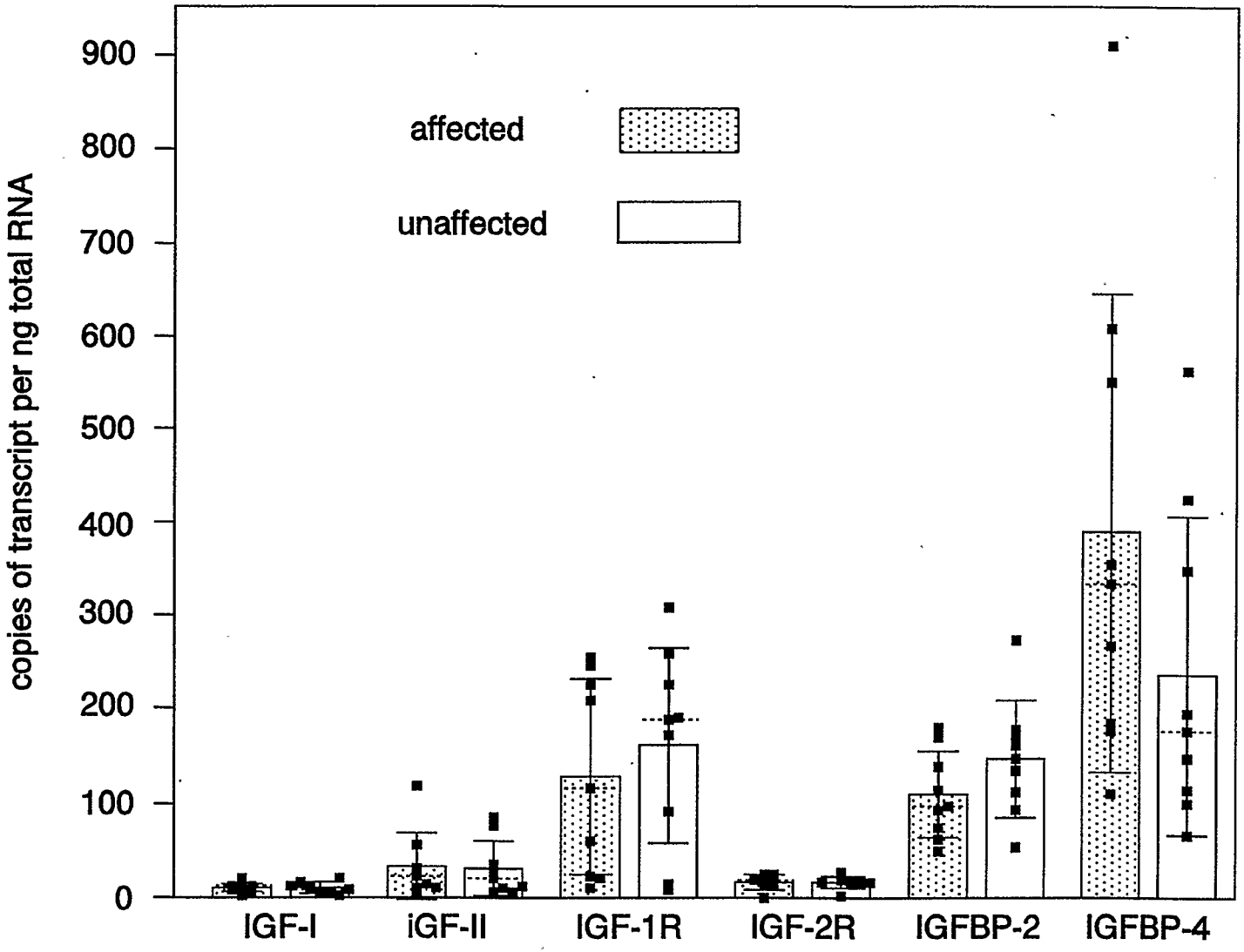
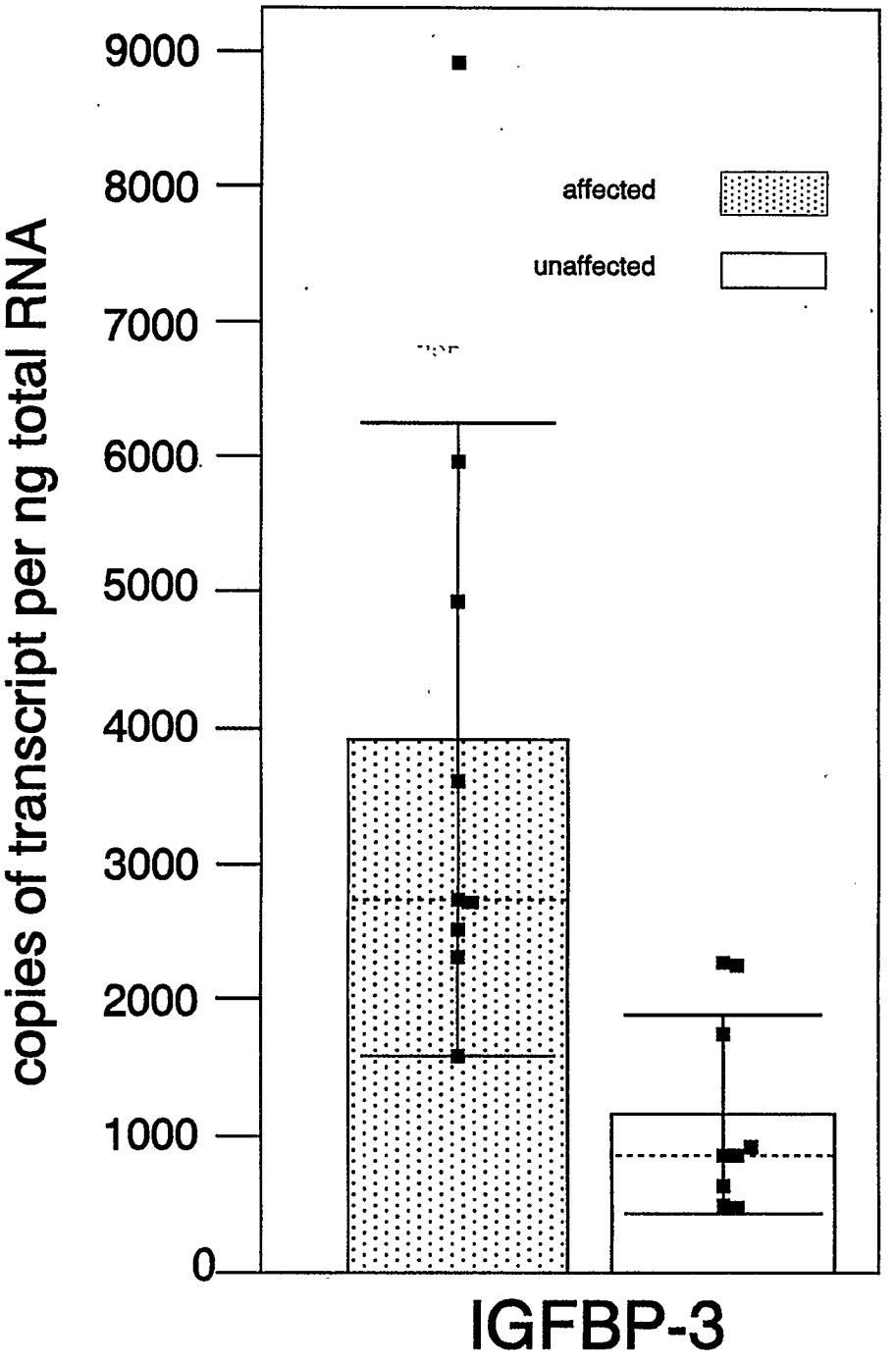

copies of transcript per ng total RNA
900
800
700
600
500
400
300
200
100
0
affected
unaffected
IGF-I
iGF-II
IGF-1R
IGF-2R
IGFBP-2
IGFBP-4

Figure 15. Graph of IGFBP-3 transcript levels in psoriatic affected and unaffected skin. (n=9)

Bars denote the mean of each sample. Error bars denote the sample standard deviation of the sample. Dashed lines denote the median of each sample.


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
copies of transcript per ng total RNA
affected
unaffected
IGFBP-3

In summary, three key results were derived from the IGF profile in psoriatic and normal skin. First, the IGFBP-3 transcript is the only one showing markedly higher levels in psoriatic lesional skin. Second, the magnitude of this increase is comparable to that of another carefully measured transcript encoding a factor involved in regulating the growth of the skin, TGF-α (Elder et al 1989, 1990, and my results). Third, the levels of steady-state IGF-1R transcripts are not elevated when normalized to the amount of total RNA, even though IGF-I binding and IGF-1R immunolocalization are more widespread in psoriatic epidermis when compared to normal epidermis (Krane et al 1991, 1992).

DISCUSSION

To further the molecular characterization of psoriatic skin, a highly sensitive assay for measuring specific gene transcripts was developed. This assay was designed to optimize sensitivity, convenience, and speed without compromising precision and accuracy. Each of the four main steps in the assay, RNA sample preparation, reverse transcription, the PCR, and PCR product quantification, was tested for possible errors. Experiments testing for errors will be discussed according to the step of the assay where they arise.

Validation of the RT-PCR assay

- RNA sample preparation

The amount of gene transcript measured by the RT-PCR assay depends upon the efficiency of the method used to extract and purify RNA from the skin. Thus RNA is extracted from all biopsies in a simple, consistent manner.. The chosen method of lysis and extraction uses the strong denaturant guanidinium thiocyanate. The presence of this strongly chaotropic salt at high concentrations ensures reproducible and consistent cell lysis, which in turn leads to consistent efficiencies of RNA extraction from all punch biopsy samples. The extraction protocol involves extensive vortexing, which reproducibly lysed and extracted RNA from all epidermal cells and likely all cells from the epidermal-dermal junction and papillary dermis of the skin. A possible error in this procedure was that RNA from cells deep in the dermis may not be efficiently extracted. Homogenization, which has been tried by myself and others, does not seem to release significantly more RNA from the dermis (Nickoloff et al 1991). Homogenization often reduces the quality of the RNA, however, possibly by releasing more contaminating proteoglycans. Homogenization would thus increase variability of sample quality, which could have unpredictable effects on transcript measurements. Hence, homogenization was avoided even though the vortexing protocol may not release all of the RNA from the dermis.

The degradation of RNA was minimized during the initial extraction by using the strongly chaotropic salt guanidinium thiocyanate to inactivate and remove ribonuclease. The additional extractions and precipitations during RNA purification helped to prepare a nearly ribonuclease-free RNA sample which was stable at -70°C. Some samples degraded over time, especially when held for several months at -20°C, but at the time of assay, all samples were checked for intactness by denaturing gel electrophoresis. It was also found by experiment that the assay is not sensitive to even significant amounts of degradation, because the target analysed is relatively small in comparison to the intact transcripts needed for Northern blot analysis, for example.

- Reverse transcription

The reverse transcription step was evaluated carefully by reverse transcribing RNA transcripts synthesized *in vitro* and measuring the yield of cDNA product by applying the PCR under strictly controlled conditions. The conditions of the reverse transcription reaction were designed to facilitate the quantitative synthesis of cDNA from mRNA template. An excess of reverse transcriptase, primers, and deoxynucleotides and optimized amounts of magnesium ion, buffer, salt and ribonuclease inhibitor were included in the reaction to ensure that the only component limiting product yield would be the amount of starting template.

Random hexamer primers, as opposed to oligo-dT or the downstream PCR primer, were used to initiate cDNA synthesis from RNA transcripts. At the concentrations used in the assay, 10 ng/μL or 1 ng/1.2 ng total RNA, random hexamer primers would be expected to anneal over most exposed stretches of RNA. Thus, to initiate the synthesis of a functional cDNA for the subsequent PCR, it is only required that a single primer anneals a short distance away from the 3' end of the PCR amplicon of the target RNA transcript. Minimizing the distance between the initiation of cDNA synthesis and the PCR amplicon is advantageous in a quantitative assay, because increasing this distance increases the probability that secondary structures in the transcript

of interest or the effects of limited RNA degradation will prevent the synthesis of a functional cDNA copy of the PCR amplicon. Indeed, random primers clearly yield more cDNA than oligo-dT for several transcripts studied (my observations, Noonan and Roninson 1990). The higher product yield presumably reflects the increased degree of interference from RNA secondary structures when oligo-(dT) primes cDNA synthesis, since the oligo-dT primer necessarily initiates synthesis a farther distance away from the PCR amplicon than the random hexamers. Thus, the use of oligo-dT for initiating the synthesis of cDNA may increase the chance of undersetimating the actual amount of transcript in a sample, because oligo-dT likely initiates cDNA synthesis at a more distant location for the native transcript than the synthetic, standard transcript. Using random hexamers as primers for cDNA synthesis provides the additional advantage that a single batch of cDNA could be used for the analysis of a number of different species by using different primers during the PCR. This latter advantage makes random hexamers a better choice for primers than the downstream or 3' PCR primer, as suggested by others (for example Robinson and Simon 1991).

Another feature of the RT-PCR assay that was designed to ensure accuracy was the use of RNA transcripts as the standards for the assay. Standard transcripts are produced by *in vitro* run-off transcription reactions. The templates for run-off transcription consist of a PCR product cloned downstream of a coliphage T7 RNA polymerase promoter and upstream of a polyadenosine sequence which lies just upstream of a number of restriction enzyme sites. These clones can be quickly constructed by standard subcloning methods over two days. An appropriate clone for each RNA species is then prepared as a template for run-off transcription by digestion at a restriction site downstream from the polyadenosine stretch and then gel-purification. The resulting linearized plasmid allows run-off transcription to begin at the T7 promoter and end just after the polyadenosine sequence. The polyadenosine sequence is thus incorporated only into full-length run-off transcripts, which in turn allows full-length transcripts to be purified to

homogeneity by chromatography on oligo-dT cellulose (Figure 9). The molar concentration of the homogeneous transcript preparation is then determined by spectrophotometric absorption at 260 nm.

Standard transcripts are diluted into a dilute RNase-free buffer to prepare a series of standards containing concentrations of transcripts comparable to those from patient samples. The standard samples are prepared in the same buffer as the patient samples, and contain an amount of carrier RNA, poly(I), equal to the total amount of patient sample RNA in each assay. The total mass of RNA in each reverse transcription reaction is carefully regulated to ensure that efficiencies in this step were not affected by differences in the ratio of polymerase and primer concentrations to the concentration of total RNA in the reaction. In short, the RNA standards used to calibrate the assay are nearly identical to the native transcripts found in real samples, and processing aliquots of standard transcripts in parallel with native transcripts through the steps of reverse transcription and the PCR maximizes the accuracy of comparisons between samples and standards.

The results of several tests of reverse transcription confirm that the yields of product from reverse transcription are proportional to the amount of input template when the same master mix of reagents is used. The absolute efficiency of cDNA synthesis, which was estimated by comparing the yields from quantitative PCR assays of reverse transcription reactions containing known amounts of RNA transcript with PCR assays of known amounts of denatured plasmid, was found to vary from 60 to 80% (data not shown). Though quite high, efficiencies are clearly less than 100%, suggesting that not all transcripts are being converted to cDNA. This inefficiency is likely due to the complex interplay of RNA secondary structure and competing enzyme activities in the reaction. More important to the quantitative assay, efficiencies can vary up to 20% of the mean for reverse transcription reactions using different reagent mixes on different days. Fortunately, for a set of reverse transcription reactions from the same master mix of

reagents, the standard deviation of the PCR product yields after RT and PCR does not exceed 10% of the mean, which is just slightly above the level of variation expected from pipetting errors and the PCR alone. Moreover, the correlation coefficient between product yield from the RT-PCR assay for standards over a 20-fold range in transcript amounts is reproducibly over 0.95 if all reactions use the same reagent mix. These results suggest that the product yields from separate reverse transcription reactions reflect the amount of template present in a sample if the same master mix of reagents is used for all reverse transcription reactions.

- Quantitative PCR

Theoretically, the yield of short product generated by a PCR can be precisely predicted if the only limiting component is the amount of template present. If primers, polymerase, and nucleotides are in excess, and if primer annealing is quantitative, the amount of long product and the amount of short product generated from each cycle of the PCR is a function of the amount of template added at the beginning of the reaction. These conditions are met if the conditions of the PCR have been optimized, and if the reaction is terminated before the product yield reaches a high enough level to prevent primer annealing or extension to occur to completion. Increased variability in product yields due to inefficient denaturation, primer annealing, and product extension have been measured by myself and others (Syvanen et al 1988, Linz 1990).

The maximum amount of short product that is synthesized during each cycle is equal to the amount of short product carried over from the last cycle plus the amount of long product carried over (Figure 2). Because the contribution from the long product is insignificant for all PCRs starting with a low number (less than 10^8 copies) of starting templates, however, the amount of short product should roughly double if primer annealing and extension occur to completion in each cycle of the reaction: this is the definition of 100% efficiency in the PCR.

Potentially, however, not all sites may be saturated with primers during the annealing step, and not all annealed primers may be successfully extended by the polymerase. These are likely the main mechanisms which can decrease the efficiency of the PCR (Syvanen et al 1988). Variations in the extent of primer annealing or polymerase extension from reaction to reaction would affect the efficiencies of different reactions and obscure the proportionality between product yield and the initial amount of template. These variations are aggravated in particular by the lack of homogeneity in most temperature cyclers (Linz 1990, Hoelzel 1990), especially if the temperatures, and ramp and soak times are not optimized and are insufficient to complete denaturation, annealing, and extension reactions. Variations also become noticeable in the later cycles of the PCR, when large amounts of short product accumulate, thus preventing primer annealing and polymerase extension from proceeding to completion and in turn decreasing the reaction's amplification efficiency. Variations are expected at these late stages, because decreases in reaction efficiency would be proportional to the amount of initial template, thus leading to a non-linear proportionality between the amount of starting template and the product yield. This proportionality would not be easily predictable, because the degree to which primer annealing and polymerase extension were compromised would be very sensitive to the exact copy number present in the reaction as well as the exact concentrations of primer, polymerase, and deoxynucleotides, and the exact times and temperatures of reaction incubation (Syvanen et al 1988, Linz 1990).

Thus, the extent of variations introduced by the PCR were measured in a number of experiments. First, the efficiency of each step in the PCR was established by directly measuring the accumulation of short product during the PCR. This efficiency is near 100% for all cycles where accumulations of short product remained below plateau levels. A high efficiency is expected in optimized reactions (Wang et al 1989, Murphy et al 1990, Robinson and Simon 1991): excess DNA polymerase, deoxynucleotides, and oligonucleotide primers are added to

ensure that the amount of short product in each cycle of the reaction would double, and denaturation, annealing, and extension temperatures and times were carefully tested to ensure complete denaturation, annealing at maximal specificity and to a saturating degree, and the complete extension of all annealed primers.

Once these conditions had been established to facilitate reproducible and maximal product synthesis, the late stages of the PCR were examined. A decrease in exponential product accumulation was not noticed until product yields reached roughly 4 ng/μL under standard reaction conditions. Since radioactive labeling could be easily detected at 1/20th of this amount, radioactive tracer labeling of short product provides enough sensitivity to quantify product yields over at least a twenty-fold range before the PCRs entered plateau. Using a more sensitive but much more expensive phosphorimager system for measuring radioactivity, Robinson and Simon (1991) can measure a one-thousand-fold linear range in product yields by extending the lower limit of detection.

Clearly, the RT-PCR assay demands that careful attention be paid to the accumulation of product levels in each reaction. Thus, range-finding experiments are typically carried out prior to accurate quantification to deduce the number of cycles of amplification which will yield sufficient product for detection without driving any reaction into plateau. Using tracer radioactivity, a range of product yields and hence of initial template number of no more than 20-fold can be accurately measured if all reactions are compared after the same number of cycles of amplification. However, with standards to compare against sample product yields, the absolute numbers of transcripts in different samples can be determined over an enormous range. Ending the assay at 21 cycles allows the determination of samples with roughly 10^5 copies of transcripts per ng total RNA, as was done in measuring cyclophilin in the skin. Samples with only 10 copies of transcripts per ng total RNA can also be accurately measured by stopping the PCR after 31 cycles, like for IGF-2R in the skin.

The reproducibility of separate PCRs containing reagents from the same master mix was evaluated rigourously by measuring the product yields from separate reactions containing equal amounts of starting template. The cumulative variation due to reverse transcription and the PCR led to a standard deviation in product yields of less than 10% of the mean. This suggests that reactions containing known amounts of standard RNA transcripts could be analysed in parallel with samples containing unknown but comparable amounts of transcripts, and then the amounts in the unknown samples could be inferred by comparing product yields. It was found that if the standard reactions did not enter plateau, all reactions showed a linear dose response to the amount of initial template. Consequently, the initial amounts of template in experimental samples could be inferred by the linear interpolation of product yields from known standards.

Comparisons of RT-PCR measurements with measurements obtained from hybridization assays

While the RT-PCR assay performs very consistently with standard transcripts, the measurements from real samples may vary due to the presence of contaminants in different samples or due to variables in sample preparation. Thus, measurements from the RT-PCR assay were compared with measurements from more commonly used assays for quantifying gene transcripts. The transcripts that were chosen for comparison were housekeeping transcripts that were expected to establish baseline levels, and growth factor and receptor transcripts that had previously been shown to be expressed at altered levels in psoriatic skin. Notably, all of these transcripts are comparatively more abundant than most in the skin, because these were the only ones that could be conveniently measured by the less sensitive hybridization methods.

First, skin RNA samples were electrophoresed and capillary blotted onto nylon membrane. These samples were hybridized with high specific activity RNA probes and washed at high stringency. The amount of specifically hybridized probe was quantified by densitometry

and these amounts were compared to the amounts of cyclophilin transcript measured by the RT-PCR assay. The results, which are only on a relative scale on the Northern blots, compare very favorably to the RT-PCR measurements. A correlation coefficient of 0.89 was calculated for five separate RNA samples.

Besides this direct test of the RT-PCR assay, results obtained from the sample of patients in the present studies were compared with the results obtained by other groups. Thus, the entire experimental procedure of patient selection, biopsy sampling, RNA preparation, and the RT-PCR assay could be compared to the slightly different protocols followed by other groups.

For this analysis, two housekeeping transcripts and a cytokine and receptor pair of transcripts were chosen for comparison. Measurements on the housekeeping genes ß-actin and cyclophilin were intended to provide a baseline for measurements of other transcripts. These transcripts encode a cytoskeletal protein and an enzyme involved in protein folding, which seem to be housekeeping functions that would be expressed at nearly the same levels in all cells. These transcripts provided the additional attractions of being abundantly expressed in most tissues and being previously measured by Northern blotting and densitometry in both psoriatic and normal skin.

My results show that the levels of both transcripts are increased by marginally significant amounts in psoriatic affected skin when compared with psoriatic unaffected skin, after normalization for total RNA. The relative degrees of increase are consistent with a previous report on cyclophilin expression in psoriatic skin (Elder et al 1990a), but conflict with a report on ß-actin levels (Kobayashi et al 1990) (Table 3). The latter discrepancy is not surprising, because the sample size in the previous report on ß-actin levels was very small, and the levels of variation noted in both of our studies suggest that a larger sample size may be needed for a more confident estimate of relative levels. Unfortunately, the fact that the levels of these transcripts in psoriatic lesional skin are elevated when normalized to either total or ribosomal

RNA prevents their use as convenient reference transcripts for normalizing the measurements of other transcript levels. Apparently, both genes are specifically activated in psoriatic skin, possibly because of their roles in the perturbed psoriatic skin physiology.

The well-studied transcripts for TGF-α and the epidermal growth factor receptor were also chosen for testing the assay. Previously, TGF-α transcripts were reported to be abundant in the skin and to be increased in psoriatic skin compared to normal and unaffected psoriatic skin by 4.5-fold and 3-fold, respectively, using Northern and slot-blot hybridization assays (Elder et al 1989, 1990a). A report using quantitative *in situ* hybridization methods also found that TGF-α transcripts were increased in psoriatic affected skin by similar amounts (Turbitt et al 1990). The only known receptor for TGF-α, the EGF-R, is also expressed at high levels in the epidermis, and the patterns of EGF-R protein and ligand-binding distribution have been shown to be altered in psoriasis (Nanney et al 1986). However, several reports have also shown that the total levels of transcripts were not significantly increased in psoriatic skin, and may in fact be slightly depressed after normalization to total RNA (Kobayashi et al 1990, Elder et al 1990). Using the RT-PCR assay, the levels of TGF-α were found to be increased 2.6-fold in psoriatic affected skin compared to psoriatic unaffected skin. Also, EGF-R transcripts were slightly depressed in affected skin to 0.9 times the level of unaffected skin. These results are consistent with previous reports using hybridization techniques and once again validate the RT-PCR assay in assessing differences in the levels of transcripts in skin.

Insulin-like growth factor ligand, receptor, and binding protein expression in psoriatic skin

The initial results from subtractive hybridization and Northern blot analyses of insulin-like growth factor binding protein-3 expression in psoriatic skin fibroblasts and psoriatic skin suggested that the insulin-like growth factors may be involved in skin physiology and the pathogenesis of psoriasis. The levels of the transcripts encoding this protein were accurately

quantified by the RT-PCR assay on both an absolute and relative scale in both psoriatic affected and unaffected skin. In all patients, IGFBP-3 transcripts were significantly increased, and increases ranged from 2 to 10-fold, with a mean of 3.4 and a median of 4. The difference between medians for IGFBP-3 levels in affected and unaffected skin was shown to be highly significant ($p < 0.05$ that medians are the same) using the Mann-Whitney U-test, and assuming a normal distribution, the difference between means was also significant ($p < 0.01$ that means are the same) (Table 4).

Since IGFBP-3 is a major regulator of IGF activity and transport in the body, it was asked whether other members of the IGF family were also expressed in the skin and whether these other members were expressed at altered levels in psoriatic affected skin. Both IGF ligands were found to be expressed at very low levels in the skin, with IGF-II levels at roughly one-fortieth that of TGF-α, and IGF-I several fold even lower (Table 4, Figures 12 and 13). These results are not surprising, since the IGF's are only known to be produced at high levels in the liver, several other mesodermally derived tissues, and the brain in the adult, and may exert endocrine and paracrine functions primarily (Han et al 1988, Moller et al 1991). Reports of IGF-I and IGF-II expression deep in the dermis of human fetuses are interesting if they reflect the patterns of expression in the adult, too (Han et al 1987a,b; Hill et al 1988). Transcripts which are expressed deep in the dermis of my own samples would likely not have been measured by my assay, because the bulk of cellular RNA analyzed by my assay comes from the epidermis and not the dermis.

A second study reports contradicting observations. Gray et al (1987) detected high levels of IGF-II transcripts in adult skin as estimated by Northern blot hybridization. They do not elaborate how skin RNA was extracted, especially with respect to the compartment of skin extracted, and they also do not elaborate on the age of the donor and the part of the body from which the skin originated. One possible explanation is that IGF ligands are being synthesized

deep in the dermis of the skin, as in human fetuses (Han et al 1987a,b; Hill et al 1988), and that these transcripts were not extracted by my protocol but were extracted by Gray's protocol. Recent immunocytochemical studies of skin show significantly lower IGF-I and IGF-II expression in normal adult skin than neonatal foreskins and fetal skin, however (Ahmed et al 1992). My results confirm these latter observations and suggest that the IGF ligands are not produced in large amounts in the adult skin, especially the epidermis, and thus the ligands interacting with binding proteins and receptors in the skin must come from paracrine or endocrine sources. In rats, IGF ligands in circulation have been shown to efficiently accumulate in the skin by as yet unknown mechanisms (Ballard et al 1991).

Of the two known IGF receptors, only one was found to be expressed at significant levels (Table 4, Figure 14). The IGF-1R is expressed at moderate levels of roughly one-sixth of the EGF-R, while the IGF-2R is almost absent in the skin. These results are consistent with immunological and ligand-binding studies on keratinocytes and skin which reveal moderate IGF-1R activity (Krane et al 1991, 1992). Curiously, the levels of IGF-1R transcripts are not significantly increased, despite the fact that perturbations are evident in the distribution of the protein and ligand binding activity in psoriatic skin. IGF-1R is normally found only in the basal layer of the epidermis, but in psoriasis, the receptor extends to several suprabasal layers of cells. Moreover, anti-IGF-1R immunoprecipitates from affected psoriatic skin yield more autophosphorylated IGF-1R-ß subunit than immunoprecipitates from unaffected skin (Krane et al 1991, 1992). The immunolocalization result suggests that more receptor molecules are present in affected epidermis, while the autophosphorylation assays suggest that either more receptor is present or receptor activity is increased. These results can be reconciled with the lack of a noticeable increase in IGF-1R transcript levels after normalization to either total RNA or ribosomal RNA. Transcript levels may be increased overall in the psoriatic lesional skin, but when averaged over the larger number of cells in the psoriatic epidermis, this increase does not

appear. Furthermore, as in the case of the EGF-R, whose protein and ligand binding levels are both increased in affected psoriatic skin, but whose transcript levels clearly are not, the steady-state levels of receptor protein may be specifically increased without a corresponding increase in steady-state receptor transcript levels (Nanney et al 1986, Kobayashi et al 1990).

The levels of expression of several other binding proteins were also examined. IGFBP-1, a binding protein abundant in embryos and the placenta, was not detected by the assay, but this is not surprising, since the production of IGFBP-1 in adult tissues has yet to be demonstrated by any studies, and the serum levels of IGFBP-1 are undetectable (Humbel 1990). However, the transcripts encoding IGFBP-2 and IGFBP-4 were found to be present at moderate levels that were comparable to the level of IGF-1R expression but several fold lower than IGFBP-3. The fact that at least three different binding proteins are expressed at significant levels in the skin is intriguing, since different binding proteins may allow the specific regulation of IGF ligand availability to specific tissues or compartments of tissues that are juxtaposed. This is perhaps most clearly demonstrated by studies of IGFBP expression in the rat ovarian follicles during oogenesis. Extensive *in situ* hybridization analyses of the different cells in the rat ovary during oogenesis suggest that juxtaposed tissues can control the activities of IGF ligands in their vicinity by the kind and amounts of IGFBP being expressed (Nakatani et al 1991, Erickson 1992). The patterns of IGFBP expression in the skin may lead to similar results. Because IGFBP-3 levels are also clearly increased in psoriatic skin, and IGFBP-2 may also be marginally increased, the different binding proteins may play a major role in mediating IGF metabolism in psoriasis and normal skin growth and differentiation.

Studies on cultured cells suggest that the roles of IGFBP-3 and several other IGFBPs can modulate many of the effects of IGF binding to its signal-transducing receptors. Though they have no signal-transducing activities themselves, IGFBP-3 and other IGFBPs are secreted from cells and bind IGF's with high affinity (K_d = 0.5 nM), apparently even higher than the signal-

transducing receptors (Martin and Baxter 1986). When in the clutches of a binding protein, however, the IGF ligand is unable to bind to and stimulate the known signal-transducing receptors. Thus, one predicted function of the IGFBPs is to decrease the effect of IGF's on a target cell. A negative effect on growth due to the presence of soluble binding proteins has been demonstrated in some cultured cell systems (for example Campbell and Novak 1990).

However, conflicting observations have also been made, whereby IGFBPs can stimulate DNA synthesis and growth in cultured cells (de Mellow and Baxter 1988). This stimulatory activity may result from the ability of IGFBPs to buffer the amount of ligand surrounding a cell by competing with signal-transducing receptors for the IGF ligands. Buffering the amount of IGF ligands around a cell with exogenously added IGFBP-3 actually prevents receptor downregulation and hence desensitization in another cell line (Conover and Powell 1991). Cell-derived IGFBP-3 may thus buffer the amount of IGF available for the IGF-1R to enhance the effects of the IGF's by preventing receptor downregulation increasing the duration of receptor stimulation.

Yet another effect of IGFBPs on IGF metabolism has been observed. IGFBP-2 has an RGD motif suspected to be involved in attaching the protein to extracellular molecules. If so, the binding protein may localize and accumulate cytokines around the cells or tissue from which they are produced by simultaneously binding cell surfaces and IGF ligands. Recently, this phenomenon has been demonstrated by using high resolution immunocytochemistry to localize IGFBP-3 and 5 to the extracellular matrix and cell surfaces (Jones et al 1992). IGFBP-3 and 5 lack RGD motifs, but may have other binding site determinants. Potentially, surface-bound IGFBPs may store IGF ligands around a cell until secreted proteases release intact ligands by specifically cleaving the binding protein. Recent work has identified proteases which can specifically cleave the binding protein without damaging the ligand (Campbell et al 1992, Davies et al 1991). This mechanism would allow cells to accumulate a store of IGF ligands which can be released on demand by secreting proteases.

Most of the activities of IGFBPs suggested by the above studies have not yet been shown to operate in human keratinocytes. However, there are several observations about psoriasis which suggest that IGF's and IGFBP-3 may be very important in the skin and in psoriasis. First, an old but rather disputed observation is that increases in growth hormone may be linked to the onset of psoriasis (Weber et al 1981). Psoriasis often appears during puberty, when growth hormone transiently rises, and it was claimed that psoriatics may have elevated levels of growth hormone even after puberty. A strict statistical correlation has not been reproducible, although several studies do suggest that some psoriatic patients may have growth hormone levels significantly above the norm (Priestley et al 1984). Incidentally, somatostatin, which among its many hormonal functions represses growth hormone secretion from the pituitary, and the somatostatin analogue octreotide, which more specifically represses growth hormone secretion, both help resolve psoriatic skin lesions (Camisa 1989).

Observations relating perturbed growth hormone levels and psoriasis are interesting, because growth hormone is known to stimulate IGF-I synthesis in the liver, and because both IGF-I and IGFBP-3 serum levels reflect growth hormone levels (Luna et al 1983, Baxter and Martin 1984). While the stimulation of IGF-I by GH is a direct mechanism, the mechanisms correlating IGFBP-3 and GH are not known. For example, GH actually represses IGFBP-3 production in a cultured squamous cell carcinoma cell line that resembles basal keratinocytes (Andreatta-van Leyen et al 1992).

The high levels of IGFBP-3 transcript in skin and the elevated levels of IGFBP-3 in psoriasis may thus play one or more very important roles in both normal and pathological skin physiology. First, high levels of IGF ligands from the circulation are likely available to the basal and possibly higher level keratinocytes. These ligands may be transferred to the skin by either the passive equilibration of binding sites in the skin and those in blood, or through a more active system yet to be discovered. The binding sites in skin may either be the IGF-receptors or

IGFBPs. The most abundant kind of binding site is likely provided by IGFBP-3, since its transcript levels are by far the highest. Moreover, its binding affinities for both IGF-I and IGF-II are also higher than those of the IGF-receptors. Once transferred to the skin, the ligands may then be released by proteases in the skin. Normal skin has low levels of plasmin, but psoriatic skin actually has 10-fold higher levels of plasminogen activator and thus also significantly higher plasmin levels (Jensen et al 1990). Thus, it appears that normal skin and psoriatic skin in particular have all of the components needed for responding to IGF-ligand signals.

Then, once IGF is recruited to the skin through this hypothetical pathway, the keratinocytes may respond in a number of interesting ways. First, higher IGFBP-3 levels combined with high plasmin levels may result in higher than normal amounts of IGF ligands in skin. IGF's are known to stimulate growth in a number of fibroblast, osteoblast, and epithelial cell lines, including keratinocytes, so one expected result would be increased growth in cells expressing IGF-receptors. Thus, the increased growth of the basal and suprabasal cells of the psoriatic epidermis may in part be due to IGF stimulation. In combination with other cytokines, IGF stimulation may also be responsible for some of the abnormal differentiation seen in the psoriatic epidermis. Of course, IGFBP-3 is only one of many players in the complex physiology of skin and in the pathogenesis of psoriasis.

Prospects for further study

- Further application of the RT-PCR assay

The RT-PCR assay is efficient and accurate if controls are carefully attended to. Amplification conditions are easy to optimize if primers are properly designed, standard transcripts can be made quickly by standard subcloning, *in vitro* transcription, and oligo-dT cellulose chromatography methods. A typical assay with standards and six to eight samples takes less than a day, and only a few ng of total RNA are consumed for one assay. The greatest

limitation to the assay is the cost of making primers. Fortunately, ongoing advances in the methods for synthesizing oligonucleotides of specific sequences promise lower costs for primer synthesis in the future. Moreover, the small amounts of primers consumed in the RT-PCR assay (10 pmol per sample) allows primers to be shared among different investigators for quick screening analyses of transcripts of suspected interest.

Many cytokines, binding proteins, and receptors of great potential interest remain to be quantitatively investigated in psoriasis and other human diseases. A prime example is the transforming growth factor-ß's, their binding proteins, the betaglycans, and their serine-threonine kinase signal transducing receptors. Several of these species have recently been cloned and they are likely to have major roles in controlling the growth, development, and physiology of many cell types *in vitro*. The patterns of expression of TGF-ß1, TGF-ß2, and TGF-ß3 in embryonic mouse skin suggest possible roles of each factor in controlling the growth and development of the epidermis (Pelton et al 1991). TGF-ß1 expression has also been evaluated by both Northern blotting and immunocytochemistry in psoriatic and normal skin (Elder et al 1990b, Kane et al 1990). Although the levels of TGF-ß1 in psoriatic skin do not appear abnormal, the absolute levels and the levels of the other known TGF-ß ligands and TGF-ß receptors have not been studied. The immunocytochemical work also suggested a redistribution of TGF-ß1 in psoriatic skin and subtle alterations in the structures of TGF-ß1 molecules from both unaffected and affected psoriatic skin. The potency of TGF-ß's in regulating keratinocyte growth and differentiation, and in regulating the expression of proteases, protease inhibitors, and other factors controlling the formation and destruction of the extracellular matrix may be particularly important in diseases like psoriasis, where great changes in cell-cell interactions arise during the inflammatory response and cellular hyperproliferation.

The expression of keratinocyte growth factor and other members of the fibroblast growth factor family have also yet to be quantitatively studied in psoriasis, even though these proteins are known to exert very potent, specific, and distinctive effects on the growth and differentiation of cultured keratinocytes (Aaronson et al 1990, Marchese et al 1990). What are the absolute levels of expression of any of these growth factor genes, and are the levels of expression of any of these genes altered in psoriasis? Answers to these questions could identify targets for further studies on the involvement of a particular growth factor on the progress of psoriasis.

Besides looking at known cytokines, the RT-PCR assay can also evaluate the expression of other proteins suspected to affect skin physiology, especially in psoriasis. A prime example is the proteases and protease inhibitors. Many different genes encoding proteases and protease inhibitors are known and some of these have already been biochemically identified as being altered in psoriatic lesions. For example, plasminogen activator is increased at the enzymatic, protein, and transcript levels (Jensen et al 1990). Are perturbations in other proteases and protease inhibitors also involved in the development of the disease? Identification of specific proteases and inhibitors may focus further studies on the targets of those proteases and the effects of those proteases on skin biology.

The sensitivity and efficiency of the RT-PCR assay can also be exploited to investigate previously inaccessible aspects of psoriasis and other diseases. Since only a small amount of RNA is needed, small biopsies are taken. Given the cooperation of interested clinicians, there are limitless possibilities for monitoring selected transcripts during treatment and during the devlopment of a lesion. Many casual and clinical observations of lesions during their development and resolution can now be elaborated by evaluating the expression of selected molecular markers. For example, the expression of TGF-α, IL-1, IL-6, IL-8, and IGFBP-3 could be followed during the temporal and spatial progression of a lesion. The temporal order of changes in each marker could indicate the course of biochemical changes in both immune

response and cellular hyperproliferation during the disease. The known spatial heterogeneity of lesions could also be evaluated to correlate biochemical markers with different tissue morphology. As more markers are identified, the specific pathways of immune response and cellular hyperproliferation could be dissected with the sensitive and quantitative assay provided by the RT-PCR method.

- Further characterization of transcripts with perturbed levels of expression in psoriatic skin

The two goals of studying the molecular pathogenesis of psoriasis are to reveal the basic underlying defects that cause the disease and to reveal the pathways by which normal skin physiology is subverted into the disease state so that more specific and thus less toxic treatments can be devised. Identifying genes which are aberrantly expressed in psoriatic skin as opposed to normal or normal-looking unaffected psoriatic skin will undoubtedly advance the knowledge base needed for achieving these two goals.

The identification of IGFBP-3 as a consistently upregulated transcript in affected psoriatic skin suggests several further avenues for study that will likely provide useful information about the development of psoriasis. As mentioned previously, growth hormone has been implicated in the development of psoriasis in at least some patients, and IGFBP-3 is one of the major circulating factors responding to growth hormone. Potentially, then, an increase in IGFBP-3 expression may be a primary causal factor in the development of psoriasis. Specifically, a primary defect in the regulation of IGFBP-3 expression may be an important aspect of the pathogenesis of psoriasis. Thus, defects in the mechanisms which normally regulate IGFBP-3 expression should be screened with the hope of identifying one of the genetic factors involved in the development of psoriasis. Studies of IGFBP-3 gene structure and promoter activity are already under way and may provide links to basic pathogenetic mechanisms.

Another obvious approach to investigating transcripts whose levels of expression are significantly altered in psoriatic skin is to further characterize specific patterns of expression within the compartments of the skin. Skin is composed of several different compartments, with different functions (reviewed in Eckert 1989). Psoriatic skin contains several morphological changes specific to each compartment. Most strikingly, the keratinocytes above the basal layer of the epidermis hyperproliferate and undergo abnormal patterns of differentiation. Previous studies have shown that more psoriatic keratinocytes are recruited into mitosis and that these recruited cells complete the cell cycle much more quickly. The main perturbation does not, however, lie in the basal layer. Rather, the suprabasal layers seem to contain the main defects, dividing and migrating upwards much more rapidly than normal. Suprabasal keratinocytes in psoriatic lesions also fail to express or aberrantly express structural proteins like the intermediate filament keratins, the intermediate filament organizer filaggrin, and the major protein in corneocytes, involucrin (Bernard et al 1988).

Thus, after identifying an up-regulated ligand, receptor, or binding protein, it would thus be particularly interesting to see where in the normal skin and where in psoriatic skin that factor was being expressed. For example, according to the hypothesis that IGFBP-3 in skin is recruiting circulating IGF's to stimulate growth in keratinocytes, one would predict that IGFBP-3 is expressed only in the basal layer of the normal epidermis, but is expressed in both the basal and the suprabasal layers of psoriatic epidermis. These predictions are easily tested by *in situ* hybridization and immunocytochemistry studies which could specifically localize both the RNA transcripts and the protein in the skin. High resolution immunolocalization of IGFBP-3 may even reveal whether the binding protein specifically binds to the extracellular matrix and sequesters IGF ligands around cell surfaces before being released, possibly by a protease system. The cellular distribution of the IGF receptors, ligands, and other binding proteins, though apparently at much lower levels than IGFBP-3, may also be very important, and should also be assessed by

both immunocytochemistry and *in situ* hybridization. Particularly interesting would be to assess how the other binding proteins are distributed in the skin. Are domains of expression for each binding protein mutually exclusive or do certain cells co-express one or more binding proteins at significant levels.

Other experiments should test the function of the IGFBPs and IGF receptors in keratinocytes. First, the ability of skin or keratinocyte IGFBP-3 to bind to the IGF ligands should be assessed. Are the binding affinities of skin IGFBP-3 similar to the recombinant protein and protein isolated from fibroblasts? IGFBP-3 from the skin and cultured keratinocytes should be purified and biochemically characterized to test for post-translational modifications that could affect binding activity and cellular localization. Is the acid-labile subunit, which increases the affinity of the IGF ligands to the ß-subunit of IGFBP-3, also produced in keratinocytes? When the gene for this factor is cloned, this answer should be readily ascertained. Alternatively, existing antibodies for this protein may be used to test for its presence in the skin.

After establishing the form of IGF-IGFBP complexes in the skin, one could then add recombinant or keratinocyte-derived IGFBP-3 and maybe also the acid-labile α-subunit to keratinocyte cultures to test for their effects on the activities of IGF ligands. In a more sophisticated experiment, keratinocytes could be transfected with IGFBP-3 constructs that raise the amount of IGFBP-3 produced and the subsequent effects on growth and differentiation could be tested. A stably transfected cell line overexpressing IGFBP-3 could be further tested by introducing varying levels of IGF's, IGF-IGFBP complexes, and IGF-IGFBP complexes in the presence of proteases to test the hypothetical network of IGF ligands, binding proteins, and proteases previously suggested.

According to the hypothesis that proteases mediate the transfer of IGF's from the circulation to the skin, the quantities of proteases in the skin, and their effects on IGFBPs and the IGF ligands should be thoroughly characterized. Protease activities and their sites of synthesis should be localized by immunocytochemistry and *in situ* hybridization in both normal and psoriatic skin. The patterns would of course reflect the other roles of these enzymes in inflammation and extracellular matrix remodeling, but their presence in the basal cells of normal skin, and in the basal and suprabasal layers of psoriatic skin would be consistent with their hypothetical roles in regulating IGF ligand levels in skin.

CONCLUSIONS

With the goal of advancing the molecular characterization of psoriasis, a method for measuring minute amounts of RNA was developed, tested, and applied. The method uses reverse transcription coupled to the polymerase chain reaction to provide exquisite sensitivity, convenience, and accuracy. After optimization and testing, the assay provides sensitivity down to 100 copies per assay and a high level of precision such that the standard deviation of replicate measurements are less than 10% of the mean. The method's accuracy was tested against the conventional hybridization methods for measuring transcript levels. Measurements on four different gene transcripts by hybridization methods were similar to those made by the RT-PCR assay developed here. The method was then used to measure the expression of the genes encoding the IGF family of ligands, binding proteins, and signal-transducing receptors. Confirming a previous finding from subtractive hybridization screening studies of psoriatic fibroblasts, IGFBP-3 transcript levels were found to be roughly three-fold higher in psoriatic affected skin than psoriatic unaffected skin after normalization for total RNA. IGFBP-3 levels were also found to be very high, while those for the IGF ligands and the type 2 IGF receptor were very low. The type 1 IGF receptor was expressed at a moderate level, confirming previous studies. However, the levels of IGF-1R expression in both affected and unaffected psoriatic skin were very similar and suggest that previous observations of the overexpression and overactivation of this receptor in psoriatic skin are not the result of changes in transcript levels. The studies performed here demonstrate the utility of the RT-PCR method for quantifying gene transcripts. Furthermore, the results of initial investigations into the expression of IGF ligands, binding proteins, and signal-transducing receptors using the RT-PCR assay suggest many productive avenues for further investigation which may shed light on the molecular pathogenesis of psoriasis.

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