GENE TRANSFER AND EXPRESSION

A LABORATORY MANUAL

GENE TRANSFER AND EXPRESSION

A Laboratory Manual

Michael Kriegler

M
stockton press

New York London Tokyo Melbourne Hong Kong

Published in the United States and Canada by Stockton Press
15 East 26th Street, New York, N.Y. 10010

Library of Congress Cataloging-in-Publication Data

Kriegler, Michael P., 1954–
Gene transfer and expression : a laboratory manual / Michael P. Kriegler
p. cm.
Includes bibliographical references.
Includes index.
ISBN 0-935859-89-6 (soft cover) :
1. Genetic transformation—Laboratory manuals. 2. Gene expression—Regulation—Laboratory manuals. I. Title.
[DNLM: 1. Gene Expression Regulation—laboratory manuals. 2. Transfection—laboratory manuals. QH 450 K92g]
QH442.K73 1990
574.87'322'078—dc20
DNLM/DLC
for Library of Congress 90-10164
CIP

Published in the United Kingdom by
MACMILLAN PUBLISHERS LTD (Journals Division), 1990
Distributed by Globe Book Services Ltd.,
Brunel Road, Houndmills, Basingstoke,
Hants RG21 2XS, England

British Library Cataloguing in Publication Data
Kriegler, M.
Gene Transfer and Expression:
A Laboratory Manual
1. Mammals. Genetic Engineering
I. Title
599.015

ISBN 0-333-53543-X

Printed in the United States of America
9 8 7 6 5 4 3 2 1

Acknowledgments

The writing of this laboratory manual reflects the efforts of a number of dedicated individuals without whose assistance I could not have completed the task. Many of the protocols were developed and virtually all of the protocols were tested with the assistance of the personnel in my laboratory, Carl Perez, Kim DeFay, Iris Albert, and Sharon Hubby, as well as my colleagues from the PCR division, John Lyons and Ernie Kawasaki. M. J. Poklar and George McGregor assisted with the library research. Jan Tuttleman critically reviewed the manuscript and made many useful suggestions regarding content and organization. Denise Ramirez and Edna McCallan typed the manuscript and Ward Ruth drew the figures. I am deeply indebted to those individuals for their help. I am also indebted to my colleagues who encouraged me to take on this project, expressing the need for a manual of this type and to those individuals who developed many of the techniques contained herein and who shared their experiences with me. I also wish to thank Ingrid Krohn for her encouragement and support.

Michael Kriegler

How to Use This Manual

This manual is composed of three parts: Part I, Gene Transfer/Background Information; Part II, Gene Transfer/Methods; and Part III, Assays for Gene Transfer and Expression. Part I provides background information on three subjects: (1) eukaryotic regulatory elements, (2) gene transfer vectors, and (3) protein synthesis, processing and glycosylation. If you are unfamiliar with any of these topics, I suggest you familiarize yourself with the information in Part I. It reflects our current understanding of the variety and function of eukaryotic (mammalian) *cis*-acting regulatory elements, the variety of gene transfer vectors currently available, as well as what is currently understood about protein synthesis, processing and glycosylation. If you are familiar with these topics, proceed to Part II. The techniques described in this section will enable you to introduce the gene or genes of interest into cells in culture. Once gene transfer has been attempted, you will want to assay for transfer and expression; Part III describes how to accomplish this.

Contents

I

GENE TRANSFER

This section of the manual contains a description and analysis of the genetic components, molecular tools, and biochemical processes that you, the experimentalist, must manipulate and understand to design incisive and convincing gene transfer studies. I have included a description of *cis*-acting elements involved in the regulation of expression of eukaryotic genes, a description with diagrammatic representation of the various gene expression vector systems that currently exist as well as a discussion of their potential applications, and a description of the post-translational mechanisms that alter protein structure. Relevant references are included to serve as a starting point for further reading on the particular *cis*-acting element, vector system, or post-translational process you are interested in studying.

1

EUKARYOTIC CONTROL ELEMENTS

INTRODUCTION

As you attempt to express genes transferred into cells or into an animal, you must deal intelligently with a plethora of information about *cis*-acting DNA elements that, either directly or indirectly, affect gene expression. Each gene appears to have not only a promoter but in many cases a matched enhancer, as well as splice signals, polyadenylation signals, and signals that determine the messenger RNA half-life. These elements exert their influence in concert in a cell-type-specific manner and serve to determine where and when in an organism the gene is expressed. These elements can also be experimentally mixed and matched with each other, resulting in recombinant elements that manifest new biologies. Thus, before a single test tube is raised, you must familiarize yourself with these elements. Despite the apparent complexity of the interaction of these *cis*-acting elements during the regulation of gene expression, there are a number of rules you should keep in mind to guide genetic design.

The information that follows is provided to illustrate the range and behavior of *cis*-acting elements in viral and cellular cistrons available to you. In this background section, data arising from the experimental manipulation of regulatory sequences of eukaryotic genes are compiled. In most cases, specific sequence information is not included, but references are cited.

First, promoters are described, and a variety of enhancers, both viral and cellular, are discussed in detail. Then the effects of splicing on gene expression are described, mRNA stability elements are examined, and both *cis*- and *trans*-acting elements that affect translational control are discussed. At the end of each section, the mechanisms by which these elements exert their influences are explored. You should note that the section on enhancers is but a partial listing of the viral and cellular enhancers identified to date. New elements are described monthly, and the identifications of their *trans*-acting enhancer-binding protein congeners follow shortly thereafter. What should be apparent from this listing is that enhancers are highly varied and function in a variety of ways. The enhancer section contains an abundance of detail because changes in enhancer sequences, even single base-pair changes, can dramatically alter the function of the enhancer.

Adapted and reprinted by permission of the publisher from "Assembly of Enhancers, Promoters, and Splice Signals to Control Expression of Transferred Genes" by Michael Kriegler in GENE EXPRESSION TECHNOLOGY (METHODS IN ENZYMOLOGY, Volume 185) edited by David V Goeddel.

Note also that promoters, enhancers, splice signals, and other *cis*-acting elements do not function as fully independent elements. It appears that there are preferred and nonfunctional combinations. In many cases, appropriate time- and tissue-specific expression is dependent on the assembly of an appropriate expression ensemble: ideally matching enhancer with promoter and promoter with splice signal and mRNA degradation signal. The selection and arrangement of *cis*-acting elements in a recombinant cistron is thus critical in achieving the desired expression phenotype. Therefore, prior to attempting gene transfer, take your time in engineering your recombinant molecule. Don't let convenient restriction sites dictate your design parameters. If necessary, destroy or create new sites via either site-directed mutagenesis or PCR-based gene assembly to ensure the inclusion or exclusion of any potentially important or detrimental DNA sequences in your construction. If you pay attention to these details, you can expect to succeed. Failure to do so may lead to an uninterpretable result and thus a waste of time and resources.

PROMOTERS AND ENHANCERS

The genetic dissection, through recombinant DNA techniques, of promoter elements, initially viral, led to a description of the molecular structure of eukaryotic promoters and to the discovery of a new class of *cis*-acting elements that appeared to modulate gene expression in a time- and tissue-specific fashion. These elements have come to be called enhancers. Enhancers appear to form part of the structural framework for DNA/protein interactions that may result in the formation of higher-order chromatin structures that may, in turn, serve to regulate transcription of subject genes from RNA polymerase II promoters. The identification of enhancer elements has led to the isolation and characterization of *trans*-acting factors that either bind to or in some other way affect the promoters or enhancers with which they interact. For a compilation of transcription-regulating proteins and an analysis of their mode of action, see Wingender (1988), Mitchell and Tjian (1989), and Ptashne and Gann (1990). What has become clear from site-directed mutagenesis of eukaryotic expression elements is that both enhancers and promoters play pivotal roles in the regulation of gene expression.

The elegant early studies of the herpes simplex thymidine kinase (HSVtk) promoter (McKnight and Kingsbury, 1982; McKnight et al., 1984) and the human β-globin promoter (Breathnach and Chambon, 1981) demonstrate that the structure of RNA polymerase II promoter elements is relatively set. One critical feature of all promoter elements is that they contain mRNA cap sites, the point at which the mRNA transcript actually begins. Yet another frequently occurring homology, the so-called TATA box, is centered around position -25, just upstream of the mRNA cap site. The TATA box accurately positions the start of transcription. Further upstream, the relative positions of promoter sequence homologies become more variable. They can, as is the case of the CCAAT homology in the HSVtk and human β-globin promoters, actually reside in different positions on different strands around 70 to 80 nucleotides upstream from the mRNA cap site. Another structural component of many housekeeping genes consists of multiple copies of GC-rich regions upstream of the mRNA cap site. There appears to be an absolute requirement for these sequences in eukaryotic promoters, so they must be included in all engineered recombinant cistrons.

With few exceptions, the most critical variable in the design of chimeric expression cistrons is the selection of an enhancer element or elements for

inclusion in the recombinant molecule. First identified in the genomes of SV40 and murine retroviruses, enhancers are the most peculiar of all known expression elements. Little is known about their mechanism of action. The key properties that characterize an enhancer are as follows:

- They are relatively large elements and may contain repeated sequences that can function independently.
- They may act over considerable distances, up to several thousand base pairs (bp).
- They may function in either orientation.
- They may function in a position-independent manner and can be within or downstream of the transcribed region, but they can function only in *cis*. If several promoters lie nearby, the enhancer may preferentially act on the closest.
- They may function in a tissue-specific manner or at a particular stage of differentiation in a given cell.

Enhancers have been identified in association with both viral and cellular genes. To minimize confusion, I will treat these two classes separately, and in yet another section discuss the class of inducible enhancers and promoters. A listing of viral enhancers can be found in Table 1-1, cellular enhancers in Table 1-2, and inducible promoters and enhancers in Table 1-3.

Viral Enhancers

The SV40 Enhancer

The first enhancing DNA sequence identified was of viral origin and resides in the genome of SV40 near the origin of replication (Banerji et al., 1981; Moreau et al., 1981). The SV40 enhancer is composed of three functional units—A, B, and C—each of which cooperates with the others or with duplicates of themselves to enhance transcription. When element C, containing the so-called enhancer core consensus sequence, is inactivated by point mutations, revertants with reduced enhancer function can be isolated that contain duplications of either one or both of the elements A or B (Herr and Clarke, 1986; Firak and Subramanian, 1986). In addition, each element can act autonomously when present in multiple tandem copies. Amplified copies of the B and C elements exhibit different cell-specific activities (Ondek, et al., 1987).

An analysis of the effects that varying the position of the SV40 enhancer had on the expression of multiple transcription units in a single plasmid revealed two types of position effects. The first, promoter occlusion, results in reduced transcription at a downstream promoter if transcription is initiated at a nearby upstream promoter. This effect does not involve enhancer elements directly, even though the effect is most pronounced when the downstream promoter lacks an enhancer element. The second effect stems from the ability of promoter sequences to reduce the effect of a single enhancer element on other promoters in the same plasmid. This effect is mediated by either promoters adjacent to the enhancer element or promoters interposed between the enhancer element and the other promoters on the plasmid (Kadesch and Berg, 1986). Perhaps one of the more controversial observations regarding the SV40 enhancer is that enhancer-binding factors are required for the establishment but not the maintenance of enhancer-dependent transcriptional activation (Wang and Calame, 1986). This conclusion has been elegantly contested (Schaffner et al., 1988), and the disagreement has not yet been resolved. Such enhancer-binding factors may be induced or activated, either directly or indirectly, in a human hepatoma cell line by the tumor promoter TPA. In this cell line,

TABLE 1-1 Viral Enhancers

Enhancer	*References*
SV40	Banerji et al., 1981; Moreau et al., 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur et al., 1980; Katinka et al., 1980, 1981; Tyndall et al., 1981; Dandolo et al., 1983; deVilliers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a,b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander and Haseltine, 1987; Thiesen et al., 1988; Celander et al., 1988; Choi et al., 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo et al., 1983; Lusky et al., 1983; Spandidos and Wilkie, 1983; Spalholz et al., 1985; Lusky and Botchan, 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al., 1987; Stephens and Hentschel, 1987; Gius et al., 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing et al., 1987; Hauber and Cullan, 1988; Jakobovits et al., 1988; Feng and Holland, 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989; Sharp and Marciniak, 1989; Braddock et al., 1989
Cytomegalovirus	Weber et al.,1984; Boshart et al., 1985; Foecking and Hofstetter 1986
Gibbon Ape Leukemia Virus	Holbrook et al., 1987; Quinn et al., 1989

TPA induces the transcriptional stimulatory activity of the SV40 enhancer (Imbra and Karin, 1986).

Similarly, F9 embryonal carcinoma (EC) cells, when forced to differentiate, exhibit a marked increase in transcription from a transiently transfected genome whose transcription is driven from an SV40 promoter. Deletion of the enhancer from this plasmid ablates the effect. Further, in both undifferentiated and differentiated F9 EC cell types, the level of transcription was found to be limited by the availability and/or activity of cellular factors necessary for enhancer function (Sleigh and Lockett, 1985). Chimeras constructed with the SV40 enhancer and the virus responsive elements (VREs) of the interferon α promoter indicate that the two subsequences, Rep A and Rep B of the VRE, when individually inserted between the TATA box of the α-interferon promoter and the SV40 enhancer, serve to silence that promoter. Such promoter silencing is fully reversible after induction. Silencing is not observed when the intact VRE is placed between the TATA box and the SV40 enhancer (Kuhl et al., 1987).

Thus, the SV40 enhancer element is a complex structure whose function is subject to some position effects, and whose cell-type-specific activation is dependent, in part, on the absence or presence of active cellular factors or proximal sequences.

The Polyoma Enhancer

The polyoma virus enhancer, also found near the viral origin of replication (Tyndall et al., 1981; Veldman et al., 1985), is similar in many ways to the SV40 enhancer. However, there are observations unique to this regulatory element. The polyoma virus enhancer is normally not active in undifferentiated F9 EC cells, but it is active in these cells after differentiation (Swartzendruber and Lehman, 1975). Nevertheless, polyoma virus mutants that can express and replicate their genomes in both cell types have been described (Katinka et al., 1980, 1981; Vasseur et al., 1980). The sequence alterations responsible for this phenotype consist of duplications and mutations around the polyoma

virus origin of replication and enhancer. These mutations appear to dramatically increase the transcription of the viral genes (Dandolo et al., 1983). Recently, an enhancer point mutant has been isolated that is fully functional in both cell types. Mutant F441 carries a single base mutation at nucleotide 5233 of the viral genome. Enhancers of mutants displaying a similar phenotype contain a duplicated segment of viral DNA encompassing nucleotide 5233. Furthermore, duplication of the point-mutated segment results in an even higher level of expression in the undifferentiated cell type. In addition, co-transfection of the F441 oligonucleotide, but not the wild-type sequence, inhibits the activity of the enhancer fragment of F441 attached to a reporter gene. Thus, it appears that the point mutation is a target for a cellular factor or factors that act in a positive manner to increase the transcription of a gene in undifferentiated F9 EC cells (Satake et al., 1988).

Studies have shown that both the SV40 enhancer and the wild-type polyoma enhancer can be repressed by adenovirus E1A gene products. Other work has shown that although the wild-type polyoma enhancer cannot function in undifferentiated F9 EC cells, the SV40 enhancer can, at least to a limited extent. On the basis of these observations, it has been postulated that undifferentiated embryonal carcinoma F9 cells contain an E1A-like activity, and that this activity is responsible for the lack of polyoma virus enhancer activity in F9 EC cells. Hen et al. (1986) report that E1A gene products do not repress a point mutant of the polyoma virus enhancer that is active in undifferentiated F9 EC cells. Their result is consistent with the notion that undifferentiated F9 EC cells contain a cellular repressor that blocks the polyoma virus enhancer and that this repressor has the same target sequence as the E1A proteins.

However, the polyoma story is even more complex. The polyoma virus is normally not permissive for replication in most lymphoid lines. Nevertheless, deletion of the PvuII D fragment from the wild-type polyoma genome (spanning the F441 point mutation) facilitated replication in some T-cells and mastocytoma cell lines; therefore, the PvuII D fragment can act as both a positive and a negative regulatory element. Substitution of the polyoma enhancer with the MuLV enhancer facilitates replication in 3T6 and B lymphoid cells. Substitution with the immunoglobulin heavy-chain enhancer facilitates replication in B lymphoid cells but not 3T6 cells (deVilliers et al., 1984) or mastocytomas (Campbell and Villarreal 1986, 1988).

Retroviral Enhancers

Retroviruses also carry potent transcriptional enhancers (Kriegler and Botchan, 1982, 1983; Levinson et al., 1982). I will consider the enhancers of the murine leukemia and sarcoma viruses simultaneously because it makes for a more interesting story when one compares their activities. The type of tissue in which these retroviruses induce disease often, though not always, reflects the cell-type specificity of the enhancers resident in the long terminal repeats (LTRs) of the integrated provirus. The enhancers in these viruses are quite similar to one another, so mix-and-match experiments between elements of their enhancers can reveal the nucleotide differences that determine tissue-type expression specificity. The transcriptional enhancers of Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV) exhibit different cell-type expression specificities from the enhancer of Friend murine leukemia virus. Although the three enhancers are approximately equally active in erythroid cells, the MoMuSV and MoMuLV enhancers are 20–40-fold more active than the Friend MuLV enhancer in T-lymphoid cells. There appears to be an element, repeated several times within these enhancers, that modulates

the activity of the enhancer in T-cells without affecting it in erythroid cells. This element thus seems to be one of the determinants of the tissue specificity of the enhancer (Bosze et al., 1986; Li et al., 1987; Thiesen et al., 1988).

Enhancer elements within the sarcoma virus MoMuSV and the non-leukemogenic (AKV) and T-cell leukemogenic (SL3-3) murine retroviruses also exhibit strong cell-type preferences in transcriptional activity. These elements are additionally regulated by the glucocorticoid dexamethasone. Mapping studies in combination with DNAse I footprinting experiments define the presence of glucocorticoid regulatory elements at the promoter-proximal ends of each enhancer repeat. These elements behave like inducible enhancers. Their regulatory activity is independent of position and orientation when they are linked in *cis* to a heterologous promoter. The sequences required for dexamethasone regulation for both AKV and SL3-3 include a 17-nucleotide consensus sequence termed the glucocorticoid-responsive element (GRE), located at the promoter-proximal ends of each enhancer repeat. Although the GREs are identical for these enhancers the sequences surrounding these elements differ (Miksicek et al., 1986; Celander and Haseltine, 1987; Celander et al., 1988).

Our experiments with both infected and transfected retroviral genomes have led us to some interesting observations regarding promoter and enhancer strengths in specific cell types. First, we and others observed that infected retroviral genomes express their gene products at substantially higher levels than do the same transfected genomes present in the same copy number (Hwang and Gilboa, 1984). Second, comparison of the synthetic capabilities of a wild-type SV40 virus and an engineered murine retrovirus (MuLV) expressing SV40 T antigen revealed that, in mouse fibroblasts, an engineered SV40 retrovirus could produce approximately 10 times the amount of T antigen as its wild-type SV40 equivalent. These observations hold true for a variety of structural genes we have analyzed (Kriegler and Botchan, 1982, 1983; Kriegler et al., 1983, 1984a,b, 1988; Choi et al., 1988).

Papilloma Virus Enhancers

The papilloma viruses, both bovine and human, induce tissue-specific diseases in their hosts and have complex enhancer architectures. Cells transformed by these viruses maintain the viral DNA as nuclear plasmids. Three enhancer elements modulate the transcriptional activity of the bovine papilloma virus (BPV). They map to a 59 bp region 3′ to the early polyadenylation sequence at the end of the early viral gene transcripts (Campo et al., 1983; Lusky et al., 1983), the 31 percent non-transforming, late region (Lusky and Botchan, 1986), and the 5′, 900 bp non-coding region of the viral genome (Spalholz et al., 1985). The 59 bp 3′ enhancer is host specific (Spandidos and Wilkie, 1983). The 5′ enhancer is located in the vicinity of the viral promoter and can be *trans*-activated by the viral protein E2. Human papilloma virus (HPV) type 18 also contains three different enhancer domains, two of which are inducible and one of which is constitutive. The inducible enhancers are responsive to papilloma virus-encoded *trans*-acting factors, whereas the constitutive enhancer requires only cellular factors for activity. Inducible enhancer IE2 is located proximal to the E6 cap site and responds to the E2 *trans*-activator. Inducible enhancer IE6 is located 500 bp upstream of the E6 cap and responds to the E6 gene product. The third, constitutive enhancer lies between the two inducible enhancers. Each enhancer functions independently of the others and may function at different stages of the viral life cycle (Gius et al., 1988). Human papilloma virus type 16 (HPV16) contains a keratinocyte-dependent enhancer in a sequence 5′ to the P97 coding region. The E2 *trans*-activator of HPV16, as well as that of the related bovine papilloma