THE FUNCTIONS OF THE V-REL ONCOGENE PRODUCT OF RETICULOENDOTHELIOSIS VIRUS, REV-T

A Thesis Submitted to the

School of Graduate Studies

University of Ottawa

In Partial fulfilment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

Faculty of Medicine

By

Mahnhoon Park

© Mahnhoon Park, Ottawa, Canada, 1996

National Library of Canada
Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services Branch
Direction des acquisitions et des services bibliographiques

395 Wellington Street
Ottawa, Ontario
K1A 0N4

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-16451-9

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

v-rel is the transforming gene of the avian reticuloendotheliosis virus, strain T (REV-T), which causes fatal lymphomas in young birds and also transforms avian lymphoid cells *in vitro*. The v-*rel* oncogene codes for a protein of 503 amino acids. The v-*rel* protein exhibits extensive homology within its N-terminal 300 amino acids with several mammalian κB enhancer binding proteins including 50-kDa and 65-kDa subunits of NF-κB transcription factor. Previously, the transforming domains of the v-*rel* oncogene were localized to the N-terminus by testing the transforming activities of five deletion mutants and twelve linker insertion mutants of v-*rel*. In this study, an array of biochemical functions of these mutant v-*rel* proteins was investigated in order to understand the mechanism of transformation by v-*rel* oncogene.

Since the v-*rel* protein shows striking homology with NF-κB subunits, wild-type v-*rel* or deletion and linker insertion mutants of v-*rel* were expressed in insect cells using the baculovirus system to determine the κB-specific DNA binding activity by electrophoretic mobility shift assay. C-terminal deletions of up to 100 amino acids, or deletion of 11 N-terminal amino acids had no effect on DNA binding activity of v-*rel* protein, whereas N-terminal deletion of 99 amino acids abolished DNA binding activity completely. Seven mutant v-*rel* proteins with linker insertions that mapped between amino acid residues 29 to 275 lost DNA binding activity, but five mutant v-*rel* proteins that contained a linker insertion mutation between amino acid residues 332 to 459 retained the DNA binding activity. These results showed that all of the mutant v-*rel*

proteins with transforming activity also retained the DNA binding activity like wild-type v-*rel* protein, whereas all of the mutant v-*rel* proteins which were nontransforming lost DNA binding activity. Therefore, it is clear that the κB-specific DNA binding activity is necessary for transformation by v-*rel*.

The active nuclear form of NF-κB transcription factor is composed of p50 and p65 subunits that contact DNA as a heterodimer. Both subunits of NF-κB are found to be homologous within the N-terminal 300 amino acids, which appeared to be required for DNA binding and dimerization. The similarities between NF-κB subunits and the v-*rel* protein in this region prompted us to investigate the interaction of the v-*rel* protein with NF-κB subunits by coimmunoprecipitation analysis. All of the v-*rel* proteins with deletion and linker insertion mutations, regardless of their transforming activities, were able to interact with p50 and p65 subunits of NF-κB. However, the interaction of non-DNA binding mutant v-*rel* proteins with NF-κB subunits abrogated the DNA binding activities of NF-κB subunits resulting in complexes that were unable to bind to DNA, whereas all of the DNA binding mutant v-*rel* proteins and wild-type v-*rel* protein were able to form functional complexes with NF-κB subunits. These results showed that all of the non-DNA binding, non-transforming mutants of v-*rel* exhibited a transdominant negative effect. Taken together, these results suggest that the formation of DNA binding complexes by v-*rel* protein with NF-κB subunits is necessary for transformation.

The NF-κB complex is maintained in an inactive state in the cytoplasm through its association with an inhibitor protein, referred to as IκB, which prevents its

translocation into the nucleus *in vivo* and interferes with its binding to DNA *in vitro*. Interestingly, pp40, which is the one of the cellular proteins associated with the v-*rel* protein in REV-T transformed cells, has been identified as avian IκB. In order to determine the possible role of pp40 association in transformation, pp40 association with v-*rel* protein was examined by coimmunoprecipitation analysis in insect cells. All of the v-*rel* proteins with deletion and linker insertion mutations, regardless of their transforming activities, were shown to associate with pp40. Association of pp40 with the DNA binding mutant v-*rel* proteins including wild-type v-*rel* protein resulted in inhibition of DNA binding activity. These results imply that the physical association with pp40 itself might not be sufficient for transformation by v-*rel*.

Since the v-*rel* protein directly binds to the κB site, the κB-dependent transcriptional activity of v-*rel* protein was investigated by cotransfection of a v-*rel* expression vector with a CAT reporter plasmid containing κB sites in front of the minimal promoter in Cos1 cells. All of the transforming mutants of v-*rel* and wild-type v-*rel* induced κB-dependent transcription in a dose-dependent manner, whereas all of the non-transforming mutants of v-*rel* failed to show any transactivation. Furthermore, all of the non-transforming mutants of v-*rel* completely inhibited the κB-dependent transactivation mediated by p65 or c-*rel* protein, demonstrating that the non-DNA binding, non-transforming mutants function as transdominant inhibitors of the *rel* family of transcription factors. These results support a model in which activation of gene expression directly by v-*rel* protein is required for its ability to induce oncogenic transformation.

ACKNOWLEDGEMENT

I would like to extend my gratitude to Dr. C. Yong Kang for his support and guidance and for providing me with an excellent scientific environment to conduct my research. Specially, his advice and encouragement were invaluable for me to carry out my Ph.D. studies.

I thank all of my Thesis Advisory Committee members for their advice and discussions. Special thanks go to Dr. Earl Brown for providing me with the chicken embryos.

I also thank the members of Dr. Kang's laboratory and of the Department of Microbiology and Immunology for their cooperation.

I would like to thank Dr. H. M. Temin for providing me with a clone of the chicken *c-rel* cDNA and Dr. H. R. Bose for giving me the pp40 clone. I also thank Dr. S. Y. Kim for providing me with the p50 and p65 clones of NF-κB. These clones were indispensable to carry out the experiment for this thesis.

I would like to acknowledge the financial support from Mogam Biotechnology Research Institute in Korea. I also acknowledge the financial support of the University of Ottawa.

DEDICATION

This thesis is dedicated to Meehae

TABLE OF CONTENTS

PAGE

ABSTRACT ii

ACKNOWLEDGEMENT v

DEDICATION vi

TABLE OF CONTENTS vii

LIST OF TABLES xiv

LIST OF FIGURES xv

LIST OF ABBREVIATIONS xviii

CHAPTER 1: GENERAL INTRODUCTION 1

1. RETICULOENDOTHELIOSIS VIRUS, STRAIN T 1

   Classification 1

   Origin 2

   Pathogenicity 3

   Genome organization 4

2. THE ONCOGENES OF RNA TUMOR VIRUSES 5

   Historical perspective 5

   Mechanisms of cell transformation by retroviruses 8

   Functions of oncogenes 9

   The transforming potential of oncogenes 14

3. THE REL ONCOGENE 15

v-*rel* 15

c-*rel* 17

Homology with other genes 18

4. THE REL GENE PRODUCT 22

Detection and characterization of the v-*rel* and c-*rel* proteins 22

Cellular localization of the v-*rel* protein 24

Complex formation of the v-*rel* protein with other proteins 25

5. TRANSFORMING DOMAINS OF V-REL ONCOGENE 26

6. STATEMENT OF OBJECTIVES 30

CHAPTER 2: MATERIALS AND METHODS 31

1. CELLS AND VIRUSES 31

Maintenance 31

Transfections 31

Production and titration of REV-T and related viruses 33

Selection and purification of recombinant baculoviruses 33

In vitro transformations 34

2. MOLECULAR CLONING AND RELATED TECHNIQUES 35

Preparation of competent *E. coli* 35

Transformation of *E. coli* 36

Purification of plasmid DNA 36

Electrophoresis of DNA 37

Purification of DNA from gels 37

Manipulation and modification of DNA 38

Polymerase chain reaction (PCR) 38

DNA sequencing 39

Labeling of oligonucleotides 39

Electrophoretic mobility shift assay (EMSA) 40

UV cross-linking 40

3. PREPARATION OF ANTIBODIES 41

Expression and purification of recombinant v-*rel* protein 41

Immunization 42

Collection of sera 42

Other antibodies used in this study 43

4. PROTEIN ANALYSIS 43

Preparation of cell extracts 43

SDS polyacrylamide gel electrophoresis (SDS-PAGE) 44

Western blots 45

Metabolic labeling of intracellular proteins 45

Immunoprecipitation 46

Immunofluorescence assay 46

5. BIOCHEMICAL ASSAYS 47

Protein quantitation 47

ß-galactosidase assay 47

Chloramphenicol acetyltransferase (CAT) assay 48

CHAPTER 3: DNA BINDING ACTIVITY OF THE V-REL ONCOGENE PRODUCT 50

1. INTRODUCTION 50

2. RESULTS 51

Characterization of DNA binding activity in the REV-T transformed CSC 51

Expression of deletion and linker insertion mutants of v-*rel* in insect cells 57

DNA binding activities of mutant v-*rel* proteins expressed in insect cells 60

Deletion mapping of the DNA binding domain of the v-*rel* protein . . . 62

3. DISCUSSION 64

The DNA binding domain of the v-*rel* protein 64

The relationship between DNA binding activity and transforming activity of the v-*rel* protein 67

CHAPTER 4: INTERACTIONS OF THE V-REL ONCOGENE PRODUCT WITH NF-κB SUBUNITS AND IκB (pp40) 71

1. INTRODUCTION 71

2. RESULTS 73

Association of the v-*rel* protein with cellular proteins in transformed CSC 73

Expression of NF-κB subunits in insect cells 76

Interaction of p105 and p50 with the *rel* proteins in insect cells 79

DNA binding activities of heterodimers of p50 with the *rel* proteins . . 82

Interaction of p65 with the *rel* proteins 88

The association of pp40 with the v-*rel* protein in transformed CSC . . . 92

The association of pp40 with the *rel* proteins in insect cells 95

3. DISCUSSION 99

Complex formation of the v-*rel* protein with NF-κB subunit and IκB in transformed CSC 99

Interaction of mutant v-*rel* proteins with NF-κB subunits 103

Association of pp40 with the mutant v-*rel* proteins 106

CHAPTER 5: THE TRANSACTIVATION ACTIVITY OF THE V-REL ONCOGENE PRODUCT 108

1. INTRODUCTION 108

2. RESULTS 109

Transient expression of *rel* family genes in Cos1 cells 109

DNA binding and transcriptional activities of *rel* family proteins expressed in Cos1 cells 113

The κB-dependent transcriptional activities of mutant v-*rel* proteins expressed in Cos1 cells 118

Functional effect of v-*rel* expression on κB-dependent transactivation activities of p65 and the c-*rel* protein 121

The functional effect of pp40 on κB-dependent transactivation activity . 124

3. DISCUSSION 128

The κB-dependent transcriptional activity of the v-*rel* protein 128

Functional effects of protein-protein interactions on κB-dependent transactivation 130

The relationship between transactivation activity and transforming activity of the v-*rel* protein 132

CHAPTER 6: MECHANISM OF TRANSFORMATION BY THE V-REL ONCOGENE 136

1. STRUCTURE OF THE V-REL ONCOGENE 136
2. STRUCTURE AND EXPRESSION OF THE c-*rel* PROTO-ONCOGENE 137
3. ACTIVATION OF THE ONCOGENIC POTENTIAL OF THE C-REL PROTO-ONCOGENE 139
4. FUNCTIONAL DOMAINS OF THE V-REL PROTEIN REQUIRED FOR ONCOGENIC TRANSFORMATION 141
5. MODELS FOR TRANSFORMATION BY THE v-*rel* ONCOGENE . 144

REFERENCES 148

APPENDIX 164

LIST OF TABLES

PAGE

1. Functional groups of retroviral oncogenes 11

2. Sequences recognized by NF-κB 21

3. Transforming activity of v-*rel* mutants 29

4. Transforming and DNA binding activities of v-*rel* mutants 68

5. Transforming, DNA binding, and complex formation activity of v-*rel* mutants 104

6. Transforming, DNA binding and transactivation activities of v-*rel* mutants . 134

7. The functional characteristics of v-*rel* mutants 142

LIST OF FIGURES

PAGE

1. Genome organization of REV-T and REV-A 6
2. Oncoprotein function as components of mitogenic signalling chains 12
3. Comparison of the predicted amino acid sequences of the chicken and turkey c-*rel* proteins and the turkey-derived v-*rel* protein 16
4. Sequence homologies among *rel* gene family 20
5. Activation of NF-κB complex 23
6. Deletion and linker insertion mutants of v-*rel* 28
7. Expression and DNA binding activity of the v-*rel* protein in transformed CSC 53
8. The competitive EMSA using mutant and wild-type κB oligonucleotides .. 55
9. The identification of protein(s) responsible for κB binding activity in transformed CSC extracts 56
10. Strategy of the construction of recombinant baculoviruses 59
11. Expression and DNA binding activities of mutant v-*rel* proteins in insect cells 61
12. Deletion mapping of the DNA binding domain of the v-*rel* protein 63
13. Immunoprecipitation analysis of transformed CSC 75
14. Expression and DNA binding activities of NF-κB subunits in insect cells . 77
15. Interaction of p105 and p50 with the *rel* proteins 81

16. Interaction of p105 with the mutant v-*rel* proteins 83

17. DNA binding activities of heterodimers of p50 with the *rel* proteins 85

18. DNA binding activities of heterodimers of p50 with the mutant v-*rel* proteins 87

19. Interaction of p65 with the *rel* proteins 89

20. DNA binding activities of heterodimers of p65 with *rel* proteins 91

21. The association of pp40 with the v-*rel* protein in transformed CSC 94

22. The association of pp40 with NF-κB subunits or *rel* proteins in insect cells 96

23. The association of pp40 with the mutant v-*rel* proteins in insect cells 98

24. The effect of pp40 association on DNA binding activities of NF-κB subunits and the *rel* proteins 100

25. Strategy for the characterization of transactivation activity of NF-κB subunits or the *rel* proteins 110

26. The transient expression of *rel* family genes in Cos1 cells 112

27. DNA binding activities of *rel* family proteins expressed in Cos1 cells 114

28. The κB-dependent transcriptional activities of *rel* family proteins expressed transiently in Cos1 cells 117

29. The κB-dependent transcriptional activities of mutant v-*rel* proteins expressed transiently in Cos1 cells 120

30. Divergent functional effects of the wild-type v-*rel* protein and ΔN99 proteins on transactivation by p65 or the c-*rel* protein 123

31. The effects of pp40 on DNA binding activities of *rel* family proteins in Cos1 cells 125

32. The effect of pp40 on the transactivation activities of *rel* family proteins . . 127

33. The effect of pp40 binding on transactivation by mutant v-*rel* proteins . . . 129

34. Models for transformation by the v-*rel* protein 145

LIST OF ABBREVIATIONS

μCi	microcuries
μg	microgram
μl	microlitre
ALSV	avian leucosis-sarcoma virus
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt
Bis	N'N'-Bis-methylene-acrylamide
BrdU	5'-bromo-2'-deoxyuridine 5'-triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CAT	chloramphenicol acetyltransferase
CBMC	REV-T transformed chicken bone marrow cells
cDNA	complementary deoxyribonucleic acid
CEF	chicken embryo fibroblasts
CIP	calf intestinal phosphatase
CSC	chick spleen cells
CsCl	cesium chloride
CSV	chick syncytial virus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate

ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E	encapsidation signal
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
g	gram
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HIFCS	heat inactivated fetal calf serum
hr(s)	hour(s)

IFA	immunofluorescence assays
IPTG	isopropyl ß-D-thio-galactoside
kDa	kilodaltons
kb	kilobases
kbp	kilobase pairs
kg	kilogram
L	litre
LB	Luria broth
LTR	long terminal repeats
MEM	minimal essential media
mg	milligram
ml	millilitre
mM	millimolar
MMC-1	an endogenous type C virus of the rhesus monkey
MOI	multiplicity of infection
NBT	p-nitro blue tetrazolium chloride
ng	nanogram
NP	non-virus producer
NP40	Nonidet p40
ONPG	o-nitrophenyl-ß-D-galactopyranoside
PBS	phosphate buffered saline
pbs	primer binding site

PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PFU	plaque forming unit
PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl methylsulfonylfluoride
ppt	polypurine tract
QEF	quail embryo fibroblasts
R	repeat region
REV-A	competent reticuloendotheliosis associate virus
REV-T	defective transforming reticuloendotheliosis virus
REV	reticuloendotheliosis virus, strain T, original isolate
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RSV	Rous sarcoma virus
sa	splice acceptor
sd	splice donor
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SV40	simian virus 40
TAE	tris-acetate buffer

TBE	tris-borate buffer
TBS	tris buffered saline
TLC	thin layer chromatography
TPB	tryptose phosphate broth
Tris	tris (hydroxymethyl) aminomethane
ts	temperature sensitive
TTBS	tris buffered saline containing 0.05%(v/v) Tween-20
UV	ultraviolet

CHAPTER 1: GENERAL INTRODUCTION

1. RETICULOENDOTHELIOSIS VIRUS, STRAIN T

Classification

The discovery of a great number of different retroviruses in many different species led to the development of the classification system outlined by Fenner (1975). The family Retroviridae includes all RNA viruses that are associated with reverse transcriptase. Based on the types of disease that they cause Retroviridae is further divided into the subfamilies Oncovirinae, Lentivirinae and Spumavirinae. The RNA tumour viruses or Oncovirinae are classified into genera based on their morphology. Type B oncoviruses have electron-dense cores which are located eccentrically in the particle. Precursor particles (type A) are observed in the cytoplasm of infected cells. Type C oncoviruses include most of the currently studied RNA tumour viruses and are characterized by centrally located electron-lucent cores. In addition, particles are not observed in the cytoplasm of infected cells. The type C oncoviruses are further divided into the mammalian type C subgenus and the avian type C subgenus. The avian type C subgenus is composed of two species: the avian leukosis-sarcoma viruses (ALSV) and the avian reticuloendotheliosis viruses. The avian reticuloendotheliosis viruses consist of four strains: chick syncytial virus (CSV), duck infectious anemia virus (DIAV), duck spleen necrosis virus (SNV) and reticuloendotheliosis virus, strain T (REV). REV is the prototype of the reticuloendotheliosis viruses.

Origin

REV is both a generic term for a group of highly related horizontally transmitted viruses of avian species and the type name for the single acute-acting oncogenic member of the group (Purchase and Witter 1975). REV was isolated from a turkey with gross lesions similar to those of lymphoid leukosis in 1958 (Robinson and Twiehaus 1974). We now recognize that REV preparations contain a non-transforming helper virus, designated REV-A, as well as a replication-defective transforming virus, referred to as REV-T, which contains the oncogene designated *rel* (Hoezler *et al.* 1979).

The REV group has been shown to be distinct from the ALSV group of viruses in several criteria. First, morphological studies on REV revealed that the structure of mature particles resembled the mammalian leukemia-sarcoma viruses more than the ALSVs (Kang *et al.* 1975). Second, serological comparison of reverse transcriptase of these two groups detected only very weak cross reactivity (Mizutani and Temin 1973, Mizutani and Temin 1974). Furthermore, the divalent cation preference of REV and mammalian retrovirus reverse transcriptase was for manganese in contrast to the requirement for magnesium by the ALSV (Kang 1975, Waite and Allen 1975). Third, significant cross hybridization between the two viruses was not observed (Kang and Temin 1973). In addition, unlike many of the ALSVs, REV sequences were largely absent from normal uninfected avian DNA (Kang and Temin 1974). These experiments taken together have led to the suggestion that the REV group may have originated from mammalian type C retroviruses (Kang *et al.* 1975, Waite and Allen 1975). Subsequently, in support of this suggestion, amino acid and nucleic acid

sequence homology were noted between REV and MMC-1, an endogenous type C virus of the rhesus monkey (Oroszlan *et al.* 1981, Rice *et al.* 1981).

Pathogenicity

REV induces a rapidly fatal lymphoma in young birds characterized by the appearance of neoplastic lesions in a wide variety of organs including liver, spleen, pancreas, kidney, thymus, and bursa of Fabricius (Purchase and Witter 1975). The target cell for transformation has been sought by analysis of hematopoietic cells transformed *in vitro* and not producing virus (NP cells), using a variety of immunological, functional, and biochemical markers (Hoelzer *et al.* 1980, Beug *et al.* 1981, Lewis *et al.* 1981). These studies have led to the conclusion that the target for REV is the primitive lymphocyte, possibly committed to B-cell development. More recent studies have indicated that the *in vivo* targets for transformation may be influenced by the pathogenicity of the helper virus, REV-A (Barth and Humphries 1988a, 1988b). The immunosuppression and bursal atrophy induced by infection with REV-A may eliminate more differentiated B-cell targets (Barth and Humphries 1988a). The use of the related chick syncytial virus (CSV), which does not demonstrate the above noted pathogenicity, to coinfect with REV-T resulted in the isolation of transformed cells which express IgM (Barth and Humphries 1988b). *In vitro* transformed cells were also shown to have undergone different stages of B-cell development (Chen *et al.* 1986). Some clones had the germ line configuration of immunoglobulin genes representing a very early class of lymphoid cells, whereas others

had notable immunoglobulin gene rearrangements. Assuming that such rearrangements had occurred prior to immortalization by v-*rel*, the potential target cells for transformation by REV-T may extend over several stages of early B-cell development.

Genome organization

As a genome, each virus particle of REV contains two copies of a positive sense RNA molecule annealed at their 5' ends (Halpern *et al.* 1973, Bender *et al.* 1978). The presence of two copies of genomic RNA is typical for retroviruses and appears to be of functional importance for proviral DNA synthesis (Panganiban and Fiore 1988).

In vitro transformation of quail embryo fibroblasts (QEF) by REV follows two hit kinetics (Hoelzer *et al.* 1979). This indicated that REV contained two viral particles: an oncogenic virus (REV-T), defective for replication, and a competent helper virus (REV-A). Subsequent analysis of the genomic RNAs of REV confirmed the presence of two RNA species of different sizes (Breitman *et al.* 1980, Gonda *et al.* 1980, Hoelzer *et al.* 1980). The genomic RNA of REV-A contained a single species which corresponded in size to the larger species of REV RNA. Characterization of these RNAs revealed that REV-T contained sequences not represented in the REV-A genome. Analysis of these sequences by RNase T1 mapping (Breitman *et al.* 1980) and cDNA hybridization (Wong and Lai 1981) indicated that such sequences were different from any of the other known transformation specific sequences. These unique sequences of REV-T, termed *rel*, hybridized with avian DNA (Chen *et al.* 1981, Lewis *et al.* 1981, Wong and Lai 1981).

The REV-T genome has been mapped in comparison with the REV-A genome by ordered oligonucleotide fingerprinting (Cohen *et al.* 1981), heteroduplex mapping (Hu *et al.* 1981) and restriction enzyme analysis of proviral DNA clones (Chen *et al.* 1981, Rice *et al.* 1982). Based on restriction analysis of proviral DNA, the REV-A and REV-T genomes were estimated to be 8.3 - 8.6 kilobases (kb) and 5.5 - 5.8 kb in length, respectively. Comparisons of the proviral DNA of REV-A with REV-T indicated that REV-T has a large deletion of approximately 3 kb predominantly from the *pol* region and has a substitution of 0.8 - 1.5 kb in the *env* gene. The REV-A and REV-T genome organizations presented by Chen and Temin (1982) are shown in Figure 1.

2. THE ONCOGENES OF RNA TUMOR VIRUSES

Historical perspective

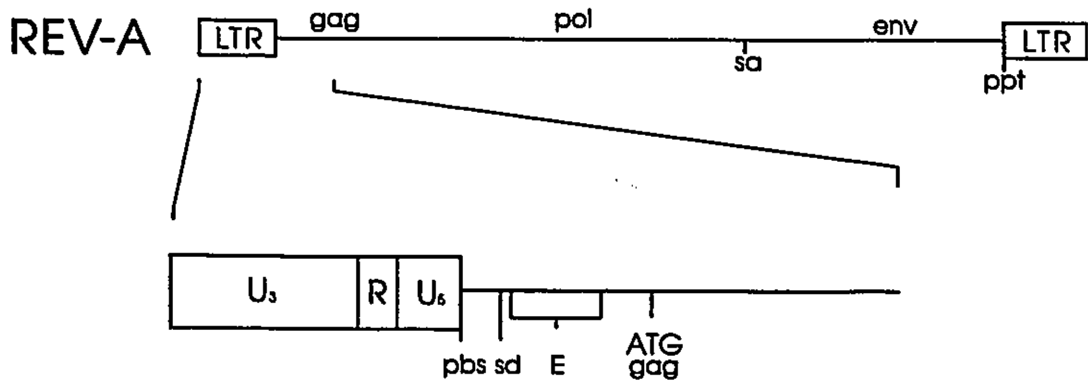
Since early in this century, retroviruses have been known to cause cancer in animals. Lymphoid tumors in chickens were among the first diseases to be recognized as having a viral etiology; shortly thereafter, virus-induced sarcoma in fowl was discovered (Rous 1910). But these tumors were regarded as somewhat of an oddity, having doubtful relevance for our understanding of cancer in higher animals, let alone cancer in humans. Only when mammary tumors, leukemias, and sarcomas in mice were traced to virus infections, the idea of virus-induced tumors gained some measure of acceptance and respectability as a worthwhile and relevant subject matter for cancer research.

The single most important development that opened the field of tumor virology

Figure 1. Genome organization of REV-T and REV-A. The deletion of *gag-pol* sequences in REV-T is indicated by the dashed line. Cell-derived sequences, *rel*, represented by a thick line replace *env* sequences present in REV-A. The location of viral genes, *gag*, *pol* and *env*, the viral long terminal repeats (LTR), the *env* splice acceptor site (sa) and polypurine tract (ppt) are indicated on the genomic map of REV-A. The unique regions of the LTRs (U_3 and U_5), the repeat region(R), the primer binding site (pbs), the *env* splice donor site (sd), the encapsidational signal(E) and the initiation codon for the *gag* gene are illustrated on the enlarged map of the 5' end of REV-A.

REV-T

REV-A



to quantitative experimentation came from advances in cell culture. They made it possible to induce oncogenic transformation of individual cells inoculated with cell-free virus. These *in vitro* transformation assays, first established for Rous sarcoma virus (Temin and Rubin 1958), provided the basis for cellular and molecular studies that distinguished RNA from DNA tumor viruses and set RNA tumor viruses apart as a unique group of microbes (Varmus 1988). The salient features of an RNA tumor virus life cycle are (a) reverse transcription of the single-strand RNA viral genome into double-strand DNA, (b) integration of this DNA into the host chromosome, and (c) expression of the integrated provirus under the control of viral transcriptional regulator sequences. Of particular importance for oncogenesis are two additional features of the retroviral growth cycle that are direct consequences of integration into the host genome: (i) the ability to acquire and transduce cellular genetic material and (ii) the insertional activation (and occasional inactivation) of cellular genes by the integrated provirus. Today, retroviruses are recognized as significant natural carcinogens in several animal species: fowl, mice, cats, cattle, and monkeys. The analysis of retrovirus-induced transformation in cell culture and in animals has led to the discovery of a set of cellular genes, called *oncogenes,* which are called viral oncogenes when they are transduced into the viral genome but their cellular counterparts are called proto-oncogenes, that function as effectors of viral carcinogenesis and play key roles in the control of cell growth and differentiation (Bishop 1987). Retroviruses, through their interaction with oncogenes, have provided independent and often exclusive access to these important regulatory elements of the cell.

<u>Mechanisms of cell transformation by retroviruses</u>

Retroviruses induce tumors and transform cells through two distinct mechanisms. Most retroviruses affect oncogenesis through the action of cellular oncogenes. This majority comprises two well-defined groups: (i) retroviruses that carry a cellular oncogene within their genomes, called transducing retroviruses, and (ii) retroviruses that lack cellular information but transform by integrating in the vicinity of a cellular oncogene, *cis*-activating retroviruses. Almost all of the transducing retroviruses have lost some viral coding information in exchange for cellular sequences. Consequently, they are defective with respect to the production of progeny and depend on a closely related helper virus for reproduction. They are also efficient carcinogens that transform cells in culture and cause tumors within short latent periods, often in a matter of days. All of the *cis*-activating retroviruses retain the full complement of viral genes and multiply efficiently in solitary infection. Characteristically, they induce tumors more slowly, within weeks or months. In cell culture, these *cis*-activating retroviruses fail to cause oncogenic transformation. Although the establishment of infection by transducing and by *cis*-activating retroviruses is dependent on viral coding sequences, such viral coding information does not play an essential and direct role in the processes of oncogenesis induced by these viruses. Transducing retroviruses often have none of their basic coding domain intact, and in *cis*-activating retroviruses these also become expendable once the provirus intergrates near a cellular oncogene.

Functions of oncogenes

Oncogenes found in retroviral genomes are derived from the cell. Most cellular proto-oncogenes are conserved over long evolutionary distances. For instance, the *ras* oncogene and parts of the *jun* oncogene are found in eukaryotes ranging from yeast to man. This durability of proto-oncogene sequences suggests that they are indispensable for a broad spectrum of life forms, fulfilling some fundamental functions that permit little change. All oncogenes work through protein products, referred to as *oncoproteins*. There are no cell-derived oncogenes that consist only of noncoding, regulatory sequences. Oncoproteins must, by definition, harbor the potential for inducing oncogenic cellular transformation.

To understand the molecular basis for oncogenesis, it is necessary to know the functions of proto-oncogene products. There are three principal approaches toward discovering the function of a proto-oncogene. First, with the nucleotide and amino acid sequence in hand, one can search for a revealing homology to a cellular gene of known function. Second, one can study the expression of the proto-oncogene in the hope that a characteristic developmental or tissue-specific pattern emerges, which might provide a clue as to function. Third, it is possible to look for specific biochemical properties of the oncoprotein such as (a) an enzymatic activity, (b) binding of a hormone, growth factor, or low-molecular-weight ligand, or (c) sequence-specific affinity for nucleic acid.

These approaches have been applied with varying degrees of success. Landmark discoveries have identified several oncogenes with known cellular genes and have

determined the normal functions of these genes. Over the past 10 years, since the function of the *src* gene product was elucidated, it has become clear that proto-oncogene products are elements of a cellular signalling network whose identities range from external ligands and growth factors, through cytoplasmic kinases and GTP-binding proteins, to nuclear transcription factors (Varmus 1988). These general ideas can best be illustrated by considering a typical mitotic signal chain. The signal originates with an extracellular substance (i.e., a growth factor, hormone, or low-molecular-weight ligand) that binds to a specific cell-surface receptor, inducing an allosteric activating change in the receptor. Many receptors are integral membrane proteins with an intracytoplasmic protein kinase domain that is activated by ligand binding. The signal is then propagated through protein phosphorylation; with some receptors the signal is channeled through a specific GTP-binding transducer protein. It may be amplified by the generation of second messengers. Often, this process is linked to increased phospholipid turnover. Second messengers activate cytoplasmic effectors, commonly protein kinases. By mechanisms as yet unknown, the signal reaches the nucleus, where it affects the function and activity of proteins that control DNA transcription and replication. Table 1 contains a list of the principal retroviral oncogenes, arranged according to function and Figure 2 is a schematic summary of this information.

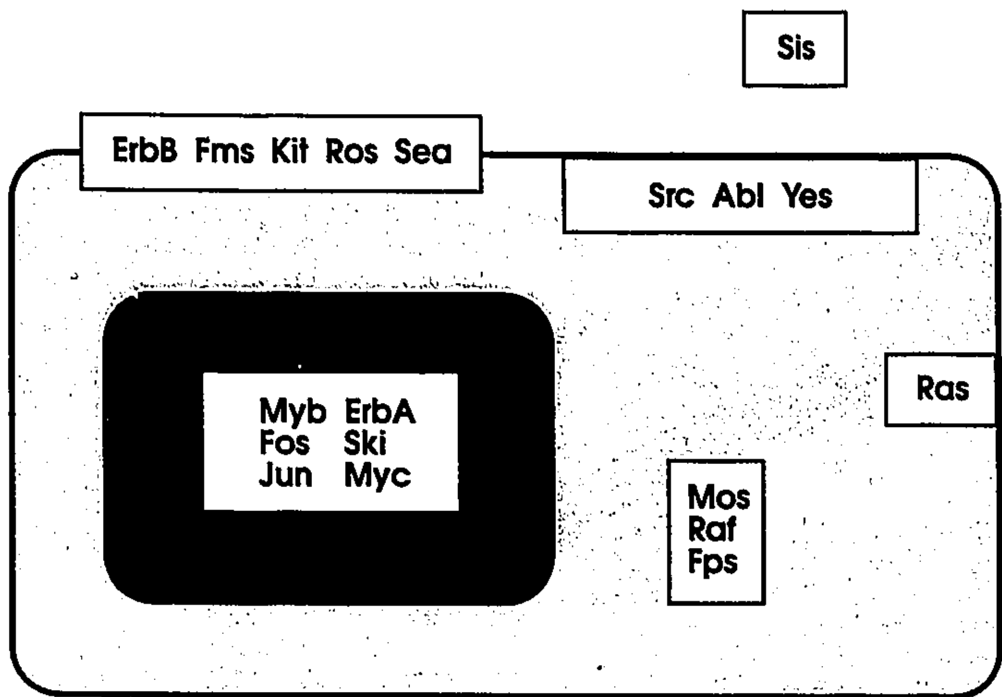
To provide some logic to the classification, I have started from the outside of the cell and worked toward the nucleus. The oncogene *sis* is derived from the gene for platelet-derived growth factor (Waterfield *et al.* 1983). The *int*-2 oncogene, which has

TABLE 1

Functional groups of retroviral oncogenes

Oncogene	Retrovirus	Function of cellular homologue
sis	Simian sarcoma virus (SSV)	PDGF
*erb*B	Avian erythroblastosis virus (AEV)-ES4, AEV-R, AEV-H	EGF receptor
fms	McDonough feline sarcoma virus (FeSV)	CSF-1 receptor
sea	S13 avian erythroblastosis virus	Cell surface receptor; ligand unknown
kit	Hardy-Zuckerman-4 FeSV	Hematopoietic receptor; product of the mouse W locus
ros	UR2 avian sarcoma virus (ASV)	Cell surface receptor; ligand unknown
*erb*A	AEV-ES4, AEV-R	Thyroid hormone receptor
H-*ras*	Harvey murine sarcoma virus (MSV)	GTP binding protein
src	Rous sarcoma virus (RSV)	Tyrosine-specific protein kinase
abl	Abelson murine leukemia virus (MuLV)	Tyrosine-specific protein kinase
fps	Fujinami ASV, PRC II ASV	Tyrosine-specific protein kinase
fes	Snyder-Theilen FeSV Gardner-Arnstein FeSV	Tyrosine-specific protein kinase
fgr	Gardner-Rasheed FeSV	Tyrosine-specific protein kinase
yes	Y73 ASV, Esh ASV	Tyrosine-specific protein kinase
mos	Moloney MSV	Cytostatic factor
raf	3611-MSV	Serine-threonine-specific kinase
mil	MH2 avian myelocytoma virus	
myc	MC29 avian myelocytoma virus	Nuclear proteins
	CM II avian myelocytoma virus	
	OK10 avian leukemia virus	
	MH2 avian myelocytoma virus	
myb	Avian myeloblastosis virus (AMV) BAI/A, AMV-E26	Transcription factor
jun	ASV17	AP-1 transcription factor
ski	SKV ASV	Nuclear proteins
fos	Finkel-Biskis-Jenkins MSV	Transcription factor
rel	Avian reticuloendotheliosis virus	Transcription factor (?)

Figure 2. Oncoprotein function as components of mitogenic signalling chains. The figure groups important examples into six categories: growth factor (*sis*); integral plasma membrane proteins with the structure of growth factor receptors that are also tyrosine kinases (*erb*B, *fms*, *kit*, *ros*, *sea*); membrane-bound tyrosine kinase (*src*, *abl*, *yes*); transducer G protein (*ras*); cytosolic protein kinases (*mos*, *raf*, *fps*); and nuclear proteins that include transcription factors (*jun*, *fos*, *myb*) and hormone receptors (*erb*A) (adapted from Benjamin and Vogt 1991).


Sis
ErbB Fms Kit Ros Sea
Src Abl Yes
Myb ErbA
Fos Ski
Jun Myc
Ras
Mos
Raf
Fps

not been found in a retrovirus genome but which can be *cis*-activated by mouse mammary tumor virus, codes for a protein related to the fibroblast growth factor (Dickson and Peters 1987). The second class of oncogenes encode the cell surface growth factor receptors. Many of these are derived from receptor protein-tyrosine kinase genes, which encode either receptors for known growth factors (e.g., *erb*B and the epidermal growth factor receptor; *fms* and the colony-stimulating factor 1 receptor) (Downward *et al.* 1984, Sherr *et al.* 1985) or receptor-like proteins with unknown ligands (e.g., *ros*, *sea*). The third class of oncogenes encode proteins associated with the inner face of the cytoplasmic membrane. These oncoproteins are of two main types: tyrosine kinase in the *src* family (e.g., *src*, *abl*, *fps*) and proteins in the *ras* family (e.g., H-*ras*, K-*ras*). There is evidence that *ras* family proteins, which are GTP-binding GTPases, are signal transducers for cell surface growth factor receptors (Shih *et al.* 1988). The *src* family proteins may have a role in the amplification stage of growth signals (Courtneidge and Herber 1987). The fourth class of oncoproteins are cytoplasmic serine/threonine kinases. Both *mos* and *raf* protein-serine/threonine kinases appear to occupy a relatively proximal position in mitotic signaling (Smith *et al.* 1986). The final class of oncogenes encode nuclear proteins. Among the nuclear oncoproteins, *jun* has been identified as a major component of the transcription factor AP-1; the *fos* and *myb* proteins also function as transcription factors (Angel *et al.* 1987, Bohmann *et al.* 1987).

The transforming potential of oncogenes

Proto-oncogenes expressed in the appropriate cell type under normal cellular control are not oncogenic. What are the conditions that activate the latent oncogenic potential of these genes? A comparison between activated oncogenes and their cellular counterparts often reveals structural and functional changes. The definition of relevant structural changes is straightforward, whereas that of the important functional changes is more difficult. Transduced oncogenes are usually truncated at one or both ends (Bishop 1987). The remaining coding sequences carry point mutations and sometimes carry deletions. They are often fused with viral sequences that are themselves truncated. Commonly, the viral sequences constitute the 5' end of the chimeric transcription unit, providing splice acceptor site and initiation codon. If a proto-oncogene inserted into a retroviral vector and expressed at levels comparable to the viral gene fails to transform cells, then specific structural changes in the viral version must be responsible for eliciting the transforming potential. An example of an oncogene that is activated by structural changes is *src*. The cellular *src* gene, overexpressed in a retrovirus vector, does not induce oncogenic transformation (Parker *et al.* 1984). Specific changes in c-*src* are necessary to reveal its oncogenic trait. The activating structural changes of cellular oncogenes can be roughly defined by studying the properties of reciprocal recombinants between the viral and the cellular genes. Greater resolution is achieved by site-directed mutagenesis and nucleotide sequencing. This approach has defined oncogenically relevant structural changes for a number of oncogenes (e.g., *src*, *ras*, and *fms*). The structural changes cause functional changes.

The role of these functional changes in oncogenesis remains the central open question for the understanding of many oncogenes. Where such functional changes have been studied, they have turned out to be quantitative (e.g., the increase or decrease of a catalytic activity). Qualitative changes have not been documented, but they conceivably exist (e.g., changes in the substrate specificity of an oncoprotein).

3. THE REL ONCOGENE

v-*rel*

Nucleotide sequencing of v-*rel* and adjoining regions has defined the structure of the predicted protein product of v-*rel* (Stephens *et al.* 1983, Wilhelmsen *et al.* 1984). There is a single long open reading frame throughout the approximate 1.5 kb of v-*rel* and there are in-frame ATG and TGA triplets slightly up and downstream, respectively. All ATG triplets even further upstream are closely followed by termination triplets. Inspection of the REV-A *env* sequence revealed that the deduced initiator for the v-*rel* protein is also the *env* initiator, and that the v-*rel* protein begins with the 11 amino-terminal amino acids of the envelope polyprotein signal sequence. Termination of the v-*rel* protein also occurs within REV-A-derived envelope sequences, but the 19 amino acids are translated out-of-frame with respect to *env*.

The complete amino-acid sequence of the predicted v-*rel* protein is shown in Figure 3. The protein is expected to contain 503 amino acids (Stephens *et al.* 1983, Wilhelmsen *et al.* 1984). About 10% of the residues are acidic and 13% are basic, which are values close to those of the average protein. Hydrophobic amino acids are rather

Figure 3. Comparison of the predicted amino acid sequences of the chicken and turkey c-*rel* proteins and the turkey-derived v-*rel* protein. The predicted amino acid sequence of turkey c-*rel* protein (middle line) is given in single-letter code, and the dashed lines above and below indicate that identical residues are present in the chicken c-*rel* protein (top line) and turkey-derived v-*rel* protein (bottom line), respectively. Symbols: x, deletions of one and three amino acids that present in the v-*rel* protein; ¶, C terminus. The *env*-derived amino acids at the N and C termini of the v-*rel* protein are underlined.

```
         MAG----C---------------------------------------------
         MAGISEPYIEIFEQPRQRGMRFRYKCEGRSAGSIPGEHSYDNNKTFPSIQILN 50
MDFLTNLRFTEG----------------T---------------------------------

         --------------------L-----------------------------
         YFGKVKIRTTLVTKNEPYKPHPHDLVGKDCRDGYYEAEFGPERRVLSFQN  100
         ----------------------------G--------------E------

         --------------------------------------------------
         LGIQCVKKKDLKESISLRISKKINPFNVPEEQLHNIDEYDLNVVRLCFQA  150
         --------------------------------------------------

         --------------------------------------------------
         FLPDEHGNYTLALPPLISMPIYDNRAPNYAELRICRVNFNCGSVKGGDEI  200
         --------------------------------------------------

         --------------------------------------------------
         FLLCDKVQKDDIENRFVLDNWEAKGSFSQADVHRQVARVFRTPPFLRDIT  250
         --------------------------------------------------

         -----------------------------K-P------------------
         EPITVKMQARRPSDQEVSEPMDFRYLPDEEDSYGNKAKRQRSTLAWQKLI  300
         -------------A-----------------PS---------------P-

         -------------T-------------------------------T----
         QDCGSAVTERPKAAPIPTVNPEGKLIKKEPNMFSPTLMLPGLGTLASSSQ  350
         -------------------------x-------------------x----

         ---P-----H------P------P------F-S-----------P-----
         MYPACSQMPTQPAQLGLGKQDTLHSCWQQLYSPSPSASSLLSMHSHNSFT  400
         ------------------------------------------L---S---

         ------N---------F--------------------S-N-----M----
         AEVPQPGAQGSSSLPAYHDNPLNWPDEKDSSFYRNFGNTHGMGAALVSAA  450
         ----------------xxx---------N---------------------

         ----A--N---HA-----------V-------------------V-----
         DMQSVSSSSIVQGTHQASATAASIMNMETNDMNCTSLNFEKYTQMLNVSN  500
         G-------------------T----T-PRTPGQVPFLRQQVGYRS¶

         ----------A-----------------L------N-------I------
         HRQQLHQAPATCPPVAAPGSTPFSSQPNVADTAVYSSFLDQDVLSDSRLS  550

         ---------------------------------------------¶
         TNPLQNHQNSLTLTDNQFYDTDGVHTDELYQSFQLDTNILQSYNH¶  595
```

evenly distributed throughout the molecule and there are no extended hydrophobic regions characteristic of membrane attachment sites. The protein is rich in proline and serine. The total proline content is predicted to be 8.7%, more than 60% higher than average. Most of these prolines are found in the 179-residue segment between amino-acid position 255 and 433, where the proline content is 14.5%. Total serine content is 9.3%, about 30% higher than average, and these residues are even more highly clustered than are the prolines. About 75% of the 47 serine residues are in the carboxy-terminal half of the molecule and a 97-residue segment between amino acid 385 and 481 contains 23 serines: a serine content of 24%.

c-*rel*

The cellular counterpart to the v-*rel* oncogene of REV-T, proto-oncogene c-*rel*, has been cloned from chickens (Chen *et al.* 1983) and turkeys (Wilhelmsen *et al.* 1984, Wilhelmsen and Temin 1984). Turkey c-*rel* contains at least nine exons which are separated by introns ranging in size from 0.1 to 10.8 kb. The cellular gene thus covers at least 24 kb of chromosomal DNA.

Nucleotide sequencing of these genomic clones and chicken c-*rel* cDNA clones (Hannink and Temin 1989, Capobianco *et al.* 1990) has revealed that there are multiple nucleotide differences between the v-*rel* and c-*rel* genes, resulting in 14 single-amino acid differences and three small deletions of one or three amino acids in the v-*rel* protein in comparison with the product of the c-*rel* gene (Wilhelmsen *et al.* 1984) as shown in Figure 3. The recombination with *env* sequences during the formation of v-*rel*

occurred at amino acid 478, with the result that the v-*rel* protein lacks 118 residues at its C terminus that are present in both the turkey and chicken c-*rel* proteins. At the N terminus, the v-*rel* protein lacks 3 amino acids that are present in the chicken and turkey c-*rel* proteins (Capobianco *et al.* 1990).

The turkey and chicken c-*rel* proteins are more than 95% identical, differing in only 28 of 595 residues. In particular, the N-terminal regions of the two proteins are highly conserved, with differences at only 4 of the first 300 amino acid positions. The C-terminal regions of the two c-*rel* proteins are more divergent, with 24 of 295 residues differing between the chicken and turkey c-*rel* proteins (Hannink and Temin 1989). The c-*rel* genes of mouse (Grumont and Gerondakis 1989) and human (Brownell *et al.* 1989) also have been cloned, and it was found that both genes show almost 80% amino acid homology with v-*rel* in their N termini.

<u>Homology with other genes</u>

Although it was shown that the *rel* oncogene is essentially unrelated to any of the other oncogenes, Steward (1987) has revealed striking homology between *rel* and *dorsal*, an embryonic polarity gene in *Drosophila*. Sequence analysis of *dorsal* cDNAs predicts a 677 amino-acid *dorsal* protein of molecular weight 75.6 kilodaltons (kDa). Beginning at *dorsal* residue 42 (v-*rel* residue 16) and extending about 295 amino acids, the sequence shows 46% identities with either the v-*rel* or turkey c-*rel* proteins. Allowing for conservative amino-acid changes, the homology increases to close to 80%. Carboxy-terminal to this extensive homologous region the two proteins appear unrelated. The

function of *dorsal* is not known, though it is one of 11 maternal effect genes required for the establishment of normal dorsal-ventral polarity in the embryo. Steward (1987) suggests that the products of these genes may constitute an activation cascade resulting in the asymmetric distribution of a morphogen, possibly the *dorsal* protein itself. The morphogen would then activate zygotic genes which establish polarity.

Recently the cloning of cDNAs encoding the DNA binding subunits of transcription factor NF-κB (Bours *et al.* 1990, Ghosh *et al.* 1990, Kieran *et al.* 1990, Meyer *et al.* 1991, Nolan *et al.* 1991 and Ruben *et al.* 1991) revealed that the DNA binding subunits of NF-κB show remarkable homology for over 300 amino acids at the N-terminal end to the v-*rel*, c-*rel*, and *dorsal* proteins. The sequences show up to 65% homology in N termini, but C-terminal halves of these proteins are highly divergent (Fig. 4). While NF-κB was originally described as a factor that bound to a short DNA sequence (κB site) important for the activity of the enhancer of the κ light chain immunoglobulin gene (Sen and Baltimore 1986), it is now clear that NF-κB has a more general role in transcription. NF-κB binding sites have been found in a number of lymphoid-specific, nonlymphoid, and viral promoters and enhancers, including the long terminal repeat of HIV-1 (Lenardo and Baltimore 1989) (Table 2). The active nuclear form of NF-κB is composed of 50- (p50) and 65-kDa (p65) proteins (Kawakami *et al.* 1988, Baeuerle and Baltimore 1989). The p50 subunit of NF-κB is known to be processed from the precursor molecule, p105 (Bours *et al.* 1990 and Ruben *et al.* 1991). The NF-κB complexes are maintained in an inactive state in the cytoplasm through their association with either the inhibitor protein of ~37 kDa termed IκB (Baeuerle

Figure 4. Sequence homologies among *rel* gene family. v-*rel* is a truncated form of c-*rel* that lacks the C-terminal region and has about 4% point substitution. The *rel* proteins are related to the transcription factor NF-κB and to the morphogen *dorsal*. These *rel* gene family members share extensive sequence homology in their N-terminal 320 amino acids as illustrated in a dotted box, but show no sequence similarity in C-terminal region. The size of each gene product is represented by the total number of amino acids. In case of p50, the sizes of precursor and processed forms are shown. The shaded boxes in p50 represent the ankyrin repeats. **P** and N represent the possible phosphorylation site and the nuclear localizing sequence respectively.

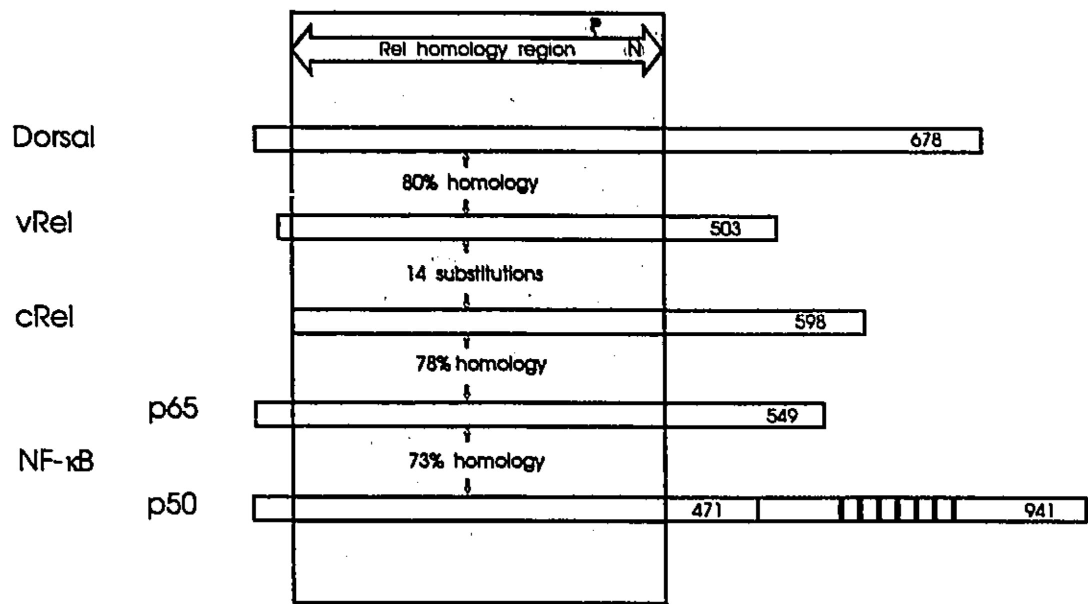

Rel homology region
N
P
Dorsal
678
80% homology
vRel
503
14 substitutions
cRel
598
78% homology
p65
549
NF-κB
73% homology
p50
471
941

TABLE 2

Sequences recognized by NF-κB

Gene	Sequence
Mouse Ig κ enhancer HIV-1(-91) CMV(4)	(G)GGGACTTTCC
HIV-1(-109) HIV-2 CMV(1) β_2 microglobulin Serum Amyloid A	(A)GGGACTTTCC
Human Ig κ enhancer CMV(3)	GGGGATTTCC
ß-IFN(PRD II)	GGGAAATTCC
CMV(2)	GGGACTTTCC
MHC class II	GGGACTTCCC
IL-2	GGGATTTCAC
Mouse IL-2R	GGGGATTCCT
Human IL-2R	GGGAATCTCC
MHC class I HLA	GGGATTCCCC
Consensus	C GGGRATYYCC T

and Baltimore 1988) or the precursor p105 (Blank *et al.* 1991, Liou *et al.* 1992, Rice *et al.* 1992). Activation causes either the phosphorylation of IκB or processing of p105, and this liberates the active NF-κB complexes (Ghosh and Baltimore 1990). Free NF-κB complex migrates to the nucleus, where it binds to the κB sites in promoters or enhancers of target genes (Fig. 5).

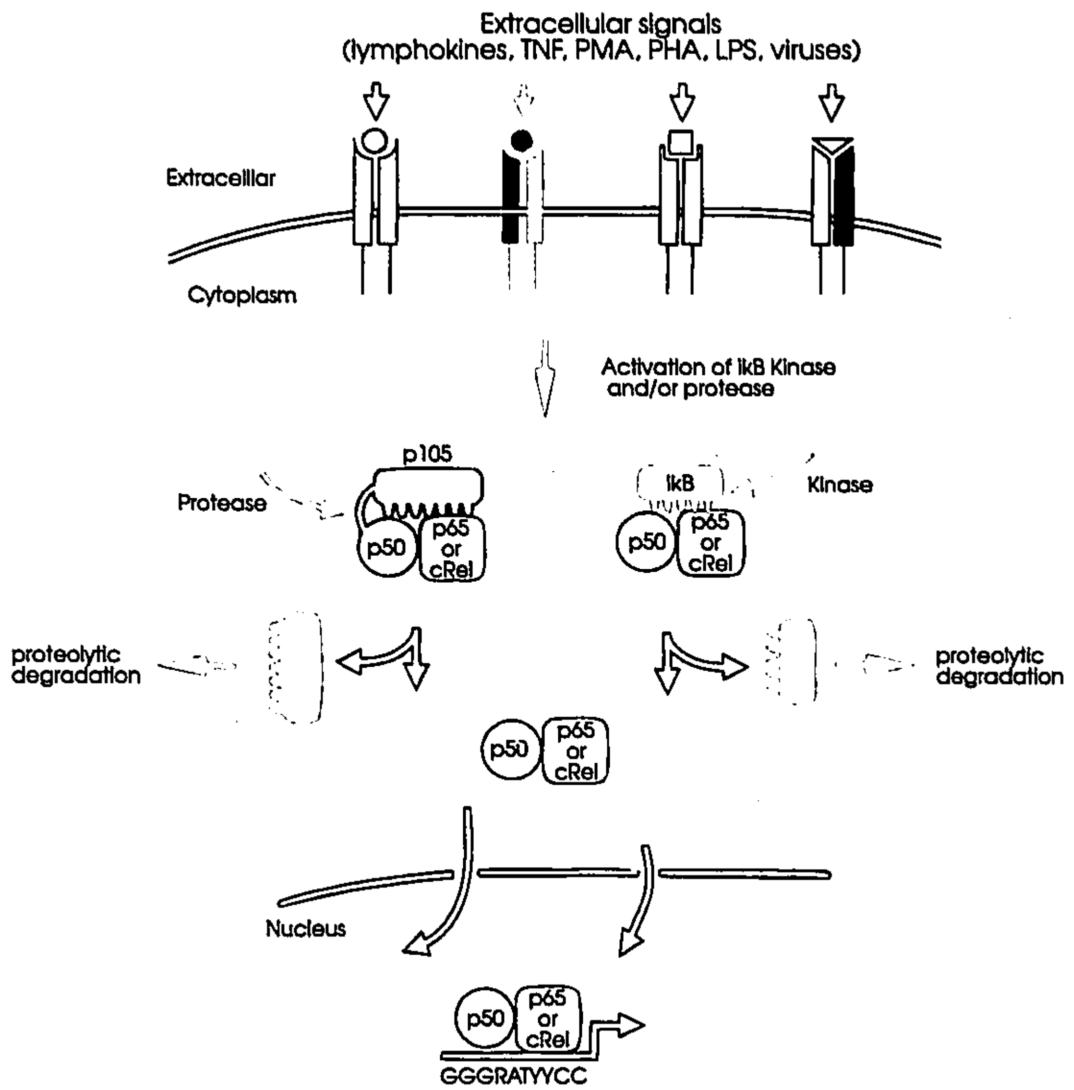
4. THE REL GENE PRODUCT

Detection and characterization of the v-*rel* and c-*rel* proteins

Two different strategies were employed for the detection of the v-*rel* protein. In the first, antisera were raised against synthetic peptides whose sequences were predicted by the v-*rel* sequence (Garson and Kang 1986, Rice *et al.* 1986). In the second, antisera were raised to segments of the v-*rel* gene expressed in bacteria (Gilmore and Temin 1986, Herzog and Bose 1986, Simek *et al.* 1986, Warlo *et al.* 1987). In each case, these antisera precipitated a protein of 55-59 kDa from lysates of REV-T-transformed cells. The v-*rel* protein was not found in uninfected cells and was not precipitated from infected cells with pre-immune sera. The v-*rel* protein has been shown to be phosphorylated mainly on serine residues (Gilmore and Temin 1986, Rice *et al.* 1986, Warlo *et al.* 1987), but no other modifications have yet been detected.

The *rel*-specific antisera precipitated a 68-kDa protein from the lysates of MSB-1 cells (Marek's-disease-transformed chicken lymphoid cell line) which were shown to express relatively high levels of c-*rel* mRNA (Simek and Rice 1988a). Since limited proteolysis of this protein by *Staphylococcus aureus* V8 protease yielded a number of

Figure 5. Activation of NF-κB complex. NF-κB contains subunits of 50 kDa (p50) and 65 kDa (p65). p50 is processed from the precursor p105. The cytoplasmic NF-κB complexes exist in two forms. One is a complex containing the precursor p105. The other is a complex with an IκB. These two types of complexes may respond to different stimuli. By activation of IκB kinase or protease, two types of complexes are processed to functional NF-κB, which migrates to nucleus where it binds to κB sites in promoters or enhancers of target genes (adapted from Liou and Baltimore 1993).



Extracellular signals
(lymphokines, TNF, PMA, PHA, LPS, viruses)
Extracellilar
Cytoplasm
Activation of IkB Kinase
and/or protease
p105
Protease
IkB
Kinase
p50
p65
or
cRel
proteolytic
degradation
Nucleus
GGGRATYYCC

fragments also found in the v-*rel* protein digests, it was concluded that this protein is the product of the chicken c-*rel* gene. Its size is in good agreement with the 67 kDa predicted by the DNA sequence of full-length c-*rel* cDNA (Capobianco *et al.* 1990).

Cellular localization of the v-*rel* protein

In REV-T-transformed chicken spleen cells, the v-*rel* protein is predominantly a cytosolic protein. When cells were labeled with [^{35}S]methionine or [^{32}P]orthophosphate and the lysates fractionated by differential centrifugation, the great majority of the v-*rel* protein was found by immune precipitation in the high-speed supernatant (Gilmore and Temin 1986, Simek *et al.* 1986, Warlo *et al.* 1987). This result has been confirmed by indirect immunofluorescence experiments. Staining of the transformed spleen cells occurred throughout the cytoplasm with no observable concentration in either the nuclear or plasma membrane (Gilmore and Temin 1986, Simek *et al.* 1986). Staining of nuclei in about 10% of the cells has also been observed (Gilmore and Temin 1986).

Quite a different result was obtained when REV-T-infected chick embryo fibroblasts (CEF) were examined. In these cells which are not transformed by REV-T, immunofluorescent staining was found exclusively in the nucleus (Gilmore and Temin 1986). The same result was obtained when two other chicken hematopoietic cell lines were examined. In MSB-1 cells and in chicken myeloid cells transformed by E26 virus, superinfection with REV-T resulted in nuclear localization of the v-*rel* protein (Gilmore and Temin 1986).

With the goal of localizing the sequences in the v-*rel* protein responsible for its

behavior in CEF, Gilmore and Temin (1988) tested a variety of altered v-*rel* proteins for subcellular distribution. They found that deletion of a *Stu* I - *Hinc* II fragment (171 base pairs, extending from amino acid position 275 to 332) resulted in a protein distributed approximately equally between nucleus and cytoplasm in CEF. When this same fragment was linked to a portion of the ß-galactosidase gene, it was found primarily in the nucleus in both CEF and spleen cells. Thus, the *Stu* I - *Hinc* II fragment contains a signal for nuclear localization. Within this fragment there is a sequence encoding Lys-Ala-Lys-Arg-Gln-Arg, which resembles the known nuclear localization signal in its high content of basic residues. When the internal Lys-Arg of the wild-type protein was changed by site-directed mutagenesis to Asn-Trp, the resulting protein behaved the same in CEF as the *Stu* I - *Hinc* II deletion. In addition, the corresponding ß-galactosidase fusion protein was no longer nuclear. Thus, the conclusion is that the v-*rel* protein contains a putative nuclear localization signal in this segment.

Complex formation of the v-*rel* protein with other proteins

The v-*rel* oncogene encodes a 59-kDa phosphoprotein located principally in the cytosol of REV-T-transformed lymphoid cells. The v-*rel* protein in immune precipitates from transformed cells is associated with at least five other proteins, approximately 124, 115, 68 and 40 kDa, which together form high-molecular-weight complexes (Simek and Rice 1988b, Tung *et al.* 1988, Davis *et al.* 1990b). The 124-, 115-, and 40-kDa proteins are apparently unrelated to the v-*rel* protein in sequence, and their

coprecipitation suggests that they are complexed with the v-*rel* protein. The coprecipitating 68-kDa protein was found to be the c-*rel* protein (Simek and Rice 1988b, Tung *et al.* 1988). The complex appeared to be stable under a variety of conditions, including a wide range of ionic strengths and chelators, and through multiple cycles of immune precipitation and elution (Simek and Rice 1988b, Davis *et al.* 1990b).

The 40-kDa protein is the most abundant cellular protein associated with the v-*rel* protein. Immunoprecipitations of extracts from REV-T-transformed lymphoid cells labeled with [^{32}P]orthophosphate using anti-v-*rel* antiserum demonstrated that p40 is highly phosphorylated, mainly on serine residues. Therefore, it is designated as pp40. In light of the discovery that NF-κB subunits show homology with the v-*rel* and c-*rel* proteins in their N-terminal 300 amino acids, it is intriguing that pp40 also shares some characteristics with IκB. The size of pp40 is very similar to that of IκB (36-40 kDa) and the association of pp40 with the v-*rel* protein is sensitive to detergents (Davis *et al.* 1990a), much like the association of IκB with NF-κB (Baeuerle and Baltimore 1988). Also, both proteins are located exclusively in the cytoplasm.

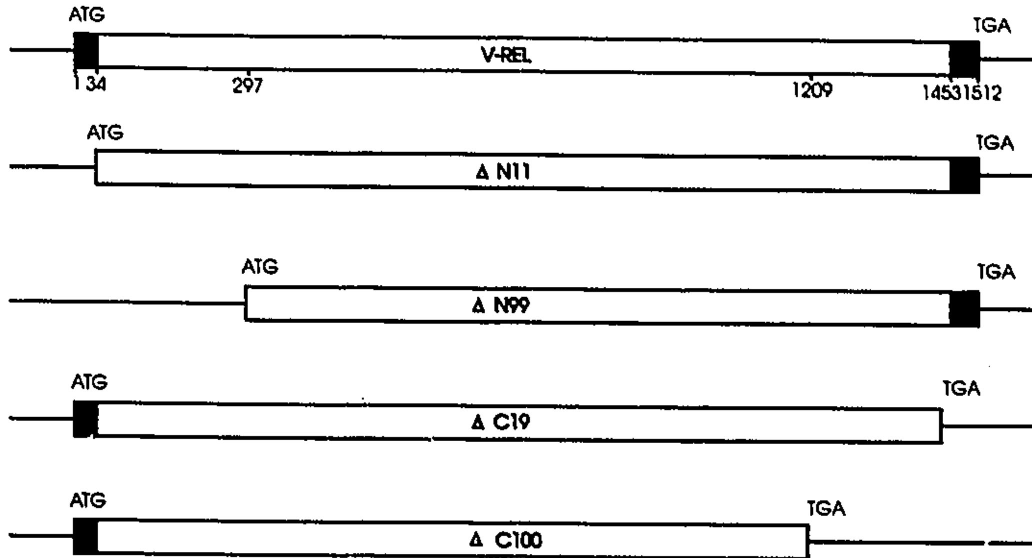
5. TRANSFORMING DOMAINS OF V-REL ONCOGENE

Several mutants of the v-*rel* oncogene have been constructed to determine which regions of v-*rel* are necessary for transforming activity. Sylla and Temin (1986) constructed chimeras between c-*rel* and v-*rel* to determine which changes contribute to the activation of this oncogene. The v-*rel*/c-*rel* chimeras with the central coding region

of v-*rel* replaced with the corresponding c-*rel* sequences, failed to transform lymphoid cells, indicating that changes in this region, at least in part, were required for the oncogenic activation of c-*rel*. Subsequently, Hannink and Temin (1989) replaced the v-*rel* C-terminal sequences with corresponding C-terminal sequences of c-*rel* and demonstrated that the transforming activity was not impaired. Thus changes in this region, including the 18 C-terminal *env*-derived amino acids of v-*rel* protein and 6 of the 14 amino-acid differences between v-*rel* and c-*rel*, were not required for the activation of c-*rel*. An analogous alteration of the N terminus of v-*rel* protein did abolish transforming activity. Thus the N-terminal residues contributed by the REV-A *env* gene and the first 20 residues of v-*rel* contribute to the oncogenicity of the v-*rel* protein.

Similar results were obtained using deletion mutants of v-*rel* (Fig. 6A). Garson *et al.* demonstrated that an N-terminal deletion of 11 *env*-derived amino acids dramatically reduced the transforming activity and a further deletion of 99 N-terminal amino acids abolished the transforming activity of v-*rel* even though C-terminal deletion of up to 100 amino acids had no effect on transforming activity (Garson *et al.* 1990) (Table 3). In addition, 12 linker insertion mutants (Fig. 6B) were constructed in order to localize the transforming domain (Garson and Kang 1990). Seven linker insertion mutants that mapped between amino acid residues 29 and 275 abolished transforming activity, but the remaining 5 mutants which contained linker insertion mutations between amino acid residues 332 and 459 retained the full-transforming activity of the wild-type v-*rel* (Table 3). The results of this analysis localized the transforming domains of the v-*rel*

Figure 6. Deletion and linker insertion mutants of v-*rel*. Deletion mutants(A) were constructed by crossover linker mutagenesis using specific double stranded oligonucleotides. Noncoding sequence is indicated by black lines. The dotted regions represent 5' and 3' *env* sequences. The nucleotide position of each deletion is marked on the map of the wild-type v-*rel*. Linker insertion mutants(B) were constructed by insertion of *Xho* I linkers, which maintained the v-*rel* reading frame, but added 4 new amino acids to the available restriction enzyme sites. The restriction enzyme site, the amino acid position and the sequence of amino acids added by linker insertion are shown (Garson and Kang 1990, Garson *et al.* 1990).


A
ATG
TGA
V-REL
1 34
297
1209
1453 1512
ATG
TGA
Δ N11
ATG
TGA
Δ N99
ATG
TGA
Δ C19
ATG
TGA
Δ C100

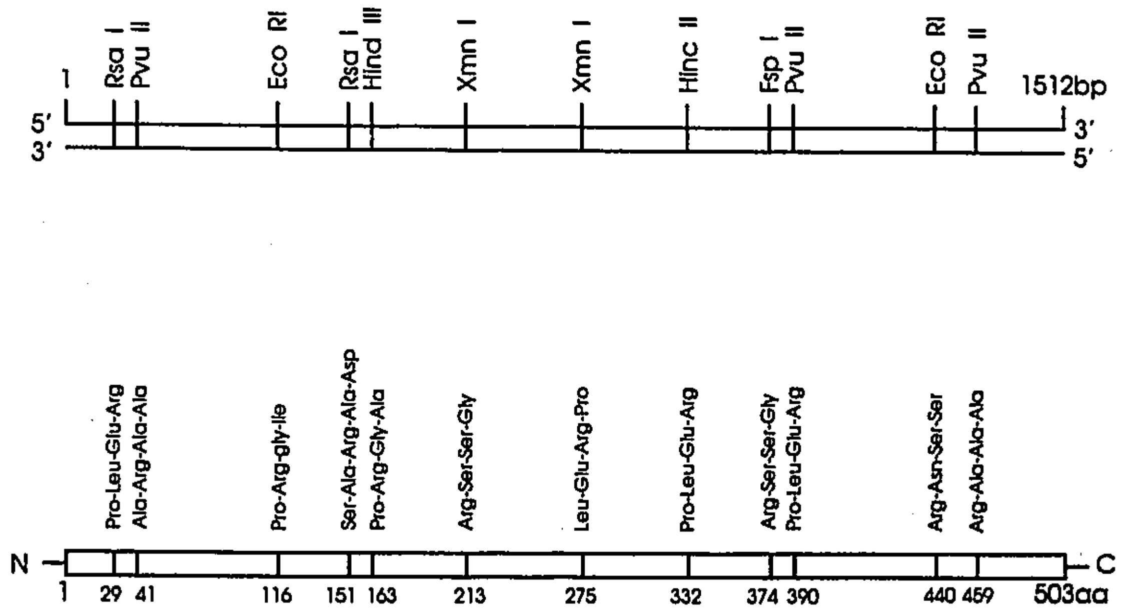

B
1
Rsa I
Pvu II
Eco RI
Rsa I
Hind III
Xmn I
Xmn I
Hinc II
Fsp I
Pvu II
Eco RI
Pvu II
1512bp
5'
3'
3'
5'
Pro-Leu-Glu-Arg
Ala-Arg-Ala-Ala
Pro-Arg-gly-Ile
Ser-Ala-Arg-Ala-Asp
Pro-Arg-Gly-Ala
Arg-Ser-Ser-Gly
Leu-Glu-Arg-Pro
Pro-Leu-Glu-Arg
Arg-Ser-Ser-Gly
Pro-Leu-Glu-Arg
Arg-Asn-Ser-Ser
Arg-Ala-Ala-Ala
N
C
1
29
41
116
151
163
213
275
332
374
390
440
459
503aa

TABLE 3

Transforming activity of v-*rel* mutants[a]

Mutant	Transforming activity[b]
REL	+++++
ΔN11	+/-[c]
ΔN99	-
ΔC19	+++++
ΔC100	+[d]
L29	-
L41	-
L116	-
L151	-
L163	-
L213	-
L275	-
L332	+++++
L374	+++++
L390	+++++
L440	+++++
L459	+++++

[a]adapted from Garson and Kang (1990), and Garson *et al.* (1990)

[b]transforming activity represents the average number of colonies obtained from at least 10 seperate transformation assays; +, transforming; -, nontransforming; +/-, abortive transformation

[c]transforming activity of ΔN11 was about 5% of wild type v-*rel*; transformed colonies were observed, but they could not be propagated

[d]transforming activity of ΔC100 was about 10% of wild type v-*rel*

oncogene to the N terminus.

6. STATEMENT OF OBJECTIVES

When I started my Ph. D. program, no definite function of the v-*rel* protein was established partly because the *rel* oncogene was not known to be related to any of the other oncogenes and the function of the related *dorsal* protein was unknown. However, cloning of NF-κB subunits revealed that the v-*rel* protein shows homology with NF-κB subunits and also suggested that the v-*rel* protein acted as a transcriptional regulator. Therefore, the overall objectives of this thesis were to investigate the functions of the v-*rel* protein as a transcriptional regulator using the deletion and linker insertion mutants of v-*rel* and to relate its function to the possible mechanism(s) of transformation. Specific objectives were;

a. To examine the DNA binding activity of the v-*rel* protein, and to determine its relationship with transforming activity
b. To examine complex formation of the v-*rel* protein with NF-κB subunits and IκB (pp40), and to determine its relationship with transforming activity
c. To investigate the transcriptional activity of the v-*rel* protein, and to determine its relationship with transforming activity

CHAPTER 2: MATERIALS AND METHODS

1. CELLS AND VIRUSES

Maintenance

All cells except insect cells (Sf9) were grown at 37°C, 5% CO_2 in a humidified Shel-lab incubator (Sheldon Manufacturing Inc.) in media supplemented with 10% heat inactivated fetal calf serum (HIFCS), penicillin-streptomycin (100 U/ml penicillin G, 100 μg/ml streptomycin sulphate) and 2 mM glutamine. Media, media supplements, and trypsin were purchased from Gibco (Gibco/BRL) unless otherwise mentioned.

Cos1 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM). Chicken spleen cells (CSC) transformed by wild-type v-*rel* or v-*rel* mutants were grown in suspension culture in RPMI 1640. Primary cultures of chicken embryo fibroblast (CEF) were prepared from 11 day old embryos from S-SPF/COFAL/MAREK'S/gs/chf-39 eggs (SPAFAS). *Spodoptera frugiperda* (Sf9) cells were maintained as monolayer cultures in TNM-FH medium (Summers and Smith 1987) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml, kanamycin 100 mg/ml), 10% HIFCS and 2 mM glutamine at 27°C.

Transfections

Cos1 cells were transfected by the DEAE-dextran method (Lévesque *et al.* 1991) with minor modification. Cos1 cells were seeded at 4 x 10^5 cells/35 mm plate 20 hrs prior to transfection. Cos1 cells were washed twice with Opti-MEM (Gibco/BRL) and

then incubated with 0.5 ml Opti-MEM containing 250 μg/ml DEAE-Dextran (Mr = 500,000, Sigma Chemical) and DNA. After 30 min incubation at room temperature, the medium was removed and replaced by 1 ml Opti-MEM containing 100 μM chloroquine diphosphate (Sigma Chemical). After 4 hr incubation at 37°C, the medium was removed and replaced by 5 ml fresh DMEM-FCS for a further 40-48 hr incubation at 37°C. The ß-galactosidase expression plasmid (pCH110) was included in every transfection at a concentration of 0.5 μg/35 mm plate in order to standardize the transfection efficiency. The total DNA concentration was maintained at 3-5 μg/35 mm plate for all experiments by adding pSVL plasmid as carrier DNA.

CEF were transfected using the polybrene method (Kawai and Nishizawa 1984). CEF were seeded at 8 x 10^5 cells/60 mm plate 18 hrs prior to transfection. Medium was removed and replaced with 1 ml of media containing 30 μg/ml polybrene (Aldrich Chemical), 5 μg/ml pREV-T or related DNA and 1 μg/ml of pREV-A. Transfected plates were incubated at 37°C for 6 hrs with occasional agitation. Transfection media were then removed and replaced with medium containing 30% (v/v) DMSO. After 4 minutes of DMSO shock, monolayers were washed and replaced with fresh medium and then incubated at 37°C for 36-48 hrs.

Sf9 cells were transfected using Lipofectin™ (Gibco/BRL) as described by Kitts and Possee (1993). Sf9 cells were seeded at 5 x 10^5 cells / 35 mm plate 2 hrs prior to transfection. One hundred nanograms of *Bsu*36 I-digested BacPAK6 viral DNA (Clontech) were mixed with 500 ng of pBacPAK1 transfer vector (Clontech) containing v-*rel*, c-*rel*, p50, p65, or pp40 gene and the volume was adjusted to 90 μl with serum-

free TNM-FH medium in a polystyrene tube. Ten microliters of Lipofectin™ (1 mg/ml) were gently mixed with the DNA solution, and the mixture was incubated at room temperature for 15-30 min. Meanwhile, the medium was removed from the cell monolayers, and the cells were washed twice with 2 ml of serum-free TNM-FH medium. Serum-free TNM-FH (1 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After 5 hr incubation at 27°C, 1 ml TNM-FH containing 10% FCS was added to each dish and the incubation at 27°C continued for 4 days.

Production and titration of REV-T and related viruses

All REV-T and related viruses were recovered from CEF following polybrene transfection of CEF. Transfected cells were fluid changed on day 5 or 6 following transfection and virus was harvested 24 hrs later. Titers of virus were determined by immunofluorescence assay of infected cells at 24-36 hr post infection.

Selection and purification of recombinant baculoviruses

Sf9 cells were plated into 35 mm tissue culture dishes in serum-free TNM-FH at a density of 1.2 x 10^6 viable cells and incubated at 27°C for 30 minutes. After medium was removed, 200 μl of the serial 10-fold dilutions of transfection supernatant were inoculated into each dish. After 1 hr incubation at 27°C, 2 ml of overlay agar (1.5% low melting agarose in TNM-FH medium) were added. When the overlay was solidified, 1 ml of medium was added and plates were incubated at 27°C for 5-6 days

in a humid environment. The plaques were visually examined by looking against a black background with a fluorescent light source and were picked and purified again by repeating the plaque assay as described above to obtain the pure recombinant viruses.

In vitro transformations

Preparation of spleen cells and *in vitro* transformations were performed essentially according to Hoelzer *et al.* (1980). Spleens were aseptically removed from 1-3 week old leghorn chicks (Animal Disease Research Institute, Agriculture Canada) and bathed in RPMI 1640, containing 1% glutamine and 2% HIFCS. A pestle was used to force spleens through the steel mesh of a tea strainer while washing with the above media. The resulting cellular suspension was passed through several layers of cheese cloth to remove aggregates. The filtrate containing spleen cells was pelleted at 400 x g for 5 minutes and cells were resuspended in the above medium at a concentration of 5 x 10^8 lymphoid cells/ml. Spleen cells (5 x 10^7) were infected with 2 ml of REV-T or REV-T mutant in the presence of 2 μg/ml polybrene for 1 hr at 37°C. After infection, spleen cells were pelleted at 400 x g for 5 minutes and were resuspended in 5 ml of overlay agar (RPMI 1640, 15% HIFCS, 1% beef embryo extract, 1% heat inactivated chicken serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate, 60 μg/ml tylocine and 0.3% noble agar) and poured onto 60 mm tissue culture plates. Transformed cells which are able to form colonies in the agar were scored 7-10 days after infection. Selected colonies were propagated in suspension culture as described above.

2. MOLECULAR CLONING AND RELATED TECHNIQUES

Maintenance of bacterial strains, general techniques for the handling and manipulation of DNA and for the preparation of necessary media and reagents are thoroughly discussed by Maniatis *et al.* (1982). These methods were used unless otherwise specified.

E. coli strains RRI, HB101, JM101 and DH5α were purchased from New England Biolabs. The following list indicates the source of all plasmids required for this work: pUC8, pUC18 and pUC19 (New England Biolabs), pSVL and pCH110 (Pharmacia), pBacPAK1 (Clontech), pBSK (Stratagene), pREV-T and chicken c-*rel* cDNA clone (kindly provided by Dr. H.M. Temin, University of Wisconsin, Madison, WI), pp40 clone (kindly provided by Dr. H.R. Bose, University of Texas, Austin, TX), pREV-A (ATCC), p50 and p65 clones (kindly provided by Dr. S.Y. Kim, Seoul National University, Seoul, Korea).

Preparation of competent *E. coli*

Bacteria were rendered competent for the uptake of plasmid DNA by treatment with $CaCl_2$. First, 10 ml of an overnight culture of bacteria grown in 2 x YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) were inoculated into 100 ml of P-medium (15.9 mM K_2HPO_4, 6.3 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1.8 μM $FeSO_4$, 1% casamino acids and 0.25% glucose) and grown to an OD_{600} of 0.4 as measured in a Beckman Du-8B spectrophotometer. Bacteria were pelleted at 6,000 x g for 10 minutes in a JA-10 rotor. After washing in 100 ml of 10 mM $CaCl_2$ at 4°C, cells were repelleted. Bacteria were

resuspended in 100 ml of 50 mM $CaCl_2$ and incubated at 4°C for 15 minutes. Finally, bacteria were pelleted and resuspended in 10 ml of 50 mM $CaCl_2$, 16% (v/v) glycerol, aliquotted and quickly frozen in a dry ice/ethanol bath before being stored at -80°C.

Transformation of *E. coli*

Transformations were performed essentially according to the method of Hanahan (1983) with several modifications. Competent *E.coli* were thawed slowly on ice. DNA in no more than 10 μl was mixed with 200 μl of competent cells in 17 mm x 100 mm polypropylene tubes and incubated at 4°C for 30 minutes. The mixture was heated in a 42°C water bath for 90 seconds and then returned to ice for several minutes. 800 μl of LB (1% bacto-tryptone, 0.5% yeast extract, 15 NaCl) broth were added and tubes were agitated (200 rpm) at 37°C for 1 hr. Cells were then spread onto LB plates containing ampicillin (50 μg/ml) and incubated overnight at 37°C.

Purification of plasmid DNA

Small scale (minipreparations) purification of plasmid DNA for screening of recombinant plasmids was done as described by Maniatis *et al.* (1982).

Large scale purification of plasmid DNA (maxipreparation) was done essentially according to the method of Maniatis *et al.* (1982) with minor modifications. Bacteria from a 500 ml culture were pelleted at 6,000 x g in a JA-10 rotor and resuspended in 7.5 ml of solution 1 (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA, 5 mg/ml lysozyme). 15 ml of solution 2 (0.2 M NaOH, 1% SDS) and finally 12 ml of 5 M

potassium acetate (pH 4.8) were added before centrifugation at 12,000 x g in a JA-20 rotor for 30 minutes. Isopropanol precipitation of DNA was done at 12,000 x g for 30 minutes in a JA-20 rotor. Samples prepared for cesium chloride gradients were precleared of precipitated proteins by centrifuging at 12,000 x g for 10 minutes prior to centrifugation to equilibrium in a VTi 65 rotor at 290,000 x g for at least 10 hrs. The band of closed circular plasmid DNA was collected and ethidium bromide and salt were removed from DNA by butanol extraction and dialysis.

Electrophoresis of DNA

DNA was separated by horizontal gel electrophoresis using 0.8% to 1.5% agarose and Tris-acetate buffer (TAE, 40 mM Tris-acetate (pH 8.0), 2 mM EDTA). Electrophoresis was usually performed with HE33 Mini submarine gel apparatus (Hoefer Scientific Instruments). Samples were mixed with 2.5 μl of 5 x loading buffer (25% glycerol, 5 x TAE, 0.06% BPB) and electrophoresed at 100 volts. The DNA was visualized under ultraviolet (UV) illumination after staining with EtBr (0.5 μg/ml in TAE). Gels were photographed using Polaroid type 667 film (Polaroid).

Purification of DNA from gels

Gels were stained briefly with ethidium bromide (0.5 μg/ml) and observed with long wave ultraviolet light to permit precise excision of DNA fragments for purification. DNA was purified from gel slices using Gene clean (BIO 101).

Manipulation and modification of DNA

All DNA modifying enzymes were purchased from Pharmacia, New England Biolabs or Boehringer Mannheim. All methods were performed essentially according to techniques described by Maniatis *et al.* (1982) or by the manufacturers.

Polymerase chain reaction (PCR)

DNA was amplified using the GeneAmp DNA Amplification Reagent Kit and DNA thermal cycler (Perkin-Elmer Cetus). The reaction mixtures consisted of 20 ng of template DNA in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin, 1.0 μM of each of the two primers, 200 μM of each of the four dNTPs and 5 units of Taq DNA polymerase (5 U/μl) in a total volume of 100 μl. Samples were overlayed with 100 μl of mineral oil and amplified using the Step-Cycle file. The initial template denaturation step was 5 minutes at 94°C and afterwards the cycle profile was 1 minute at 94°C (denaturation), 2 minutes at 45 - 50°C (annealing) and 3 minutes at 72°C (extension). At the end of the 30th cycle, the 72°C extension step was prolonged for an additional 7 minutes and samples were then cooled to 4°C. The bottom aqueous phase was removed and extracted once with chloroform/isoamyl alcohol.

All primers used for PCR (see APPENDIX) were synthesized by an Applied Biosystem 380B DNA synthesizer in the University of Ottawa Biotechnology Research Institute.

DNA sequencing

DNA sequencing by the chain-termination method (Sanger *et al.* 1977) was performed using the modified T7 DNA polymerase (Sequenase) sequencing system (United States Biochemical Corporation) with [α-^{35}S]dATP according to the manufacturer's instructions. About 5 μg of plasmid DNA (pBSK clone, pUC clone) and 30 ng of primer (M13/pUC forward sequencing primer, T3 sequencing primer, T7 sequencing primer) were used for reactions.

Sequencing reactions were electrophoresed using the IBI model STS 45 Thermoplate Sequencing Apparatus on 0.2 mm 6% polyacrylamide gels (5.7% acrylamide, 0.3% bis, 8 M urea in 1 x TBE). Electrophoresis was run at 50 watts constant power until the bromophenol blue reached the bottom of the gel (sequences less than 180 nucleotides from the primer). After electrophoresis, gels were lifted onto Whatman 3 MM paper and dried in a Bio-Rad model 583 gel dryer. Gels were exposed to X-ray film at -80°C.

Labeling of oligonucleotides

Double stranded κB oligonucleotides were labeled by annealing each complementary strand and phosphorylating the 5'-end with T_4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (6,000 Ci/mmol). Labeled oligonucleotides were purified by ethanol precipitation.

Photoreactive [^{32}P]-labeled oligonucleotides were prepared by annealing coding strand templates (27 bases, 5'-CAACGGCAGGGGAATCTCCCTCTCCTT-3') to a complementary 10 base primer (5'-AAGGAGAGGG-3') and filling in the overhang

with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP, [α-^{32}P]dCTP, dGTP and equimolar amounts of dTTP and 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdU).

Electrophoretic mobility shift assay (EMSA)

DNA binding reactions were carried out in 20 μl of binding buffer (25 mM HEPES (pH 7.9), 10% glycerol (v/v), 0.1 mM EDTA, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM DTT, 100 μg/ml poly (dI - dC)). Cellular extracts were incubated without [^{32}P]-labeled κB oligonucleotides at room temperature for 15 minutes, and then approximately 0.02 ng of κB oligonucleotides (20,000 - 40,000 cpm) were added and equilibration was achieved by incubating at room temperature for 20 minutes. To the reaction, 5 μl of binding buffer containing 0.1% bromophenol blue was added, and then the mixture was immediately loaded onto native 5 - 6% polyacrylamide gel (59 parts acrylamide; 1 part bisacrylamide) and run in 0.25 x TBE buffer at 100 volts for 1 hr. Gels were then dried and exposed to X-ray film.

For competition EMSA, the unlabeled competitors were incubated with cell extracts for 15 minutes at room temperature before addition of labeled κB oligonucleotides. For super-shift assays using antibody, the antiserum was added after the DNA binding reaction.

UV cross-linking

The cell extracts were incubated with approximately 1 ng of [^{32}P]-labeled

photoreactive κB probe (10^6 cpm) in 96-well plates and the DNA binding reactions were irradiated with short wave ultraviolet light using a UV illuminator (Fotodyne) at a distance of 5 cm for 15 min. The irradiated samples were analyzed by immunoprecipitation.

3. PREPARATION OF ANTIBODIES

Expression and purification of recombinant v-*rel* protein

Approximately 3 x 10^7 Sf9 cells, in a T-75 flask (Corning), were infected with recombinant baculovirus containing the v-*rel* gene at a MOI of 5 PFU/cell and incubated for 72 hrs at 27°C. Cells were harvested and washed once with cold PBS. The cell pellets were resuspended in 10 ml of PBS containing 0.5% NP-40 and 10% glycerol (v/v) and lysed by vortexing and incubating at 4°C for 15 minutes. Nuclei were isolated by centrifugation and washed twice with cold PBS. The nuclear pellets were resuspended in 2 ml of H_2O, lysed by addition of an equal volume of 2 x SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 10% ß-mercaptoethanol, 10% SDS, 25% glycerol, 0.04% bromophenol blue) and heated at 100°C for 5 minutes. Preparative electrophoresis was performed using the Bio-Rad Protean II Dual Slab system, using 1.5 mm thick 10% polyacrylamide gels containing SDS. Electrophoresis was carried out at a maximum of 40 mA per gel. After electrophoresis, gels were rinsed briefly in distilled water at 4°C and then stained in 0.25 M KCl at 4°C for 10 minutes (Hager and Burgess 1980). The appropriate protein band was excised from the gel, and soaked in distilled water at room temperature for 15-30 minutes until no

further staining could be seen. Protein was electroeluted from the gel using the Bio-Rad model 422 Electro-Eluter following the manufacturer's instructions. Electroelution was done at 32 mA for 4 hrs. The electroeluted protein was diluted to 2 ml with PBS and then concentrated using microconcentrators (Centricon-30, Amicon) at 4,500 x g for 1 hr in a JA-21 rotor.

Immunization

Gel purified v-*rel* protein (20 μg) in PBS was emulsified in an equal volume of Freund's complete adjuvant for primary immunization or Freund's incomplete adjuvant for secondary immunization. Antigens were administered intramuscularly in the leg and boosts were performed at three week intervals using alternating legs for immunization. Sera were collected on the tenth day following the last injection. Each serum sample was monitored for its ability to react with the v-*rel* protein in Western blot assays.

Collection of sera

All rabbits were bled from the ear immediately prior to immunization and on the tenth day following primary or subsequent immunization. At the completion of immunization rabbits were bled by heart puncture after sedation with Innovar-Vet (0.22 ml/kg). Sacrifice of the rabbits was ensured by cervical dislocation after collection of blood. Whole blood was incubated at 37°C to allow complete clotting and spun at 400 x g for 10 minutes to remove cells and clots from the serum. All sera

were aliquoted and stored at -80°C.

Other antibodies used in this study

Antibodies against p50, p65, and human IκB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-pp40 antiserum was kindly provided by Dr. H. R. Bose (University of Texas, Austin, TX).

4. PROTEIN ANALYSIS

Preparation of cell extracts

Whole cell extracts from infected Sf9 cells were prepared essentially as described by Lacoste *et al.* (1990). Briefly, cells were washed with PBS and suspended in whole cell extraction buffer (20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 10% glycerol, 1 mM DTT, and 1 mM PMSF). Cells were lysed by slowly adding 5 M NaCl to a final concentration of 0.5 M. Lysates were gently mixed for 45 min at 4°C, and cellular debris was removed by centrifugation (15,000 rpm, 15 min, 4°C). Supernatants were then diluted to 0.1 M NaCl by adding extraction buffer.

The nuclear extracts from transformed CSC were prepared essentially as described by Gilmore and Temin (1986). Transformed CSC were washed with PBS, swollen in hypotonic buffer (10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 10 mM NaCl, 1 mM PMSF), and broken by Dounce homogenization. The homogenate was centrifuged at 1,000 x g for 5 minutes and the supernatant was removed. Whole cells were removed

by resuspending the crude nuclear pellets in 10 mM Tris buffer containing 40% sucrose and pelleting the nuclei through a 50% sucrose cushion at 50,000 x g. The nuclear extracts were prepared from the purified nuclei with high-salt extraction buffer (20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF).

The nuclear extracts from transfected Cos1 cells were prepared as described by Dignam *et al.* (1983) with minor modifications. Cells were washed twice with PBS, harvested, and resuspended in lysis buffer (20 mM HEPES (pH 7.9), 20 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM $MgCl_2$, 1 mM PMSF). Cells were left on ice for 5 minutes and centrifuged for 1 minute at top speed in a microcentrifuge at room temperature. The crude nuclear pellet was resuspended in high-salt extraction buffer, left on ice for 15 minutes, resuspended again, and left for a further 15 minutes. Nuclear debris was removed by centrifugation for 15 minutes at 4°C.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE (Laemmli 1970) using the Bio-Rad Mini-Protean II system (Bio-Rad). Gels were made with a 10% running gel (10% acrylamide, 0.13% bis, 0.1% SDS, 0.75 M Tris, pH 8.8) and a 5% stacking gel (5% acrylamide, 0.07% bis, 0.1% SDS, 63 mM Tris, pH 6.8) and were electrophoresed in 192 mM glycine, 25 mM Tris and 0.1% SDS at 150 volts until the bromophenol blue reached the bottom of the gel. Prior to electrophoresis, protein samples were denatured by boiling for 5 minutes in SDS-PAGE sample buffer.

Western blots

Proteins were separated by 10% SDS-PAGE on the Bio-Rad Mini Protean II gel system. Following electrophoresis, proteins were transferred to PVDF membrane (Millipore) using the Bio-Rad Mini Trans-blot assembly. Transfer was carried out at 100 volts for 40 minutes in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3 (Burnette 1981).

Immunological detection was carried out at room temperature with agitation. Briefly, membranes were washed for 10 minutes in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and then blocked in TBS containing 3% gelatin for 30 minutes. Membranes were washed with TBS containing 0.05% (v/v) Tween-20 (TTBS) before a 1 hr incubation with antiserum, diluted 1 : 500 in TTBS containing 1% gelatin. The first antibody solution was removed and after two washes, membranes were incubated for 1 hr with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad), diluted 1 : 3000 in TTBS containing 1% gelatin. Following two washes, 5 minutes each in TTBS and one wash in TBS, the membrane was developed in carbonate buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8) containing 0.30 mg of NBT (p-nitro blue tetrazolium chloride, Bio-Rad) and 0.15 mg of BCIP (5-bromo-4-chloro-3-indoxyl phosphate toluidine salt, Bio-Rad). The colour reaction was terminated by washing the membrane with distilled water.

Metabolic labeling of intracellular proteins

For labeling with [^{35}S]methionine, cells were first starved of methionine for 1 hr

in methionine-free media; Minimum essential media (MEM) without methionine (Flow Laboratories) for Cos1 cells, RPMI 1640 without methionine for transformed CSC, and Grace media without methionine for Sf9 cells. All media were supplemented with 5% dialyzed HIFCS. Starving media were replaced with fresh methionine-free media containing L-[^{35}S]methionine (NEN) at 100 μCi/ml and incubated for 1-4 hrs.

Immunoprecipitation

All manipulations were performed at 4°C unless otherwise indicated. The cellular extracts from labeled cells (CSC, Cos1 and Sf9 cells) were prepared in RIPA buffer (50 mM Tris (pH 7.2), 150 mM NaCl, 0.5% Triton X-100, 1 mM benzamidine HCl, 1 mM PMSF) and the cell debris was removed by centrifugation. The supernatant was rotated overnight with 1-5 μl of antiserum, and then incubated with 2.5 mg of protein A Sepharose CL-4B (Pharmacia) for 1 hr. The beads were washed 5 times with RIPA buffer, and resuspended in 30 μl of SDS-PAGE sample buffer. After boiling, the beads were spun down and samples were analyzed by SDS-PAGE. Following electrophoresis, gels were stained, destained, and then subjected to fluorography with Amplify (Amersham). Gels were dried using a Bio-Rad model 583 gel dryer and exposed to Cronex 4L film at -80°C until proper exposures were obtained.

Immunofluorescence assay

CEF cells were seeded on Flow multitest slides (Flow Laboratories) and then infected 24 hrs later. Immunofluorescence assays (IFA) were done at 24 hr post

infection. Immunofluorescence assays were performed on Cos1 cells at 36-48 hr post transfection. IFA slides were washed for 5 minutes in PBS prior to fixation in 100% acetone at -20°C for 5 minutes. Slides were dried and then washed for 5 minutes in PBS. Antiserum, diluted 1:250 in PBS, was added to each well of the slides and the slides were incubated for 1 hr at 37°C. Following incubation with the first antibody, the slides were washed 3 times. Donkey anti-rabbit IgG linked with fluorescein (Amersham), diluted 1:20 in PBS, was added to each well and the slides were incubated for 1 hr at 37°C. Slides were then washed 3 times, 5 minutes each in PBS. After drying, number 1 coverslips were mounted with glycerol/PBS. Samples were viewed using a Leitz Laborlux K Fluorescent microscope.

5. BIOCHEMICAL ASSAYS

Protein quantitation

Determination of protein concentration in samples was performed by the methods described by Bradford (1984) using the Bio-Rad protein microassay kit (Bio-Rad). Bovine serum albumin was used as a standard.

ß-galactosidase assay

To standardize the transfection efficiency of Cos1 cells, 500 ng of ß-galactosidase expression plasmid (pCH110) were included in every transfection and the ß-galactosidase activity was examined by the method of Lim and Chae (1989) with minor modifications. Briefly, transfected cells were harvested by scraping from two 35 mm

plates and washed twice with PBS. The cell pellets were resuspended in 200 μl of 25 mM Tris-HCl (pH 8.0) and disrupted by five cycles of freezing and thawing and the cell debris was removed by centrifugation. One third of each cell lysate was incubated at 37°C for 1 hr in 0.5 ml of PBS containing 3.5 mM ONPG (0-nitrophenyl-ß-D-galactopyranoside; Sigma) and 0.5% NP-40, and the absorbance of the supernatant was read at 420 nm. Purified ß-galactosidase (Sigma) was used as a standard. The remainder of each cell lysate was standardized according to ß-galactosidase activity and used for immunoprecipitation or CAT assay.

Chloramphenicol acetyltransferase (CAT) assay

CAT assay of Cos1 cells was performed as described by Neumann *et al.* (1987). The lysates of transfected Cos1 cells, which were standardized according to ß-galactosidase activity, were incubated at 65°C for 5 minutes and clarified by centrifugation. The supernatant was mixed with 75 μl of CAT reaction buffer (25 mM Tris-HCl (pH 8.0), 0.05 μCi of [^{14}C]chloramphenicol (60 mCi/mmol, NEN), 75 μg of n-butyryl Coenzyme A (Promega)) and incubated at 37°C for 1-2 hrs. After incubation, half of the total reaction mixture was extracted with 1 ml of ethyl acetate. The organic layer was dried and taken up in 20 μl of ethyl acetate, spotted on silica gel thin-layer plates (Brinkmann), and run with chloroform-methanol (95:5). The plates were dried for autoradiography. CAT (0.05 unit, Promega) was used as a positive control and 25 mM Tris was used as a negative control.

The remaining half of the reaction mixture was mixed with 0.5 ml of xylene in

order to extract only the acetylated forms of chloramphenicol. The organic layer was counted with scintillant using β scintillation counter (Beckman). The percentage of acetylated chloramphenicol in each sample was determined by comparing the counts per minute of acetylated forms as a fraction of total counts per minute in the acetylated and nonacetylated forms of chloramphenicol. All assays were performed at least three times. Standard errors were within 15%.

CHAPTER 3: DNA BINDING ACTIVITY OF THE V-REL ONCOGENE PRODUCT

1. INTRODUCTION

The v-*rel* protein was shown to be related to the *dorsal* protein of *Drosophila*. The extent of homology in a 295-amino acid stretch at the amino terminus is 75% when conservative amino acid changes are taken into account (Steward 1987). The carboxyl termini are unrelated. Reminiscent of the v-*rel* protein, the *dorsal* protein is also found either in the cytoplasm or nucleus. The *dorsal* protein is located in the cytoplasm of cleavage stage embryos. In the cellular blastoderm, it is still uniformly distributed throughout the embryo; however, it is relocalized to nuclei in a graded fashion, such that the *dorsal* protein is most concentrated in ventral nuclei but remains cytoplasmic in cells on the dorsal side of the embryo (Roth *et al.* 1989, Rushlow *et al.* 1989, Steward 1989). The cloning of κB site binding transcription factor NF-κB, which plays a crucial role in the expression of immunoglobulin κ light chain gene, also revealed homology with the v-*rel* and *dorsal* proteins (Bours *et al.* 1990, Ghosh *et al.* 1990, Kieran *et al.*, 1990, Meyer *et al.* 1991, Nolan *et al.* 1991, Ruben *et al.* 1991). Biochemical evidence suggests further similarities between these proteins. NF-κB is found in the cytoplasm and nucleus and also associates in complexes with other cellular proteins (Lenardo and Baltimore 1989).

Functional studies support the suggestion that NF-κB, *dorsal* and v-*rel* proteins are closely related. In cotransfection studies, v-*rel* and *dorsal* proteins can activate certain

promoters (Gélinas and Temin 1988, Hannink and Temin 1989, Rushlow *et al.* 1989). In addition, the *dorsal* and v-*rel* proteins bound to DNA through heterologous DNA binding domains can specifically activate transcription from plasmids containing artificial operator-promoter elements (Bull *et al.* 1990, Kamens *et al.* 1990).

The biochemical and functional similarities of the v-*rel* protein and NF-κB strongly suggest that the v-*rel* protein might act as a transcription factor through κB sites of target genes. Therefore, the first objective of this study was to examine the DNA binding activity of the v-*rel* protein, especially to an NF-κB binding site. The second objective was to relate the DNA binding activity of the v-*rel* protein to transforming activity.

To address these questions, the DNA binding activities of v-*rel* proteins expressed by deletion and linker insertion mutants in transformed CSC or insect cells were determined by electrophoretic mobility shift assay (EMSA) using a double-stranded oligonucleotide containing NF-κB binding sites.

2. RESULTS

Characterization of DNA binding activity in the REV-T transformed CSC

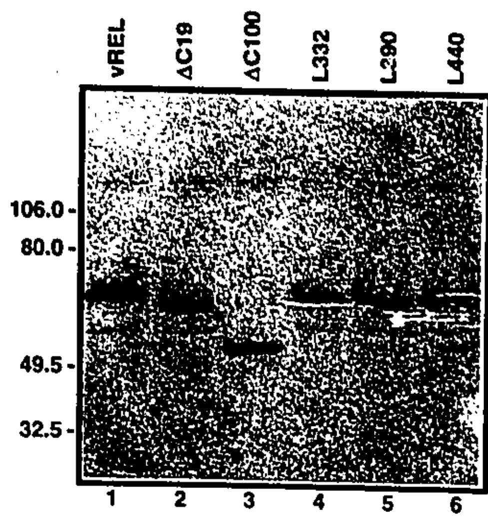
Deletion and linker insertion mutants of v-*rel* (Fig. 6) were constructed previously and tested for transforming activity by *in vitro* transformation assays using primary chicken spleen cells (Garson *et al.* 1990, Garson and Kang 1990). Colonies from transforming mutants (wild-type v-*rel*, ΔN11, ΔC19, ΔC100, L332, L374, L390, L440, or L459) were selected and were grown in suspension culture. Almost all of the

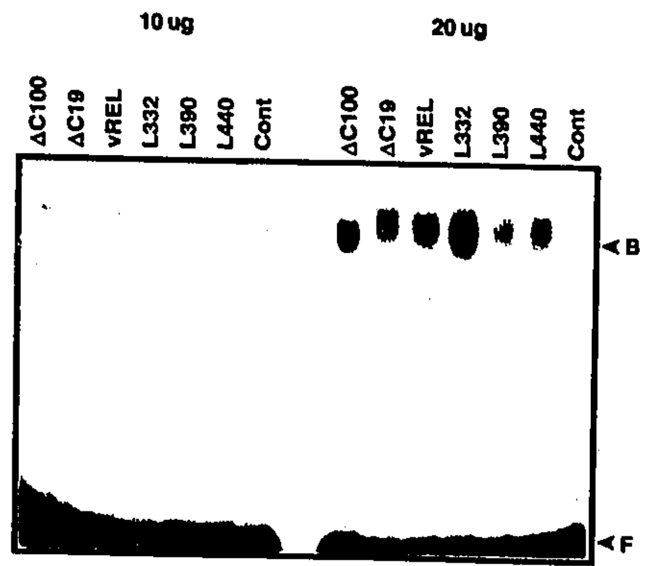
colonies from the transforming mutants, except ΔN11, were successfully established as permanent cell lines. CSC transformed by the wild-type v-*rel*, deletion mutants (ΔC19 and ΔC100), or linker insertion mutants (L332, L374, L390, L440, and L459) were used throughout this thesis.

To determine if the v-*rel* transformed cells contain κB site binding activity and ultimately to determine whether the v-*rel* protein has κB site binding activity, nuclear extracts were prepared from CSC transformed by wild-type v-*rel* or deletion and linker insertion mutants of v-*rel*. The presence of v-*rel* proteins in nuclear extracts was confirmed by Western blot using anti-v-*rel* antiserum. As shown in Figure 7A, all of the transformed CSC expressed the same level of the v-*rel* proteins of appropriate sizes. Similar amounts (10 μg or 20 μg) of nuclear extracts were used in EMSA. EMSA was performed using a [^{32}P]-labeled oligonucleotide containing two tandem copies of the κB sequence present in the human immunodeficiency virus (HIV) long terminal repeat (LTR) (Nabel and Baltimore 1987). One major protein-DNA complex (indicated by arrowhead B in Fig. 7B) was observed after incubation of [^{32}P]-labeled oligonucleotide with the nuclear extracts of all transformed CSC and the intensity of the DNA-protein complex was proportional to the amount of extract used in DNA binding reaction (Fig. 7B), suggesting that the nuclear extracts of CSC transformed by the wild-type v-*rel*, deletion, or linker insertion mutants of v-*rel* indeed contained NF-κB-like DNA binding activity.

To demonstrate the specificity of this protein-DNA complex for the κB oligonucleotide, competitive EMSA was performed using the nuclear extracts prepared

Figure 7. Expression and DNA binding activity of the v-*rel* protein in transformed CSC. (A) Expression of the v-*rel* protein in transformed CSC. Nuclear extracts from CSC transformed by wild-type v-*rel* (vREL; lane 1), ΔC19 (lane 2), ΔC100 (lane 3), L332 (lane 4), L390 (lane 5), or L440 (lane 6) were subjected to electrophoresis through an SDS-10% polyacrylamide gel and transferred to Immobilon membranes. The v-*rel* proteins were detected by using a colorimetric Western blot assay employing anti-v-*rel* antiserum and secondary goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Positions of prestained protein markers (in kilodaltons) are shown on the left. (B) DNA binding activity of transformed CSC extracts. Nuclear extracts from transformed CSC (10 μg or 20 μg) were incubated with [^{32}P]-labeled κB oligonucleotides. DNA binding reactions were resolved on a native 5% polyacrylamide gel. Arrowhead B indicates the protein-bound oligonucleotides and arrowhead F represents the free oligonucleotides.


A
vREL
ΔC19
ΔC100
L332
L390
L440
106.0
80.0
49.5
32.5
1
2
3
4
5
6


B
10 ug
20 ug
ΔC100
ΔC19
vREL
L332
L390
L440
Cont
ΔC100
ΔC19
vREL
L332
L390
L440
Cont
B
F

from transformed CSC in the presence of wild-type and mutant NF-κB oligonucleotides containing three point mutations (Fig. 8A). The mutant NF-κB oligonucleotide, which has been reported to have a 100 times lower affinity for NF-κB (Kawakami *et al.* 1988), showed no competition at a 100-fold molar excess (Fig. 8B) even though the wild-type oligonucleotide competed effectively at a 50-fold molar excess (Fig. 8C). These data demonstrate that the nuclear extracts of transformed CSC contain a specific NF-κB-like binding activity.

To investigate whether the specific DNA-protein complex contains the v-*rel* protein, EMSA was performed in the presence of anti-v-*rel* antibody. The nuclear extracts were incubated with antibodies after DNA binding reaction (Fig. 9A). A new slower migrating complex (indicated by an arrow) and a corresponding decrease in the main DNA-complex was observed in the presence of anti-v-*rel* antiserum (Fig. 9A, lane 3). Presumably the anti-v-*rel* antibodies bind to v-*rel* or v-*rel*-related proteins in this complex and thus cause a further retardation of mobility of the complex. The appearance of a super-shifted band and a corresponding decrease in the main complex was detected only in the presence of anti-v-*rel* antiserum but not with preimmune serum, anti-pp40 antiserum made against pp40 protein which is complexed with the v-*rel* protein, or anti-VSV antiserum made against disrupted vesicular stomatitis virus (Fig. 9A, lane 2, 4, 5). Thus these results suggest that the v-*rel* protein is at least one component of κB binding activity detected in the nuclear extracts of transformed CSC.

To show definitely that the v-*rel* protein is included in the DNA-protein complex, UV cross-linking experiments were perfomed. The nuclear extracts from CSC

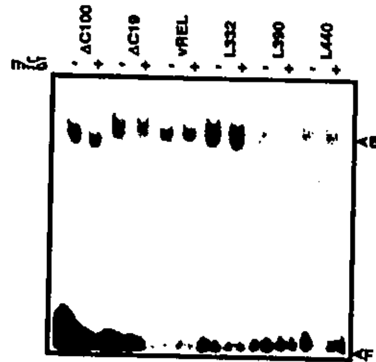
Figure 8. The competitive EMSA using mutant and wild-type κB oligonucleotides. (A) The sequences of double stranded wild-type κB (wtκB) and mutant κB (mκB) oligonucleotides. The lighter letter portions of mκB represent the substituted sequences of wtκB. (B) The competitive EMSA using mκB. Twenty micrograms of nuclear extracts from CSC transformed by v-*rel* mutants (ΔC100, ΔC19, L332, L390, L440) or by wild-type v-*rel* (vREL) were incubated with(+) or without(-) mκB, and followed by incubation with [^{32}P]-labeled κB probe. For competition, fifty times molar excess of unlabeled mκB was used. EMSA was performed on a native 5% polyacrylamide gel. (C) The competitive EMSA using wtκB. The same extracts were used for DNA binding reaction except wtκB as a competitor. EMSA was also performed on a native 5% polyacrylamide gel. Arrowhead B indicates the protein-bound oligonucleotides and arrowhead F represents the free oligonucleotides.

A

wtkB: GATCAGGGACTTTCCGCTGGGGACTTTCC
TCCCTGAAAGGCGACCCCTGAAAGGCTAG

mkB: GATCA[illegible]ACTTTCCGCTG[illegible]ACTTTCC
T[illegible]TGAAAGGCGAC[illegible]TGAAAGGCTAG

B



C

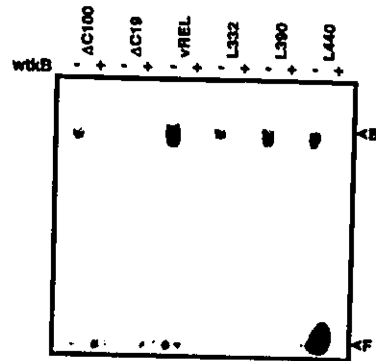
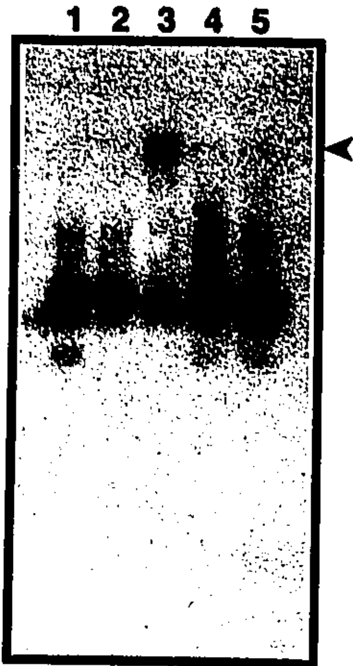
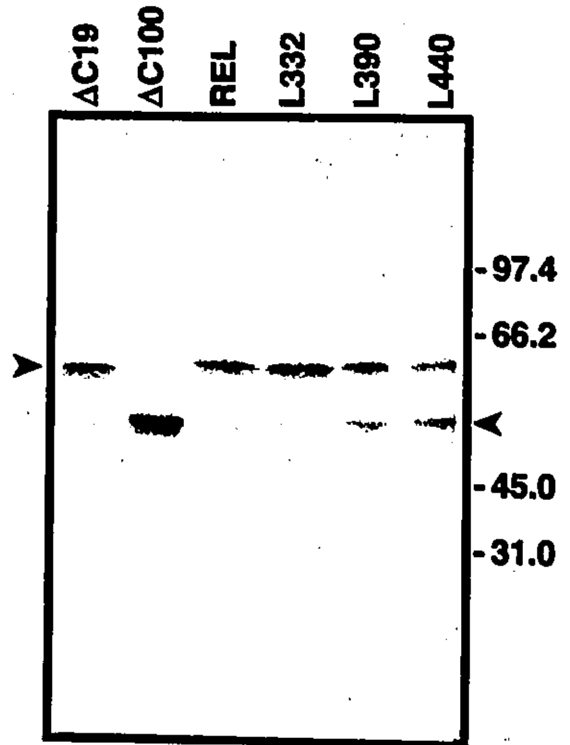


Figure 9. The identification of protein(s) responsible for κB binding activity in transformed CSC extracts. (A) EMSA in the presence of antibody. Nuclear extracts from CSC transformed by wild-type v-*rel* were incubated with [^{32}P]-labeled κB oligonucleotide and followed by incubation with antiserum (lane 1; without antiserum, lane 2; pre-immune serum, lane 3; anti-v-*rel* antiserum, lane 4; anti-pp40 antiserum, and lane 5; anti- VSV antiserum). The arrow indicates a band with retarded migration after addition of antibody directed to the v-*rel* protein. (B) UV cross-linking analysis of κB binding protein(s) in transformed CSC extracts. Nuclear extracts from CSC transformed by ΔC19, ΔC100, wild-type v-*rel* (REL), L332, L390, or L440 were incubated with a [^{32}P]-labeled photoreactive κB probe. DNA binding reactions were irradiated with UV-light, immunoprecipitated with anti-v-*rel* antiserum, and analyzed on a denaturing SDS-10% polyacrylamide gel. Arrows indicate the v-*rel* protein (upper arrow) or coimmunoprecipitated proteins (lower arrow). Position of protein markers are also shown on the right.

A



B



transformed by wild-type v-*rel* or by deletion and linker insertion mutants of v-*rel* were incubated with labeled NF-κB oligonucleotide containing bromodeoxyuridine, and irradiated with UV light to covalently cross-link the κB probe and protein. As shown in Figure 9B, immunoprecipitation of DNA-protein adducts with anti-v-*rel* antiserum demonstrated that the major DNA-protein adduct was composed of the v-*rel* protein. The DNA-v-*rel* protein adducts in CSC transformed by deletion mutants (ΔC19, ΔC100) were smaller than those in CSC transformed by wild type or linker insertion mutants of v-*rel*. An additional 50-kDa adduct was also detected in all transformed CSC, suggesting that this adduct may represent a v-*rel* related protein or another protein complexed with the v-*rel* protein. These results clearly show that the wild-type v-*rel* protein itself has κB binding activity. Furthermore, these results also demonstrate that all of the v-*rel* proteins encoded by transforming mutants (ΔC19, ΔC100, L332, L374, L390, L440, L459) show κB binding activity like the wild-type v-*rel* protein.

Expression of deletion and linker insertion mutants of v-*rel* in insect cells

Since all of the v-*rel* proteins encoded by transforming mutants of v-*rel* had the same κB binding activity as the wild-type v-*rel* protein, the κB binding activity of the v-*rel* proteins encoded by non-transforming mutants of v-*rel* (ΔN99, L29, L41, L116, L151, L163, L213, L275) was examined in order to investigate the relationship between DNA binding activity and transforming activity of the v-*rel* protein.

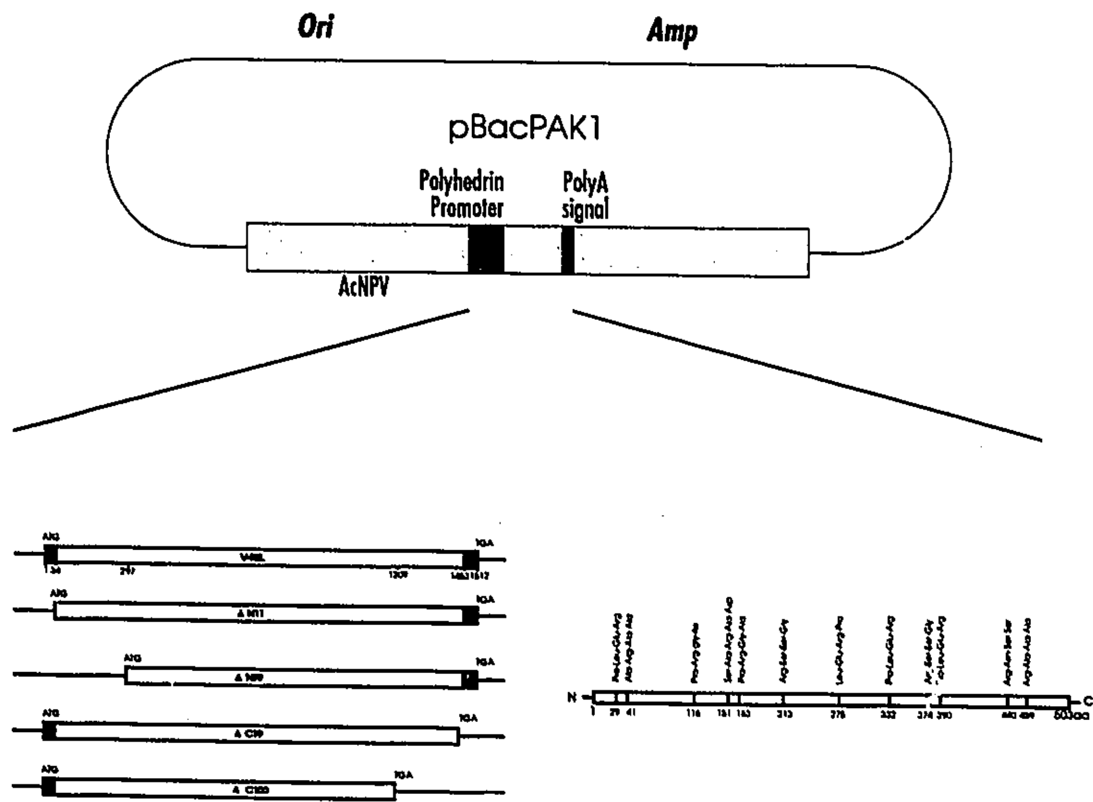
The baculovirus system has been used to express a variety of mammalian and viral proteins in insect cells (Kang 1988, Luckow and Summers 1988). This system takes

advantage of the strong polyhedrin promoter, which drives expression of the polyhedrin gene or foreign genes to high levels. Besides the high level of expression, this system offers the opportunity to study the function of the expressed protein because of its capability for proper post-translational modifications as would occur in a mammalian cell expression system. Therefore, the deletion and linker insertion mutants of v-*rel* were expressed in insect cells using the baculovirus system in order to investigate possible relationships between the DNA binding activity and transforming activity of the v-*rel* protein.

To construct deletion and linker insertion mutants of v-*rel* which are suitable for expression in insect cells, the pUC-V-REL clones (Fig. 6) were used as templates for PCR amplification. PCR primers were designed to contain the *Bgl* II site followed by the translation initiation codon (for 5'-primer) or the translation termination codon (for 3'-primer). Primer vR1 and vR6 were used for amplification of wild-type v-*rel* and all of the linker insertion mutants, vR2 and vR6 for ΔN11, vR3 and vR6 for ΔN99, vR1 and vR4 for ΔC100, or vR1 and vR5 for ΔC19 (see APPENDIX). The PCR products were digested with *Bgl* II, and cloned into the *Bam*H I site of the baculovirus transfer vector, pBacPAK1, resulting in recombinant plasmids containing mutant v-*rel* sequences (Fig. 10).

To produce recombinant baculoviruses which contain v-*rel* sequences in their genomes, Sf9 cells were cotransfected with a mixture of *Bsu*36 I-digested BacPAK6 viral DNA and recombinant plasmids using Lipofectin™, and the cotransfected cells were incubated at 27°C for 4 days. During the incubation period, the rare event of

Figure 10. Strategy of the construction of recombinant baculoviruses. In the pBacPAK transfer vector the dotted areas represent the AcNPV sequences and black boxes represent the promoter and polyA segments of polyhedrin gene. Deletion and linker insertion mutants of v-*rel* were amplified by PCR, and cloned into the *Bam*H I site of pBacPAK1. The recombinant plasmids containing v-*rel* mutants were cotransfected into Sf9 cells with *Bsu*36 I-digested BacPAK6 viral DNA, and the recombinant baculoviruses containing mutant v-*rel* sequences were purified by the plaque assay.



Recombinant plasmids

Bsu36 I-digested
BacPAK6 viral DNA

Cotransfection into Sf9 cells

Plaque assay

Recombinant virus

homologous recombination between the polyhedrin gene sequences on the plasmid and the baculovirus genome is expected to occur. As a result, the v-*rel* sequences become part of recombinant baculovirus genomes which will be packaged into virions. The supernatants of cotransfected cells were used as inocula for plaque assays on Sf9 cells. Recombinant baculoviruses were purified by plaque assay and amplified for production of recombinant virus stocks.

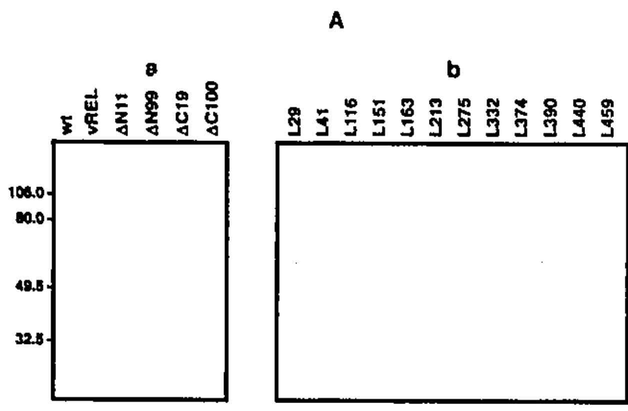
To examine the expression of the deletion and linker insertion mutants of v-*rel* in insect cells, Sf9 cells were infected with the recombinant baculoviruses containing mutant v-*rel* sequences. Cells were harvested 48 hr after infection, and whole cell lysates from infected cells were analyzed by Western blot using anti-v-*rel* antisera (Fig. 11A). The deletion and linker insertion mutants of v-*rel* were expressed approximately the same amounts (Fig. 11A.a and Fig. 11A.b).

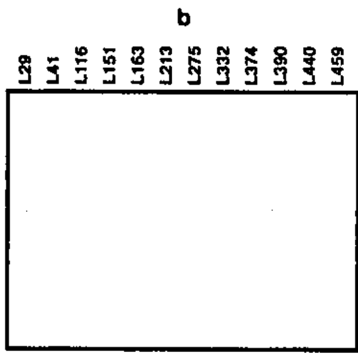
DNA binding activities of mutant v-*rel* proteins expressed in insect cells

The whole cell extracts prepared from Sf9 cells infected with recombinant baculoviruses were used for EMSA with labeled κB oligonucleotide to determine the κB binding activities of mutant v-*rel* proteins expressed in insect cells. As shown in Figure 11B, no specific DNA-protein complex is detected in wild-type AcNPV infected cell extracts (lane wt in Fig. 11B.a), whereas the specific DNA binding activity is readily detected in whole cell extracts of Sf9 cells infected with recombinant baculoviruses containing the wild-type v-*rel* (lane vREL in Fig. 11B.a), demonstrating that the v-*rel* protein is solely responsible for the κB binding activity detected in the

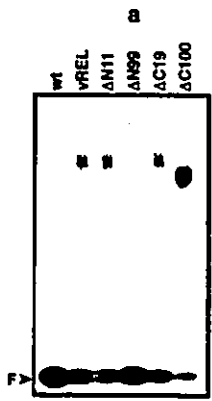
Figure 11. Expression and DNA binding activities of mutant v-*rel* proteins in insect cells. (A) Expression of mutant v-*rel* proteins in insect cells. Sf9 cells were infected with recombinant baculoviruses containing deletion(a) or linker insertion mutants(b) of v-*rel*. Whole cell extracts were prepared from infected Sf9 cells at 48 hr post infection and were analyzed by Western blot using anti-v-*rel* antiserum. Positions of prestained protein markers are shown on the left. (B) DNA binding activities of mutant v-*rel* proteins expressed in insect cells. The same sets of whole cell extracts as above were incubated with [^{32}P]-labeled κB probe and DNA binding reactions were resolved on native 5% polyacrylamide gels. F indicates the free oligonucleotides.

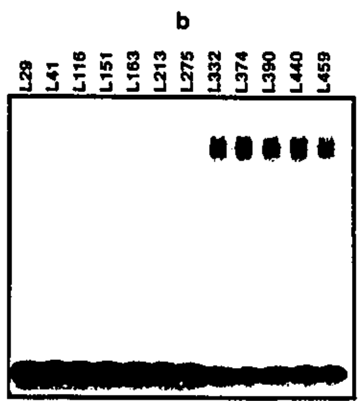
A





B





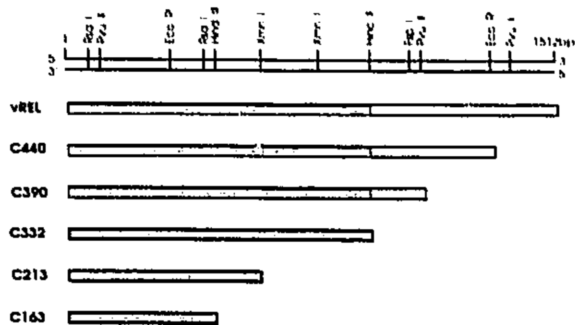
whole cell extracts of Sf9 cells infected with recombinant baculoviruses. The specificity of κB-binding activity and the involvement of the v-*rel* protein in DNA-protein complex were also examined by competitive EMSA and by immunoprecipitation analysis of DNA-protein adducts respectively as described previously (data not shown). The κB binding activity of the v-*rel* protein is unaffected by the deletion of as many as 100 C-terminal amino acids (ΔN19, ΔN100) or by the deletion of 11 N-terminal amino acids (ΔN11), but the deletion of 99 N-terminal amino acids (ΔN99) abolishes the κB binding activity of the v-*rel* protein even though the expression of v-*rel* proteins of all deletion mutants is almost at the same level as wild type v-*rel* (Fig. 11B.a). These results show the importance of the N-terminal region, except for the *env*-derived 11 amino acids, for DNA binding activity of the v-*rel* protein. Analyses of κB binding activities of mutant v-*rel* proteins encoded by linker insertion mutants also indicated that changes to the N-terminus (amino acid 29 - 275) abolished DNA binding activity, whereas changes to the C-terminus (amino acids 332 - 459) showed no effect on this activity (Fig. 11B.b). Therefore, these results localize the DNA binding domain of the v-*rel* protein to the N-terminus excluding *env*-derived N-terminal 11 amino acids.

<u>Deletion mapping of the DNA binding domain of the v-*rel* protein</u>

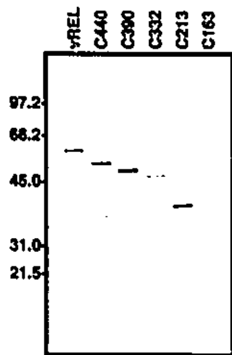
In order to map the DNA binding domain of the v-*rel* protein more precisely, serial C-terminal deletions (Fig. 12A) were made using the *Xho* I restriction enzyme site which was inserted into the linker insertion mutants (L440, L390, L332, L213, L163; see the legend of Fig. 6). The PCR products from these linker insertion mutants were

Figure 12. Deletion mapping of the DNA binding domain of the v-*rel* protein. (A) Schematic presentation of serial C-terminal deletion mutants. Serial C-terminal deletions were created using the *Xho* I restriction enzyme site which was inserted into the corresponding enzyme site to make linker insertion mutants (L440, L390, L332, L213, L163: see Fig. 6). The deletion fragments were cloned into the pBacPAK transfer vector and the corresponding recombinant baculoviruses were purified by plaque assay. (B) Expression of C-terminal deletion mutants of v-*rel* in insect cells. Sf9 cells were infected with the recombinant baculoviruses containing wild-type v-*rel* (vREL) or the indicated C-terminal deletion mutants (C440, C390, C332, C213, C163). Whole cell extracts were prepared from infected Sf9 cells at 48 hr post infection and were analyzed by Western blot using anti-v-*rel* antiserum. (C) DNA binding activities of C-terminal deletion mutant v-*rel* proteins expressed in insect cells. The same whole cell extracts used in (B) were incubated with [^{32}P]-labeled κB probe and DNA binding reactions were resolved on a native 5% polyacrylamide gel. F represents the free oligonucleotides.

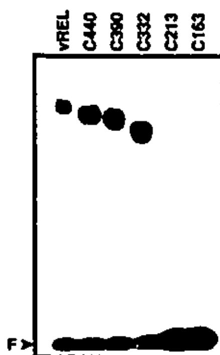
A



B



C



first digested with *Xho* I, gap-filled using Klenow fragment, ligated to *Bgl* II linkers, digested with *Bgl* II, and subcloned into the modified pBacPAK1 transfer vector. This vector contains three termination codons followed by a *Bam*H I site so that the inserted gene can be terminated in any reading frame. The recombinant baculoviruses containing these serial C-terminal deletions were purified by plaque assay.

To examine the expression of these serial C-terminal deletion mutants, Sf9 cells were infected with the recombinant baculoviruses and the whole cell extracts from infected cells were analyzed by Western blot using anti-v-*rel* antiserum (Fig. 12B). Serial C-terminal deletion mutants were expressed in similar amounts and were of the appropriate sizes.

The DNA binding activities of the C-terminal deletion mutants of v-*rel* were tested by EMSA using [^{32}P]-labeled κB probe (Fig. 12C). C-terminal deletion of up to 172 amino acids (C332) had no effect on DNA binding activity of the v-*rel* protein, but further deletion completely abolished the DNA binding activity. These results confirm that the DNA binding domain of the v-*rel* protein is located within the N-terminal 332 amino acids.

3. DISCUSSION

The DNA binding domain of the v-*rel* protein

Data presented in this study have established a functional and structural link between the v-*rel* protein and the entire NF-κB family of enhancer binding proteins. First, EMSA using nuclear extracts of transformed CSC with labeled κB

oligonucleotides confirmed that nuclear extracts of v-*rel* transformed cells contain NF-κB-like binding activity as suggested by others (Kabrun *et al.* 1991, Kochel and Rice 1992). However, the super-shift assay using anti v-*rel* antibody (Fig. 9A) and UV cross-linking experiments (Fig. 9B) clearly demonstrated that the v-*rel* protein directly binds to the κB site. Second, EMSA using the v-*rel* protein expressed in the baculovirus system definitely showed that the v-*rel* protein expressed in a heterologous system is a functional κB binding protein since whole cell extracts prepared from Sf9 cells infected with the wild-type baculovirus displayed no κB binding activity (Fig. 11B.a).

The deletion and linker insertion mutants of v-*rel* were expressed in the baculovirus system to determine which regions are necessary for the DNA binding activity. The expression of mutants in the baculovirus system permitted me to determine the DNA binding activities by EMSA using the labeled κB oligonucleotides. C-terminal deletion of up to 100 amino acids, or deletion of the N-terminal 11 amino acids had no effect on DNA binding activity of the v-*rel* protein, whereas N-terminal deletion of 99 amino acids abolished the DNA binding activity (Fig. 11B.a). Seven linker insertion mutants that mapped between amino acid residues 29 to 275 abolished the DNA binding activity, but the remaining five mutants which contained a linker insertion mutation between amino acid residues 332 and 459 retained the DNA binding activity (Fig. 11B.b). The results of this analysis localize the DNA binding domain of the v-*rel* protein to the N-terminus. Further analysis using serial C-terminal deletion mutants (Fig. 12) confirmed that the DNA binding domain of the v-*rel* protein is located within the N-terminal 320 amino acids (amino acids 12 - 332). Similar results were obtained

by Morrison *et al.* (1992) using a series of internal deletion mutants of v-*rel*. They found that deletion of the N-terminal 11 amino acids (residue 2 - 12) had no effect on DNA binding activity, but any deletions between amino acid residue 13 to 292 abolished the DNA binding activity of the v-*rel* protein. Another report (Kumar *et al.* 1992) also demonstrated that C-terminal deletions up to amino acid residue 293 showed no effect on DNA binding activity. Taken together with our results, it is clear that the DNA binding domain of the v-*rel* protein is located between amino acid residues 13 to 292.

Recently two groups analyzed the tertiary structure of the p50 subunit of NF-κB bound to the κB site (Ghosh *et al.* 1995, Müller *et al.* 1995). The homodimer of the p50 subunit (amino acid residues 39 to 350) bound to the 10 base pair κB site. The structure of p50 bound to DNA is unrelated to that of previously identified DNA-binding proteins. As opposed to the usual DNA recognition by short α-helices, p50 interacts with DNA through loops. The *rel* homology region (RHR) of p50 consists of two domains, both of which bear a striking resemblance to an immunoglobulin domain. Although only the carboxy terminal domain contributes to the dimer interface, both domains are involved in important DNA interactions. Making about 38 individual contacts with target DNA, the p50 dimer wraps itself around about two-thirds of the cylindrical surface of a double helix (Ghosh *et al.* 1995, Müller *et al.* 1995). This structural analysis of RHR coincides with our results showing that the DNA binding domain of the v-*rel* protein is located between amino acid residues 12 to 332, which correspond to amino acid residues 39 to 359 of p50. Also, deletion of the N-

terminal 99 amino acids (ΔN99), or the four amino acids present in the N-terminal linker insertion mutant of v-*rel* (L29, L41, L116, L151, L163, L213, L275) might have enough structural impact on the DNA binding loops directly or indirectly to abolish the DNA binding activity.

The relationship between DNA binding activity and transforming activity of the v-*rel* protein

The analysis of DNA binding activities of mutant v-*rel* proteins expressed in insect cells (Fig. 11B) showed that all of the transforming mutant proteins (ΔN11, ΔC19, ΔC100, L332, L374, L390, L440, L459) retained the DNA binding activity of the wild-type v-*rel* protein, whereas, all of the non-transforming mutant v-*rel* proteins (ΔN99, L29, L41, L116, L151, L163, L213, L275) lost the DNA binding activity (Table 4). This result clearly demonstrates that the κB site binding activity is necessary for transformation by the v-*rel* protein. Several groups have also indicated the importance of DNA binding by the v-*rel* protein for oncogenesis (Ballard *et al.* 1990, Morrison *et al.* 1992, Walker *et al.* 1992, Diehl and Hannink 1993, Diehl *et al.* 1993, Mosialos and Gilmore 1993). So far, none of the v-*rel* mutants, which lost the DNA binding activity as a result of deletion or point mutation in the N-terminus, has been reported to retain transforming activity, revealing that DNA binding activity is directly related to the mechanism of transformation by v-*rel*.

Even though the importance of DNA binding activity of the v-*rel* protein for transformation (Table 4) was established, DNA binding activity itself doesn't seem to

TABLE 4

Transforming and DNA binding activities of v-*rel* mutants

Mutant	Transforming activity[a]	DNA binding activity
REL	+++++	+
ΔN11	+/−[b]	+
ΔN99	−	−
ΔC19	+++++	+
ΔC100	+	+
L29	−	−
L41	−	−
L116	−	−
L151	−	−
L163	−	−
L213	−	−
L275	−	−
L332	+++++	+
L374	+++++	+
L390	+++++	+
L440	+++++	+
L459	+++++	+

[a]adapted from Garson and Kang (1990), and Garson *et al.* (1990)

[b]transformed colonies were observed, but they could not be propagated

be sufficient for full transforming activity of v-*rel*, for the following reasons. First, deletion of 11 N-terminal *env*-derived amino acids (ΔN11) reduced the transforming activity to 1% of wild type v-*rel* (Table 3, Garson *et al.* 1990) although the ΔN11 mutant protein retained DNA binding activity similar to the wild-type v-*rel* protein (Fig. 11B.a). Furthermore, chicken spleen cells transformed by ΔN11 failed to become an immortalized cell line, suggesting that the 11 N-terminal *env*-derived amino acids are required for full transforming activity. These results are consistent with the observation that the addition of short coding sequences to the 5'-end of v-*rel* abolished transformation (Gilmore and Temin 1988). At present, it is not fully understood how the 11 N-terminal *env*-derived amino acids function in the transforming activity of v-*rel*. However, it is possible that the acquisition of the *env*-derived amino acids could change the N-terminal conformation of v-*rel* and modify a c-*rel* function contributing to the activation of proto-oncogene. Second, deletion of 100 C-terminal amino acids (ΔC100) reduced the transforming activity to 10% of wild-type v-*rel* (Garson *et al.* 1990) despite the observation that DNA binding activity of the ΔC100 mutant protein was retained (Fig. 11B.a). My results are consistent with other findings that indicate an important role for the C-terminus of v-*rel* in transformation using serial C-terminal deletions (Sarkar and Gilmore 1993), or using C-terminal replacement mutants (Diehl and Hannink 1993). All of these mutants retain DNA binding activity, but show reduced transforming activity, suggesting that a C-terminal function is necessary for full transforming activity of v-*rel*, in addition to DNA binding activity. In contrast to the results obtained using C-terminal deletion mutants, C-terminal linker insertion mutants

(L332, L374, L390, L440, and L459) retained full transforming activity like wild-type v-*rel* (Garson and Kang 1990). These results may suggest that the C-terminal function doesn't necessarily require a highly ordered structure like the N-terminal DNA binding domain (Ghosh *et al.* 1995, Müller *et al.* 1995), which is susceptible to even slight changes induced by linker insertion (Fig. 11B.b). Therefore, the insertion of four amino acids into several positions in the C-terminus of v-*rel* is not enough to disrupt this C-terminal function.

CHAPTER 4: INTERACTIONS OF THE V-REL ONCOGENE PRODUCT WITH NF-κB SUBUNITS AND IκB (pp40)

1. INTRODUCTION

The pleiotropic nuclear factor NF-κB, which was originally described as a protein binding to the immunoglobulin κ enhancer (Sen and Baltimore 1986), also binds to the several similar sequences which are found in the regulatory regions of certain cytokine genes, an acute phase response gene, and several viral enhancers, including HIV-1 (Baeuerle 1991). NF-κB is present in an active form in the nucleus of a restricted set of cell types (mature B cells, differentiated monocytes, and some T cell lines). In most other cell types, it is present in an inactive form in the cytoplasm. Following activation by various stimuli (cytokines, phorbol ester, the tax protein of HTLV-1, and double stranded RNA), NF-κB is translocated into the nucleus.

The active nuclear form of NF-κB is composed of p50 and p65 subunits (Kawakami *et al.* 1988, Baeuerle and Baltimore 1989) that contact DNA as a heterodimer (Urban *et al.* 1991). Cloning of cDNA encoding the p50 subunit revealed that p50 is synthesized in the form of a non-DNA binding precursor p105 (105 kDa) and must be proteolytically cleaved from the N-terminal portion of p105 (Bours *et al.* 1990, Ghosh *et al.* 1990, Kieran *et al.* 1990, Meyer *et al.* 1991). Recently another related p100 gene, which is highly homologous to p105, was cloned (Neri *et al.* 1991, Schmid *et al.* 1991, Bours *et al.* 1992). p100 is processed to p52, a factor close in size and with properties similar to p50. The C-terminal portion of p105 or p100 is shown to contain the IκB-

like function (Blank *et al.* 1991, Hatada *et al.* 1992, Henkel *et al.* 1992, Liou *et al.* 1992, Rice *et al.* 1992). The p65 subunit is not cleaved from a precursor but is translated into its final form (Nolan *et al.* 1991, Ruben *et al.* 1991). p65 contains an acidic amino acid rich carboxyl terminus that mediates transcriptional activation. Both subunits of NF-κB are found to be homologous within their N-terminal 300 amino acid sequence, which appears to be required for DNA binding and dimerization (Logeat *et al.* 1991). The similarities of NF-κB subunits and the v-*rel* and c-*rel* proteins in this region also suggest that NF-κB subunits could form heterodimeric complexes with the v-*rel* and c-*rel* proteins. In fact, it has been demonstrated that the p50 subunit of NF-κB can interact with the v-*rel* and c-*rel* proteins *in vitro* (Kieran *et al.* 1990, Logeat *et al.* 1991). Therefore, the first objective of this study was to investigate the interaction between NF-κB subunits and the mutant v-*rel* proteins, and to examine the functional effects of the heterodimerization on DNA binding activity.

The NF-κB complex is maintained in an inactive state in the cytoplasm through its association with an inhibitory protein, referred to as IκB, which prevents its translocation into the nucleus *in vivo* and interferes with its binding to DNA *in vitro* (Baeuerle and Baltimore 1988). Some of the various stimuli that can activate NF-κB appear to trigger the phosphorylation of IκB, which is then released from the NF-κB - IκB complex (Ghosh and Baltimore 1990). Interestingly, pp40, which is one of the cellular proteins associated with the v-*rel* protein in REV-T transformed cells (Davis *et al.* 1990a), shares some biochemical characteristics with IκB. The size of pp40 is very similar to that of IκB (36 - 40 kDa) and the association of pp40 with the v-*rel* protein

is sensitive to detergents (Davis *et al.* 1990a), much like the association of IκB with NF-κB (Baeuerle and Baltimore 1988). The cloning of pp40 revealed that pp40 indeed shows functional, immunological, and structural homologies with mammalian IκB (Davis *et al.* 1991, Haskill *et al.* 1991, Inoue *et al.* 1991, Kerr *et al.* 1991), suggesting that NF-κB and the v-*rel* protein share the IκB/pp40 subunit. Therefore, the second objective of this study was to determine whether pp40 can directly associate with the mutant v-*rel* proteins, and to examine the functional effects of pp40 association on DNA binding activity of v-*rel* proteins.

To address these questions, p50, p65, c-*rel* and pp40 genes were cloned into the pBacPAK1 transfer vector, and the corresponding recombinant baculoviruses were selected for expression of these genes in insect cells. The dimerization between NF-κB subunits and the mutant v-*rel* proteins, or the association of pp40 with the mutant v-*rel* proteins were investigated by coimmunoprecipitation analysis using specific antisera. The functional effects of these protein-protein interactions on DNA binding activity were examined by EMSA using [^{32}P]-labeled κB oligonucleotides. The possible links between transformation and these protein-protein interactions are also discussed.

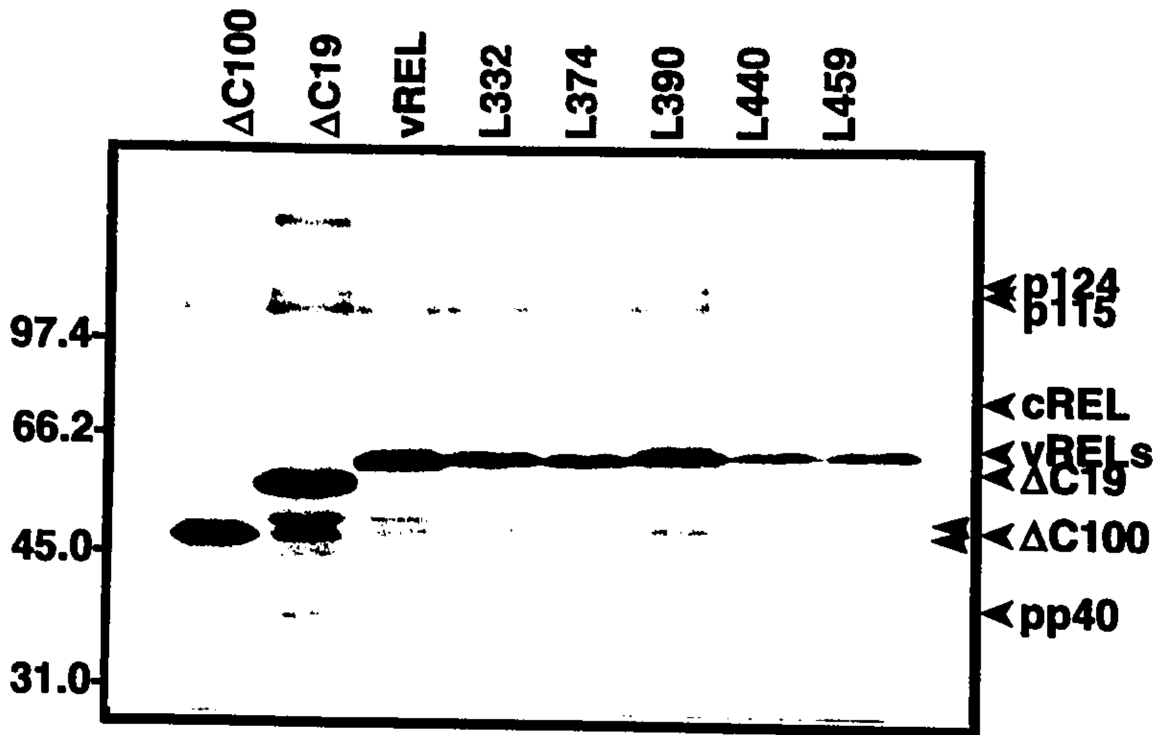
2. RESULTS

Association of the v-*rel* protein with cellular proteins in transformed CSC

Previous studies have shown that the v-*rel* protein exists in a high molecular weight complex with several cellular proteins in the cytoplasm of transformed chicken lymphoid cells (Simek and Rice 1988a, Tung *et al.* 1988, Morrison *et al.* 1989, Davis *et*

al. 1990b, Lim *et al.* 1990). In addition to the v-*rel* protein, the complex includes proteins of about 40 kDa, 68 kDa, 115 kDa, and 125 kDa. The 68 kDa protein has been shown to be the c-*rel* protein (Simek and Rice 1988b). By the subsequent cloning of associated proteins, the 40-, 115-, and 124-kDa proteins have been identified as avian IκB, avian NF-κB p100, and avian NF-κB p105, respectively (Davis *et al.* 1991, Capobianco *et al.* 1992, Sif and Gilmore 1993). Therefore, complex formation of the v-*rel* protein with NF-κB and IκB is likely to be of functional importance. In order to determine whether the mutant v-*rel* proteins are also associated with these proteins in CSC transformed by deletion (ΔC19, ΔC100) or linker insertion mutants (L332, L374, L390, L440, L459) of v-*rel*, radioimmunoprecipitation experiments were performed with extracts prepared from [^{35}S]methionine-labeled CSC. Antiserum directed against the v-*rel* protein co-immunoprecipitated the same set of three proteins (p40, p115, and p124) in addition to c-*rel* protein and the corresponding v-*rel* proteins in all of the transformed CSC (Fig. 13). Additional two proteins in the size range 45-50 kDa (marked by two arrows inside the box) were also coprecipitated. Because neither of these two additional proteins were precipitated with pre-immune serum nor detected by Western blot using anti-v-*rel* antiserum (data not shown), it is likely that the 45- to 50-kDa proteins are also cellular proteins complexed with the v-*rel* protein. These results demonstrated that all of the transforming v-*rel* mutant proteins (ΔC19, ΔC100, L332, L374, L390, L440, L459) are associated with the same set of proteins as those complexed to the wild-type v-*rel* protein (vREL in Fig. 13) in transformed CSC, indicating their possible role in transformation. However, it is necessary to examine

Figure 13. Immunoprecipitation analysis of transformed CSC. The chicken spleen cells transformed by indicated v-*rel* mutants (ΔC100, ΔC19, vREL, L332, L374, L390, L440, or L459) were metabolically labeled with [^{35}S]methionine. The extracts prepared from labeled cells were immunoprecipitated with anti-v-*rel* antiserum and the immunoprecipitates were resolved on an SDS-10% polyacrylamide gel. Positions of protein markers are shown on the left and the coimmunoprecipitated proteins are indicated on the right. Two arrows inside the box also indicate the two additional coimmunoprecipitated proteins.



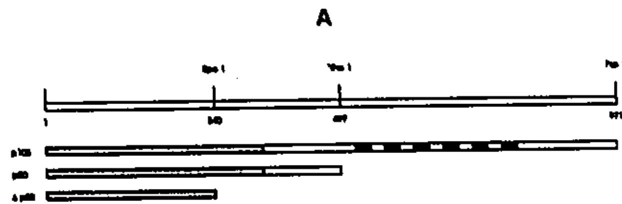
ΔC100
ΔC19
vREL
L332
L374
L390
L440
L459
97.4
66.2
45.0
31.0
p124
p115
cREL
vRELs
ΔC19
ΔC100
pp40

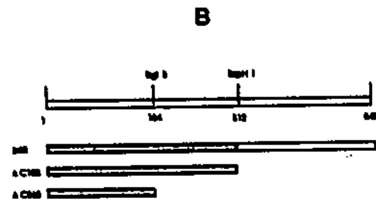
whether the non-transforming v-*rel* mutant proteins can also associate with NF-κB and IκB in order to determine the possible role of complex formation in transformation.

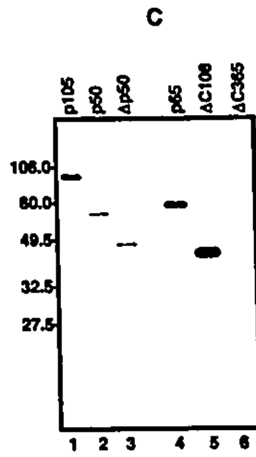
Expression of NF-κB subunits in insect cells

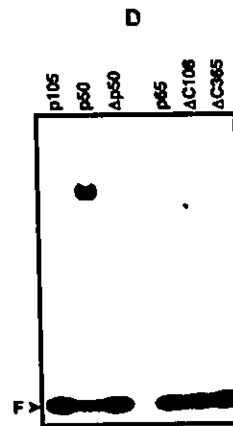
Since I had already selected all of the recombinant baculoviruses containing deletion or linker insertion mutants of v-*rel* (Fig. 11), it was decided to express NF-κB subunits in insect cells in order to examine the dimerization between NF-κB subunits and the mutant v-*rel* proteins encoded by deletion and linker insertion mutants. To construct NF-κB p105 and its deletion mutants which are suitable for expression in insect cells, the rabbit p105 cDNA clone (Ghosh *et al.* 1990) in the pBSK vector was used as a template for PCR amplification. PCR primers were designed to contain the *Bam*H I site followed by the translation initiation codon (for 5'-primer) or the translation termination codon (for 3'-primer). Primers 505 and 503 (see APPENDIX) were used for amplification of full-length p105. The PCR products were digested with *Bam*H I and subcloned directly into the pBacPAK1 transfer vector (p105 in Fig. 14A). For the deletion mutants of p105, PCR products were digested with *Xho* I or *Spe* I, gap-filled using the Klenow fragment, ligated to *Bam*H I linkers, digested with *Bam*H I, and then cloned into the modified pBacPAK1 transfer vector, which was described in Chapter 3 (Fig. 14A). We named these deletion mutants as p50 or Δp50 respectively. It should be mentioned that deletion mutant p50 is bigger than naturally processed p50, which is assumed to be 417 amino acid long (Ghosh *et al.* 1990). To construct p65 and its deletion mutants, the rabbit p65 cDNA clone in the pBSK vector (Nolan *et al.* 1991)

Figure 14. Expression and DNA binding activities of NF-κB subunits in insect cells. (A) Schematic presentation of p105 and its deletion mutants. Deletions were made by cutting the p105 cDNA with indicated restriction enzymes. The shaded boxes and the black boxes represent *rel* homology regions and ankyrin repeats respectively. (B) Schematic presentation of p65 and its deletion mutants. Deletions were made by using the indicated restriction enzyme sites and the shaded boxes represent *rel* homology regions. (C) Expressions of NF-κB subunits and their deletion mutants in insect cells. Whole cell extracts were prepared from Sf9 cells infected with recombinant baculoviruses containing the indicated form of each cDNA (lane 1; p105, lane 2; p50, lane 3; Δp50, lane 4; p65, lane 5; ΔC108, or lane 6; ΔC365) and were analyzed by Western blot using anti-p50 antiserum (lane 1-3) or anti-p65 antiserum (lane 4-6). (D) DNA binding activity of NF-κB subunits and their mutants. The same extracts used in (C) were incubated with [^{32}P]-labeled κB probe and EMSA was performed on a native 5% polyacrylamide gel. F represents the free oligonucleotides.


A


B


C
p105
p50
Δp50
p65
ΔC106
ΔC365
106.0
80.0
49.5
32.5
27.5
1
2
3
4
5
6


D
p105
p50
Δp50
p65
ΔC106
ΔC365
F

was used for PCR amplification. PCR primers were designed to contain a *Bcl* I site followed by the translation initiation codon (5'-primer) or the translation termination codon (3'-primer). Primer 655 and 653 (see APPENDIX) were used for amplification of full-length p65. The PCR products were digested with *Bcl* I and cloned into the pBacPAK1 transfer vector (p65 in Fig. 14B). For the deletion mutants of p65, PCR products were digested with *Bsp*H I or *Bgl* II, gap-filled using the Klenow fragment, ligated to *Bcl* I linkers, digested with *Bcl* I, and then cloned into the modified pBacPAK1. The resulting deletion mutants were named ΔC108 and ΔC365 (Fig. 14B). Also, the chicken c-*rel* cDNA (Capobianco *et al.* 1990) was used as template for PCR amplification with primers cR5 and cR3, and cloned into the pBAcPAK transfer vector (data not shown). The recombinant baculoviruses containing NF-κB subunits and their deletion mutants were purified by plaque assay.

To examine the expression of NF-κB subunits and their deletion mutants, Sf9 cells were infected with the corresponding recombinant baculoviruses and the whole cell extracts from infected cells were analyzed by Western blot using either anti-p50 antiserum (lane 1 to 3 in Fig. 14C) or anti-p65 antiserum (lane 4 to 6 in Fig. 14C). NF-κB subunits and their deletion mutants expressed comparable levels of proteins of appropriate sizes (Fig. 14C). It was noted that p105 was not processed to p50 as observed when p105 was transiently expressed in eukaryotic cells (Fan and Maniatis 1991). This is possibly due to the lack of a proteolytic activity in insect cells which is necessary for a normal processing of p105 or due to a reduced level of protease expression in insect cells caused by baculovirus-mediated inhibition of host protein

synthesis.

In order to examine the DNA binding activities of NF-κB subunits and their deletion mutants expressed in insect cells, the same whole cell extracts used in Western blots were tested by EMSA using the [^{32}P]-labeled κB probe (Fig. 14D). NF-κB p105 showed no DNA binding activity, but p50, which has a deletion of 480 C-terminal amino acids of p105, showed strong DNA binding activity (Fig. 14D). However, further deletion of up to N-terminal amino acid residues 340 abolished the DNA binding activity (Fig. 14D). These results confirmed that DNA binding domain of p50 is located in N-terminal 360 amino acids (Ghosh *et al.* 1990, Kieran *et al.* 1990) and the C-terminal half of p105 has IκB-like function (Blank *et al.* 1991, Hatada *et al.* 1992, Henkel *et al.* 1992, Liou *et al.* 1992, Rice *et al.* 1992). Full-length p65 and ΔC108 showed specific DNA binding activity according to their sizes, but further deletion of up to N-terminal amino acid residues 184 abolished the DNA binding activity (Fig. 14D), confirming that DNA binding domain of p65 is located within the 300 N-terminal amino acids (Nolan *et al.* 1991). Therefore, these results demonstrated that NF-κB subunits and their deletion mutants expressed in insect cells exhibit functional DNA binding activity.

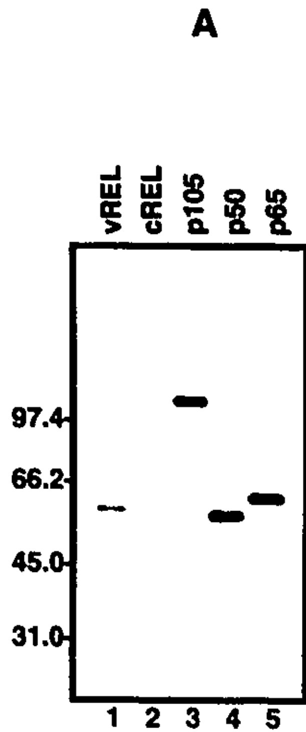
<u>Interaction of p105 and p50 with the *rel* proteins in insect cells</u>

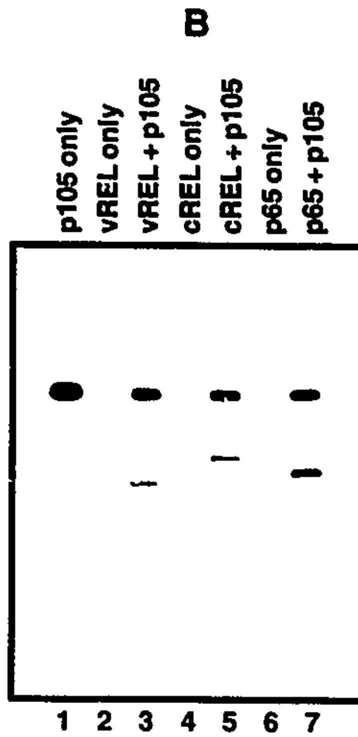
Prior studies have demonstrated that the v/c-*rel* proteins are complexd with NF-κB p105 and NF-κB p100 proteins in transformed or normal cells (Capobianco *et al.* 1992, Sif and Gilmore 1993). Furthermore, it was shown that the v/c-*rel* proteins and the

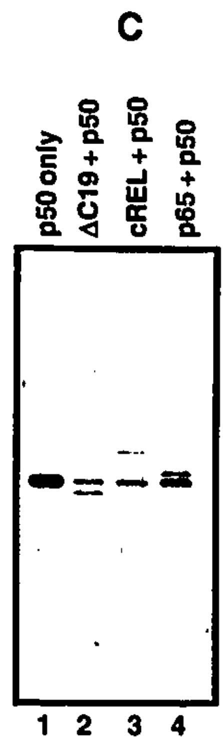
p50 subunit of NF-κB can interact *in vitro* and form stable heterodimeric complexes (Kieran *et al.* 1990, Logeat *et al.* 1991, Walker *et al.* 1992). In order to examine whether p105 or p50 can interact with the v/c-*rel* proteins or p65 in insect cells, Sf9 cells were infected singly with recombinant baculoviruses containing individual genes (v-*rel*, c-*rel*, or p65) or coinfected with recombinant baculoviruses containing p105 or p50. Immunoprecipitation analysis of [^{35}S]methionine labeled extracts from individually infected cells (Fig. 15A) using specific antisera (lane 1 and 2; anti-v-*rel* antiserum, lane 3 and 4; anti-p50 antiserum, lane 5; anti-p65 antiserum) showed that each protein was expressed at comparable levels in insect cells. Further immunoprecipitation analysis of individually infected cells with anti-p50 antiserum (lanes 1, 2, 4, 6 in Fig. 15B) demonstrated that the v/c-*rel* proteins and p65 lacked reactivity with anti-p50 antiserum. However, when coinfected with the recombinant baculoviruses containing p105, the v/c-*rel* protein or p65 formed complexes with p105, as evidenced by their coimmunoprecipitation with anti-p50 antiserum (lanes 3, 5, 7 in Fig. 15B). The same coimmunoprecipitation patterns were observed using anti-p50 antiserum when coinfected with recombinant baculoviruses containing p50 (Fig. 15C), confirming that the dimerization domain of p50 is located in the N terminus (Kieran *et al.* 1990, Logeat *et al.* 1991, Walker *et al.* 1992). The ΔC19 mutant (lane 2 in Fig. 15C) was used in this experiment instead of the wild-type v-*rel* since the p50 protein is very similar in size to that of the wild-type v-*rel* protein.

Since the wild-type v-*rel* protein was shown to interact with p105 in insect cells, I further examined whether the mutant v-*rel* proteins encoded by deletion and linker

Figure 15. Interaction of p105 and p50 with the *rel* proteins. (A) Expression of NF-κB subunits and the *rel* proteins in insect cells. Sf9 cells were infected with recombinant baculoviruses containing v-*rel* (lane 1), c-*rel* (lane 2), p105 (lane 3), p50 (lane 4), or p65 (lane 5) coding sequences and were labeled metabolically with [^{35}S]methionine at 24 hr post infection. The labeled cell extracts were immunoprecipitated with anti-v-*rel* antiserum (lane 1 and 2), anti-p50 antiserum (lane 3 and 4), or anti-p65 antiserum (lane 5). The immunoprecipitates were resolved on an SDS-10% polyacrylamide gel. (B) Coimmunoprecipitation of p105 with the *rel* proteins. Sf9 cells were infected with individual recombinant baculoviruses as indicated (lane 1; p105, lane 2; v-*rel*, lane 4; c-*rel*, lane 6; p65) or coinfected with recombinant virus containing p105 (lane 3; v-*rel* + p105, lane 5; c-*rel* + p105, or lane 7; p65 + p105). The infected cells were labeled with [^{35}S]methionine and the labeled extracts were immunoprecipitated anti-p50 antiserum. (C) Coimmunoprecipitation of p50 with the *rel* proteins. Sf9 cells were coinfected with the indicated recombinant baculoviruses and the recombinant baculovirus containing p50 (lane 1; p50, lane 2; ΔC19 + p50, lane 3; c-*rel* + p50, or lane 4; p65 + p105). The infected cells were labeled and the labeled extracts were immunoprecipitated with anti-p50 antiserum.


A
vREL
cREL
p105
p50
p65
97.4
66.2
45.0
31.0
1 2 3 4 5


B
p105 only
vREL only
vREL + p105
cREL only
cREL + p105
p65 only
p65 + p105
1 2 3 4 5 6 7


C
p50 only
ΔC19 + p50
cREL + p50
p65 + p50
1 2 3 4

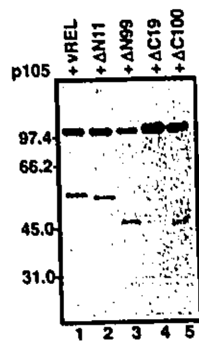
insertion mutants of v-*rel* can also interact with p105 in order to determine the possible role of complex formation in transformation. Sf9 cells were coinfected with recombinant baculoviruses containing the mutant v-*rel* sequences and recombinant baculovirus containing the p105 sequences, and were metabolically labeled with [^{35}S]methionine. Immunoprecipitation analysis performed with labeled extracts from coinfected cells demonstrated that all of the mutant v-*rel* proteins encoded by deletion (lanes 2 - 5 in Fig. 16A), or linker insertion mutants (lanes 7 - 18 in Fig. 16A) formed complexes with p105 like the wild-type v-*rel* protein (lanes 1, 6 in Fig. 16A), as shown by their coimmunoprecipitation with anti-p50 antiserum (Fig. 16A). The same coimmunoprecipitation patterns were also observed with anti-v-*rel* antiserum (Fig. 16B), which showed no cross-reactivity with p105 (data not shown). Therefore, these results clearly demonstrated that all of the mutant v-*rel* proteins, regardless of their transforming activities (Table 3) or their DNA binding activities (Table 4), can interact with p105, and also with p50 (Fig. 15C).

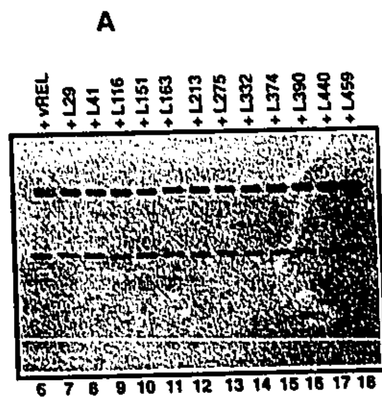
<u>DNA binding activities of heterodimers of p50 with the *rel* proteins</u>

Active NF-κB complexes bind to the κB site as heterodimer of p50 and p65 *in vivo* (Baeuerle and Baltimore 1989, Urban *et al.* 1991) even though the homodimer of p50 or p65 can also bind to the κB site *in vitro* (Nolan *et al.* 1991, Ruben *et al.* 1991). The v-*rel* protein expressed in insect cells can bind to the κB site as a homodimer (Fig. 11B) and the v-*rel* protein can also interact with p105 or p50 in insect cells (Fig. 15B). Furthermore, p105 or p50 can interact with all of the mutant v-*rel* proteins (Fig. 16),

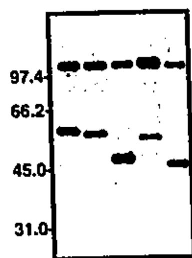
Figure 16. Interaction of p105 with the mutant v-*rel* proteins. Sf9 cells were coinfected with recombinant baculoviruses containing the indicated mutant v-*rel* sequences (lane 1-18) and the recombinant baculovirus containing p105. Infected Sf9 cells were labeled metabolically with [^{35}S]methionine at 24 hr post infection. Half of the each cell extracts were immunoprecipitated with anti-p50 antiserum(A) and the other half of the extracts were immunoprecipitated with anti-v-*rel* antiserum(B). The immunoprecipitates were resolved on SDS-10% polyacrylamide gels.

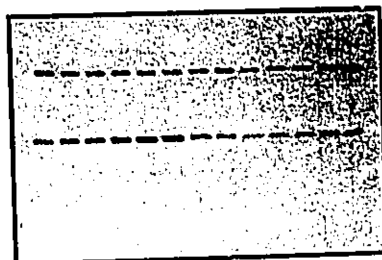
A





B

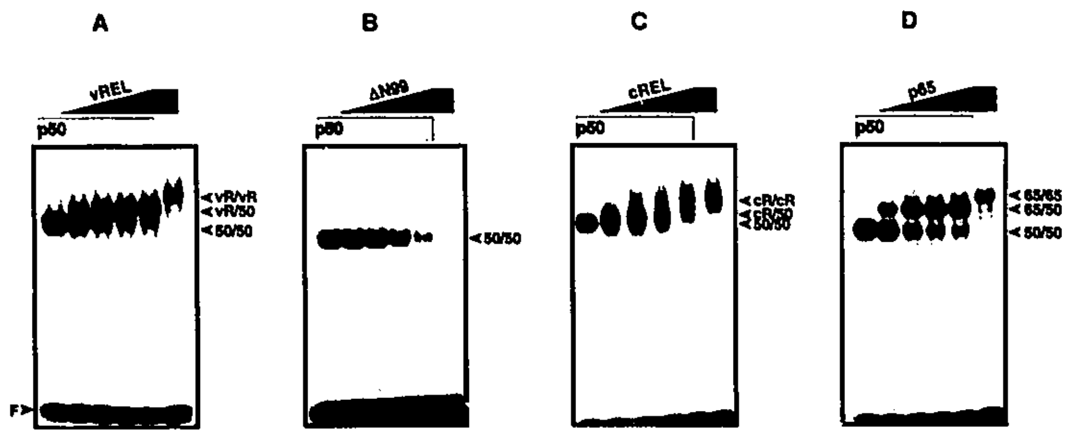




regardless of their DNA binding activities. These results thus prompted an investigation of the DNA binding activities of heterodimers of p50 with the *rel* proteins.

In order to demonstrate the DNA binding activity of heterodimer between p50 and the v-*rel* protein more dramatically, constant amounts of extracts prepared from Sf9 cells infected with recombinant baculovirus containing the p50 sequences were mixed with increasing amounts of extracts prepared from Sf9 cells infected with recombinant baculovirus containing the wild-type v-*rel* sequences and EMSA was performed using these mixed extracts. As shown in Fig. 17A, mixing of extracts containing p50 and the v-*rel* protein resulted in an additional complex with intermediate mobility (lane 2 to 5) relative to complexes seen when only the v-*rel* protein (lane 6) or p50 (lane 1) were present. These results indicated that the v-*rel* protein can bind DNA as a homodimer but also as a heterodimer with p50. Figure 17A also showed that the relative amounts of the p50/v-*rel* protein heterodimers were dependent on the amount of the v-*rel* protein present in the mixed extracts. The same experiments performed with the extracts containing the c-*rel* protein (Fig. 17C) or p65 (Fig. 17D) demonstrated that the c-*rel* protein and p65 can bind DNA as a homodimer or as a heterodimer with p50. In contrast, the DNA binding activity of the p50 homodimer was gradually diminished with increasing amount of the ΔN99 mutant v-*rel* protein (Fig. 17B). Since it has been shown that the ΔN99 mutant v-*rel* protein had no DNA binding activity (Fig. 11B) but still dimerized with p50 (Fig. 16), these results demonstrated that the DNA binding activity of p50 was abolished by heterodimerization with the ΔN99 mutant v-*rel*

Figure 17. DNA binding activities of heterodimers of p50 with the *rel* proteins. Whole cell extracts were prepared from Sf9 cells infected with recombinant baculoviruses containing p50, v-*rel*, ΔN99, c-*rel*, or p65 sequences. Constant amount of extracts containing p50 was mixed with increasing amounts of extracts containing the *rel* protein (A; vREL, B; ΔN99, C; cREL, or D; p65). Total protein was kept constant by adding extracts prepared from Sf9 cells infected with wild-type AcNPV. The mixed extracts were incubated with [^{32}P]-labeled κB probe and resolved on native 5% polyacrylamide gels. The first lane in each panel shows the result obtained with extracts containing only p50 and the last lane of each panel shows the results obtained with extracts containing the individual *rel* protein. The positions of homodimers or heterodimers are shown on the right. F indicates the free oligonucleotides.

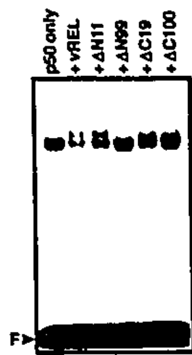

A
B
C
D
vREL
ΔN99
cREL
p65
p50
p50
p50
p50
vR/vR
vR/50
50/50
50/50
cR/cR
cR/50
50/50
65/65
65/50
50/50
F

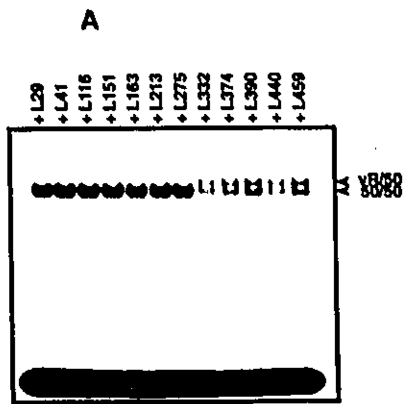
protein. Therefore, the ΔN99 mutant v-*rel* protein can function as a transdominant inhibitor of κB-specific DNA binding (Herkowitz 1987).

Next, I further investigated the DNA binding activities of heterodimers between p50 and the mutant v-*rel* proteins. The extracts containing p50 were mixed with extracts from Sf9 cells infected with recombinant baculoviruses containing the individual mutant v-*rel* sequences and EMSA was performed. When the amount of p50 in the extracts was in excess to that of the mutant v-*rel* protein (Fig. 18A), two bands were seen for all of the mutant v-*rel* proteins with the DNA binding activity, which presumably represented the heterodimers of p50 with the mutant v-*rel* proteins and p50 homodimer. All of the mutant v-*rel* proteins without DNA binding activity somewhat reduced the DNA binding activity of the p50 homodimer. When the amounts of the mutant v-*rel* proteins were equal to or in excess to the amount of p50 (Fig. 18B), all of the mutant v-*rel* proteins with DNA binding activity formed heterodimers with p50 exclusively, whereas all of the mutant v-*rel* proteins without DNA binding activity completely abolished the DNA binding activity of p50 by heterodimerization. In case of ΔC100, only one band was observed in both conditions since the mobility of p50 homodimer and that of heterodimer of p50 with ΔC100 were indistinguishable. These results clearly demonstrated that all of the mutant v-*rel* proteins with DNA binding activity can form active heterodimers with p50, whereas all of the mutant v-*rel* proteins that have no DNA binding activity can form inactive heterodimers with p50.

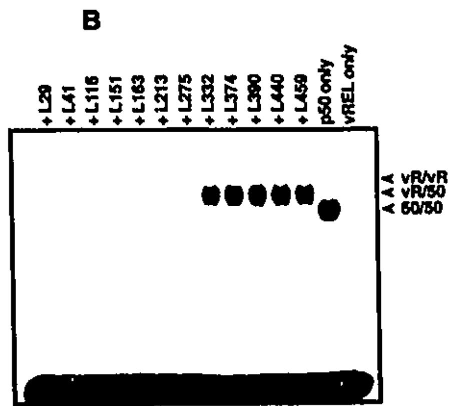
Figure 18. DNA binding activities of heterodimers of p50 with the mutant v-*rel* proteins. Whole cell extracts from Sf9 cells infected with recombinant baculoviruses containing p50 were mixed with the extracts prepared from Sf9 cells infected with recombinant baculoviruses containing the indicated mutant v-*rel* sequences. The amount of p50 is in excess to the amount of mutant v-*rel* proteins in (A) and the amounts of mutant v-*rel* proteins are in excess to the amount of p50 in (B). The mixed extracts were used for EMSA. The positions of homodimers and heterodimers are shown on the right. F indicates the free oligonucleotides.

A





B

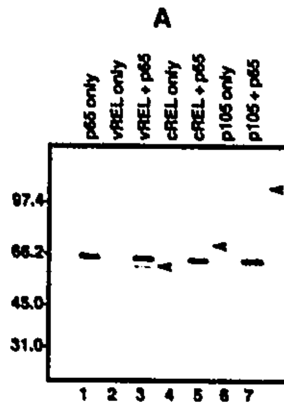


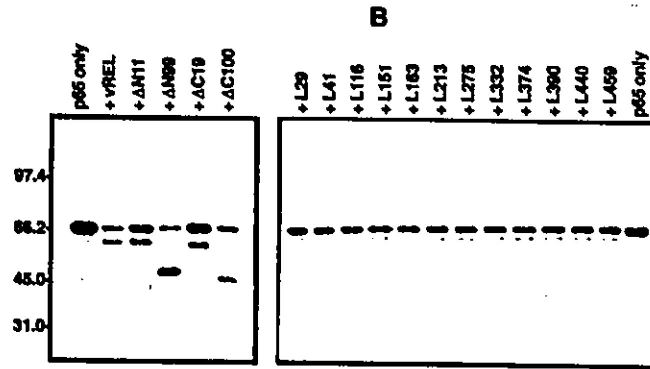
Interaction of p65 with the *rel* proteins

Active NF-κB complex is a heterodimer of p50 and p65 (Baeuerle and Baltimore 1989) and it is believed that the dimerization domain is located in the N-terminal *rel* homology region (Logeat *et al.* 1991), which is shared with the v/c-*rel* proteins (Ghosh *et al.* 1990, Nolan *et al.* 1991). Since it was shown that the v-*rel* protein can interact with p50 in insect cells (Fig. 15 and 16), p65 is also expected to interact with the v-*rel* protein. In order to examine whether p65 can interact with the v/c-*rel* proteins or p105 in insect cells, Sf9 cells were infected singly with recombinant baculoviruses containing individual genes (lane 1; p65, lane 2; v-*rel*, lane 4; c-*rel*, lane 6; p105 in Fig. 19A) or cotransfected together with the recombinant baculovirus containing p65 sequences (lanes 3, 5, 7 in Fig. 19A). Immunoprecipitation analysis of [^{35}S]methionine labeled extracts from singly infected cells with anti-p65 antiserum showed that the v/c-*rel* proteins and p105 lacked reactivity with anti-p65 antiserum (lanes 2, 4, 6 in Fig. 19A). However, when coexpressed with p65, the v/c-*rel* proteins and p105 formed complexes with p65, as shown by their coimmunoprecipitation with anti-p65 antiserum (lanes 3, 5, 7 in Fig. 19A). Therefore, these results directly demonstrated that the v-*rel* or c-*rel* protein can interact with p65.

Since the wild-type v-*rel* protein was shown to interact with p65 in insect cells, I further examined whether the mutant v-*rel* proteins can also interact with p65 in insect cells in order to determine the possible role of complex formation in transformation. Immunoprecipitation of labeled proteins prepared from Sf9 cells coinfected with recombinant baculovirus containing p65 sequences and recombinant baculoviruses

Figure 19. Interaction of p65 with the *rel* proteins. (A) Coimmunoprecipitation of p65 with the *rel* proteins. Sf9 cells were infected with recombinant baculoviruses expressing NF-κB subunits and *rel* proteins (lane 1; p65, lane 2; vREL, lane 4; cREL, or lane 6; p105) or coinfected with recombinant baculoviruses expressing p65 (lane 3; vREL + p65, lane 5; cREL + p65, or lane 7; p105 + p65). Infected Sf9 cells were labeled with [^{35}S]methionine at 24 hr post infection and the labeled proteins were immunoprecipitated with anti-p65 antiserum. Arrowheads indicate the coimmunoprecipitated *rel* proteins. (B) Coimmunoprecipitation of p65 with the mutant v-*rel* proteins. Sf9 cells were coinfected with recombinant baculovirus expressing p65 sequences and recombinant baculoviruses expressing individual mutant v-*rel* proteins. Labeled proteins were immunoprecipitated with anti-p65 antiserum and were resolved on SDS-10% polyacrylamide gels.

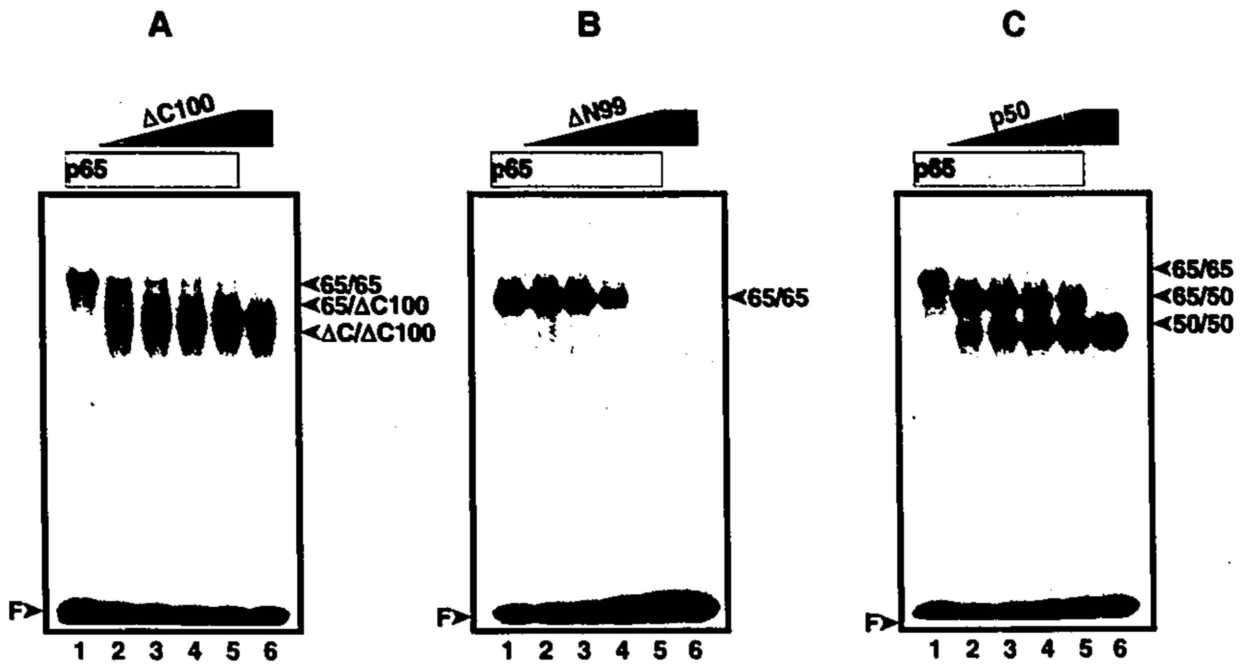

A
p65 only
vREL only
vREL + p65
cREL only
cREL + p65
p105 only
p105 + p65
97.4
66.2
45.0
31.0
1 2 3 4 5 6 7


B
p65 only
+ vREL
+ ΔN11
+ ΔN99
+ ΔC19
+ ΔC100
+ L29
+ L41
+ L116
+ L151
+ L163
+ L213
+ L275
+ L332
+ L374
+ L390
+ L440
+ L459
p65 only
97.4
66.2
45.0
31.0

containing individual mutant v-*rel* sequences showed that all of the mutant v-*rel* proteins formed complexes with p65 like the wild-type v-*rel* protein, as evidenced by their coimmunoprecipitation by anti-p65 antiserum (Fig. 19B). These results indicated that all of the mutant v-*rel* proteins, regardless of their transforming activities or their DNA binding activities, can interact with p65.

Next, the effect of interaction between p65 and the v-*rel* protein on DNA binding activity was investigated. In order to examine the DNA binding activity of a heterodimer of p65 with the v-*rel* protein, a constant amount of whole cell extracts containing p65 was mixed with increasing amounts of whole cell extracts containing ΔC100 mutant v-*rel* protein and EMSA was performed using these mixed extracts. The ΔC100 mutant v-*rel* protein was chosen instead of the wild-type v-*rel* protein because the mobility of the p65 homodimer and that of the v-*rel* homodimer were indistinguishable. As shown in Fig. 20A, the mixture containing p65 and ΔC100 v-*rel* protein resulted in an additional complex with intermediate mobility relative to that of p65 (lane 1) or ΔC100 (lane 6) complexes. These results indicated that ΔC100 v-*rel* protein can bind DNA as a heterodimer with p65. Figure 20A also demonstrated that the amounts of p65/ΔC100 heterodimers were dependent on the amount of ΔC100 v-*rel* protein present in the mixed extracts. The same experiments performed with whole cell extracts containing p50 (Fig. 20C) confirmed the previous results (Fig. 17D) showing that p65 can bind DNA as a heterodimer with p50. However, the DNA binding activity of the p65 homodimer was gradually reduced in the presence of increasing amounts of ΔN99 v-*rel* protein in the mixture (Fig. 20B). Since it was

Figure 20. DNA binding activities of heterodimers of p65 with *rel* proteins. Whole cell extracts were prepared from Sf9 cells infected with recombinant baculoviruses expressing p65, ΔC100, ΔN99, or p50. A constant amount of extract containing p65 was mixed with increasing amounts of extracts containing ΔC100(A), ΔN99(B), or p50(C). The total protein amount of mixed extracts was kept constant by adding extracts from Sf9 cell infected with wild-type AcNPV. The mixed extracts were used for EMSA. Lane 1 shows the results obtained with extracts containg p65 only and lane 6 shows the results obtained with extracts containing only the *rel* proteins. Positions of homodimers and heterodimers are shown on the right. F indicates the free oligonucleotides.


A
B
C
ΔC100
ΔN99
p50
p65
p65
p65
65/65
65/ΔC100
ΔC/ΔC100
65/65
65/65
65/50
50/50
F
F
F
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6

shown that ΔN99 v-*rel* protein had no DNA binding activity (Fig. 11B) but still retained the dimerization ability with p65 (Fig. 18B), these results suggested that the DNA binding activity of p65 is abolished by heterodimerization with ΔN99 v-*rel* protein. The same results were obtained when the other mutant v-*rel* proteins that lacked DNA binding activity were used (data not shown). These results indicated that all of the mutant v-*rel* proteins with DNA binding activity can form active heterodimers with p65, but all of the mutant v-*rel* proteins with no DNA binding activity can form inactive heterodimers with p65.

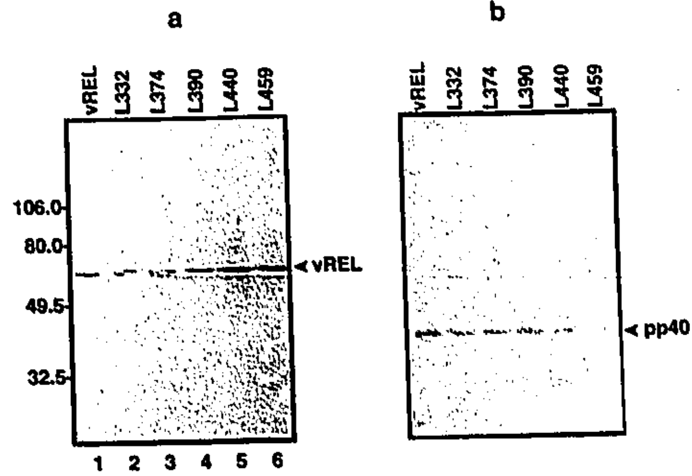
<u>The association of pp40 with the v-*rel* protein in transformed CSC</u>

Previous studies show that the viral and cellular *rel* proteins associate with several cellular proteins in transformed and normal cells (Simek and Rice 1988b, Tung *et al.* 1988, Morrison *et al.* 1989, Davis *et al.* 1990b, Lim *et al.* 1990). The v/c-*rel* complexes include proteins of about 124 kDa, 115 kDa, and 40 kDa. The most abundant cellular protein associated with the complex is a 40-kDa protein, pp40, that is extensively phosphorylated on serine residues (Tung *et al.* 1988). pp40 has been identified as avian IκB and shows functional, immunological, and structural similarities with mammalian IκB (Davis *et al.* 1991, Haskill *et al.* 1991, Inoue *et al.* 1991, Kerr *et al.* 1991). Although it was demonstrated by coimmunoprecipitation analysis using anti-v-*rel* antiserum that all of the transforming mutant v-*rel* proteins were able to associate with pp40 in transformed CSC as was the wild-type v-*rel* protein (Fig. 13), the association of pp40 with the v-*rel* proteins was further investigated using antiserum specific for pp40 (Davis

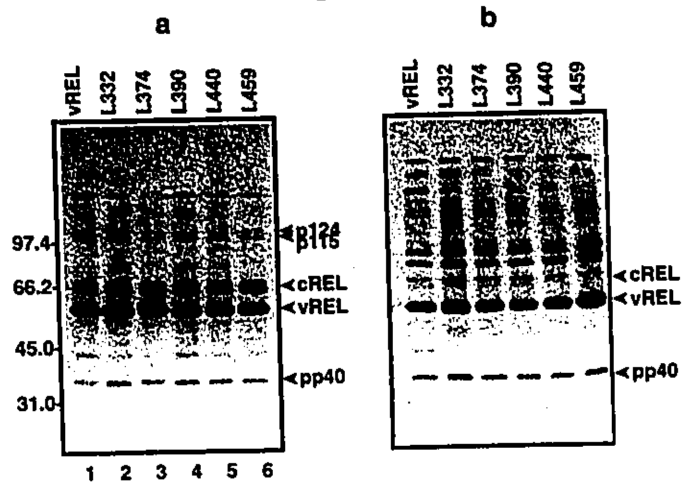
et al. 1990b). The specificity of pp40 antiserum was determined by Western blot analysis, using whole cell extracts obtained from CSC transformed by wild-type v-*rel* (vREL) or linker insertion mutant (L332, L374, L390, L440, L459). The pp40 antiserum recognized a single protein in all of the transformed CSC (Fig. 21A.b) and failed to recognize the v-*rel* protein, which was detected by anti-v-*rel* antiserum (Fig. 21A.a). In order to determine whether cellular proteins associated with the v-*rel* protein can be coprecipitated by anti-pp40 antiserum, immunoprecipitations were performed using extracts obtained from [^{35}S]methionine-labeled CSC with anti-v-*rel* antiserum (Fig. 21B.a) or anti-pp40 antiserum (Fig. 21B.b). The pp40 antisera immunoprecipitated pp40, v-*rel* and c-*rel* proteins (Fig. 21B.b) confirming the physical association of pp40 with v-*rel* or c-*rel* protein. The two high-molecular-weight proteins (p115 and p124), which coprecipitated with anti-v-*rel* antiserum (Fig. 21B.a), did not coprecipitate with anti-pp40 antiserum, suggesting that the transformed CSC may have contained two distinct types of v-*rel* complexes. The amounts of the v-*rel* protein immunoprecipitated from extracts of all of transformed CSC by antisera specific for pp40 and v-*rel* protein were essentially equivalent, suggesting that the complex containing p115 and p124 that lacks pp40 represents a minor complex containing the v-*rel* protein in transformed CSC. These results are consistent with the finding that the c-*rel* protein in normal cells also forms two distinct complexes either with pp40, or with p124 and p115 (Kochel *et al.* 1991).

Figure 21. The association of pp40 with the v-*rel* protein in transformed CSC. (A) Western blot analysis of transformed CSC extracts. Whole cell extracts were prepared from CSC transformed by wild-type v-*rel* (vREL; lane 1), L332 (lane 2), L374 (lane 3), L390 (lane 4), L440 (lane 5), or L459 (lane 6). Whole cell extracts were analyzed by Western blot using either anti-v-*rel* antiserum(a) or anti-pp40 antiserum(b). Arrowheads show positions of the v-*rel* protein or pp40. (B) Immunoprecipitation analysis of transformed CSC. Transformed CSC were labeled with [^{35}S]methionine and the labeled proteins were immunoprecipitated with anti-v-*rel* antiserum(a) or anti-pp40 antiserum(b). Arrowheads indicate the coimmunoprecipitated proteins in addition to the v-*rel* protein and pp40.

A



B



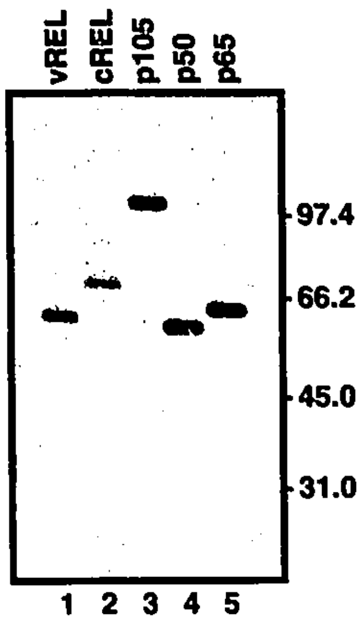
<u>The association of pp40 with the *rel* proteins in insect cells</u>

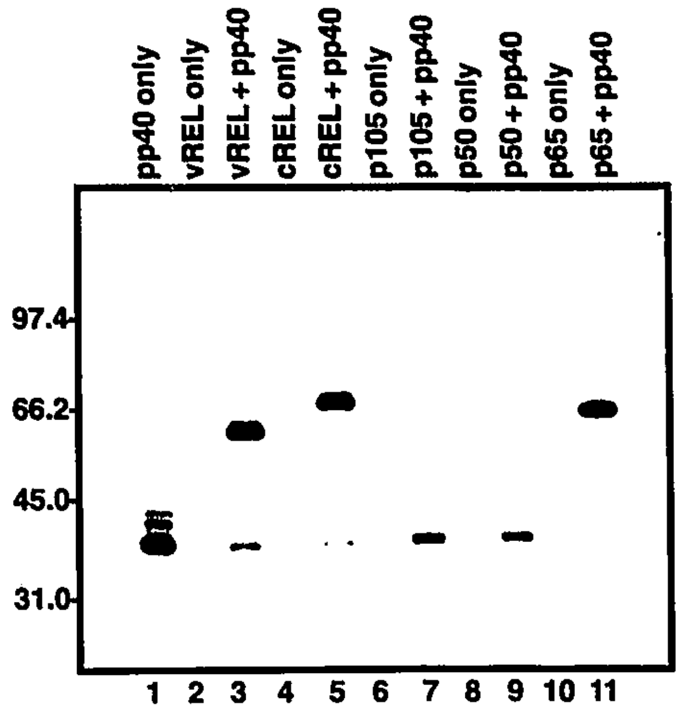
Since all of the transforming mutant v-*rel* proteins associated with pp40 in transformed CSC, the physical association of pp40 with NF-κB subunits, the c-*rel* protein, or the mutant v-*rel* proteins were examined in insect cells in order to determine whether the association with pp40 is critical for transformation.

First, the pp40 gene was amplified by PCR from the pp40 clone (Davis *et al.* 1991) using the 405 and 403 primers (see APPENDIX) which contained *Bam*H I sites. The PCR products were digested with *Bam*H I and were cloned into the pBacPAK1 transfer vector. Recombinant baculoviruses containing the pp40 gene were purified by plaque assay. The expression of pp40 in insect cells was confirmed by immunoprecipitation using extracts prepared from [^{35}S]methionine-labeled Sf9 cells infected with a recombinant baculovirus (lane 1 in Fig. 22B). In addition to the major 40 kDa protein, two more slowly migrating bands were observed following immunoprecipitation with anti-pp40 antiserum (lane 1 in Fig. 22B). These two extra bands probably represent differentially phosphorylated forms of pp40 since these bands disappeared after CIP treatment (data not shown).

Second, in order to examine whether pp40 can directly associate with NF-κB subunits or the v/c-*rel* proteins, Sf9 cells were infected singly with recombinant baculoviruses containing individual genes (v-*rel*, c-*rel*, p105, p50, or p65) or coinfected together with the recombinant baculovirus containing the pp40 gene. Immunoprecipitation analysis of [^{35}S]methionine labeled extracts from individually infected cells using specific antisera (lane 1 and 2; anti-v-*rel* antiserum, lane 3 and 4;

Figure 22. The association of pp40 with NF-κB subunits or *rel* proteins in insect cells. (A) Expression of NF-κB subunits and *rel* proteins in insect cells. Sf9 cells were infected with recombinant baculoviruses containing v-*rel* (vREL; lane 1), c-*rel* (cREL; lane 2), p105 (lane 3), p50 (lane 4), or p65 (lane 5) sequences. Infected cells were labeled with [^{35}S]methionine and labeled proteins were immunoprecipitated using anti-v-*rel* antiserum (lane 1 and 2), anti-p50 antiserum (lane 3 and 4), or anti-p65 antiserum (lane 5). The immunoprecipitates were resolved on an SDS-10% polyacrylamide gel. (B) Coimmunoprecipitation of pp40 with NF-κB subunits or the *rel* proteins. Sf9 cells were infected with recombinant baculoviruses containing the individual genes (lane 1; pp40, lane 2; vREL, lane 4; cREL, lane 6; p105, lane 8; p50, or lane 10; p65) or coinfected with the recombinant baculovirus containing the pp40 gene (lane 3; vREL + pp40, lane 5; cREL + pp40, lane 7; p105 + pp40, lane 9; p50 + pp40, or lane 11; p65 + pp40). Infected cells were labeled and proteins were immunoprecipitated using anti-pp40 antiserum.


A
vREL
cREL
p105
p50
p65
97.4
66.2
45.0
31.0
1 2 3 4 5

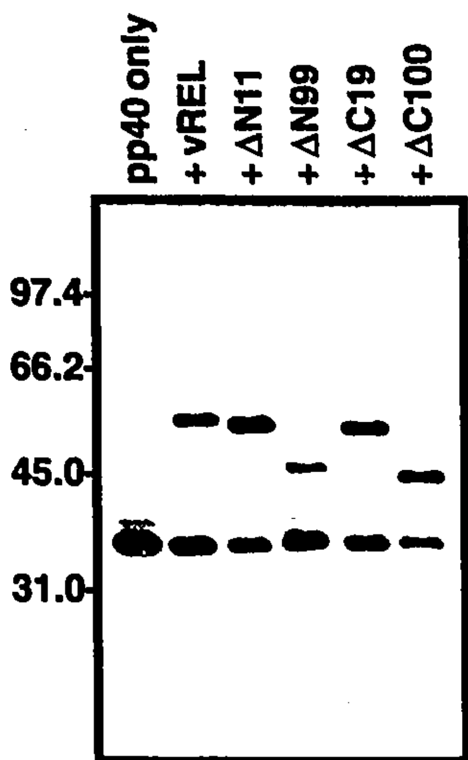

B
pp40 only
vREL only
vREL + pp40
cREL only
cREL + pp40
p105 only
p105 + pp40
p50 only
p50 + pp40
p65 only
p65 + pp40
97.4
66.2
45.0
31.0
1 2 3 4 5 6 7 8 9 10 11

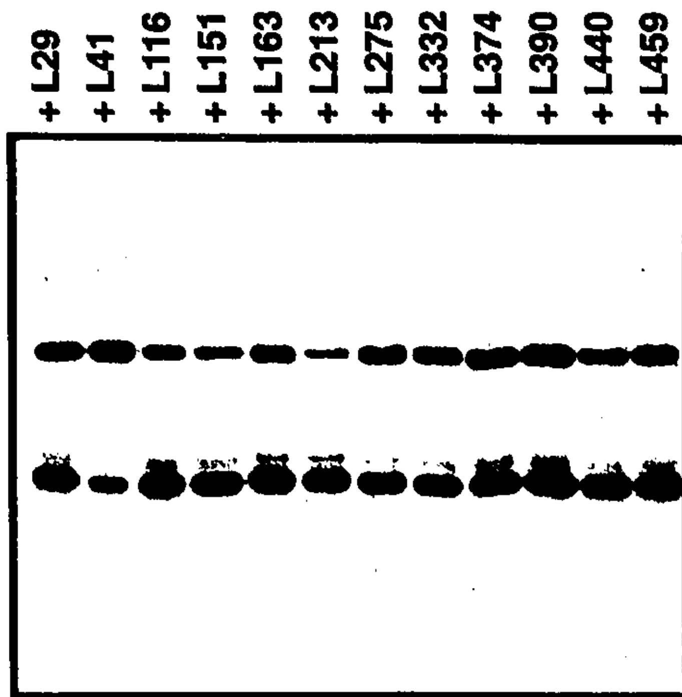
anti-p50 antiserum, lane 5; anti-p65 antiserum) (Fig. 22A) showed that each protein was expressed at comparable levels in insect cells. Further immunoprecipitation analysis of individually infected cells with anti-pp40 antiserum (lanes 2, 4, 6, 8, 10 in Fig. 22B) demonstrated that anti-pp40 antisera lacked reactivity with NF-κB subunits or the v/c-*rel* proteins. However, when these proteins were coexpressed with pp40, pp40 associated with the v/c-*rel* proteins and p65, as evidenced by their coimmunoprecipitation with anti-pp40 antiserum (lanes 3, 5, 11 in Fig. 22B). p105 did not associate with pp40 at all (lane 7 in Fig. 22B), but very little p50 seemed to associate with pp40 (lane 9 in Fig. 22B). These results showed that the v/c-*rel* proteins directly associated with pp40 and pp40 associated mainly with p65 rather than with p50 as did the mammalian IκB (Haskill *et al.* 1991, Inoue *et al.* 1991, Kerr *et al.* 1991).

Third, it was examined whether pp40 can associate with the mutant v-*rel* proteins in order to understand a possible role of pp40 association for transformation. Sf9 cells were coinfected with recombinant baculoviruses containing individual mutant v-*rel* sequences and the recombinant baculovirus containing the pp40 gene, and were metabolically labeled with [^{35}S]methionine. Immunoprecipitation analysis performed with labeled extracts from coinfected cells demonstrated that all of the mutant v-*rel* proteins directly associated with pp40 like the wild-type v-*rel* protein (vREL), as shown by their coimmunoprecipitation with anti-pp40 antiserum (Fig. 23). These results showed that all of the mutant v-*rel* proteins, regardless of their DNA binding activities or their transforming activities, can associate with pp40.

Finally, the effect of pp40 association on DNA binding activity was examined. A

Figure 23. The association of pp40 with the mutant v-*rel* proteins in insect cells. Sf9 cells were coinfected with recombinant baculoviruses containing the indicated mutant v-*rel* sequences and the recombinant baculovirus containing the pp40 gene. Infected cells were labeled with [^{35}S]methionine and the labeled proteins were immunoprecipitated using anti-pp40 antiserum. The immunoprecipitates were resolved on SDS-10% polyacrylamide gels. The bottom bands represent pp40 and upper bands represent the mutant v-*rel* proteins.


pp40 only
+ vREL
+ ΔN11
+ ΔN99
+ ΔC19
+ ΔC100
97.4-
66.2-
45.0-
31.0-


+ L29
+ L41
+ L116
+ L151
+ L163
+ L213
+ L275
+ L332
+ L374
+ L390
+ L440
+ L459

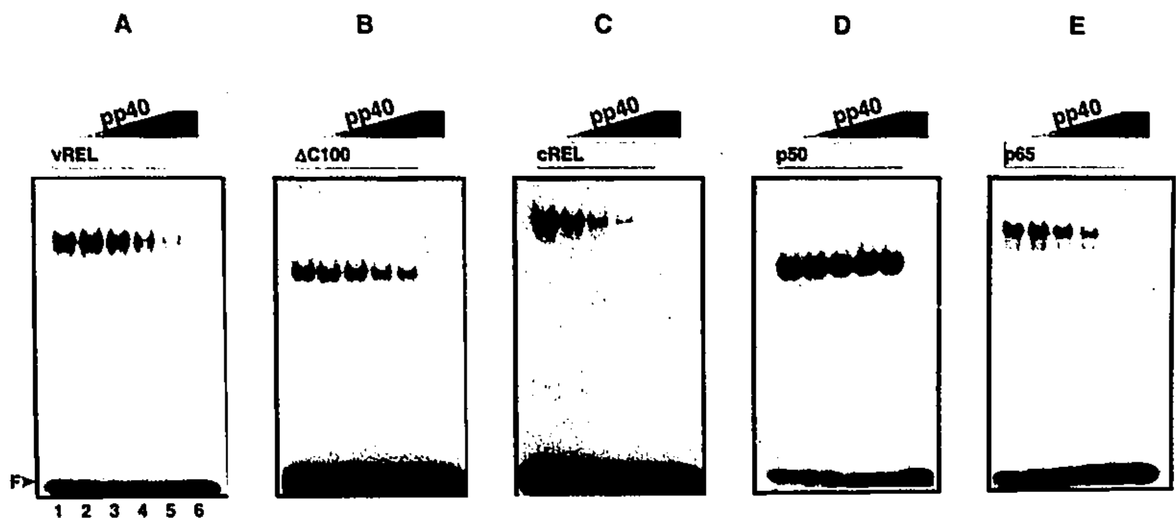
constant amount of whole cell extracts containing v-*rel* protein, ΔC100 v-*rel* protein, c-*rel* protein, p50, or p65 was mixed with increasing amounts of extracts containing pp40 and EMSA was performed with these mixed extracts. As shown in Fig. 24, the DNA binding activities of the v-*rel* protein(A), ΔC100 v-*rel* protein(B), the c-*rel* protein(C), and p65(E) were gradually reduced by the presence of increasing amounts of pp40 in the mixed extracts, indicating that the association of pp40 inhibits the DNA binding activities of *rel* family gene products as does the mammalian IκB (Davis *et al.* 1991, Haskill *et al.* 1991, Inoue *et al.* 1991, Kerr *et al.* 1991). However, no significant inhibition of DNA binding by p50(D) was observed, reflecting the weak physical association of pp40 with p50. Association of pp40 with the other mutant v-*rel* proteins that have DNA binding activity also inhibited their binding to DNA (data not shown).

3. DISCUSSION

Complex formation of the v-*rel* protein with NF-κB subunit and IκB in transformed CSC

In addition to the common *rel* homology region in the N terminus of NF-κB subunits and the c-*rel* protein, complex formation is another feature shared by NF-κB subunits and the c-*rel* protein. The cytoplasmic complex of NF-κB is composed of at least four proteins; p105, p50, p65, and IκB (Baeuerle and Baltimore 1988, Ghosh and Baltimore 1990, Rice *et al.* 1992, Naumann *et al.* 1993). p105 and IκB probably regulate the subcellular distribution and DNA binding activity of two active DNA binding subunits, p50 and p65 (Haskill *et al.* 1991, Kerr *et al.* 1991, Hatada *et al.* 1992, Henkel

Figure 24. The effect of pp40 association on DNA binding activities of NF-κB subunits and the *rel* proteins. Whole cell extracts were prepared from Sf9 cells infected with recombinant baculoviruses containing v-*rel*, ΔC100, c-*rel*, p50, p65, or pp40 genes. A constant amounts of extracts containing NF-κB subunits or *rel* proteins (A; vREL, B; ΔC100, C; cREL, D; p50, or E; p65) were mixed with increasing amounts of extracts containing pp40 . The mixed extracts were used for EMSA. The first lane (lane 1) shows the results obtained with extracts containing only the *rel* proteins and the last lane (lane 6) shows the results obtained with extracts containing only pp40. F indicates the free oligonucleotides.


A
B
C
D
E
pp40
pp40
pp40
pp40
pp40
vREL
ΔC100
cREL
p50
p65
F
1 2 3 4 5 6

et al. 1992). The c-*rel* protein also associates with cellular proteins in the cytoplasm of normal hematopoietic cells (Morrison *et al.* 1989, Davis *et al.* 1990a, Kochel *et al.* 1991). The c-*rel* complex includes proteins of about 40 kDa, 115 kDa, and 124 kDa. These 40-, 115-, and 124-kDa proteins have been identified as avian IκB, avian NF-κB p100, and avian NF-κB p105, respectively (Davis *et al.* 1991, Capobianco *et al.* 1992, Neumann *et al.* 1992, Rice *et al.* 1992, Sif and Gilmore 1993). Taken together, these findings suggested that the subcellular distribution and DNA binding activity of *rel* family gene products are tightly regulated by IκB, p105, and p100. The v-*rel* protein has also been shown to associate with NF-κB p100, NF-κB p105, and IκB in the cytoplasm of transformed cells like the c-*rel* protein (Simek and Rice 1988b, Tung *et al.* 1988, Davis *et al.* 1990b, Lim *et al.* 1990). Furthermore, the results presented here show that all of the transforming mutant v-*rel* proteins containing deletions (ΔC19, ΔC100) or linker insertions (L332, L374, L390, L440, L459) associate with the same set of proteins in transformed cells that complexes with the wild-type v-*rel* protein (Fig. 13), indicating the possible importance of complex formation in transformation. Therefore, it is possible that the v-*rel* protein might transform lymphoid cells by associating with and interfering with the activity of NF-κB and IκB, which are normally complexed with the c-*rel* protein.

The ability of a specific antiserum to precipitate an antigenically unrelated protein is suggestive but not conclusive evidence that the proteins associate to form a heterocomplex. In addition to the ability of anti-v-*rel* antiserum to coprecipitate p124, p115 and pp40, the ability of pp40-specific antiserum to coprecipitate the antigenically

unrelated v-*rel* protein provides conclusive evidence that pp40 and the v-*rel* protein associate to form a heterocomplex in the cytoplasm of transformed cells (Fig. 21). Furthermore, anti-pp40 antiserum permitted the identification of two v-*rel* complexes in the cytoplasm of transformed cells. Since the minor v-*rel* complex contains p124, p115 and the c-*rel* protein but lacks pp40, the pp40-specific antiserum selectively immunoprecipitates the major complex that contains the v-*rel*, c-*rel* proteins and pp40 (Fig. 21). These results are consistent with the data obtained by separation of v-*rel* complexes through gel filtration (Davis *et al.* 1990b). Davis *et al.* (1990b) found that the majority of cytoplasmic v-*rel* protein (80%) is complexed with pp40 and the remaining cytoplasmic v-*rel* protein (20%) is complexed with p124 and p115. In contrast to the v-*rel* protein, chicken c-*rel* protein in normal lymphoid cells is primarily complexed with p124 and p115, and only a small percentage is complexed with pp40 (Morrison *et al.* 1989, Davis *et al.* 1990a, Kochel *et al.* 1991). Thus, the ability of the v-*rel* protein to form complexes with NF-κB and IκB may be different from that of the c-*rel* protein *in vivo*.

It is quite clear that the NF-κB precursors, p100 and p105, can complex with the v-*rel* protein in the cytoplasm of transformed cells. However, the processed forms of p50 and p52 have never been detected in immunoprecipitates from v-*rel* transformed cells with anti-v-*rel* antibody. This is probably because the majority of p50 or p52 in unstimulated cells is likely to be in the precursor form, and therefore the amount of the v-*rel* protein complexed with p50 or p52 may be quite low. On occasion, we have detected the two bands of approximately 50 kDa in v-*rel* immunoprecipitates (indicated

by two arrows inside the box in Fig. 13), and it is likely that these two bands represent p50 and p52 complexed with the v-*rel* protein since it was shown that nuclear extracts of transformed CSC also contain DNA binding proteins of approximately 50 kDa besides the v-*rel* protein (Fig. 9B).

Interaction of mutant v-*rel* proteins with NF-κB subunits

Since the v-*rel* protein was shown to interact with p105 in transformed cells (Fig. 13) and *in vitro* (Kieran *et al.* 1990, Logeat *et al.* 1991), the ability of mutant v-*rel* proteins to interact with NF-κB subunits was investigated in order to determine the importance of complex formation for transformation. Coexpression followed by coimmunoprecipitation analysis in insect cells revealed that all of the mutant v-*rel* proteins, regardless of their transforming activities, were able to interact with NF-κB subunits like the wild-type v-*rel* protein (Table 5). These results clearly demonstrate that interaction of the v-*rel* protein with NF-κB subunits is not sufficient for transformation. Furthermore, the c-*rel* protein also interacts with p105, p50, or p65 (Fig. 15 and Fig. 19), yet the c-*rel* protein is not highly transforming when overexpressed in avian spleen cells (Mosialos *et al.* 1991). However, these results do not exclude the possibility that interaction of the v-*rel* protein with NF-κB subunits is still necessary for transformation.

In theory, *rel* proteins with mutations that interrupt the DNA binding domain but still permit protein interaction could heterodimerize with the *rel* family proteins. The resulting heterodimers would be unable to bind to DNA since they would lack a

TABLE 5

Transforming, DNA binding, and complex formation activity of v-*rel* mutants

Mutant	Transforming activity	Dimerization with NF-κB subunits	Association with pp40	DNA binding activity		
				homo-dimer	hetero-dimer	inhibition by pp40 association
REL	+++++	+	+	+	+	+
ΔN11	+	+	+	+	+	+
ΔN99	–	+	+	–	–	U[a]
ΔC19	+++++	+	+	+	+	+
ΔC100	+	+	+	+	+	+
L29	–	+	+	–	–	U
L41	–	+	+	–	–	U
L116	–	+	+	–	–	U
L151	–	+	+	–	–	U
L163	–	+	+	–	–	U
L213	–	+	+	–	–	U
L275	–	+	+	–	–	U
L332	+++++	+	+	+	+	+
L374	+++++	+	+	+	+	+
L390	+++++	+	+	+	+	+
L440	+++++	+	+	+	+	+
L459	+++++	+	+	+	+	+

[a]U, undetectable

functional DNA binding domain (Herkowitz 1987). Therefore, this type of mutant could trans-dominantly inhibit NF-κB/c-*rel* activity by heterodimerization. In fact, several p50 mutants, which were unable to bind to DNA but were able to form homo- or heterodimers, were shown to function as transdominant inhibitors of NF-κB activity (Logeat *et al.* 1991, Bressler *et al.* 1993). These findings prompted an investigation of the effect of heterodimerization on DNA binding activity since it was shown that all of the mutant v-*rel* proteins, regardless of their DNA binding activities (Table 4), could heterodimerize with NF-κB subunits (Table 5). The analysis of DNA binding activities of heterodimers demonstrated that all of the transforming-, DNA binding mutant v-*rel* proteins formed active heterodimers with NF-κB subunits, whereas all of the non-transforming, non-DNA binding mutant v-*rel* proteins formed inactive heterodimers with NF-κB subunits (Fig. 18 and Fig. 20). Furthermore, these non-transforming, non-DNA binding mutant v-*rel* proteins also formed inactive heterodimers with the wild-type v-*rel* or c-*rel* proteins (data not shown). Therefore, the non-transforming mutant v-*rel* proteins functioned as transdominant inhibitors of *rel* family proteins. Taken together, these results suggest that the formation of active heterodimers by the v-*rel* protein with NF-κB subunits is necessary for transformation. These results are also consistent with the fact that the transforming v-*rel* proteins exist as heterodimers with 50-kDa proteins in nuclear extracts of all of the transformed CSC (Fig. 9B).

The results also indicate that the v-*rel* subregion involved in dimerization is distinct from the functional DNA binding domain since all of the mutant v-*rel* proteins, regardless of their DNA binding activities, were shown to heterodimerize with NF-κB

subunits. Previously, several mutational analyses of p50, p65, c-*rel*, and *dorsal* located the dimerization domain to the C-distal part of the *rel* homology region (RHR) (Ip *et al.* 1991, Logeat *et al.* 1991, Ruben *et al.* 1992, Bressler *et al.* 1993, Doerre *et al.* 1993). More recently, studies of tertiary structure of p50 homodimers revealed that the 300 amino acid RHR consists of two distinct domains (Ghosh *et al.* 1995, Müller *et al.* 1995). Domain I includes the N-terminal 200 amino acids and plays an important role mainly in DNA binding. Domain II includes the remaining 100 amino acids and plays important roles both in DNA binding and dimerization. Therefore, only two of the v-*rel* mutants, L213 and L275, contained mutations in the dimerization domain. Both of these mutant v-*rel* proteins retained dimerization activity, but lost the DNA binding and transformation activity. These results imply that the four amino acid insertion in this region might have enough structural impact on the DNA binding loops, directly or indirectly, to abolish the DNA binding activity, but not enough structural impact on the dimerization α-helix to disrupt dimerization.

Association of pp40 with the mutant v-*rel* proteins

pp40, a 40-kDa phosphoprotein, was originally identified as a major v-*rel*-associated protein in chicken lymphoid cells transformed by REV-T (Davis *et al.* 1990b). The cloning of pp40 revealed that pp40 has functional, immunological, and structural similarities with mammalian IκB (Davis *et al.* 1991, Haskill *et al.* 1991, Inoue *et al.* 1991, Kerr *et al.* 1991), which prevents translocation of NF-κB into the nucleus and inhibits the binding of NF-κB to DNA by association with NF-κB (Baeuerle and

Baltimore 1988). It was shown that pp40 can directly associate with NF-κB subunits and the v/c-*rel* proteins in insect cells (Fig. 22B) as shown *in vitro* (Inoue *et al.* 1991, Kerr *et al.* 1991), and the association of pp40 with *rel* family proteins inhibits their binding to the κB site (Fig. 24). Furthermore, all of the mutant v-*rel* proteins were physically associated with pp40 in insect cells like wild-type v-*rel* protein (Table 5). First, these results suggest that the association of v-*rel* protein with pp40 alone is not sufficient for transformation since all of the mutant v-*rel* proteins, regardless of their transforming activities (Table 3), were shown to associate with pp40 (Table 5). Second, these results indicate that the v-*rel* subregion involved in pp40 association is distinct from the functional DNA binding domain since all of the mutant v-*rel* proteins, regardless of their DNA binding activities (Table 4), associated with pp40 (Table 5). These results are consistent with previous reports suggesting that pp40 (or IκB) associates with the nuclear localization signal (NLS) of *rel* family proteins (Beg *et al.* 1992, Ganchi *et al.* 1992, Kumar and Gélinas 1993) since all of the v-*rel* mutants described here contain the intact NLS of wild-type v-*rel*.

CHAPTER 5: THE TRANSACTIVATION ACTIVITY OF THE V-REL ONCOGENE PRODUCT

1. INTRODUCTION

The mechanisms governing cell transformation by oncogenes remain to be established. Some oncoproteins have been shown to alter the expression of various reporter genes. It is likely that altered transcriptional regulation of specific cellular genes by these oncoproteins plays a key role in the transformation process. By use of transient transfection assays, it was shown that expression of v-*rel* protein in rodent fibroblasts resulted in activation of certain promoters, including the polyomavirus early and late promoters, the simian virus 40 early promoter, and the long terminal repeats of both avian and murine retroviruses (Gélinas and Temin 1988, Hannink and Temin 1989). The chicken and mouse c-*rel* proteins have subsequently been shown to activate gene expression in yeast when bound to DNA through Gal4 or LexA repressor/operator interactions (Bull *et al.* 1990, Kamens *et al.* 1990).

Although the transactivation activities of the *rel* proteins were detected either using transient transfection assays or using LexA and Gal4 systems, the target DNA sequence for the *rel* proteins has not been defined until the discovery that transcription factor NF-κB shows homology with the *rel* proteins in their 300 N-terminal amino acids (Bours *et al.* 1990, Ghosh *et al.* 1990, Kieran *et al.* 1990, Meyer *et al.* 1991, Nolan *et al.* 1991, Ruben *et al.* 1991). This finding suggests that the *rel* proteins like NF-κB might act as transcription factors through the κB site of target genes. Since it was shown that

the v-*rel* protein directly binds to the κB site, the first objective of this study was to examine κB-dependent transcriptional activity of the v-*rel* protein. Also, the v-*rel* protein could form heterodimers with NF-κB subunits and could associate with IκB (pp40), the second objective of this study was to determine the effects of heterodimerization and IκB association on κB-dependent transcriptional activity.

To address these questions, p50, p65, c-*rel*, v-*rel*, and pp40 genes were cloned into the pSVL vector for the expression of these genes in Cos1 cells. The transcriptional activities of these gene products were examined by cotransfection of a CAT reporter plasmid containing κB sites in front of promoter. The effects of heterodimerization and IκB association on transcriptional activity were determined by coexpression of these genes in the presence of the CAT reporter plasmid. The relationship between the transcriptional activity and transformation is also discussed.

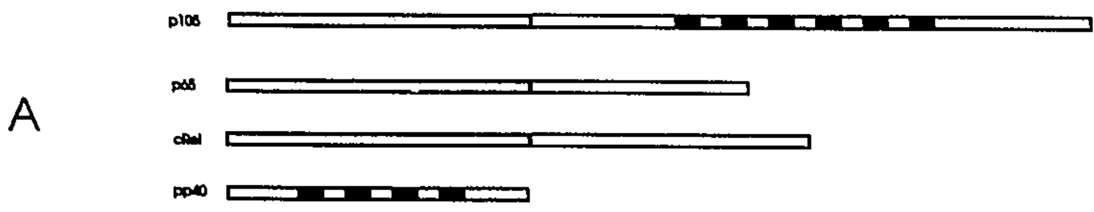
2. RESULTS

Transient expression of *rel* family genes in Cos1 cells

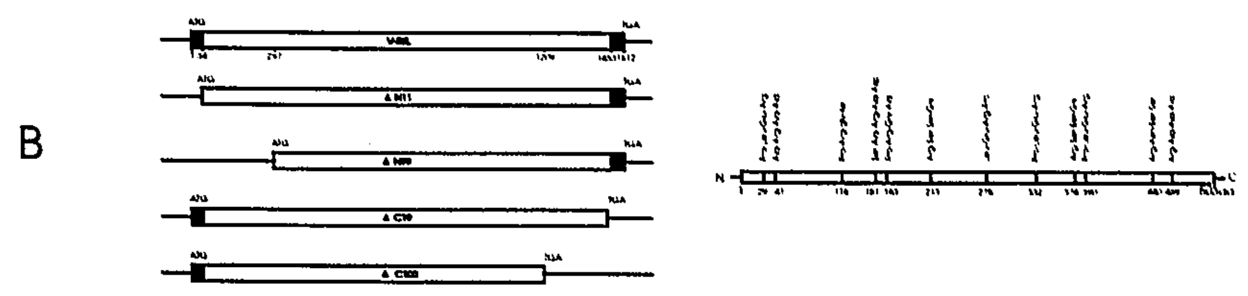
In order to examine the transcriptional activities of *rel* family proteins in Cos1 cells, we first constructed the producer plasmids which direct the expression of *rel* family genes in Cos1 cells. As described in Chapter 3 and Chapter 4, the same PCR products of NF-κB p105, NF-κB p65, c-*rel*, v-*rel* and its mutants used for cloning into pBacPAK vector were cloned into the *Bam*H I site of the pSVL vector, which contains the replication origin of SV40 for high level episomal replication in cell lines expressing the SV40 large T antigen, such as Cos1 cell (Fig. 25). The expression of the inserted

Figure 25. Strategy for the characterization of transactivation activity of NF-κB subunits or the *rel* proteins. (A) The schematic presentation of p105, p65, c-*rel*, and pp40 gene. The shaded boxes and black boxes represent the *rel* homology region and ankyrin repeats respectively. (B) The schematic presentation of deletion and linker insertion mutants of v-*rel*. (C) The structure of pSVL vector which is used for expression of *rel* family genes in Cos1 cells. (D) The reporter plasmid which has wild type (J16) or mutant (J32) κB motif in 5'-upstream region of CAT gene. The boxed portions of sequences indicate the wild type κB motifs and altered bases in mutant κB are shown below the wild type sequences.

A

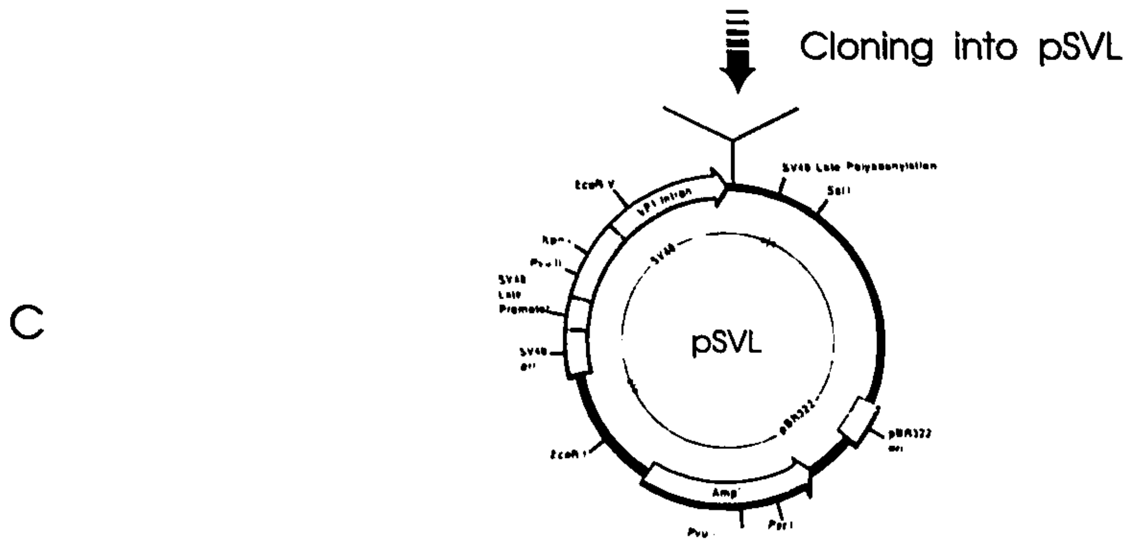


B

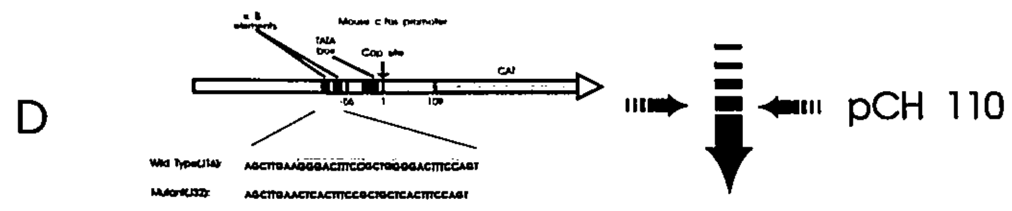


Cloning into pSVL

C



D



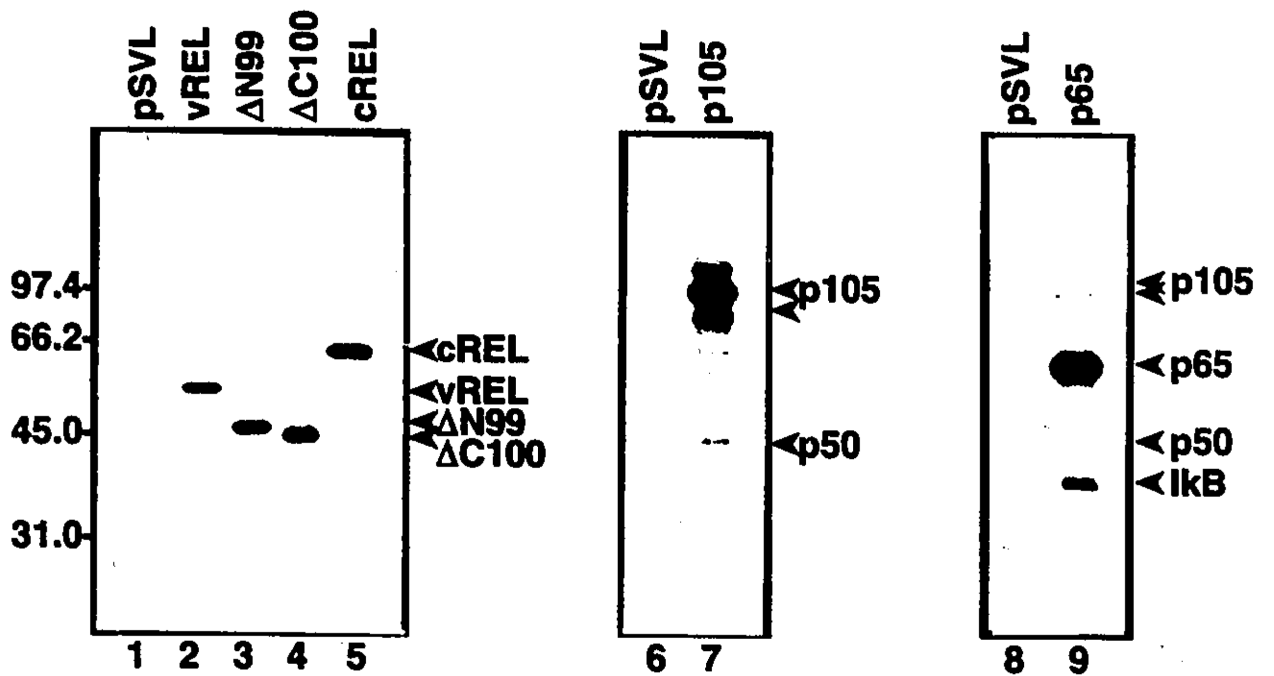
pCH 110

Cotransfection into Cos1 cells

CAT Assay

gene is directed by the strong SV40 late promoter. The pSVL clone containing wild-type v-*rel*, ΔN99, ΔC100, or c-*rel* gene was expressed transiently in Cos1 cells by transfection. Immunoprecipitation analysis of labeled extracts prepared from transfected Cos1 cells using anti-v-*rel* antiserum confirmed that the wild-type v-*rel* protein, mutant v-*rel* proteins (ΔN99, ΔC100) and the c-*rel* protein were expressed at comparable levels (Fig. 26, lane 2 to 5). The expression of NF-κB p105 was also confirmed by immunoprecipitation analysis of Cos1 cells transfected with pSVL clone containing p105 using anti-p50 antiserum (Fig. 26, lane 7). However, several additional bands were observed in addition to p105 and p50. These bands may correspond to differently processed forms of p105, which were also observed by several groups (Blank *et al.* 1991, Henkel *et al.* 1992, Liou *et al.* 1992). In the case of p65, immunoprecipitation using anti-p65 antiserum coprecipitated several proteins of 105-, 100-, 50-, 36-kDa in addition to p65 (Fig. 26, lane 9). p105, p100, p50 were subsequently identified as NF-κB p105 related proteins and p36 was identified as IκB using immunoprecipitation with anti-p50 antiserum and anti-IκB antiserum respectively (data not shown). Since neither of these gene products were detected in Cos1 cells transfected with pSVL vector alone nor were these gene products produced by transfection, these results indicated that the transient expression of p65 induced the expression of endogenous p105 and IκB. These results are consistent with previous data suggesting that the expression of p65 induces the expression of p105 and IκB, which contain κB sites in their promoters, for autoregulation of NF-κB activity (Ten *et al.* 1992, Brown *et al.* 1993, Le Bail *et al.* 1993, Rice and Ernst 1993, Scheinman *et al.* 1993,

Figure 26. The transient expression of *rel* family genes in Cos1 cells. Cos1 cells were transfected with pSVL without an insert (lane 1, 6, 8) or pSVL containing v-*rel* (lane 2), ΔN99 (lane 3), ΔC100 (lane 4), c-*rel* (lane 5), p105 (lane 7) or p65 (lane 9) genes. Transfected Cos1 cells were metabolically labeled with [^{35}S]methionine at 40 hr post transfection and the labeled proteins were immunoprecipitated using anti-v-*rel* antiserum (lanes 1-5), anti-p50 antiserum (lane 6 and 7), or anti-p65 antiserum (lane 8 and 9). The immunoprecipitates were resolved on SDS-10% polyacrylamide gels. Positions of immunoprecipitated and coimmunoprecipitated proteins are shown on the right.


pSVL
vREL
ΔN99
ΔC100
cREL
97.4
66.2
45.0
31.0
cREL
vREL
ΔN99
ΔC100
1
2
3
4
5
pSVL
p105
p105
p50
6
7
pSVL
p65
p105
p65
p50
IkB
8
9

Scott *et al.* 1993, Sun *et al.* 1993, Chiao *et al.* 1994).

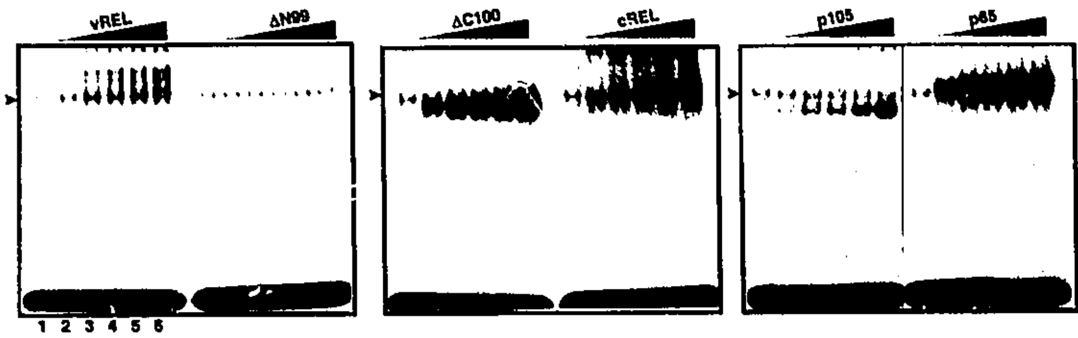
<u>DNA binding and transcriptional activities of *rel* family proteins expressed in Cos1 cells</u>

In order to confirm the DNA binding activities of *rel* family proteins expressed in Cos1 cells, Cos1 cells were transfected with graded amounts (lane 1; 0, Lane 2; 0.5 μg, lane 3; 1 μg, lane 4; 1.5 μg, lane 5; 2 μg, lane 6; 2.5 μg) of each pSVL clone containing mutant (ΔN99, ΔC100) or wild-type v-*rel*, c-*rel*, NF-κB p105, or NF-κB p65 gene, and EMSA was performed with nuclear extracts of transfected Cos1 cells using [^{32}P]-labeled κB oligonucleotides. As shown in Fig. 27A, all of the nuclear extracts from transfected cells including nuclear extracts from cells transfected with pSVL without insert (lane 1) contained a common DNA-protein complex which was presumed to represent DNA binding by endogenous NF-κB of Cos1 cells (marked by arrowhead). In addition, each *rel* family protein except ΔN99 displayed an additional DNA-protein complexes in a dose-dependent manner. These complexes were assumed to represent DNA binding activity of the v-*rel*, ΔC100, c-*rel* protein, processed form of p105, or p65 respectively. However, no additional complexes other than endogenous NF-κB complex was detected in nuclear extracts from cells transfected with ΔN99, reflecting the inability of the ΔN99 protein to bind DNA.

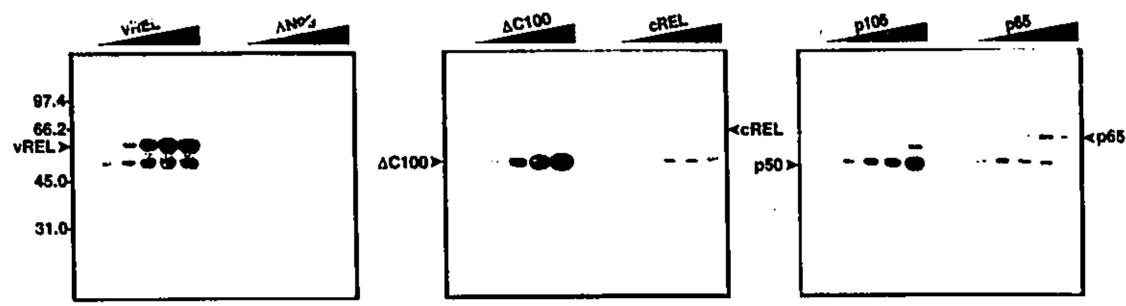
To further determine the identities of proteins involved in the unique DNA-protein complexes detected by EMSA, UV cross-linking experiments were performed. The same nuclear extracts used for EMSA were incubated with [^{32}P]-labeled photoreactive

Figure 27. DNA binding activities of *rel* family proteins expressed in Cos1 cells. (A) EMSA of NF-κB subunits and the *rel* proteins expressed in Cos1 cells. Cos1 cells were transfected with pSVL alone (lane 1) or with increasing amounts of pSVL containing the v-*rel* gene (lane 2; 0.5 μg, lane 3; 1 μg, lane 4; 1.5 μg, lane 5; 2 μg, or lane 6; 2.5 μg). The total amount of transfected DNA was held constant by addition of varying amounts of the pSVL vector. The same conditions were used for transfection with pSVL containing ΔN99, ΔC100, c-*rel*, p105, or p65 genes. Nuclear extracts were prepared from transfected Cos1 cells at 48 hr post transfection and were used for EMSA. The arrowhead indicates a complex that is believed to represent endogenous NF-κB. (B) UV cross-linking analysis of κB binding proteins in transfected Cos1 cells. The extracts used in (A) were incubated with [^{32}P]-labeled photoreactive κB probe, irradiated with UV-light, and immunoprecipitated with anti-v-*rel* antiserum (for vREL, ΔN99, ΔC100, and cREL), anti-p50 antiserum (for p105), or anti-p65 antiserum (for p65). The immunoprecipitates were resolved on SDS-10% polyacrylamide gels. Arrowheads indicate the κB-bound proteins.

A



B

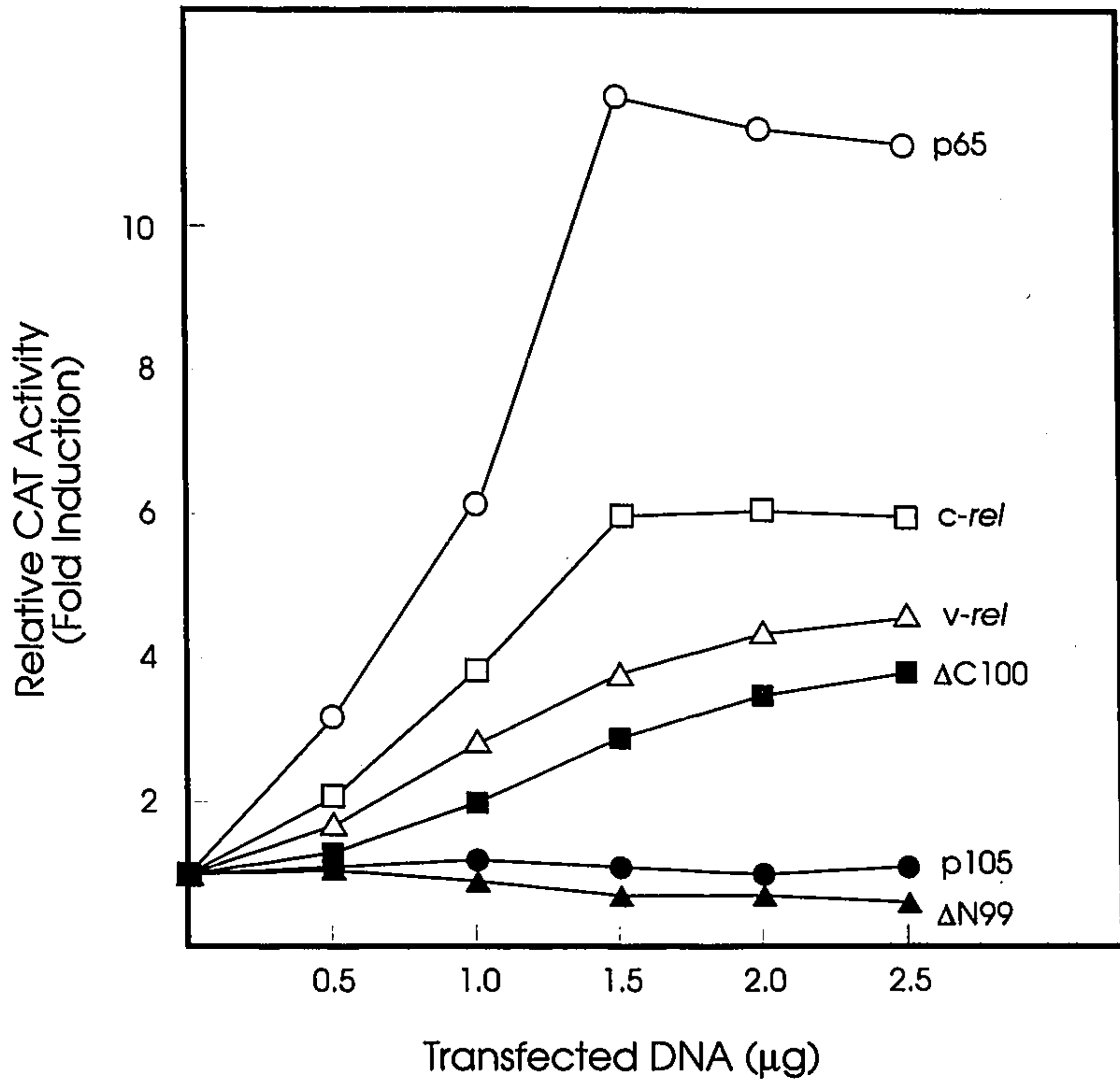


κB oligonucleotides, irradiated with UV-light, and immunoprecipitated with specific antisera. As shown in Fig. 27B, immunoprecipitation analysis of nuclear extracts from Cos1 cells transfected with graded amounts of pSVL containing the wild-type v-*rel*, ΔC100, or c-*rel* genes using anti-v-*rel* antiserum demonstrated that the major DNA-protein adducts were composed of wild-type v-*rel*, ΔC100, or c-*rel* proteins respectively. Furthermore, the amounts of these major DNA-protein adducts immunoprecipitated with anti-v-*rel* antiserum were dose-dependent. However, no DNA-protein adduct was detected in nuclear extracts from cells transfected with the ΔN99 construct, confirming that ΔN99 protein has no DNA binding activity. Immunoprecipitation of nuclear extracts from cells transfected with p105 or p65 genes using anti-p50 antiserum or anti-p65 antiserum also showed that the major DNA-protein adducts composed of p50 or p65 respectively. An additional 50-kDa adduct was detected in all of the immunoprecipitates of nuclear extracts from cells transfected with v-*rel*, ΔC100, c-*rel*, and p65 genes. In the case of ΔC100, this 50-kDa adduct was overlapped with ΔC100 protein because of their similar sizes. The 50-kDa adduct was subsequently identified as endogenous p50 by immunoprecipitation using anti-p50 antiserum (data not shown). Therefore, these results demonstrated that transiently expressed p65, v-*rel*, ΔC100, and c-*rel* proteins bind DNA as heterodimers with endogenous p50 in Cos1 cells.

Since the κB-specific DNA binding activities of *rel* family proteins expressed transiently in Cos1 cells had been confirmed, the κB-dependent transcriptional activities of *rel* family proteins using CAT reporter plasmid (Fig. 25D) were examined. The reporter plasmid contained a truncated c-*fos* promoter, which included only 59 bp

upstream from the transcription start site, linked to the CAT gene. Two tandem copies of wild-type NF-κB motifs (J16) or mutant NF-κB motifs (J32) were inserted at the 5'-end of the truncated promoter (Pierce *et al.* 1988, Lenardo *et al.* 1989). Because the inserted oligonucleotide did not interrupt the transcribed region, differences in CAT activity were assumed to reflect differences in the frequency of transcription. In order to determine the κB-dependent transcriptional activities of *rel* family proteins, Cos1 cells were cotransfected with CAT reporter plasmid J16 and the graded amounts of pSVL containing wild-type or mutant v-*rel* (ΔC100, ΔN99), c-*rel*, p105, or p65 genes. Cotransfection of CAT reporter plasmid J16 and pSVL vector gave a background of 5-6% conversion of chloramphenicol to its acetylated form, which was assumed to reflect the endogenous NF-κB activity of Cos1 cells. The activity of *rel* family proteins was compared to this basal level to determine relative CAT activity. As shown in Fig. 28, p65 markedly induced transcription up to 10-fold above the basal level in a dose-dependent manner, whereas p105 failed to significantly activate transcription even at high doses of input expression plasmid. These results confirmed that the p65 subunit of NF-κB, but not the p50 subunit of NF-κB, functions as a potent activator of κB-dependent transcription since p65 contains a C-terminal transcriptional activation domain (Ballard *et al.* 1992, Ruben *et al.* 1992). Consistent with prior reports (Bull *et al.* 1990, Kamens *et al.* 1990, Inoue *et al.* 1991), the c-*rel* protein induced transcription significantly (up to 6-fold) in a dose-dependent manner. The v-*rel* and ΔC100 proteins also stimulated transcription up to 4- or 3-fold above the basal level, respectively in a dose-dependent manner, whereas ΔN99 protein showed no enhancement of

Figure 28. The κB-dependent transcriptional activities of *rel* family proteins expressed transiently in Cos1 cells. Cos1 cells were cotransfected with CAT reporter plasmid J16 and increasing amounts of pSVL containing v-*rel*, ΔN99, ΔC100, c-*rel*, p105, or p65 genes. The total amount of transfected DNA was held constant by addition of varying amounts of the pSVL vector. Whole cell extracts were prepared from transfected cells at 48 hr post transfection, and were incubated with [^{14}C]chloramphenicol and n-butyl CoA. Acetylated forms of chloramphenicol were extracted by xylene, and cpm were counted in a β-scintillation counter. Results of four independent experiments are expressed as the mean fold increase in CAT activity relative to the value of CAT reporter induced by pSVL vector only. In all cases, standard errors of the mean were less than 15%.


p65
c-rel
v-rel
ΔC100
p105
ΔN99
Relative CAT Activity
(Fold Induction)
10
8
6
4
2
0.5
1.0
1.5
2.0
2.5
Transfected DNA (μg)

transcription at all. These results demonstrated that the v-*rel* protein could act as a transactivator of κB-dependent transcription like the c-*rel* protein, even though the v-*rel* protein might be a less potent activator than the c-*rel* protein. The weak transactivation activity of ΔC100 protein compared to the wild-type v-*rel* protein indicated the importance of the C-terminal sequences of v-*rel* for transactivation since both the v-*rel* and ΔC100 protein showed comparable κB-specific DNA binding activities (Fig. 27). The inability of ΔN99 protein to induce the κB-dependent transcription seemed to be due to lack of κB-specific DNA binding activity of ΔN99. The κB-dependence of transactivation by p65, the c-*rel* protein, the wild-type v-*rel* or mutant (ΔC100) proteins was further suggested by the fact that transactivation by *rel* family proteins were completely abolished when CAT reporter plasmid J32 which contained mutant NF-κB motif was used (data not shown).

The κB-dependent transcriptional activities of mutant v-*rel* proteins expressed in Cos1 cells

Since the wild-type v-*rel* protein expressed in Cos1 cells showed κB-dependent transactivation, I next examined the κB-dependent transcriptional activities of mutant v-*rel* proteins in order to examine the importance of transactivation activity for transformation. For this purpose, the deletion and linker insertion mutants of v-*rel* were cloned into the *Bam*H I site of the pSVL vector (Fig. 25B).

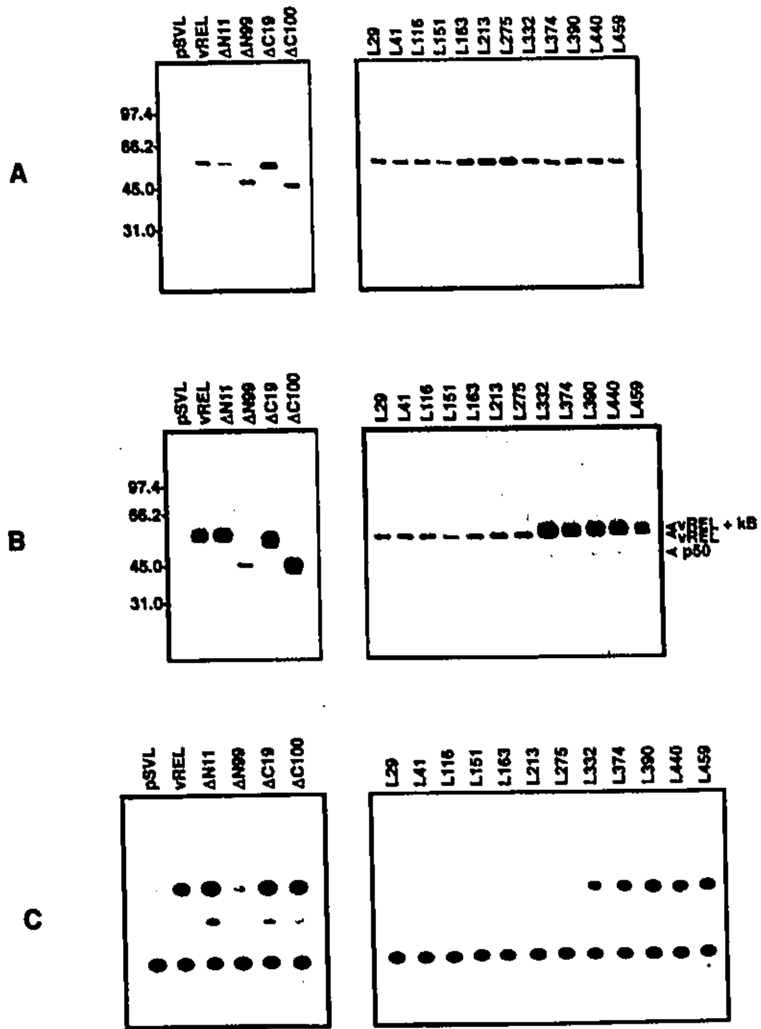
First, Cos1 cells were transfected with pSVL containing deletion or linker insertion mutants of v-*rel* in order to demonstrate the transient expression of mutant v-*rel*

proteins. Transfected Cos1 cells were metabolically labeled with [^{35}S]methionine and labeled proteins were immunoprecipitated with anti-v-*rel* antiserum. As shown in Fig. 29A, transfected Cos1 cells expressed the wild-type or mutant v-*rel* proteins of appropriate sizes at comparable levels.

Second, UV cross-linking experiments were performed in order to confirm the κB-specific DNA binding activities of mutant v-*rel* proteins transiently expressed in Cos1 cells. The labeled extracts prepared from transfected Cos1 cells were incubated with [^{32}P]-labeled photoreactive κB probe, irradiated with UV-light, and immunoprecipitated with anti-v-*rel* antiserum. As shown in Fig. 29B, all of the transforming mutant v-*rel* proteins formed the DNA-protein adducts approximately 5 kDa larger than labeled v-*rel* proteins that were also specifically immunoprecipitated by anti-v-*rel* antiserum. An additional 50-kDa adduct, which was identified as endogenous p50, was detected by UV cross-linking analysis for all of the transforming mutant v-*rel* proteins. In contrast, no DNA-protein adduct was detected by UV cross-linking analysis of the non-transforming mutant v-*rel* proteins. These results confirmed that all of the transforming mutant v-*rel* proteins retained DNA binding activity of the wild-type v-*rel* protein, whereas all of the non-transforming mutant v-*rel* proteins lost DNA binding activity. Furthermore, these results demonstrated that, like the wild-type v-*rel* protein, all of the transforming mutant v-*rel* proteins bind DNA as heterodimers with endogenous p50 in Cos1 cells (Fig. 27B).

Finally, Cos1 cells were cotransfected with CAT reporter plasmid J16 and pSVL containing deletion or linker insertion mutants of v-*rel* to investigate the κB-dependent

Figure 29. The κB-dependent transcriptional activities of mutant v-*rel* proteins expressed transiently in Cos1 cells. (A) Expression of v-*rel* mutants in Cos1 cells. Cos1 cells were transfected with pSVL, or pSVL containing the indicated mutant v-*rel* genes and were labeled with [^{35}S]methionine at 40 hr post transfection. Half of labeled extracts from transfected Cos1 cells were immunoprecipitated with anti-v-*rel* antiserum and the immunoprecipitates were resolved on SDS-10% polyacrylamide gels. (B) DNA binding activities of mutant v-*rel* proteins. The other half of labeled extracts from (A) were first incubated with [^{32}P]-labeled photoreactive κB probe, irradiated with UV and then immunoprecipitated with anti-v-*rel* antiserum. The immunoprecipitates were resolved on SDS-10% polyacrylamide gels. Arrowheads indicate the κB-bound vREL, vREL only, or coimmunoprecipitated p50. (C) The transactivation activities of mutant v-*rel* proteins. Cos1 cells were cotransfected with CAT reporter plasmid J16 and pSVL containing the indicated mutant v-*rel* genes. Cells were harvested at 48 hr post transfection and whole cell extracts were prepared by three cycles of freezing and thawing. CAT assays were performed by incubation of whole cell extracts with [^{14}C]chloramphenicol and n-butyl CoA. CAT activity was analyzed by TLC.



A
pSVL vREL ΔN11 ΔN99 ΔC19 ΔC100
L29 L41 L116 L151 L163 L213 L275 L332 L374 L390 L440 L459
97.4
66.2
45.0
31.0
B
pSVL vREL ΔN11 ΔN99 ΔC19 ΔC100
L29 L41 L116 L151 L163 L213 L275 L332 L374 L390 L440 L459
97.4
66.2
45.0
31.0
vREL + kB
vREL
p50
C
pSVL vREL ΔN11 ΔN99 ΔC19 ΔC100
L29 L41 L116 L151 L163 L213 L275 L332 L374 L390 L440 L459

transcriptional activities of mutant v-*rel* proteins. As shown in Fig. 29C, all of the transforming mutant v-*rel* proteins induced transcription up to 3-4 fold above the basal level, whereas all of the non-transforming mutant v-*rel* protein showed no enhancement of transcription. Transactivation by the transforming mutant v-*rel* proteins was completely abolished when the CAT reporter plasmid J32, which contained mutant NF-κB motif was used (data not shown). These results showed that all of the transforming mutant v-*rel* proteins retained the κB-dependent transactivation activity of the wild-type v-*rel* protein, whereas all of the non-transforming mutant v-*rel* proteins lost transactivation activity. Also, these results suggested that the activation of gene expression directly by v-*rel* protein might be required for oncogenic transformation.

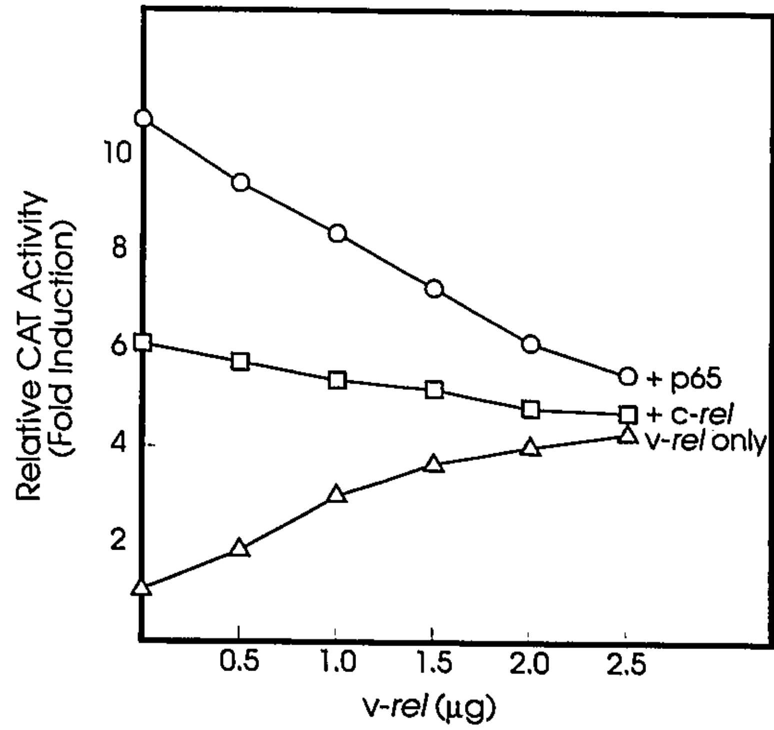
Functional effect of v-*rel* expression on κB-dependent transactivation activities of p65 and the c-*rel* protein

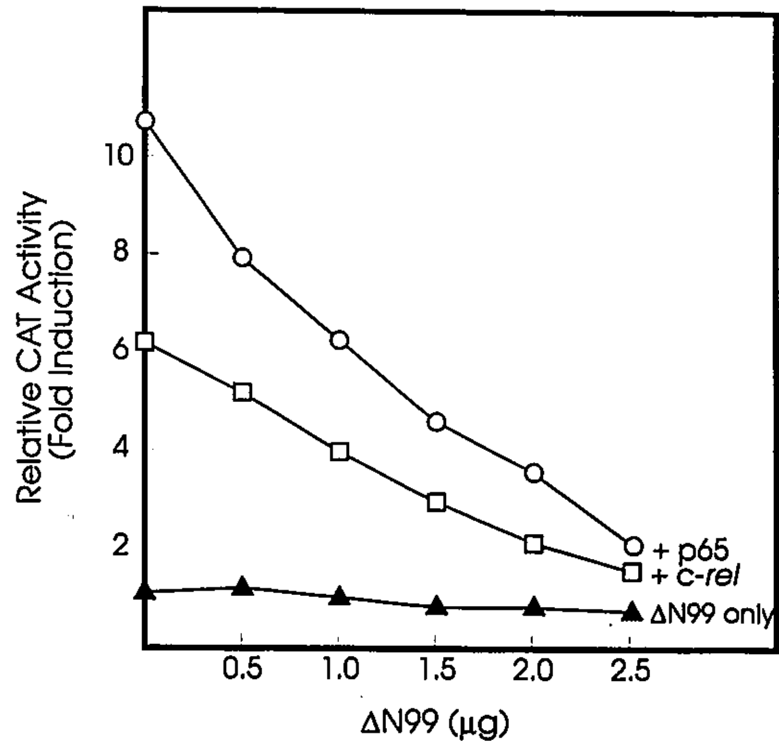
Although the v-*rel* protein expressed in Cos1 cells showed transactivation activity through κB site binding, it displayed the lower transactivation potential than the normal cellular transcription factors, p65 and the c-*rel* protein (Fig. 28). Furthermore, the v-*rel* protein was shown to heterodimerize with NF-κB subunits (Fig. 18 and Fig. 19) and possibly with the c-*rel* protein. These results prompted an investigation of whether the expression of the v-*rel* protein could interfere with κB-dependent transactivation activity mediated by p65 or the c-*rel* protein. To explore the functional consequences of simultaneous expression of the v-*rel* protein and p65, Cos1 cells were cotransfected with CAT reporter plasmid J16, a fixed amount of the p65 expression

plasmid, and graded amounts of the v-*rel* expression plasmid (Fig. 30A). In the absence of the v-*rel* protein, p65 activated transcription up to 10-fold over basal levels. However, coexpression of the v-*rel* protein in these cells markedly repressed transactivation by p65 down to 4-fold over basal levels in a dose-dependent manner. Similar antagonistic effects of the v-*rel* protein were observed with the c-*rel* protein (Fig. 30A). In spite of the antagonistic effects of the v-*rel* protein, transactivation mediated by p65 or the c-*rel* protein was not repressed lower than the level of transactivation stimulated by the v-*rel* protein alone (Fig. 30A). All of the transforming mutant v-*rel* proteins like the wild-type v-*rel* protein showed similar inhibitory effects on κB-dependent transactivation by p65 or the c-*rel* protein (data not shown).

In contrast to the limited inhibitory effects of the transforming mutant v-*rel* proteins or the wild-type v-*rel* protein on transactivation by p65 and the c-*rel* protein, non-transforming mutant ΔN99 protein completely repressed the transactivation by p65 and the c-*rel* protein down to basal levels in a dose-dependent manner (Fig. 30B). All of the non-transforming mutant v-*rel* proteins also showed complete inhibition of κB-dependent transactivation activity mediated by p65, the c-*rel* protein, or even the wild-type v-*rel* protein (data not shown). Since all of the non-transforming mutant v-*rel* proteins were shown to function as dominant negative mutants and formed non-DNA binding heterodimers with p50 or p65, the inhibitory effects of these mutants on κB-dependent transactivation appeared to be the result of complex formation by heterodimerizations. UV cross-linking analysis confirmed that coexpression of the non-transforming v-*rel* mutants with p65, or c-*rel* abrogated the DNA binding activity of

Figure 30. Divergent functional effects of the wild-type v-*rel* protein and ΔN99 proteins on transactivation by p65 or the c-*rel* protein. Cos1 cells were cotransfected with CAT reporter plasmid J16 and increasing amounts of pSVL containing v-*rel*(A) or ΔN99(B) sequences in the presence of plasmids expressing p65 or c-*rel*. The total amount of transfected DNA was held constant by addition of varying amounts of the pSVL vector. Results of these independent experiments are expressed as the mean fold increase in CAT activity relative to the value of CAT reporter plasmid induced by the pSVL vector only. In all cases, standard errors of the mean were less than 15%.


A
Relative CAT Activity
(Fold Induction)
10
8
6
4
2
0.5
1.0
1.5
2.0
2.5
v-rel (μg)
+ p65
+ c-rel
v-rel only


B
Relative CAT Activity
(Fold Induction)
10
8
6
4
2
0.5
1.0
1.5
2.0
2.5
ΔN99 (μg)
+ p65
+ c-rel
ΔN99 only

p65, or the c-*rel* protein (data not shown). Taken together, these results demonstrated that all of the non-transforming, non-DNA binding mutant v-*rel* proteins can function as transdominant inhibitors of *rel* family transcription factors.

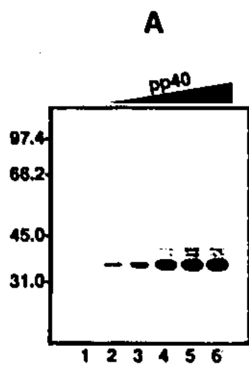
<u>The functional effect of pp40 on κB-dependent transactivation activity</u>

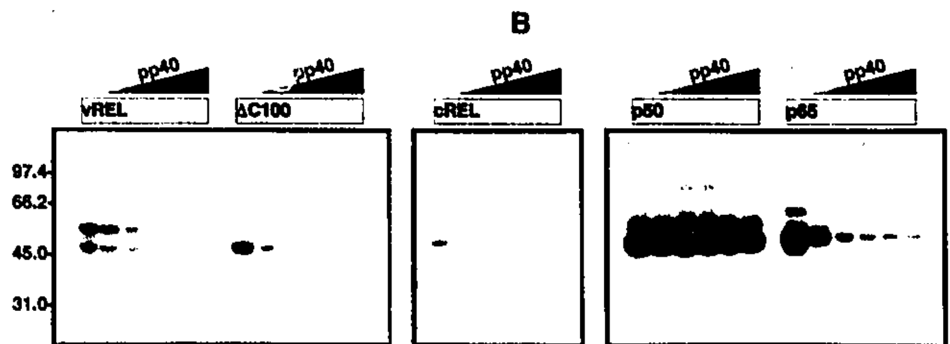
Since pp40 was shown to associate with p65, c-*rel*, and v-*rel* proteins (Fig. 21 and Fig. 22), and was also shown to inhibit the κB-specific DNA binding activities of these *rel* family proteins *in vitro* (Fig. 24), the functional effects of pp40 on κB-dependent transactivation activity *in vivo* were investigated.

First, pp40 gene was cloned into pSVL vector for transient expression in Cos1 cells. Cos1 cells were transfected with the graded amounts of the pSVL containing the pp40 gene and expression of pp40 was examined by immunoprecipitation using anti-pp40 antiserum. As shown in Fig. 31A, pp40 was expressed in a dose-dependent fashion. In addition to the major 40-kDa protein, two more slowly migrating proteins were also detected by immunoprecipitation. These two extra bands probably represent the differentially phosphorylated forms of pp40 since these two bands disappeared on CIP treatment (data not shown).

Second, Cos1 cells were cotransfected with fixed amounts of pSVL containing *rel* family genes (v-*rel*, ΔC100, c-*rel*, p50 or p65) and with graded amounts of pSVL containing the pp40 gene in order to examine the effect of pp40 on κB-specific DNA binding activities of *rel* family proteins. UV cross-linking analysis followed by immunoprecipitation of nuclear extracts of cotransfected Cos1 cells demonstrated that

Figure 31. The effects of pp40 on DNA binding activities of *rel* family proteins in Cos1 cells. (A) Expression of pp40 in Cos1 cells. Cos1 cells were transfected with pSVL without an insert (lane 1), or with increasing amount of pSVL containing the pp40 gene (lane 2; 0.5 μg, lane 3; 1 μg, lane 4; 1.5 μg, lane 5; 2.0 μg, or lane 6; 2.5 μg). Cos1 cells were labeled with [^{35}S]methionine at 40 hr post transfection and labeled extracts were immunoprecipitated with anti-pp40 antiserum. (B) The effect of pp40 association with NF-κB subunits or the *rel* proteins on DNA binding activity. Cos1 cells were cotransfected with a constant amount of pSVL containing the indicated gene (v-*rel*, ΔC100, c-*rel*, p105, or p65) and with increasing amounts of pSVL containing the pp40 gene. The total amount of transfected DNA was held constant by addition of the pSVL vector. Nuclear extracts were prepared from cotransfected cells at 48 hr post transfection. The extracts were incubated with [^{32}P]-labeled photoreactive κB, irradiated with UV, and immunoprecipitated with anti-v-*rel* antiserum (for vREL, ΔC100, cREL), anti-p50 antiserum (for p105), or anti-p65 antiserum (for p65).


A
pp40
97.4
66.2
45.0
31.0
1 2 3 4 5 6

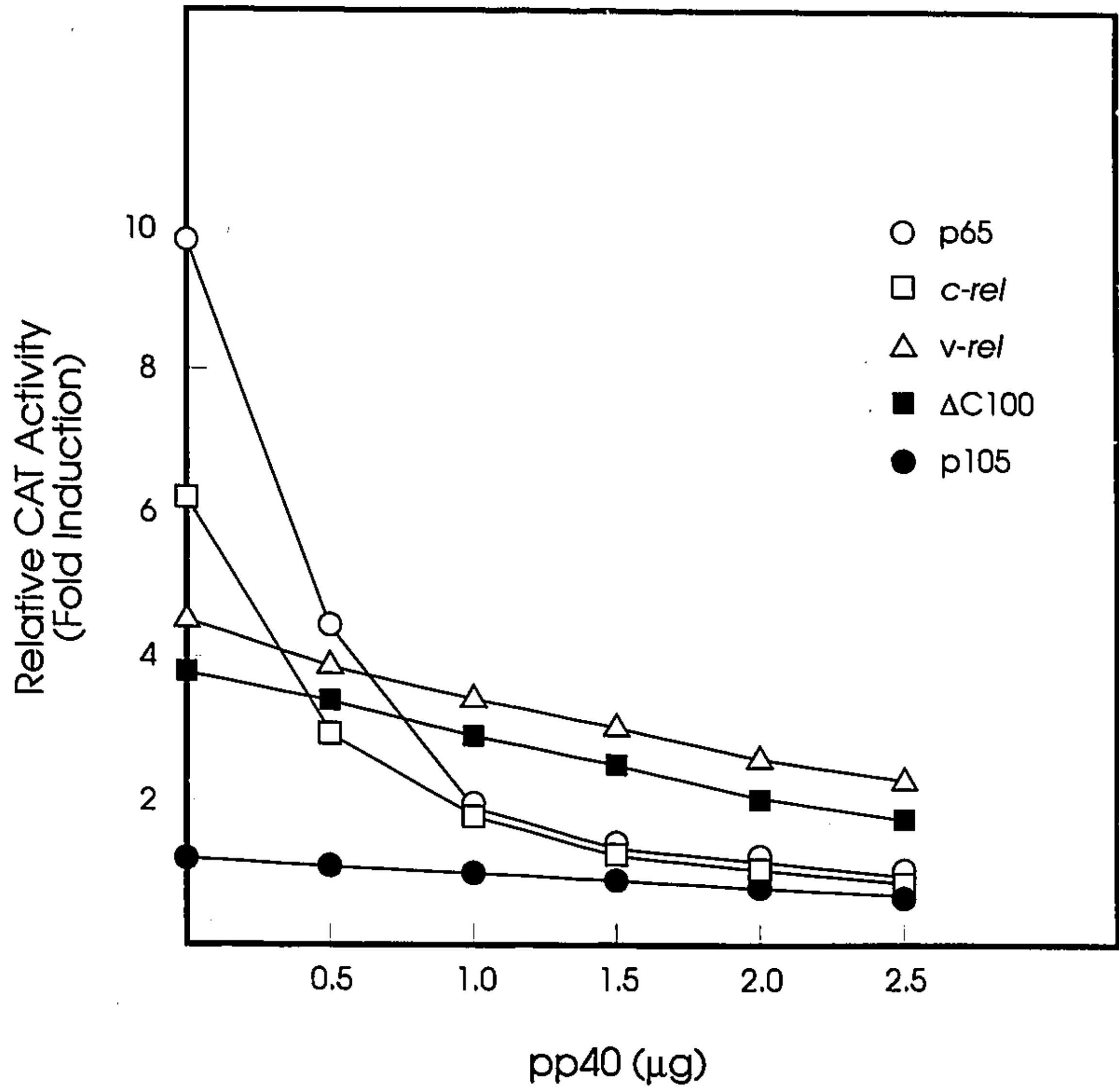

B
pp40
vREL
ΔC100
cREL
p50
p65
97.4
66.2
45.0
31.0

the κB-specific DNA binding activities of the wild-type (vREL) or mutant (ΔC100) v-*rel* proteins, the c-*rel* protein, and p65 were inhibited dramatically by the expression of pp40 in a dose-dependent manner (Fig. 31B). However, the DNA binding activity of p50 was not affected by the presence of pp40 (Fig. 31B). These results were consistent with *in vitro* studies (Fig. 24), and confirmed that pp40 inhibits the DNA binding activities of *rel* family proteins, except p50, by its physical association with them. These results also suggested that pp40 might inhibit the DNA binding activities of *rel* family proteins *in vivo* by sequestering *rel* family proteins in the cytoplasm through its physical association (Baeuerle and Baltimore 1988, Davis *et al.* 1991, Haskill *et al.* 1991, Inoue *et al.* 1991).

Third, Cos1 cells were cotransfected as above along with the CAT reporter plasmid J16 to determine the effect of pp40 on κB-dependent transactivation by *rel* family proteins. As shown in Fig. 32, the κB-dependent transactivation by the wild-type (vREL) or mutant v-*rel* (ΔC100) proteins, c-*rel* protein, and p65 were significantly suppressed by the expression of pp40, in a dose-dependent manner. However, transactivation by p105 was not significant in the presence or absence of pp40 (Fig. 32). Combined together with the effect of pp40 on κB-specific DNA binding activity, these results demonstrated that pp40 inhibits the κB-specific DNA binding activities of p65, v-*rel*, and c-*rel* proteins resulting in suppression of κB-dependent transactivation by these *rel* family proteins.

Finally, Cos1 cells were cotransfected with CAT reporter plasmid J16 and pSVL containing deletion or linker insertion mutants of v-*rel* in the absence or in the

Figure 32. The effect of pp40 on the transactivation activities of *rel* family proteins. Cos1 cells were cotransfected with CAT reporter plasmid J16, pSVL containing the indicated gene (v-*rel*, ΔC100, c-*rel*, p105, or p65), and with increasing amounts of pSVL containing the pp40 gene. The total amount of transfected DNA was held constant by addition of varying amounts of the pSVL vector. Whole cell extracts were prepared from transfected cells at 48 hr post transfection, and were incubated with [^{14}C]chloramphenicol and n-butyl CoA. Acetylated forms of chloramphenicol were extracted by xylene, and cpm were counted in a β-scintillation counter. Results of three independent experiments are expressed as the mean fold increase in CAT activity relative to the value of CAT reporter induced in the presence of pSVL vector only. In all cases, standard errors of the mean were less than 15%.



Relative CAT Activity
(Fold Induction)
10
8
6
4
2
0.5
1.0
1.5
2.0
2.5
pp40 (μg)
p65
c-rel
v-rel
ΔC100
p105

presence of the pSVL containing the pp40 gene to determine the effect of pp40 on κB-dependent transactivation by mutant v-*rel* proteins since all of these mutant v-*rel* proteins were shown to associate with pp40 (Fig. 24). As shown in Fig. 33, transactivation by transforming mutant v-*rel* proteins were significantly suppressed in the presence of pp40. However, transactivation by the non-transforming mutant v-*rel* proteins was not significant in the presence or absence of pp40.

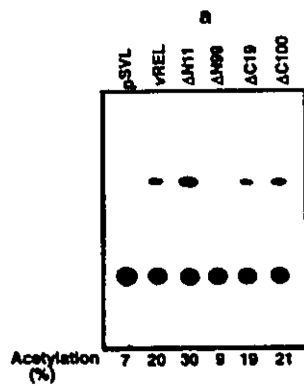
3. DISCUSSION

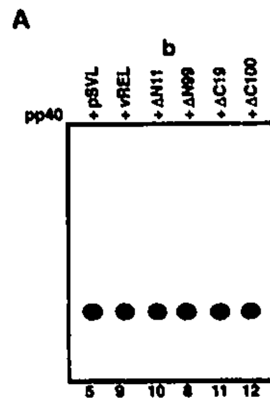
The κB-dependent transcriptional activity of the v-*rel* protein

The v-*rel* protein has been implicated as a transcriptional activator on the basis of its ability to modulate the activity of certain viral promoters (Gélinas and Temin 1988, Hannink and Temin 1989) or its ability to transactivate when bound to DNA through LexA repressor/operater interaction (Kamens *et al.* 1990). The findings that the v-*rel* protein shows homology with transcription factor NF-κB and binds directly to κB sites prompted the investigation of κB-dependent transcriptional activity of the v-*rel* protein. Transient gene expression studies with Cos1 cells revealed that the v-*rel* protein functions as a transcriptional activator through κB sites. Although the transactivation potential of the v-*rel* protein was shown to be less potent than that of p65 or the c-*rel* protein, the correlations between the expression of the v-*rel* protein, the κB-specific DNA binding activity, and dose-dependent transactivation clearly demonstrated that the v-*rel* protein can directly activate transcription from promoters containing κB sites as suggested by several groups (Gélinas and Temin 1988, Boehmelt *et al.* 1992, Walker

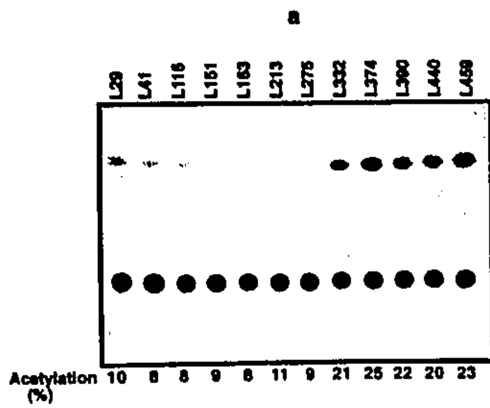
Figure 33. The effect of pp40 binding on transactivation by mutant v-*rel* proteins. (A) Cos1 cells were cotransfected with CAT reporter plasmid J16 and pSVL containing the indicated v-*rel* deletion mutant in the absence(a) or presence(b) of pSVL containing the pp40 gene. CAT assays were performed using cell extracts prepared from cotransfected cells and CAT activities were analyzed by TLC. Acetylation percentages are shown on the bottom. (B) Cos1 cells were cotransfected with CAT reporter plasmid J16 and pSVL containing the indicated v-*rel* linker insertion mutant in the absence(a) or presence(b) of pSVL containing the pp40 gene. CAT activities were analyzed by TLC and acetylation percentages are shown on the bottom.

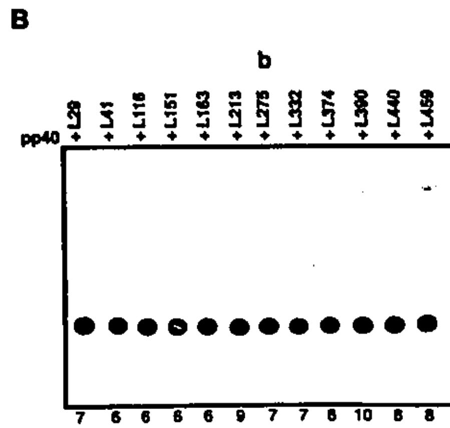
A





B





et al. 1992, Capobianco and Gilmore 1993). Our results are also consistent with several reports showing that the v-*rel* protein induces expression of endogenous MHC class I, MHC class II, IL-2 receptor, or HMG-14b, all of which contain κB sites in their promoters (Boehmelt *et al.* 1992, Hrdličková *et al.* 1994, Nehyba *et al.* 1994).

<u>Functional effects of protein-protein interactions on κB-dependent transactivation</u>

The active nuclear form of NF-κB is composed of a heterodimer between p50 and p65 (Urban *et al.* 1991), and the c-*rel* protein binds to DNA as a heterodimer with p50 or p65 (Hansen *et al.* 1992, Rice *et al.* 1992). We have also shown that the v-*rel* protein exists as a heterodimer with a 50-kDa protein, which is assumed to be the chicken p50, in the nucleus of transformed CSC (Fig. 9B). Furthermore, UV cross-linking analysis of nuclear extracts prepared from transfected Cos1 cells has demonstrated that transiently expressed *rel* family proteins (p65, v-*rel*, and c-*rel* proteins) bind to DNA as heterodimers with endogenous p50 (Fig. 27B), and these heterodimers are responsible for κB-dependent transactivation (Fig. 28). These findings suggest that the expression of the v-*rel* protein could interfere with κB-dependent transactivation by normal *rel* family transcription factors as a result of heterodimerization. The coexpression studies revealed that the expression of the v-*rel* protein partially inhibits κB-dependent transactivation mediated by p65 or the c-*rel* protein in a dose-dependent manner (Fig. 30A). Since the v-*rel* protein was shown to form a transcriptionally active heterodimer with endogenous p50, like p65 or the c-*rel* protein (Fig. 27), and this heterodimer shows less potent transactivation compared to the heterodimers containing p65 or c-*rel*

protein (Fig. 28), the limited inhibitory effects of v-*rel* expression on κB-dependent transactivation by p65 or the c-*rel* protein appear to be the consequence of competition for heterodimer formation. These results are consistent with a previous report which demonstrated that overexpression of the c-*rel* protein attenuates κB-dependent transactivation by p65 (Doerre *et al.* 1993). In contrast to the limited inhibitory effect of the wild-type v-*rel* protein on transactivation mediated by p65 or the c-*rel* protein, the expression of non-transforming mutant v-*rel* protein (for example, ΔN99) completely inhibits κB-dependent transactivation by p65 or the c-*rel* protein by forming the inactive heterodimers with p65, the c-*rel* protein, and p50 (Fig. 30B). These results confirm that all of the non-transforming mutant v-*rel* proteins that are unable to bind DNA function as transdominant inhibitors of *rel* family transcription factors. Taken together, these results imply that the formation of transcriptionally active heterodimers by the v-*rel* protein with other *rel* family proteins is necessary for transformation.

There are now two well-defined functions for IκB protein. One is inhibition of DNA binding of NF-κB/*rel* proteins, which was demonstrated in *in vitro* DNA binding studies (Zabel and Baeuerle 1990, Haskill *et al.* 1991, Kerr *et al.* 1991, Inoue *et al.* 1992). The second function is inhibition of nuclear translocation of NF-κB/*rel* proteins *in vivo* by masking the nuclear localization signals of NF-κB/*rel* proteins (Beg *et al.* 1992, Zabel *et al.* 1993). The participation of IκB in regulation of NF-κB activity and identification of the *rel*-associated protein pp40 as the avian IκB (Davis *et al.* 1991) suggest an important role for pp40 in gene regulation and oncogenesis by v-*rel*. Since pp40 can associate with NF-κB/*rel* proteins in insect cells (Fig. 21 and Fig. 22) and the

physical association of pp40 inhibits the DNA binding activities of NF-κB/*rel* proteins *in vitro* (Fig. 24), the effect of pp40 *in vivo* was examined. The coexpression studies in Cos1 cells demonstrated that pp40 inhibits κB-dependent transactivation by p65, v-*rel*, and c-*rel* proteins in a dose-dependent manner (Fig. 32) by inhibiting κB-specific DNA binding of NF-κB/*rel* proteins in nuclear extracts of cotransfected Cos1 cells (Fig. 31). Furthermore, coexpression of pp40 showed similar inhibitory effects on κB-dependent transactivation mediated by transforming mutant v-*rel* proteins (Fig. 33). Taken together with indirect immunofluorescence experiments (data not shown), these results suggest that pp40 prevents the nuclear translocation of NF-κB/*rel* proteins by associating with these proteins, resulting in inhibition of κB-dependent transactivation. In the case of p50, neither significant reduction of κB-specific DNA binding activity in nuclear extracts (Fig. 31) nor the inhibition of nuclear translocation (data not shown) was observed in the presence of pp40, reflecting a weak physical association of p50 with pp40 (Fig. 22). Also, it was noticed that the v-*rel* protein is more resistant to pp40-mediated inhibition of nuclear DNA binding and transactivation than the c-*rel* protein or p65 (Fig. 31B and Fig. 32). Similar results were also obtained by *in vitro* studies (Fig. 24). The reduced pp40-mediated inhibition of the v-*rel* protein compared to the c-*rel* protein *in vivo* might be the result of a reduced affinity of the v-*rel* protein for pp40 (Diehl *et al.* 1993).

The relationship between transactivation activity and transforming activity of the v-*rel* protein

Transient gene expression studies with Cos1 cells revealed that the v-*rel* protein functions as a κB-dependent transcriptional activator. Furthermore, it was also shown that all of the transforming mutant v-*rel* proteins retained κB-dependent transactivation activity, whereas all of the non-transforming mutant v-*rel* proteins failed to transactivate transcription (Fig. 29C). These results establish the relationship between κB-dependent transactivation and transforming activity of the v-*rel* protein (Table 6), and suggest that activation of gene expression directly by the v-*rel* protein is required for oncogenic transformation.

Deletion of the C-terminal 100 amino acids of v-*rel* (ΔC100) reduces transforming activity to 10% of the wild-type v-*rel* (Garson *et al.* 1990), and also reduces κB-dependent transactivation (Fig. 28). Since both the wild-type v-*rel* and ΔC100 proteins show comparable κB-specific DNA binding activities (Fig. 27), these results indicate the importance of C-terminal sequences of v-*rel* for transactivation of gene expression and oncogenesis. Larger deletions of up to C-terminal 172 amino acids of the v-*rel* protein was shown to abolish both transforming activity and transactivation activity but not DNA binding activity (Walker *et al.* 1992, Sarkar and Gilmore 1993). Taken together, these results localize a transcriptional activation domain in the C-terminus of the v-*rel* protein that is required for oncogenic transformation (Sarkar and Gilmore 1993). In contrast to the results obtained by C-terminal deletion mutants, the C-terminal linker insertion mutants (L332, L374, L390, L440, and I 459) retain full transforming activity (Garson and Kang 1990). These results suggest that the transactivation domain doesn't necessarily require a highly ordered structure as does the N-terminal DNA binding

TABLE 6

Transforming, DNA binding and transactivation activities of v-*rel* mutants

Mutant	Transforming activity	DNA binding activity	Transactivation activity
REL	+++++	+	++
ΔN11	+	+	++
ΔN99	–	–	–
ΔC19	+++++	+	++
ΔC100	+	+	+
L29	–	–	–
L41	–	–	–
L116	–	–	–
L151	–	–	–
L163	–	–	–
L213	–	–	–
L275	–	–	–
L332	+++++	+	++
L374	+++++	+	++
L390	+++++	+	++
L440	+++++	+	++
L459	+++++	+	++

domain (Ghosh *et al.* 1995, Müller *et al.* 1995). Therefore, the insertion of four amino acids at several sites in the C-terminus of v-*rel* is not enough for disruption of the transcriptional activation domain.

CHAPTER 6: MECHANISM OF TRANSFORMATION BY THE V-REL ONCOGENE

The discovery of structural and functional relationships between the c-*rel* protein and the NF-κB transcription factor provides a new insight into the mechanism of transformation by v-*rel* protein. The v-*rel* oncogene was originally isolated as the transforming gene of reticuloendotheliosis virus strain T, REV-T, acutely transforming avian retrovirus (Theilen *et al.* 1966). REV-T causes a rapid and fatal lymphoproliferative disease in young galliform birds *in vivo* and transforms avian lymphoid cells *in vitro* (Theilen *et al.* 1966, Hoelzer *et al.* 1980, Stephens *et al.* 1983, Barth and Humphries 1988a). Thus, inappropriate regulation of the activity of a member of the NF-κB family could lead to neoplastic transformation.

1. STRUCTURE OF THE V-REL ONCOGENE

The v-*rel* oncogene, like many other retroviral oncogenes, is a recombinant between transduced cellular sequences and viral coding sequences (Wilhelmsen *et al.* 1984). In the case of v-*rel*, the transduction of c-*rel* sequences into REV-T resulted in the substitution of *env*-derived sequences for both N- and C-terminal c-*rel* derived sequences (Stephens *et al.* 1983, Wilhelmsen *et al.* 1984). The v-*rel* oncogene encodes a protein of 503 amino acids which is detectably phosphorylated on serine and threonine residues and has an apparent molecular size of 59-kDa (Wilhelmsen *et al.* 1984, Garson and Kang 1986, Gilmore and Temin 1986, Herzog and Bose 1986, Rice

et al. 1986). At the N-terminus, the v-*rel* protein contains 11 amino acids encoded by *env*-derived sequences in place of the first 2 amino acids of the c-*rel* protein. At the C-terminus, the v-*rel* protein contains 18 amino acids encoded by out-of-frame *env*-derived amino acids in place of 118 C-terminal c-*rel*-derived amino acids. In addition, the central *rel*-derived region of the v-*rel* protein contains many differences from the corresponding region of the c-*rel* protein, as a result of the high mutation rate of retroviruses (Dougherty and Temin 1986). These differences include 14 single amino acid substitutions and three small deletions of one or three amino acids in the v-*rel* protein relative to the c-*rel* protein. Finally, an important aspect of transformation by the v-*rel* oncogene is that the v-*rel* oncogene is expressed from strong promoter/enhancer elements in the REV-T LTR.

2. STRUCTURE AND EXPRESSION OF THE c-*rel* PROTO-ONCOGENE

The v-*rel* oncogene is derived from its cellular homolog, the c-*rel* proto-oncogene. Molecular cloning of cDNA sequences encoding the chicken and turkey c-*rel* proteins has demonstrated that the c-*rel* proto-oncogene encodes a protein of 598 amino acids with an apparent molecular size of 68-kDa (Hannink and Temin 1989, Capobianco *et al.* 1990). The coding exons of the c-*rel* proto-oncogene are distributed over more than 30 kbp of genomic DNA, and they are expressed primarily as an mRNA of approximately 4 kbp (Chen *et al.* 1983, Moore and Bose 1989). This mRNA contains a short G/C-rich 5' noncoding region, approximately 1.8 kbp of c-*rel* coding sequence, and a long A/T-rich 3' nontranslated region.

The 4 kbp c-*rel* mRNA is expressed at low levels from a promoter without a classical TATA box (Hannink and Temin 1990). Multiple start sites are used in the initiation of transcription from the c-*rel* promoter, a common feature of non-TATA promoters. The c-*rel* promoter is a relatively weak promoter, approximately 100-fold less active than the promoter/enhancer of REV-T. The 5' flanking sequences that are responsible for the basal level of c-*rel* expression have been mapped to within 97 bp of the major transcription site. Potential binding sites for several transcription factors, including SP1 (Briggs *et al.* 1986), Krox-24 (Lemaire *et al.* 1990), HIP-1 (Means and Farnham 1990), and NF-κB, are contained within this region. The presence of a putative NF-κB site within the minimal c-*rel* promoter is intriguing since the promoters that regulate expression of NF-κB p105, NF-κB p100, and IκB also contain the putative NF-κB sites (Ten *et al.* 1992, Chiao *et al.* 1994, Liptay *et al.* 1994). Furthermore, activation of NF-κB has been shown to upregulate the transcription of c-*rel*, p105, p100 and IκB through the putative NF-κB sites present in promoters. These results suggest that autoregulation is a common mechanism for controlling the level of production of these ubiquitous transcription factors.

The c-*rel* protein is widely expressed in many tissues, but the highest levels have been detected in lymphoid tissues (Moore and Bose 1989). Most studies indicate that c-*rel* stimulates immune-response-linked proliferation in lymphoid cells. Increased levels of the c-*rel* protein are found in spleen cells during an immune response (Hrdličková *et al.* 1994). Several reports suggest that the c-*rel* protein induces expression of proteins known to be more abundant during immune responses, including interleukin 2,

interleukin 2 receptor α, gamma interferon, and MHC class I and II by κB-dependent transactivation (Inoue *et al.* 1991, Sica *et al.* 1992, Tan *et al.* 1992, Ghosh *et al.* 1993, Hrdličková *et al.* 1994). These results suggest that one of roles of the c-*rel* protein in the induction of proliferative immune response is to function as a κB-specific transactivator.

3. ACTIVATION OF THE ONCOGENIC POTENTIAL OF THE C-REL PROTO-ONCOGENE

In the formation of REV-T, the avian retrovirus that contains the v-*rel* oncogene, several deletions occurred in the *gag* and *pol* genes in addition to the transduction of c-*rel* sequences in place of the majority of the *env* gene. The necessity of these deletions in the viral *gag* and *pol* sequences in addition to the acquisition of the v-*rel* sequences for the transforming property of REV-T has been demonstrated (Chen and Temin 1982, Miller and Temin 1986). The presence of the *gag* and *pol* regions suppresses the transforming property of REV-T by decreasing the level of expression of the v-*rel* oncogene, most likely by interfering with efficient splicing of the v-*rel* viral mRNA (Miller and Temin 1986). In addition to providing an insight into mechanisms that regulate the production of retroviral mRNA molecules, these results indicate that high levels of expression of the v-*rel* oncogene are required for oncogenic transformation. Since the c-*rel* promoter is a relatively weak promoter, approximately 100 times less active than the LTR of REV-T, this result indicates that transcriptional activation of the c-*rel* proto-oncogene is a necessary event for activation of its oncogenic

potential.

However, transcriptional activation of the c-*rel* proto-oncogene is not sufficient for activation of its oncogenic potential since the c-*rel* protein, when expressed from retroviral LTR, transforms avian lymphoid cells with a frequency only 1% or less that of the v-*rel* oncogene (Kamens *et al.* 1990). As the v-*rel* oncogene is also a multiply-mutated form of the c-*rel* proto-oncogene, some of these differences are important for activation of the oncogenic potential of c-*rel*. The important differences between the v-*rel* and c-*rel* proteins that are sufficient to activate the oncogenic potential of c-*rel* have been localized to the N-terminal 400 amino acids of the v-*rel* protein (Hannink and Temin 1989). A recombinant retrovirus containing N-terminal v-*rel* sequences and C-terminal c-*rel* sequences is able to transform and immortalize avian lymphoid cells with an efficiency equal to that of retrovirus encoding the v-*rel* oncogene.

However, the presence of N-terminal v-*rel* sequences is not an absolute prerequisite for transformation since a retrovirus containing the c-*rel* proto-oncogene with a C-terminal 55 amino acid deletion is highly transforming (Kamens *et al.* 1990). The C-terminal 118 amino acids of avian c-*rel* protein contain a cytoplasmic retention domain and part of a gene activation domain (Capobianco *et al.* 1990). The v-*rel* protein, which is missing 118 C-terminal amino acids of the c-*rel* protein, is a nuclear protein when overexpressed in CEF, whereas the overexpressed c-*rel* protein is entirely cytoplasmic in CEF. Furthermore, removing 55 to 103 C-terminal amino acids of c-*rel* protein allows the proteins to enter the nucleus as efficiently as the v-*rel* protein. These results indicate that deletion of the cytoplasmic retention domain activates the oncogenic

potential of c-*rel*. Its presence, however, does not suppress the transforming potential of N-terminal v-*rel* sequences since a recombinant retrovirus containing the N-terminal v-*rel* sequences along with 118 C-terminal amino acids of c-*rel* protein transforms as readily as wild-type v-*rel* (Hannink and Temin 1989).

4. FUNCTIONAL DOMAINS OF THE V-REL PROTEIN REQUIRED FOR ONCOGENIC TRANSFORMATION

The domains of v-*rel* protein that are required for transformation have been defined by several studies (Gilmore and Temin 1988, Garson and Kang 1990, Garson *et al.* 1990, Morrison *et al.* 1992). One domain consists of the first 300 amino acids of the v-*rel* protein which are homologous to the subunits of NF-κB and *dorsal*. The DNA binding activity of the v-*rel* protein is contained within this N-terminal domain. Any perturbation of this region of the v-*rel* protein except the N-terminal *env*-derived 11 amino acids, either by insertion or deletion, abolishes both DNA binding activity and transformation activity of the v-*rel* protein (Table 7), demonstrating that DNA binding activity of the v-*rel* protein is necessary for transformation. The N-terminal *env*-derived 11 amino acids are required for full transforming activity (Garson *et al.* 1990), but deletion of these N-terminal 11 amino acids exhibits no effect on DNA binding activity (Table 7). At present, it is not fully understood how N-terminal *env*-derived amino acids affect the transforming activity of the v-*rel* protein.

Another function contained in this N-terminal domain is the protein-dimerization activity. Based upon the tertiary structure of NF-κB p50 homodimer bound to the κB

TABLE 7

The functional characteristics of v-*rel* mutants

Mutant	Trans-forming activity	Dimeriza-tion with NF-κB subunits	Association with pp40	DNA binding activity			Trans-activation activity
				homo-dimer	hetero-dimer	inhibition by pp40 association	
REL	+++++	+	+	+	+	+	++
ΔN11	+	+	+	+	+	+	++
ΔN99	–	+	+	–	–	U[a]	–
ΔC19	+++++	+	+	+	+	+	++
ΔC100	+	+	+	+	+	+	+
L29	–	+	+	–	–	U	–
L41	–	+	+	–	–	U	–
L116	–	+	+	–	–	U	–
L151	–	+	+	–	–	U	–
L163	–	+	+	–	–	U	–
L213	–	+	+	–	–	U	–
L275	–	+	+	–	–	U	–
L332	+++++	+	+	+	+	+	++
L374	+++++	+	+	+	+	+	++
L390	+++++	+	+	+	+	+	++
L440	+++++	+	+	+	+	+	++
L459	+++++	+	+	+	+	+	++

[a]U, undetectable

site (Ghosh *et al.* 1995, Müller *et al.* 1995), the N-terminal *rel* homology region is predicted to consist of two distinct domains. Domain I includes 200 N-terminal amino acids and plays an important role mainly in DNA binding. Domain II includes the remaining 100 amino acids and plays an important role in DNA binding and protein dimerization. Two linker insertion mutants, (L213, L275), which have mutations in the dimerization domain, abolish DNA binding and transformation activity, but retain dimerization activity (Table 7). These results probably imply that DNA binding has a more stringent structural requirement than dimerization. All of the deletion and linker insertion mutants of v-*rel*, regardless of their transforming activities, dimerize with NF-κB subunits and IκB, like wild-type v-*rel* (Table 7). However, dimerizations of the non-DNA binding mutants with NF-κB subunits abrogate the DNA binding activities of NF-κB subunits, whereas DNA binding mutants and wild-type v-*rel* are able to form functional heterodimers with NF-κB subunits. These results demonstrate that all of the non-DNA binding, non-transforming mutants of v-*rel*, which retain the dimerization activity, function as transdominant negative mutants of NF-κB/c-*rel* transcription factors.

The remaining C-terminal amino acids comprise a second domain of the v-*rel* protein that is required for both oncogenic transformation and transactivation of gene expression. Deletion of the *env*-derived 19 C-terminal amino acids of the v-*rel* protein displays no effects on either transformation or transactivation activity, but further deletion of 100 amino acids from the C-terminal of v-*rel* significantly reduces both transformation and transactivation activity (Table 7, Garson *et al.* 1990). Larger

deletions of 172 C-terminal amino acids of the v-*rel* protein completely abolishes both transformation and transactivation activity (Walker *et al.* 1992, Sarkar and Gilmore 1993). Therefore, these results suggest that transformation activity of the v-*rel* protein results from its ability to activate the expression of target genes.

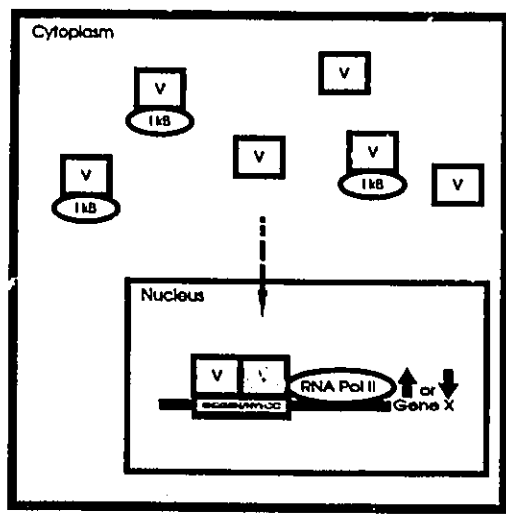
5. MODELS FOR TRANSFORMATION BY THE v-*rel* ONCOGENE

The mechanism by which the v-*rel* oncogene transforms avian lymphoid cells is still poorly understood. Based on our knowledge about the biochemical characteristics of NF-κB transcription factor, the v-*rel* and c-*rel* proteins, it is likely that transformation by v-*rel* oncogene is mediated by altered gene expression, most probably involving genes normally regulated by c-*rel* and other members of the NF-κB/Rel family. However, there are several possible mechanisms by which transcription of such genes could be affected (Fig. 34).

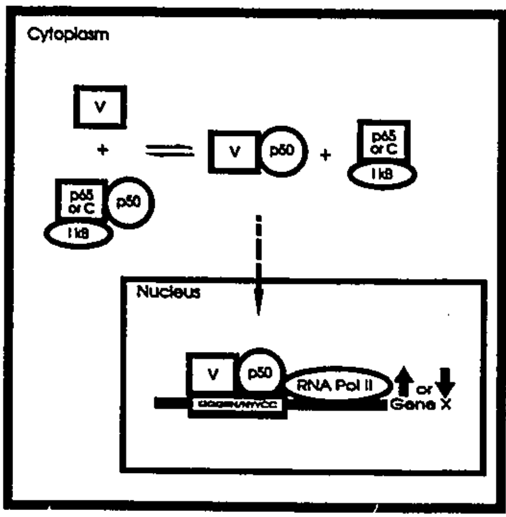
The first hypothesis is that the DNA binding activity of the v-*rel* protein is critically required for transformation. Transformation is then the result of an altered pattern of gene expression resulting from the direct action of the v-*rel* protein as a DNA-bound transcriptional modulator (Fig. 34A). At least some of the genes whose expression is altered by v-*rel* might be normally regulated by c-*rel* or NF-κB, given the apparent similarity in the recognition sequence of c-*rel*/NF-κB and v-*rel*. There are two lines of evidence that support this direct model for transformation by v-*rel*. First, the v-*rel* protein is the predominant κB-binding protein present in nuclear extracts from CSC transformed by mutant or wild-type v-*rel* (Fig. 9). Second, analysis of deletion

Figure 34. Models for transformation by the v-*rel* protein. In each model, the v-*rel* protein is indicated by the dotted box, the v-*rel* associated protein of 40 kDa (or IκB) is indicated by an oval, the p50 subunit of NF-κB is indicated by a circle, and the p65 (or c-*rel*) subunit of NF-κB is indicated by a box. The horizontal double arrows represent possible interactions between the various proteins, and the single bold arrow crossing the nuclear envelope indicates nuclear translocation of an active NF-κB or NF-κB-related complex. The cellular transcriptional machinery is simplistically represented by the oval labeled "RNA pol II". In these models, transformation by the v-*rel* protein is thought to be the result of altered regulation of one or more genes, represented by "gene X". The effect of v-*rel* on the expression of these genes could be either activating or inhibitory, as indicated by a pair of vertical arrows. (A) The v-*rel* protein as an independently acting DNA-bound transcriptional modulator. In this model, the majority of the v-*rel* protein is present in the cytoplasm, perhaps by association with pp40 (IκB). However, a sufficient amount of the v-*rel* protein is able to enter the nucleus and act as a DNA-bound transcriptional activator or repressor. The NF-κB/c-*rel* complex is not shown since it is not directly involved in gene regulation in this model. (B) Formation of heterodimeric complexes with the v-*rel* protein and p50. In this model, the v-*rel* protein forms a dimer with the p50 subunit of NF-κB/c-*rel*, displacing p65/c-*rel* and IκB. The p50/v-*rel* complex then translocates to the nucleus and is able to alter the expression of certain genes. (C) Activation of NF-κB/c-*rel* by the v-*rel* protein. In this model, the v-*rel* protein binds to IκB, resulting in the production of NF-κB/c-*rel* complex that is not bound to IκB and is free to enter the nucleus and bind DNA.

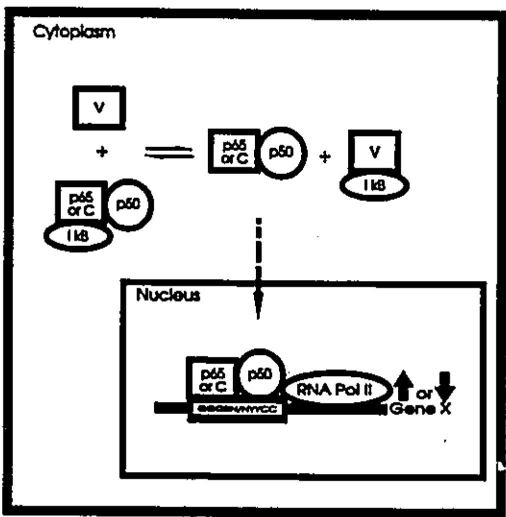
A



B



C



and linker insertion mutants of v-*rel* provides a strong correlation between the ability of mutant v-*rel* proteins to bind DNA and their transforming activities (Table 7).

A second hypothesis is that NF-κB/c-*rel* transcription factor activity is altered by protein-protein interactions between v-*rel* and the NF-κB/c-*rel* complex. One version of this hypothesis is that the v-*rel* protein displaces one of the subunits of NF-κB/c-*rel*, resulting in novel heterodimers whose transcriptional properties are different from the normal complex (Fig. 34B). For example, the v-*rel* protein forms a heterodimer with the p50 subunit of NF-κB/c-*rel*, displacing p65/c-*rel* and IκB. The heterodimer between the v-*rel* protein and p50 then translocates to the nucleus and is able to alter the expression of certain genes. There are several pieces of evidence to support this hypothesis. First, the v-*rel* protein is complexed with p105 and p100 precursor of p50 in the cytoplasm of CSC transformed by the mutant or wild-type v-*rel* (Fig. 13). Second, all of the transforming mutants of v-*rel* heterodimerize with p50 *in vitro*, resulting in functional heterodimers which bind specifically to the κB site. In contrast, all of the non-transforming mutants of v-*rel* heterodimerize with p50 *in vitro*, resulting in non-DNA binding heterodimers (Fig. 18 and Table 7). Third, the v-*rel* protein binds to DNA as a heterodimer with p50 in the nuclei of transformed CSC and transfected Cos1 cells (Fig. 9 and Fig. 27).

Another version of this second hypothesis is that the v-*rel* protein associates with IκB (pp40), sequestering IκB with the subsequent formation of increased level of active NF-κB/c-*rel* (Fig. 34C). Transformation would then be the result of altered gene regulation by constitutively active NF-κB/c-*rel*. There is substantial circumstantial

evidence to support this type of indirect model for transformation by v-*rel*. First, the v-*rel* protein is located predominantly in the cytoplasm of REV-T transformed cells (Gilmore and Temin 1986). Second, pp40 (IκB) is associated with the v-*rel* protein in transformed cells (Tung *et al.* 1988), and *in vitro* association of pp40 with the v-*rel* protein is able to inhibit DNA binding of the v-*rel* protein (Davis *et al.* 1991, Fig. 25). However, our results showing that all of the deletion and linker insertion mutants of v-*rel*, regardless of their transforming activities, can associate with pp40 (Fig. 24) strongly argue against this model.

REFERENCES

Angel, P., M. Imagawa, R. Chiu, B. Stein, R.J. Imbra, H.J. Rahmsdorf, C. Jonat, P. Herrlich and M. Karin. 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49:** 729-739.

Barth, C.F. and E.H. Humphries. 1988a. A nonimmunosuppressive helper virus allows high efficiency induction of B cell lymphomas by reticuloendotheliosis virus strain T. *J. Exp. Med.* **167:** 89-108.

Barth, C.F. and E.H. Humphries. 1988b. Expression of v-*rel* induces mature B-cell lines that reflect the diversity of avian immunoglobulin heavy- and light-chain rearrangements. *Mol. Cell. Biol.* **8:** 5358-5368.

Baeuerle, P.A. and D. Baltimore. 1988. IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **242:** 540-546.

Baeuerle, P.A. and D. Baltimore. 1989. A 65-kd subunit of active NF-κB is required for inhibition of NF-κB by IκB. *Genes Dev.* **3:** 1689-1698.

Baeuerle, P.A. 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. *Biochem. Boiphys. Acta* **1072:** 63-80.

Ballard, D.W., W.H. Walker, S. Doerre, P. Sista, J.A. Molitor, E.P. Dixon, M.J. Peffer, M. Hannink and W.C. Greene. 1990. The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. *Cell* **63:** 803-914.

Ballard, D.W., E.P. Dixon, N.J. Peffer, H. Bogerd, S. Doerre, B. Stein and W.C. Greene. 1992. The 65-kDa subunit of human functions as a potent transcriptional activator. *Proc. Natl. Acad. Sci. USA* **89:** 1875-1879.

Beg, A.A., S.M. Ruben, R.I. Scheinman, S. Haskill, C.A. Rosen and A.S. Baldwin, Jr. 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. *Genes Dev.* **6:** 1899-1913.

Bender, W., Y.H. Chien, S. Chattopadhyay, P.K. Vogt, M.B. Gardner and N. Davidson. 1978. High-molecular-weight RNAs of AKR, NZB and wild mouse viruses and avian reticuloendotheliosis virus all have similar dimer structures. *J. Virol.* **25:** 888-896.

Benjamin, T. and P.K. Vogt. 1991. Cell transformation by viruses. In *Fundamental virology*. 2nd ed. Edited by Fields, B.M. and D.M. Knipe. Raven Press, New York. p. 304.

Beug, H., H. Muller, S. Grieser, G. Doederlein and T. Graf. 1981. Hematopoietic cells transformed *in vitro* by REV-T avian reticuloendotheliosis virus express characteristics of very immature lymphoid cells. *Virology* **115:** 295-309.

Bishop, J.M. 1987. The molecular genetics of cancer. *Science* **235:** 305-311.

Blank, V., P. Kourilsky and A. Israël. 1991. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. *EMBO J.* **10:** 4159-4167.

Boehmelt, G., A. Walker, N. Kabrun, G. Mellitzer, H. Beug, M. Zenke and P.J. Enrietto. 1992. Hormone-regulated v-*rel* estrogen receptor fusion protein: reversible induction of cell transformation and cellular gene expression. *EMBO J.* **11:** 4641-4652.

Bohmann, D., T.J. Bos, A. Admon, T. Nishimura, P.K. Vogt and R. Tjian. 1987. Human proto-oncogene c-*jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* **238:** 1386-1392.

Bours, V., J. Villalobos, P.R. Burd, K. Kelley and U. Siebenlist. 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell cycle motifs. *Nature* **348:** 76-79.

Bours, V., P.R. Burd, K. Brown, J. Villalobos, S. Park, R.P. Ryseck, B.R.K. Kelly and U. Siebenlist. 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. *Mol. Cell. Biol.* **12:** 685-695.

Bradford, M.M. 1984. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Breitman, M.L., M.M.C. Lai and P.K. Vogt. 1980. The genomic RNA of avian reticuloendotheliosis virus REV. *Virology* **100:** 450-461.

Bressler, P., K. Brown, W. Timmer, V. Bours, U. Siebenlist and A.S. Fauci. 1993. Mutational analysis of the p50 subunit of NF-κB and inhibition of NF-κB activity by *trans*-dominant p50 mutants. *J. Virol.* **67:** 288-293.

Briggs, M.R., J.T. Kadonaga, S.T. Bell and R. Tjian. 1986. Purification and biochemical characterization of the promoter-specific transcription factor SP1. *Science* **234:** 47-52.

Brown, K., S. Park, T. Kanno, G. Franzoso and U. Siebenlist. 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. *Proc. Natl. Acad. Sci. USA* 90: 2532-2536.

Brownell, E., N. Mitterder and N.R. Rice. 1989. A human *rel* protooncogene cDNA containing an *Alu* fragment as a potential coding exon. *Oncogene* 4: 935-942.

Bull, P., K.L. Morley, M.F. Hoekstra, T. Hunter and I.M. Verma. 1990. The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. *Mol. Cell. Biol.* 10: 5473-5485.

Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112: 195-203.

Capobianco, A.J., D.L. Simmons and T.D. Gilmore. 1990. Cloning and expression of chicken c-*rel* cDNA: unlike $p59^{v\text{-}rel}$, $p68^{c\text{-}rel}$ is a cytoplasmic protein in chicken embryo fibroblasts. *Oncogene* 5: 257-265.

Capobianco, A.J., D. Chang, G. Mosialos and T.D. Gilmore. 1992. p105, the NF-κB p50 precursor protein, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. *J. Virol.* 66: 3758-3767.

Capobianco, A.J. and T.D. Gilmore. 1993. A conditional mutant of vRel containing sequences from the human estrogen receptor. *Virology* 193: 160-170.

Chen, H.R., L.-S. Yeh and W.C. Barker. 1986. Similarity between oncogenic v-*rel* protein and regulatory *fnr* protein. *Nucleic Acids Res.* 14: 3977.

Chen, I.S.Y., T.W. Mak, J.J. O'Rear and H.M. Temin. 1981. Characterization of reticuloendotheliosis virus strain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. *J. Virol.* 40: 800-811.

Chen, I.S.Y. and H.M. Temin. 1982. Substitution of 5' helper virus sequences into non-*rel* portion of reticuloendotheliosis virus strain T suppresses transformation of chicken spleen cells. *Cell* 31: 111-120.

Chen, I.S.Y., K.C. Wilhelmsen and H.M. Temin. 1983. Structure and expression of c-*rel*, the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. *J. Virol.* 45: 104-113.

Chiao, P.J., S. Miyamoto and I.M. Verma. 1994. Autoregulation of IκBα activity. *Proc. Natl. Acad. Sci. USA* 91: 28-32.

Cohen, R.S., T.C. Wong and M.M.C. Lai. 1981. Characterization of transformation- and replication-specific sequences of reticuloendotheliosis virus. *Virology* 113: 672-685.

Courtneidge, S.A. and A. Herber. 1987. An 81 kd protein complexed with middle T antigen and pp60^{c-src}: a possible phosphatidylinositol kinase. *Cell* 50: 1031-1037.

Davis, N., W. Bargmann and H.R. Bose, Jr. 1990a. Identification of protein complexes containing the c-*rel* proto-oncogene product in avian hematopoietic cells. *Oncogene* 5: 1109-1115.

Davis, N., W. Bargmann, M.Y. Lim and H.R. Bose, Jr. 1990b. Avian reticuloendotheliosis virus-transformed lymphoid cells contain multiple pp59^{v-rel} complexes. *J. Virol.* 64: 584-591.

Davis. N., S. Ghosh, D.L. Simmons, P. Tempst, H.C. Liu, D. Baltimore and H.R. Bose, Jr. 1991. Rel-associated pp40: an inhibitor of the *rel* family of transcription factors. *Science* 253: 1268-1271.

Dickson, C. and G. Peters. 1987. Potential oncogene product related to growth factors. *Nature* 326: 833.

Diehl, J.A. and M. Hannink. 1993. Heterologous C-terminal sequences disrupt transcriptional activation and oncogenesis by p59^{v-rel}. *J. Virol.* 67: 7161-7171.

Diehl, J.A., T.A. McKinsey and M. Hannink. 1993. Differential IκB-β inhibition of DNA-binding by *rel* proteins. *Mol. Cell. Biol.* 13: 1769-1778.

Dignam, J.D., R.M. Lebovitz and R.G. Roeber. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11: 1475-1489.

Doerre, S., P. Sista, S.-C. Sun, D.W. Ballard and W.C. Greene. 1993. The c-*rel* protooncogene product represses NF-κB p65-mediated transcriptional activation of the long terminal repeat of type 1 human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 90: 1023-1027.

Dougherty, J.D. and H.M. Temin. 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. *Mol. Cell. Biol.* 6: 4387-4395.

Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger and M.D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and v-*erb*B oncogene protein sequence. *Nature* 307: 521-527.

Fan, C.M. and T. Maniatis. 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. *Nature* 354: 395-398.

Fenner, F. 1975. The classification and nomenclature of viruses. *Intervirology* 6: 1-12.

Ganchi, P.A., S.-C. Sun, W.C. Greene and D.W. Ballard. 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. *Mol. Cell. Biol.* 3: 1339-1352.

Garson, K. and C.Y. Kang. 1986. Identification of the v-*rel* protein in REV-T transformed chicken bone marrow cells and expression in Cos1 cells. *Biochem. Biophys. Res. Commun.* 134: 716-722.

Garson, K. 1989. Identification and functional mapping of the protein product of the v-*rel* oncogene. Ph. D. Thesis, University of Ottawa.

Garson, K. and C.Y. Kang. 1990. Mapping of the functional domains of the v-*rel* oncogene. *Oncogene* 5: 1431-1434.

Garson, K., H. Percival and C.Y. Kang. 1990. The N-terminal *env*-derived amino acids of v-*rel* are required for full transforming activity. *Virology* 177: 106-115.

Gélinas, C. and H.M. Temin. 1988. The v-*rel* oncogene encodes a cell-specific transcriptional activator of certain promoters. *Oncogene* 3: 349-355.

Ghosh, G., G. Van Duyne, S. Ghosh and P.B. Sigler. 1995. Structure of NF-κB p50 homodimer bound to a κB site. *Nature* 373: 303-310.

Ghosh, P., T.-H. Tan, N.R. Rice, A. Sica and H.A. Young. 1993. The interleukin 2 CD28-responsive complex contains at least three members of the NF-κB family: c-Rel, p50, and p65. *Proc. Natl. Acad. Sci. USA* 90: 1696-1700.

Ghosh, S. and D. Baltimore. 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. *Nature* 344: 678-682.

Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan and D. Baltimore. 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. *Cell* 62: 1019-1029.

Gilmore, T.D. and H.M. Temin. 1986. Different localization of the product of the v-*rel* oncogene in chicken fibroblast and spleen cells correlates with transformation by REV-T. *Cell* 44: 791-800.

Gilmore, T.D. and H.M. Temin. 1988. v-*rel* oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. *J. Virol.* **62:** 703-714.

Gonda, M.A., N.R. Rice and R.V. Gilden. 1980. Avian reticuloendotheliosis virus: characterization of the high-molecular-weight viral RNA in transforming and helper virus populations. *J. Virol.* **34:** 743-751.

Grumont, R.J. and S. Gerondakis. 1989. Structure of a mammalian c-*rel* protein deduced from the nucleotide sequence of murine cDNA clones. *Oncogene Res.* **4:** 1-8.

Hager, D.A. and R.R. Burgess. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *E. coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Anal. Biochem.* **109:** 76-86.

Halpern, M.S., E. Wade, E. Rucker, K.L. Baxter-Gabbard, A.S. Levine and R.R. Friis. 1973. A study of the relationship of reticuloendotheliosis virus to the avian leukosis-sarcoma complex of viruses. *Virology* **53:** 287-299.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557-580.

Hannink, M. and H.M. Temin. 1989. Transactivation of gene expression by nuclear and cytoplasmic *rel* proteins. *Mol. Cell. Biol.* **9:** 4323-4336.

Hannink, M. and H.M. Temin. 1990. Structure and autoregulation of the c-*rel* promoter. *Oncogene* **5:** 1843-1850.

Hansen S.K., C. Nerlov, U. Zabel, P. Verde, M. Johnsen, P.A. Baeuerle and F. Blasi. 1992. A novel complex between the p65 subunit of NF-κB and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. *EMBO J.* **11:** 205-213.

Haskill, S., A.A. Beg, S.M. Tompkins, J.S. Morris, A.D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph and A.S. Baldwin, Jr. 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. *Cell* **65:** 1281-1289.

Hatada, E.N., A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T.W. McKeithan and C. Scheidereit. 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene *bcl-3* act as specific inhibitors of NF-κB DNA binding. *Proc. Natl. Acad. Sci. USA* **89:** 2489-2493.

Henkel, T., U. Zabel, K. Van Zee, J.M. Müller, E. Fanning and P.A. Baeuerle. 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. *Cell* **68:** 1121-1133.

Herskowitz, I. 1987. Functional inactivation of genes by dominant negative mutations. *Nature* **329:** 219-222.

Herzog, N.K and H.R. Bose, Jr. 1986. Expression of the oncogene of avian reticuloendotheliosis virus in *E. coli* and identification of the transforming protein in reticuloendotheliosis virus T-transformed cells. *Proc. Natl. Acad. Sci. USA* **83:** 812-816.

Hoelzer, J.D., R.B. Franklin and H.R. Bose, Jr. 1979. Transformation by reticuloendotheliosis virus: development of a focus assay and isolation of a nontransforming virus. *Virology* **93:** 20-30.

Hoelzer, J.D., R.B. Lewis, C.R. Wasmuth and H.R. Bose, Jr. 1980. Hematopoietic cell transformation by reticuloendotheliosis virus: characterization of the genetic defect. *Virology* **100:** 462-474.

Hrdličková, R., J. Nehyba and E.H. Humphries. 1994. v-*rel* induces expression of three avian immunoregulatory surface receptors more efficiently than c-*rel*. *J. Virol.* **68:** 308-319.

Hu, S.S.F., M.M.C. Lai, T.C. Wong, R.S. Cohen and M. Sevoian. 1981. Avian reticuloendotheliosis virus: characterization of genome structure by heteroduplex mapping. *J. Virol.* **37:** 899-907.

Inoue, J.I., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter and I.M. Verma. 1991. c-*rel* activates but v-*rel* suppresses transcription from κB sites. *Proc. Natl. Acad. Sci. USA* **88:** 3715-3719.

Inoue, J.I., L.D. Kerr, D. Rashid, N. Davis, H.R. Bose, Jr. and I.M Verma. 1992. Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. *Proc. Natl. Acad. Sci. USA* **89:** 4333-4337.

Ip, Y.T., R. Kraut, M. Levin and C.A. Rushlow. 1991. The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in Drosophila. *Cell* **64:** 439-446.

Kabrun, N., J.W. Hodgson, M. Doemer, G. Mak, B.R. Franza, Jr. and P.J. Enrietto. 1991. Interaction of the v-*rel* protein with an NF-κB DNA binding site. *Proc. Natl. Acad. Sci. USA* **88:** 1783-1787.

Kamens, J., P. Richardson, G. Mosialos, R. Brent and T. Gilmore. 1990. Oncogenic transformation by vRel requires an amino-terminal activation domain. *Mol. Cell. Biol.* 10: 2840-2847.

Kang, C.Y. and H.M. Temin. 1973. Lack of sequence homology among RNAs of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and chicken endogenous RNA-directed DNA polymerase activity. *J. Virol.* 12: 1314-1324.

Kang, C.Y. and H.M. Temin. 1974. Reticuloendotheliosis virus nucleic acid sequences in cellular DNA. *J. Virol.* 14: 1179-1188.

Kang, C.Y. 1975. Characterization of endogenous RNA-directed DNA polymerase activity of reticuloendotheliosis viruses. *J. Virol.* 16: 880-886.

Kang, C.Y., T.C. Wong and K.V. Holmes. 1975. Comparative ultrastructural study of four reticuloendotheliosis viruses. *J. Virol.* 16: 1027-1038.

Kang, C.Y. 1988. Baculovirus vectors for expression of foreign genes. *Adv. Virus. Res.* 35: 177-192.

Kawai, S. and M. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* 4: 1172-1174.

Kawakami, K., C. Scheidereit and R.G. Roeder. 1988. Identification and purification of a human immunoglobulin enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter *in vitro*. *Proc. Natl. Acad. Sci. USA* 85: 4700-4704.

Kerr, L.D., J. Inoue, N. Davis, E. Link, P.A. Baeuerle, H.R. Bose, Jr. and I.M. Verma. 1991. The *rel*-associated pp40 protein prevents DNA binding of *rel* and NF-κB: relationship with IκBβ and regulation by phosphorylation. *Genes Dev.* 5: 1464-1476.

Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M.B. Urban, P. Kourilsky, P.A. Baeuerle and A. Israël. 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* 62: 1007-1018.

Kitts, P.A. and R.D. Possee. 1993. A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* 14: 810-816.

Kochel, T., J.F. Mushinski and N.R. Rice. 1991. The v-*rel* and c-*rel* proteins exist in high molecular weight complexes in avian and murine cells. *Oncogene* 6: 615-626.

Kochel, T. and N.R. Rice. 1992. v-*rel* and c-*rel* protein complexes bind to the NF-κB site *in vitro*. *Oncogene* 7: 567-572.

Kumar, S., A.B. Rabson and C. Gélinas. 1992. The RxxRxRxxC motif conserved in all Rel/κB proteins is essential for the DNA-binding activity and redox regulation of the v-Rel oncoprotein. *Mol. Cell. Biol.* 12: 3094-3106.

Kumar, S. and C. Gélinas. 1993. IκBα-mediated inhibition of v-Rel DNA binding requires direct interaction with the RxxRxRxxC Rel/κB DNA-binding motif. *Proc. Natl. Acad. Sci. USA* 90: 8962-8966.

Lacoste, J., M. D'Addario, A. Roulston, M.A. Wainberg and J. Hiscott. 1990. Cell-specific differences in activation of NF-κB regulatory elements of human immunodeficiency virus and beta interferon promoters by tumor necrosis factor. *J. Virol.* 64: 4726-4734.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Le Bail, O., R. Schmidt-Ullrich and A. Israël. 1993. Promoter analysis of the gene encoding the IκBα (MAD3) inhibitor of NF-κB: positive regulation by members of the *rel*/NF-κB family. *EMBO J.* 12: 5043-5049.

Lemaire, P., C. Vesque, J. Scmitt, H, Stunnenberg, R. Frank and P. Charnay. 1990. The serum-inducible mouse gene Krox-24 encodes a sequence specific transcriptional activator. *Mol. Cell. Biol.* 10: 3456-3467.

Lenardo, M.J. and D. Baltimore. 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58: 227-229.

Lenardo, M.J., C.-M. Fan, T. Maniatis and D. Baltimore. 1989. The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell* 57: 287-294.

Lévesque, J.P., P. Sansilvestri, A. Hatzfeld and J. Hatzfeld. 1991. DNA transfection in Cos cells: a low-cost serum-free method compared to lipofection. *Biotechniques* 11: 313-318.

Lewis, R.B., J. McClure, B. Rup, D.W. Niesel, R.F. Garry, J.D. Hoelzer, K. Nazerian and H.R. Bose, Jr. 1981. Avian reticuloendotheliosis virus: identification of the hematopoietic target cell for transformation. *Cell* 25: 421-431.

Lim, K. and C.B. Chae. 1989. A simple assay for DNA transfection by incubation of the cells in culture dishes with substrate for ß-galactosidase. *Biotechniques* 7: 576-579.

Lim, M.Y., N. Davis, J. Zhang and H.R. Bose, Jr. 1990. The v-*rel* oncogene product complexed with cellular proteins including its proto-oncogene product and heat shock protein 70. *Virology* **175:** 149-160.

Liou, H.C., G.P. Nolan, S. Ghosh, T. Fujita and D. Baltimore. 1992. The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50. *EMBO J.* **11:** 3003-3009.

Liou, H.C. and D. Baltimore. 1993. Regulation of the NF-κB/*rel* transcription factor and IκB inhibitor system. *Current Opinion in Cell Biology* **5:** 477-487.

Liptay, S., R.M. Schmid, E.G. Nabel and G.J. Nabel. 1994. Transcriptional regulation of NF-κB2: Evidence for κB-mediated positive and negative autoregulation. *Mol. Cell. Biol.* **14:** 7695-7703.

Logeat, F., N. Israël, R. Ten, V. Blank, O. LeBail, P. Kourilsky and A. Israël. 1991. Inhibition of transcription factor belonging to the *rel*/NF-κB family by a trans dominant negative mutant. *EMBO J.* **7:** 1827-1832.

Luckow, V.A. and M.D. Summers. 1988. Trends in the development of baculovirus expression vectors. *Bio/Technology* **6:** 47-55.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular cloning; a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor.

McDonnell, P.C., S. Kumar, A.B. Rabson and C. Gélinas. 1992. Transcriptional activity of *rel* family proteins. *Oncogene* **7:** 163-170.

Means, A.L and P.J. Farnham. 1990. Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. *Mol. Cell. Biol.* **10:** 653-661.

Meyer, R., E.N. Hatada, H.-P. Hohmann, M. Heiker, C. Bartsch, U. Rüthlisberger, H.-W. Lahm, E.J. Schläger, A.P.G.M. van Loon and C. Scheidereit. 1991. Cloning of the DNA binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. *Proc. Natl. Acad. Sci. USA* **88:** 966-970.

Miller, C.K and H.M. Temin. 1986. Insertion of several different DNAs in reticuloendotheliosis virus strain T suppresses transformation by reducing the amount of subgenomic RNA. *J. Virol.* **58:** 75-80.

Mizutani, S. and H.M. Temin. 1973. Lack of serological relationship among DNA polymerases of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and chicken cells. *J. Virol.* 12: 440-448.

Mizutani, S. and H.M. Temin. 1974. Specific serological relationships among partially purified DNA polymerases of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and avian cells. *J. Virol.* 13: 1020-1029.

Moore, B.E. and H.R. Bose, Jr. 1989. Expression of the c-*rel* and c-*myc* proto-oncogenes in avian tissues. *Oncogene* 4: 845-852.

Morrison, L.E., N. Kabrun, S. Mudri, M.J. Hayman and P.J. Enrietto. 1989. Viral *rel* and cellular *rel* associate with cellular proteins in transformed and normal cells. *Oncogene* 4: 677-683.

Morrison, L.E., G. Boehmelt and P.J. Enrietto. 1992. Mutations in the *rel*-homology domain alter the biochemical properties of v-*rel* and render it transformation defective in chicken embryo fibroblasts. *Oncogene* 7: 1137-1147.

Mosialos, G., P. Hamer, A.J. Capobianco, R.A. Laursen and T.D. Gilmore. 1991. A protein kinase A recognition sequence is structurally linked to transformation by $p59^{v\text{-}rel}$ and cytoplasmic retention of $p68^{c\text{-}rel}$. *Mol. Cell. Biol.* 11: 5867-5877.

Mosialos, G. and T.D. Gilmore. 1993. vRel and c-Rel are differentially affected by mutations at a consensus protein kinase recognition sequence. *Oncogene* 8: 723-730.

Müller, C.W., F.A. Rey, M. Sodeoka, G.L. Verdine and S.C. Harrison. 1995. Structure of the NF-κB p50 homodimer bound to DNA. *Nature* 373: 311-317.

Nabel, G. and D. Baltimore. 1987. An inducible factor activates expression of human immunodeficiency virus in T cells. *Nature* 326: 711-713.

Naumann, M., F.G. Wulczyn and C. Scheidereit. 1993. The NF-κB precursor p105 and the proto-oncogene product Bcl-3 are IκB molecules and control nuclear translocation of NF-κB. *EMBO J.* 12: 213-222.

Nehyba, J., R. Hrdličková and E.H. Humphries. 1994. Evolution of the oncogenic potential of v-*rel*: *rel*-induced expression of immunoregulatory receptors correlates with tumor development and in vitro transformation. *J. Virol.* 68: 2039-2050.

Neri, A., C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A.T. Maiolo, R.S. Chaganti and R. Dalla-Favera. 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene, *lyt*-10, Homologous to NF-κB p50. *Cell* 67: 1075-1087.

Neumann, J.F., C. Morency and K. Russian. 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* 5: 711-713.

Neumann, M., K. Tsapos, J.A. Scheppler, J. Ross and B.R. Franza, Jr. 1992. Identification of complex formation between two intracellular tyrosine kinase substrates: Human c-Rel and the p105 precursor of p50 NF-κB. *Oncogene* 7: 2095-2104.

Nolan, G.P., S. Ghosh, H.-C. Liou, P. Tempst and D. Baltimore. 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. *Cell* 64: 961-969.

Oroszlan, S., M. Barbacid, T.D. Copeland, S.A. Aaronson and R.V. Gilden. 1981. Chemical and immunological characterization of the major structural protein (p28) of MMC-1, a rhesus monkey endogenous type C virus: homology with the major structural protein of avian reticuloendotheliosis virus. *J. Virol.* 39: 845-854.

Panganiban, A.T. and D. Fiore. 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. *Science* 241: 1064-1069.

Parker, R., H.E. Varmus and J.M. Bishop. 1984. Expression of v-*src* and chicken c-*src* in rat cells demonstrates qualitative differences between $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. *Cell* 37: 131-139.

Pierce, J.W., M. Lenardo and D. Baltimore. 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. *Proc. Natl. Acad. Sci. USA* 85: 1482-1486.

Purchase, H.G. and R.L. Witter. 1975. The reticuloendotheliosis viruses. *Curr. Top. Microbiol. Immunol.* 71: 103-124.

Rice, N.R., T.I. Bonner and R.V. Gilden. 1981. Nucleic acid homology between avian and mammalian type C viruses: relatedness of reticuloendotheliosis virus cDNA to cloned proviral DNA of endogenous colobus virus CPC-1. *Virology* 114: 286-290.

Rice, N.R., R.R. Hiebsch, M.A. Gonda, H.R. Bose, Jr. and R.V. Gilden. 1982. Genome of reticuloendotheliosis virus: characterization by use of cloned proviral DNA. *J. Virol.* 42: 237-252.

Rice, N.R., T.D. Copeland, S. Simek, S. Oroszlan and R.V. Gilden. 1986. Detection and characterization of the protein encoded by the v-*rel* oncogene. *Virology* 149: 217-229.

Rice, N.R., M.L. MacKichan and A. Israël. 1992. The precursor of NF-κB p50 has IκB-like functions. *Cell* **71:** 243-253.

Rice, N.R. and M.K. Ernst. 1993. *In vivo* control of NF-κB activation by IκBα. *EMBO J.* **12:** 4685-4695.

Richardson, P.M. and T.D. Gilmore. 1991. vREL is an inactive member of the Rel family of transcriptional activating proteins. *J. Virol.* **65:** 3122-3130.

Rijsewijk, F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel and R. Nusse. 1987. The *Drosophila* homolog of mouse mammary oncogene *int*-1 is identical to the segment polarity gene *wingless*. *Cell* **50:** 649-657.

Robinson, F.R. and M.J. Twiehaus. 1974. Isolation of the avian reticuloendothelial virus (strain T). *Avian Disease* **18:** 278-288.

Roth, S., D. Stein and C. Nüsslein-Volhard. 1989. A gradient of nuclear localization of the *dorsal* determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59:** 1189-1202.

Rous, P. 1910. A transmissible avian neoplasm: sarcoma of the common fowl. *J. Exp. Med.* **12:** 696-705.

Ruben, S., P.J. Dillon, R. Schreck, T. Henkel, C.-H. Chen, M. Maher, P.A. Baeuerle and C. Rosen. 1991. Isolation of a *rel*-related human cDNA that potentially encodes the 65-kd subunit of NF-κB. *Science* **251:** 1490-1493.

Ruben, S., R. Narayanan, J. Klement, C. Chen and C.A. Rosen. 1992. Functional characterization of NF-κB p65 transcriptional activator and an alternatively spliced derivative. *Mol. Cell. Biol.* **12:** 444-454.

Rushlow, C.A., K. Han, J.L. Manley and M. Levine. 1989. The traded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in *Drosophila*. *Cell* **59:** 1165-1177.

Sanger, F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463-5467.

Sarkar, S. and T.D. Gilmore. 1993. Transformation by the vRel oncoprotein requires sequences carboxy-terminal to the Rel homology domain. *Oncogene* **8:** 2245-2252.

Scheinman, R.I., A.A. Beg and A.S. Baldwin, Jr. 1993. NF-κB p100 (Lyt-10) is a component of H2TF1 and can function as an IκB-like molecule. *Mol. Cell. Biol.* **13:** 6089-6101.

Schmid, R., N.D. Perkins, C.S. Duckett, P.C. Andrews and G.J. Nabel. 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. *Nature* **352**: 733-736.

Scott, M.L., T. Fujita, J.-C. Liou, G.P. Nolan and D. Baltimore. 1993. The p65 subunit of NF-κB regulates IκB by two distinct mechanisms. *Genes Dev.* **7**: 1266-1276.

Sen, R. and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46**: 705-716.

Sherr, C.J., C.W. Rettenmier, R. Sacca, M.F. Roussel, A.T. Look and E.R. Stanley. 1985. The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* **41**: 665-676.

Shih, C.C., J.P. Stoye and J.M. Coffin. 1988. Highly preferred targets for retrovirus integration. *Cell* **53**: 531-537.

Sica, A., T.-H. Tan, N. Rice, M. Kretzschmar, P. Ghosh and H.A. Young. 1992. The c-*rel* protooncogene product c-Rel but not NF-κB binds to the intronic region of the human interferon-γ gene at a site related to an interferon-stimulable response element. *Proc. Natl. Acad. Sci. USA* **89**: 1740-1744.

Sif, S. and T.D. Gilmore. 1993. NF-κB p100 is one of the high-molecular-weight proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. *J. Virol.* **67**: 7612-7617.

Simek, S., R.M. Stephens and N.R. Rice. 1986. Localization of the v-*rel* protein in reticuloendotheliosis virus strain T-transformed lymphoid cells. *J. Virol.* **59**: 120-126.

Simek, S. and N.R. Rice. 1988a. Detection and characterization of the Protein encoded by the chicken c-*rel* protooncogene. *Oncogene Res.* **2**: 103-119.

Simek, S. and N.R. Rice. 1988b. p59$^{v\text{-}rel}$, the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed chicken lymphoid cells. *J. Virol.* **62**: 4730-4736.

Smith M.R., S.J. DeGudicibus and D.W. Stacey. 1986. Requirement for c-*ras* proteins during viral oncogene transformation. *Nature* **320**: 540-543.

Stephens, R.M., N.R. Rice, R.R. Heibsch, H.R. Bose, Jr. and R.V. Gilden. 1983. Nucleotide sequence of v-*rel*: the oncogene of reticuloendotheliosis virus. *Proc. Natl. Acad. Sci. USA* **80**: 6229-6233.

Steward, R. 1987. *Dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-*rel*. *Science* **238:** 692-694.

Steward, R. 1989. Relocalization of the *dorsal* proteins from the cytoplasm to the nucleus correlates with its function. *Cell* **59:** 1179-1188.

Summers, R.G. and G.E. Smith. 1987. A manual of methods for baculovirus vector and insect cell culture procedures. *Tex. Agric. Exp. Stn. Bull.* **1555.**

Sun, S.-C., P.A. Ganchi, D.W. Ballard and W.C. Greene. 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science* **259:** 1912-1915.

Sylla, B.S. and H.M. Temin. 1986. Activation of oncogenicity of the c-*rel* proto-oncogene. *Mol. Cell. Biol.* **6:** 4709-4716.

Tan, T.-H., G.P. Huang, A. Sica, P. Ghosh, H.A. Young, D.L. Longo and N.R. Rice. 1992. κB site-dependent activation of the interleukin-2 receptor α-chain gene promoter by human c-Rel. *Mol. Cell. Biol.* **12:** 4067-4075.

Temin, H.M. and H. Rubin. 1958. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. *Virology* **6:** 669-688.

Ten, R.M., C.V. Paya, N. Israël, O. Le Bail, M.-G. Mattei, J.-L. Virelizier, P. Kourilsky and A. Israël. 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. *EMBO J.* **11:** 195-203.

Theilen, G.H., R.F. Ziegel and M.J. Twiehaus. 1966. Biological studies with RE virus (strain T) that induces reticuloendotheliosis in turkeys, chickens and Japanese quail. *J. Natl. Cancer Inst.* **37:** 731-743.

Tung, H.Y.L., W.J. Bargmann, M.Y. Lim and H.R. Bose, Jr. 1988. The v-*rel* oncogene product is complexed to a 40-kDa phosphoprotein in transformed cells. *Proc. Natl. Acad. Sci. USA* **85:** 2479-2483.

Urban, M.B., R. Schreck and P.A. Baeuerle. 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* **10:** 1817-1825.

Varmus, H. 1988. Retroviruses. *Science* **240:** 1427-1435.

Waite, M.R.F. and P.T. Allen. 1975. RNA-directed DNA polymerase activity of reticuloendotheliosis virus: characterization of the endogenous and exogenous reactions. *J. Virol.* **16:** 872-879.

Walker, W.H., B. Stein, P.A. Ganchi, J.A. Hoffman, P.A. Kaufman, D.W. Ballard, M. Hannink and W.C. Greene. 1992. The v-*rel* oncogene: insights into the mechanism of transcriptional activation, repression, and transformation. *J. Virol.* **66:** 5018-5029.

Walro, D.S., N.K. Herzog, J. Zhang, M.Y. Lim and H.R. Bose, Jr. 1987. The transforming protein of avian reticuloendotheliosis virus is a soluble cytoplasmic protein which is associated with a protein kinase activity. *Virology* **160:** 433-444.

Waterfield, M.D., G.T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, B. Westermark, C.H. Heldin, J.S. Huang and T.F. Deuel. 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. *Nature* **304:** 35-39.

Wilhelmsen, K.C., K. Eggleton and H.M. Temin. 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. *J. Virol.* **52:** 172-182.

Wilhelmsen, K.C. and H.M. Temin. 1984. Structure and dimorphism of c-*rel* (turkey), the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. *J. Virol.* **49:** 521-529.

Wong, T.C. and M.M.C. Lai. 1981. Avian reticuloendotheliosis virus contains new class of oncogene of turkey origin. *Virology* **111:** 289-293.

Zabel, U. and P.A. Baeuerle. 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. *Cell* **61:** 255-265.

Zabel, U., T. Henkel, M.S. Silva and P.A. Baeuerle. 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. *EMBO J.* **12:** 201-211.

APPENDIX

OLIGONUCLEOTIDE SEQUENCES OF PCR PRIMERS

Primers used for v-*rel*

vR1: 5'-GGAGATCTACCATGGACTTTCTCACCAAC-3'

vR2: 5'-GGAGATCTACCATGATCTCAGAGCCCTAC-3'

vR3: 5'-GGAGATCTACCATGGAGTTTGGGCCCGAA-3'

vR4: 5'-GGAGATCTTCACAAGCTGAGCAGGCTGCT-3'

vR5: 5'-GGAGATCTTCAGGTCATGATGCTTGCAGT-3'

vR6: 5'-GGAGATCTTTGTCACGAACGATACCC-3'

Primers used for c-*rel*

cR5: 5'-GGAGATCTACCATGGCGGGTATCTCAGAG-3'

cR3: 5'-GGAGATCTGGCTCAATGGTTATAGCT-3'

Primers used for p50

505: 5'-GGGGATCCACCATGGCAGACGATGAT-3'

503: 5'-GGGGATCCAGCAGGCTAAATTTTGCC-3'

Primers used for p65

605: 5'-GGTGATCAACCATGGACGATCTGTTT-3'

603: 5'-GGTGATCAACCTTAGGAGCTGATCTG-3'

Primers used for pp40

405: 5'-CGGGATCCACCATGCTCAGCGCCCA-3'

403: 5'-GGGGATCCCGTTTAAAATGTCAGCTG-3'