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Molecular Cloning of Avian Sarcoma Virus CT10 and Characterization of its Protein Product

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MOLECULAR CLONING OF AVIAN SARCOMA VIRUS CT10

AND CHARACTERIZATION OF ITS PROTEIN PRODUCT

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Finally, I would like to dedicate this to Julie, with love. I'd never forget twice.

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LIST OF ABBREVIATIONS

anti-ptyr: antiserum that specifically recognizes phosphotyrosine-containing proteins

ASV: avian sarcoma virus

CEF: chicken embryo fibroblasts

CSF-1: colony-stimulating factor-1 (macrophage colony-stimulating factor)

DAG: 1,2-diacylglycerol

DMSO: dimethylsulfoxide

DTT: dithiothreitol (Cleland's reagent)

EDTA: disodium ethylenediaminetetraacetic acid

EGF: epidermal growth factor

env: retroviral gene encoding envelope glycoproteins

gag: retroviral gene encoding virion core proteins

GAP: *ras* GTPase activator protein (see page 10)

IP_3: inositol-(1,4,5)trisphosphate

kb: kilobase pairs

kd: kilodaltons (relative molecular mass)

LTR: long terminal repeat (region of retroviral genome containing promoter and polyadenylation signal)

MA: retroviral matrix protein (most 5' of gene products encoded by the *gag* gene; same as avian retroviral p19)

MOI: multiplicity of infection

MTag: polyomavirus middle tumor antigen

PDGF: platelet-derived growth factor

pfu: plaque-forming unit

PI: phosphatidylinositol

PIP_2: phosphatidylinositol-(4,5)bisphosphate

PI-PLC: phosphoinositide-specific phospholipase C

PLC: phospholipase C

PMSF: phenylmethylsulfonylflouride

pol: retroviral gene encoding RNA-dependent DNA polymerase, integrase, and RNAse H activities

RIPA buffer: radioimmunoprecipitation assay buffer; see page 22

RSV: Rous sarcoma virus

SDS: sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SSC: standard saline citrate

TGF-α: transforming growth factor-α

UR2AV: UR2-associated virus (a nontransforming avian helper retrovirus)

GLOSSARY OF TERMS

anchorage independence: the ability of tissue culture cells to proliferate while unattached to a solid substratum; for most cell types, including fibroblasts, a property of transformed cells and not of normal cells.

avian sarcoma virus: an avian retrovirus that carries an oncogene and rapidly induces a transformed phenotype in infected cells *in vitro* and growth of infected mesodermally-derived cells to form solid tumors *in vivo*.

domain: a discrete region of a larger protein that confers a specific structure or function, such as catalytic activity or ability to bind to other cellular components; used loosely in this thesis to denote a region of a protein that, by sequence comparison, is likely to confer a common (though in some cases unknown) function to other proteins containing a homologous region.

G-protein: one of a family of guanine nucleotide-binding proteins that couple transmembrane receptors to intracellular effector enzymes, leading to modulation of effector activity upon receptor stimulation (see Gilman, 1987).

growth control: a broad term for the mechanisms that govern when and under what circumstances a cell will proliferate.

helper virus: a replication-competent virus that can provide in *trans* essential functions which are lacking in replication-defective viruses, allowing transmission of the defective virus.

homology: used loosely in this thesis to denote significant amino acid sequence similarity, presumably indicating a common structure and/or function. Philosophically, however, such similarity probably implies a common evolutionary origin and thus homology in the strict sense.

immunoblotting: a technique in which proteins are separated by SDS-PAGE and transferred to nitrocellulose filters, which are then probed with antibodies that bind to specific proteins on the filter.

immunoprecipitation: a technique in which antibodies are used to bind to specific proteins in a cell lysate; the antibody-antigen complexes are then collected by binding the immunoglobulins to a solid support such as protein A-sepharose.

in vitro kinase assay: a technique to detect protein kinases, in which antigen-antibody complexes are washed, then incubated with γ-labeled ATP and divalent cation; if a kinase is present in the immunoprecipitate, the labeled γ-phosphate will be transferred to proteins which can then be detected by SDS-PAGE.

northern blotting: a technique in which RNA is separated by denaturing agarose gel electrophoresis and transferred to nitrocellulose filters; the filters are then hybridized with specific, labeled nucleic acid probes which bind to homologous RNA present on the filters.

oncogene: a gene that can confer a transformed phenotype *in vitro* or induce tumors *in vivo* when expressed in appropriate cells. With the exception of several

genes from DNA tumor viruses, oncogenes are derived by mutation of normal cellular genes, or are normal cellular genes that can become oncogenic if inappropriately expressed.

oncogene transduction: the process whereby cellular proto-oncogene sequences are stably incorporated into a retroviral genome, generating an infectious, acutely transforming retrovirus.

phospholipase C: an enzyme that catalyzes the hydrolysis of the phosphodiester bond of phospholipids to generate diacylglycerol and phosphorylated head group.

protein-serine kinase (serine/threonine kinase): an enzyme that catalyzes the transfer of the γ-phosphate of ATP to the hydroxyl group of serine and/or threonine residues on substrate proteins.

protein-tyrosine kinase (tyrosine kinase): an enzyme that catalyzes the transfer of the γ-phosphate of ATP to the hydroxyl group of tyrosine residues on substrate proteins.

proto-oncogene: a normal cellular gene that can confer a transformed phenotype *in vitro* or cause tumor formation *in vivo* if mutated and/or inappropriately expressed.

RNA tumor virus: a retrovirus carrying an oncogene, which can rapidly transform appropriate infected cells *in vitro* and induce neoplastic disease *in vivo*.

signal transduction: used in this thesis to denote the process whereby signals are transmitted from the extracellular environment into the cell to generate intracellular responses.

Southern blotting: a technique in which DNA fragments are separated by agarose gel electrophoresis, denatured, and transferred to nitrocellulose filters. The filters are then hybridized with specific nucleic acid probes which bind to homologous DNA fragments on the filters.

transfection: a technique in which cloned DNA is introduced into tissue culture cells, generally by calcium phosphate coprecipitation. The transfected DNA becomes incorporated into the genomes of a small percentage of the transfected cells and can be expressed.

transformation: a term that describes a wide variety of phenotypic changes observed in tissue culture cells that are thought to reflect the changes responsible for the aberrant growth of tumor cells *in vivo*. Some of the parameters of transformation are altered morphology, decreased growth factor dependence, increased saturation density, lack of contact inhibition, increased metabolic rate, and anchorage independence (see Hanafusa 1977).

ABSTRACT

Four previously uncharacterized avian sarcoma viruses were screened and two of these, RPL30 and CT10, were found to encode apparently novel oncogenes. Biologically active CT10 DNA was molecularly cloned and the nucleotide sequence was determined. The CT10 genome encodes a *gag*-fusion polypeptide of 47 kilodaltons, termed p47$^{gag\text{-}crk}$. This protein contains blocks of sequence similarity to a noncatalytic, potentially regulatory region found in the nonreceptor tyrosine kinases, a phosphoinositide-specific phospholipase C, and the *ras* GTPase activator protein; no homology was found to any known catalytic domain. Potential roles for the homologous domains, termed SH2 and SH3, in normal signal transduction and in the biological activity of p47$^{gag\text{-}crk}$ are discussed.

Biochemical data demonstrated that phosphotyrosine levels on at least three cellular proteins were greatly elevated in CT10-infected cells, and that a tyrosine kinase activity was immunoprecipitated in association with p47$^{gag\text{-}crk}$. A specific antiserum that recognizes the c-*crk*-derived portion of the oncogene product was generated, and a number of proteins were identified that were phosphorylated in *in vitro* kinase reactions of v-*crk* immunoprecipitates or were coimmunoprecipitated with the v-*crk* protein. All of the phosphoproteins that specifically coprecipitate with p47$^{gag\text{-}crk}$ contained high levels of phosphotyrosine; these proteins include the three major phosphotyrosine-containing proteins of CT10-infected cells. Preliminary experiments demonstrated that p47$^{gag\text{-}crk}$, the *crk*-associated tyrosine kinase activity, and the major phosphotyrosine-containing proteins of infected cells were localized on membranes in the nonionic detergent-insoluble cytoskeletal matrix.

A series of 10 v-*crk* mutants were constructed and their biological ac-

tivity was assessed. Lesions within the SH2 or SH3 domains decreased or abolished biological activity, and viral *gag* sequences were also required for full transforming activity. Mutations in the nonhomologous regions between the SH2 and SH3 domains had no effect. Biochemical analysis of mutant-infected cells demonstrated that in all cases increased phosphotyrosine levels correlated with transformation.

SECTION ONE

INTRODUCTION

1-1 *General introduction*

The acutely transforming retroviruses, or RNA tumor viruses, provide an invaluable biological system for studying the molecular mechanisms of malignant transformation. Each RNA tumor virus expresses at high levels one, or in some cases more than one, viral oncogene which had originally been derived from the host genome. The molecular cloning of these transforming viruses has led to the identification of numerous genes that have the ability to induce uncontrolled cell growth when mutated and/or inappropriately expressed—the cellular proto-oncogenes (see Bishop, 1985 for review). The historical role of the RNA tumor viruses in shaping how we understand carcinogenesis and growth control on the molecular level is indisputable; what I hope to demonstrate in this thesis is that there's life in the old system yet. The study of previously uncharacterized sarcoma viruses is perhaps the most efficient way to identify novel oncogenes, and with each new oncogene the task of elucidating the pathways of normal and abnormal growth control becomes less daunting.

The RNA tumor viruses rapidly induce tumors in infected animals (generally within two weeks in the case of avian sarcoma viruses) and are able to cause transformation of appropriate cultured cells with single-hit kinetics (reviewed by Teich et al., 1982). Therefore each viral oncogene, when expressed in a retroviral context, is sufficient to induce transformation and tumorigenesis. While this probably does not reflect the situation in spontaneous tumors, which may involve the gradual accumulation of genetic alterations under the fierce selection of growth in an organism, it greatly simplifies biochemical analysis. The

molecular consequences of expression of a single gene product can be assessed relative to matched controls that do not express the oncogene. An added advantage of the viral system is the ease with which large numbers of primary cultured cells can be rapidly induced to express the oncogene via the viral replication pathway.

Since each viral oncogene is derived from a normal (and obviously nontransforming) cellular proto-oncogene present in the genome of every cell of the host organism, comparison with the normal homolog provides important clues to the mechanism of transformation. The two most important potential differences between the viral and cellular genes are in expression level and structural mutations that may have arisen during or subsequent to transduction of the proto-oncogene into the viral genome. In some cases, such as the *fos* gene, it appears that constitutive expression via the strong viral promoter is sufficient to induce transformation. This is consistent with the presumptive role of the c-*fos* protein as a component of the transcriptional activator complex AP-1, and the observation that expression of c-*fos* is rapidly and transiently induced in cultured cells after stimulation with a variety of mitogens and growth factors (Verma and Sassone-Corsi, 1987; Curran and Franza, 1988). In the case of the *src* oncogene, on the other hand, structural mutations are generally required for transformation. The discovery that the v-*src* gene product is many times more active than that of c-*src* as a protein-tyrosine kinase implicates this enzymatic activity in transformation and has initiated a great deal of research into potential mechanisms for negatively regulating the activity of tyrosine kinases *in vivo* (Hanafusa, 1986).

The wide variety of viral oncogenes isolated to date is a reflection of the complexity of the signal transduction pathways involved in normal growth control and the many ways that these pathways can be subverted in the transformed cell. In many cases, the role of the normal proto-oncogene in signal

transduction has been deduced in part by the aberrant action of the corresponding viral oncogene. The viral oncogenes (reviewed in Bishop, 1985 and Bishop and Varmus, 1985) that have been characterized include a growth factor, *sis* (the beta chain of platelet-derived growth factor); several growth factor receptors with intrinsic tyrosine kinase activity including *erbB* (the EGF/TGF-α receptor), *fms* (the CSF-1 receptor), *kit* (the mouse W locus), *sea*, and *ros*; protein-tyrosine kinases of the nonreceptor class *src*, *yes*, *fgr*, *fps/fes*, and *abl*; the serine kinases *mos* and *mil/raf*; G-protein analogs ^{H}*ras* and ^{K}*ras*; and nuclear proteins *fos*, *jun* (both of which are components of the AP-1 transcription activator complex), *myb*, *myc*, *erbA*, *ski*, *ets*, and *rel*. While it certainly has not been demonstrated that the normal homologs to each of these viral oncogenes are involved in normal growth control (this is especially true of the nonreceptor tyrosine kinases, for which no biological role has yet been demonstrated), the ability of the oncogenic form to induce uncontrolled growth indicates that these genes can short-circuit the normal growth-control pathways. In addition, the implication of several of the cellular homologs to the viral oncogenes in human tumors (reviewed in Marshall, 1985; Bishop, 1987) indicates that the virus transduction can in some cases mimic mutational events that can lead to spontaneous tumors.

Analysis of RNA tumor viruses is not the only method to identify oncogenes; given the existence of such viruses, however, the system has unique advantages. Oncogenes have been identified by transfection of DNA from tumor cells into recipient "normal" cell lines such as NIH 3T3; the transforming sequences can be rescued, often with great difficulty, from transformed foci or from tumors induced by recipient cells injected into nude mice (see Parada et al., 1982 and Fasano et al., 1984 for examples). The success of this technique depends on high transfection efficiency, a relatively small size for the genomic transforming gene, and the ability of the transforming gene to transform the recipient cell to

an obvious phenotype. To date, this technically demanding method has identified few proto-oncogenes that had not been previously identified as viral oncogenes. In addition, the RNA tumor viruses may provide a more realistic view of genes with oncogenic potential since the end result of viral infection is a tumor, often in outbred animals, as opposed to a transformed focus on a dish of immortalized tissue-culture cells.

The mechanisms that control the ability of each cell in a multicellular organism to proliferate only at the appropriate time in development or adult life are likely to be extremely complex. To identify the important components of such a mechanism from purely biochemical and genetic approaches would be a formidable task. The discovery of viral oncogenes and their normal cellular counterparts, however, has identified many of the proteins and mechanistic motifs involved in growth control pathways. The characterization of novel viral oncogenes is perhaps the single most efficient way to fill in the numerous gaps in our present understanding of eukaryotic growth control.

1-2 *Retroviral life cycle and oncogene transduction*

The genetic information of a retroviral particle consists of two identical strands of (+)-strand RNA. Upon penetration of the host cell plasma membrane, the genomic RNA is reverse transcribed by the viral RNA-dependent DNA polymerase packaged in the virion core. The two copies of genomic information may allow generation of a complete cDNA copy even if one or both RNA molecules has been damaged; this ability of the polymerase to "jump" from strand to strand may be important to oncogene transduction, as described below. After reverse transcription, the viral genome (now in double-stranded DNA form with long terminal repeats, or LTRs, at both ends) integrates into the host cell chromosome via the integrase activity encoded by the viral *pol* gene. This pro-

virus, once integrated, directs synthesis of viral genomic and subgenomic mRNAs to initiate the next infectious cycle (the replication cycle is reviewed in Varmus and Swanstrom, 1985).

The exact mechanisms involved in oncogene transduction by retroviruses are still controversial; however, a reasonable hypothesis can be constructed based on peculiarities of the retroviral life cycle (see Bishop and Varmus, 1985). A first step might involve integration of a provirus upstream from or within the coding sequence of a proto-oncogene. A DNA rearrangement resulting in deletion of the 3' LTR (where the transcription termination and polyadenylation signals reside) would result in transcription of a hybrid viral-proto-oncogene mRNA via the still-intact 5' LTR. Since the *cis*-acting sequences required for packaging viral RNA into virions are in the 5' portion of the genome, the hybrid RNA could be packaged into a virion along with an intact viral genome. (This assumes that the cell has been infected with at least two retroviruses, only one of which has suffered a rearrangement). During reinfection of a cell by the "heterozygous" virion, the ability of the reverse transcriptase to jump from one mRNA to another would allow the generation of a viral-oncogene-viral recombinant provirus with LTRs at both ends. This provirus would contain all of the *cis*-acting sequences required for transcription, packaging, reverse transcription, and integration of the hybrid viral genome.

Although the exact mechanism of generation of the RNA tumor viruses is unimportant to this discussion, a few general properties of the oncogene-carrying retroviruses should be noted. First, the transduction process generally results in the deletion of viral structural gene sequences, rendering the sarcoma viruses replication-defective. In order to produce infectious progeny virus, they therefore require coinfection with replication-competent helper viruses to provide the missing essential function in *trans*. To date, of all RNA tumor

viruses only certain strains of Rous sarcoma virus have been shown to be replication-competent, and there is evidence that the original Rous virus was itself defective (Dutta et al., 1985). The second important point is that the process of transduction often results in truncation of the cellular proto-oncogene and insertion into viral coding sequences. Consequently, many viral oncogene products are hybrid proteins, with viral structural gene sequences fused to sequences derived from the proto-oncogene. A practical implication of this is that the oncogene products can often be immunoprecipitated by antibodies that recognize viral structural proteins, allowing preliminary characterization of the transforming protein in the absence of a specific antibody.

1-3 *Introduction to signal transduction mechanisms*

The central problem of eukaryotic growth control is the elucidation of mechanisms of signal transduction: how positive and negative stimuli from the environment can be transmitted through the plasma membrane to the nucleus, where they are ultimately reflected in altered transcriptional activity. The bare outlines of this system are beginning to be understood, but in many places there are crucial gaps. There are also whole classes of molecules, such as the nonreceptor tyrosine kinases and the *ras* proteins, which are implicated in signal transduction but for which no normal cellular function has yet been demonstrated. In this section I will briefly review some of what is known about the mechanisms of signal transduction, with an emphasis on how oncogene products may fit into these pathways.

One mechanism that is quite well understood is that used by the steroid and thyroid hormones (reviewed in Beato, 1989). Since these hormones are lipid-soluble, the problem of transmitting a signal across the plasma membrane is circumvented by simple diffusion. Once in the cytoplasm, the hormones

bind to specific receptors which in turn can bind to DNA and influence the transcription of various hormone-responsive genes. The viral oncogene *erb*A, which potentiates the transforming potential of the *erb*B oncogene in some cell types (Damm et al., 1987), is a homolog of the normal cellular thyroid hormone (T_3 and T_4) receptor (Sap et al., 1986; Weinberger et al., 1986). While this mechanism is simple and direct, it cannot account for the biological activity of peptide hormones, neurotransmitters, growth factors, and other cytokines, which are unable to diffuse through the plasma membrane and must initiate their biological responses by binding extracellularly to transmembrane receptors.

A second well-characterized signal transduction system involves the activation of protein kinases via cyclic nucleotide second messengers. In the classic example, binding of β-adrenergic agonists to specific cell-surface receptors leads to the cytoplasmic activation of a GTP-binding protein, or G-protein; this activation is thought to involve exchange of bound GDP for GTP and dissociation of the α subunit of the G-protein from its β and γ subunits. The GTP-bound α subunit can then activate the enzyme adenylyl cyclase, thereby causing an increase in the intracellular concentration of the second messenger, cAMP (reviewed in Gilman, 1987). This compound in turn binds to the regulatory subunit of cAMP-dependent protein kinase, causing dissociation of an active catalytic subunit and leading to the phosphorylation of substrate proteins on serine and threonine residues (see Edelman et al., 1987). This is a good paradigm for other signal transduction pathways, in that there are several steps involving signal amplification and many levels at which the signal can be downregulated. That this pathway ultimately results in the activation of a protein kinase reflects the fact that phosphorylation is the most commonly used cellular mechanism to rapidly and reversibly alter the conformation and activity of proteins. While the cAMP-dependent pathway is not generally associated with positive growth con-

trol, an oncogene that had been isolated by transfection, *mas*, was found to have a structure similar to the β-adrenergic receptor and other receptors coupled to G-proteins, suggesting that this oncogene may transform by a G-protein-mediated pathway (Young et al., 1986). In addition, the *ras* oncogene products bind GDP and GTP and have sequence similarity to classical G-proteins (Marshall, 1986; Barbacid, 1987). However, as will be discussed below, there is evidence that the *ras* proteins are functionally quite different from true G-proteins.

Growth control mechanisms have been studied most often with tissue culture cells, and many peptide growth factors and mitogens have been isolated from serum. These growth factors bind to specific transmembrane receptors on the cell surface; in many cases, the cytoplasmic domains of these receptors possess an intrinsic protein-tyrosine kinase activity (Yarden and Ullrich, 1988). The seminal discovery that the v-*src* oncogene product has tyrosine kinase activity (Collett et al., 1980; Hunter and Sefton, 1980; Levinson et al., 1980) and the subsequent finding of the same activity in many growth factor receptors has led to a great deal of interest in the substrates of normal and oncogenic tyrosine kinases. It is presumed that ligand binding causes the specific tyrosine phosphorylation by the receptors of intracellular substrate proteins, initiating a cascade of events culminating in cell division, and that the tyrosine kinase oncogenes may cause uncontrolled cell growth by phosphorylating these same substrates in the absence of exogenous signals. The central importance of tyrosine kinases in growth control can be inferred from the large number of viral and other oncogenes that encode tyrosine kinases (Bishop and Varmus, 1985; Bishop, 1987). While there is good evidence that the tyrosine kinase activity of the growth factor receptors is required for biological activity, the major substrate of these receptors is often the receptor itself; it has been suggested in the case of the insulin receptor that it is a

change in conformation brought about by autophosphorylation, and not phosphorylation of heterologous proteins, that is important for at least some aspects of signal transmission (Forsayeth et al., 1987)

A great deal of recent work has established the importance of phospholipase C (PLC) in signal transduction. The PLC-mediated cleavage of phosphatidylinositol-bisphosphate (PIP_2) into two intracellular second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG), is involved in the cellular responses to many extracellular stimuli, including mitogens (Majerus, et al., 1986; Berridge, 1987). DAG activates the serine/threonine kinase activity of the protein kinase C family (reviewed in Nishizuka, 1986), while IP_3 causes the release of calcium from intracellular stores. Increased cellular calcium concentrations can affect many cellular processes, including the activation of Ca^{++}-dependent serine/threonine kinases (see Edelman et al., 1987). The DAG-dependent activation of protein kinase C has been implicated in a wide range of intracellular responses, including alteration of growth properties (Nishizuka, 1986). The phorbol ester tumor promoters are thought to exert their biological activity by binding specifically to protein kinase C and abrogating its DAG requirement.

The involvement of phosphoinositide-specific phospholipase C (PI-PLC) in the cellular response to hormones and mitogens has led to a great deal of work on the mechanism of activation of PI-PLCs. Pharmacological data have in many cases implicated a G-protein in the activation of PLC activity (Majerus, 1986; Berridge, 1987). It was once thought that the *ras* family of GTP-binding proteins might be involved in the coupling of PI-PLCs to cell surface receptors; however, experimental evidence has not borne this out (see Downward, et al., 1988; Yu et al., 1988). Recently, there have been some indications that at least one PI-PLC is phosphorylated on tyrosine in response to treatment of cells with

epidermal growth factor (EGF); this raises the possibility that a PI-PLC could be directly phosphorylated and activated by the intrinsic protein-tyrosine kinase activity of the EGF receptor and other growth factor receptors (Wahl et al., 1988; Wahl et al., 1989). However, the existence of many types of PI-PLCs suggest that there may be many different mechanisms involved in responses to different stimuli (Rhee et al., 1989).

The normal function of the *ras* family of proteins and the mechanism whereby their oncogenic variants transform remains enigmatic. The fact that mutated *ras* genes can transform, and that antibodies that inactivate normal *ras* function can block the serum-stimulated entry of quiescent cells into the cell cycle (Mulcahy et al., 1985) suggests a central role for the *ras* gene products in mediating the signals of growth control. Oncogenic forms of *ras* have lower intrinsic GTPase activities compared to the normal homologs (reviewed in Barbacid, 1987), suggesting that, as in the case of classical G-proteins, the GTP-bound form is the active signal transducer. Recently, McCormick and colleagues have characterized an activity from normal cells that stimulates the GTPase activity of the normal, but not the oncogenic, forms of *ras* (Trahey and McCormick, 1987). This GTPase Activator Protein, or GAP, has been shown to still interact with oncogenic *ras* molecules, however; genetic evidence suggests that interaction of *ras* with GAP is required for biological activity (reviewed in McCormick, 1989). It has therefore been proposed that GAP is a component of the ultimate target, or effector, of *ras* action, analogous to adenylyl cyclase in the case of the classical G-proteins, as outlined above.

Although *ras* appears to play a crucial role in growth control, it must be emphasized that neither the stimulus that activates *ras* nor the enzyme activity ultimately stimulated by *ras* are presently understood. Antibody injection experiments have shown that *ras* activity is required for growth of cells transformed

by the tyrosine kinase oncogenes *fes*, *fms*, and *src*, but not for growth of cells transformed by the serine kinase oncogenes *mos* and *raf* (Smith et al., 1986). This is consistent with a requirement for *ras* activity downstream from the tyrosine kinases and upstream from the serine kinases in growth control pathways. Coupled with the evidence that *ras* is not involved in regulation of PI-PLC (and may actually function downstream of PI-PLCs—see Yu et al., 1988), this suggests that there might be a poorly understood *ras*-dependent pathway governing growth control which might ultimately lead to the activation of serine kinases such as c-*mos* and c-*raf*.

There has been some recent evidence that supports the idea that serine kinases such as *mil/raf* and *mos* might play a role in transmitting signals involved in growth control, as might be expected given that they were originally isolated as viral oncogenes. One group has found that tyrosine phosphorylation of cellular *Raf-1* kinase is increased in cells stimulated with mitogens or transformed with tyrosine-kinase oncogenes, concomitant with an increase in *in vitro Raf-1* kinase activity (Morrison et al., 1988b). *In vitro* studies have also shown that insulin stimulation leads to an increase in the activity of the serine/threonine kinase, MAP-2 kinase, toward its substrate, S6 kinase; there is evidence that the activation of MAP-2 kinase is via tyrosine phosphorylation (Sturgill et al., 1988). Furthermore, the higher eukaryotic homolog of the yeast cell-cycle gene cdc2, a serine/threonine kinase, was shown to be equivalent to the factor identified in *Xenopus* as MPF (maturation promoting factor), whose activity is required for oocyte maturation (Arion et al., 1988). MPF/cdc2 has been shown to be phosphorylated on tyrosine in a cell-cycle-dependent manner (Draetta et al., 1988), and there is indirect evidence that the serine kinase activity of the cellular homolog to the *mos* oncogene is required for MPF activity during oocyte maturation (Sagata et al., 1988).

The data above suggest that cascades of tyrosine and serine kinases might be involved in the regulation of growth control; this is not particularly surprising. It is likely that tyrosine kinases work proximal to the membrane in the early stages of signal transduction, either as growth factor receptors or through a poorly understood pathway involving the nonreceptor tyrosine kinases. The serine kinases are probably the intracellular, downstream effectors, ultimately transmitting the signal to the nucleus. Consistent with this, evidence is emerging that phosphorylation may regulate the activity of some transcription factors (Hoeffler et al., 1988; Sorger and Pelham, 1988; Prywes et al., 1988). Another intriguing observation is that the transcription factor NF-KB can exist in a cytoplasmic, inactive form; upon stimulation, the active factor dissociates from an inhibitor and migrates to the nucleus, where it binds to DNA (Baeuerle, et al., 1988). Phosphorylation would be an excellent way to trigger such a dissociation and activation.

It would be virtually impossible to comprehensively review the huge volume of work on the mechanisms of signal transduction. Although this field has grown explosively over the past decade, however, the complexity of the pathways involved has precluded a coherent understanding of the the mechanisms and interrelationships important to growth control. Different cell types probably respond in different ways to a given stimulus, depending on what pathways are activated, what proteins are available to respond to the output of these pathways, and how different pathways interconnect and cross-regulate each other. Clearly, to understand these regulatory networks it would be helpful to isolate and identify the proteins involved; quite a few of the critical mediators remain to be identified. The oncogenes isolated from the RNA tumor viruses have in many cases guided the questions being asked and the experimental approaches used to elucidate signal transduction pathways, since the viral oncogenes are demonstr-

ably able to distort these pathways. In this thesis I will describe the identification of a previously uncharacterized viral oncogene that transforms cells through an apparently novel mechanism. This work, by identifying unforeseen ways in which signal transduction might be regulated in normal and transformed cells, opens a new perspective on the field and may be expected to provide an intellectual and experimental starting point for novel approaches aimed at a more comprehensive understanding of the mechanisms of growth control.

SECTION TWO

MATERIALS AND METHODS

2-1 *Cell culture and DNA transfection*

Cell culture. Chicken embryo fibroblasts (CEF) were cultured and infected with viruses essentially as described (Hanafusa, 1969). CEF were prepared from 11-day embryos and were subcultured for infection or transfection 5-9 d after primary culture in either Ham's F-10 medium supplemented with 10% tryptose phosphate broth (TPB), 5% calf serum, and 1% chicken serum or Scherer's medium supplemented with 10% TPB and 2 or 5% calf serum. All media contained 100 U/ml penicillin, 50 μg/ml streptomycin, and 1 μg/ml fungizone. Cells were routinely maintained at 37°.

Secondary CEF were infected 4-12 h after subculture with virus stock after the addition of DEAE-dextran to 5 μg/ml. To select for transformed cells, infected or transfected dishes were often overlaid with F-10, Scherer's, or F-10:Scherer's (1:1) medium as above further supplemented with 0.5% bovine embryo extract and 0.375% agar (soft agar medium) and incubated at 40°.

Colony formation assay. To assay for anchorage-independent growth of infected or transfected CEF, approximately 10^6 CEF (including less than 10^3 virus-infected cells) were plated in 10 cm dishes in Minimum Essential Medium supplemented with 10% TPB, 10% calf serum, 1% chicken serum, 1% DMSO, and 0.4% agar on a layer of the same medium containing 0.7% agar. Colonies were scored 3-4 weeks after plating.

Tumorigenicity and virus rescue. Tumors were induced in SPAFAS white leghorn chickens by injecting 0.1 ml virus stock into each wing web of newborn chicks (2-5 d after hatching).

Virus was recovered from tumors by mincing fresh tumor tissue, dissociating in buffered saline containing 0.25% trypsin, and plating tumor cells on fresh secondary CEF.

DNA transfection. Transfection of molecularly cloned viral DNA was performed essentially as described (Wigler et al., 1979; Cross and Hanafusa, 1983). For CT10 and CT10-derived mutants, 2 μg DNA was cleaved with *Sca*I and mixed with 2 μg *Sac*I-cleaved pUR2AV helper virus DNA (Neckameyer and Wang, 1984). DNA was resuspended in 10 μl ligase buffer along with diluted T4 ligase and incubated at 14^{o} for 30 min. After heating to 65^{o} for 10 min, the DNA was added to 0.5 ml Buffer A (0.25 M $CaCl_2$, 25 mM HEPES pH 7.15, 50 μg/ml sheared calf thymus DNA). 0.5 ml Buffer B (280 mM NaCl, 25 mM HEPES pH 7.15, 1.5 mM Na_2HPO_4) was added dropwise while vortexing. The precipitate was allowed to form at room temperature for 20 min, then was added to 2 6 cm dishes seeded the previous night with $7x10^5$ CEF in F-10 medium; the medium was changed 2 h before transfection to F-10 lacking TPB. The precipitate was washed off after 4 h and fresh medium was added. Plates were transferred 2 d after transfection, overlaid with soft agar medium, and incubated at 40^{o}. With wild-type CT10, transformation was generally evident after the second transfer (10-14 d post-transfection).

For mutants (10-NRC, 10-NSC) expressed in replication-competent Rous sarcoma virus (RSV)-derived vectors, 2 μg pSR-XD2-derived DNA and 2 μg pBH-rep DNA (Cross and Hanafusa, 1983; Iba et al., 1984) were cleaved with *Sal*I, mixed, ligated, and transfected as above. Cells were completely infected (judged by transformation of parallel transfections with pSR-XD2, which encodes v-*src*) 5-7 d post-transfection.

2-2 *"Oncogene blot" screen of novel viruses*

DNA probes. Probes specific for 19 known viral oncogenes were prepared from molecularly cloned DNA. The restriction fragments chosen (except positive control) contain no helper virus-derived sequences that might hybridize nonspecifically to the retroviral genome. For each oncogene, the plasmid and specific restriction fragment used, species of origin, and reference are listed below. *fms*: pSM3, 1.4 kilobase (kb) *Pst*I insert, cat (Donner et al., 1982 and C. Sherr, personal communication). *ros*: pros, 0.85 kb *Eco*RI-*Pvu*II insert, chicken (Neckameyer and Wang, 1985). *erbB*: perb5, 0.56 kb *Bam*HI insert, chicken (Yamamoto et al., 1983 and J. Samarut, personal communication). *abl*: pAB3sub3, 1.6 kb *Sac*I-*Hin*dIII fragment, mouse (Reddy et al., 1983 and S. Goff, personal communication). *fgr*: pGR-FeSV, 0.93 kb *Sma*I-*Kpn*I fragment, cat (Naharro et al., 1984). *src*: pTT107, 0.87 kb *Pvu*II fragment, chicken (Takeya and Hanafusa, 1982). *fps*: pBR-FO4, 0.41 kb *Bam*HI insert, chicken (Foster et al., 1986). *yes*: pMR/YA, 0.43 kb *Bam*HI fragment, chicken (Sudol and Hanafusa, 1986). *mos*: pcmos, 0.92 kb *Ava*I-*Hin*dIII fragment, mouse (Van Beveren et al., 1981). *mil*: pMBS28, 0.58 kb *Bam*HI-*Sph*I fragment, chicken (Kan et al., 1984 and D. Stehelin, personal communication). ^{H}*ras*: pBS9, 0.46 kb *Eco*RI insert, rat (Ellis et al., 1980). ^{K}*ras*: pHiHi3, 0.38 kb *Sac*II-*Xba*I fragment, rat (Ellis et al., 1981). *sis*: pSSV, 0.92 kb *Pst*I-*Xba*I fragment, woolly monkey (Devare et al., 1982). *erbA*+*erbB*: perbA, 1.2 kb *Sal*I-*Bam*HI insert, chicken (Debuire et al., 1984). *rel*: pEcoRI-rel, 0.97 kb *Eco*RI insert, turkey (Chen et al., 1981). *ski*: pSRski-1, 1.2 kb *Sac*I insert, chicken (Li et al., 1986 and E. Stavnezer, personal communication). *fos*: pfos1, 1.0 kb *Pst*I fragment, mouse (Curran et al., 1982). *myc*: pcmyc1, 0.8 kb *Cla*I-*Eco*RI fragment, chicken (Alitalo et al., 1983 and W. Hayward, personal communication). *myb*: pvm2, 0.35 kb *Eco*RI-*Sal*I fragment, chicken (Klempnauer et al., 1982). Avian retrovirus positive control: pSRA2, 3.6 kb *Eco*RI fragment of RSV (DeLorbe et al., 1980).

Filter binding. 50 ng each fragment was denatured by boiling for 5 min in 0.1 M NaOH, neutralized by adding an equal volume of 1M TRIS pH 6.8, diluted five-fold with 15X SSC (Standard Saline Citrate) and blotted to nitrocellulose using a slot-blot manifold. Filters were baked at 80^{o} for 3 h *in vacuo* before prehybridization

Viral RNA preparation. ^{32}P-labeled avian sarcoma virus cDNA was generated by reverse transcription of viral genomic RNA harvested from infected cell culture fluids. Medium was collected at 4 h intervals (4 ml/10 cm dish), prespun to remove cells and debris, and spun 90 min, 40,000 rpm in a Ti60 rotor to pellet virus. Pellets were resuspended in 10mM TRIS pH 7.4, 5 mM EDTA, 0.5% SDS, 1 mg/ml proteinase K (1 ml extraction buffer/25 ml original volume) and digested at 37^{o} for 1 h. The RNA solution was extracted 3 times with phenol:chloroform:isoamyl alcohol (50:50:1), once with chloroform:isoamyl alcohol (99:1) and ethanol precipitated. The pellet was resuspended in 1 M LiCl and precipitated with 7 volumes ethanol to remove SDS. 5 to 10 μg RNA were recovered from 100 ml culture fluid.

Reverse transcription. 1 μg viral RNA was suspended in 10 μl 50 mM TRIS pH 8.3, 10 mM $MgCl_2$, 150 mM NaCl, 10 mM DTT, and 0.5 μg/μl random hexadeoxynucleotide primer and heated to 80^{o} for 2 min. After cooling to room temperature, RNA was diluted to 20 μl to a final concentration of 100 μM deoxynucleotide triphosphates lacking dCTP, 2.5 μCi/μl [α-^{32}P]dCTP (3000 Ci/mmol), and 1 U/μl AMV reverse transcriptase. The reaction was incubated at room temperature for 10 min, then 42^{o} for 1 h. EDTA was added to stop the reaction, NaOH was added to 50 mM, and the solution was incubated at 65^{o} for 1 h to hydrolyze RNA. After neutralization with 1 M TRIS pH 6.8, the cDNA was separated from unincorporated label by gel filtration on sephadex G-25. Approximately 10^{7} cpm labeled cDNA was recovered in the excluded volume.

2-3 *Nucleic acid analysis*

RNA preparation. CEF were pelleted and resuspended in water (1 ml/10 cm dish). Cells were lysed by adding an equal volume of 2X extraction buffer (20mM TRIS pH 7.4, 10 mM EDTA 1% SDS, 2 mg/ml proteinase K), DNA was sheared by repeated passage through a 22G needle, and the lysate was incubated at 37° for 1 h. After protease digestion, the lysate was extracted with phenol-chloroform, then with chlololform, and was ethanol precipitated.

Northern blotting. Gel buffer was 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1mM EDTA. RNA samples (usually 10 μg RNA/lane) were suspended in gel buffer containing 50% formamide, 6.3% formaldehyde, 10% glycerol, and bromophenol blue, and were heated at 95° for 2 min prior to loading. Samples were run on 1% agarose gels containing 2% formaldehyde and 1 μg/ml ethidium bromide.

After electrophoresis, the gels were washed in two changes of 10X SSC for 40 min to remove formaldehyde and were blotted by capillary action to nitrocellulose filters in 10X SSC. Transfer efficiency and RNA integrity were monitored after transfer by visualization of 18S and 28S rRNA under UV illumination.

DNA electrophoresis and southern blotting. DNA was separated on agarose gels in TRIS-acetate buffer, usually containing 1 μg/ml ethidium bromide to allow visualization under UV. If DNA was to be recovered, bands were excised, electroeluted into dialysis bags, and purified by ion-exchange chromatography on Elutip-D columns (Schleicher and Schuell) and ethanol precipitation.

For southern blotting, gels were incubated after electrophoresis in 0.5 M NaOH, 1.5 M NaCl at 37° to denature DNA, then neutralized in 0.5 M TRIS pH 7.4, 3 M NaCl, 5 mM EDTA. DNA was blotted to nitrocellulose by capillary action in 10X SSC.

Hybridization conditions. For "oncogene blot," southern blots, and

bacteriophage plaque lifts, the hybridization buffer consisted of 20mM TRIS pH 7.4, 0.5% SDS, 50% formamide, 5X SSC, 100 μg/ml yeast tRNA, 20 μg/ml sheared single-stranded DNA, 1 mM EDTA, and 0.5-3x10^6 cpm/ml denatured, nick-translated ^{32}P-labeled probe (or ^{32}P-labeled cDNA for oncogene blot). Filters were prehybridized at least 4 h at 37^o in buffer without probe and were hybridized at least 12 h at 37^o with gentle rocking. Oncogene blots and southern blots were washed in 2X SSC, 0.1% SDS at 37^o for 40 min followed by 0.5X SSC, 0.1% SDS at 50^o for 40 min and exposed (moderate stringency); the second wash in some cases was 0.1X SSC, 0.1% SDS at 50^o (high stringency). Northern blot hybridization buffer was 50mM sodium phosphate pH 6.5, 0.1% SDS, 50% formamide, 5X SSC, 1mM EDTA, 100 μg/ml sheared single-stranded DNA, 1X Denhardt's (50X Denhardt's is 1% each ficoll, polyvinylpyrrolidone, and bovine serum albumin), and 1-4x10^6 cpm denatured, nick-translated probe. Filters were prehybridized and hybridized as above and washed in 2X SSC, 0.1% SDS for 15 min at room temperature, then 0.1X SSC, 0.1% SDS at 37^o for 30 min, then 5X SSC, 0.1% SDS, 50% formamide at 37^o for 40 min and exposed. To strip the probe from filters for rehybridization, filters were incubated in 1X elution buffer (15X elution buffer contains 50mM TRIS pH 7.4, 0.5% SDS, 2 mM EDTA, and 1X Denhardt's) at 65^o for 1-2 h.

Nick translation. DNA was labeled at a concentration of 10 μg/ml in 50 mM TRIS pH 7.4, 5 mM $MgCl_2$, 20 uM deoxyribonucleotides lacking dCTP, 5 mCi/ml [α-^{32}P]dCTP (3000 Ci/mmol), 0.09 U/ml DNAse I, 125 U/ml *E. coli* DNA polymerase I. Reactions were incubated for 1 h at 14^o and terminated by adding EDTA to 10 mM and heating to 65^o for 10 min. Probe was separated from unincorporated label by chromatography on Sephadex G-25 minispin columns. Typical specific activities were 2-5x10^8 cpm/μg DNA.

Viral DNA isolation. Unintegrated CT10 viral DNA was prepared from approximately $2x10^8$ quail QT6 cells 24 h after infection with CT10 virus at a MOI of >1 transforming unit/cell. Hirt supernatant DNA was prepared as described (Hirt, 1967) by lysing QT6 cells in 1% SDS, adding NaCl to 1 M, and precipitating high molecular weight DNA at 4^o overnight. The supernatant was digested with pronase (0.5 mg/ml) and RNAse A (100 μg/ml), and ethanol precipitated. Viral DNA was purified by gel filtration on Biogel A-5M in 20 mM TRIS pH 7.4, 100mM NaCl, 5 mM EDTA, and fractions containing viral DNA were identified by southern blotting. The pooled fractions contain a mixture of mitochondrial DNA, linear viral DNA, and closed circular viral DNA with one or two LTRs.

Cloning into lambda phage. Hirt supernatant DNA was cleaved with *Eco*RI and approximately 150 ng DNA was ligated to 1 μg dephosphorylated, *Eco*RI-cut λ-ZAP arms (Stratagene) in 5 μl volume. 1 μl of the ligation was packaged using the Stratagene Gigapack system following the manufacturer's protocol and titered on *E. coli* BB4. A library of $6x10^5$ recombinant phage was obtained.

Plaque screening. $5x10^4$ recombinant phage were screened by infecting BB4 cells and plating on 10 15 cm LB plates. Duplicate nitrocellulose filters were lifted from each plate, then placed on 3MM paper saturated with 0.5 M NaOH, 1.5 M NaCl for 10 min, then transferred to paper saturated with 0.2 M TRIS pH 7.4, 2X SSC. Filters were rinsed in 6X SSC, baked, prehybridized, and hybridized with probes prepared from cloned UR2AV DNA (Neckameyer and Wang, 1984) as described in section 3-3. For the first screen, plaques were hybridized with an LTR probe (#9). For subsequent screening of positive plaques, one filter was hybridized with a mixed "CT10" mixed probe (#1,2,9) while the other filter was hybridized with a mixed "helper" probe (#3,4,5,6,7,8). Four phage

clones were obtained that hybridized only to the "CT10" probe and not to the "helper" probe.

Plasmid rescue. pBluescriptSK(-) plasmids containing viral inserts were rescued from plaque-purified recombinant λ-ZAP phage as described by the manufacturer (Stratagene). 200 μl XL1-Blue bacteria (OD_{595}=1.0 in 10 mM $MgSO_4$) were mixed with approximately $5x10^6$ pfu recombinant phage and 10^9 pfu M13 R408 helper phage and incubated at 37° for 15 min. 5 ml 2X YT medium was added and cells were shaken at 37° for 5 h. The mixture was heated at 70° for 20 min to kill bacteria and inactivate lambda phage, debris and cells were spun out, and 200 μl of the supernatant was added to 200 μl fresh XL1-Blue cells. After phage adsorption, cells were plated onto ampicillin-containing LB plates. Ampicillin-resistant colonies were grown up, minipreps of plasmid DNA were obtained (Maniatis et al., 1982), and DNA was retransformed into *E. coli* HB101 to remove any contaminating M13 DNA. Two plasmids containing 2.4 kb inserts in opposite orientation in the vector, p10-211 and p10-282, were chosen for further analysis.

DNA sequencing. DNA was sequenced from closed circular plasmid DNA (Chen and Seeburg, 1985) using the Sequenase system (U.S. Biochemical). 1-3 μg miniprep plasmid DNA was dissolved in 20 μl 0.2 M NaOH, 2 mM EDTA and incubated at room temperature for 5 min to denature. 2 μl 2 M ammonium acetate pH 4.5 was added and DNA was precipitated with 45 μl ethanol. Pelleted DNA was sequenced by following the Sequenase dideoxy chain-termination protocol exactly, using [α-^{35}S]dATP to label and the universal or reverse sequencing primers. Reaction products were separated on 5% or 8% acrylamide (19:1 acrylamide:bis) gels containing 50% urea in TRIS borate-EDTA buffer.

Deletion subcloning. Nested, unidirectional deletions of the original p10-211 and p10-282 plasmids were generated by the exonuclease III-mung bean

nuclease method exactly as outlined by Stratagene. Briefly, each plasmid was cleaved with *Kpn*I (3' overhang) and *Hin*dIII (5' overhang) then digested with 20 U/μg exonuclease III for 1 to 5 min at 37^{o}. After termination of the reaction, digested DNA was treated with mung bean nuclease to remove single-stranded regions, was self-ligated, and transformed into *E. coli*. Colonies were rapidly screened as follows: colonies were streaked out on LB plates, allowed to grow at least 6 h, then a loop of bacteria was suspended in 40 μl of 20 mM TRIS pH 7.4, 100 mM NaCl, 5 mM EDTA. The bacteria were extracted once with an equal volume of phenol-chloroform, the aqueous was treated with 100 μg/ml RNAse A for 2 min at 37^{o}, and an aliquot was run on a minigel to visualize supercoiled plasmid DNA. Clones with appropriate deletions were grown up and miniprep DNA was prepared for sequencing.

By generating deletions of the two original CT10 clones, all regions of the insert were sequenced at least once from each strand. For several regions with ambiguities, fragments were cloned into M13 vectors (Messing, 1983) for sequencing from single-stranded templates or specific deletions were generated by restriction digestion and religation of the parental plasmids.

2-5 *Protein analysis*

Lysis, labeling, and SDS-PAGE. CEF were subcultured in 6 cm dishes and cell pellets were made by washing dishes 3X with ice-cold buffered saline, scraping cells into eppendorf tubes, spinning briefly to pellet cells, and freezing at -70^{o} after removing aqueous supernatant. Pellets were lysed on ice in RIPA buffer containing 150 or 300 mM NaCl (RIPA buffer consists of 10 mM TRIS pH 7.4, 5 mM EDTA, 10, 150, or 300 mM NaCl, 10% glycerol, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 KIU/ml aprotinin; PMSF was added to 1 mM, leupeptin to 0.5 μg/ml, and fresh aprotinin to 1 μg/ml just before lysis to

inhibit proteases). Lysates were spun in a microfuge for 10 min at 4° to pellet insoluble material and the supernatant was taken for immunoprecipitation. For western blotting, cell pellets were generally lysed by boiling for 5 min in Sol buffer (10 mM TRIS pH 7.4, 1% SDS, with or without 1 mM Na_3VO_4 and 0.1 mM Na_2MoO_4). The lysate was cleared by centrifugation in a microfuge at room temperature for 10 min. When necessary, protein concentrations were determined by the Bradford method, using the Bio-Rad assay reagent.

CEF were labeled by starving for 2 h in medium containing 5% dialyzed calf serum and lacking leucine, phosphate, or methionine, and labeling for 4-5 h with 1 ml/6 cm dish the same medium supplemented with 0.25-2.0 mCi/ml [^{3}H]leucine (60 Ci/mmol), 0.5-5.0 mCi/ml carrier-free [^{32}P]orthophosphate, or 0.25-1.0 mCi/ml [^{35}S]methionine (800 Ci/mmol) respectively.

Gel electrophoresis. Proteins were separated by standard discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). Stacking gel was 4% acrylamide (37.5:1 acrylamide:bis) and separating gel 7.5, 8.5, or 10% acrylamide (37.5:1) After electrophoresis, ^{32}P-labeled gels were fixed for at least 1 h in 7% acetic acid, 20% methanol and dried; to enrich for phosphotyrosine, some gels were rehydrated in 1 M KOH and incubated at 55° for 2 h, then fixed for at least 2 h and dried again (Cooper and Hunter, 1981). for ^{3}H- or ^{35}S-labeled samples, gels were rinsed with water for 5 min then treated with flour (Amplify, Amersham) for 30 min at room temperature and dried.

Immunoprecipitation. Aliquots of cell lysates (generally 1/6 to 1/3 of 6 cm plate) were diluted to 1 ml in RIPA buffer containing 150 mM NaCl (RIPA-150) and antibody was added. Lysates were incubated on ice for at least 1 h, then 50 μl protein A-sepharose beads (preincubated with rabbit anti-mouse immunoglobulins for monoclonals) were added and lysates incubated for at least 30 min at 4° while rotating. Beads were washed 3X with RIPA containing 300mM

NaCl (RIPA-300), 2X with RIPA containing 10 mM NaCl (RIPA-10), then beads were boiled for 3 min in 30 μl sample buffer (125 mM TRIS pH 6.8, 2% SDS, 5% β-mercaptoethanol, 50% glycerol, and bromophenol blue) prior to loading on gels.

Kinase assay. Lysates were immunoprecipitated as above, and beads were washed 3X with RIPA-300, followed by 2X with kinase buffer (50 mM HEPES pH 7.4, 10 mM $MnCl_2$). Beads were resuspended in 30 μl kinase buffer containing 10-20 μCi [γ-^{32}P]ATP (3000 Ci/mmol) and incubated at room temperature for 10 min. Reactions were terminated by washing 2X with RIPA-10, then samples were boiled in sample buffer and loaded on gels.

Immunoblotting. Lysates were prepared by boiling in Sol buffer as previously described and equal amounts of protein (30-100 μg) were mixed with an equal volume of 2X sample buffer (0.25 M TRIS pH 6.8, 4% SDS, 10% β-mercaptoethanol, 50% glycerol), boiled for 3 min, and separated by SDS-PAGE. Proteins were transferred electrophoretically to nitrocellulose in 20mM TRIS base, 150 mM glycine, 20% methanol. Filters were blocked in wash buffer (0.9% NaCl, 10 mM TRIS pH 7.4, 0.1% triton X-100, 0.02% sodium azide) containing 1% ovalbumin, then incubated with antibody in the same buffer, generally 1/500 dilution anti-*crk* antiserum or 1/1000 dilution affinity-purified antiphosphotyrosine antibody (Wang, 1985; Hamaguchi et al., 1988), for at least 1 h at room temperature. Filters were washed, blocked again, and incubated with 0.1 μCi/ml [^{125}I]protein A (40 mCi/mg) for at least 1 h at room temperature. Filters were then washed again and exposed.

Glycerol gradient sedimentation. Glycerol gradients were essentially as described (Brugge et al., 1981). Cells were lysed in 0.5 ml RIPA-150 lacking glycerol, cleared, and 0.4 ml was layered on a 4.6 ml 10-30% linear glycerol gradient (in RIPA-150). Tubes were spun at 40,000 rpm for 18 h in a SW50.1 rotor. 0.4 ml fractions were mixed with an equal volume of RIPA-150 lacking glycerol

and immunoprecipitated as previously described.

Partial proteolytic mapping. Labeled protein bands were mapped by partial proteolysis with *S. aureus* V8 protease as described (Cleveland et al., 1977). Rehydrated gel bands were soaked in sample buffer (125 mM TRIS pH 6.8, 0.1% SDS, 0.5% DTT, 1mM EDTA), applied to gel slots, and overlaid with sample buffer containing glycerol and V8 protease (generally 100 ng/slot). Samples were electrophoresed into the stacking gel, then switched off for 15 min before resuming electrophoresis. For V8 analysis, gels and running buffer contained 1 mM EDTA, stacking gel was 5% acrylamide, and separating gel was 12.5% acrylamide.

Phosphoamino acid analysis. ^{32}P-labeled bands were rehydrated and washed in 10% methanol and lyophilized. Bands were rehydrated in a total volume of 1.2 ml 50 mM NH_4HCO_3 containing 50 μg/ml proteinase K and incubated overnight at 37^{o}. The supernatant was lyophilized and washed by resuspending in 0.5 ml water and relyophilizing 3X. The pellet was dissolved in 10 μl 6 N HCl, sealed in a glass capillary tube, and heated to 100^{o} for 2 h. The acid hydrolysate was ejected into 1 ml water, lyophilized, and washed as above.

The pellet was dissolved in 10 μl water containing 1 mg/ml each phosphoserine, phosphothreonine, and phosphotyrosine, and was spotted onto cellulose-coated glass TLC plates. For 1-dimensional analysis, plates were electrophoresed with buffer containing 5% acetic acid, 0.5% pyridine (pH 3.5) at 1000 V for 90 min. For 2-dimensional analysis, plates were first electrophoresed with buffer containing 7.8% acetic acid, 2.5% formic acid (pH 1.9) at 1000 V for 1 h, dried, then rotated 90^{o} and electrophoresed with pyridine-acetate pH 3.5 buffer for 1 h at 1000 V. Phosphoamino acid spots were visualized by spraying plates with 0.5% ninhydrin, 0.07% pyridine in acetone and heating briefly.

Membrane/soluble fractionation. Cells from a 10 cm plate were col-

lected, washed, and suspended in 3 ml hypotonic buffer (10 mM NaCl, 20 mM TRIS pH 7.4, 1 mM EDTA, and protease inhibitors) and allowed to swell for 10 min on ice. The cells were broken by 20-25 strokes in a dounce homogenizer and spun at 1000xG to pellet unbroken cells and nuclei. The supernatant was centrifuged at 35,000 rpm for 30 min in a Ti60 rotor. The pellet (P100, or crude membrane fraction) was dissolved in RIPA-150 buffer, the supernatant (S100 fraction) was adjusted to RIPA-150 composition, and fractions were immunoprecipitated as described above.

Cytoskeletal matrix fractionation. CEF were separated into nonionic detergent-soluble and insoluble fractions as described (Hamaguchi and Hanafusa, 1987). Briefly, dishes were incubated with 0.5 ml CSK buffer (10mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose, 1% triton X-100, plus protease and phosphatase inhibitors) and incubated on ice with gentle rocking for 3 min. The detergent-resistant structures remaining on the plate were reextracted with CSK buffer for 1 min, and the supernatants were pooled as the soluble fraction. The two fractions were adjusted to RIPA-150 composition (immunoprecipitation), or were mixed with sample buffer and boiled (immunoblotting).

2-6 *Generation of* crk-*specific antibody*

Construction of expression vector. The T7 RNA polymerase-dependent expression system developed by Studier (Studier and Moffat, 1986; Rosenberg et al., 1987) was used to express a truncated v-*crk* protein, which contains no *gag* sequences, in *E. coli*. The *Sty*I-*Hae*II fragment of pCT10 (see figure 1) was end-filled with T7 DNA polymerase and ligated to an 8-mer *Bam*HI linker. After digestion with *Bam*HI and gel purification, the fragment was ligated to *Bam*HI-cut pET-3a (Rosenberg et al., 1987). Clones were isolated containing the

crk-specific insert in the correct orientation for transcription and in-frame translation from the vector's T7 promoter and translation initiation sites.

Bacterial expression. The *crk* expression plasmid (termed pETC-2) was transformed into *E. coli* strain BL21 (DE3) pLysS, which contains an integrated bacteriophage T7 RNA polymerase gene under the control of the *lac* promoter and a plasmid that transcribes low levels of T7 lysozyme (a specific inhibitor of T7 RNA polymerase). Transformants were grown under ampicillin and chloramphenicol selection to maintain both the expression plasmid and the lysozyme plasmid. IPTG was added to 0.4 mM to late log phase cells (OD_{595}=1.0) to induce expression of the *crk* peptide. After 3 h maximal accumulation was observed of a 26 kd protein, not present in control bacteria, which is presumed to be the *crk* peptide (calculated MW=27 kd). This protein is the most prominent band on a Coomassie blue-stained gel, and constitutes several percent of total bacterial protein.

Antigen preparation and antibody production. 15 ml of bacteria expressing the *crk* peptide were pelleted and resuspended in 0.5 ml 50mM TRIS pH 8.0, 1 mM EDTA. 0.5 ml 2X protein sample buffer was added and the lysate was boiled for 5 min. The entire lysate was electrophoresed on a preparative 12% SDS-PAGE gel and the *crk* peptide was visualized by Coomassie blue staining. The band corresponding to the *crk* peptide was excised, washed extensively in 10% methanol, and lyophilized. The dried band was ground with a mortar and pestle in 4 ml phosphate-buffered saline, was mixed with 2 ml complete Freund's adjuvant, and was injected intramuscularly into 2 rabbits. By staining, I estimate that approximately 50 μg of peptide was injected per rabbit. 5 weeks later, rabbits were boosted by intramuscular injection of 1/4 the amount of the original immunization in incomplete Freund's adjuvant, and serum was collected 1 week later.

2-7 *Construction of v-crk mutants*

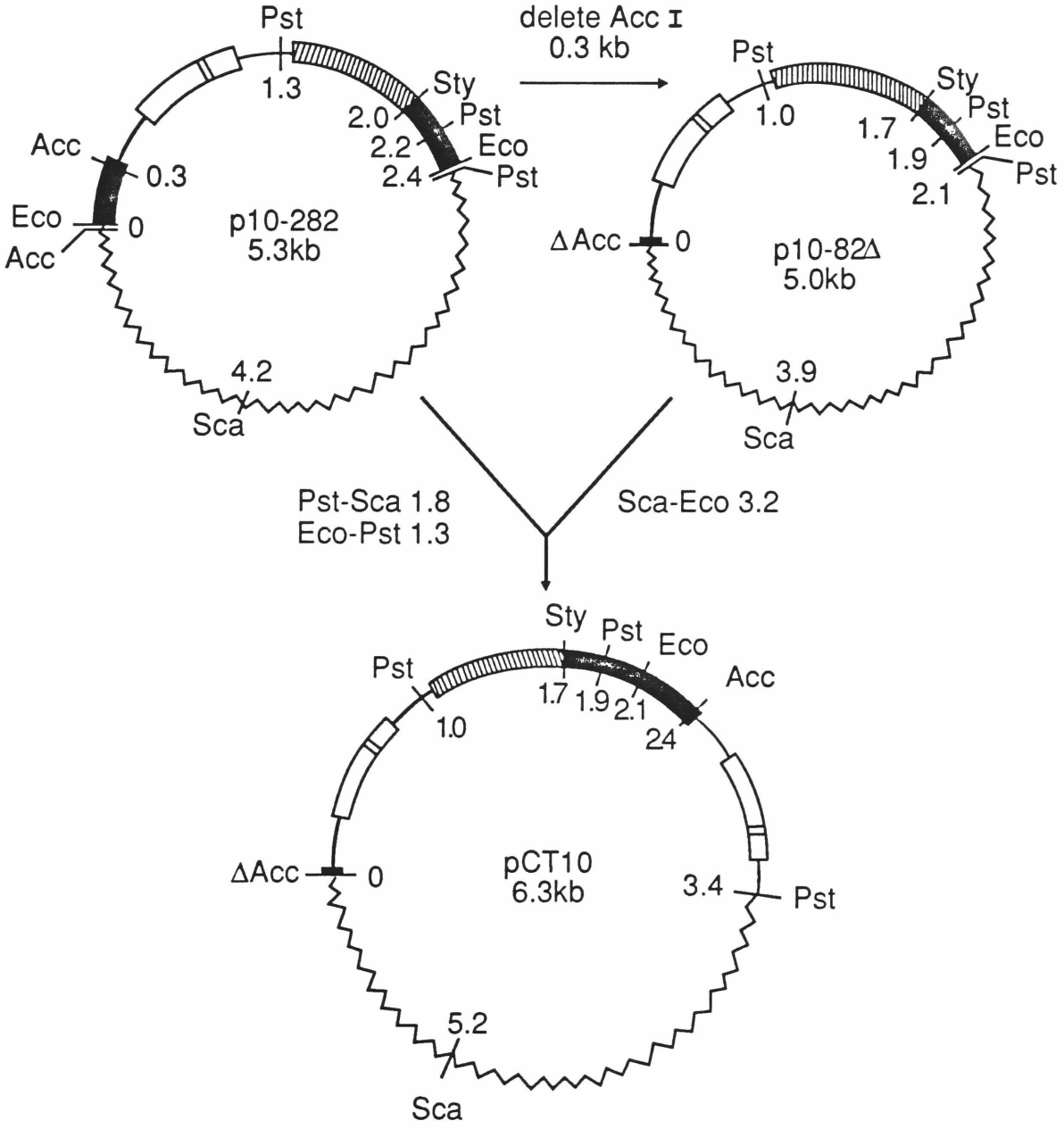
General techniques. Standard molecular biological techniques (Maniatis et al., 1982) were used to construct *in vitro* mutants of the wild-type CT10 genome. Manufacturer's instructions or standard protocols were followed when cleaving DNA with restriction endonucleases, end-filling with T7 DNA polymerase, dephosphorylating with bacterial alkaline phosphatase, phosphorylating with T4 polynucleotide kinase, and ligating with T4 DNA ligase. DNA fragments were purified by agarose gel electrophoresis as described (section 2-3), and constructs were transformed using $CaCl_2$ into *E. coli* strain HB101.

Construction of pCT10. Since the original molecular clones of ASV CT10, p10-211 and p10-282, are circularly permuted, a nonpermuted clone was constructed to facilitate *in vitro* mutagenesis. This construction is outlined in figure 1. First, the 0.3 kb *Acc*I fragment of p10-282 was deleted by restriction digestion, end-filling, and religation to generate p10-82Δ. The 3.2 kb *Sca*I-*Eco*RI fragment of p10-82Δ was combined in a three-part ligation with the 1.3 kb *Eco*RI-*Pst*I and the 1.8 kb *Pst*I-*Sca*I fragments of p10-282 to generate pCT10. This construct contains the entire nonpermuted CT10 genome with two LTRs in a pBluescriptSK(-) vector. Biological activity was retained as demonstrated by transfection of CEF with *Sca*I-cleaved pCT10 along with UR2AV helper virus DNA.

Deletion mutants. p10-ΔSH2 was constructed by ligating an *Sph*I 8-mer linker to the end-filled 5.9 kb *Eco*RI-*Sty*I fragment of pCT10. p10-ΔSH3 was constructed by blunt-end self-ligation of the end-filled 6.0 kb *Acc*I-*Eco*RI fragment of pCT10.

Linker insertion mutants. p10-BSP was made by ligation of an *Sph*I 8-mer linker to end-filled, *Bgl*II-cut pCT10 DNA. Similarly, p10-ESP was made by insertion of an *Sph*I 8-mer linker into the end-filled *Eco*RI site of pCT10, p10-

Figure 1. Construction of pCT10. Wavy line represents pBluescriptSK(-) vector sequences; black boxes denote cellular proto-oncogene-derived sequences, cross-hatched boxes *gag* sequences, and open boxes the viral long terminal repeat. Numbers denote kilobase pairs from the original *Acc*I site in the vector. Restriction enzyme sites shown are Acc, *Acc*I; ΔAcc, destroyed *Acc*I site; Pst, *Pst*I; Eco, *Eco*RI; Sty, *Sty*I; Sca, *Sca*I.


Pst
1.3
Sty
2.0
Pst
2.2
Eco
2.4
Pst
Acc
0.3
Eco
0
Acc
p10-282
5.3kb
4.2
Sca
delete Acc I
0.3 kb
Pst
1.0
Sty
1.7
Pst
1.9
Eco
2.1
Pst
ΔAcc
0
p10-82Δ
5.0kb
3.9
Sca
Pst-Sca 1.8
Eco-Pst 1.3
Sca-Eco 3.2
Sty
Pst
Eco
Pst
1.7
1.9
2.1
Acc
1.0
2.4
ΔAcc
0
pCT10
6.3kb
3.4
Pst
5.2
Sca

MH was made by insertion of a *Hin*dIII 12-mer linker into the end-filled *Mst*II site of pCT10, and p10-SMH was made by insertion of a *Hin*dIII 12-mer linker into one of the two *Sma*I sites of pCT10.

Oligonucleotide-directed mutagenesis. Point mutants were generated using the bacterial strain and protocols provided by the Bio-Rad Mutagene kit. The 0.23 kb *Eco*RI-*Pst*I fragment of pCT10 was cloned into M13mp18 and M13mp19 RF DNA that had been cut with *Eco*RI and *Pst*I. Single-stranded recombinant M13 phage DNA was prepared by infection of *E. coli* strain CJ236, which has a *dut, ung* phenotype resulting in uracil-substituted progeny phage. This substituted DNA, containing the 0.23 kb fragment of the *crk* gene, was used as the template for oligonucleotide-directed synthesis of second-strand DNA as outlined by the manufacturer. The mutagenic oligonucleotides were: 5'-CTTCCTGGT*TAA*CGACTCGGG-3', which creates a novel *Hpa*I site and changes Arg 273 of v-*crk* to Asn; and 5'-GACGATGTA*TC*GCGAGACGC-3', which creates a novel *Nru*I site and changes His 294 to Arg. The nucleotides in italics are different from the wild-type CT10 sequence.

Mutagenized DNA was transformed into *E. coli* JM101, resulting in degradation of the parental uracil-substituted DNA strand, and progeny virus clones were grown up and screened by digestion of RF DNA with *Hpa*I or *Nru*I. Positive clones were sequenced to confirm the mutations. The 0.23 kb *Pst*I-*Eco*RI fragment was cut from the RF DNA of mutated clones and reinserted into pCT10 by ligation to the 6.1 kb *Eco*RI-partial *Pst*I fragment of pCT10.

Gag-minus constructs. Mutants from which viral *gag* sequences were deleted were constructed using a replication-competent RSV-derived vector system (Cross and Hanafusa, 1983). pNRC was made by ligating *Bam*HI linkers to the 0.8 kb *Sty*I-*Hae*II fragment of pCT10 and inserting into the *Bgl*II site of the the vector pNR200 (Kornbluth et al., 1986). pNSC was made by first construct-

ing a hybrid vector, termed pNR111, consisting of the 5.8 kb *Bgl*II-*Cla*I fragment of pNR200 ligated to the 1.8 kb *Cla*I-*Bgl*II fragment of pXD11-1 (Cross and Hanafusa, 1983). The *Bam*HI-linkered 0.8 kb *Sty*I-*Hae*II fragment of pCT10 was then inserted into the *Bgl*II site of pNR111 to generate pNSC.

SECTION THREE

PRELIMINARY CHARACTERIZATION OF FOUR AVIAN SARCOMA VIRUSES

3-1 *Introduction*

As outlined in the section 1, RNA tumor viruses have proven to be a rich source of oncogenes. There is no reason to believe that all possible oncogenes have already been identified, or more specifically that we have identified all of the genes that can be transduced into retroviruses and can, in a viral context, induce transformation. Given that it is useful to identify as many oncogenes as possible, this search can be facilitated by analysis of previously uncharacterized RNA tumor viruses.

Avian sarcoma viruses (ASVs) have played a pivotal role in tumor virology since the early years of this century, when Peyton Rous discovered the sarcoma virus that bears his name (Rous, 1911). In the ensuing decades, numerous transmissible tumor agents were isolated from fowl, primarily chickens (Claude and Murphy, 1933; Rous, 1936; Beard, 1963; Bishop and Varmus, 1985). Novel avian sarcoma viruses are still being identified from spontaneous tumors in chicken stocks or tumors induced by infection with high titers of nontransforming avian leukosis viruses (Hayman et al., 1985; Ikawa et al, 1986; Maki et al, 1987). The identification of novel oncogenes from sarcoma viruses does not necessarily require fresh virus isolates, however; given the somewhat haphazard way in which existing sarcoma viruses were cloned after the advent of modern molecular biology and the subsequent discovery of viral oncogenes, there are still a number of "classical" avian sarcoma viruses lurking in freezers around the world that have yet to be characterized genetically.

I chose to screen four previously isolated avian sarcoma viruses for the presence of novel oncogenes. Three of these viruses, RPL25, RPL28, and RPL30, were obtained from a series of viruses studied in the 1950's and 1960's by Burmester's group at the Regional Poultry Research Center in East Lansing, Michigan (Frederickson et al., 1964). Five viruses (RPL25, RPL26, RPL27, RPL28, and RPL30) were injected into newly hatched chickens. In three cases, RPL25, RPL28, and RPL30, sarcomas were induced and acutely transforming avian sarcoma viruses were recovered from tumor tissue (H. Hanafusa and T. Hanafusa, unpublished). The fourth virus, CT10, was obtained from the American Type Culture Collection. Termed chicken tumor number 10, or Claude's agent, it was isolated in 1927 from an apparently spontaneous chicken tumor by Murphy and Claude at the Rockefeller Institute and was one of the prototype avian sarcoma viruses studied in the 1930's and 1940's (Burk, et al., 1941; Claude, et al., 1947). I injected the ATCC stock into chickens and was able to obtain high-titer sarcoma virus stocks from tumor explants.

3-2 *"oncogene blot" screen*

The first step in analyzing these uncharacterized viruses was to determine whether they encoded oncogenes that had already been identified in other RNA tumor viruses. I developed a screening method that allows rapid comparison of any novel RNA tumor virus to the known oncogenes. I isolated oncogene-specific DNA fragments from a panel of 19 plasmids (originally collected by D. Foster and C. Simon) containing known viral oncogenes. Each DNA probe was carefully chosen so that it contained no virus-derived sequences that could hybridize nonspecifically with retroviral replicative gene sequences. These probes were bound to nitrocellulose filters and were hybridized under moderate stringency with ^{32}P-labeled cDNA made by reverse transcription of sarcoma virus genomic

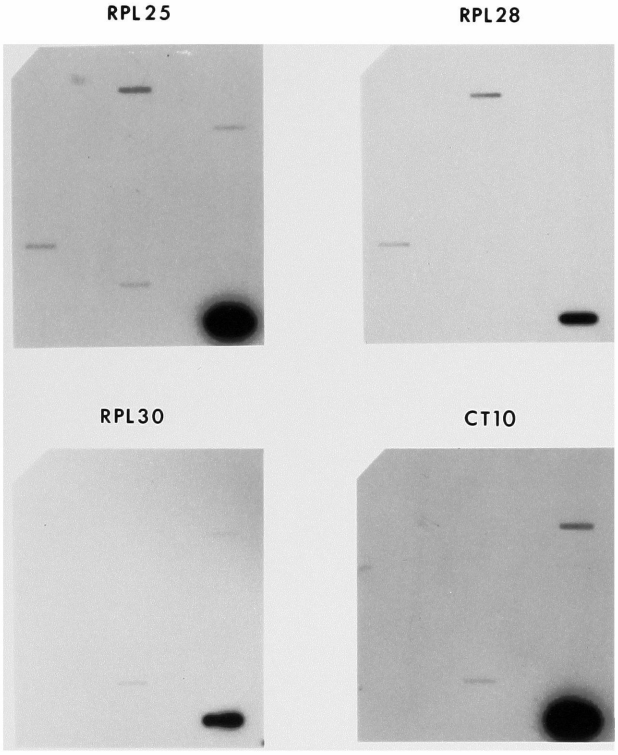
RNA. The RNA template was obtained from virion particles released by chicken embryo fibroblasts (CEF) transformed with the uncharacterized sarcoma viruses. If these ASVs encode a known oncogene, the [^{32}P]cDNA would be expected to hybridize to the corresponding DNA fragment on the "oncogene blot."

The results of this screening are shown in figure 2. RPL25 and RPL28 cDNA hybridized to an ALV positive control, as expected; more importantly, it also hybridized to the slots corresponding to the *erbB* and *erbA+erbB* probes. Southern blotting of the restriction-digested original plasmids with the viral cDNA probes demonstrated that the hybridization was specific to *erbB* sequences. Southern blotting also indicated that the low-level hybridization observed to the *src* and *myb* probes was due to contamination of the probe fragment with non-oncogene-derived viral sequences. Furthermore, northern blots of RNA from cells infected with RPL25 and RPL28 hybridized with an *erbB* probe gave specific hybridization to bands not present in control cell RNA (not shown). These data demonstrate that RPL25 and RPL28 encode the *erbB* oncogene, whose cellular homolog is the EGF/TGF-α receptor (Downward et al., 1984). Unfortunately, numerous isolates of avian sarcoma viruses encoding *erbB* have already been characterized, so little could be learned from further study of these two viruses.

A more interesting result was seen in the case of RPL30 and CT10. No specific hybridization was seen to any of the oncogene probes, although the positive ALV control hybridized strongly. Again, the low-level signal in the *src* and *myb* slots was shown by Southern blotting to be nonspecific. Since the CT10 and RPL30 culture fluids used to prepare the cDNA probe contained high titers of sarcoma virus (as demonstrated by infection of fresh CEF), the lack of hybridization observed strongly suggests that these viruses encode novel oncogenes.

3-3 *Preliminary characterization of ASV genomes*

Figure 2. Hybridization of avian sarcoma virus cDNA to oncogene probes. DNA probes were isolated from known oncogenes and bound to nitrocellulose filters as diagrammed on the right (see section 2-2 for precise identity of probes). ^{32}P-labeled cDNA prepared from RPL25, RPL28, RPL30, or CT10 virions was hybridized to filters as described in text.



ros	erbB	fms
abl	fps	src
yes	fgr	mil
mos	H_{ras}	K_{ras}
erbA+B	rel	sis
myc	myb	fos
ski		ALV

The RPL30 and CT10 genomes were initially characterized by northern blotting of RNA prepared from infected CEF. Figure 3 shows hybridization of such a blot with a probe prepared from the highly conserved long terminal repeat (LTR) of avian leukosis virus UR2AV (Neckameyer and Wang, 1984). Since virtually all RNA tumor viruses are defective (see section 1-2), infectious sarcoma virus stocks contain replication-competent helper virus as well as sarcoma virus; the 7.5 kb genomic RNA and the 3.0 kb *env* subgenomic mRNA of the helper virus are seen in all lanes. In the case of RPL30, a large message of approximately 9 kb is also observed; this is presumed to be the RPL30 sarcoma virus genome. In the case of CT10, a strong additional band is seen at approximately 2.5 kb, and again is presumed to be the sarcoma virus genomic message.

To determine what, if any, viral genetic information had been deleted from these viruses during the process of oncogene transduction, I prepared nine probes from cloned UR2AV DNA (figure 4). In the case of RPL30, each of the nine fragments hybridized to the 9 kb putative sarcoma virus genomic message. This indicates that any deletion that occurred during transduction is very small, consistent with the large genome size observed. Since RPL30 is replication-defective (unpublished observation), oncogene sequences have presumably been inserted into the coding sequences of one of the viral replicative genes. In the case of CT10, only the LTR probe (#9) and the two most 5' *gag* probes (#1,2) hybridized to the 2.5 kb message. This indicates that at least 5 kb of viral genetic information was lost during transduction, consistent with the extremely small size of the CT10 genome. It should also be noted that probe #2 is from the region downstream of the splice donor used to generate viral subgenomic messages, demonstrating that the 2.5 kb CT10 message is the full-length genomic mRNA and not the spliced product of a putative larger genomic message that might be obscured by helper virus bands.

Figure 3. Viral mRNA in cells infected with avian sarcoma viruses. Total cellular RNA from CEF infected with RPL25, RPL28, RPL30, or CT10 was northern blotted and hybridized with a UR2AV LTR probe.

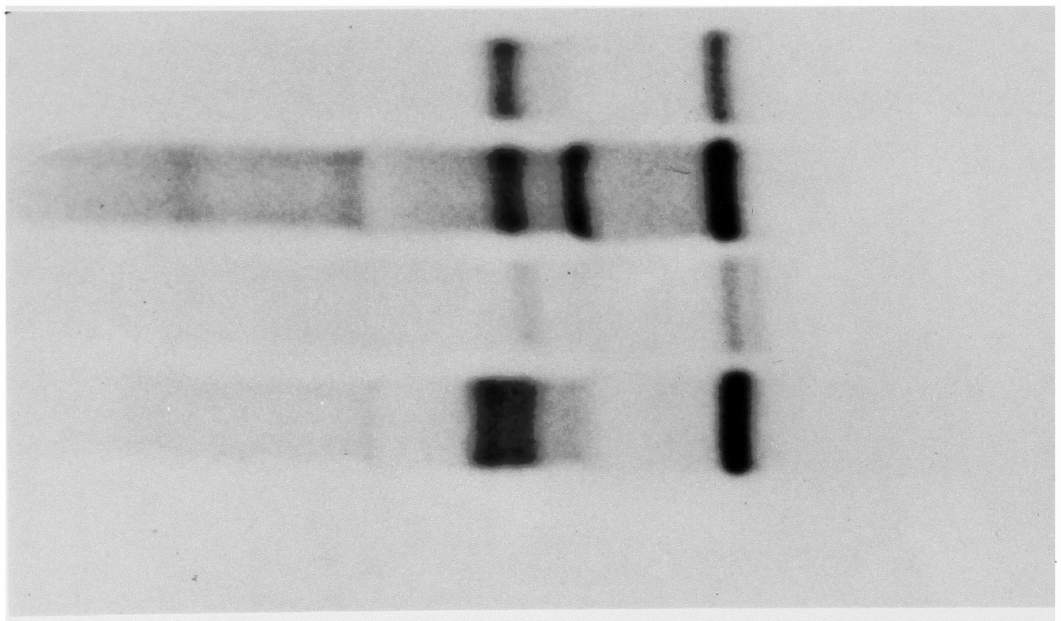
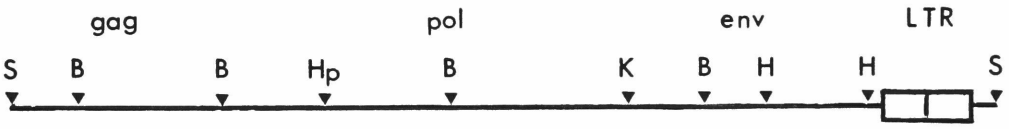

RPL25
RPL28
RPL30
CT10
Uninfected

Figure 4. Hybridization of RPL30 and CT10 genomic RNA with various probes derived from molecularly-cloned UR2AV helper virus DNA. The genome of UR2AV is diagrammed at the top. Open box denotes the LTR; the approximate positions of the viral *gag, pol* and *env* genes are indicated. Restriction enzyme sites used to generate individual probes are shown: S, *Sac*I; B, *Bam*HI; Hp, *Hpa*I; K, *Kpn*I; H, *Hin*dIII. Positive hybridization of a given probe to RPL30 or CT10 genomic RNA, assayed by northern blotting, is indicated by "+".

Hybridization to ALV probes



probe	1	2	3	4	5	6	7	8	9
RPL30	+	+	+	+	+	+	+	+	+
CT10	+	+	–	–	–	–	–	–	+

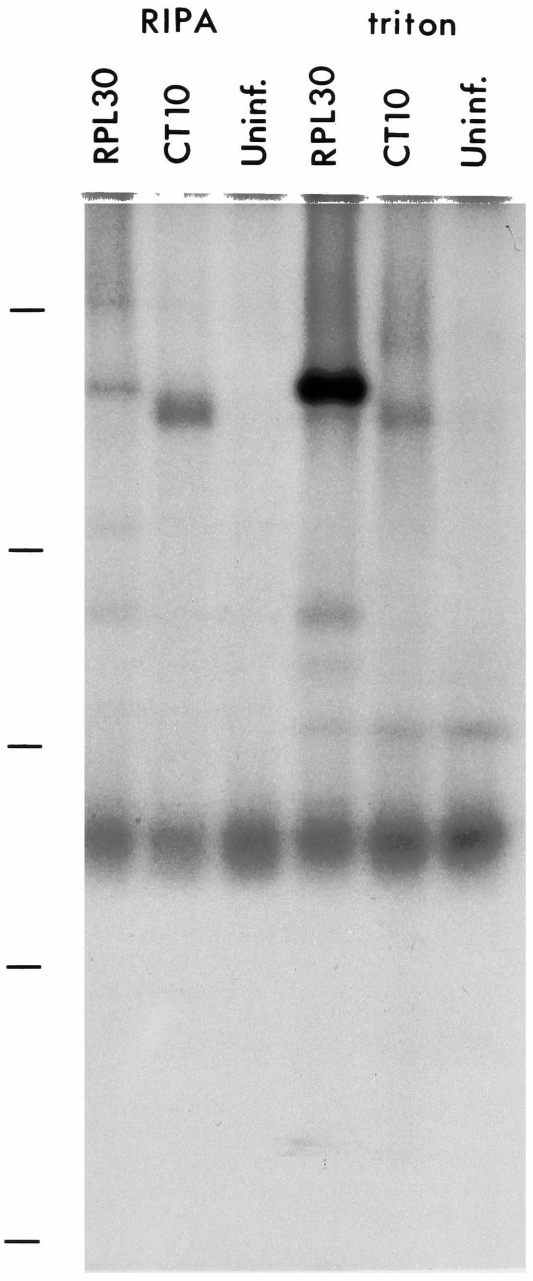
3-4 *Preliminary protein characterization*

As mentioned in section 1-2, viral oncogene products are often fusion proteins composed of sequences derived from both viral structural genes and a cellular proto-oncogene, and can therefore be immunoprecipitated by antibodies that recognize viral structural proteins. Since many oncogenes encode protein kinases, I assayed the *in vitro* kinase activity of proteins immunoprecipitated from RPL30- and CT10-infected cell lysates (Collett and Erikson, 1978). The antibody used was TBR serum (Brugge and Erikson, 1977), which was raised in rabbits bearing RSV-induced tumors. This serum recognizes avian retroviral structural proteins, and very weakly recognizes $p60^{c\text{-}src}$.

Figure 5 demonstrates that TBR serum immunoprecipitates of CT10- and RPL30-infected CEF lysates contain *in vitro* kinase activity. Two different protein extraction buffers were used: RIPA buffer contains the ionic detergents SDS and sodium deoxycholate, while triton buffer contains only the nonionic detergent triton X-100. RPL30 immunoprecipitates phosphorylated *in vitro* a band of approximately 150 kilodaltons (kd). Phosphorylation of this protein was much stronger when cell lysates are prepared in the absence of ionic detergents, suggesting that the kinase is relatively labile to detergent denaturation. CT10 immunoprecipitates phosphorylate a broad band of approximately 135-155 kd. In contrast to the RPL30 activity, more CT10 kinase activity was observed in lysates prepared with ionic detergents. In both cases, Mn^{++} gave much higher *in vitro* kinase activity than Mg^{++}, a common observation for many tyrosine kinases (not shown). Phosphoamino acid analysis demonstrated that for both CT10 and RPL30 phosphate was transferred primarily to tyrosine residues (not shown). These results suggest that both CT10 and RPL30 encode or encode proteins that are associated with protein-tyrosine kinases.

To address whether cellular phosphotyrosine levels on proteins of cells

Figure 5. *In vitro* kinase activity of TBR immunoprecipitates of RPL30- or CT10-infected cell lysates. Infected or uninfected (Uninf.) CEF were lysed in RIPA buffer or triton extraction buffer (RIPA buffer lacking SDS and sodium deoxycholate) as indicated. Gel was 8% acrylamide, and was washed with alkali (see section 2-4) to enrich for phosphotyrosine. Approximate molecular weight markers are indicated to left; from top, 220, 100, 68, 43, and 27 kd.



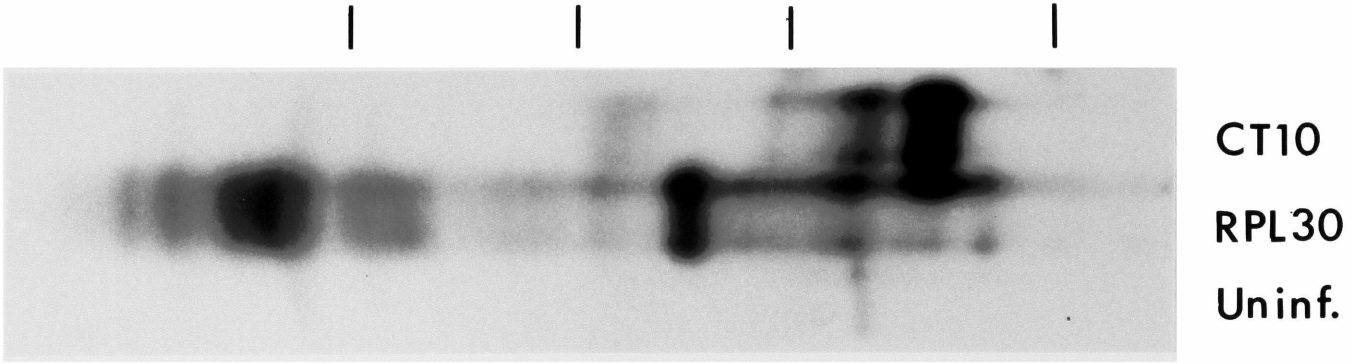
In Vitro Kinase

infected with CT10 and RPL30 are elevated, as would be expected if these viruses encode active tyrosine kinases, anti-phosphotyrosine immunoblots were performed. In this experiment, proteins from infected cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an antibody that specifically recognizes phosphotyrosine (Hamaguchi et al., 1988). Figure 6 demonstrates that there is a gross elevation of phosphotyrosine on a subset of cellular proteins in cells infected by both RPL30 and CT10. The pattern observed in the case of CT10 is particularly interesting, since only 3 proteins (approximate molecular weights 135-155, 120, and 70 kd) contain detectably increased phosphotyrosine. In the case of RPL30 and other tyrosine kinase oncogenes, a much broader spectrum of proteins are phosphorylated (see Hamaguchi et al., 1988). These *in vivo* phosphotyrosine results are consistent with the *in vitro* kinase assay data, and again suggest that these two viruses encode tyrosine kinase oncogene products.

3-5 *Conclusion*

The data presented in this section demonstrate that it is possible to rapidly screen uncharacterized RNA tumor viruses and identify those encoding novel oncogenes. While a sample size of four can hardly be considered statistically significant, the fact that two out of four viruses apparently contain novel oncogenes suggests that there is a good chance that any uncharacterized RNA tumor virus will contain a novel oncogene, and that this is still an efficient method to identify such genes. This is consistent with reasonable assumptions about the complexity of growth control pathways; the multiplicity of growth factor receptors, as only one example, each of which might be expected to stimulate uncontrolled growth if deregulated, suggests that numerous genes might have the potential to be transduced by retroviruses as viral oncogenes.

Figure 6. Antiphosphotyrosine immunoblot of proteins from cells infected with CT10 and RPL30. Approximately 30 μg protein from CT10-infected, RPL30-infected, or uninfected CEF was separated by SDS-PAGE, transferred to nitrocellulose, and probed with phosphotyrosine-specific antibody. Gel was 8.5% acrylamide; markers, 220, 100, 68, and 43 kd.


CT10
RPL30
Uninf.

The fact that both CT10 and RPL30 increase cellular phosphotyrosine content is interesting in light of the fact that many other tyrosine kinase oncogenes have been isolated from avian sarcoma viruses (*src, yes, fps, ros, erbB, sea*). An intriguing possibility is that tyrosine phosphorylation somehow enhances viral replication and provides a selective advantage to avian viruses encoding tyrosine kinases. Anindya Dutta in this laboratory has found that *src* kinase activity stimulates transcription from the RSV LTR, especially in low serum (A. Dutta, manuscript in preparation), and some previous studies have suggested that *src* activity increases viral yield (Leong et al., 1972; Bell et al., 1975). If tyrosine kinase activity does have a positive effect on viral transcription, this has practical implications, as discussed more fully in section 6-3; it may explain why non-transforming mutants replicate poorly relative to wild-type transforming viruses.

SECTION FOUR

MOLECULAR CLONING AND GENETIC STRUCTURE OF AVIAN SARCOMA VIRUS CT10

4-1 *Introduction*

The molecular cloning and nucleotide sequencing of previously unidentified oncogenes provides an experimental and conceptual basis for studies on the mechanisms of normal and abnormal growth control. Since the oncogene product has the ability to produce malignant transformation, the first and most important contribution of a new oncogene is in further defining the types of proteins that can alter growth control pathways. While adding to the list of oncogenes within a given class is important to a full and comprehensive view of growth control, it is the identification of new classes of oncogenes that holds the greatest promise for redefining the ways that we think about the problem of growth control. In this section I will describe the structure of the CT10 transforming gene and its protein product. The surprising nature of this protein establishes a new class of oncogene and provides new insights into how various enzymes implicated in growth control and signal transduction may be coordinately regulated.

4-2 *Molecular cloning of ASV CT10*

The cloning of avian sarcoma viruses (ASVs) involves technical considerations that led to my decision to first clone CT10 and not RPL30. Obviously there is no cloned DNA probe specific for a novel oncogene, although in the past it has been possible to generate a specific probe by hybridizing ^{32}P-labeled cDNA synthesized from sarcoma virus genomic RNA to an excess of non-

transforming helper virus RNA and removing the annealed hybrids by hydroxyapatite chromatography (see Shibuya et al., 1980). It is much easier to use a cloned DNA probe from conserved retroviral sequences; however, since defective sarcoma viruses such as CT10 and RPL30 are actually mixtures of sarcoma virus and helper virus (which generally predominates in the population), it is essential to have some way to distinguish between the sarcoma virus and helper genomes. For this reason, the small genome size of CT10 relative to its helper (2.5 kb vs. 7.5 kb) and the fact that CT10 is lacking at least 5 kb of helper virus genetic information made this virus more convenient to clone than RPL30.

Although the rest of this thesis will concern my work on ASV CT10, I would like to mention the strategy that I developed to clone the oncogene of RPL30. Since its genome size is similar to that of helper virus and it hybridizes to every helper-derived DNA probe that I tested, it was necessary to separate the sarcoma virus from its associated helper. This was accomplished by generating non-producer cell clones (Hanafusa et al., 1963) by infecting CEF at very low multiplicity and replating cells in agar suspension within a few hours after infection. I recovered transformed colonies from the agar, grew them up, and was able to isolate a cell clone that contained the RPL30 genome in the absence of helper virus. I constructed a cDNA library from mRNA isolated from these cells, which can now be screened with helper-derived probes to isolate the RPL30 genome.

To molecularly clone ASV CT10, I used the technique of isolating viral replication intermediates from cells infected at high multiplicity of sarcoma virus (see Mayer et al., 1986). Hirt supernatant DNA (Hirt, 1967) prepared 24 h after infection contains a mixture of viral replication intermediates and byproducts: linear DNA with two LTRs (the true precursor to the integrated provirus), and covalently closed circular DNA containing one or two LTRs (Varmus

and Swanstrom, 1985). The complete sarcoma virus genome can be cloned in circularly permuted form by digesting the circular viral DNA with a restriction enzyme that has a unique site in the genome and by ligating the cleaved DNA to a lambda phage vector.

I prepared Hirt supernatant DNA from quail QT6 cells infected with high-titer CT10 virus and used southern blotting to identify a suitable restriction enzyme for molecular cloning. By hybridizing southern blots of restriction endonuclease-digested Hirt supernatant DNA with the various UR2AV probes diagramed in figure 4, it was possible to distinguish fragments derived from digestion of helper virus DNA from those derived from CT10 (not shown). This analysis suggested that there was a unique *Eco*RI site in the CT10 genome, so this enzyme was used for cloning.

I digested Hirt supernatant DNA with *Eco*RI and ligated it to *Eco*RI arms of the phagemid vector λ-ZAP (Stratagene), packaged the ligated DNA *in vitro*, and obtained a library of $6x10^5$ recombinant phage. I then took advantage of the fact that CT10 has suffered a large deletion of viral sequences, and by screening duplicate filters was able to isolate lambda clones with inserts that hybridized to probes 1,2, and 9 but not to probes 3,4,5,6,7, and 8 (see figure 4 for nomenclature of DNA probes), as would be expected of *bona fide* CT10 clones. Two molecular clones, containing 2.4 kb inserts in opposite orientation in the vector (and therefore derived from independent cloning events) were chosen for further study.

Prior to sequencing these molecular clones, it was important to prove that they had the same biological activity as the parental CT10 virus. A potential pitfall would be two closely spaced *Eco*RI sites in the CT10 genome, which would result in the DNA cloned by *Eco*RI digestion lacking a small segment of genetic information. When DNA from the CT10 clones was digested with *Eco*RI,

ligated, and transfected into CEF along with UR2AV helper virus DNA as described in section 2-1, however, the transfected cells became transformed with a similar phenotype to those infected with the parental virus. Figure 7 shows that CEF transfected with the cloned CT10 DNA grew to high density and were more fusiform and refractile than cells transfected with helper alone, and were able to grow in agar suspension as anchorage-independent colonies. In addition, culture fluids from transfected dishes induced sarcomas within 2 weeks after injection into newborn chickens (not shown). These data demonstrate that the cloned CT10 DNA retains the full transforming activity of the parental virus.

4-3 *Nucleotide sequence of ASV CT10*

The nucleotide sequence of the two independent CT10 clones was determined by standard methods, as outlined in section 2-4, and is presented in figure 8. The two molecular clones differed in sequence at only 3 positions, all in the *gag* leader region; in these cases, the base that was identical to that of the corresponding position of the published RSV sequence (Schwartz et al., 1983) was chosen for the figure. The complete CT10 genome is 2407 nucleotides long, making it the smallest naturally isolated retrovirus so far identified. It contains a 521 base-pair (bp) LTR, unusually long for an avian retrovirus, as well as all canonical sequences required in *cis* for viral replication (Coffin, 1985).

The sequence contains a single large open reading frame that encodes a *gag*-fusion polypeptide of 440 amino acids with a calculated molecular weight of 47.1 kilodaltons (kd). Translation begins with the normal *gag* initiator methionine, followed by 207 additional amino acids nearly identical to previously sequenced avian retroviral *gag* (Van Beveren et al., 1985). The sequence then diverges, encoding 238 novel amino acids presumably transduced from a cellular proto-oncogene. The last three amino acids and stop codon are apparently pro-

Figure 7. Biological activity of molecularly-cloned CT10 DNA. Molecularly-cloned CT10 DNA plus UR2AV helper virus DNA (panels A and C) or UR2AV DNA alone (panels B and D) were transfected into CEF. Photomicrographs are of monolayer cultures (panels A and B) or agar suspension cultures (panels C and D).

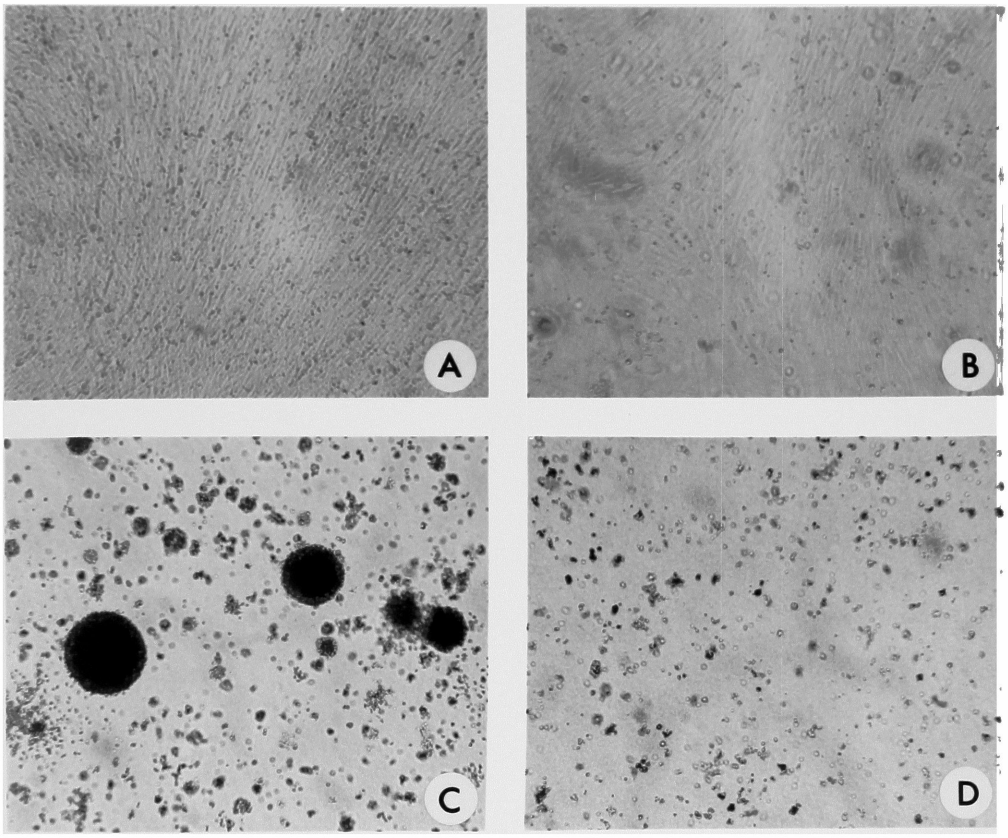
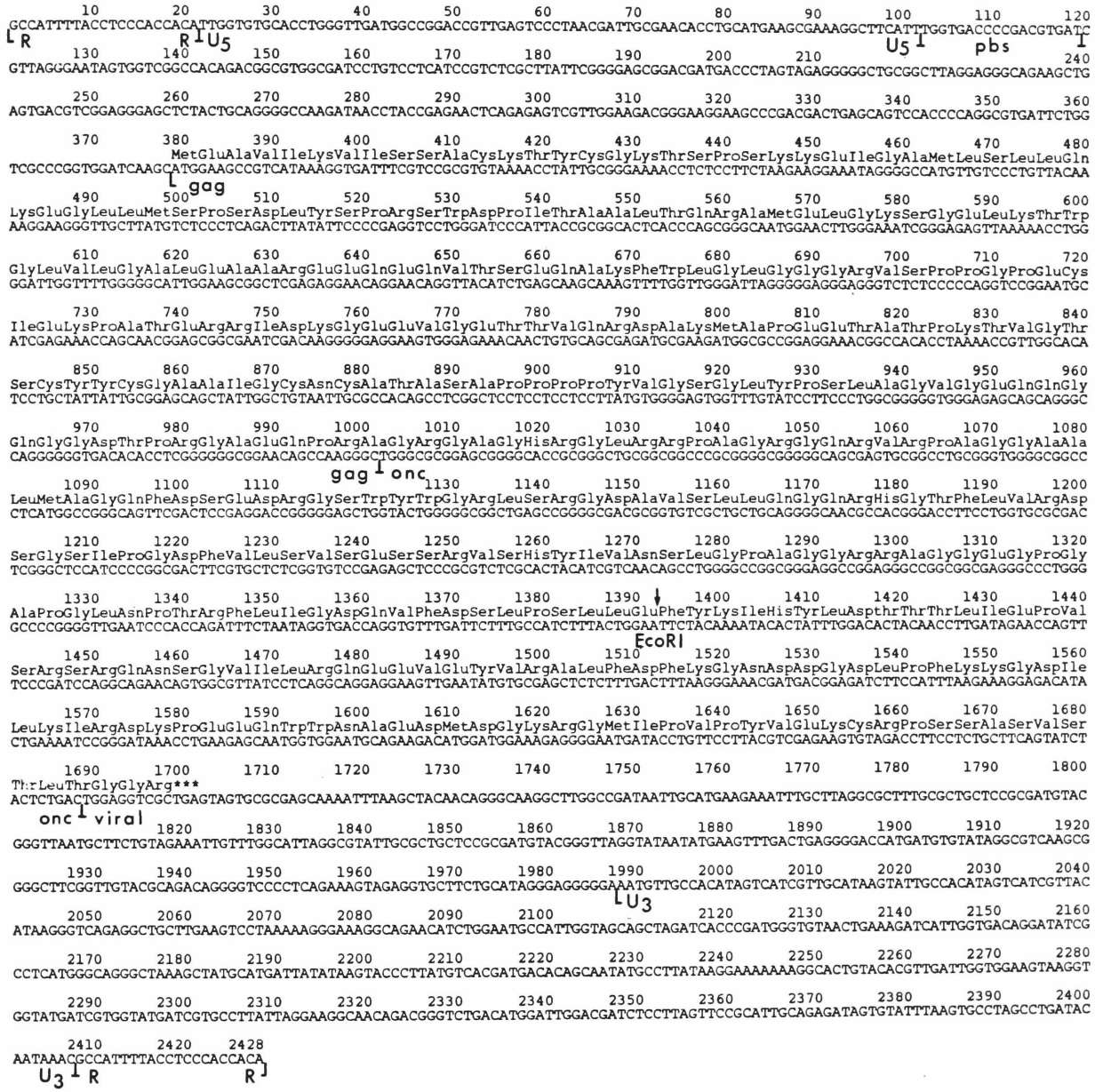

A
B
C
D

Figure 8. Nucleotide sequence of avian sarcoma virus CT10 and predicted amino acid sequence encoded by the major open reading frame. Sequence presented begins and ends with expected initial and terminal nucleotides of the viral genomic RNA. R, U_3, and U_5 regions of viral LTR are indicated. pbs: tRNA primer binding site. gag: sequences encoding viral *gag* protein. onc: transduced oncogene sequences. *Eco*RI site used for molecular cloning is indicated.

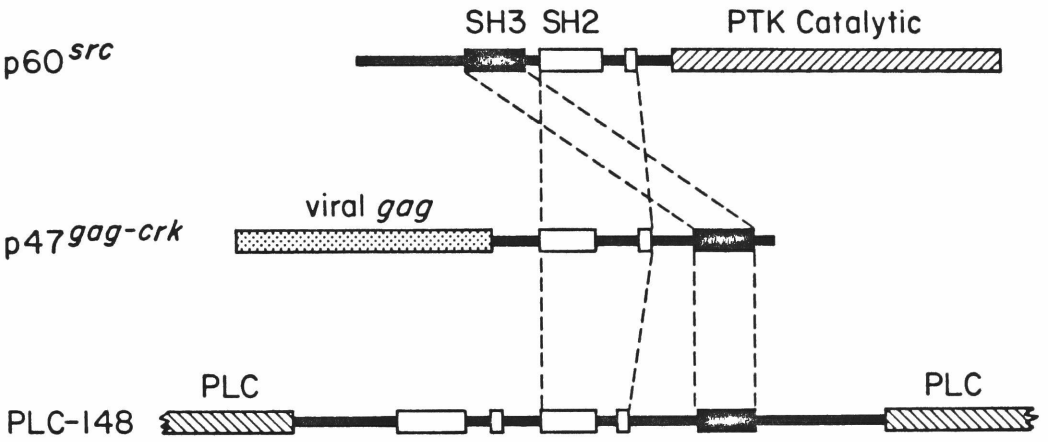

R
U5
U5
pbs
gag
gag
onc
EcoRI
onc
viral
U3
U3
R
R

vided by viral sequences; interestingly, the same virally derived nucleotide sequence is found at the 3' recombination junction of RSV (Takeya et al., 1982; Schwartz et al., 1983; Lerner and Hanafusa, 1984; Bizub et al., 1984). Since both translation start and stop signals are provided by viral sequences, I initially assumed that the CT10 transforming protein was truncated at both ends relative to its normal cellular counterpart. However, recent data from this laboratory have demonstrated that the 5' recombination occurred in upstream noncoding sequences of the cellular proto-oncogene, while the 3' recombination does result in carboxy-terminal truncation of the viral oncogene product relative to its cellular homolog (C. Reichman, unpublished).

Comparison of the transduced oncogene sequence to the protein database revealed 3 blocks of amino acid sequence similarity to the nonreceptor class of protein-tyrosine kinases. Two of these blocks, one of approximately 50 amino acids and one of approximately 10, are found in all nonreceptor tyrosine kinases so far identified and had been previously termed the "SH2" domain (for *S*rc *H*omology 2) by Pawson and colleagues (Sadowski et al., 1986; Pawson, 1988) Since the short 10 amino acid block is separated from the larger block by a region with no homology when the tyrosine kinases and CT10 protein are compared, I have termed this the SH2' region. The remaining block of homology, consisting of approximately 50 amino acids, is present in all the cellular nonreceptor tyrosine kinases so far identified with the exception of c-*fps* (Huang et al., 1985). I have termed this the SH3 domain, for third region of *src* homology.

The homology between the CT10 transforming gene and the tyrosine kinases is extremely surprising for two reasons (see figure 9). First, the CT10 transforming protein does not contain any amino acid homology to the well-conserved catalytic domain of the protein kinases. Numerous lines of evidence have demonstrated that the catalytic activity of tyrosine kinases resides entirely

Figure 9. Comparison of the structure of $p60^{src}$, CT10 transforming protein $p47^{gag\text{-}crk}$, and PLC-148. Black box denotes SH3 homology, large white box SH2 homology, and small white box SH2' homology. Tyrosine kinase (PTK) and presumed phospholipase C (PLC) catalytic domains are denoted by hatched boxes.


SH3 SH2
PTK Catalytic
p60 src
viral gag
p47 gag-crk
PLC
PLC
PLC-148

in a domain (the carboxy-terminal half of $p60^{src}$) that is entirely absent from the CT10 protein (reviewed in Hunter and Cooper, 1985). Second, the SH2 and SH3 blocks of homology are transposed and spaced quite differently when the CT10 protein and the nonreceptor tyrosine kinases are compared. Since the CT10 protein does not encode a catalytic kinase domain, and since I had previously shown that cellular phosphotyrosine levels in CT10-transformed CEF were elevated compared to normal controls and that protein-tyrosine kinase activity can be immunoprecipitated along with the CT10 transforming protein, I termed the CT10 oncogene *crk*, for *C*T10 *R*egulator of *K*inase, and the protein product $p47^{gag\text{-}crk}$.

4-4 *Homology of* crk *to phospholipase C and GAP*

The recent cloning of a phosphatidylinositol-specific phospholipase C (PI-PLC), termed PLC-148 (Stahl et al., 1988; Suh et al., 1988a), revealed a region of striking homology to the *crk* gene (figure 9). PLC-148 contains two SH2+SH2' blocks and one SH3 block; when the amino-acid sequence of PLC148 containing the second SH2+SH2' block and the SH3 block is aligned with the CT10 sequence, the order of the blocks is the same and the spacing between them very similar. The regions surrounding the SH2, SH2', and SH3 blocks have no detectable similarity, however, strongly suggesting that the *crk* gene is not a chicken homolog to the bovine PLC-148 gene.

The structural similarity between PLC-148 and $p47^{gag\text{-}crk}$ raised the possibility that CT10 might encode a truncated member of the PI-PLC family that might possess deregulated PI-PLC activity. This was an attractive possibility considering the considerable evidence implicating the breakdown of phosphatidylinositol-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) by PI-PLCs in the transduction of cellular signals including those involved in growth control (see section 1-3; reviewed in Majerus et al.,

1986; Berridge, 1987). Several lines of evidence have subsequently indicated that this is not the case. First, four other PI-PLC's have recently been cloned and sequenced, none of which contains SH2 or SH3 homology, suggesting that these regions are not involved in PLC catalytic activity (Suh et al., 1988b; Katan et al, 1988; Bennett et al., 1988; Bloomquist et al., 1988). This was demonstrated directly in the case of PLC-148 by construction of an *in vitro* mutant from which the *crk*-homologous domain had been deleted; this deleted protein retained PI-PLC catalytic activity (Bristol et al., 1989 and J. Knopf, personal communication). In addition, in experiments performed in collaboration with A. Aderem and Y. Fukui, I was unable to detect significant PI-PLC activity in TBR immunoprecipitates of *crk*-transformed CEF or a significant increase in PI-PLC activity in membranes prepared from cells expressing *crk* protein (not shown). Finally, Y. Fukui and K. Barker in this laboratory have not found any striking differences in phosphoinositide metabolism in CT10-transformed CEF relative to controls (unpublished observations).

The recent cloning of the *ras* GTPase Activator Protein (GAP) has demonstrated that this protein contains two copies of the SH2+SH2' domain, although no SH3 homology is observed (Vogel et al., 1988; Trahey et al., 1988). GAP was isolated by its ability to stimulate the intrinsic GTPase activity of the cellular *ras* proteins (Trahey and McCormick, 1987; McCormick, 1989). The *ras* family consists of a group of 21 kd GTP-binding proteins analogous to the G-proteins (Gilman, 1987); two *ras* genes have been isolated as viral oncogenes, and *ras* mutations are frequently observed in human tumors (reviewed in Marshall, 1986; Barbacid, 1987). In addition, microinjection of an antibody that binds to and blocks normal *ras* activity has been shown to block the growth of quiescent 3T3 cells after serum stimulation (Mulcahey et al., 1985). These data imply that *ras* proteins are intimately involved in the transmission of growth-control signals.

There is considerable circumstantial evidence that GAP is a component of the "effector" of *ras* action (reviewed in McCormick, 1989), the cellular target that responds to *ras*-mediated signals. GAP may therefore play a central role in normal and abnormal growth control.

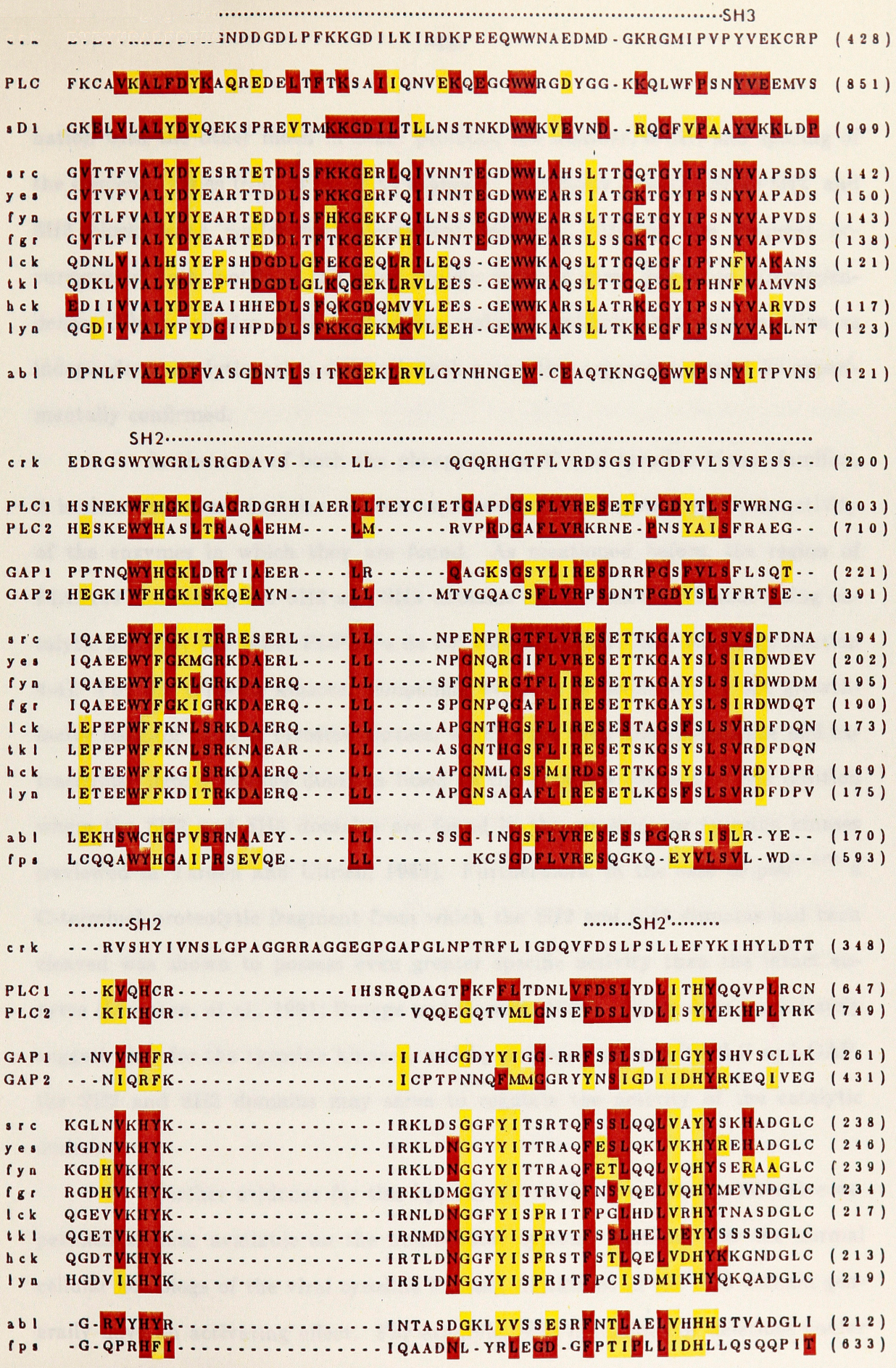
In addition to the nonreceptor tyrosine kinases, PLC-148, and GAP, a chicken spectrin gene that has been recently sequenced was found to have homology to *crk* (Lehto et al., 1988). In this case a single SH3 block is present. It is difficult to imagine what an intermediate filament protein might have in common with the other proteins with *crk* homology; however, it is possible that there are binding sites on the cytoskeletal matrix for tyrosine kinases and/or their substrates, since it has been shown that binding of $p60^{v\text{-}src}$ mutants to the nonionic detergent-insoluble matrix of CEF correlates with their ability to transform (Hamaguchi and Hanafusa, 1987; Loeb et al., 1987).

Figure 10 compares the amino acid sequences of all the proteins identified to date that contain SH2 or SH3 domains. In all cases, the percent amino acid identity with the *crk* gene within the conserved domains is approximately 40%; when conservative amino acid changes are considered, similarity increases to greater than 50%. This degree of sequence similarity is the same as that seen when other SH2- or SH3-containing proteins are compared to each other (PLC vs. *abl*, for example). It can also be seen that several amino acids are 100% conserved, implying important functional roles for these residues.

4-5 *Properties of SH2 and SH3 domains*

The presence of strongly conserved SH2 and SH3 domains in diverse enzymes implicated in signal transduction invites speculation on the possible function of these domains (see Katan and Parker, 1988 and Pawson, 1988). One clear structural implication is that the SH2+SH2' and SH3 motifs are modular and independent. Each motif is present alone in at least one case, and in combi-

Figure 10. Comparison of amino acid sequences of conserved SH3, SH2, and SH2' domains in various proteins. Orange boxes denote identity with *crk* sequence, yellow boxes similar amino acids. Dashes indicate gaps imposed to maximize sequence alignment. Numbers in parentheses on right correspond to the last amino acid shown on each line, numbered from the amino terminus of each protein. crk, p47$^{gag\text{-}crk}$; PLC, bovine PLC-148 (Stahl et al., 1988); PLC1 and PLC2, first and second SH2 blocks, respectively; sD1, chicken spectrin D1 (Lehto et al., 1988); src, chicken c-*src* (Takeya and Hanafusa, 1983); yes, chicken c-*yes* (Sudol et al., 1988); fyn, human c-*fyn* (Semba et al., 1986; Kawakami et al., 1986); fgr, human c-*fgr* (Katamine et al., 1988); lck, murine c-*lck* (Marth et al., 1985; Voronova and Sefton, 1986); tkl, chicken c-*tkl* (Strebhardt et al., 1987); hck, human c-*hck* (Quintrell et al., 1987; Ziegler et al., 1987); lyn, human c-*lyn* (Yamanishi et al., 1987); abl, murine c-*abl* (Reddy et al., 1983; Ben-Neriah et al., 1986); GAP, bovine GTPase activator protein (Vogel et al., 1988); GAP1, GAP2, first and second SH2 blocks, respectively; fps, chicken c-*fps* (Huang et al., 1985).


SH3
NDDGDLPFKKGDILKIRDKPEEQWWNAEDMD-GKRGMIPVPYVEKCRP (428)
PLC FKCAVKALFDYKAQREDELTFTKSAIIQNVEKQEGGWWRGDYGG-KKQLWFPSNYVEEMVS (851)
sD1 GKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWWKVEVND--RQGFVPAAYVKKLDP (999)
src GVTTFVALYDYESRTETDLSFKKGERLQIVNNTEGDWWLAHSLTTGQTGYIPSNYVAPSDS (142)
yes GVTVFVALYDYEARTTDDLSFKKGERFQIINNTEGDWWEARSIATGKTGYIPSNYVAPADS (150)
fyn GVTLFVALYDYEARTEDDLSFHKGEKFQILNSSEGDWWEARSLTTGETGYIPSNYVAPVDS (143)
fgr GVTLFIALYDYEARTEDDLTFTKGEKFHILNNTEGDWWEARSLSSGKTGCIPSNYVAPVDS (138)
lck QDNLVIALHSYEPSHDGDLGFEKGEQLRILEQS-GEWWKAQSLTTGQEGFIPFNFVAKANS (121)
tkl QDKLVVALYDYEPTHDGDLGLKQGEKLRVLEES-GEWWRAQSLTTGQEGLIPHNFVAMVNS
hck EDIIVVALYDYEAIHHEDLSFQKGDQMVVLEES-GEWWKARSLATRKEGYIPSNYVARVDS (117)
lyn QGDIVVALYPYDGIHPDDLSFKKGEKMKVLEEH-GEWWKAKSLLTKKEGFIPSNYVAKLNT (123)
abl DPNLFVALYDFVASGDNTLSITKGEKLRVLGYNHNGEW-CEAQTKNGQGWVPSNYITPVNS (121)
SH2
crk EDRGSWYWGRLSRGDAVS-----LL------QGQRHGTFLVRDSGSIPGDFVLSVSESS-- (290)
PLC1 HSNEKWFHGKLGAGRDGRHIAERLLTEYCIETGAPDGSFLVRESETFVGDYTLSFWRNG-- (603)
PLC2 HESKEWYHASLTRAQAEHM----LM------RVPRDGAFLVRKRNE-PNSYAISFRAEG-- (710)
GAP1 PPTNQWYHGKLDRTIAEER----LR------QAGKSGSYLIRESDRRPGSFVLSFLSQT-- (221)
GAP2 HEGKIWFHGKISKQEAYN-----LL-----MTVGQACSFLVRPSDNTPGDYSLYFRTSE-- (391)
src IQAEEWYFGKITRRESERL----LL-----NPENPRGTFLVRESETTKGAYCLSVSDFDNA (194)
yes IQAEEWYFGKMGRKDAERL----LL-----NPGNQRGIFLVRESETTKGAYSLSIRDWDEV (202)
fyn IQAEEWYFGKLGRKDAERQ----LL-----SFGNPRGTFLIRESETTKGAYSLSIRDWDDM (195)
fgr IQAEEWYFGKIGRKDAERQ----LL-----SPGNPQGAFLIRESETTKGAYSLSIRDWDQT (190)
lck LEPEPWFFKNLSRKDAERQ----LL-----APGNTHGSFLIRESESTAGSFSLSVRDFDQN (173)
tkl LEPEPWFFKNLSRKNAEAR----LL-----ASGNTHGSFLIRESETSKGSYSLSVRDFDQN
hck LETEEWFFKGISRKDAERQ----LL-----APGNMLGSFMIRDSETTKGSYSLSVRDYDPR (169)
lyn LETEEWFFKDITRKDAERQ----LL-----APGNSAGAFLIRESETLKGSFSLSVRDFDPV (175)
abl LEKHSWCHGPVSRNAAEY-----LL-----SSG-INGSFLVRESESSPGQRSISLR-YE-- (170)
fps LCQQAWYHGAIPRSEVQE-----LL--------KCSGDFLVRESQGKQ-EYVLSVL-WD-- (593)
SH2
SH2'
crk ---RVSHYIVNSLGPAGGRRAGGEGPGAPGLNPTRFLIGDQVFDSLPSLLEFYKIHYLDTT (348)
PLC1 ---KVQHCR----------IHSRQDAGTPKFFLTDNLVFDSLYDLITHYQQVPLRCN (647)
PLC2 ---KIKHCR----------VQQEGQTVMLGNSEFDSLVDLISYYEKHPLYRK (749)
GAP1 --NVVNHFR----------IIAHCGDYYIGG-RRFSSLSDLIGYYSHVSCLLK (261)
GAP2 ---NIQRFK----------ICPTPNNQFMMGGRYYNSIGDIIDHYRKEQIVEG (431)
src KGLNVKHYK----------IRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLC (238)
yes RGDNVKHYK----------IRKLDNGGYYITTRAQFESLQKLVKHYREHADGLC (246)
fyn KGDHVKHYK----------IRKLDNGGYYITTRAQFETLQQLVQHYSERAAGLC (239)
fgr RGDHVKHYK----------IRKLDMGGYYITTRVQFNSVQELVQHYMEVNDGLC (234)
lck QGEVVKHYK----------IRNLDNGGFYISPRITFPGLHDLVRHYTNASDGLC (217)
tkl QGETVKHYK----------IRNMDNGGYYISPRVTFSSLHELVEYYSSSSDGLC
hck QGDTVKHYK----------IRTLDNGGFYISPRSTFSTLQELVDHYKKGNDGLC (213)
lyn HGDVIKHYK----------IRSLDNGGYYISPRITFPCISDMIKHYQKQADGLC (219)
abl -G-RVYHYR----------INTASDGKLYVSSESRFNTLAELVHHHSTVADGLI (212)
fps -G-QPRHFI----------IQAADNL-YRLEGD-GFPTIPLLIDHLLQSQQPIT (633)

nation with the other motif in other proteins; the number, order, and spacing of the elements varies from example to example. It is likely that the SH2+SH2' and SH3 blocks each confer an independent function, although the frequent occurrence of both motifs in the same protein suggests some degree on interdependence. The small size of these domains makes it unlikely that they function as independent catalytic units, although such a negative argument cannot be experimentally confirmed.

In the case of both the phospholipase C and tyrosine kinase families, it is clear that these domains are not required for the recognized catalytic activity of the enzymes in which they are found. As mentioned before, the region of PLC-148 containing the SH2 and SH3 domains can be deleted without losing catalytic activity, and other PI-PLC's do not contain SH2 or SH3 homology (section 4-4). For the tyrosine kinases, compelling evidence is presented by the growth-factor receptor class of tyrosine kinases, which have their transmembrane and extracellular ligand-binding domains fused to the catalytic domain in the position where the SH2 and SH3 domains are found in the nonreceptor tyrosine kinases (reviewed in Yarden and Ullrich, 1988). Furthermore, in the case of $p60^{v\text{-}src}$ a C-terminal proteolytic fragment from which the SH2 and SH3 domains had been cleaved was shown to possess even greater specific activity than the intact enzyme (Levinson, et al., 1981; Brugge and Darrow, 1984). These data immediately suggest that for the tyrosine kinases, and by analogy perhaps PI-PLC and GAP, the SH2 and SH3 domains may serve to regulate the activity of the catalytic domain.

Further evidence for this hypothesis can be found in the extensive experimental data available on the nonreceptor tyrosine kinases. For the normal cellular homologs of the viral tyrosine kinases, mutations in the SH3 domain generally have an activating effect. For example, SH3 mutations or deletions confer

transforming ability on the normally nontransforming products of the c-*src* and c-*abl* genes (Kato et al., 1986; Potts et al., 1988; Frantz et al., 1989; Jackson and Baltimore, 1989). In the case of v-*abl* and v-*fgr*, the SH3 domain was entirely deleted during oncogene transduction (Ben Neriah et al., 1986; Katamine et al., 1988), while v-*src* and v-*yes* contain amino acid changes in SH3 relative to their normal homologs (Hanafusa, 1986; Sudol et al., 1988). Furthermore, in neuronal cells an alternatively spliced form of $p60^{c\text{-}src}$ is synthesized containing a six amino acid insertion in the SH3 domain (Martinez, et al., 1987; Levy et al., 1987); the neuronal form of $p60^{c\text{-}src}$ has elevated specific kinase activity (Brugge et al., 1985). These results, along with the proteolytic activation studies mentioned in the previous paragraph, suggest that SH3 may be involved in negatively regulating the activity of the catalytic domain. This is consistent with the general observation that the nonreceptor tyrosine kinases are under tight negative regulation in normal cells (reviewed for the *src* family by Hunter, 1987; Cooper, 1989).

Conversely, deletions, insertions, and point mutations in the amino termini of v-*src* and v-*fps*, especially within the SH2 domain, generally induce a less transformed morphology in infected cells and cause a decrease in *in vitro* kinase activity compared with the wild type (Kitamura and Yoshida, 1983; Cross et al., 1985; Sadowski et al., 1986; Jove et al., 1986a; Jove et al., 1986b; Raymond and Parsons, 1987; Wang and Parsons, 1989). The lesions of several temperature-sensitive mutants of v-*src* and v-*fps* (Bryant and Parsons, 1982; DeClue et al., 1987) and host range mutants of v-*src* and v-*fps* (Sadowski et al., 1986; DeClue et al., 1987; DeClue and Martin, 1989; Verderame et al., 1989) are located in the SH2 domain. Taken in sum, these results suggest a positive role for the SH2 region in transformation by these tyrosine kinases. It has been suggested, based on the host-range mutants that map within SH2, that this domain is involved in binding positive-acting cellular factors or critical substrates (Sa-

dowski et al., 1986; reviewed in Pawson, 1988).

Results are also beginning to accumulate that implicate tyrosine phosphorylation of SH2 and SH3 domains in the regulation of $p60^{c\text{-}src}$ and perhaps phospholipase C. It has been shown that $p60^{c\text{-}src}$ complexed with polyomavirus middle T antigen (which has elevated kinase activity *in vivo* and *in vitro* compared with the uncomplexed form) is phosphorylated on one or more amino-terminal tyrosines (Yonemoto et al., 1985; Cartwright et al., 1985). The most likely sites of phosphorylation are at positions 90 and/or 92 (Cooper, 1989), at the N-terminal boundary of SH3. Interestingly, every protein that contains an SH3 domain has a tyrosine in at least one of the two analogous positions with the exception of v-*crk*, which encodes phenylalanine in both positions (see figure 10). Stimulation of CEF with platelet-derived growth factor (PDGF) also results in amino-terminal tyrosine phosphorylation of $p60^{c\text{-}src}$, with a concomitant increase in specific activity (Ralston and Bishop, 1985). An intriguing recent result has indicated that stimulation of cells with epidermal growth factor results in tyrosine phosphorylation of a PI-PLC, as assayed by immunoaffinity purification with antiphosphotyrosine antibodies (Wahl et al., 1988). Furthermore, this phosphorylation occurs only on PLC-148, which contains SH2 and SH3 domains, and not on other forms of PI-PLC that lack this homology (Wahl et al., 1989; Rhee et al., 1989). While these results are fragmentary, they raise the exciting possibility that tyrosine phosphorylation of SH2 and SH3 domains might be a general mechanism for the regulation of enzymes containing these domains during normal signal transduction.

There is also circumstantial evidence that the amino terminal regulatory domain and the catalytic domain of the nonreceptor tyrosine kinases are actually in close apposition to each other. A monoclonal antibody that recognizes v-*src*, v-*yes*, and v-*fgr* appears to recognize an epitope assembled from both N-

terminal and C-terminal determinants (McCarley et al., 1987), suggesting close association of the two domains. Also, a 45 kd fragment containing both the SH2 and catalytic domains can be generated from the v-*fps* protein by limited proteolysis, suggesting a compact structure (Weinmaster, 1983) and there appears to be an autophosphorylation site in SH2, implying accessibility to the catalytic domain (Sadowski and Pawson, 1987). These data, along with the evidence for negative and positive effects of N-terminal mutations, suggest that the SH2 and SH3 domains may serve as a *cis*-acting regulatory subunit of the nonreceptor tyrosine kinases. In the case of the second messenger-responsive serine/threonine kinases, a regulatory subunit is present in either *cis* or *trans*; inhibition of catalytic activity in the unstimulated state may be effected by binding of a "pseudosubstrate" sequence present on the regulatory domain that effectively competes for substrate binding (House and Kemp, 1987; Pearson et al., 1988; reviewed by Hardie, 1988). It is possible that a prototope within the SH2 or SH3 domains may normally bind tightly to the catalytic domain of the nonreceptor tyrosine kinases and inhibit its activity.

4-6 *Models for SH2 and SH3 function and transformation by v-*crk

The evidence presented in the last section demonstrates that the SH2 and SH3 domains are small, modular elements implicated in the regulation of enzyme activity in *cis*. Perhaps the most important clue to the function of these domains is provided by the *crk* gene product itself. $p47^{gag\text{-}crk}$ is composed almost entirely of viral *gag* sequences and the SH2+SH2' and SH3 domains. The short stretches of proto-oncogene sequence surrounding these domains are in two cases extremely rich in proline and glycine residues, consistent with a turn or coil structure and not with the α-helix or β-sheet structures common to the globular domains found in enzymes (Chou and Fasman, 1978). The v-*crk* gene has no

significant sequence similarity to any known catalytic domain, and as mentioned previously the small size and modular nature of the SH2 and SH3 domains makes it unlikely that they possess an intrinsic catalytic activity. The most compelling hypothesis that explains the biological activity of the v-*crk* gene product and the presumed regulatory function of the homologous SH2 and SH3 domains on other proteins is that these domains bind to cellular components that normally function in the regulation of the activity of enzymes that contain them.

If this is true, expression of p47$^{gag\text{-}crk}$ might be expected to drastically alter the intracellular equilibrium of putative SH2- and SH3-binding factors. In this light, I would like to briefly reexamine the two pieces of biochemical evidence that I have so far presented concerning *crk* transformation. First, there is a large increase in protein phosphotyrosine levels in CT10-infected cells. Since p47$^{gag\text{-}crk}$ is obviously not itself a tyrosine kinase, this implies that the activity of one or more normal cellular protein-tyrosine kinases is elevated. Given the large body of evidence implicating SH2 and SH3 domains in regulating the activity of nonreceptor tyrosine kinases, a reasonable hypothesis is that p47$^{gag\text{-}crk}$ binds to and sequesters a putative cellular factor that normally binds to and represses the activity of cellular kinases. Since it is expressed via the strong viral promoter, the *crk* gene product would be expected to be present at much higher concentration than the cellular tyrosine kinases. Second, I have shown that a relatively weak tyrosine kinase activity coprecipitates with p47$^{gag\text{-}crk}$. I have presented evidence from the literature that the SH2 and SH3 domains may be closely apposed to the catalytic domains of the nonreceptor tyrosine kinases and that the nature of this interaction may regulate catalytic activity. It is therefore possible that this type of regulation could occur in *trans*, and that by binding to the catalytic domains p47$^{gag\text{-}crk}$ could activate by competing away a kinase's own potentially inhibitory SH2 and/or SH3 domains. This would be analogous to

the activity of polyoma middle T, which binds to the c-*src*, c-*yes*, and c-*fyn* tyrosine kinases and can elevate their specific kinase activity (Cheng, et al., 1986; Kornbluth et al., 1987; Kypta et al, 1988; Cheng et al., 1988). While I think that this is a less likely mechanism for kinase activation than sequestration of inhibitors, this model provides at least a reasonable mechanistic basis for the presence of kinase activity in v-*crk* immunoprecipitates. I will discuss these and other models in greater detail in Section 5.

What does the presence of SH2 and SH3 domains on PI-PLC, tyrosine kinases, and GAP tell us about signal transduction mechanisms in normal cells? The most reasonable hypothesis is that these enzymes interact with, and are perhaps coordinately regulated by, common protein factors. PLC, nonreceptor tyrosine kinases, and GAP are all enzymes implicated in the intracellular amplification of receptor-mediated signals transduced through the plasma membrane. In the case of PI-PLCs, a wide variety of hormones and growth factors have been shown to stimulate PI-PLC activity and pharmacological evidence implicates a G protein in receptor coupling (Majerus et al., 1986; Berridge, 1987). It should be kept in mind, however, that in no case has a specific PLC been shown to be coupled to a specific receptor or G-protein. The recent results demonstrating that PLC-148 is phosphorylated on tyrosine in response to EGF or PDGF (Wahl, et al., 1987; Wahl et al., 1988; Rhee et al., 1989; J. Knopf, personal communication) suggest that it may be a direct substrate for the kinase activity of the receptors for these growth factors. However, the observed tyrosine phosphorylation could be the result of feedback; it is not yet known what effect tyrosine phosphorylation has on the specific activity of PLC-148. In the case of GAP, it is clear that this protein interacts with members of the *ras* family of GTP-binding proteins and is probably a component of the effector of *ras* action (McCormick, 1989). In this case, both the signal input (what activates *ras*, presumably by

stimulating exchange of bound GDP for GTP) and the nature of the output from the effector are unclear. Significantly, data from microinjection of *ras* antibodies imply that *ras* works downstream from tyrosine kinase oncogenes and phospholipases, and upstream from the serine kinase oncogenes (Smith et al., 1986; Yu et al, 1988). The normal nonreceptor tyrosine kinases have not yet been shown convincingly to be coupled to to any signal transduction event, though recent evidence has implicated $p56^{lck}$ in T-cell activation via an association with the CD4 and CD8 antigens (Veillette et al., 1988). The presence of high levels of nonreceptor tyrosine kinases in terminally differentiated cells, especially those of neural, neuroendocrine, and hematopoietic origin, suggests that they may be normally involved not in growth control but in a specialized cell function such as secretion (Cooper, 1989).

In all three cases cited above, a common denominator is that these enzymes are thought to respond to or propagate signals from unknown cellular components. The discovery of the *crk* oncogene, which drastically alters the growth properties of cells in which it is expressed, has focused attention on the SH2 and SH3 domains. In a sense, all that $p47^{gag\text{-}crk}$ can do is bind other cellular factors—factors that might also bind to tyrosine kinases, PI-PLCs, and GAP. It is reasonable to assume that the identification of proteins that interact with $p47^{gag\text{-}crk}$ will help to fill in some of the poorly understood interrelationships between components of the signal transduction pathways involved in growth control.

SECTION FIVE

CHARACTERIZATION OF p47$^{gag\text{-}crk}$ AND ASSOCIATED PROTEINS

5-1 *Introduction*

As I have outlined in the previous section, ASV CT10 encodes an oncogene product with amino acid sequence similarity to a putative regulatory domain found in the nonreceptor tyrosine kinases, a PI-specific phospholipase C, and GAP. Preliminary experiments indicated that there were elevated levels of phosphotyrosine on proteins in CT10-infected CEF, and that a protein-tyrosine kinase activity could be immunoprecipitated along with the CT10 transforming protein. Given the extreme unlikelihood that the v-*crk* gene product, p47$^{gag\text{-}crk}$, encodes a catalytic activity, it is apparent that this oncogene uses a novel mechanism to transform cells in which it is expressed, presumably via its interaction with normal cellular enzymes or factors that normally regulate such enzymes. Since p47$^{gag\text{-}crk}$ is derived from a normal cellular proto-oncogene, there is a good chance that studying the aberrant action of the *crk* oncogene might directly lead to mechanisms and factors that are involved in normal signal transduction.

It is clear that to understand the mechanism by which the v-*crk* gene transforms, it is necessary to study the protein product. While such work is by necessity descriptive and phenomenological, it provides the necessary background for future work involving mutants and aimed at determining what is necessary and sufficient for transformation by this oncogene. In this section I will describe the generation of a specific antiserum that recognizes p47$^{gag\text{-}crk}$, studies to address what proteins and enzymatic activities may be associated with p47$^{gag\text{-}crk}$, and some preliminary work on the subcellular localization of the transforming

protein and proteins with which it interacts. I will also discuss models for the biological activity of the v-*crk* oncogene based on this work and the sequence data presented in the previous section.

5-2 *Specificity of increased phosphotyrosine*

One of the initial observations that made the genetic structure of the v-*crk* gene so intriguing was the elevation of phosphotyrosine in CT10-infected cells, which implied a deregulation of cellular protein-tyrosine kinase activity (figure 6). One could imagine, however, that increased phosphotyrosine was an obligatory byproduct of malignant transformation, and not a specific consequence of expression of $p47^{gag\text{-}crk}$. Many growth-factor receptors possess an intrinsic protein-tyrosine kinase activity which is stimulated by binding of the appropriate ligand (Yarden and Ullrich, 1988), so one could imagine a scheme whereby an autocrine loop could be established involving the secretion of growth factors by transformed cells. For example, many transformed cells secrete TGF-α, which is known to bind to and activate the EGF receptor (see Dernyck, 1986 for review). Such a loop might be expected to elevate cellular phosphotyrosine compared to normal controls. There is also some evidence that transformation by the ^{H}ras oncogene leads to increased phosphorylation of at least one protein on tyrosine (Morrison et al., 1988a).

To address this possibility, I examined in collaboration with M. Hamaguchi the phosphotyrosine levels in CEF transformed with oncogenes that do not encode tyrosine kinases. For these experiments the Harvey *ras* oncogene, a G-protein analogue (Barbacid, 1987), and the v-*mos* oncogene, a protein-serine kinase (Blair, 1986), were expressed in CEF using a RSV-derived expression vector (Hughes et al., 1987). Infected chicken cells grew to high density and were capable of anchorage-independent growth in agar suspension, demonstrating that

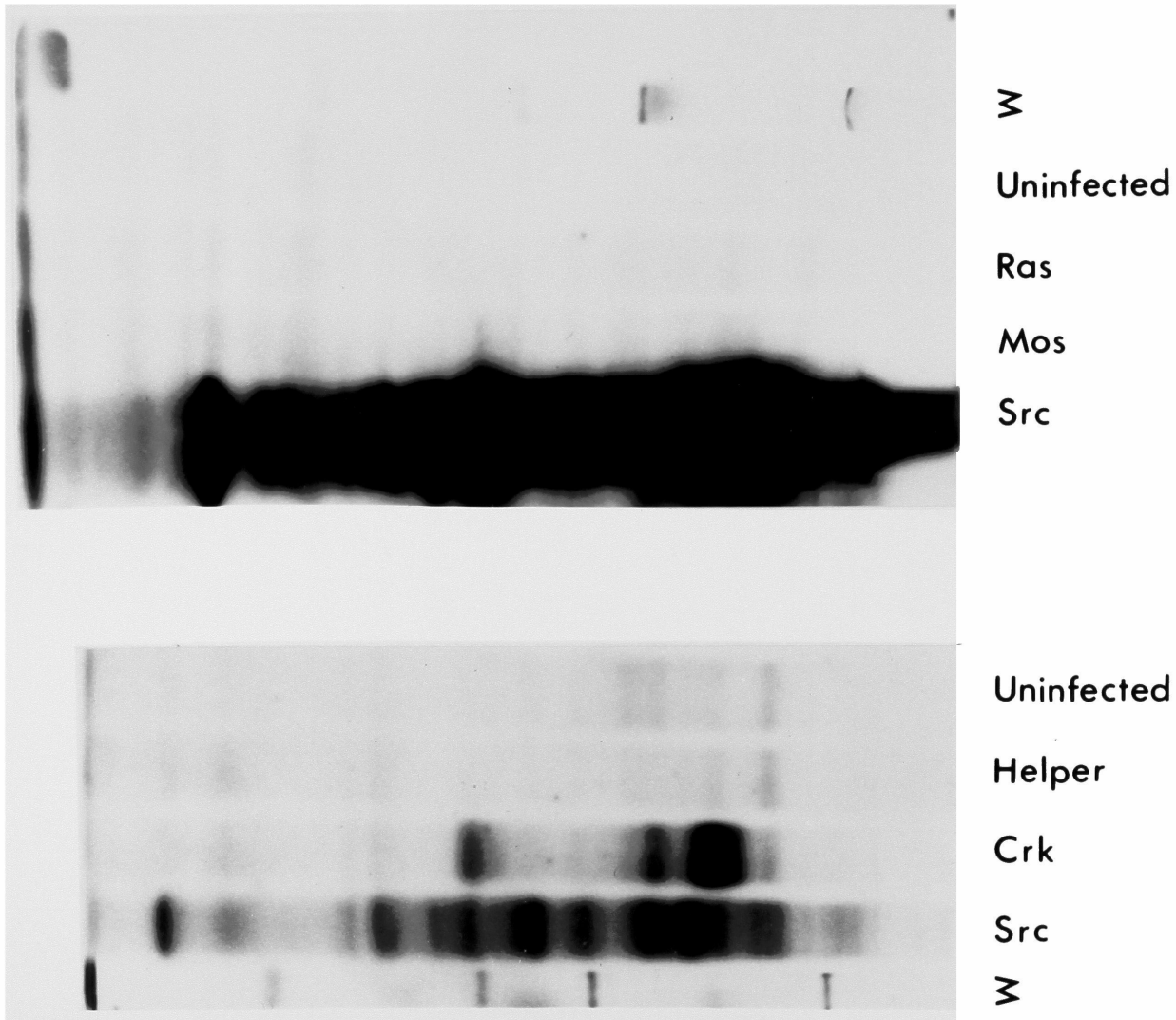
these murine virus-derived oncogenes are capable of transforming chicken cells (not shown). Lysates from these cells were immunoblotted with an antibody (anti-ptyr) that specifically recognizes phosphotyrosine on cellular proteins (figure 11). It is clear that even when the filter is overexposed there was no obvious increase in phosphotyrosine in chicken cells transformed by the *mos* or *ras* oncogenes. In contrast, there was a very marked increase in phosphotyrosine on at least three proteins in cells expressing $p47^{gag\text{-}crk}$. These data suggest that the increase seen in the case of v-*crk* is specific, not a general property of transformed CEF, and may play a causative role in transformation by this oncogene. I would like to mention that the anti-phosphotyrosine antibody used in this and subsequent experiments was prepared in rabbits by injection of lysates of bacteria expressing the active v-*abl* tyrosine kinase (Wang, 1985), while the antibody used for figure 6 was prepared by injection of phosphotyramine coupled to carrier protein (Hamaguchi et al., 1988). Since the pattern and extent of labeling are similar, it is unlikely that the apparent increase in phosphotyrosine is an antibody artifact.

5-3 *Generation of a* crk-*specific antibody*

Although TBR serum and other sera that recognize viral structural proteins recognize $p47^{gag\text{-}crk}$ via the protein's *gag* moiety, these antisera recognize many highly expressed viral proteins in infected cells and are therefore unsuitable for many experiments. In addition, such antisera would not recognize the cellular proto-oncogene product or the product of *in vitro* mutants from which the viral *gag* sequences had been deleted. I therefore generated an antiserum that specifically recognizes the proto-oncogene-derived portion of $p47^{gag\text{-}crk}$.

Initial attempts to generate antisera using synthetic peptides

Figure 11. Antiphosphotyrosine immunoblots of cells expressing different oncogenes. **A**: Equal amounts of protein from uninfected CEF or CEF transformed by v-Ha-*ras* (Ras), v-*mos* (Mos) or Rous sarcoma virus (Src) probed with antiphosphotyrosine antibody. **B**: Uninfected CEF or CEF infected with UR2AV (Helper), CT10 plus UR2AV (Crk), or Rous sarcoma virus (Src) probed as in A. Gels were 7.5% acrylamide. M: molecular weight markers; from top, 220, 100, 68, 43, and 27 kd.


A
W
Uninfected
Ras
Mos
Src
B
Uninfected
Helper
Crk
Src
W

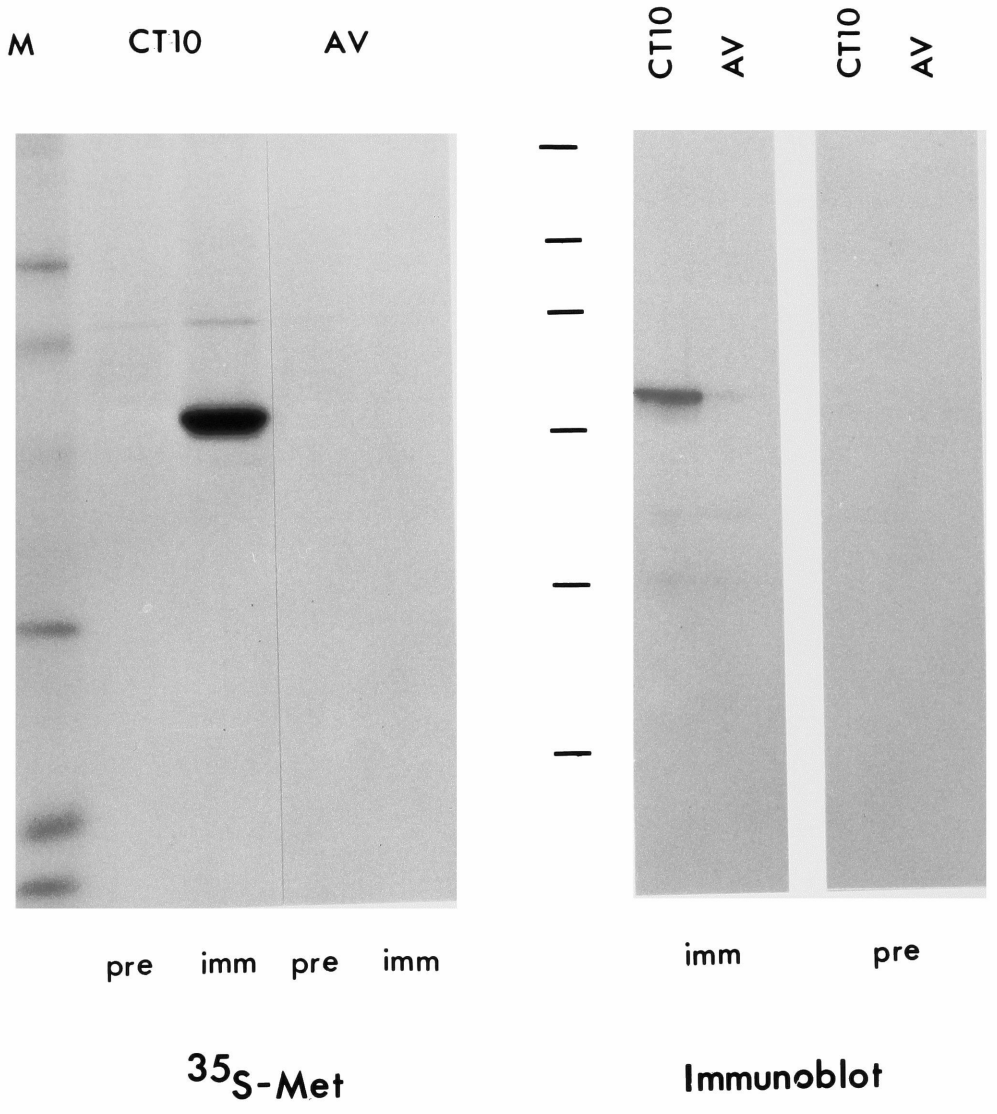
corresponding to hydrophilic regions of the v-*crk* protein were unsuccessful. I therefore turned to expression of the *crk*-specific portion of the protein in *E. coli*. I used the T7 RNA polymerase-dependent bacterial expression system developed by W. Studier and colleagues (Studier and Moffat, 1986; Rosenberg et al., 1987). In this system, gene fragments are cloned in-frame downstream from a T7 polymerase-specific promoter and short leader peptide. When introduced into bacteria carrying the T7 polymerase gene under the control of the *lac* promoter, high levels of protein synthesis can be induced by adding the lactose analog IPTG. I cloned the 0.8 kb *Sty*I-*Hae*II fragment of pCT10 (see figure 1), which encodes the entire c-*crk*-derived 238 amino acids and 2 amino acids of viral *gag*, into the appropriate vector and immunized rabbits with the crude bacterially-expressed protein prepared by SDS-PAGE (see section 2-6).

As shown in figure 12, left panel, a specific band of approximately 47 kd was immunoprecipitated by immune sera in [^{35}S]methionine-labeled CT10-infected lysates, but not in lysates prepared from cells infected with helper virus alone. This antiserum also recognized a 47 kd protein in immunoblots of lysates from CT10-infected cells (figure 12, right panel). These results demonstrate that the antiserum recognizes both native and denatured p47$^{gag\text{-}crk}$ (predicted MW 47.1 kd) and does not recognize any helper virus proteins. This antiserum will be referred to as anti-*crk* serum. While not evident on short exposure, the antiserum also recognized a 38 kd protein in both immunoprecipitates and western blots that corresponds to the predicted molecular weight of the proto-oncogene product (C. Reichman and B. Mayer, unpublished).

5-4 crk-*associated tyrosine kinase activity*

I have shown that TBR sera immunoprecipitate a protein-tyrosine kinase activity that phosphorylates a 135-155 kd band from lysates of CT10-

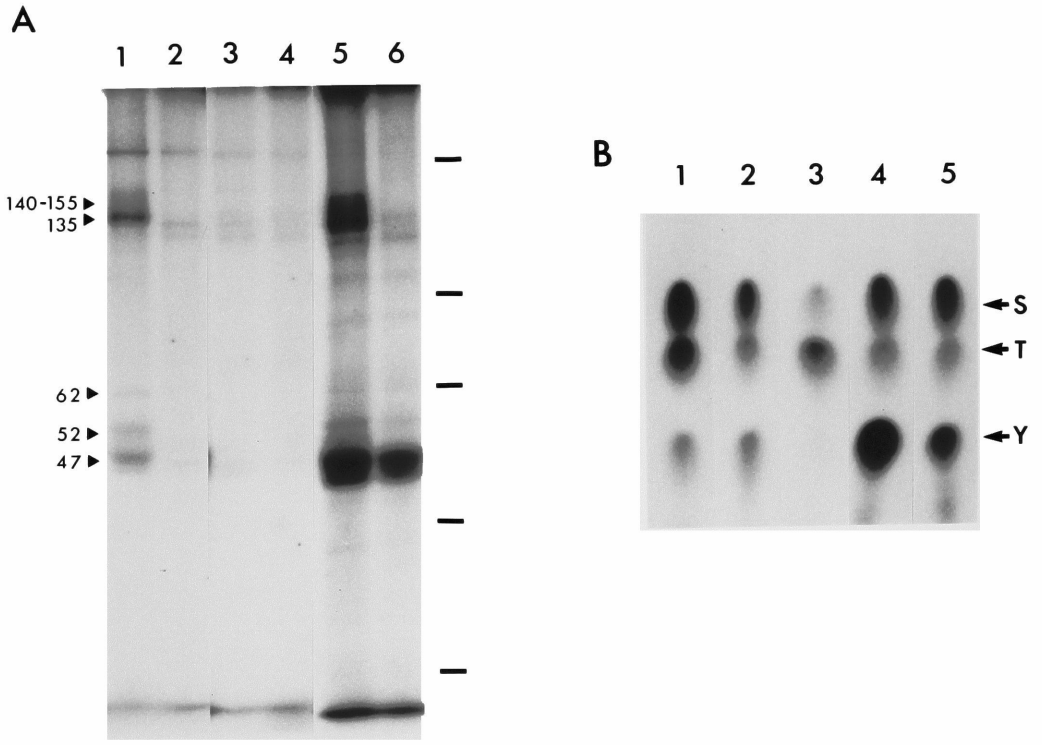
Figure 12. Anti-*crk* antiserum recognizes v-*crk* oncogene product. Left panel: [^{35}S]methionine-labeled CEF lysates from cells transfected with CT10+UR2AV (CT10) or UR2AV (AV) DNA, immunoprecipitated with anti-*crk* (imm) or preimmune (pre) serum. Right panel: immunoblots of CT10+UR2AV (CT10) or UR2AV (AV) infected cell lysates probed with anti-*crk* (imm) or preimmune (pre) serum. Gels were 10% acrylamide; markers, 220, 100, 68, 43, 27, and 18 kd.


M
CT10
AV
CT10
AV
CT10
AV
pre
imm
pre
imm
imm
pre
35S-Met
Immunoblot

infected CEF (see figure 5). Since TBR sera were raised in rabbits bearing RSV-induced tumors and contain antibodies that recognize $p60^{v\text{-}src}$ (Brugge and Erikson, 1977), it was possible that the kinase activity observed was due to c-*src* or c-*src*-related kinases directly recognized by the sera. Also, the virus used for the previous experiment had not been molecularly cloned, raising the possibility that there was low-level contamination of the CT10 stock with a virus carrying a tyrosine kinase oncogene. (All protein work other than that in Section 3 was performed on cells transfected with cloned CT10 DNA). As seen in figure 13A, however, when CEF infected with molecularly cloned CT10 virus were immunoprecipitated with anti-*crk* serum and subjected to *in vitro* kinase assay, a 135 kd protein was phosphorylated. This protein was not seen when anti-*crk* antiserum was used to immunoprecipitate lysates from CEF infected with helper virus alone, or when CT10-infected cells were precipitated with preimmune serum. In addition to the prominent 135 kd band, three other bands, with approximate molecular weights of 62, 52, and 47 kd, were specifically phosphorylated in anti-*crk* immunoprecipitates of CT10-infected CEF lysates.

The amino acids to which phosphate was transferred *in vitro* were determined by acid hydrolysis of the ^{32}P-labeled bands and thin-layer electrophoresis. As shown in figure 13B, the 135 kd band and the 140-155 kd region above it were phosphorylated predominantly on tyrosine, although there was a moderate amount of phosphoserine and phosphothreonine as well. The 47 kd protein (which is $p47^{gag\text{-}crk}$ itself—see section 5-5) and the 52 kd protein both contain lower levels of phosphotyrosine, estimated at 15-30%, compared to phosphoserine and phosphothreonine. The 62 kd protein, in contrast, was phosphorylated predominantly on threonine, with trace levels of phosphotyrosine visible only upon long exposure. These data confirm that a protein-tyrosine kinase activity coprecipitates with $p47^{gag\text{-}crk}$, presumably via association; they also sug-

Figure 13. *In vitro* kinase activity in *crk* immunoprecipitates and phosphoamino acid analysis of proteins phosphorylated *in vitro*. **A**: *In vitro* kinase assays were performed on cells transfected with CT10+UR2AV (lanes 1, 3, 5) or UR2AV DNA alone (lanes 2, 4, 6) and immunoprecipitated with anti-*crk* antiserum (lanes 1, 2), preimmune rabbit serum (lanes 3, 4) or TBR serum (lanes 5, 6). Approximate molecular weights of major proteins phosphorylated *in vitro* are indicated on the left in kb. Gel was 8.5% acrylamide; markers, 220, 100, 68, 43, and 27 kd. **B**: phosphoproteins indicated in panel A were analyzed for phosphoamino acid content. Lane 1, 47 kd; lane 2, 52 kd; lane 3, 62 kd; lane 4, 135 kd; lane 5, 140-155 kd. Positions of phosphoamino acid standards are indicated on right: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.


A
1 2 3 4 5 6
140-155
135
62
52
47
B
1 2 3 4 5
S
T
Y

gest that a serine kinase activity may also be associated with p47$^{gag\text{-}crk}$, although serine/threonine kinases are much more ubiquitous in cell lysates than tyrosine kinases so the phosphorylation seen could be due to a nonspecific activity.

I have used a wide variety of antisera to assess the specificity of the apparent *crk*-associated tyrosine kinase activity. An antiserum raised against detergent-disrupted helper virus particles (anti-virion serum; Feldman et al., 1980) and a monoclonal antibody to the MA portion of *gag* (Potts et al., 1987) both gave phosphorylation of the 135 kd band in *in vitro* kinase assays of CT10-infected cell immunoprecipitates (not shown). These antisera are directed against the *gag* portion of p47$^{gag\text{-}crk}$, entirely distinct from the c-*crk*-derived region used to generate the anti-*crk* serum, strongly suggesting that the kinase activity is specific and not an antibody artifact. Sera that recognize p60^{src} (MAb 327, Lipsich et al., 1983; MAb GD11, Parsons et al., 1986; anti-bacterially expressed p60^{src}, Gilmer and Erikson, 1983), c-*abl* proteins (Wang, 1985), p62$^{c\text{-}yes}$ (Sudol and Hanafusa, 1986), the EGF receptor (Decker, 1984), v-*ros* (Jong and Wang, 1987), and a conserved tyrosine kinase catalytic domain (MAb 443, Lipsich et al., 1983) did not give any specific, increased phosphorylation *in vitro* when CT10-infected lysates were compared to controls (not shown), again arguing that the kinase activity observed is due to a specific interaction with p47$^{gag\text{-}crk}$. Interestingly, one monoclonal antibody that recognizes viral MA protein and efficiently immunoprecipitates p47$^{gag\text{-}crk}$ (Lee et al., 1985) did not give any specific phosphorylation *in vitro*. It is difficult to explain this result, although the antiserum may contain an inhibitor of kinase activity or be sterically hindered from binding to the putative kinase-associated fraction of p47$^{gag\text{-}crk}$.

One curious aspect of the 135 kd protein phosphorylation is that all TBR sera tested that recognize p47$^{gag\text{-}crk}$ (3 different rabbits) gave strong phos-

phorylation of a protein that migrates in the 135-155 kd range, while the anti-virion serum, anti-MA monoclonal, and anti-*crk* serum gave strong phosphorylation only in the 135 kd region and much weaker 140-155 kd phosphorylation (compare lanes 1 and 5 in figure 13A). Partial proteolytic mapping of *in vitro* phosphorylated proteins from TBR immunoprecipitates demonstrated that the 140-155 kd material has a very similar protease map to that of the 135 kd protein (not shown); the more slowly migrating material may represent a more highly phosphorylated form of the 135 kd band. One of the differences between TBR sera and the other antisera that recognize $p47^{gag\text{-}crk}$ is that the TBR serum weakly recognizes $p60^{c\text{-}src}$ or c-*src*-related kinases; a band of approximately 60 kd, presumably *src*-related, was phosphorylated in TBR immunoprecipitates of both normal and CT10-infected CEF (see figure 13A, lanes 5 and 6). It is possible that the *src*-related tyrosine kinase recognized by TBR serum contributes to the 140-155 kd *in vitro* phosphorylation. This hypothesis is supported by a preclearance experiment, in which I precleared CT10 lysates of all c-*src* kinase activity by sequential immunoprecipitations with a *src*-specific monoclonal antibody; subsequent immunoprecipitation with TBR serum gave less 140-155 kd phosphorylation than parallel TBR immunoprecipitation with uncleared lysate (not shown).

The tyrosine kinase activity associated with $p47^{gag\text{-}crk}$ is very weak compared to that of other protein-tyrosine kinases. For example, the phosphate transferred to the 135-155 kd substrate in TBR immunoprecipitates is much less than the autophosphorylation of endogenous $p60^{c\text{-}src}$ immunoprecipitated and assayed from the same cell lysates, clearly demonstrating that only a small fraction of the total tyrosine kinase activity in CT10-infected cells can be immunoprecipitated in association with $p47^{gag\text{-}crk}$ (not shown). In addition, the extent of kinase activity varies significantly from experiment to experiment, at

times being barely detectable; the magnitude of the kinase activity appears to correlate with the levels of $p47^{gag\text{-}crk}$ in infected cells, but I have not addressed this rigorously. As shown earlier in figure 6, more kinase activity was recovered if ionic detergents were present in extraction buffers; further experiments showed that optimal recovery was obtained when 300 mM NaCl instead of the normal 150 mM was present (not shown). These data suggest that the kinase activity is strongly associated with insoluble cellular components, perhaps the cytoskeletal matrix (see also section 5-7). As with many tyrosine kinases, more activity was seen if Mn^{++} was present in the reaction buffer than if Mg^{++} was the divalent cation (not shown). The presence of 1mM Ca^{++} in extraction or kinase buffers had no effect on activity (not shown). The kinase activity was drastically reduced if cell lysates were incubated at 37^{o} for 10 min before immunoprecipitation (not shown), demonstrating that the activity is cell-derived and not a property of the antiserum or due to chemical reaction.

The identity of the tyrosine kinase associated with the *crk* gene product is unclear. I attempted to label TBR immunoprecipitates with ^{14}C-labeled FSBA, an ATP analog that covalently binds to ATP-binding proteins (Pal et al., 1975; Kamps et al., 1984), but no specific labeling was observed (not shown). Presumably the level of kinase in the immunoprecipitates is too low to detect by this method. Most tyrosine kinases autophosphorylate in *in vitro* kinase reactions, so the most likely candidate for the actual kinase is the 135 kd protein. It is unlikely that this protein is a glycosylated growth-factor receptor, since the kinase activity did not bind to wheat germ or lentil lectins (not shown). The only nonreceptor tyrosine kinases with a comparable molecular weight so far identified are the c-*abl* gene products (Ben-Neriah et al., 1986; Jackson and Baltimore, 1989); I have proteolytically mapped the 135-155 kd band phosphorylated in TBR immunoprecipitates of CT10-infected cells against the approximately 150

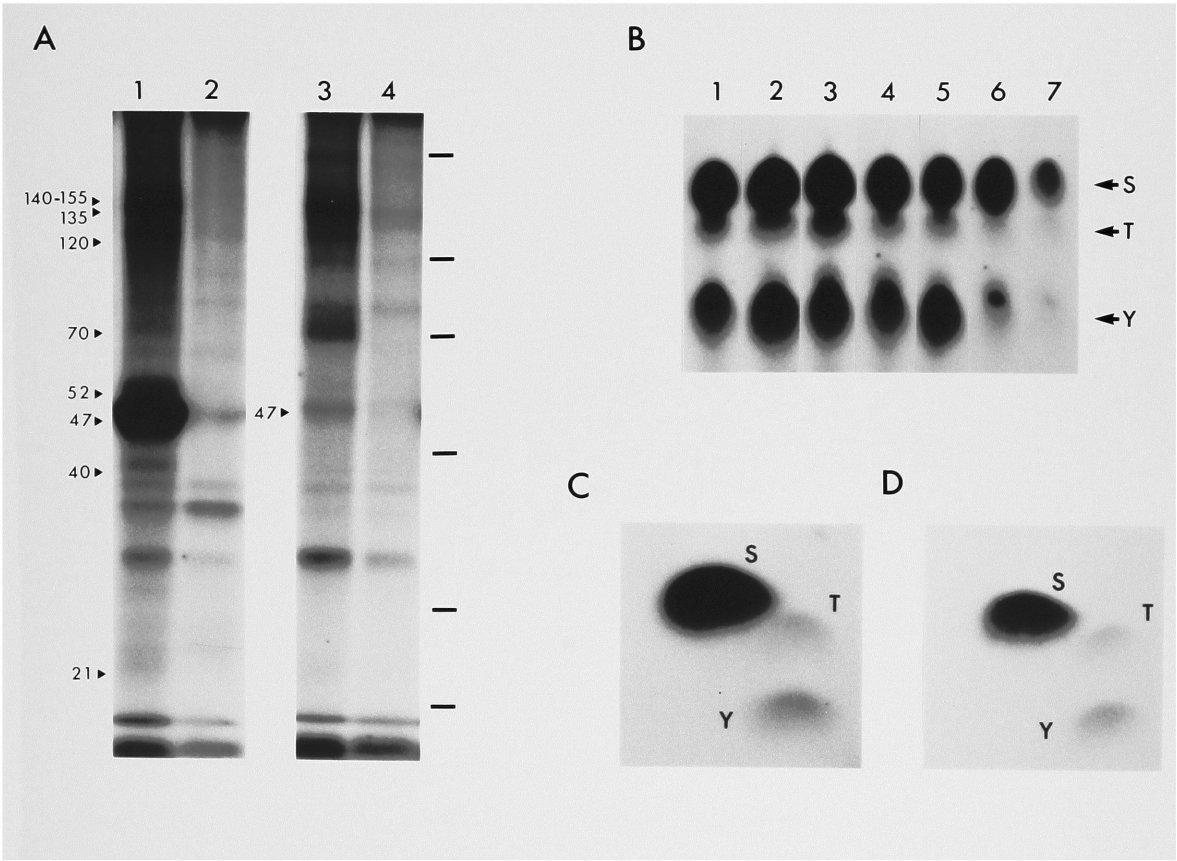
kd protein phosphorylated in CEF lysates immunoprecipitated with an *abl*-specific antiserum (Wang, 1985) and the patterns were quite different (not shown). Therefore, if the 135 kd protein is in fact the tyrosine kinase, it is most likely a presently uncharacterized protein. Of course, the kinase could be the 52 or 62 kd protein phosphorylated *in vitro*, or one of the other phosphoproteins identified in the next section.

5-5 crk-*associated phosphoproteins*

The predicted structure of the *crk* gene product and its biological activity suggest that p47$^{gag\text{-}crk}$ is associated with other cellular proteins in infected cells. The finding of an associated kinase activity (section 5-4) is consistent with this hypothesis. Since phosphorylation is implicated in many signal transduction phenomena, and since the SH2 and SH3 domain-containing tyrosine kinases and PLC-148 may be regulated by phosphorylation (section 4-5), and furthermore since p47$^{gag\text{-}crk}$ causes a demonstrable increase in the tyrosine phosphorylation of several cellular proteins (section 5-2), I examined whether any phosphoproteins coprecipitate with p47$^{gag\text{-}crk}$ in CT10-infected cells. Using ^{32}P as a label enhances detection of proteins present at low levels; while this approach biases my study of putative *crk*-associated proteins, I feel that it is reasonable to assume that potentially interesting associated proteins will be phosphorylated.

I labeled CEF infected with CT10 or helper virus alone with [^{32}P]orthophosphate and immunoprecipitated RIPA lysates with anti-*crk* serum. As seen in figure 14A, the major ^{32}P-labeled band was a 47 kd protein which is p47$^{gag\text{-}crk}$ itself (see below). In addition, several other phosphoproteins were specifically immunoprecipitated from CT10-infected cells but not from helper-infected controls. These proteins have apparent molecular weights of 135-155, 120, 52, 40, and 21 kd. By phosphoamino acid analysis, each of these proteins

Figure 14. Phosphoproteins coimmunoprecipitating with p47$^{gag\text{-}crk}$. **A**: RIPA lysates of $^{32}P_i$-labeled CEF infected with CT10+UR2AV (lanes 1 and 3) or UR2AV alone (lanes 2 and 4) immunoprecipitated with anti-*crk* serum (lanes 1 and 2) or anti-ptyr serum (lanes 3 and 4). Approximate apparent molecular weights in kd of major phosphoproteins are indicated. Gel was 9% acrylamide. Markers 220, 100, 68, 43, 27, and 18 kd. **B**: One-dimensional phosphoamino acid analysis of phosphoproteins immunoprecipitated by anti-*crk* serum. Lane 1, 140-155 kd; lane 2, 135 kd; lane 3, 120 kd; lane 4, 70 kd; lane 5, 52 kd; lane 6, 40 kd; lane 7, 21 kd. **C** and **D**: two-dimensional phosphoamino acid analysis of p47$^{gag\text{-}crk}$ immunoprecipitated by anti-*crk* serum (panel C) or anti-ptyr serum (panel D). For panels B, C, and D, the positions of phosphoamino acid standards are indicated: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

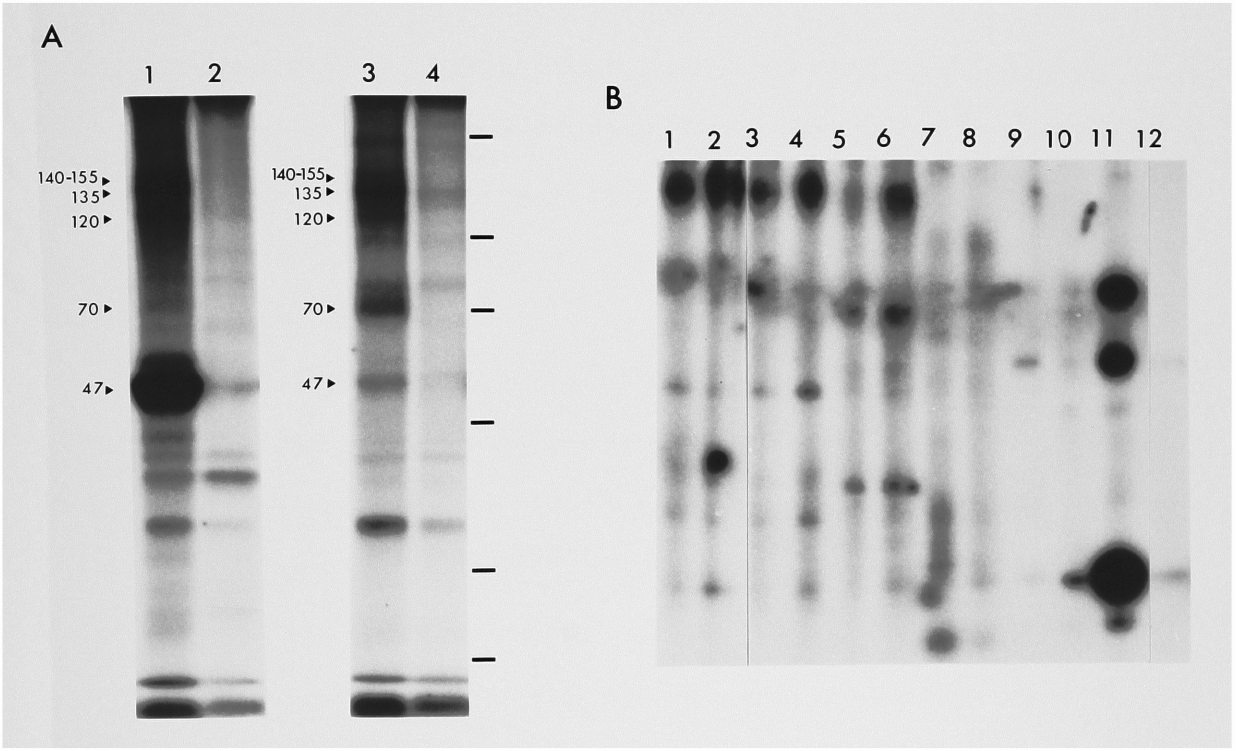

A
1
2
3
4
140-155
135
120
70
52
47
40
21
47
B
1
2
3
4
5
6
7
S
T
Y
C
S
T
Y
D
S
T
Y

contained detectable amounts of phosphotyrosine, in most cases approaching 50% of total phosphoamino acids (figure 14B). Phosphotyrosine is an extremely rare modification compared to serine or threonine phosphorylation. In normal chicken cells, less than 0.1% of phosphate on proteins is on tyrosine; in RSV-infected cells, which have greatly elevated phosphotyrosine levels, this percentage is still less than 1% (Sefton et al., 1980). The finding that each of the coprecipitating proteins contains high levels of phosphotyrosine is therefore highly significant. It is statistically improbable that such a correlation would occur by chance.

The v-*crk* protein product itself was highly phosphorylated. Phosphoamino acid analysis on the 47 kd band showed that the majority of this phosphorylation was on serine; less than 6% of total phosphate was on tyrosine (figure 14C). When ^{32}P-labeled lysates of CT10-infected CEF were immunoprecipitated with an antibody specific for phosphotyrosine (anti-ptyr), a band was also seen at 47 kd. This protein is p47$^{gag\text{-}crk}$ (see below), and phosphoamino acid analysis demonstrated that this fraction of p47$^{gag\text{-}crk}$ had very similar relative levels of phosphotyrosine compared to the whole population immunoprecipitated by anti-*crk* (compare figures 14C and 14D). Since p47$^{gag\text{-}crk}$ has less than 10% of its total phosphate on tyrosine, this implies that there are at least 10 serine phosphorylations per molecule if precipitation by anti-ptyr is mediated only by the tyrosine phosphorylations on p47$^{gag\text{-}crk}$ itself. Since many proteins that coprecipitate with p47$^{gag\text{-}crk}$ contain high levels of phosphotyrosine, it is likely that at least some of the p47$^{gag\text{-}crk}$ that is precipitated by anti-ptyr serum is precipitated by virtue of its association with other phosphotyrosine-containing proteins.

When the pattern of phosphoproteins that are precipitated by anti-*crk* serum from CT10-infected cells is compared with those precipitated by anti-ptyr serum, several striking similarities are apparent (figure 15A). In each case, ^{32}P-labeled bands are seen at 135-155 kd, 120 kd, and 70 kd. These three bands

Figure 15. Partial proteolytic mapping of phosphoproteins immunoprecipitated by anti-*crk* and anti-ptyr sera. **A**: Same gel as in figure 14A. $^{32}P_i$-labeled proteins from CEF infected with CT10+UR2AV (lanes 1 and 3) or UR2AV (lanes 2 and 4) immunoprecipitated with anti-*crk* serum (lanes 1 and 2) or anti-ptyr serum (lanes 3 and 4). Apparent molecular weights in kd of major common phosphoproteins are indicated. **B**: Partial proteolytic mapping of proteins indicated in panel A, and p47$^{gag\text{-}crk}$. Lanes 1, 3, 5, 7, and 10 from anti-ptyr immunoprecipitates (see panel A, lane 3); lanes 2, 4, 6, 8, and 11 from anti-*crk* immunoprecipitates (see panel A, lane 1). Lanes 1 and 2, 140-155 kd; lanes 3 and 4, 135 kd; lanes 5 and 6, 120 kd; lanes 7 and 8, 70 kd; lanes 10 and 11, 47 kd. Lane 9, 47 kd protein phosphorylated in *in vitro* kinase reactions of anti*crk* immunoprecipitate (see figure 13A). Lane 12, [^{35}S]methionine-labeled p47$^{gag\text{-}crk}$ immunoprecipitated by anti-*crk* serum (see figure 12).


A
1
2
3
4
140-155
135
120
70
47
140-155
135
120
70
47
B
1 2 3 4 5 6 7 8 9 10 11 12

correspond to the three major bands seen in anti-ptyr immunoblots of CT10-infected cell lysates (figure 11); immunoprecipitation with anti-ptyr, while less sensitive, has the advantage that the ptyr-containing proteins can be recovered from the gel and proteolytically mapped. I compared the profiles generated by partial proteolysis with V8 protease (Cleveland et al., 1977) of the 140-155, 135, 120, and 70 kd phosphoproteins immunoprecipitated by anti-ptyr and anti-*crk* sera (figure 15B). In each case the proteolytic maps were essentially identical, demonstrating that the three major phosphotyrosine-containing proteins of CT10-infected cells can be coimmunoprecipitated with $p47^{gag\text{-}crk}$. The data in figure 15B also demonstrate that in both anti-*crk* and anti-ptyr immunoprecipitates, the 135 kd phosphoprotein and the 140-155 kd material above it are closely related if not identical.

The similarity seen between the anti-*crk* and anti-ptyr immunoprecipitates implies that the v-*crk* transforming protein is physically associated with those proteins in infected cells that have aberrantly high levels of phosphotyrosine. This suggests that $p47^{gag\text{-}crk}$ is in some way directly involved in the tyrosine phosphorylation of these proteins. Of course, it is impossible to infer from this data that *crk*-associated proteins are phosphorylated by virtue of their association with $p47^{gag\text{-}crk}$ (the reverse may be true, that association with the *crk* protein may depend on tyrosine phosphorylation), but this result focuses attention on a class of protein that is highly phosphorylated on tyrosine and may associate with $p47^{gag\text{-}crk}$. These proteins are candidates for the direct cellular mediators of the altered growth properties of cells transformed by CT10.

One further inference can be drawn from these data. Some of the phosphotyrosine-containing, *crk*-associated proteins, for example the 52 kd and 40 kd species, are not seen in anti-ptyr immunoblots or anti-ptyr immunoprecipitates, suggesting that they are present at much lower levels than the 135-155,

120, and 70 kd species. Their relative enrichment in the anti-*crk* immunoprecipitates suggests that they may be more specifically associated with p47$^{gag\text{-}crk}$; for example, a greater proportion of the total 52 kd protein in infected cells may be associated with p47$^{gag\text{-}crk}$ than the proportion of 135-155 kd protein. By using anti-ptyr serum to immunoblot anti-*crk* immunoprecipitates in parallel with whole cell lysates from the same cells, I estimate that less than 5% of the total 135-155, 120, and 70 kd proteins were immunoprecipitated with anti-*crk* (not shown). It is possible that the proportion of the 52 kd protein, for example, may be much higher. It is important to consider this in any model for the biological activity of the v-*crk* gene that involves sequestration of regulatory factors.

Because the anti-*crk* antibody might recognize conserved SH2 or SH3 epitopes on p47$^{gag\text{-}crk}$, and since SH2 and SH3 domains are found on phosphotyrosine-containing proteins such as the nonreceptor tyrosine kinases, it is possible that the anti-*crk* serum is directly recognizing the phosphoproteins rather than immunoprecipitating them by virtue of their association with p47$^{gag\text{-}crk}$. To address this question, I immunoprecipitated ^{32}P-labeled CT10-infected lysates with other antibodies that recognize p47$^{gag\text{-}crk}$. As shown in figure 16, TBR serum and a monoclonal antibody that recognizes the viral MA protein also immunoprecipitated the 135-155 kd and 120 kd proteins as well as p47$^{gag\text{-}crk}$ (the other putative associated proteins are obscured by helper virus-encoded phosphoproteins). Since the anti-*gag* monoclonal must recognize an entirely different epitope than the *crk*-specific serum, these results strongly suggest that the immunoprecipitation of these proteins is not the result of direct antibody recognition. In addition, antibodies that do not recognize p47$^{gag\text{-}crk}$, including preimmune sera, did not immunoprecipitate the *crk*-associated phosphoproteins (not shown), demonstrating that these phosphoproteins do not bind nonspecifically to protein A-sepharose or immunoglobulins.

Figure 16. Immunoprecipitation of phosphoproteins from CT10-infected CEF. Protein from CEF infected with CT10+UR2AV (C) or UR2AV helper virus (H) labeled *in vivo* with [^{32}P]-orthophosphate and immunoprecipitated with antibodies as marked. Anti-p19: monoclonal antibody to retroviral MA protein. Anti-ptyr+TBR: immunoprecipitation with anti-ptyr, elution from immune complexes with phenylphosphate, and reprecipitation with TBR serum. Arrows indicate 135-155 kd phosphoprotein and p47$^{gag\text{-}crk}$. Gel was washed with alkali to enhance detection of phosphotyrosine-containing proteins. Markers: 220, 100, 68, 43, and 27 kd.

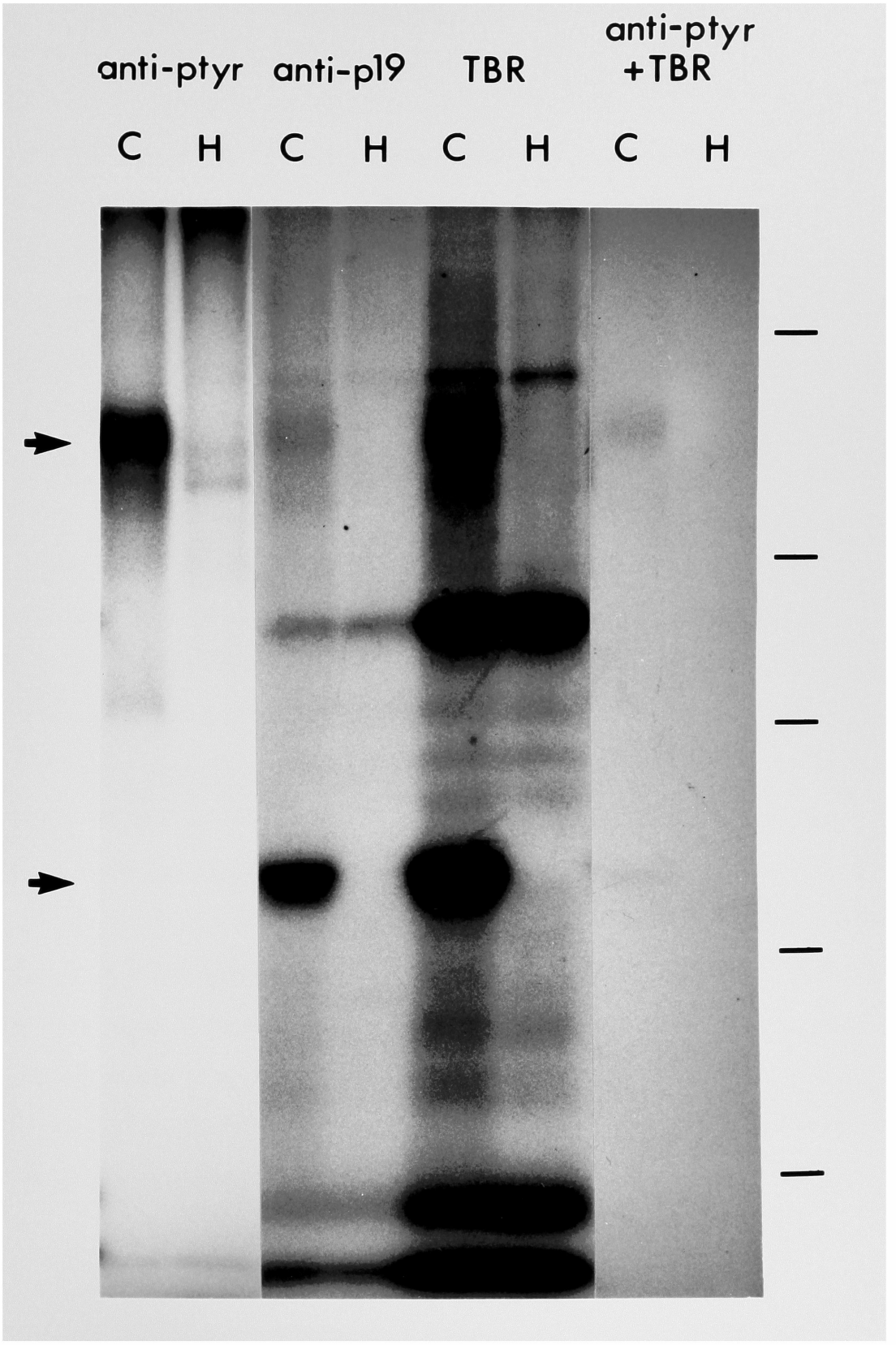

anti-ptyr
anti-p19
TBR
anti-ptyr
+TBR
C
H
C
H
C
H
C
H

Figure 16 also demonstrates that when ^{32}P-labeled CT10-infected cell lysates were immunoprecipitated with anti-ptyr serum, eluted from the immune complexes with phenyl phosphate, then reprecipitated with TBR serum, p47$^{gag\text{-}crk}$ and the 135-155 kd phosphoprotein were recovered in roughly equal amounts. While such an experiment is in no way conclusive, this result is consistent with an association between these proteins, and suggests that it may be possible to purify the complexes. The low recovery of ^{32}P-labeled 135-155 kd protein relative to immunoprecipitation with TBR serum alone may reflect non-quantitative immunoprecipitation by the anti-ptyr serum and/or incomplete elution from the anti-ptyr antibody prior to reprecipitation with TBR.

Partial proteolytic mapping of the ^{32}P-labeled 47 kd bands phosphorylated in *in vitro* kinase assays, immunoprecipitated by anti-*crk*, or immunoprecipitated by anti-ptyr sera demonstrated that they were identical to authentic ^{35}S-labeled p47$^{gag\text{-}crk}$ immunoprecipitated by anti-*crk* serum (figure 15B). It is interesting that p47$^{gag\text{-}crk}$ can be phosphorylated *in vitro* on tyrosine, albeit at low levels, suggesting that it might be a substrate for the *crk*-associated tyrosine kinase *in vivo* as well. I also mapped the 135-155 kd proteins phosphorylated in *in vitro* kinase reactions or immunoprecipitated from ^{32}P-labeled cells by TBR serum; the maps were similar but not identical (not shown). It should be remembered that the sites of phosphorylation *in vitro* may differ from those used *in vivo*, leading to some differences in the proteolytic maps. Since the 135-155 kd protein is highly phosphorylated on tyrosine *in vivo* and is demonstrably present in anti-*crk* or TBR immunoprecipitates, it is likely that this is the same protein as the one that is phosphorylated in *in vitro* kinase reactions.

One strange aspect of the *crk*-associated proteins is that the 135-155 kd and the 120 kd proteins was still immunoprecipitated by TBR serum or anti-MA monoclonal when lysates were boiled in 1% SDS, 10 mM TRIS, then diluted

to RIPA buffer composition on ice before addition of antiserum (not shown). I have not yet repeated this experiment with the anti-*crk* serum, so I can not comment on the 70, 52, 40, or 21 kd associated proteins. This result might suggest that the 135-155 and 120 kd proteins are precipitated by virtue of direct antibody recognition or nonspecific binding; however, as I mentioned previously, control antibodies do not precipitate these proteins (even after boiling), and the epitopes recognized by anti-MA monoclonal and anti-*crk* sera are entirely distinct. In addition, it seems extremely unlikely that only those proteins highly phosphorylated on tyrosine (as opposed to those phosphorylated predominantly on serine and/or threonine, which should be much more abundant) would be nonspecifically precipitated or directly recognized by all sera that recognize $p47^{gag\text{-}crk}$. The coprecipitation of the 135-155 kd and the 120 kd phosphoproteins is unaffected by the presence of 1 mM DTT or 0.5% β-mercaptoethanol in the lysis buffer (not shown), so it is also unlikely that these proteins are precipitated by virtue of spurious disulfide linkages formed during lysis.

A possible explanation for this may be that the SH2 and/or SH3 domains, which I speculate are the regions to which the *crk*-associated proteins bind, are capable of renaturing into their proper conformations after dilution to RIPA buffer conditions on ice. There are precedents for regeneration of enzyme activity after SDS-PAGE of proteins (Spanos and Hubscher, 1982; see Itoh et al., 1987 for an example), and presumably it would be easier to refold a small (less than 50 amino acids) binding domain than an entire catalytically active enzyme. There are also numerous precedents for generating specific associations between proteins *in vitro*, either by adding together purified proteins (see Ludlow et al., 1989) or cotranslating *in vitro* (see Schuermann et al., 1989). I feel that the weight of circumstantial evidence argues that the presumed association between $p47^{gag\text{-}crk}$ and the phosphotyrosine-containing proteins that coprecipitate with it

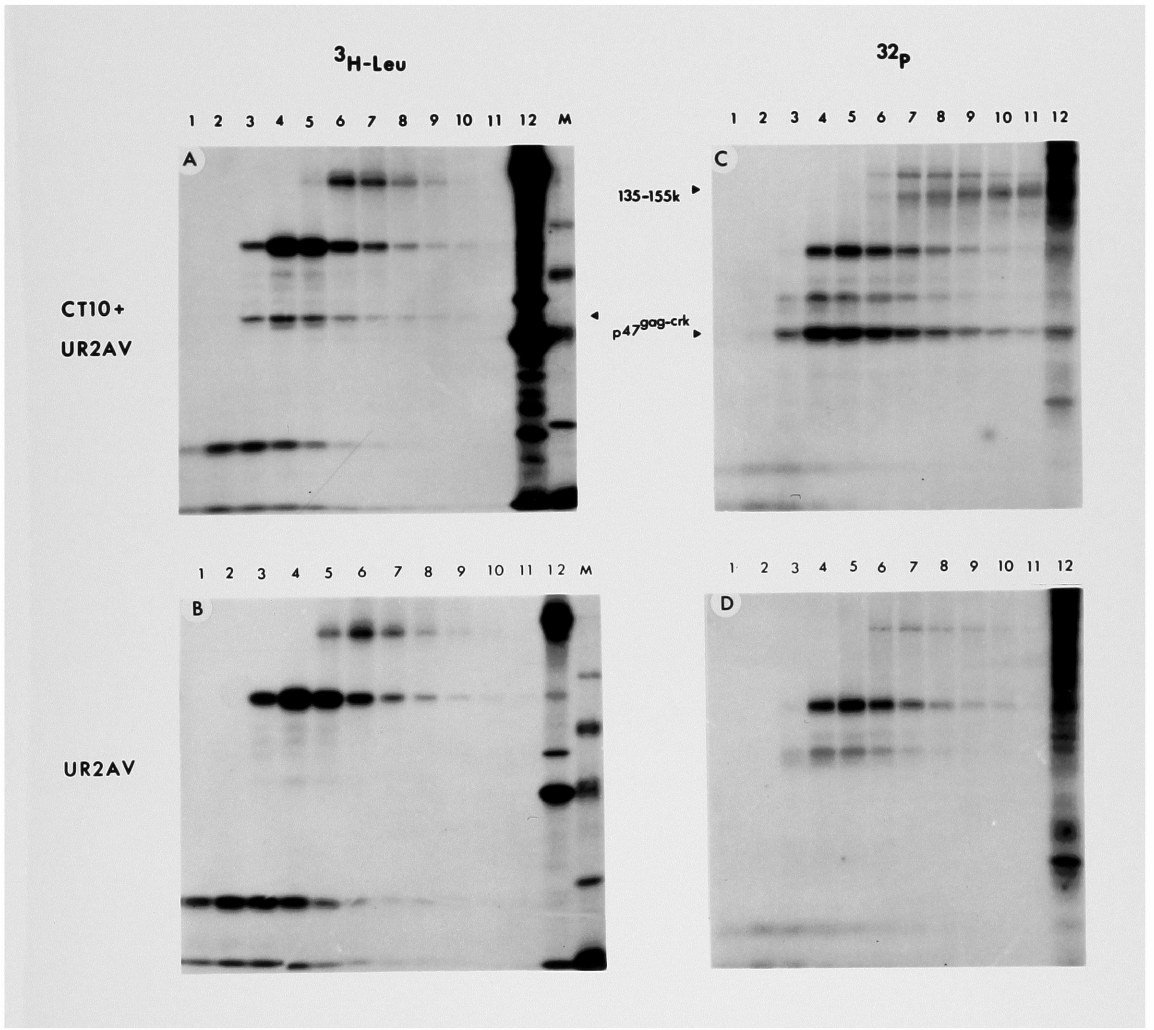
is significant, but that this apparent association should be viewed with caution. Future work, especially the molecular cloning of these putative *crk*-associated proteins, will be required to settle this issue.

5-6 *Glycerol gradient sedimentation*

If the phosphoproteins identified above are actually associated with p47$^{gag\text{-}crk}$ *in vivo*, they would be expected to sediment in glycerol gradients more rapidly than monomer, uncomplexed proteins. To test this, I subjected RIPA lysates of [^{3}H]leucine labeled, [^{32}P]orthophosphate-labeled, or unlabeled CT10-infected and helper-infected CEF to sedimentation on 10-30% glycerol gradients. In this experiment I used TBR serum for immunoprecipitation since the viral structural protein precursors pr180 and pr76 provide convenient internal molecular weight controls. Gradients immunoprecipitated with anti-*crk* serum gave comparable results, though the lack of molecular weight standards makes interpretation more difficult. One caveat of this type of experiment is that the lysates are in SDS-containing RIPA buffer for at least 22h, and some types of interactions or enzyme activities may be modified by prolonged exposure to detergent. Unfractionated lysates kept at 4^{o} in RIPA buffer for this period of time generally showed less specific kinase activity *in vitro*, less specific ^{32}P-labeled associated proteins, and higher nonspecific background than lysates treated under more typical conditions (not shown).

As seen in figure 17A, the majority of [^{3}H]leucine-labeled p47$^{gag\text{-}crk}$ sedimented on glycerol gradients as expected for its monomer molecular weight, though some of the *crk* protein trailed into the heaviest fractions of the gradient. In *in vivo* ^{32}P-labeled lysates (figure 17C), a similar pattern was seen for p47$^{gag\text{-}crk}$. The 135-155 kd *crk*-associated phosphoprotein first appeared in fraction 5, corresponding to a monomer molecular weight of approximately 180 kd,

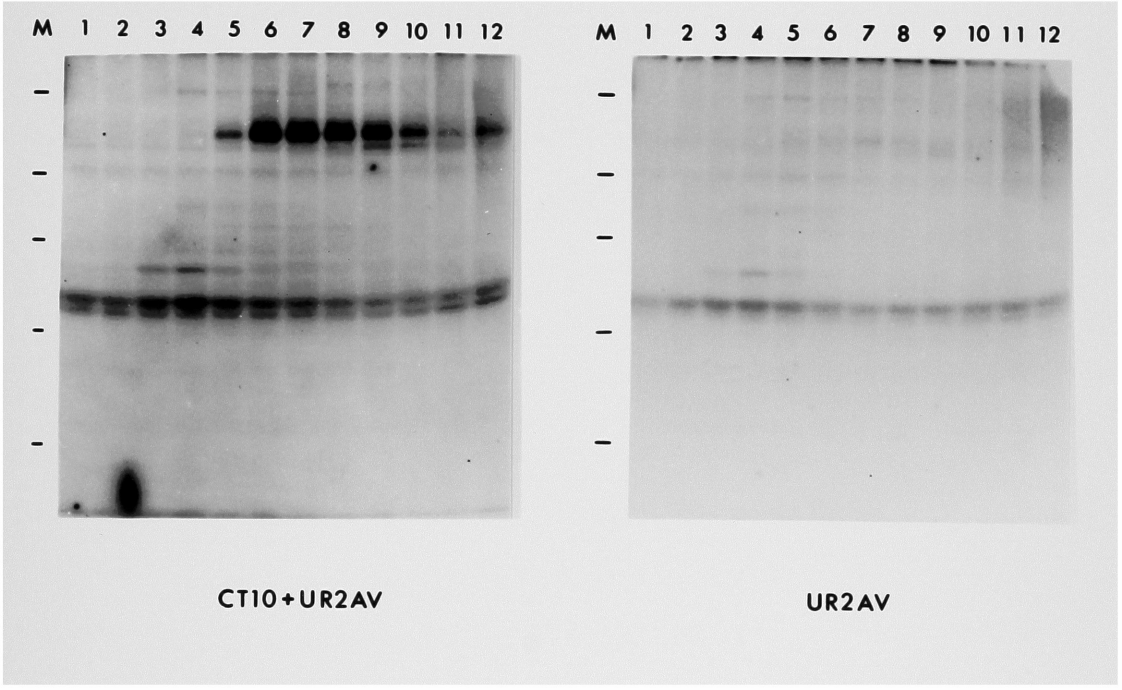
Figure 17. Glycerol gradient sedimentation analysis of CT10-infected or helper virus-infected CEF proteins. CT10-infected (panels A and C) or helper-infected (panels B and D) cell lysates labeled *in vivo* with [^{3}H]-leucine (panels A and B) or [^{32}P]-orthophosphate (panels C and D) separated on 10-30% glycerol gradients. Individual fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right. p47$^{gag\text{-}crk}$ and the 135-155 kd phosphoprotein are indicated. ^{32}P-labeled gels were washed with alkali. Markers: 220, 100, 68, 43, and 27 kd.


^{3}H-Leu
^{32}P
1 2 3 4 5 6 7 8 9 10 11 12 M
A
B
C
D
CT10+
UR2AV
UR2AV
135-155k
p47gag-crk

based on the cosedimentation of the viral precursor protein pr180. This is consistent with the association of the 135 kd phosphoprotein with a small proportion of p47$^{gag\text{-}crk}$, although the resolution of this type of analysis is not very sharp. The 135-155 kd phosphoprotein was evident in all of the fractions of the gradient heavier than fraction 4, suggesting a wide range of sizes for complexes containing this protein. Curiously, the apparent molecular weight of the 135 kd protein in SDS-PAGE increases in the heavier fractions of the gradient; this suggests that the more slowly migrating form of the 135-155 kd protein is present in heavier complexes, perhaps containing more components or oligomerized forms. The 120 kd phosphoprotein is barely visible in this experiment, but can be seen in the three heaviest fractions of the gradient. In another experiment using anti-*crk* serum, it was clear that this protein was present only in very heavy (fractions 10-12) complexes.

When unlabeled lysates were separated on similar gradients and individual fractions were subjected to *in vitro* kinase assay, activity (assessed by phosphorylation of the 135-155 kd protein) first appeared in fraction 5 and peaked in fractions 6-7 (figure 18A). In contrast to *in vivo* ^{32}P-labeled 135-155 kd protein, however, *in vitro* phosphorylation of this protein decreased and was absent from the heaviest fractions. Thus *in vitro* phosphorylation of this protein does not directly correspond to the presence of *in vivo* ^{32}P-labeled material. There are several possible explanations for this data. If the actual tyrosine kinase in the *crk* immunoprecipitates is the 135-155 kd protein itself, then perhaps the higher molecular weight form may not be kinase-active in autophosphorylation reactions (it may, for example, be already highly phosphorylated or complexed with inhibitors). Another possibility is that the actual kinase is another protein present in immunoprecipitates that phosphorylates the 135-155 kd protein in *trans*; this other protein may not be present in the heaviest fractions of the gra-

Figure 18. *In vitro* kinase assay of fractions separated by glycerol gradient sedimentation. CT10-infected or helper-infected cell lysates were fractionated as in figure 17, immunoprecipitated with TBR serum, and subjected to *in vitro* kinase assay. Sedimentation is from left to right. Gels were washed with alkali. Markers: 220, 100, 68, 43, and 27 kd.


M 1 2 3 4 5 6 7 8 9 10 11 12
M 1 2 3 4 5 6 7 8 9 10 11 12
CT10+UR2AV
UR2AV

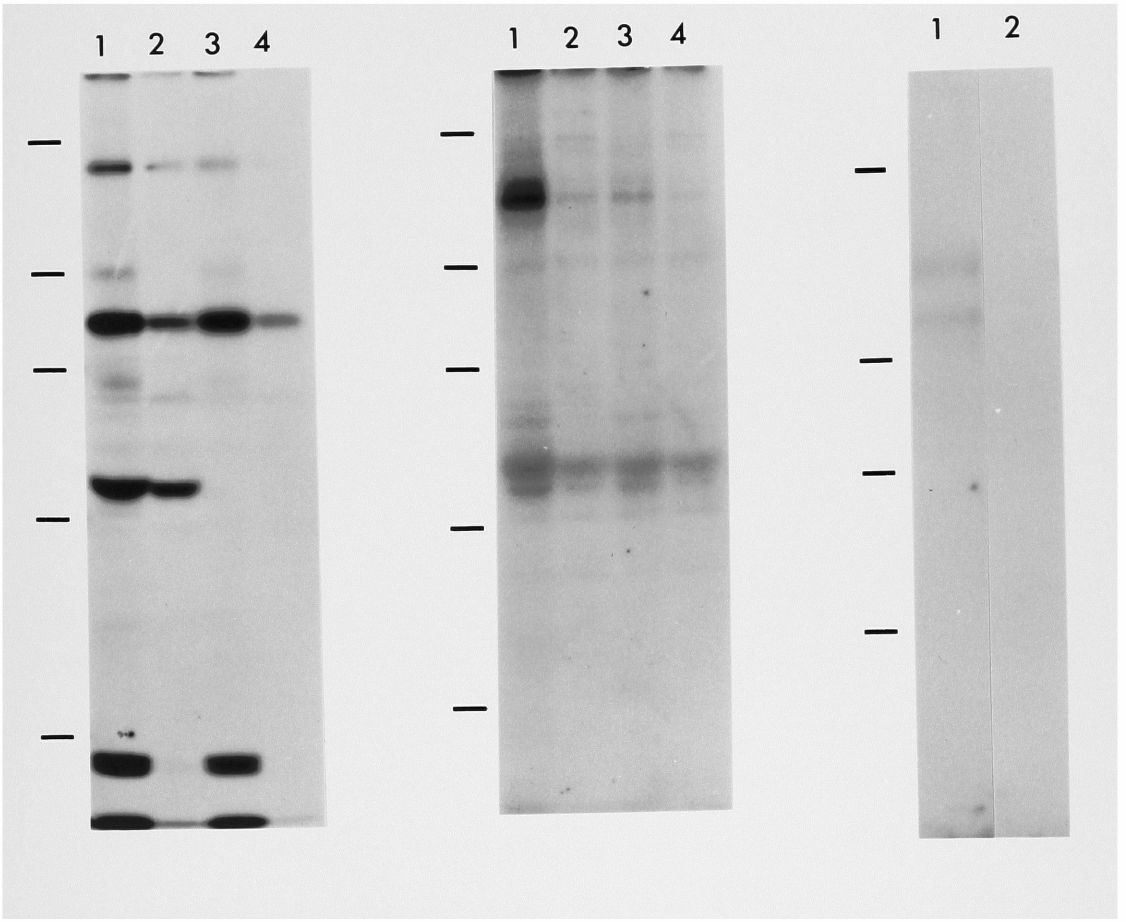
dient. I have performed a mixing experiment in which I combined fractions 1-4 of the gradient with fractions 9-12; no increase in kinase activity was observed compared to these fractions alone (not shown), implying that the relatively low kinase activity seen in the heaviest fractions is not due to the lack of a low-molecular weight kinase (for example, uncomplexed $p60^{c\text{-}src}$).

5-7 *Subcellular localization of* crk*-associated kinase activity and phosphoproteins*

Experiments with mutants of $p60^{v\text{-}src}$ have led to the proposal that essential substrates for the tyrosine kinase activity of this oncogene product are localized on the plasma membrane and associated with nonionic detergent-insoluble cellular structures. $p60^{src}$ and presumably other members of the *src* family of kinases contain a short amino-terminal sequence that directs the addition of the long-chain fatty acid myristic acid to glycine at position 2 (see Cooper, 1989 for review). This amino-terminal domain was shown to be necessary and in most cases sufficient for membrane localization (Cross et al., 1984; Pellman et al., 1985a). *Src* mutants with lesions in this domain, while retaining high kinase activity, are unable to induce transformation (Cross et al., 1984; Kamps et al., 1985; Buss et al., 1986). Presumably these mutants are unable to phosphorylate plasma membrane substrates critical to the transformed phenotype. Furthermore, it was found that $p60^{v\text{-}src}$ and deletion mutants that retain transforming activity are tightly associated with the triton X-100-insoluble cytoskeletal matrix, while $p60^{c\text{-}src}$ and nontransforming v-*src* mutants are relatively soluble in nonionic detergents (Hamaguchi and Hanafusa, 1987; Loeb et al., 1987), suggesting that substrates critical for transformation may be localized on the cytoskeletal matrix.

I have examined the localization of $p47^{gag\text{-}crk}$, its associated tyrosine kinase activity, and the phosphotyrosine-containing proteins of CT10-infected

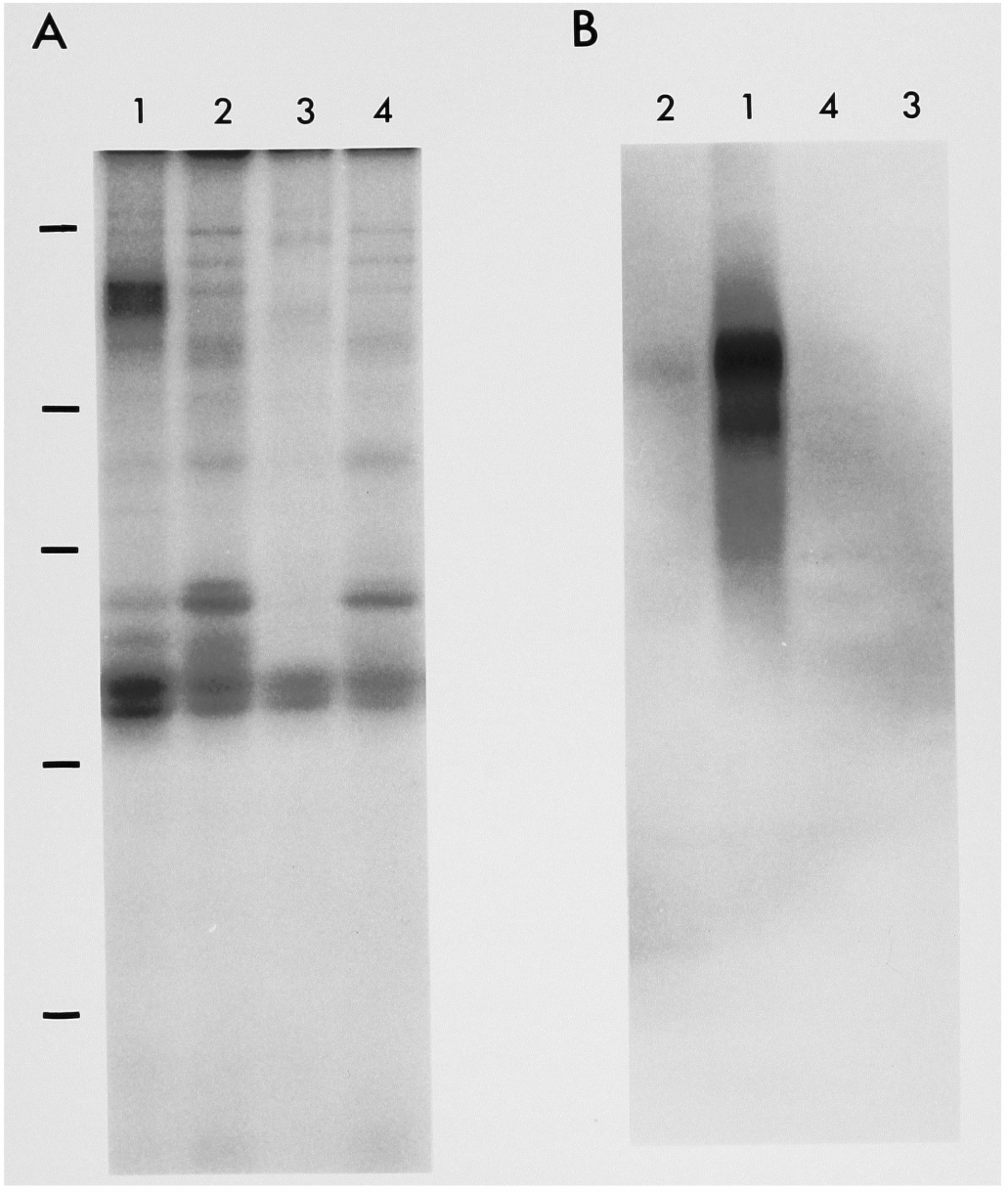
Figure 19. Cytosolic and membrane fractionation of CT10-infected CEF. Left panel: [^{3}H]leucine-labeled CEF immunoprecipitated with TBR serum. Middle panel: *in vitro* kinase assay of unlabeled TBR immunoprecipitates. Right panel: anti-ptyr immunoblot. Lanes 1 and 2, CEF infected with CT10+UR2AV; lanes 3 and 4, UR2AV-infected CEF. Lanes 1 and 3, P100 crude membrane fraction; lanes 2 and 4, S100 cytosolic fraction. Gels: left and middle panels, 8.5% and right panel, 7.5% acrylamide; markers, 220, 100, 68, 43, and 27 kd. ^{32}P-labeled gel was washed with alkali.


1 2 3 4
1 2 3 4
1 2

cells. As shown in figure 19A, upon hypotonic lysis more than half of the [^{3}H]leucine-labeled p47$^{gag\text{-}crk}$ was found in the crude membrane fraction ("P100"), while a significant percentage was found in the soluble, cytoplasmic fraction ("S100"). Although I have not examined this in greater detail, this is consistent with the loose, salt-dependent association with membranes seen with other *gag*-fusion oncogene products (Feldman et al., 1983). When TBR immunoprecipitates of fractionated cell lysates were subjected to *in vitro* kinase assay, all of the *crk*-associated tyrosine kinase activity, assessed by 135-155 kd protein phosphorylation, was found in the crude membrane pellet (figure 19B). Consistent with this *in vitro* result, the 135-155 kd and 120 kd phosphotyrosine-containing proteins (which I have shown in section 5-5 to be coprecipitated with p47$^{gag\text{-}crk}$) were localized by anti-ptyr immunoblotting to the membrane pellet (figure 19C). The 70 kd phosphotyrosine-containing protein is not visible in this light exposure. These results are consistent with the *src* mutant data, and suggest that, as is the case for p60$^{v\text{-}src}$, phosphorylation on tyrosine of membrane-associated proteins may be involved in transformation by the v-*crk* oncogene product.

In collaboration with M. Hamaguchi, I have also examined whether the *crk*-associated tyrosine kinase activity and phosphotyrosine-containing proteins are soluble in nonionic detergent. As shown in figure 20A, all of the TBR-precipitable kinase activity capable of phosphorylating the 135-155 kd protein was recovered in the detergent-insoluble matrix fraction. As an internal control, the c-*src*-related kinase activity (the 60 kd band immunoprecipitated by TBR serum in both CT10-infected and control lysates) was relatively soluble, consistent with previous results (Hamaguchi and Hanafusa, 1987; Loeb et al., 1987). By anti-ptyr immunoblotting, the 135-155, 120, and 70 kd phosphotyrosine-containing proteins were also localized in the insoluble fraction. In addition, im-

Figure 20. Association of major phosphotyrosine-containing proteins and *crk*-associated kinase activity with detergent-insoluble cytoskeletal matrix. **A**: *in vitro* kinase assays of TBR immunoprecipitates. **B**: anti-ptyr immunoblot. Lanes 1 and 2, CT10+UR2AV-infected CEF; lanes 3 and 4, UR2AV-infected CEF. Lanes 1 and 3, triton X-100-insoluble matrix fraction; lanes 2 and 4, soluble fraction. Gels were 8.5% (panel A) or 7.5% (panel B) acrylamide; markers, 220, 100, 68, 43, and 27 kd.


A
1
2
3
4
B
2
1
4
3

munoprecipitation of *in vivo* ^{32}P-labeled lysates demonstrated that approximately 50% of ^{32}P-labeled p47$^{gag\text{-}crk}$ and all of the 135-155 and 120 kd *crk*-associated proteins were in the cytoskeletal fraction (not shown). Again, these results are consistent with the data from v-*src* mutants which implicate tyrosine phosphorylation of cytoskelatal matrix-associated proteins in transformation.

5-8 *Models for biological activity of p47*$^{gag\text{-}crk}$

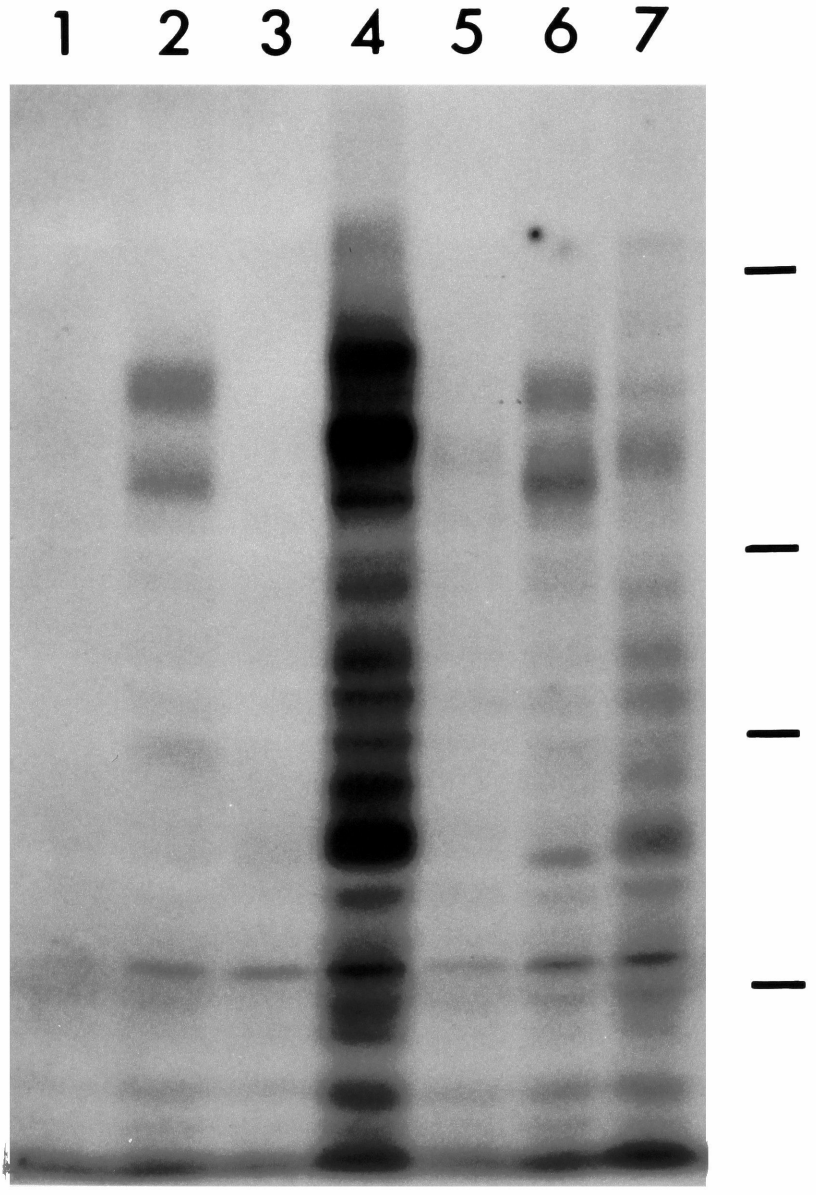
I have shown in this section that p47$^{gag\text{-}crk}$ has some suggestive biochemical properties. High-level expression of this protein causes a drastic elevation in the tyrosine phosphorylation of at least three cellular proteins (or, less likely, actually induces their synthesis); a small proportion of these three proteins can be immunoprecipitated with p47$^{gag\text{-}crk}$, presumably via association. Several other phosphoproteins coprecipitate with p47$^{gag\text{-}crk}$, each of which contains high levels of phosphotyrosine; since these proteins are not evident in anti-ptyr immunoblots, a relatively high proportion may be associated with p47$^{gag\text{-}crk}$. A weak protein-tyrosine kinase activity and possibly a serine/threonine kinase activity are also immunoprecipitated by antibodies that recognize p47$^{gag\text{-}crk}$. The *crk*-associated tyrosine kinase and the three major phosphotyrosine-containing proteins in CT10-infected cells are localized in a subcellular compartment where critical substrates for transformation by tyrosine kinase oncogenes are thought to reside.

Since many growth factor receptors and oncogene products have tyrosine kinase activity, the most striking result of v-*crk* expression is the elevation in cellular phosphotyrosine levels, especially since only a few cellular proteins seem to be affected. If we assume that this is due to the increased activity of an endogenous cellular tyrosine kinase or kinases (there are other possibilities, as discussed below), two mechanisms could explain this. First, p47$^{gag\text{-}crk}$ could ac-

tivate a tyrosine kinase or kinases indirectly by binding to factors that normally inhibit the catalytic activity of the kinase(s) in normal cells; alternatively, p47$^{gag\text{-}crk}$ could bind to and directly activate the kinase(s) itself. There is precedent in the literature for the latter model: polyomavirus middle T antigen (MTag) is thought to transform cells in which it is expressed by binding to and in some cases increasing the specific activity of the *src*-family kinases p60$^{c\text{-}src}$, p62$^{c\text{-}yes}$, and p59$^{c\text{-}fyn}$ (Courtneidge and Smith, 1983; Kornbluth et al., 1987; Kypta et al., 1988; Cheng et al., 1988).

There are several important biochemical differences between transformation by MTag and p47$^{gag\text{-}crk}$, however. First, the tyrosine kinase activity associated with p47$^{gag\text{-}crk}$ is very weak; as I mentioned earlier, it is weaker than the *in vitro* kinase activity of endogenous p60$^{c\text{-}src}$ in uninfected cells. Immunoprecipitates of MTag have much higher kinase activity associated with them, due at least in part to an estimated 100-fold increase in the specific activity of p60$^{c\text{-}src}$ molecules with which it is associated (Bolen et al., 1984; Courtneidge, 1985). While it is possible that I have not used optimal conditions to assess the *crk*-associated kinase activity, I have used the conditions that have been shown to give high activity for other members of the nonreceptor tyrosine kinase family and MTag-associated tyrosine kinases. The second difference is that intracellular phosphotyrosine levels in *crk*-transformed cells are much higher than in cells transformed by MTag (Yonemoto et al, 1987; Kaplan et al., 1987). I have confirmed this by anti-ptyr immunoblotting in collaboration with M. Hamaguchi. Figure 21 compares the phosphotyrosine-containing proteins of CEF expressing MTag, p47$^{gag\text{-}crk}$, of p60$^{v\text{-}src}$. Under normal culture conditions, MTag-expressing CEF had no detectable increase in phosphotyrosine relative to helper-infected controls, while CT10-infected cells showed a marked elevation in phosphotyrosine on the 135-155, 120, and 70 kd proteins. Only after the addition to

Figure 21. Phosphotyrosine-containing proteins of CEF expressing various oncogenes, and the effect of phosphatase inhibitor. Lysates from CEF infected with RSV (lane 4), expressing polyoma MTag (lanes 3 and 7), infected with CT10+UR2AV (lanes 2 and 6) or UR2AV alone (lanes 1 and 5) cultured normally (lanes 1-4) or overnight in the presence of 50 uM sodium orthovanadate (lanes 5-7) were separated on 7.5% gel and immunoblotted with anti-ptyr serum. Markers: 220, 100, 68, and 43 kd.


1
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the culture medium of sodium orthovanadate, a phosphatase inhibitor, could an increase in phosphotyrosine-containing proteins be seen in MTag-expressing cells (Yonemoto et al., 1987). In contrast, CT10-infected cells showed little increase in phosphotyrosine after vanadate treatment; if anything, there was a decrease in the case of the 70 kd protein.

I feel that this result, in combination with the extremely weak *in vitro* activity of the *crk*-associated kinase, argues that the mechanism of transformation by p47$^{gag\text{-}crk}$ is fundamentally different from that of MTag. The variability I have observed in the level of *crk*-associated kinase activity from experiment to experiment also suggests that this associated activity may not be crucial for transformation. Since I have presented evidence (section 4-5) that the SH2 and/or SH3 domains of the nonreceptor tyrosine kinases may be tightly associated with the catalytic domains of these enzymes, a possible explanation for the weak *crk*-associated kinase would be that occasional bimolecular complexes may form between the SH2 and/or SH3 domains of p47$^{gag\text{-}crk}$ and the catalytic domain of a nonreceptor tyrosine kinase. It would be possible to assess this hypothesis by cotranslation *in vitro* of p47$^{gag\text{-}crk}$ and nonreceptor tyrosine kinases.

If p47$^{gag\text{-}crk}$ does not activate kinases directly, then it might do so by interacting with and titrating away potential inhibitors of these kinases. The evidence presented in section 4-5 suggests that the SH2- and SH3-containing amino termini of the nonreceptor tyrosine kinases can inhibit the activity of the associated catalytic domain; this inhibition might be modulated by *trans*-acting cellular factors. If p47$^{gag\text{-}crk}$ is in fact activating cellular kinases by sequestering inhibitors, one would expect such an inhibitor to be present at low levels in the cell and to be quantitatively associated with p47$^{gag\text{-}crk}$. For example, only a small percentage of the 135-155, 120, and 70 kd phosphoproteins are immunoprecipitated along with the v-*crk* gene product (section 5-5); these proteins are therefore

unlikely to be titratable inhibitors. More likely candidates would be those phosphotyrosine-containing proteins visible in anti-*crk* immunoprecipitates, but not evident in anti-ptyr immunoprecipitates or immunoblots. Of course, the lysis and immunoprecipitation conditions that I have used in these experiments might dissociate a putative inhibitor-v-*crk* complex that may exist *in vivo*.

A mechanism used *in vivo* to regulate the activity of $p60^{c\text{-}src}$ and other *src* family kinases suggests an intriguing candidate for a titratable inhibitor. It has been found that phosphorylation of tyrosine 527 of $p60^{c\text{-}src}$ inhibits its kinase activity (reviewed in Hunter, 1987); dephosphorylation of this residue causes an apparent 10-fold increase in *in vitro* specific activity (Cooper and King, 1986), and *in vitro* mutagenesis of this residue to phenylalanine causes the normally nontransforming c-*src* protein to acquire transforming potential (Cartwright et al., 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987; Reynolds et al., 1987). Residue 527 is thought to be phosphorylated in *trans* by an unknown cellular kinase (Jove et al., 1987). If such a "527 kinase" bound to the SH2 and/or SH3 domains of the *src* family kinases, it could be sequestered from its normal substrates by binding to $p47^{gag\text{-}crk}$ (and might be expected to give low-level *in vitro* kinase to anti-*crk* immunoprecipitates). However, experimental evidence does not yet support this hypothesis. First, I was unable to detect an increase in the *in vitro* kinase activity of $p60^{c\text{-}src}$ or $p62^{c\text{-}yes}$ in CT10-infected cells, as would be expected if these proteins were underphosphorylated at the regulatory tyrosine (not shown). Second, I have overexpressed the amino-terminal half of $p60^{c\text{-}src}$, which contains the SH2 and SH3 domains, in CEF and was unable to detect the increase in cellular phosphotyrosine levels expected if a "527 kinase" were being competed away from $p60^{c\text{-}src}$ (not shown). However, more definitive experiments are required to rule out this hypothesis.

A further potential mechanism to explain increased cellular phospho-

tyrosine involves a role for p47$^{gag\text{-}crk}$ in bringing cellular kinases and potential substrates together. The SH2 and SH3 domains of the nonreceptor tyrosine kinases may contain substrate-recognition domains in addition to potential regulatory domains that may be tightly associated with the catalytic domain; the evidence to date is consistent with a regulatory role for SH3 and the involvement of SH2 in binding critical substrates (Section 4-5). One could imagine formation of a trimolecular complex mediated by p47$^{gag\text{-}crk}$, involving a tyrosine kinase (bound to the SH3 domain of the v-*crk* protein) and a kinase substrate (bound to the SH2 domain of p47$^{gag\text{-}crk}$), leading to increased phosphorylation of the substrate. Although this model seems rather farfetched, it is consistent with the data suggesting that p47$^{gag\text{-}crk}$ binds to the three major phosphotyrosine-containing proteins in CT10-infected cells, as well as a tyrosine kinase activity. My results demonstrating low-level serine/threonine kinase activity in *crk* immunoprecipitates are also intriguing in this light. Recent evidence has suggested that tyrosine phosphorylation of serine/threonine kinases may be involved in normal and abnormal signal transduction (Sturgill et al., 1988; Morrison et al, 1988b; Draetta et al., 1988). Evaluation of this model awaits the purification and cloning of various *crk*-associated phosphoproteins.

A potential model that does not invoke the activation of tyrosine kinases involves the inhibition by p47$^{gag\text{-}crk}$ of a protein-tyrosine phosphatase. While there is as yet no direct evidence that tyrosine phosphatases might be specific for certain substrates and not merely constitutive activities, this is not an unreasonable assumption. Recently, a protein-tyrosine phosphatase was cloned that has amino acid sequence homology to the intracellular domain of the common leukocyte antigen CD45 (Charbonneau et al., 1988). Since the CD45 protein has a structure reminiscent of a transmembrane receptor, presumably the putative phosphatase activity of the receptor might be regulated by ligand binding. If

p47$^{gag\text{-}crk}$ were to bind to and inhibit a specific phosphatase activity, an accumulation of phosphotyrosine on that phosphatase's normal substrates would be expected. One piece of data that is consistent with such a model is the fact that adding the phosphatase inhibitor sodium orthovanadate to the medium of CT10-infected cells does not lead to a further increase in phosphotyrosine of the 135-155, 120, and 70 kd proteins (figure 21). Of course, the fact that a detectable tyrosine kinase activity is associated with p47$^{gag\text{-}crk}$ is difficult to explain in this model.

While the increased phosphotyrosine seen in CT10-infected cells is striking, there is as yet no evidence that it is causally involved in transformation by v-*crk*. For example, it could be a result of feedback loops attempting to shut down deregulated signaling pathways, or of an autocrine loop involving secretion of growth factors and subsequent stimulation of their receptors. As I have shown in figure 11, increased phosphotyrosine is not a general property of transformed CEF, but this does not prove that it is directly involved in transformation by the v-*crk* gene. It will be necessary to study v-*crk* mutants and make correlations between tyrosine phosphorylation and transforming activity in order to assess the relevance of the increased phosphotyrosine observed (see section 6).

The homology between *crk*, PI-PLC, and GAP is intriguing since PLC and GAP are thought to play critical roles in signal transduction. As mentioned in section 4-4, I have no evidence that phosphoinositide metabolism is altered in CT10-infected cells, but this has not been exhaustively examined. It may be significant that Y. Fukui has found that p47$^{gag\text{-}crk}$ immunoprecipitates are associated with a type I phosphotidylinositol (PI) kinase (Fukui et al., 1989). A similar PI kinase activity was identified in association with MTag-p60$^{c\text{-}src}$ complex (Kaplan et al., 1987; Courtneidge and Heber, 1987), the PDGF receptor (Kaplan et al., 1987), and the products of tyrosine kinase oncogenes (Fukui et al. 1989),

whereas little PI kinase was found in association with the products of non-transforming *src* mutants (Fukui and Hanafusa, 1989) or $p60^{c\text{-}src}$ (Piwnica-Worms et al., 1986; Fukui and Hanafusa, 1989). The relevance of this to transformation by CT10 is unclear; however, no significant increase in polyphosphoinositides has been seen in CT10-infected cells (Y. Fukui, unpublished).

The homology of the *crk* oncogene with GAP in the SH2 domain is interesting in light of the proposed role of GAP as a component of the effector of *ras* action. There is some evidence, however, that another protein might be associated with GAP in active effector complexes (McCormick, 1989); perhaps the actual enzyme activity of the effector resides in this putative second protein. If the interaction between GAP and this proposed second effector component is mediated by the SH2 domain, $p47^{gag\text{-}crk}$ might have the ability to interact with and perhaps modulate the activity of such a protein. There is also the possibility that the *ras* binding site of GAP is the SH2 domain; this would have wide-ranging implications for regulation of nonreceptor tyrosine kinases that also contain this domain. It may be significant that two of the phosphotyrosine-containing phosphoproteins found in anti-*crk* immunoprecipitates have molecular weights of 120 and 21 kd, corresponding to the molecular weights of GAP and the *ras* family, respectively (Vogel et al., 1988; Trahey et al, 1988; Marshall, 1986). It would be interesting to test anti-*crk* immunoprecipitates for GTP-binding or GAP activity, or use *ras* or GAP antibodies to probe immunoblots of *crk* immunoprecipitates.

The protein data that I have presented in this section clearly do not point to a single model that would explain the biological activity of the v-*crk* gene; many models are consistent with at least some of the data. What these data do indicate is that the mechanism whereby the v-*crk* gene product transforms is unique. The fact that $p47^{gag\text{-}crk}$ must transform by virtue of its

association with normal cellular proteins provides an experimental handle on putative cellular factors with the ability to alter the signal transduction pathways that govern growth control. By purifying and eventually cloning proteins that interact with $p47^{gag\text{-}crk}$, important links in these pathways may be elucidated.

SECTION SIX

SITE-DIRECTED MUTAGENESIS OF THE v-*crk* ONCOGENE

6-1 *Introduction*

The biochemical data presented in the previous section provides a focus for further study on the mechanism of transformation by the v-*crk* oncogene product. As suggestive as some of these results might be, however, it is impossible to conclude that any of these observations are directly relevant to the actual transforming activity of $p47^{gag\text{-}crk}$. In order to establish what is required for biological activity, it will be necessary to correlate the biochemical properties of mutants of the v-*crk* gene with their ability to induce various parameters of transformation. Ideally, one would like to generate both mutants that are fully transforming and mutants that are transformation-defective, each of which behaves differently than the wild-type in terms of the biochemical properties discussed in the last section. It then becomes possible to make the correlations between biochemical and biological activity required to establish what actually is necessary to induce transformation.

Another important aim of mutant studies is to establish structure-function relationships between the various domains of $p47^{gag\text{-}crk}$ and transforming activity. The v-*crk* gene has three recognizable blocks of sequence similarity to other proteins, the SH2, SH2', and SH3 domains. As discussed in section 4-5, there is evidence from sequence comparison that SH2+SH2' and SH3 may function independently, perhaps by binding different cellular factors. Mutating each of these domains independently may dissociate biochemical parameters in a revealing way. In addition, the transduced oncogene portion of $p47^{gag\text{-}crk}$ is fused to 208 amino acids derived from the viral *gag* gene; in the case of several

tyrosine-kinase oncogenes and their cellular counterparts, retroviral *gag* has been shown to play a role in the transforming ability of proteins to which it is fused (Foster et al., 1985; Daley et al., 1987). Since the cellular homolog of v-*crk* is obviously not fused to *gag*, removing the *gag* sequences from p47$^{gag\text{-}crk}$ might give important clues about the aberrant action of the viral oncogene.

The v-*src* gene is perhaps the most well-studied of the viral oncogenes, and serves as an excellent paradigm for the many ways in which mutants can define the properties of a protein that are important for transformation (reviewed in Jove and Hanafusa, 1987; Cooper, 1989). Initial studies with temperature-sensitive mutants established that phosphorylation of cellular proteins on tyrosine was crucial to *src* transformation (Sefton et al., 1980). Deletion and point mutants defined the extent of the catalytic domain and confirmed that *in vitro* kinase activity correlated with transformation (Cross et al., 1984; Bryant and Parsons, 1984; Cross et al., 1985; Snyder et al., 1985; Kamps et al., 1986). Amino-terminal mutants defined a domain essential for myristoylation and membrane association, and established that membrane binding was required for transforming activity (Cross et al., 1984; Pellman et al, 1985b; Kamps et al. 1985). Mutants in the SH2 and SH3 domains of both v-*src* and c-*src* established a role for these domains in regulation of kinase activity (Bryant and Parsons, 1982; Kitamura and Yoshida, 1983; Cross et al., 1984; Cross et al., 1985; Kato et al., 1986), and modification of phosphoacceptor residues established that phosphorylation of p60$^{v\text{-}src}$ is unessential for transformation (Cross and Hanafusa, 1983; Snyder et al., 1983), while phosphorylation of p60$^{c\text{-}src}$ is intimately involved in the regulation of its activity (Cartwright et al., 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987; Reynolds et al., 1987). Recent work has used *src* mutants to correlate transforming activity with association with the cytoskeletal matrix (Hamaguchi and Hanafusa, 1987; Loeb et al., 1987),

association with type 1 PI-kinase (Fukui and Hanafusa, 1989), and phosphorylation *in vivo* of certain cellular substrates (Hamaguchi et al., 1988; Reynolds et al., 1989).

6-2 *Expression system and in vitro mutagenesis*

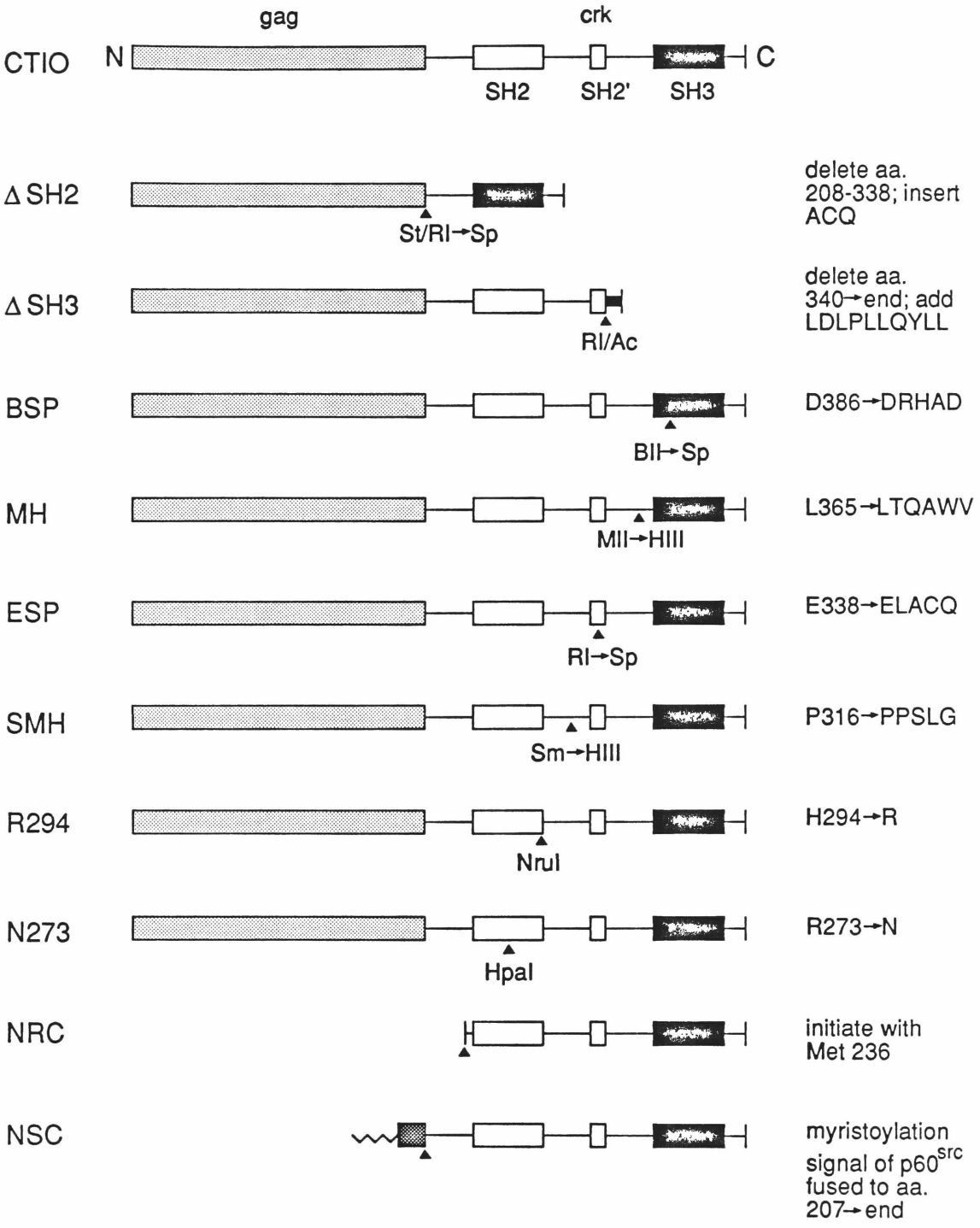
I have used molecularly cloned CT10 DNA to construct deletion, linker insertion, and oligonucleotide-directed point mutants of the v-*crk* gene. Since the original molecular clones were circularly permuted at the *Eco*RI site within the SH2' domain of v-*crk*, I constructed a plasmid containing the nonpermuted CT10 genome with two LTRs to facilitate the generation of mutants (figure 1). This plasmid, pCT10, is the parental plasmid of all the mutants described here. Biological activity of the mutants was assayed by transfection of *Sca*I-cut pCT10-derived DNA along with UR2AV helper virus DNA into CEF. In this system, expression of the mutant *crk* gene product is dependent on replication of the mutant CT10 virus. Since all mutants contain the same LTR and *cis*-acting replicative sequences (except for two mutants that are expressed in a RSV-derived vector; see below), the efficiency of replication of the mutants should be similar to that of the wild-type.

It should be kept in mind that the expression system I am using, a replicating avian retroviral vector, is quite different from mammalian cell systems using selectable, non-replicating vectors (for example, transfection of NIH 3T3 cells with expression vectors containing the neomycin-resistance gene). There are advantages and disadvantages to both strategies. Using a replicating virus allows rapid assay of biological activity—cultures are generally fully infected within two weeks of transfection. The system is such that it is the average phenotype of each mutant that is studied, since millions of cells on each plate are derived from independent infection and integration events; this avoids clonal variation with in-

dividual transfectants. Furthermore, the cells used for the assay are primary cultures, not an established cell line; it has been shown that immortalized cell lines such as 3T3 are in some senses partially transformed, and can be induced to assume a full-blown transformed phenotype by transfection of genes that cannot by themselves transform primary rodent cells (Land et al., 1983). On the other hand, replication of the viruses in culture results in the frequent generation of variants via reverse transcriptase errors and recombination with helper virus, and as will be discussed below, nontransforming or weakly transforming mutants that are replication-defective (as is CT10) are strongly selected against in culture compared with the replication-competent helper virus or fully transforming defective viruses.

The mutants that I have constructed are diagramed in figure 22. I have deleted the entire N-terminal half of the c-*crk*-derived sequences that encodes the SH2 and SH2' domains (10-ΔSH2) or the entire C-terminal portion encoding the SH3 domain and the last few amino acids of SH2' (10-ΔSH3). Where convenient restriction sites were available, I generated linker-insertion mutants resulting in the in-frame insertion of four or five amino acids. These insertions are in the amino-terminal part of SH3 (10-BSP), in the region between SH2' and SH3 (10-MH), within the SH2' block (10-ESP), and in the region between SH2 and SH2' (10-SMH). Since there are no convenient restriction sites in SH2, I created two point mutants at well-conserved residues within this domain by oligonucleotide-directed mutagenesis. I changed the histidine at position 294 to arginine (10-R294) and the arginine at position 273 to asparagine (10-N273). As can be seen in figure 10, the arginine at position 273 is absolutely conserved in all known SH2 domains, and is within the very highly conserved FLV*R*XS sequence. The histidine at position 294 is conserved in all but one SH2 domain, and is at the C-terminal boundary of the SH2 homology.

Figure 22. *In vitro*-constructed mutants of the v-*crk* gene. Viral *gag* sequences (shaded box), SH2 and SH2' domains (white boxes) and SH3 domain (black box) are indicated. Restriction sites used for or created as a result of mutagenesis are identified (see section 2-7). St, *Sty*I; RI, *Eco*RI; Sp, *Sph*I; Ac, *Acc*I; BII, *Bgl*II; MII, *Mst*II; HIII, *Hind*III; Sm, *Sma*I. Amino acid changes in the various mutants are indicated on the right using the single-letter amino acid code. Amino acid numbers are as in figures 8 and 10.


gag
crk
CTIO
N
C
SH2
SH2'
SH3
ΔSH2
St/RI→Sp
delete aa. 208-338; insert ACQ
ΔSH3
RI/Ac
delete aa. 340→end; add LDLPLLQYLL
BSP
BII→Sp
D386→DRHAD
MH
MII→HIII
L365→LTQAWV
ESP
RI→Sp
E338→ELACQ
SMH
Sm→HIII
P316→PPSLG
R294
NruI
H294→R
N273
HpaI
R273→N
NRC
initiate with Met 236
NSC
myristoylation signal of p60src fused to aa. 207→end

The linker-insertion and point mutants are expected to produce local perturbations of protein structure. Ideally, they would not change the overall conformation of the protein drastically, but it is impossible to predict how any alteration in primary sequence will affect secondary and tertiary structure of a given protein. In the specific case of p47$^{gag\text{-}crk}$, the fact that the SH2, SH2' and SH3 domains appear to be modular and are spaced quite differently in different proteins suggests that mutations in one domain will not affect the structure of the others; this is supported by the very glycine- and proline-rich nature of two of the sequences linking the conserved domains, suggesting a non-helix, non-β-sheet secondary structure (Chao and Fasman, 1978). In any case, a positive result (wild-type biological activity) unequivocally demonstrates that the mutated region can tolerate structural alteration, while a negative result (loss of transforming activity) must be viewed with more caution.

In order to assess the contribution of viral *gag* sequences to the biological activity of p47$^{gag\text{-}crk}$, I have constructed two *gag*-deleted mutants. For these mutants I have used a replication-competent RSV-derived vector (Cross and Hanafusa, 1983; Kornbluth et al., 1986) in which the truncated *crk* gene is inserted at the position occupied by the *src* gene in wild-type RSV. These constructs do not require co-transfection with helper virus to replicate, avoiding the problem of selection against the replication-defective CT10-derived virus. In addition, there is evidence that important *cis*-acting sequences are present in the 5' *gag* region of avian retroviruses (Arrigo, et al., 1987; Nishizawa et al., 1987; Carlberg et al., 1988), so a virus containing no *gag* sequences would not be expected to replicate well. In the case of the mutants that retain *gag*, I did not use this approach because the presence of two copies of 5' *gag* in a RSV vector context might result in the rapid deletion of the 3' *gag*, *pol*, and *env* sequences between these long direct repeats.

The two gag(-) constructs have very different translation start sites. C. Reichman in our lab has cloned a cDNA encoding the cellular homolog of the v-*crk* gene, and his data demonstrate that the cellular product uses the methionine homologous to that at position 236 of v-*crk* to initiate translation. The region of v-*crk* between the *gag-crk* junction and methionine 236 is derived from intron and 5' untranslated sequences, and is not found in the c-*crk* product (C. Reichman, unpublished). The mutant 10-NRC should use the normal c-*crk* initiator methionine (amino acid 236 of v-*crk*) and encode a 205 amino acid product. The second mutant, 10-NSC, was constructed to initiate translation with the amino-terminal 14 amino acids of $p60^{src}$, which are fused via linkers to the C-terminal 234 amino acids of v-*crk*. These 14 *src*-derived amino acids are sufficient to direct myristoylation of heterologous proteins (Pellman et al., 1985a), so this construct should direct the synthesis of a myristoylated, 252-amino acid product. Myristoylation is in many cases sufficient to direct tight association of proteins with the plasma membrane; I hoped that by using the *src* amino terminus I could target this *gag*(-) *crk* mutant to membranes, as well as take advantage of a translational start site that is known to work efficiently in a retroviral context.

6-3 *Biological activity of* v-crk *mutants*

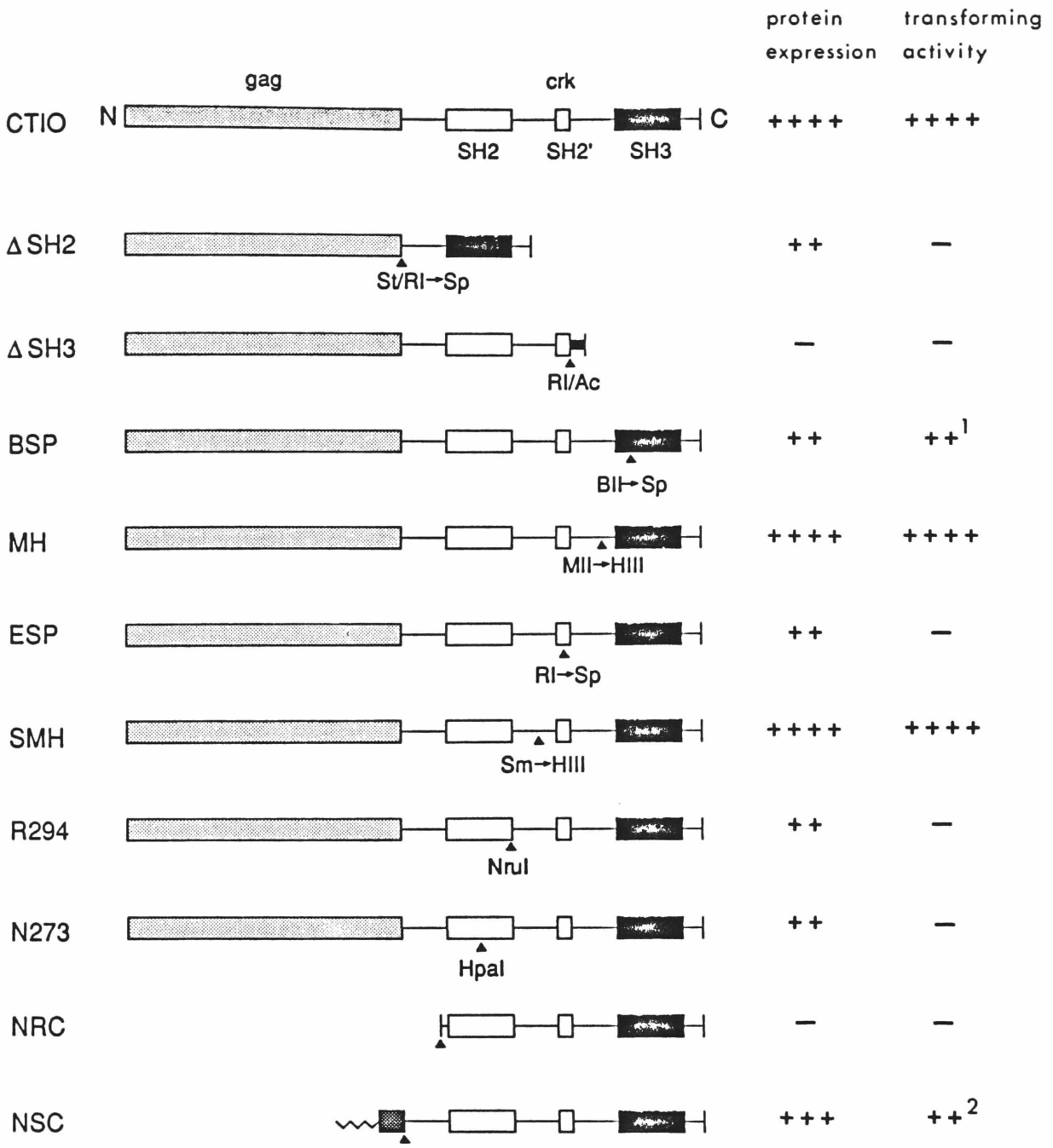
Each of the *in vitro* mutants described above was transfected into CEF along with cloned helper virus DNA where necessary and transforming activity was assessed. I used several parameters of transformation (Hanafusa, 1977) to compare the mutants with wild-type CT10. Morphological alteration is a common property of transformed cells; CT10-infected CEF are rather subtly altered relative to RSV-transformed CEF, but can be distinguished from untransformed controls by their increased refractility, more elongated, fusiform morphology, and

tendency to align into closely packed parallel arrays of cells. Concomitant with this morphological alteration is an increased cell density, which is probably due to both an increased growth rate and loss of contact-inhibition of growth in confluent cultures. A convenient early marker for transformation is acidification of the medium, most probably due to increased metabolism (Hanafusa, 1977). A less subjective and perhaps more biologically relevant assay is the ability of transformed cells to grow in agar suspension, demonstrating anchorage-independent growth. This parameter correlates quite well with tumorigenicity *in vivo*, and probably reflects an ability to grow in the absence of positive environmental signals required for the growth of normal cells. The final criterion is tumorigenicity *in vivo*, the definitive property of an RNA tumor virus. As will be discussed below, however, this parameter is difficult to assay with some mutants due to the rapid generation of variants with different biological properties than the authentic mutant.

The transforming activity of each of these viruses is summarized in figure 23. The only two mutants that retained full transforming activity, indistinguishable from that of the parental CT10 virus, were 10-MH and 10-SMH. These mutants have linkers inserted into the nonconserved regions in between the SH2' and SH3 or the SH2 and SH2' blocks, respectively. These results are consistent with the assumption that it is only the conserved homology blocks that are important for the biological activity of $p47^{gag\text{-}crk}$. Since the spacing and even the order of these blocks varies from protein to protein (see figure 9), it is not surprising that the insertion of four or five amino acids into the intervening sequences between the homology blocks does not affect their function. Further mutants will be required to assess how much flexibility is allowed in the spacing of these elements.

The remaining eight mutants all had diminished transforming ability

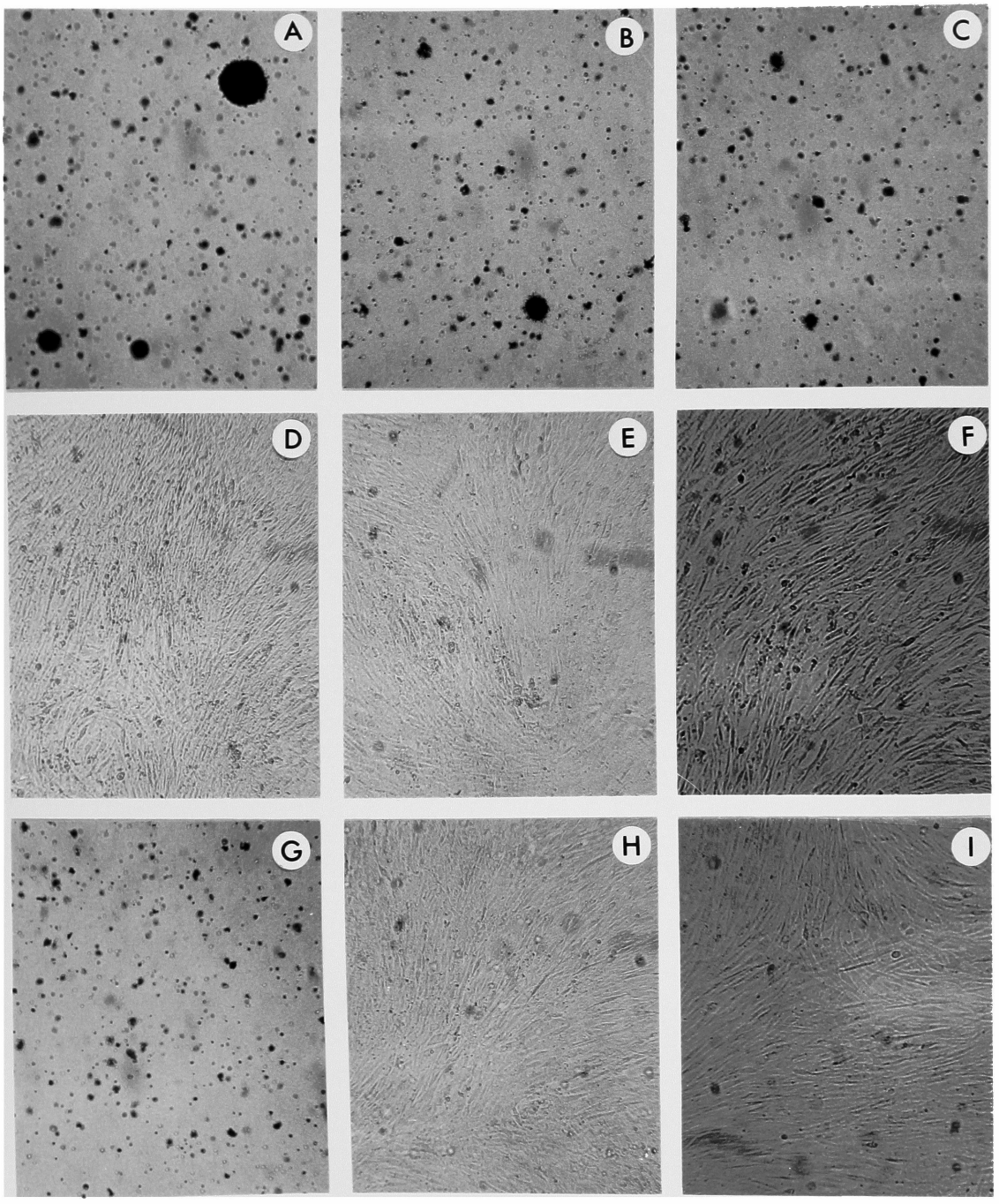
Figure 23. Relative protein expression and transforming activity of the v-*crk* mutants. Protein expression was determined by anti-*crk* immunoblotting as in figure 25. Transforming activity was assayed by induction of morphological alteration, increased cell density, and anchorage-independent growth (see text). [1], monolayer culture indistinguishable from helper-infected controls, but mutant retains some ability to induce anchorage-independent growth. [2], infected cells are morphologically altered, but do not grow to high density or in agar suspension.


protein expression
transforming activity
gag
crk
CTIO
N
C
SH2
SH2'
SH3
++++
++++
ΔSH2
St/RI→Sp
++
−
ΔSH3
RI/Ac
−
−
BSP
BII→Sp
++
++[1]
MH
MII→HIII
++++
++++
ESP
RI→Sp
++
−
SMH
Sm→HIII
++++
++++
R294
NruI
++
−
N273
HpaI
++
−
NRC
−
−
NSC
+++
++[2]

relative to the parental CT10 virus. Only two of these mutants, 10-BSP, which contains a four-amino acid insertion in the SH3 domain, and 10-NSC, which contains the 14-amino acid *src*-derived myristoylation signal in place of *gag*, retained any detectable transforming activity. The remaining six mutants were unable to transform by any criterion when mutant-infected cells were compared with helper-infected controls (not shown). As discussed below, these mutants must be considered in light of the level of expression of the mutant-encoded *crk* protein. However, the total lack of transforming activity of the deletion mutants 10-ΔSH2 and 10-ΔSH3, the linker insertion into the SH2' block 10-BSP, and the two point mutants of conserved SH2 residues 10-N273 and 10-R294, suggests that even minor perturbations in the SH2 and SH2' homology region compromises a crucial biochemical propery required for transformation by the *crk* protein.

The two most interesting mutants in terms of biological activity are 10-BSP and 10-NSC, which retain partial transforming ability. In the case of 10-BSP, infected cells were indistinguishable from helper-infected controls in monolayer culture (figure 24E and H). However, when infected cells were plated in agar suspension, they had the ability to form colonies (figure 24B). These colonies were somewhat smaller than those induced by wild-type CT10 (figure 24A), but were generated with comparable efficiency, demonstrating that the induction of anchorage-independent growth is a property of the authentic 10-BSP mutant and not of variants that arose in culture (not shown). The 10-NSC mutant has an even stranger phenotype. In monolayer culture, infected cells were refractile and somewhat fusiform (figure 24F), similar to CT10-infected cells. However, mutant-infected cells did not grow to a greater density than helper-infected controls, suggesting that growth rate and density dependence were normal in these cells. When infected cells were plated in suspension, only a few tiny colonies were observed (figure 24C), demonstrating a virtual lack of anchorage-

Figure 24. Morphological alteration and induction of anchorage-independent growth of CEF infected with v-*crk* mutants. Panels A, B, C, and G, agar colony-formation assay; micrographs were taken 28 d after plating. Panels D, E, F, H, and I, monolayer cultures; micrographs taken 7 d after transfection (panels F and I) or 14 d after transfection (panels D, E, and H). CEF were transfected with CT10+UR2AV (panels A and D), 10-BSP+UR2AV (panels B and E), 10-NSC (panels C and F), UR2AV (panels G and H) or NR200, the parental vector for 10-NSC containing no oncogene sequences (panel I).


A
B
C
D
E
F
G
H
I

independence. These two mutants display dissociation of transformation parameters: 10-BSP retains the ability to induce anchorage-independent growth in the absence of morphological alteration or altered growth properties in monolayer culture, while 10-NSC induces morphological changes in the absence of any other parameter of transformation. This latter phenotype is particularly interesting, because studies with v-*src* mutants generally show that the ability to induce morphological changes is the first parameter to be lost as mutants become less transforming (Jove et al., 1986a).

When culture fluids from mutant-infected cells were injected into newborn chickens, only 10-SMH and 10-MH were able to rapidly induce sarcomas (not shown). 10-ΔSH3, 10-ΔSH2, 10-N273, and 10-R294 did not induce any tumors after six weeks (not shown). 10-BSP, 10-ESP, 10-NRC, and 10-NSC induced tumors with a relatively long latency compared to CT10. When these tumors were excised and virus was recovered by cocultivation, cultures were rapidly morphologically transformed and grew to high density. Since this phenotype is very different from that seen in cells transfected with the authentic mutant DNA, I assume that these tumors were induced by variants that arose in culture or in the infected chickens; these could be true revertants or, more likely in the case of the linker insertions, pseudorevertants with second-site mutations. This phenomenon has been observed upon injection of partially transforming RSV-derived viruses (Cross et al., 1985; Levy et al., 1986). Immunoblotting of lysates from cells cocultured with 10-NSC- or 10-NRC-induced tumor tissue with anti-*crk* antibody demonstrated the presence of a variety of *crk*-related proteins in the 40-50 kd range, suggesting that the original *gag*-deficient mutants had recovered viral *gag* sequences by recombination. This type of recombination and activation of transforming potential has been observed in the case of *gag*-deficient c-*abl* constructs (Daley et al., 1987).

Even though these mutants contain the same replication and transcription signals as the parental CT10 or RSV, it is possible that the lack of transforming activity observed was due to poor replication and/or poor protein expression. Indeed, as is shown in the next section, all the nontransforming or partially transforming mutants express less *crk* protein than the wild-type or fully transforming mutants. By northern blotting, I have examined the levels of viral RNA expression in infected cells for many of these mutants (not shown). In the case of 10-ΔSH3, I was unable to detect any *crk*-related mRNA in infected cells. I cannot therefore conclude anything about the biological activity of this mutant, since it is possible that the deletion affected critical *cis*-acting elements, precluding replication of the mutant virus or protein expression. In the case of mutant 10-NRC, viral mRNA is present at reasonable levels but I have not been able to detect the mutant protein by immunoblotting. It is possible that the c-*crk* translation initiation site is not used efficiently, or that the encoded protein is very unstable. Again, no conclusion is possible about the activity of this mutant. In all other cases examined, levels of viral expression corresponded to the levels of protein detected by immunoblotting, suggesting that the mutant proteins are relatively stable.

In the cases where nontransforming or weakly transforming mutant protein can be detected in mass cultures (10-ΔSH2, 10-BSP, 10-ESP, 10-R294, 10-N273, and 10-NSC) it is possible to conclude that no gross defect exists in the ability of these viruses to replicate and synthesize mutant protein. It has been widely observed that nontransforming or weakly transforming avian retroviruses do not replicate in culture as well as nearly identical transforming viruses (see Foster et al., 1986 for an example). This may reflect the presumed growth advantage that transformed cells have over untransformed cells in culture, which would tend to counteract the natural selection against either a replication-

defective virus or a replication-competent virus carrying extra, oncogene-derived sequences; as discussed in section 3-5, it may also reflect a positive effect of transformation on the replication of the virus itself. To circumvent this problem with mass cultures, I took advantage of the ability of transforming viruses to induce colonies in agar suspension. I transfected CEF with the mutant viruses and two days after transfection, after at most one round of viral replication (Varmus and Swanstrom, 1985), I trypsinized the transfected cells and replated them in agar suspension. In each case, with the exception of 10-BSP and 10-NSC as described above, no colonies were observed, demonstrating that mutants with lesions in the SH2 or SH2' domains are incapable of inducing anchorage-independent growth. Since this assay does not depend on viral replication to generate a detectable transformed phenotype (transformed colonies should be induced by transfected DNA alone), I can conclude that the lack or abrogation of ability to induce anchorage-independent growth seen with 10-ΔSH2, 10-BSP, 10-ESP, 10-R294, 10-N273, and 10-NSC is due to a defect in the mutant *crk* protein and not to insufficient replication in culture.

6-4 *Expression of mutant* crk *proteins and phosphotyrosine content of infected cells*

To determine the level of protein expression of the various *in vitro* constructed *crk* mutants, I immunoblotted transfected cell lysates with anti-*crk* antibody. In each case equal amounts of total protein were loaded per lane. As can be seen in figure 25A, the two fully transforming mutants 10-SMH and 10-MH expressed levels of mutant *crk* protein comparable to wild-type CT10. The *gag*(-), myristoylation(+) mutant 10-NSC was also expressed very well. The mutants 10-ΔSH2, 10-N273, 10-R294, 10-ESP, and 10-BSP were expressed at relatively low but detectable levels. The 10-ΔSH3 and 10-NRC mutants did not pro-

Figure 25. *Crk* proteins and phosphotyrosine-containing proteins of CEF transfected with various v-*crk* mutants. Top, anti-*crk* immunoblot; bottom, anti-ptyr immunoblot. Approximately 30 μg protein from CEF transfected with mutants as indicated was separated on 8.5% gels and immunoblotted. Markers, 220, 100, 68, 43, and 27 kd.

A
CT10
ΔSH2
ΔSH3
BSP
MH
ESP
SMH
R294
N273
NRC
NSC
AV
anti·crk
B
CT10
ΔSH2
ΔSH3
BSP
MH
ESP
SMH
R294
N273
NRC
NSC
AV
anti·ptyr

duce detectable amounts of mutant protein. I have not rigorously tested the conditions for anti-*crk* immunoblotting to confirm that this assay is quantitatively linear, so the different amounts of protein observed should be interpreted in a stricly relative sense. However, as mentioned above, the ability to detect even low levels of protein demonstrates that in these cases there is no defect in the replication of the mutant viruses. This protein data also demonstrates that, where protein is detectable, the mutations that I constructed maintain the correct reading frame within v-*crk*, since the sizes of the mutant proteins are in all cases as expected.

I also examined the levels of phosphotyrosine on proteins in cells infected with the mutants by anti-ptyr immunoblotting (figure 25B). The lysates were the same as those used for anti-*crk* immunoblotting. The fully transforming mutants 10-MH and 10-SMH induced tyrosine phosphorylation of the 135-155, 120, and 70 kd proteins to levels comparable to wild-type CT10 (the CT10 lysate used here had been frozen and thawed several times, so some dephosphorylation of this sample may have occurred). In contrast, the nontransforming mutants showed no increased phosphotyrosine above the level seen in helper-infected controls. The most interesting pattern was seen with the two partially transforming mutants. 10-BSP, which retains some ability to induce anchorage-independent growth, induced a moderate increase in the tyrosine phosphorylation of the 135-155, 120, and 70 kd proteins. The 10-NSC mutant, which induces morphological alteration in the absence of altered growth properties, induced very strong tyrosine phosphorylation of the 120 kd protein, and weak phosphorylation of the 135-155 kd and 70 kd proteins.

Several conclusions can be drawn from this data. First, increased tyrosine phosphorylation is 100% correlated in these mutants with the transformed state of the cells. The *crk* proteins of 10-R294, 10-N273, and 10-

ESP are expressed at comparable levels to that of 10-BSP, which induces anchorage-independent growth. Only in the case of 10-BSP, however, is increased phosphotyrosine observed. This is consistent with increased phosphotyrosine being crucial to the mechanism of transformation by this protein, and demonstrates that it is not a meaningless byproduct of expression of the *crk* protein. The pattern observed in cells transfected with 10-NSC is particularly interesting, in that the 120 kd protein is phosphorylated on tyrosine to at least as great an extent as seen in the case of fully transforming viruses. High-level tyrosine phosphorylation of this protein is therefore not sufficient to cause the altered growth properties of transformed cells. However, high-level phosphorylation of this protein does correlate with morphological alteration, suggesting that this protein could be directly involved in alteration of cell shape. It is interesting to note that the tyrosine phosphorylation of a 120 kd protein has been correlated with morphological alteration in the case of cells expressing various *src* mutants (Reynolds et al., 1989). Tyrosine phosphorylation of the 135-155 and 70 kd proteins correlates very well with transformation by the *crk* mutants. Nontransforming mutants induce no increase, while the partially transforming mutants 10-BSP and 10-NSC induce a moderate increase; these proteins are highly phosphorylated in cells infected with wild-type CT10 or fully transforming mutants. This data is consistent with the tyrosine phosphorylation of these proteins being required for transformation by the *crk* oncogene.

Further study of the 10-NSC mutant should yield important clues to the mechanism of *crk* transformation. This mutant has no changes in the c-*crk*-derived sequences relative to wild-type CT10; the only difference is that the *gag* moiety of $p47^{gag\text{-}crk}$ has been replaced by the myristoylation signal of $p60^{src}$. This implies that viral *gag* confers some property on the fusion protein that cannot be replaced by myristoylation alone. Since the *crk*-associated tyrosine kinase

activity and the phosphotyrosine-containing proteins of *crk*-transformed cells are localized on the membrane, I simplistically assumed that the *src* myristoylation signal would direct the *gag*(-) NSC mutant to the appropriate cell compartment. There are several possible explanations for the weak transforming activity of this mutant. First, I have not yet tested whether the 10-NSC *crk* protein is in fact myristoylated; however, the N-terminal 14 amino acids of *src* have been previously shown to direct myristoylation of heterologous proteins (Pellman, 1985a). Furthermore, some myristoylated proteins appear to be soluble (Olsen et al., 1985; Garber et al., 1985), so it is possible that the 10-NSC protein may not be membrane-associated even if it is myristoylated. It is also possible that *gag* directs $p47^{gag\text{-}crk}$ to a specific cell compartment that is crucial for transformation; it is interesting that the viral MA protein, which is present in $p47^{gag\text{-}crk}$, is thought to mediate association of the virion core with the plasma membrane prior to budding of the mature virion (Dickson et al., 1985). Clearly, more detailed localization studies are required to determine the critical subcellular localization for the *crk* protein.

6-5 *Implications and prospects*

A great deal of work remains to be done on these mutants, ultimately aimed at correlating biological activity with the biochemical properties of the various mutants. For example, an analysis of phosphotyrosine-containing *crk*-associated proteins and *crk*-associated kinase activity will establish whether any of the properties of the wild-type protein correlate with parameters of transformation. One potential problem with this type of analysis is that the non-transforming and partially transforming mutant proteins are expressed at lower levels than the wild-type. Since the *crk*-associated phosphoproteins and associated tyrosine kinase activity are close to the lower limits of detection even in the

case of the wild-type protein, negative data (lack of association) will not be very compelling. As mentioned above, low-level expression of mutant proteins is a result of the viral expression system used for these studies. Experiments are currently underway to express these proteins in mammalian cell lines using a selectable vector, so that high-level expression of both wild-type and non-transforming mutant proteins can be obtained. Preliminary data demonstrate that $p47^{gag\text{-}crk}$ is able to morphologically transform NIH-3T3 cells (C. Marshall and B. Mayer, unpublished). This system will allow a more detailed correlation of biochemical and transforming parameters under conditions of equal protein expression, and should help elucidate the specific roles of various domains of $p47^{gag\text{-}crk}$ in the biological and biochemical activity of this protein.

The total lack of transformation of cells infected with linker-insertion or point mutants of the SH2 or SH2' domains implies that these regions are crucial for transformation by v-*crk*. The two point mutants at conserved SH2 amino acids are particularly suggestive, since in these cases the perturbation of the overall structure of the protein should be relatively minor. Since the residues changed, arginine-273 and histidine-294, are highly conserved in all SH2 domains (figure 10), these two positively charged amino acids may be involved in ionic interactions with SH2-binding proteins or with other residues within SH2 itself. The lack of transforming activity of the 10-ESP mutant, containing a linker in the SH2' domain, is somewhat surprising, since there is less amino acid conservation in this domain compared with the SH2 and SH3 motifs. However, insertion of four amino acids into this small domain might be expected to drastically alter its conformation.

The results above, in combination with the decreased transforming ability of the SH3 linker-insertion mutant 10-BSP, suggest that all three homologous domains are required for transformation by v-*crk*. Based on data from SH3

mutants of the c-*src* and c-*abl* genes, which are generally activated relative to the nonmutated cellular kinase, I and others have proposed that this region in the tyrosine kinases might be involved in binding to a cellular inhibitor of their activity (section 4-5). If v-*crk* transforms by sequestering such an inhibitor, one might expect SH3 mutations to be much more debilitating than other mutations to the transforming activity of this oncogene. The data presented above, especially the partially transforming phenotype of SH3 mutant 10-BSP, do not support this hypothesis; if anything, SH3 seems to be less important than either SH2 or SH2'. One caveat of this is that the linker in mutant 10-BSP is inserted within a region encoding a six-amino acid stretch of SH3 that has little sequence similarity among different SH3 domains; it is possible that this region is relatively insensitive to mutation. Mutation of individual strongly conserved SH3 residues will be required to fully assess the relative importance of the SH3 domain to transformation. In any case, the data in this section strongly suggest that a simple model involving only the SH3 domain in the biological activity of p47$^{gag\text{-}crk}$ is inaccurate.

I believe that the best approach to elucidate the mechanism of transformation by v-*crk* will be to isolate and molecularly clone proteins that interact with p47$^{gag\text{-}crk}$ in infected cells. A straightforward way to do this would be affinity chromatography of cell lysates using a matrix coupled to purified *crk* proteins (synthesized in bacteria or by baculovirus vectors). The mutants I have described in this section will be invaluable to this type of study, since presumably the nontransforming mutants are incapable of interacting with cellular factors critical for transformation. By comparing the cellular proteins which interact with the wild-type protein to those that interact with nontransforming mutant proteins, it should be possible to identify a subset of proteins whose binding correlates with transformation. Since the *gag*(-) mutant 10-NSC is only weakly

transforming, the relative subcellular localization of this protein and the wild-type gene product may suggest where in the cell to look for important *crk*-binding proteins, and may therefore suggest an initial purification scheme to enrich for potential binding proteins.

The data from various mutants strongly implicates tyrosine phosphorylation in transformation by v-*crk*. Since increased phosphotyrosine is visible on only three major proteins in CT10-infected CEF, as opposed to cells transformed by most tyrosine kinase oncogenes in which many proteins contain increased phosphotyrosine (see Hamaguchi et al., 1988), this is an attractive system from which to purify potentially interesting phosphotyrosine-containing proteins. The tyrosine kinase oncogenes phosphorylate many proteins by virtue of their extremely high activity *in vivo*; the majority of these proteins might have nothing to do with transformation, or with substrates of the normal proto-oncogene product. In the case of transformation by CT10, however, the kinase (or possibly the phosphatase) that is responsible for the increased phosphotyrosine is a normal, cellular enzyme; because of this the proteins with increased phosphotyrosine in CT10-infected cells are more likely to be biologically relevant. The mutant data suggests that tyrosine phosphorylation of the 120 kd protein may be involved in morphological alteration; this protein could be isolated relatively easily from 10-NSC-infected CEF by anti-ptyr immunoaffinity chromatography. Similarly, the other two major phosphotyrosine containing proteins could be isolated from wild-type CT10-infected CEF. This approach might actually converge with the *crk*-affinity purification scheme mentioned above, since I have shown in section 5-5 that many phosphotyrosine-containing proteins apparently associate with $p47^{gag\text{-}crk}$.

As was the case in the previous section, the data I have presented on v-*crk* mutants is suggestive, but does not as yet indicate which, if any, of the

models for the biological activity of v-*crk* are accurate. Using these mutants as tools, however, it should be possible in the near future to assemble enough correlative data to establish what molecular events are required for transformation and to begin to isolate cellular factors that mediate the profound changes induced by expression of $p47^{gag\text{-}crk}$. As I have stated before, I feel that the identification of these cellular mediators will be important to a complete understanding of normal growth control. We now have a shadowy understanding of how these pathways work, but as yet no detailed knowledge of how the many strands of the regulatory network interconnect to confer the exquisite regulation of growth control evident in the development and adult life of a metazoan organism. The action of oncogenes demonstrates how these netwoks can be twisted and broken to unleash the uncontrolled growth of cancerous cells. The novel mechanism whereby the v-*crk* gene subverts the normal growth control mechanisms in infected cells gives us a new experimental tool and a new conceptual framework with which to address the problem of eukaryotic growth control.

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