AN ANALYSIS OF PHOSPHOTYROSYL-PROTEIN PHOSPHATASES PRESENT IN SOLUBLE PROTEIN EXTRACTS PREPARED FROM CHICKEN EMBRYO BODIES

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TITLE: An Analysis of Phosphotyrosyl-Protein Phosphatases Present in Soluble Protein Extracts Prepared from Chicken Embryo Bodies

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ABSTRACT

A phosphotyrosyl-protein phosphatase, designated pTPIII, was purified to near homogeneity from soluble extracts prepared from 11-day old chicken embryo bodies. SDS-PAGE analysis suggested that this pTP activity correlated with the presence of a 58 kd protein band in affinity purified enzyme preparations. The purified protein exhibited biochemical characteristics in common with a phosphotyrosyl-protein phosphatase purified from human placental protein extracts, pTP1B (Tonks et al, 1988a; Pallen et al, 1991), and thus may represent the chicken homolog of this protein.

Two partial cDNA clones encoding phosphotyrosyl-protein phosphatases were also isolated. Both code for members of the transmembrane class of tyrosine specific protein phosphatases, and thus probably bear no relation to the pTPIII and pTPI activities purified from soluble protein extracts. One cDNA appeared to encode the COOH-terminus of the chicken homolog of LAR, while the second seemed to code for the chicken homolog of PTP-zeta. Two cDNAs encoding PTP-zeta were isolated, however the evidence presented suggested that both clones were likely derived from incompletely processed nuclear RNAs.

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LIST OF ABBREVIATIONS USED

a.a.	Amino Acid
ATP	Adenosine 5'-Triphosphate
bp	Base Pair
BSA	Bovine Serum Albumin
°C	Degrees Celcius
CAM	Calmodulin
cAMP	Cyclic Adenosine 3',5'-Monophosphate
CD	Cell Determinant
cdc	Cell Division Control
cDNA	Complementary Deoxyribonucleic Acid
CEF	Chicken Embryo Fibroblast
Ci	Curie
cm	Centimeter
CM	Carboxymethyl
COOH	Carboxy
CPM	Counts Per Minute
CsCl	Cesium Chloride
CSF-1	Colony Stimulating Factor 1
dATP	Deoxyadenosine Triphosphate
DAG	Diacylglycerol
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic Acid
FGF	Fibroblast Growth Factor
fm	Femptomolar
FN-III	Type III Fibronectin
g	Gravity
GAP	GTPase Activating Protein
GTP	Guanosine 5'-Triphosphate
HCl	Hydrochloric Acid
HEPES	N-2-hydroxyethylpiperazine-N"-2-ethanesulfonic Acid
HPTP	Human Phosphotyrosyl-Protein Phosphatase
H_2O	Water
hr	Hour
IgG	Immunoglobulin
IGF	Insulin-Like Growth Factor
IP3	Inositol 1,4,5 Triphosphate
kd	Kilodalton
l	Liter
LAR	Leukocyte Common Antigen Related Protein
LB	Luria Broth
LC	Lung Cell Carcinoma
LCA	Leukocyte Common Antigen
MAP-2	Microtubule Associated Protein 2
mCi	Millicurie

LIST OF ABBREVIATIONS (CONT.)

2-ME	2-Mercaptoehtanol
mg	Milligram
MHC	Major Histocompatibilty Complex
min	Minute
$MgCl_2$	Magnesium Cloride
ml	Milliliter
mM	Millimolar
$MnCl_2$	Manganese Chloride
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	Nanogram
NGF	Nerve Growth Factor
NH_2	Amino
nM	Nanomolar
NP-40	Nonidet P-40
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PFU	Plaque Forming Unit
PI 3	Phosphatidylinositol 3 Phosphate
PIP2	Phosphatidylinositol 4,5 bisphosphate
PKA	cAMP Dependent Protein Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
pm	Picomolar
PMSF	Phenylmethylsulfonyl Floride
PolyA	Polyadenylate
pNPP	p-Nitrophenol Phosphate
p-ser	Phosphserine
pSK	Serine Specific Protein Kinase
pSP	Phosphseryl-Protein Phosphatase
p-thr	Phosphothreonine
pTK	Tyrosine Specific Protein Kinase
pTP	Phosphotyrosyl-Protein Phosphatase
p-tyr	Phosphotyrosine
RCC	Renal Cell Carcinoma
RCM	Reduced Carboxyamidomethylated and Mayelated
RNA	Ribonucleic Acid
RSV	Rous Sarcoma Virus
SDS	Sodium Dodecyl Sulphate
sec	Second
SH2	Src Homology Domain 2
SH3	Src Homology Domain 3
TAE	Tris-Acetate Buffer
TBE	Tris-Borate Buffer
TCA	Trichloroacetic Acid
TE	Tris-EDTA
TLC	Thin Layer Chromatography

LIST OF ABBREVIATIONS (CONT.)

Tris	Tris(hydroxymethyl)aminomethane
μg	Microgram
μl	Microliter
μM	Micromolar
UV	Ultraviolet
w	weight
v	volume
V	Volt

CHAPTER 1: INTRODUCTION

Phosphorylation has become accepted as one of the major mechanisms whereby protein function is regulated within eukaryotic cells. Cellular events shown to be regulated, at least in part, by phosphorylation of key proteins in the pathway include glycolysis, gluconeogenesis, fatty acid synthesis, glycogen metabolism, cholesterol synthesis, protein synthesis, muscle contraction, and mitosis (Krebs and Beavo, 1979; Norbury and Nurse, 1989; Cohen and Cohen, 1989; Cohen 1989). Protein phosphorylation has been demonstrated to both positively and negatively regulate the activity of substrate proteins, and in some cases, to alter their subcellular localization.

The phosphorylation of substrate proteins is catalyzed by enzymes known as protein kinases, while dephosphorylation is catalyzed by protein phosphatases. For the most part, phosphorylation occurs at either serine or threonine residues. Together, phosphoserine (p-ser) and phosphothreonine (p-thr) account for over 99.9% of the phosphoamino acids found within the cell. Phosphotyrosine (p-tyr) is one of the minor phosphoamino acids detected within the cell.

Under normal conditions, p-tyr accounts for less than 0.05% of the total phosphoamino acid content (Hunter and Sefton, 1980). Over the past 10 years a great deal of information has been accumulated that demonstrates the importance of protein phosphorylation at tyrosine residues in regulating the signal transducing processes that control cell growth and differentiation (Hunter and Cooper, 1985; Hunter, 1989; Ullrich and Schlessinger, 1990; Cantley et al., 1991).

Protein phosphorylation is both rapid and reversible, qualities ideally suited for systems involved in the transmission of signal information. Through the phosphorylation of more than one key substrate protein, it would be possible to coordinately regulate several different biochemical processes in response to a single external signal, as is often necessary. The reversibility of the reaction could serve as a means of regulating the duration of signal transmission.

It has been suggested that there exists within the cell a dynamic balance between protein kinase and protein phosphatase activities, and that this balance serves to maintain a steady state level of protein phosphorylation. In response to a given external signal, this balance is somehow disrupted, leading to a net gain or loss in the number of phosphate groups attached to key substrate proteins. By altering the biological activity of these proteins, as a result of changes in their phosphorylation state, the physiological response to the signal is achieved.

1-I. TYROSINE KINASES

That protein phosphorylation at tyrosine residues plays an important role in regulating cell growth and differentiation was first suggested by the discovery that several retroviral oncogenes encode proteins possessing tyrosine specific kinase activity (Bishop, 1985; Hunter and Cooper, 1985). This hypothesis has been strongly supported by the subsequent demonstration that several growth factor receptors, when activated, possess tyrosine specific kinase activity (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990), and by the discovery that tyrosine phosphorylation also plays an important role in the regulation of both cell division (Gould and Nurse, 1989; Gould et al., 1990) and embryonic development (Chabot et al., 1988; Sprenger et al., 1989; Ruiz i Altaba and Melton, 1989; Price et al., 1989; Schejter and Shilo, 1989; Kramer et al., 1991). Tyrosine specific protein kinases (pTKs) can be divided into two main classes, the transmembrane receptor and nonreceptor pTKs.

1-Ia. Growth Factor Receptor pTKs

The receptors for epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin, insulin-like growth factor (IGF), colony stimulating factor 1 (CSF-1), and several other proteins, are all members of the growth factor receptor class of pTKs (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990).

The tyrosine kinase activity of growth factor receptor pTKs is regulated by the binding of appropriate ligands to the extracellular ligand binding domains of these proteins. Ligands may take the form of circulating peptide hormones, such as EGF and PDGF (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990), or the extracellular domains of proteins expressed on the outer membrane surface of neighboring cells, such as "bride of sevenless", the ligand of the "sevenless" transmembrane pTK (Kramer et al., 1991; Klausner and Samelson, 1991).

In the case of receptors for circulating growth factors, ligand binding generally induces a conformational change which in turn results in receptor oligomerization (Greenfield et al., 1989; Yarden and Schlessinger, 1987a, 1987b; Heldin et al., 1989; Honegger et al., 1989). Oligomerization mediated activation of the receptors has been suggested to arise from cross phosphorylation of the receptor proteins on tyrosine residues, resulting in enhanced pTK activity of the individual receptor molecules. As will be discussed later, autophosphorylation of the receptors on tyrosine residues not only increases their intrinsic pTK activity, but also serves to create binding domains for the recruitment of critical substrates involved in the transmission of signal information (Cantley et al., 1991).

1-Ib. Nonreceptor pTKs

The members of the second class of pTKs, the nonreceptor pTKs, include the proteins pp60^{c-src}, pp62^{c-yes}, p56^{lck}, p59^{fyn}, p56^{lyn}, as well as several others (Hunter and Cooper, 1985; Hunter, 1989).The prototypical nonreceptor class pTK, and probably the most extensively studied of all pTKs, is pp60^{c-src}. It contains a stretch of approximately 300 amino acids that defines the catalytic domain (Hanks <u>et al.</u>, 1988). A homologous region has been found in all pTKs identified to date. pp60^{c-src}, like most members of the nonreceptor class, is modified by the addition of myristic acid to an NH_2-terminal glycine residue (Hunter and Cooper, 1985; Perlmutter <u>et al.</u>, 1988; Resh, 1990). This modification is responsible, at least in part, for localization of these proteins to the inner surface of the plasma membrane [one exception is the c-abl protein that is localized to the nucleus (Van Etten <u>et al.</u>, 1989)]. Membrane association is required for cell transformation by oncogenic pp60^{v-src} protein (Cross <u>et al.</u>, 1985; Kamps <u>et al.</u>, 1985).

In their normal state, the nonreceptor pTKs, like the growth factor receptor pTKs, possess only very low levels of tyrosine kinase activity and must be activated in order to express full activity. While members of this class of pTKs do not contain an extracellular domain for the binding of activating molecules, they do appear to be activated in response to growth factors. Treatment of platelets with thrombin has been shown to be associated with increased tyrosine phosphorylation mediated by the activation of nonreceptor pTKs (Golden and Brugge, 1989; Ferrell and Martin, 1989). $pp60^{c-src}$ family members have also been demonstrated to be activated by PDGF treatment of human fibroblast cells (Gould and Hunter, 1988; Kypta et al., 1990). In this case, the nonreceptor and receptor kinases appear to physically associate, and activation may result from the phosphorylation of the nonreceptor kinase by the growth factor activated pTK.

It has long been proposed that the members of the $pp60^{c-src}$ family of pTKs actually represent the catalytic subunits of larger multisubunit containing cell surface receptors, and recent observations support this hypothesis. $p59^{fyn}$ has been shown to be associated with the T cell antigen receptor (Samelson et al., 1990; Cooke et al., 1991), while $p56^{lck}$ has been demonstrated to be associated with the interleukin-2 receptor (Horak et al., 1991; Hatakeyama et al., 1991), as well as both CD8 and CD4, two T cell glycoproteins involved in the recognition of class I and class II major histocompatibility complex (MHC) determinants, respectively, on antigen presenting cells (Veillette et al., 1988; Glaichenhaus et al., 1991; Chalupny et al., 1991). Evidence also suggests the involvement of a nonreceptor pTK in the transmission of signals through the B-lymphocyte antigen receptor (Gold et al., 1990). This hypothesis has received support by the recent observation that the c-lyn pTK may be associated with the B cell antigen receptor (Yamanashi et al., 1991).

Each nonreceptor pTK contains approximately 80 unique NH_2-terminal amino acids (Hunter and Cooper, 1985; Perlmutter et al., 1988). It appears that it is these amino acids that regulate the association of the $pp60^{src}$ family member pTKs with their respective receptor subunits. In the case of the association of $p56^{lck}$ with CD4 and CD8, binding has been shown to involve cysteine motifs, present in both proteins, which mediate the interaction of a region of the unique $p56^{lck}$ NH_2-terminal amino acid sequence with the cytoplasmic tails of CD4 and CD8 (Turner et al., 1990; Shaw et al., 1989).

The tyrosine kinase activity of the nonreceptor pTKs is also regulated by phosphorylation. The regulatory importance of this phosphorylation was first revealed in experiments investigating the binding of polyoma virus middle T antigen with $pp60^{c-src}$ (Courtneidge and Smith, 1983; Courtneidge, 1985). These studies showed that the increased pTK activity associated with formation of this complex was correlated with decreased phosphorylation of $pp60^{c-src}$ at a COOH-terminal site, later identified as tyrosine 527 (Cooper et al., 1986). The importance of tyrosine 527 phosphorylation in negatively regulating the pTK activity of $pp60^{c-src}$ was subsequently demonstrated by the generation of point mutations at this site (Cartwright et al., 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987). Substitution of tyrosine 527 with phenylalanine was demonstrated to increase both the pTK and transforming activities of pp60^{c-src}. Similar studies with other nonreceptor pTKs supported the hypothesis that phosphorylation of tyrosine residues equivalent to the tyrosine 527 site of pp60^{c-src} negatively regulates the pTK activity of these enzymes (Amrein and Sefton, 1988; Kawakami et al., 1988; Marth et al., 1988). Phosphorylation at this site does not appear to result from autophosphorylation, as a kinase defective pp60^{c-src} mutant is fully phosphorylated in vivo (Jove et al., 1987). Recently, a pTK that specifically phosphorylates pp60^{c-src} at tyrosine 527 has been purified from neonatal rat brain membrane extracts (Okada and Nakagawa, 1989), and a cDNA encoding this protein has been isolated (Nada et al., 1991).

The nonreceptor pTKs also appear to be regulated by autophosphorylation at a conserved tyrosine residue located within the catalytic domain. The results of studies involving both biochemical analysis and site directed mutagenesis suggested that phosphorylation at this site serves to up regulate the kinase activity of nonreceptor pTKs (Weinmaster et al., 1984; Meckling-Hanson et al., 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987; Cartwright et al., 1987).

Nonreceptor pTKs may be subject to further regulation by phosphorylation at serine residues. pp60^{c-src} has been shown to be phosphorylated at serine 12 and serine 17 by protein kinase C (PKC) and cAMP dependent protein kinase (PKA), respectively (Gould et al., 1985; Patschinsky et al., 1986). The importance of these phosphorylation events remains unclear. pp60c-src has also been shown to be phosphorylated at three NH_2-terminal sites by the $p34^{cdc2}$ kinase, a protein kinase specifically activated at, and involved in regulating, passage through mitosis (Shenoy et al., 1989; Morgan et al., 1989). While phosphorylation at these sites has been correlated with increased $pp60^{c-src}$ kinase activity during mitosis (Chackalaparampil and Shalloway, 1988), it appears that other factors are also involved. The c-abl pTK has also been shown to be phosphorylated by the $p34^{cdc2}$ kinase, however, no increase in c-abl pTK activity was observed (Kipreos and Wang, 1990). Additional tyrosine and serine phosphorylation sites in the NH_2-terminus of $pp60^{c-src}$, including the 80 amino acid unique region, have also been detected (Gould and Hunter, 1988). It has been suggested that, rather than affecting pTK activity directly, phosphorylation at these sites might influence pTK activity indirectly by determining the ability of the enzyme to interact with other proteins (Hunter, 1989).

The identification of two conserved amino acid domains, designated $pp60^{c-src}$ homology regions 2 and 3 (SH2 and SH3), that are differentially conserved amongst the nonreceptor pTKs (the SH2 domain appears to present in all nonreceptor pTKs whereas the SH3 domain is not), but not the receptor class pTKs, has uncovered yet another mechanism whereby the activities of the $pp60^{c-src}$ family of pTKs are regulated

(Pawson, 1988; Koch et al., 1991). These domains appear to regulate the association of these pTKs with other proteins, particularly those involved in signal transduction. SH2 containing polypeptides have been shown to directly associate with tyrosine phosphorylated proteins, and it is the SH2 domain that mediates this binding (Margolis et al., 1990; Moran et al., 1990; Anderson et al., 1990). It has been proposed that amino acid differences in SH2 domains determine the subset of tyrosine phosphorylated proteins with which a given SH2 containing polypeptide can interact. As will be discussed later, the presence of SH2 domains is not restricted to nonreceptor pTKs, in fact they are found in many other proteins involved in regulating signal transduction.

As well as regulating the association of nonreceptor pTKs with other proteins, SH2 domains may play a more direct role in controlling pTK activity. It has been suggested that pTK activity may be regulated by the association of the region of the protein containing the phosphorylated tyrosine 527 site, or its equivalent, with the SH2 domain within the same molecule (Cantley et al., 1991). Association of these two domains could result in the protein adopting a conformation that either inhibits kinase activity or perhaps restricts the access of substrates to the active site of the enzyme. Activation by dephosphorylation of the 527 site could result from a conformational change that deinhibits kinase activity or allows substrates access to the active site. A similar model has been proposed for regulation of the activity of serine specific protein kinases (Soderling, 1990).

1-II. TYROSINE KINASE SUBSTRATES

At present, the chain of events involved in the transmission of signal information within the cell remains poorly understood. The identification of pTK substrates has proven to be a very difficult task due to the low levels of phosphotyrosine in both normal and transformed cells. The development of antibodies specific for phosphotyrosine (Ross et al., 1981; Kamps and Sefton, 1988; Glenney et al., 1988), and the identification of SH2 and SH3 domains (Pawson, 1988; Koch et al., 1991) have proven instrumental in determining the identity of key pTK substrates. In turn, the identification of pTK substrates has helped to shed some light on possible mechanisms of signal transduction and cell transformation.

1-IIa. Phosphorylation of Serine Specific Kinases

An increase in the level of serine and threonine phosphorylated proteins has been observed within minutes following growth factor treatment of cells (Czech et al., 1988), and recently, evidence for the activation of a protein kinase cascade has been reported to result from the treatment of cells with EGF, insulin or nerve growth factor (NGF) (Ahn and Krebs, 1990; Boulton et al., 1991). Activation of such a cascade would be one mechanism whereby signal information originating at the membrane could be disseminated throughout the cell.

One of the serine/threonine protein kinases (pSKs) activated in response to growth factor treatment of cells is raf-1. This pSK has been demonstrated to be activated in cells transformed with the oncogenes v-fms, v-src, v-sis, polyoma virus middle T antigen, and Ha-ras (Morrison et al., 1988), as well as in response to the treatment of cells with insulin (Blackshear et al., 1990; Kovacina et al., 1990). While this enzyme has been shown to be directly activated through tyrosine phosphorylation by the PDGF receptor pTK (Morrison et al., 1989), activation of the raf-1 kinase in oncogene transformed cells, or cells treated with insulin, was correlated with increased serine and threonine but not tyrosine phosphorylation of the protein. This suggests the possibility of two separate mechanisms, one direct and one indirect, for the activation of raf-1 in response to growth factor treatment of cells. The mechanisms controlling the regulation of raf-1 activity in vivo have not been elucidated, however, models involving protein phosphorylation, association with activating lipid cofactors, and interaction of raf-1 with regulatory proteins such as a receptor subunit (as proposed for nonreceptor pTKs), have been proposed (Li et al., 1991).

Several observations suggest that activation of raf-1 plays an important role in the transmission of signal information within the cell. When activated by NH_2-terminal truncation, raf-1 is capable of inducing DNA synthesis following injection into serum starved cells (Rapp et al., 1987). Also, while microinjection of monoclonal antibodies directed against the $p21^{ras}$ protein were capable of blocking transformation not only by $p21^{ras}$ itself, but also by the activated pTKs v-src and v-fms, transformation by activated forms of the pSKs Raf-1 and c-mos was not blocked (Smith et al., 1986). Together with evidence suggesting translocation of raf-1 from the cytoplasm to the nucleus following mitogen treatment of cells (Olah et al., 1991), these observations supported the hypothesis that raf-1 participates in the transmission of signals generated by activated pTKs by acting as a downstream effector. Further support comes from evidence that raf-1 activity is required for transmission of signal information through torso, a transmembrane receptor tyrosine kinase, involved in Drosophila development (Ambrosio et al., 1989), and that raf-1 activity is required for mitogen stimulated growth of NIH/3T3 cells (Kolch et al., 1991). These observations underscore the importance of the demonstration that Raf-1 is a candidate substrate for activated pTKs.

Activation of pTKs by several growth factors, including EGF, PDGF, insulin, and insulin like growth factor II, has been associated with the appearance of a tyrosine phosphorylated

42 kd substrate protein, recently identified as the pSK, microtubule associated protein-2 (MAP-2) kinase (Rossomando et al., 1989). The MAP-2 kinase is also activated by mitogens acting through the serine kinase PKC, and it has been suggested that phosphorylation at both tyrosine and serine residues is required for activation of MAP-2 kinase activity (Anderson et al., 1990; Rossomando et al., 1989). MAP-2 kinase has been proposed to play a role in activating protein synthesis in mitogen stimulated cells by stimulating the phosphorylation of the ribosomal protein subunit S6, through phosphorylation of an S6 kinase activity (Sturgill et al., 1988).

Several other serine kinases appear to be activated in response to growth factor treatment of cells as part of a kinase-kinase cascade (Ahn et al., 1990). The identities of these kinases have not as yet been established. It is likely that only a few are direct substrates of activated pTKs, and that the remainder are activated by indirect mechanisms involving phosphorylation at serine residues, or perhaps through the mobilization of secondary messengers generated in response to activation of pTK activities. Further, there is a great deal of evidence suggesting the existence "crosstalk" between signal transduction pathways involving different protein kinases. PKC in particular appears to play an important role in mediating crosstalk between the different pathways (Houslay, 1991).

1-IIb. Phosphorylation of Intracellular Signalling Proteins

The hypothesis that activated pTKs promote the generation of secondary messenger molecules, that in turn activate pSKs, is supported by the observation that phospholipase C gamma (PLC gamma) physically associates with the receptors for EGF and PDGF, and that it becomes phosphorylated following treatment of cells with these mitogens (Morrison et al., 1990; Margolis et al., 1989; Meisenhelder et al., 1989). Tyrosine phosphorylation of PLC gamma has also been reported following T cell activation (Weiss et al., 1991) and NGF treatment of cells (Vetter et al., 1991). Tyrosine phosphorylation of PLC gamma has been shown to increase the catalytic activity of this enzyme (Nishibe et al., 1990; Sultzman et al., 1991), resulting in the production of the secondary messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2). These messengers have been shown to be involved in the activation of PKC and the release of intracellular calcium stores, respectively (Berridge and Irvine, 1989).

Activation of pTKs appears to give rise to another potential secondary messenger, phosphatidylinositol 3 phosphate (PI 3). PI 3-kinase activity has been found to physically associate with activated CSF1 and PDGF receptors, as well as pp60c-src/polyoma virus middle T antigen immunoprecipitates (Coughlin et al., 1989; Kazlauskas and Cooper, 1989;

Varticovski et al., 1989; Kaplan et al., 1986; Kaplan et al., 1987). The presence of PI 3-kinase activity has been correlated with the presence of an 85 kd tyrosine phosphorylated protein in the above complexes (Kaplan et al., 1987). While the roles PI 3-kinase, and its product PI 3, in stimulating cell growth are at present unresolved, the ability of polyoma middle T antigen mutants to bind PI 3-kinase does correlate well with the ability of these mutants to induce cell transformation (Courtneidge and Heber, 1987).

The finding that $p21^{ras}$ acts downstream of pTKs in signal transduction pathways (Smith et al., 1986), but is not itself phosphorylated on tyrosine residues, suggested the possibility that pTKs mediate activation of $p21^{ras}$ through phosphorylation of the $p21^{ras}$ GTPase activating protein (GAP). GAP interacts with $p21^{ras}$ and stimulates its otherwise weak intrinsic GTPase activity (Trahey and McCormick, 1987). It is well established that $p21^{ras}$ is in its active state when in the GTP bound form, and becomes inactivated when converted to the GDP bound form (DeVendittis et al., 1986). GAP has been demonstrated to form a complex with activated PDGF receptors (Malloy et al., 1990; Kaplan et al., 1990b), and to be phosphorylated on tyrosine residues following treatment of cells with PDGF and EGF, as well as in cells transformed by the v-src, v-fms, v-abl and v-fps pTKs (Ellis et al., 1990). Phosphorylation of GAP by activated pTKs has been reported to result in the formation of large molecular weight complexes containing the GAP protein and proteins of 62 kd or 190 kd, and is associated with decreased GAP activity (Moran et al., 1991). GAP forms independent complexes with p62 and p190, and formation of these complexes requires the phosphorylation of p62 and p190 on tyrosine residues (Moran et al., 1991). The role of these complexes, and of GAP and p21^{ras} themselves, in transducing signal information are at present unclear.

1-IIc. Phosphorylation of Nonreceptor pTKs

As mentioned earlier, pp60^{c-src} physically associates with the PDGF receptor to form a complex (Gould and Cooper, 1988; Kypta et al., 1990). Formation of this complex was associated with increased phosphorylation of the pp60^{c-src} protein, as well as increased pp60^{c-src} kinase activity. Thus, the nonreceptor pTKs represent potential substrates of the growth factor receptor pTKs, and may play a role in the transmission of signal information originating with the transmembrane pTKs. It is possible that activation of the nonreceptor pTKs simply serves to amplify signals in response to growth factors. Alternatively, it may be necessary to activate these pTKs, as they may possess substrate specificities different than the growth factor receptors pTKs. Tyrosine phosphorylation of critical substrates not recognized by, or perhaps inaccessible to, the receptor class pTKs may be necessary to ensure that the proper physiological effect is obtained.

1-IId. Phosphorylation of SH2 Domain Containing Proteins

With the exception of raf-1, and the other pSKs, all of the pTK substrates described above share common conserved noncatalytic amino acid sequences designated SH2 domains (Pawson, 1988; Koch et al., 1991). SH2 domains play an important role in the recruitment of substrates for the formation of signal transducing complexes with activated pTKs. These domains have been shown to be sufficient to mediate binding of proteins to activated growth factor receptors (Anderson et al., 1990). As mentioned above, SH2 domains interact with phosphotyrosine containing peptides. Binding of proteins containing SH2 domains has been shown to require the autophosphorylation of growth factor receptors on tyrosine residues (Margolis et al., 1990; Kim et al., 1991). These observations suggested that autophosphorylation of the receptor pTKs creates the site within the receptor to which the SH2 domain containing proteins can bind. Together, these observations suggest that proteins found to contain SH2 domains likely play a role in transduction of signal information, and represent potential protein substrates for activated pTKs.

1-IIe. Phosphorylation of Cytoskeletal Proteins

Another set of proteins phosphorylated on tyrosine residues in response to growth factor induction of pTK activity, or in cells transformed by activated pTKs, are cytoskeletal proteins. Focal contacts are regions of the plasma membrane involved in mediating attachment to solid substrate (Burridge et al., 1988). Several of the cytoskeletal proteins involved in the formation of focal contacts have been shown to substrates for activated pTKs, including vincullin, talin, and the beta subunit of the fibronectin receptor (Sefton et al., 1981; Pasquale et al., 1986; Hirst et al., 1986). Tyrosine phosphorylation of the beta subunit of the fibronectin receptor has been reported to correlate with reduced affinity for talin and fibronectin, two of the proteins with which it interacts (Tapley et al., 1989). Other cytoskeletal proteins phosphorylated on tyrosine residues include lipocortin I, lipocortin II, ezrin, and a set of three as yet unidentified proteins of 215 kd, 76 kd, and 22 kd (Radke and Martin, 1979a; Fava and Cohen, 1984; Gould et al., 1986; Glenney and Zokas, 1989). Phosphorylation of the above proteins on critical tyrosine residues could result in changes in the ability of cytoskeletal proteins to interact, in turn leading to a disruption of cell adhesiveness and alterations in cell shape, such as the rounding up of cells observed following transformation. This hypothesis is supported by the observation that activated pp60^{v-src} associates with focal contact points and gap junctions in transformed cells (Rohrschnieder, 1980; Henderson and Rohrschnieder, 1987), and that expression of this activated pTK results in decreased cell adhesiveness (Warren and Nelson, 1987). Activation of pp60^{c-src} at mitosis could contribute to alterations in cell shape associated with mitosis (Chackalaparampil and Shalloway, 1988).

Many cytoskeletal proteins contain regions of homology with pp60^{c-src}, known as the SH3 domain (Pawson, 1988; Koch et al., 1991). The presence of SH3 domains is not restricted to cytoskeletal proteins, however, all proteins which harbour SH3 sequences appear to be associated with membranes. This has led to the suggestion that SH3 domains might represent regions involved in regulating subcellular distribution (Koch et al., 1991).

Recently, talin, a cytoskeletal protein involved the attachment of actin filaments to the membrane at focal contact points, has been shown to contain an SH2 domain, common to proteins involved in transduction of signal information (Davis et al., 1991). This suggests that SH2 domains may also play an important role in defining pTK substrates that are localized to the cytoskeleton. Further, it suggests a role for talin in mediating changes in cytoskeletal organization associated with transformation and cell growth.

Tyrosine phosphorylation also appears to play an important role in regulating cell-cell communication. Gap junctions are channels in the membranes of adjacent cells that allow the passage of small molecules between the cells, thereby allowing for cell-cell communication (Loewenstein, 1981). Evidence suggests that cell-cell communication plays an important role in regulating cell growth and differentiation (Loewenstein, 1979; Fraser et al, 1987). As mentioned above, activated pp60^{v-src} has been demonstrated to localize to gap junctions in transformed cells. Studies employing temperature-sensitive v-src mutants have demonstrated that a loss in cell-cell communication accompanies activation of the pTK upon shifting from the nonpermissive temperature to the permissive temperature (Atkinson et al., 1981; Azarnia and Loewenstein, 1984). Recently, decreased communication in v-src transformed cells has been correlated with tyrosine phosphorylation of connexin-43, a structural component of gap junctions (Filson et al., 1990). Inhibition of junctional communication correlates with growth factor induced cell proliferation suggesting an important role for pTKs in regulating intercellular communication (Maldonado et al., 1988).

The diverse nature of the pTK substrates identified to date indicates that phosphorylation at tyrosine residues is involved in the regulation of several different cellular processes. The regulation of multiple processes by tyrosine phosphorylation is consistent with the observation that transformation of cells by activated pTKs is associated with multiple phenotypic alterations. It is possible that novel pTK substrates remain to be isolated, and that tyrosine phosphorylation will be implicated as a mechanism controlling the activation of an even greater number of cellular processes than is presently appreciated.

1-III. PHOSPHOTYROSYL-PROTEIN PHOSPHATASES

As mentioned earlier, protein phosphorylation is generally a reversible event, with the steady state level of phosphorylation of a given cellular protein being determined by a dynamic balance between the activities of the protein kinases and the protein phosphatases. The first indications of the existence of phosphotyrosine specific protein phosphatase activity (pTP) within cells came from experiments employing temperature- sensitive mutants to investigate the transforming properties of avian retroviral pTK oncogenes (Friis et al., 1979; Hunter et al., 1979; Radke and Martin 1979b). These experiments demonstrated that when cells were shifted from the permissive temperature, at which the pTK was active, to the nonpermissive temperature, at which the pTK was inactive, the cells no longer exhibited the transformed cell phenotype but rather took on characteristics of a normal cell phenotype. This reversion was correlated with a decrease in the phosphotyrosine content of the cells which could not be blocked by inhibitors of protein synthesis. These observations suggested that the decrease in phosphotyrosine content of these cells did not result from protein turnover, but rather was due to the presence of an activity within the cells that was capable of dephosphorylating proteins at phosphotyrosine residues.

Several pTP activities have now been purified from a number of sources to homogeneity, or near homogeneity. The first reported purification of a pTP to homogeneity was that of Tonks et al. (1988a). The pTP activity was purified from human placental extracts and was correlated with the presence of 37 kd protein. As will be discussed later, the isolation of cDNAs encoding this enzyme suggested the possibility that the protein isolated by Tonks et al. (1988a) may have been a truncated form of the enzyme produced by proteolysis during the purification process. A larger form of the same enzyme with a molecular weight of at least 46 kd has recently been purified to homogeneity from placental extract (Pallen et al., 1991). pTP activities of molecular weights ranging from 38 kd to 60 kd have also been purified from mouse adipocytes (Liao et al., 1991), human epidermoid carcinoma cells (Butler et al., 1989), rat spleen (Swarup and Subrahmanyam, 1989), and from the electric organ of *Torpedo californica* (Mei and Huganir, 1991). The relationship among these pTPs remains to be established, but all exhibit very similar biochemical characteristics. All require the presence of reducing agents for full activity, all are independent of divalent cations, and all exhibit other characteristics which differ from those of the serine/threonine phosphatases.

Two other forms of pTPs have also been isolated from bovine heart (Zhang and Van Etten, 1990) and bovine brain (Singh, 1990). The enzyme isolated from bovine heart has similar biochemical characteristics to the pTPs described above, however, this enzyme has a molecular weight of approximately 18 kd, and appears to be related to low molecular weight cytosolic acid phosphatases (Lawrence and Van Etten, 1981; Taga and Van Etten, 1982; Waheed et al., 1988; Chernoff and Li, 1985). The brain enzyme has a molecular weight of approximately 85 kd, is dependent upon magnesium for activity, and is inhibited by micromolar concentrations of calcium. The existence of multiple forms of pTP enzymes has been supported by the isolation of many different cDNAs encoding for pTPs. As with the pTKs, pTPs can be divided into a transmembrane receptor-like class and a nonreceptor class, each class containing several different subtypes.

1-IIIa. Transmembrane pTPs

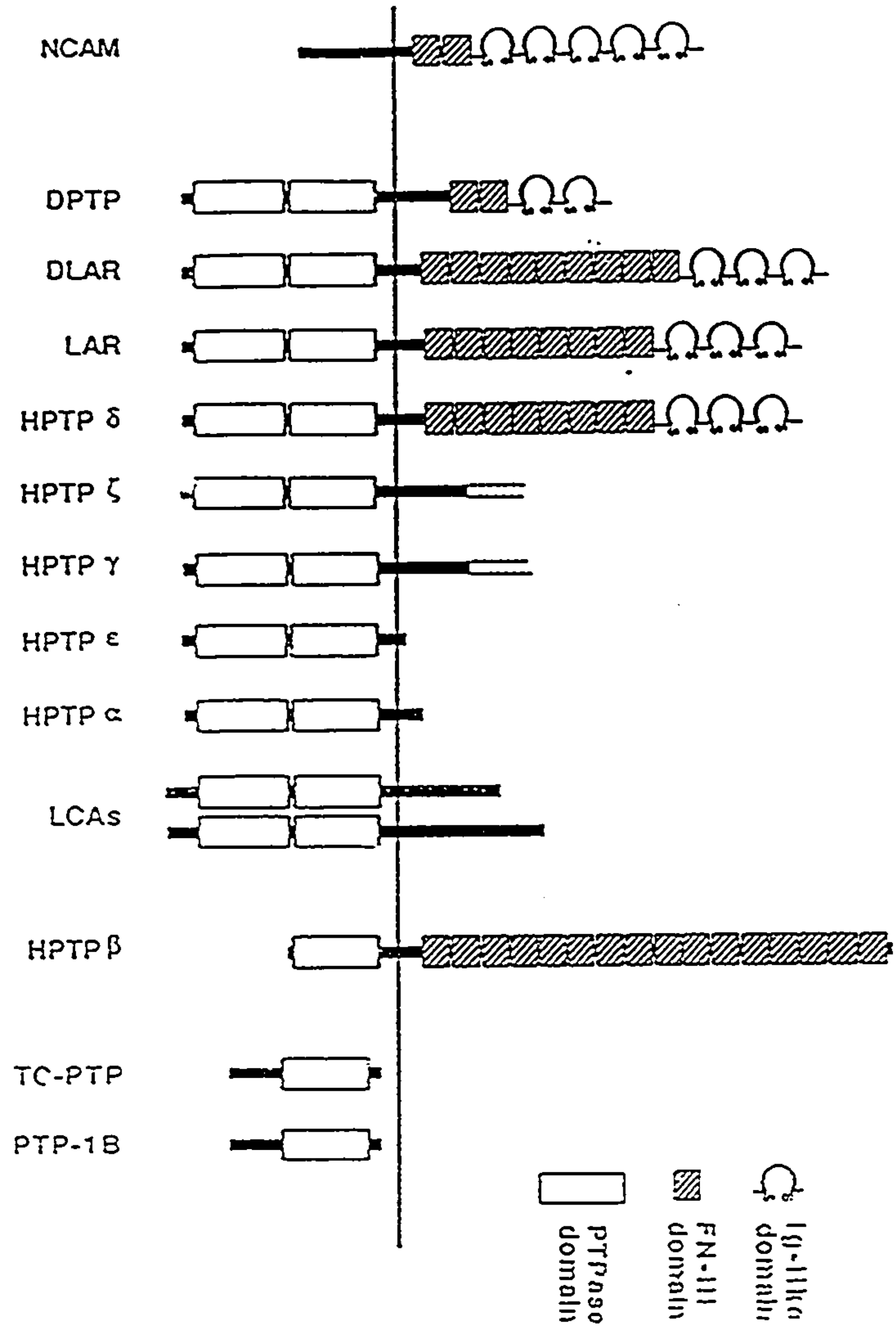
The discovery of transmembrane pTPs resulted from the determination of amino acid sequences by microsequencing peptides derived from the placental pTP isolated by Tonks et al. (1988a). Such sequences were homologous to those in the human transmembrane receptor-like proteins of unknown function, namely Leukocyte Common antigen (LCA), also known as CD45, and leukocyte antigen related protein (LAR) (Charbonneau et al., 1988; Charbonneau et al., 1989). Both of these proteins were subsequently shown to possess tyrosine specific phosphatase activity (Tonks et al., 1988c; Tonks et al., 1990; Streuli et al., 1990). Since this discovery, seven different human (Streuli et al., 1987; Streuli et al., 1988; Krueger et al., 1990; Kaplan et al., 1990), two mouse (Sap et al., 1990; Matthews et al, 1990), and two Drosophila (Streuli et al., 1989) transmembrane pTPs have been identified by cDNA cloning.

Figure IIIa shows schematically the structures of most of the pTPs that have been identified to date. The presence of extracellular domains in these pTPs suggests that they may be activated by the binding of specific ligands. As yet, no ligands for these phosphatases have been identified. Several of these pTPs, including human LAR, Drosophila LAR, and HPTP-delta, contain IgG-like and type III fibronectin-like repeats within their extracellular domains, while HPTP-beta contains only the type III fibronectin like repeats (Streuli et al., 1988; Streuli et al., 1989; Kreuger et al., 1990). These structures are characteristic of cell adhesion molecules, and thus has led to the hypothesis that the activity of these enzymes is regulated by cell-cell contact and may have a role in regulating contact inhibition of growth.

With the exception of HPTP-beta, all of the transmembrane pTPs that have been identified to date contain two potential catalytic domains separated by linker sequences

Figure IIIa. Schematic Comparison of Known pTPs

The three characteristic domain structures [the immunoglobulin-like (IgG-like), Fibronectin-III-like (FN-like), and pTP catalytic domains) are shown symbolically. Both IgG-like and FN-like domains are about 100 amino acids in length, whereas the pTP catalytic domain is about 300 amino acids long. The thick vertical lines represent segments of protein that are not related to other molecules. Two of the several LCA (CD45) isoforms generated by alternative splicing are shown. The extracellular regions of HPTP-gamma and HPTP-zeta are not yet completely sequenced. The structure of the neural cell adhesion molecule (NCAM) is included for comparison. The NCAM molecule does not have a pTP domain, but the extracellular receptor segment is similar to many of the transmembrane pTPs. The horizontal line represents the plasma membrane. [This figure and legend have been adapted from Saito and Streuli, (1991).]


NCAM
DPTP
DLAR
LAR
HPTP δ
HPTP ζ
HPTP γ
HPTP ε
HPTP α
LCAs
HPTP β
TC-PTP
PTP-1B
PTPase domain
FN-III domain
Ig-like domain

of varying length. Mutational analysis of CD45 and LAR suggested that perhaps only the NH_2-terminal domain, domain 1 (closest to the membrane), possessed catalytic activity, and that sequences in the second domain played a role in determining substrate specificity (Streuli et al., 1990). However, recent studies concerning the pTP activity of HPTP alpha against several different substrates suggested that both domains do indeed possess pTP activity. However, the two domains have quite different substrate specificities (Wang and Pallen, 1991). Further, the results of these experiments suggested that inactivation of the first domain may suppress the activity of the second domain. It is possible that in the first experiments involving LCA and LAR, the pTP activity of the second domain was not observed as a result of this phenomenon.

The most studied of all the receptor pTPs is CD45, a protein which is expressed predominantly on cells of hematopoietic origin, and is present in the membranes of these cells in multiple, differentially spliced isoforms (Streuli et al., 1988). Alternative splicing of exons 4, 5, and 6 appears to give rise to the different isoforms, and CD45 isoforms appear to be differentially expressed on different lymphoid cells (Woollett et al., 1985; Coffman and Weissman, 1981; Lefrancois and Bevan, 1985). Changes in isoform expression have also been shown to accompany T cell activation (Birkeland et al., 1989). The significance of isoform switching has not been determined, however alteration of ligand binding specificity and/or the ability to associate with other proteins to form functional signal transducing complexes have been suggested as possible outcomes of isoform switching.

Before the discovery that LCA possesses tyrosine phosphatase activity, little was known about the functional role of LCA in hematopoietic cells. Since this discovery however, much progress has been made. It is quite clear now that CD45 plays a very important role in regulating the transduction of signal information originating from several different cell surface receptors, including the T cell antigen receptor, the B cell antigen receptor, and the T cell surface molecule CD2 (Pingel and Thomas, 1989; Koretzky et al., 1990; Koretzky et al., 1991; Justement et al., 1991).

Cell surface expression of CD45 is required for coupling activation of both the T cell receptor and the CD2 protein to phosphatidylinositol turnover and the subsequent production of the secondary messengers DAG and Ca^{+2} (Koretzky et al., 1990; Koretzky et al., 1991). Production of interleukin 2 in response to CD2 and T cell activation is also blocked (Koretzky et al., 1991). Similarly, CD45 is also required for induction of Ca^{+2} mobilization in response to B cell activation (Justement et al., 1991). Evidence has been obtained indicating that CD45 is physically associated with components of both the B cell and T cell antigen receptors, thus putting it in an ideal location for mediating transduction of signal information originating from these receptors (Volarevic et al., 1990; Justement et al., 1991).

One possible mechanism through which CD45 might act involves the dephosphorylation of $p56^{lck}$ at tyrosine 505, a site that has been demonstrated to negatively regulate pTK activity (Armein and Sefton, 1988; Marth et al., 1988). As described earlier, $p56^{lck}$ is physically associated with CD4 and CD8. These cell surface proteins recognize MHC determinants on antigen presenting cells, and appear to play an important role in regulating the activation of and the transmission of signal information from the T cell antigen receptor (Rudd et al., 1989).

The hypothesis that CD45 functions as a positive regulator of $p56^{lck}$ pTK activity during T cell activation is supported by several important observations. First, crosslinking of CD4 and CD45 molecules on the cell surface has been demonstrated to potentiate T cell activation, whereas conjugation of CD45 with other cell surface markers inhibited activation (Ledbetter et al., 1988). Second, studies employing cells expressing wildtype CD45, cells expressing no CD45, and revertant $CD45^{+}$ cell lines have shown a correlation between the presence of CD45 and dephosphorylation of the tyrosine 505 site in $p56^{lck}$ (Ostergaard et al., 1989). Finally, through experiments involving the mixing immunoprecipitates containing CD4/$p59^{lck}$ and CD45, it has recently been demonstrated that CD45 can dephosphorylate tyrosine 505 *in vitro*, and this dephosphorylation results in activation of $p56^{lck}$ kinase activity (Mustelin and Altman, 1990).

Several other experimental observations have provided indirect evidence in support of the hypothesis that CD45 is involved in activation of $p56^{lck}$, and that in turn this activation is important in mediating T cell response to antigen stimulation. CD4/$p56^{lck}$ association has been demonstrated to be required for antigen induced signal transduction in the T cell activation process (Glaichenhaus et al., 1991). It has been reported that, in response to antigen, CD4 molecules move within the membrane and migrate to positions in the vicinity of or physically in contact with T cell antigen receptor, a complex which includes the tyrosine phosphatase CD45 (Saizawa et al., 1987; Rivas et al., 1988; Mittler et al., 1989; Volarevic et al., 1990). Further evidence that CD4, CD45, and the T cell receptor form a stable complex has been provided by the results of co-capping experiments (Dianzani et al, 1990). Finally, another observation that supports the above hypothesis is that the level of tyrosine phosphorylation of cellular proteins was markedly decreased following T cell receptor stimulation of a $CD45^-$ mutant cell line (Koretzky et al, 1991)

At present virtually nothing is known about mechanisms regulating the enzymatic activity of transmembrane pTPs. Complex formation with other proteins could provide one possible mechanism, however evidence in support of this has not been presented. It is possible that phosphorylation of CD45 plays a role in regulating its activity. It contains consensus recognition sites for phosphorylation by casein kinase II and PKC (Charbonneau et al., 1988). However, in vitro phosphorylation of CD45 using these enzymes did not result in any change in pTP activity (Tonks et al., 1990). Rapid and transient phosphorylation of CD45 on tyrosine residue(s) has also been reported to result in response to activation of the T cell receptor (Stover et al., 1991). Identification of this modification required stringent conditions involving the use of pTP inhibitors in the isolation buffers. The physiological significance of this phosphorylation event has not been determined.

1-IIIb. Nonreceptor pTPs

The second family of pTPs is comprised of those pTP proteins that contain neither a transmembrane nor an extracellular domain, and, as with the pTKs, is designated the nonreceptor class. The existence of several members, or subtypes, within this class of pTPs was suggested from the results of the biochemical analyses of cytoplasmic pTPs described earlier, and has been supported by the results of cDNA cloning.

Six different nonreceptor pTPs have been identified from analysis of human cDNAs (Cool et al., 1989; Chernoff et al., 1990; Brown-Shimer et al., 1990; Sadhu et al., 1990; Shen et al., 1991; Yang and Tonks, 1991; Gu et al., 1991). Included amongst these is the cDNA encoding the pTP1B protein purified by Tonks et al (1988) (Chernoff et al., 1990; Brown-Shimer et al., 1990). Other nonreceptor pTPs include four yeast pTPs (Russell and Nurse, 1986; Russell et al., 1989; Ottilie et al., 1991; Guan et al., 1991), two different rat brain pTPs (Guan et al., 1990; Lombroso et al., 1991), one mouse T cell pTP (Mosinger et al.; 1991), and one Drosophila pTP (Edgar and O'Farrell, 1989). Including the low molecular weight bovine liver acid phosphatase (Camici et al., 1989), the amino acid sequences of a total of ten different types of nonreceptor pTPs have been determined.

As with the transmembrane class, little is known about the mechanisms whereby the enzymatic activities of the nonreceptor pTPs are regulated, however, the isolation two heat stable inhibitors of bovine brain pTPs has been reported (Ingebritsen, 1989). These inhibitors were shown to be distinct from the two heat stable inhibitors of the type 1 phosphoseryl/phosphothreonyl specific protein phosphatases (pSPs).

Phosphorylation has also been implicated in the regulation of this class of pTP. Activators of PKA and PKC have been demonstrated to increase the activity of an as yet unidentified membrane associated pTP (Brautigan and Pinault, 1991). This pTP was present in the membrane fraction of monkey

CV-1 cells as a 150 kd protein complex containing a 55 kd catalytic subunit. The identity of the subunit phosphorylated in response to the pSK activators was not resolved, however dissociation of the complex was not required for the activation of pTP activity. Interestingly, PKA did not directly phosphorylate the pTP in vitro, suggesting that another kinase(s) might act as an intermediate between PKA and the pTP.

Subcellular localization likely plays a key role in determining the accessibility of the different pTPs to their respective substrates. The pTP1B protein isolated by Tonks et al. (1988) is reportedly associated with both the particulate and soluble portions of placental extracts. Association with the membrane fraction has been proposed to be mediated by isoprenylation and/or palmitoylation of the COOH-terminus of the protein (Guan et al., 1990). Similar modifications have been suggested for a rat brain pTP enriched in striatum (Lombroso et al., 1991). In this case, a potential NH_2-terminal myristoylation site was also identified.

The COOH-terminal amino acid sequences of pTP1B and the human T cell pTP may contain other regulatory elements. The two proteins share good homology over their NH_2-terminal and phosphatase catalytic domains, but diverge at their COOH-termini (Chernoff et al., 1990; Guan et al., 1990; Brown-Shimer et al., 1990; Cool et al., 1989). Removal of COOH-terminal sequences of the T cell pTP by limited trypsin digestion was found to enhance phosphatase activity (Zander et al., 1991).

Similarly, when a full length T cell pTP cDNA was expressed in baby hamster kidney cells, the protein was essentially inactive in an *in vitro* assay unless first subjected to tryptic digestion (Cool *et al.*, 1990). The full length and truncated forms of the protein also differed in their patterns of subcellular distribution. The full length product was associated solely with the particulate fraction whereas the truncated form was associated with both the soluble and particulate fractions. These results suggested that the COOH-terminal domain of the T cell pTP plays an important role in regulating the activity and subcellular localization of the protein. Because pTP1B also contains a large COOH-terminal domain, it has been suggested that this domain plays a similarly important role in regulation of pTP1B activity and intracellular localization.

When the full length T cell pTP was expressed *in vivo*, it appeared to be part of a large complex of >600 kd. Limited proteolysis was required for activation of phosphatase activity (Cool *et al.*, 1990). Similarly, the monkey cell pTP discussed above was also found to be part of a large molecular weight complex, and it too was activated by limited proteolysis (Brautigan and Pinault, 1991). In this case, activation was associated with dissociation of the catalytic fragment from the large molecular weight complex. Since digestion of either protein yielded catalytically active fragments of similar size, and, as discussed above, removal of the COOH-terminus of the T cell protein is associated with enzymatic activation, it has been suggested that the COOH- terminal sequences of these proteins may direct their association with other proteins to form large molecular weight complexes, and that association of the pTP with these other proteins may play an important role in regulating pTP activity. The other protein components of the complex could represent subunits that regulate pTP activity, or control subcellular localization. Unique COOH-terminal sequences of different pTPs may determine the proteins with which the nonreceptor class pTP can associate.

Not all nonreceptor pTPs have large COOH-terminal domains capable of directing subcellular distribution and complex formation. Some pTPs contain other types of domains that appear to play similar roles. Recently, a cDNA isolated from a human breast carcinoma line was found to encode a pTP that lacked a large hydrophobic COOH-terminal domain such as those found in the T cell and pTP1B pTPs, but instead contained an NH_2-terminal domain that exhibits homology to the SH2 domains present in nonreceptor pTKs and several other proteins involved in signal transduction (Shen et al., 1991). Little is known about the role this domain plays in regulating phosphatase activity, but its presence suggests the involvement of this pTP in signal transduction pathways, and this domain likely serves to mediate the association of this pTP with other proteins that contain phosphorylated tyrosine residues. At present, however, the subcellular distribution of the protein is not known.

Two other pTPs which lack a COOH-terminal regulatory domain have also been cloned very recently. Interestingly, these phosphatases contain an NH_2-terminal domain that bears homology to domains present in the cytoskeletal proteins band 4.1, ezrin, and talin (Gu et al., 1991; Yang and Tonks, 1991). This domain is separated from the COOH-terminal pTP catalytic domain of these proteins by approximately 300 amino acids of unknown function. Band 4.1, ezrin, and talin all localize to points of interaction between the cytoskeleton and the plasma membrane, and likely play a role in regulating cell shape (Burridge et al., 1988, Gould et al., 1986; Anderson and Marchesi, 1985). It is the NH_2-terminal domains of these proteins, with which the above pTPs share homology, that appear to regulate the subcellular localization of these proteins (Anderson and Marchesi, 1985). As discussed earlier, many cytoskeletal proteins are substrates for activated pTKs, including ezrin and talin, and phosphorylation of these proteins in transformed cells is associated with changes in cellular morphology. It is possible that the newly identified pTPs play an important role in regulating cell shape by controlling the tyrosine phosphorylation state of cytoskeletal proteins.

1-IIIc. Regulation of Cell Growth by pTPs

The accumulation of a great deal of evidence that overexpression or activation of pTKs stimulates cell growth led to the speculation that pTPs function to negatively regulate cell growth, and that underexpression or inactivation of pTPs could lead to cell transformation. In other words, it has been suggested that pTPs possess properties associated with antioncogenes or tumor suppressors. Several experimental observations have supported this hypothesis. HPTP gamma, a pTP isolated by Kaplan et al. (1990), has recently been identified as a candidate tumor suppressor gene involved in development of renal cell (RCC) and lung cell (LC) carcinomas (LaForgia et al., 1991). HPTP gamma was localized to chromosome 3, subregion band p21, at a position near the breakpoint associated with the development of RCC and LC tumors. Screening of clinical samples and several tumor cell lines derived from RCC and LC tumors demonstrated hemizygous deletion of the HPTP gamma gene in a high percentage of the samples tested. While not all samples exhibited deletion of the HPTP gamma gene, in those cases where loss was not observed, there may have been more subtle disruptions of the gene that could not be detected using the screening method employed. These observations suggest, but do not prove, that HPTP gamma has properties consistent with antioncogenes or tumor suppressors.

Suppression of cell growth due to the activation of pTPs has also been reported. Activation of membrane associated pTP activity has recently been reported to correlate with contact inhibition of growth of cultured cells (Pallen and Tong, 1991). pTP activity increased only as the rate of cell proliferation decreased and cells approached saturation density, with maximum activity being associated with the quiescent state. Serum starvation induced inhibition of growth at low cell density was not correlated with an increase in pTP activity, indicating that the stimulation of pTP activity was density dependent and likely required some level of cell contact. These observations were consistent with the existence of a regulatory mechanism that maintains pTP activity at a basal level during cell proliferation but allows for density dependent activation of pTP activity at quiescence. They were also consistent with the observations of Klarlund (1985) who observed that vanadate, a potent inhibitor of pTP activity, had no effect on cell growth during the exponential phase of cell growth but allowed cells to continue growth to high saturation densities and to overcome contact inhibition of growth. A regulatory mechanism involving an alteration in basal pTP activity levels would likely help to provide conditions more favorable for cell growth.

Tyrosine phosphorylation plays an important role in controlling the progression of cells through mitosis by negatively regulating the activity of $p34^{cdc2}$, a serine/threonine protein kinase that becomes activated during the G2 phase of cell division following DNA replication (Gould and Nurse, 1989). p80^{cdc25}, a protein which accumulates during the G2 phase of the cell cycle, is required for activation of p34^{cdc2} (Russell and Nurse, 1986; Moreno et al., 1990). The observations that p80^{cdc25} function can be complemented by the expression of a T cell pTP in yeast (Gould et al., 1990), and that it can control tyrosine dephosphorylation of p34^{cdc2} in a cell free system (Kumagai and Dunphy, 1991) suggested the possibility that p80^{cdc25} protein was itself a tyrosine phosphatase. However, no pTP activity could be directly associated with this protein, nor did the predicted amino acid sequence of p80^{cdc25} show high homology to known pTP sequences (Russell and Nurse, 1986; Russell et al., 1989; Edgar and O'Farrell, 1989; Sadhu et al., 1990). The failure to demonstrate pTP activity appears to have been due to the use of inappropriate substrates, as two groups have recently demonstrated that p80^{cdc25} does in fact contain an intrinsic tyrosine phosphatase activity (Dunphy and Kumagai, 1991; Gautier et al., 1991). This finding suggested that there may be other pTPs with very restricted substrate specificity that will require the use of suitable substrates for their identification, and that the pTP catalytic domain consensus sequences will have to be redefined.

When we began our investigation of pTP activities in chicken embryo fibroblasts several years ago, very little was known about pTPs and their involvement in regulating cellular processes. The goal of this work was to purify a tyrosine phosphatase to homogeneity, and to clone the corresponding cDNA encoding the enzyme. It was our hope that these studies would lead to a greater general understanding of pTPs. Further, it was also hoped that these studies would also provide tools that could be used in future experiments designed towards gaining a better understanding of pTP functions and their roles in regulating cell growth and differentiation.

CHAPTER 2: MATERIALS AND METHODS

2-I. CHEMICALS AND REAGENTS

All reagents used were of commercial reagent grade or better and were used without further purification. Whatman DE52 cellulose was purchased from Mandel Scientific. DNA sequencing kits, DNA restriction enzymes, dextran sulphate, Protein A-sepharose, Sephacryl S-200, Sephadex G-75, Cyanogen Bromide activated sepharose, Wheatgerm Lectin sepharose 6MB, Deoxy-nucleic acids, and nucleic acids grade agarose were purchased from Pharmacia Fine Chemicals. Oligonucleotides were purchased from the Institute for Molecular Biology at McMaster University (MOBIX). Radiolabelled compounds were purchased from New England Nuclear, DuPont. gamma-thio-ATP and Enolase were purchased from Boerhinger Mannhiem. Protein assay dye reagent, gamma globulin protein standard, and molecular weight standards for the calibration of molecular sieving resins and SDS-PAGE gels were purchased from Bio-Rad. Centriprep-10 and Centriprep-30 concentrators were purchased from Amincon. Nitrocellulose filters were purchased from Schleicher and Schuell. The total chicken embryo cDNA library packaged in lambda gt11 virus (library CL1001b) was purchased from Clontech. The pGEM 7zf(+) plasmid and thermostable Taq DNA polymerase were purchased from Promega. Rabbit anti-mouse IgG antibody was purchased from

Cappel. Strataclean resin was purchased from Stratagene. DNA nick translation labelling kit was purchased from Bethesda Research Laboratories. Purified Calcineurin (phosphatase 2B) was the generous gift of Dr. J. Wang (University of Calgary, Alberta). All remaining chemicals and reagents were purchased from Sigma.

2-II. ANIMALS

Fertilized, COFAL-negative, and chicken helper factor-negative chicken eggs used for preparation of primary chicken embryo fibroblasts (CEFs) were purchased from Hyline International. Fertilized chicken eggs used to prepare chicken embryo bodies used for phosphotyrosyl-protein phosphatase purification were purchased from Westdale Hatchery, Hamilton, Ontario.

2-III. CELL CULTURE

Primary chicken embryo fibroblasts (CEFs) were prepared as previously described (Branton and Landry-Magnan, 1979). The cell line F.2 R2-H1, which expresses wt $p130^{gag-fps}$ (Weinmaster et al, 1984), and the Rous sarcoma virus transformed field vole cells (RSV-vole) (Purchio et al., 1983) were grown in Dulbecco medium (low glucose) supplemented with 10% fetal calf serum.

2-IV. BACTERIA and BACTERIOPHAGE CULTURE

2-IVa. E. coli Growth

Cultures of E. coli were grown at 37 °C under standard conditions (Ausubel et al., 1989) in LB broth plus the appropriate antibiotic for plasmid selection, if required. LB broth was supplemented with 0.2% maltose for Y1090 cultures to be infected with bacteriophage lambda gt11.

Table 2-IVa. Bacteria and Bacteriophage Strains

Name	Genotype
MV1190	(lac-pro AB), thi, supE, (sr-recA)306::Tn10 [F':traD36, proAB, lacI^{q}Z M15]7
Y1090	Δlac$_{U169}$, proA^{+}, Δlon, araD139, strA, supF, trpC22::tn10/, pMC9 (The plasmid pMC9 directs the synthesis of large amount of the lambda repressor and carries a tetracycline resistance gene)
lambda gt11	lac5, srl3°, c1857, srl4°, nin5, srl5°, Sam100, cl^{ts}, int^{+}, red^{+}

2-IVb. Growth and Isolation of Lambda gt11 Bacteriophage

Stocks of bacteriophage were grown as liquid or plate lysates prepared using standard protocols (Ausubel et al., 1989).

2-V. PREPARATION of CHICKEN EMBRYO BODIES for PHOSPHATASE PURIFICATION

11 day old chicken embryos were removed from the egg and the head, wings, legs, and internal organs were removed by dissection. The resulting material was washed twice with ice cold phosphate buffered saline solution (PBS) and frozen at -70 °C until use.

2-VI. PREPARATION of REDUCED CARBOXYAMIDOMETHYLATED AND MALEYLATED LYSOZYME

Reduced carboxyamidomethylated and maleylated (RCM) lysozyme was prepared according to the method of Tonks et al. (1988a), with the following modification. At the final step, rather than purifying the RCM lysozyme by chromatography on Sephadex G-25, the modified protein was precipitated by the addition of 100% w/v TCA to a final concentration of 20%. The protein was collected by centrifugation at 30,000 x g for 10 min, and the pellet washed 3 times with ice cold 20% w/v TCA. The final pellet was resuspended in 25 ml 0.5 M Tris-HCl (pH 8.5), stirred overnight at 4 °C, and then dialyzed overnight versus 4.0 l 20 mM Imidazole-HCl (pH 7.2). The dialysate was clarified by centrifugation at 10,000 x g for 10 min, diluted to a final concentration of 20 mg/ml, and then stored in 5.0 ml aliquots at -20 °C.

2-VII. PREPARATION of RECEPTOR TYROSINE PROTEIN KINASES

Receptor tyrosine kinase preparations used for the generation of phosphotyrosyl-RCM lysozyme substrate were prepared by the method of Pike et al. (1984), with the exception that the synthetic peptide poly[glu:tyr (4:1)] was used as the substrate to measure kinase activity. When necessary, kinase preparations were concentrated to a final concentration of 1 - 4 units/ml (1 unit = 1 nmole ^{32}P incorporated per min) by centrifugation using Centriprep-30 concentrators. Aliquots containing at least 10 units of kinase activity were then frozen at -70 °C.

2-VIII. PREPARATION of ^{32}P-LABELLED SUBSTRATES

2-VIIIa. ^{32}P-Labelled Phosphoseryl-Casein and Phosphotyrosyl-IgG

^{32}P-Labelled phosphoseryl-casein and ^{32}P-labelled phosphotyrosyl-IgG were prepared as previously described (Nelson and Branton, 1984), with the exception that the $pp60^{v-src}$ required for IgG phosphorylation was immunoprecipitated from RSV-vole cells rather than RSV infected chicken embryo fibroblasts.

2-VIIIb. ^{32}P-Labelled Phosphotyrosyl-Enolase

^{32}P-Labelled phosphotyrosyl-enolase was prepared as follows. Approximately 10^{10} F.R2-H1 cells were disrupted in 30 mls FPS-lysis buffer [20 mM Tris-HCl (pH 7.0) containing 0.15 M NaCl, 1.0 mM EDTA, 1.0% (v/v) NP-40, and 5.0% (w/v) sodium deoxycholate]. After 20 min on ice, the lysate was centrifuged at 15,000 x g for 20 min. The supernatant was then combined with 0.75 ml packed protein-A sepharose beads, 0.3 ml rabbit anti-mouse IgG antibody, and 0.45 ml 1/100 diluted anti-$p19^{gag}$ ascites fluid (Ingman-Baker <u>et al.</u>, 1984). After 3 hrs of constant mixing at 4 °C, the beads were removed by centrifugation at 30 x g for 30 sec. The beads were then washed twice with FPS-lysis buffer and 3 times with FPS-kinase buffer

[100 mM HEPES (pH 7.0) containing 20 mM $MnCl_2$]. The beads were then incubated at 30 °C for 30 min in a 0.8 ml reaction mixture containing 0.35 ml FPS-kinase buffer, 0.15 ml ^{32}P-gamma-ATP (10.0 mCi/ml with a specific activity of 3000 Ci/mM), and 0.3 ml acid denatured enolase [0.6 mg enolase in 0.3 ml 20 mM Tris-HCl (pH 7.0) containing 2.0 mM dithiothreitol (DTT) and 2.0 mM $MnCl_2$ was incubated with 0.3 ml 50 mM acetic acid for 30 min at 30 °C. Following incubation, a further 0.3 ml 50 mM acetic acid was added. This procedure was carried cut just prior to the addition of the enolase to the kinase reaction mixture.]. The kinase reaction was terminated by the addition of 0.8 ml 2x SDS-PAGE sample buffer [0.2 mM Tris-HCl (pH 6.8) containing 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol (2-ME), and 20% (v/v) glycerol]. The samples were then boiled for 5 min and the beads removed by centrifugation. The resulting supernatant was applied to a 7.5% polyacrylamide gel with a 5.0% stacking gel and electrophoresed overnight at a constant voltage of 80 volts. ^{32}P-Labelled protein bands were visualized by autoradiography using Kodak XAR-5 X-ray film. The band corresponding to phosphorylated enolase was cut from the gel and the enolase was extracted from the gel by elution into 50 mM ammonium bicarbonate containing 0.1% SDS and 4% 2-ME. Gel debris was removed by centrifugation, and following the addition of 100 ug bovine serum albumin (BSA) as carrier protein, 100% (w/v) trichloroacetic acid (TCA) was added to a final concentration 25% (v/v). The solution was allowed to sit at 4 °C for 90 min and then centrifuged at 15,000 x g for 30 min. The pellet was resuspended in acetone containing 2% (v/v) 1.0 N HCl and then recentrifuged. The pellet was then resuspended in 3.0 ml buffer T [20 mM Tris-HCl (pH 7.0) containing 10% (v/v) glycerol, 2.0 mM EDTA, 2.0 mM EGTA, and 10 mM 2-ME], and the pH adjusted to 7.

In some cases, following the removal of the protein-A beads after the kinase reaction, 100% TCA was added to the supernatant to a final concentration of 25% and the solution allowed to sit at 4 °C for 60 min. Precipitated protein was collected by centrifugation at 15,000 x g for 5 min, resuspended in 1.0 N NaOH, and incubated at 37 °C for 90 min. 100% TCA was then added to 25% and the solution allowed to sit on ice for 60 min. The precipitate was collected by centrifugation at 15,000 x g for 5 min, resuspended in 0.5 ml acetone containing 2% (v/v) 1.0 N HCl, and recentrifuged at 15,000 x g for 5 min. The resulting pellet was resuspended in buffer T and the pH adjusted to 7. This procedure was only used to prepare large quantities of substrate for use in assaying column fractions over the course of phosphatase purification.

2-VIIIc. ^{32}-Labelled RCM-Lysozyme

A cocktail containing 2.0 ml 1.0 M Imidazole (pH 7.2), 1.5 ml glycerol, 0.5 ml 5.0 M NaCl, 0.2 M 1.0 M $MnCl_2$, 0.6 ml 1.0 M magnesium acetate, 0.1 ml 2% Triton X-100, 0.05 ml 0.2 M

EGTA pH 7.0, and 0.05 ml 0.1 M Na_3VO_4 pH 12, as well as a hormone mix containing 0.46 ml 200 μg/ml Epidermal growth factor (EGF), 0.048 ml 500 μM Insulin, 0.1 ml 1.0 M dithiothreitol (DTT), 0.25 ml 1.0 M Imidazole (pH 7.2), 15 μl 40 mg/ml BSA, and 1.627 ml H_2O, were prepared in advance of substrate preparation.

An aliquot of receptor tyrosine kinase preparation, 1.75 ml cocktail, 1.25 ml hormone mix, 5.0 ml 15.0 mM ATP, 5.0 ml 1% sodium deoxycholate containing 0.5 mM ammonium molybdate, 2.5 ml 20 mg/ml RCM-lysozyme, 4.0 mCi ^{32}P-gamma-ATP, and H_2O to a final volume of 25.0 ml were mixed and incubated overnight (18-20 hrs) at 30 °C. Ice cold 100% w/v TCA was then added to a final concentration of 20% and the mixture incubated at 4 °C for 1 hr. The precipitated protein was collected by centrifugation at 30,000 x g for 10 min and the pellet washed 3 times with ice cold 20% w/v TCA.

The final pellet was resuspended in 10 ml 0.5 M Tris-HCl (pH 8.5), stirred overnight at 4 °C, and then dialyzed overnight versus 4.0 l 20 mM Imidazole (pH 7.2). Following clarification by centrifugation, aliquots were stored at 4 °C.

2-IX. PREPARATION OF THIO-PHOSPHOTYROSYL RCM LYSOZYME SEPHAROSE COLUMN

RCM lysozyme (40 mg) was phosphorylated as described above except that ^{35}S-gamma-thio-ATP was substituted for ^{32}P-gamma-ATP. The thio-phosphorylated protein was then dialyzed overnight versus 0.1 M sodium borate (pH 8.5) containing 0.5 M NaCl, and coupled to CNBr activated sepharose according to the manufacturer's instructions. The final product was then equilibrated with buffer A2 (see section XIa) and stored at 4 °C in the presence of 0.02% sodium azide. The coupling efficiency was greater than 90%.

2-X. PROTEIN PHOSPHATASE ASSAYS

The standard phosphotyrosyl and phosphoseryl protein phosphatase assays were performed as previously described (Nelson and Branton, 1984).

p-Nitrophenol phosphatase activity was assayed in a 1.0 ml reaction volume containing 20 mM p-nitrophenolphosphate (pNPP) as substrate. Reactions performed at pH 5.0 were carried out using 100 mM sodium acetate buffer, at pH 7.0 in 100 mM Tris-HCl buffer, and at pH 10.5 in 100 mM sodium carbonate buffer. All assays were performed for 60 min at 37 °C. The reactions were terminated by the addition of 1.0 ml of 2.0 M sodium carbonate and the absorbance of the solution at 410 nm determined. The concentration of the reaction product p-nitrophenol formed was determined using a molar extinction coefficient of 17,800.

2-XI. PURIFICATION of PHOSPHOTYROSYL-PROTEIN PHOSPHATASES

All steps were performed at 4 °C and 5.0 ml fractions were collected in all cases except for the DE52 chromatography step, in which case 10 ml fractions were collected.

2-XIa. Purification of pTPIII

Approximately 400 g of chicken embryo bodies were minced, and then homogenized in 1.0 l of 100 mM Tris-HCl (pH 7.0) containing 2.0 mM EDTA, 2.0 mM EGTA, 25 mM benzamidine-HCl, 10 mM 2-ME, and 0.1 mM phenylmethylsulfonylfloride (PMSF). The homogenate was centrifuged at 40,000 x g for 30 min. The supernatant was collect and centrifuged at 100,000 x g for 60 min. The resulting supernatant, designated the cell extract, was filtered through glass wool and applied to a Whatman DE52 column (4.5 x 26 cm) previously equilibrated with buffer A (20 mM Imidazole-HCl (pH 7.2), containing 2.0 mM EDTA, 2.0 mM EGTA, 1.0 mM benzamidine-HCL, and 10 mM 2-ME). The column was washed with 1500 mls buffer A and then eluted with a 2000 ml linear gradient of 0.0-0.5 M NaCl in buffer A. Phosphotyrosyl-protein phosphatase (pTP) activity present in the column flow through fractions (pTPI) was collected and further purified as will be described later. Salt gradient fractions containing pTP activity (pTPIII) were pooled and applied directly to a Cibracon Blue 3GA-agarose column (2.2 x 27 cm) previously equilibrated with buffer A and the column washed with 500 ml buffer A. pTPIII was eluted with a 500 ml linear gradient of 0.0-1.5 M NaCl in buffer A. Active fractions were pooled and concentrated by centrifugation in Centriprep-10 concentrators.

The concentrated pTPIII sample was then applied to a Sephacryl S-200 column (2.2 x 85 cm) previously equilibrated with buffer A. Active fractions eluted from the column were pooled and applied directly to a poly-lysine Sepharose column (1.6 x 20 cm) previously equilibrated with buffer A2 (buffer A containing 10% v/v glycerol) and the column was washed with 500 ml buffer A2. pTP activity was then eluted with a 250 ml linear gradient of 0.0-0.5 M NaCl in buffer A2. Fractions containing activity were dialyzed overnight versus 4.0 l buffer A2.

The pTP III sample was then applied to a thio-phosphorylated RCM lysozyme-Sepharose affinity column (1.6 x 12) previously equilibrated with buffer A2. The column was washed with 200 ml buffer A2 and then pTP activity was eluted with a 250 ml linear gradient of 0.0-0.25 M NaCl in buffer A2. Active fractions were pooled and dialyzed overnight versus 4.0 l buffer A3 (buffer A containing 50% v/v glycerol). Aliquots were then frozen at -70 °C.

2-XIb. Purification of pTP I

Pooled fractions of pTPI (DE52 flow through) were applied directly to a Red-A Matrix gel column (1.6 x 15) previously equilibrated with buffer B [20 mM Tris-HCl (pH 6.5) containing 20% (v/v) glycerol, 1.0 mM EDTA, 1.0 mM EGTA, 10 mM 2-ME]. The column was washed with 200 mls buffer B and then developed with a 400 ml linear gradient of 0.0-1.5 M NaCl in buffer B. Active fractions were pooled and made 70% saturated by the addition of solid ammonium sulfate. Following incubation at 4 °C for 90 min with constant stirring, the solution was centrifuged at 6,000 x g for 45 min, and the pellet was resuspended in 5.0 ml buffer B. After stirring 60 min at 4 °C, the solution was clarified by centrifugation at 15,000 x g for 20 min. The supernatant was then applied to a Sephadex G-75 column (2.6 x 65 cm) previously equilibrated with buffer B. Active fractions were pooled and applied directly to a Whatman CM52 column (2.2 x 25 cm) previously equilibrated with buffer B2 (buffer B with 0.1 mM EDTA and 0.1 mM EGTA). The column was washed with 500 ml buffer B2 and then developed with a 500 ml linear gradient of 0.0-0.5 M NaCl in buffer B2. Active fractions were pooled, dialyzed overnight versus 4.0 l buffer B2.

The pTP I sample was then applied to a thio-phosphorylated RCM lysozyme-sepharose affinity column (1.6 x 12) previously equilibrated with buffer B2. The column was washed with 200 ml buffer B2 and then pTP activity was eluted with a 250 ml linear gradient of 0.0-0.25 M NaCl in buffer B2. Active fractions were pooled and dialyzed overnight versus 4.0 l buffer B3 (buffer B2 containing 50% v/v glycerol). Aliquots were then frozen at -70 °C until further use.

2-XII. CALMODULIN(CAM)-AGAROSE CHROMATOGRAPHY

Partially purified pTPIII samples (0.5 mls of preparations purified by anion exchange, Cibracon Blue 3GA agarose, and Sephacryl S-200 chromatography) were diluted 1/10 with buffer C [20 mM Tris-HCl (pH 7.0) containing 10% glycerol, 0.3 mM $CaCl_2$, 3.0 mM magnesium acetate, 4.0 mM DTT, and 2.0 mM 2-ME] and stirred at 4 °C for 30 min. The sample was then applied to a 2.0 ml bed volume CAM-agarose column equilibrated with buffer C. The column was washed with 20 ml buffer C, 10 ml buffer C containing 200 mM NaCl, and then eluted with 10 ml of buffer C containing 200 mM NaCl and 2.0 mM EGTA. 2.0 ml fractions were collected during the washes and 1.0 ml fractions were collected during elution.

2-XIII. PROTEIN DETERMINATION

Protein was determined according to the method of Bradford (1976) using the BioRad protein assay dye reagent. Gamma globulin was used as a standard for all assays.

2-XIV. ELECTROPHORESIS

2-XIVa. SDS-PAGE of Proteins

The discontinuous gel system developed by Laemmli (1970), consisting of a 5% stacking gel and a 10% separating gel, was used to resolve proteins. Protein bands were visualized by staining with either coomassie brilliant blue (Chrambach *et al.*, 1967) or silver (Oakley *et al.*, 1980).

2-XIVb. PAGE of DNA Fragments

DNA fragments were resolved on either 4% or 5% acrylamide gels electrophoresed in TAE [40 mM Tris-Acetate (pH 8.5), containing 2mM EDTA] according to standard protocols (Maniatis *et al.*, 1989).

2-XIVc. Phosphoamino Acid Analysis

Purified ^{32}P-labelled protein was hydrolysed in 6N HCl at 110 °C for 1 hr. The hydrolysate was frozen in liquid N_2, overlayed with H_2O, frozen again, and then lyophilized. The pellet was twice resuspended in 50 μl H_2O and dried under vacuum, and finally resuspended in 20 μl H_2O. An aliquot containing approximately 2000 cpm was mixed with 5 μg cold phosphoamino acid markers (p-tyr, p-ser, and p-thr). This aliquot was then spotted onto a cellulose thin layer chromatography plate (Polygram CEL300) and overlayed with 0.5 μl tracking dye (0.1% orange G and 0.1% xylene cyanol). The sample was then subjected to two dimensional electrophoresis. The first dimension involved electrophoresis at pH 1.9 (2.6% formic acid and 8.2% acetic acid) until the orange G tracking dye had travelled from the centre of the plate to the edge closest to the cathode. Following rotation of the plate by 90 °C, the second dimension, electrophoresis at pH 3.5 (0.5% pyridine, 5% acetic acid, and 1 mM EDTA) was performed. Both dimensions of electrophoresis were performed at a constant voltage of 1000 V. Cold phosphoamino acids were detected by staining with ninhydrin. Radiolabelled phosphoamino acids were detected by autoradiography.

2-XIVd. DNA Agarose Gels

Agarose gels were cast according to well established protocols (Maniatis et al., 1989) and were run submerged under TAE buffer.

2-XIVe. DNA Sequencing Gels

The products of DNA sequencing reactions were separated on 8% sequencing gels prepared from 40% (19:1) stock acrylamide solutions. The gels were cast and electrophoresed according to standard laboratory procedures using TBE [89mM Tris-borate (pH 8.3), containing 25 mM EDTA] as the running buffer.

2-XV. DNA SEQUENCING REACTIONS

All sequencing reactions utilized double stranded DNA as a template and were carried out according to the method of Sanger et al. (1977), using the protocol supplied with the T7-DNA polymerase sequencing kit. All reactions were performed using ^{35}S-alpha-dATP (500 Ci/mmole) as the radioisotope.

2-XVI. PURIFICATION of DNA FRAGMENTS

Following digestion with the appropriate restriction enzyme(s), DNA samples were applied to a 5% acrylamide gel and electrophoresed overnight. DNA fragments were then visualized under UV light following staining with ethidium bromide. Fragments of the appropriate size were excised from the gel and transferred to a sterile eppendorf tube. The acrylamide was crushed using a teflon plunger and 0.6 ml DNA elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 1% w/v SDS) was added. The sample was wrapped in tin foil and incubated overnight at 37 °C with mixing.

The acrylamide was then spun down by centrifugation and the supernatant collected. The acrylamide was washed with DNA elution buffer. The supernatants were pooled, filtered through siliconized glass wool, and the DNA collected by ethanol precipitation. The DNA pellet was resuspended in 0.2 ml 0.3 M sodium acetate and then reprecipitated with ethanol. The DNA pellet was then washed with 70% ethanol, dried briefly under vacuum, and then resuspended in 25 μl sterile H_2O.

2-XVII. PLASMID ISOLATION

2-XVIIa. Small Scale Isolation of Episomal DNA

2.0 ml bacterial cultures were grown overnight in the appropriate selection medium. 1.5 ml of the culture was transferred to a sterile eppendorf tube and the bacteria pelleted by centrifugation. The supernatant was removed, the bacteria resuspended in 0.2 ml glucose lysis buffer [25 mM Tris-HCl (ph 8.0) containing 50 mM glucose, 10 mM EDTA, and 4 mg/ml lysozyme]. Following incubation of the suspension at room temperature for 5 min, 0.4 ml freshly prepared 0.2 M NaOH/0.1% SDS was added, and the tubes mixed by inversion. After incubation on ice for 5 min, 0.3 ml 7.5 M ammonium acetate was added, the samples were mixed by vortexing for 30 sec, and then samples were returned to the ice for a further 10 min incubation. Debris was then pelleted by centrifugation for 5 min, the supernatants transferred to a fresh sterile eppendorf, and 0.6 volumes isopropyl alcohol was added. Following a 10 min incubation at room temperature, the samples were centrifuged for 10 min. The resulting pellets were washed with 0.5 ml 70% v/v ethanol, dried briefly under vacuum, and then resuspended in 75 μl sterile H_2O. A 10 μl aliquot was then subjected to restriction digestion analysis to identify the appropriate plasmids. Prior to sequencing, 5 μl strataclean resin was added to each sample. The samples were mixed by vortexing for 15 sec., incubated 1.5 min. at room temperature and then centrifuged for 2 min. The supernatants were transferred to fresh sterile eppendorfs and an 8 μl aliquot of each sample was used in subsequent sequencing reactions.

2-XVIIb. Large Scale Isolation of Episomal DNA

Large scale plasmid isolations were performed according to established protocols (Maniatis et al., 1989). Further purification was accomplished by ultracentrifugation through a 12% CsCl density gradient.

2-XVIII. UNIDIRECTIONAL DELETION of PLASMIDS

Unidirectional deletion of plasmids was performed using Exo III according to the method of Henikoff (1987). Deleted plasmids were used as templates for sequence determinations. Such deletions allow for the use of a single oligonucleotide primer to sequence across the entire length of a plasmid insert.

2-XIX. SCREENING of LAMBDA gt11 cDNA LIBRARY

A total chicken embryo (10-11 day) cDNA library was screened for phosphotyrosyl-protein phosphatase encoding sequences using standard laboratory protocols (Ausubel et al., 1989). Both single stranded synthetic oligonucleotides and double stranded DNA fragments were used as hybridization probes.

The following degenerate oligonucleotides were used as probes: PTP-A, GTICA(C/T)TG(C/T)TC(A/C/T/G)GC(A/C/T/G)GGIGT; PTP-B, GTICA(C/T)TG(C/T)AG(C/T)GC(A/C/T/G)GGIGT (I = inosine triphosphate). These sequences encode the phosphotyrosyl-protein phosphatase consensus amino acid sequence VHCSAGV. The same oligonucleotides were used by Streuli et al. (1989) to clone two Drosophila phosphotyrosyl-protein phosphatases.

The 5' ends of these oligonucleotide probes were labelled with ^{32}P-gamma-ATP (3000 Ci/mmole) according to established protocols (Ausubel et al., 1989). The probes were hybridized overnight at 48 °C in sodium citrate/sodium chloride buffer, and washed at 55 °C.

The DNA fragment probes were labelled with ^{32}P-alpha-dATP (3000 Ci/mmole) through the use of a nick translation kit. All reactions were preformed according to the instructions supplied with the kit. DNA fragments were hybridized overnight in formamide buffer at 42 °C, and washed at 65 °C.

2-XX. DNA AMPLIFICATION USING THE POLYMERASE CHAIN REACTION

DNA amplification using the polymerase chain reaction (PCR) was performed according to the method of Saiki _et al._ (1988), using the reaction buffer supplied by the thermostable Taq DNA polymerase manufacturer.

CHAPTER 3: BIOCHEMICAL ANALYSIS OF CHICKEN EMBRYO PHOSPHOTYROSYL-PROTEIN PHOSPHATASES

Previous studies by our group, using ^{32}P-labelled IgG as substrate, identified at least three separable pTP activities in soluble protein extracts prepared from cultured chicken embryo fibroblasts (CEFs) (Nelson and Branton, 1984). pTPI was present in the flow through of a DE52 (anion exchange resin) column but was retained on a CM52 (cation exchange resin) column. pTPII and pTPIII were retained by and partially separated on DE52. All of the enzymes exhibited similar biochemical characteristics and had little or no activity against a phosphoseryl-protein substrate. Various studies suggested that these enzymes were not related to any of the known phosphoseryl/phosphothreonyl-protein phosphatases.

In the hope of gaining more information about the chicken pTPs, we have extended the studies described above. In order to purify the enzymes sufficiently to allow cloning of the genes that encode them, we have expanded the purification protocol used to isolate these enzymes. The protocol now includes an affinity chromatography step as well as other procedures.

3-I. PURIFICATION OF PHOSPHOTYROSYL-PROTEIN PHOSPHATASES

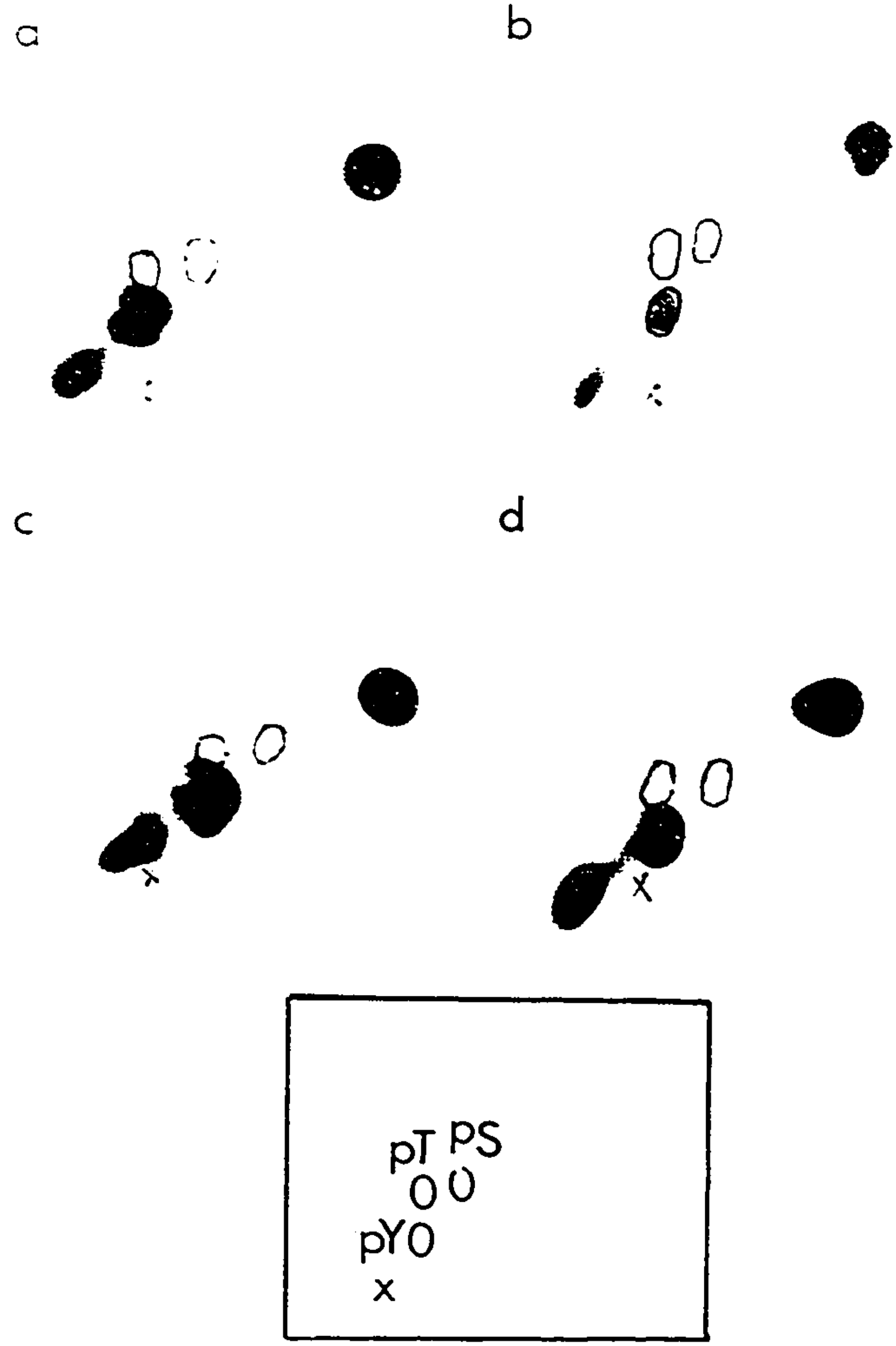
3-Ia. Phosphoamino Acid Analysis of Substrates

Expansion of the protocol used to purify the chicken pTPs required the production of greater amounts of phosphotyrosyl-protein substrate in order to assay column fractions at each step in the purification scheme. Generation of larger amounts of ^{32}P-labelled IgG proved to be impractical. It was therefore necessary to investigate the use alternative substrates. Two other substrates were used in this study. Acid denatured enolase and RCM-lysozyme were phosphorylated in vitro using the pp130$^{gag-fps}$ pTK or receptor pTK preparations (see Materials and Methods).

Figure 1 shows the results of phosphoamino acid analysis of the two alternative phosphotyrosyl-protein substrates used in this study. The results of a similar analysis of the other substrates has been reported elsewhere (Nelson and Branton, 1984). Figures 1a and 1b show the results of analyses of enolase and RCM-lysozyme phosphorylated by the fps pTK. Similarly, Figures 1c and 1d present results obtained with the same substrates phosphorylated by receptor tyrosine kinase preparations. In all cases, the substrates appeared to contain phosphotyrosine as their sole radiolabelled phosphorylated amino acid.

Figure 1. Phosphoamino Acid Analysis of Substrates.

Enolase and RCM lysozyme were phosphorylated *in vitro* with ^{32}P as described in Materials and Methods, using either fps (Figures 1a and 1b, respectively) or placental receptor kinase preparations (Figures 1c and 1d). Aliquots of the reaction mixtures were subjected to phosphoamino acid analysis. Open circles indicate the positions of cold phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) markers. X denotes the origin. The spot in the upper right of each figure represents free phosphate, while that below and to the left of the origin results from incompletely hydrolyzed peptides in the reaction mixture.



a
b
c
d
x
X
pT
pS
pY
x

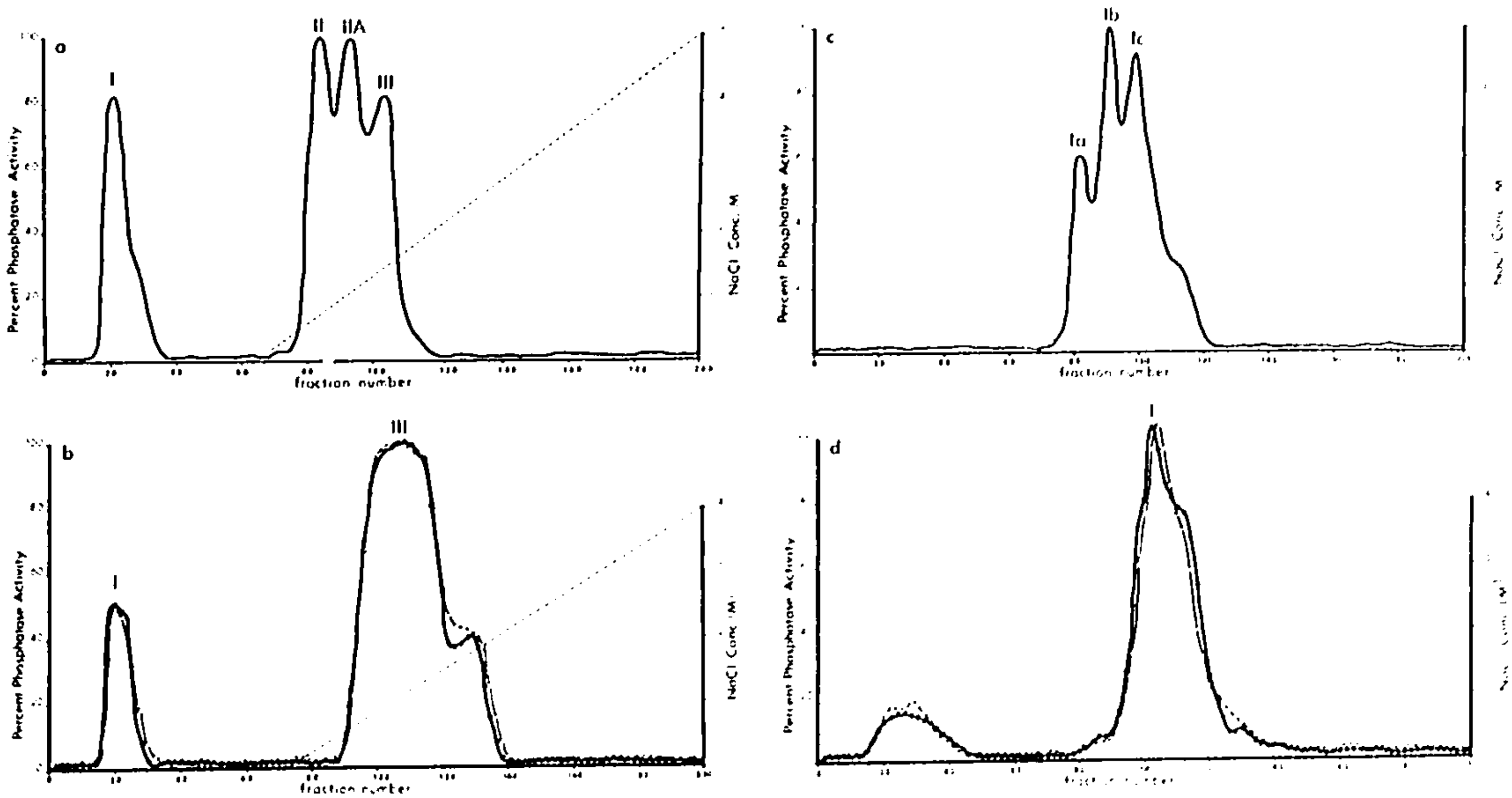
3-Ib. The Effect of Chelators on the Purification of pTPs by DEAE- and CM-Cellulose Chromatography.

Previous studies by our group, using ^{32}P-labeled IgG as substrate, identified the presence of at least three species of pTP activities in soluble extracts prepared from cultured chicken embryo fibroblasts (CEFs) (Nelson and Branton, 1984). In an attempt to separate these species more completely, larger DEAE-cellulose (an anion exchange resin) columns and shallower salt elution gradients were employed. Figure 2A shows that under these conditions, in addition to the activity present in the column flow through, three pTP activities were eluted from the column. Two of these eluted at salt concentrations typical of pTPII and pTPIII, however, a new form was detected, termed pTPIIA, which eluted at a salt concentration intermediate between the other two forms. These data suggested that at least four pTPs could be detected in CEF extracts. In the previous study (Nelson and Branton, 1984), pTPI had been shown to bind to CM-cellulose columns (a cation exchange resin). As shown in Figure 2B, under the expanded chromatography conditions, three species could also be resolved on CM-cellulose, termed pTPIA, pTPIB and pTPIC. These results indicated the presence of a total of at least six identifiable pTP activities in soluble extracts of prepared from CEFs.

Earlier studies on the isolation of serine/threonine specific phosphatases (pSPs) had demonstrated that these

Figure 2. Separation of pTPs by Ion Exchange Chromatography I.

Extracts from CEF prepared in the absence (Fig. 2a) or presence (Fig. 2b) of 2mM EGTA and 2mM EDTA were fractionated by DE52 cellulose chromatography as described in Materials and Methods. Activity present in the flow through from these extracts was rechromatographed on CM52 cellulose (Figs. 2c and 2d, respectively). In all cases aliquots from each fraction were analyzed for pTP activity using ^{32}P-labeled IgG (closed circles) or enolase (open circles) as substrate. The dotted line indicates the salt concentration within the gradient. Values represent the average of duplicate samples and have been expressed as a % of the highest activity observed.


a
I
II
IIA
III
Percent Phosphatase Activity
NaCl Conc. M
fraction number
b
I
III
Percent Phosphatase Activity
NaCl Conc. (M)
fraction number
c
Ia
Ib
Ic
Percent Phosphatase Activity
fraction number
d
I
Percent Phosphatase Activity

enzymes were extremely sensitive to proteolytic degradation, in particular, to degradation by calcium-dependent proteases (Brautigan <u>et al.</u>, 1985; Stewart <u>et al.</u>, 1983). These degradation products have been purified and in many cases they retained phosphatase activity. To determine whether pTPs were similarly sensitive to the actions of these proteases, and to further examine the origin of the multiple pTP activities detected in CEFs, cell extracts were prepared and chromatographed in the presence of 2 mM EGTA and 2 mM EDTA. These components were included in reagents in order to chelate metal ions and thus inhibit metal-dependent proteases. Figure 2C shows that in the presence of these chelators essentially only two pTP activities were separated by DEAE-cellulose chromatography, one in the column flow through (pTPI), and a second which eluted at the same salt concentration found previously with pTPIII. A small amount of activity was present in the position of pTPII. These data suggested that pTPII and pTPIIA could represent degradation products of pTPIII. Figure 2D shows that in the presence of EGTA and EDTA, pTPI activity separated by CM-cellulose chromatography was present in a single species rather than in the three forms shown in Figure 2B. These results suggested the possibility that only one form of pTPI activity exists, and that the other two species may represent proteolytic breakdown products.

Figures 2C and 2D also show that when ^{32}P labelled enolase was used as substrate, ion exchange chromatography of soluble CEF extracts resulted in profiles of pTP activity that were identical to those seen using IgG as substrate.

It was possible that these results might have been explained by the inhibition of pTP activity EGTA and/or EDTA, perhaps due to a requirement by the enzymes for calcium. To address this question directly, pTPI (DE-52 flow through, ie. a mixture of forms A, B and C), pTPII, pTPIIA and pTPIII were purified as described in Figure 2A and phosphatase activities were assayed in the presence and absence of EDTA and EGTA using ^{32}P-labelled IgG as a substrate. Table 1 shows that neither chelating agent had an effect on any of the pTP activities. These data supported the idea that several of the pTPs detected in CEF extracts may have resulted from the degradation of two principal pTP activities, now termed pTPI and pTPIII.

3-Ic. Purification of pTP Activities from Chicken Embryos.

As only relatively small amounts of protein could be obtained from extracts prepared from CEF cultures, further biochemical studies of the pTPs were both very difficult and costly. To make further investigations more feasible, studies were carried out to determine the nature of pTP activity present in chicken embryos, and in particular, those present in soluble extracts prepared from the embryo tissues normally used to generate CEF cultures. Embryos were dissected to remove

TABLE 1 EFFECT OF EGTA AND EDTA ON pTP ACTIVITY

pTP	Standard Conditions	2 mM EDTA	2 mM EGTA	2 mM EDTA + EGTA
I	100	98	100	102
II	100	98	110	103
IIA	100	102	110	93
III	100	96	104	96

Table 1

Table 1 shows the results of phosphatase assays carried out using partially purified pTPI, pTPII, pTPIIA or pTPIII preparations. Each value represents an average of two experiments done in triplicate and is presented as a % of the maximal activity observed under standard conditions. In all cases the amount of phosphate released was in the fmol range and represented about 10 to 20% of the added substrate.

heads, wings, legs and internal organs, and the resulting chick embryo bodies were homogenized. Soluble protein extracts were then prepared in a fashion similar to that used with CEFs.

Figure 3a and 3b show the profiles of pTP activities following ion exchange chromatography of such extracts on DE-52 and CM-52 resins. The levels of protein analyzed from these extracts were similar to those studied previously using extracts from CEFs. Extracts were prepared and assays performed in the presence of EDTA and EGTA using either ^{32}P-labelled enolase or ^{32}P-labelled RCM lysozyme as substrate. Both substrates gave essentially identical results. As shown previously using extracts from CEFs, pTPI and pTPIII were the major activities detected. The data presented in Figure 3a and 3b show that a single pTP activity bound to and eluted from each of the ion exchange resin. These data indicated that the pTP activities detected in chick embryos appeared comparable to those isolated from CEFs. Thus, chick embryos were used in all future experiments.

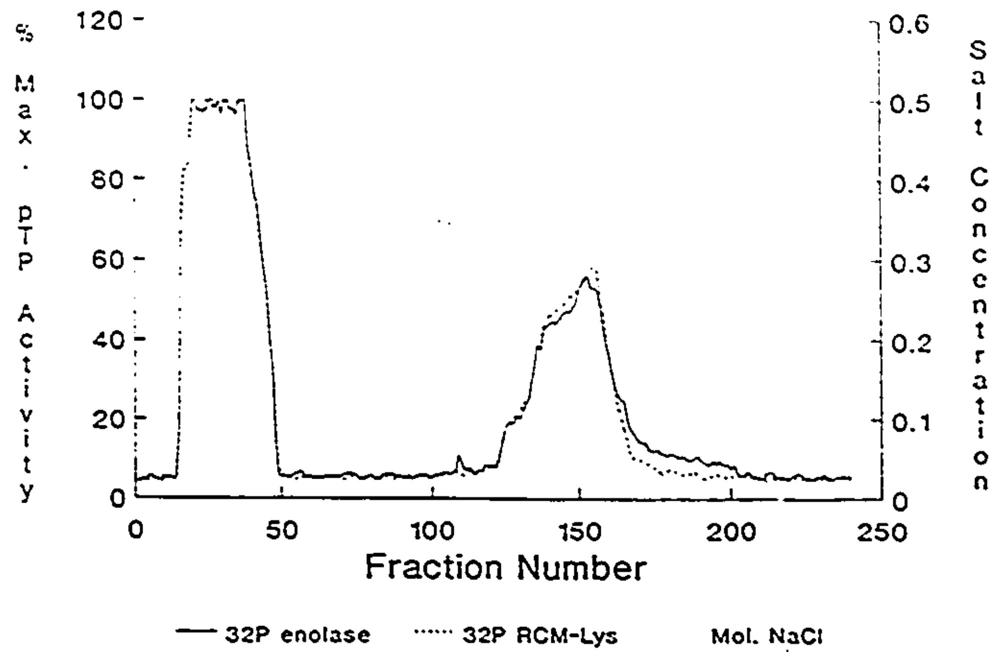
3-Id. Purification of pTPIII

To obtain more highly purified pTPIII, the material isolated by chromatography on DE52, as described in Figure 3a, was subjected to a series of further purification steps. In general, soluble protein extracts prepared from approximately

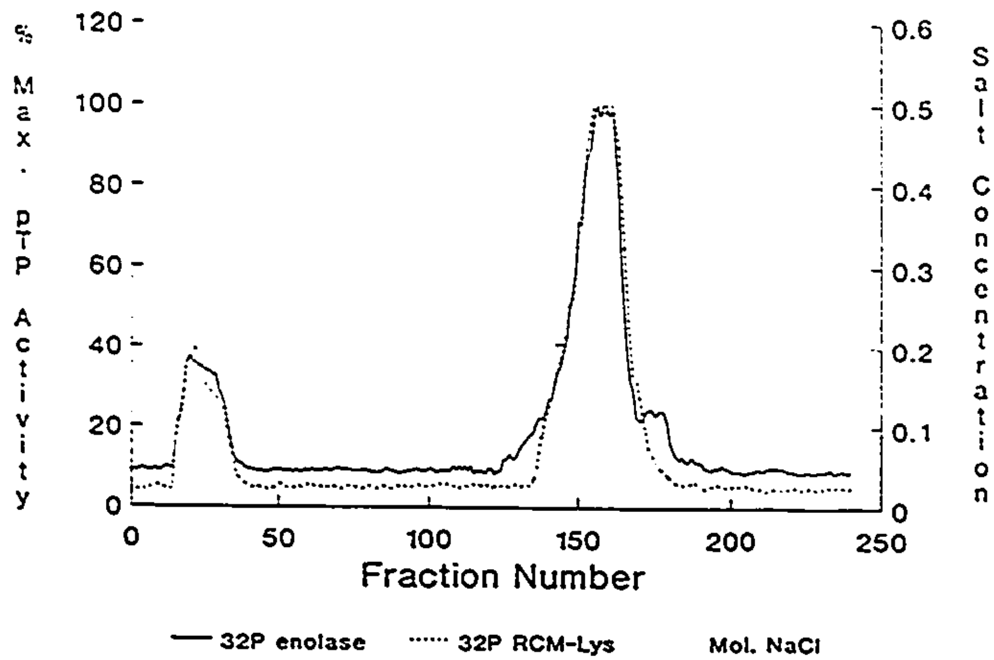
Figure 3. Separation of pTPs by Ion Exchange Chromatography.

Extracts from chicken embryo bodies prepared in the presence of 2mM EGTA and 2mM EDTA were fractionated by DE52 cellulose chromatography (Figure 3a). Activity present in the flow through from these extracts was rechromatographed on CM52 cellulose (Figure 3b). In all cases aliquots from each fraction were analyzed for pTP activity using ^{32}P-labeled enolase (solid lane) or RCM-Lys (dotted line) as substrate. The dashed line indicates the salt concentration within the gradient. Values have been expressed as a % of the highest activity observed.

A) DE-52 pTP Activity Profile



B) CM-52 pTP Activity Profile



400 g of chicken embryo bodies were used as the starting material. Identical profiles to those shown in Figure 3a were obtained when larger columns were employed to process the greater amounts of protein present in these larger volume extracts (data not shown).

To purify pTPIII further, fractions containing pTPIII activity, which eluted from the DE-52 resin at approximately 150 mM NaCl, were pooled and applied directly to a Cibracon Blue 3GA-agarose column. Figure 4 shows the pattern of activities resolved by chromatography on this resin. pTPIII activity generally eluted from the column at approximately 700 mM NaCl. As is summarized in Table 2, chromatography on DE52 typically led to a 13.6-fold purification of pTPIII, while that on Cibracon Blue 3GA agarose produced about a 2-fold enrichment.

The next step in the purification protocol involved molecular sieving chromatography on Sephacryl S-200. Aside from providing a 4 fold purification of the enzyme, this technique also provided an estimate of the molecular weight of pTPIII. In general the activity eluted from the column at a position corresponding to a molecular weight of approximately 60 kd. A typical elution profile has been presented in Figure 5.

A further 3-fold purification of pTPIII was obtained by chromatography on Poly-lysine Sepharose. pTPIII activity bound to the column and was subsequently eluted at a salt concentration of approximately 200 mM NaCl. Figure 6

Figure 4. Chromatography of pTPIII on Cibracon Blue 3GA Agarose.

DE-52 purified pTPIII was subjected to Cibracon Blue 3GA chromatography as described in Materials and Methods. Aliquots from each fraction were assayed using ^{32}P-labelled RCM lysozyme as substrate. Values have been expressed as a % of the highest activity observed. The solid line represents pTP activity, while the dashed line indicates the salt concentration within the gradient.

Cibracon Blue 3GA pTP Profile

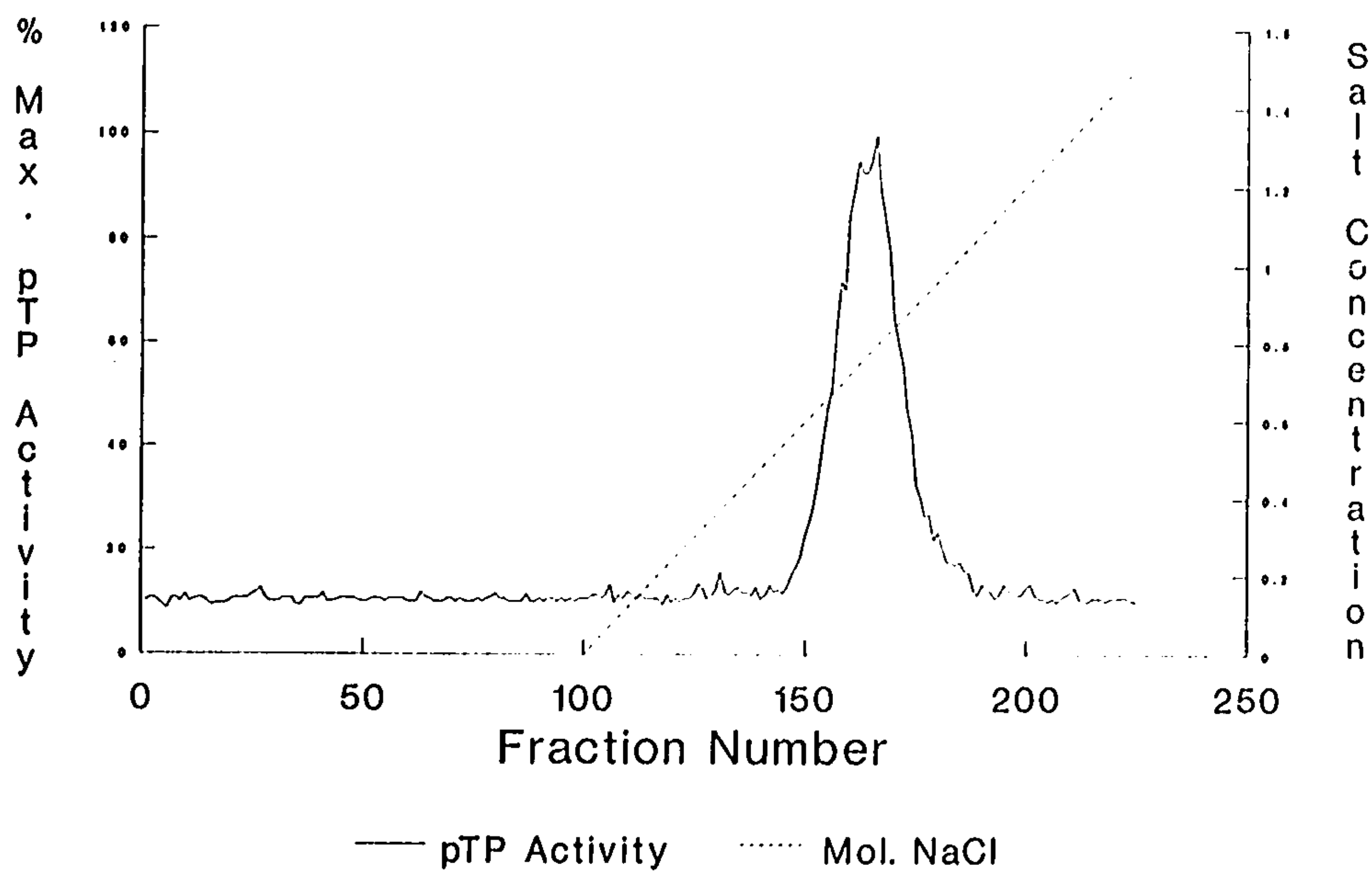


Figure 5. Chromatography of pTPIII on Sephacryl S-200.

Cibracon Blue 3GA purified pTPIII was further purified by molecular sieving chromatography on Sephacryl S-200. Aliquots were assayed for pTP activity using ^{32}P-labelled RCM lysozyme as substrate. Values have been expressed as a % of the highest activity observed. The solid line indicates the pTP activity. The arrows denote the positions at which the marker proteins eluted [1) γ-globulin (150 kd), 2) bovine serum albumin (67 kd), 3) ovalbumin (44 kd), 4) chymotrypsinogen (25 kd), 5) myoglobin (17 kd), and 6) Ribonuclease (13.5 kd)]. pTPIII eluted with an apparent molecular mass of approximately 60 kd.

Sephacryl S-200 pTP Profile

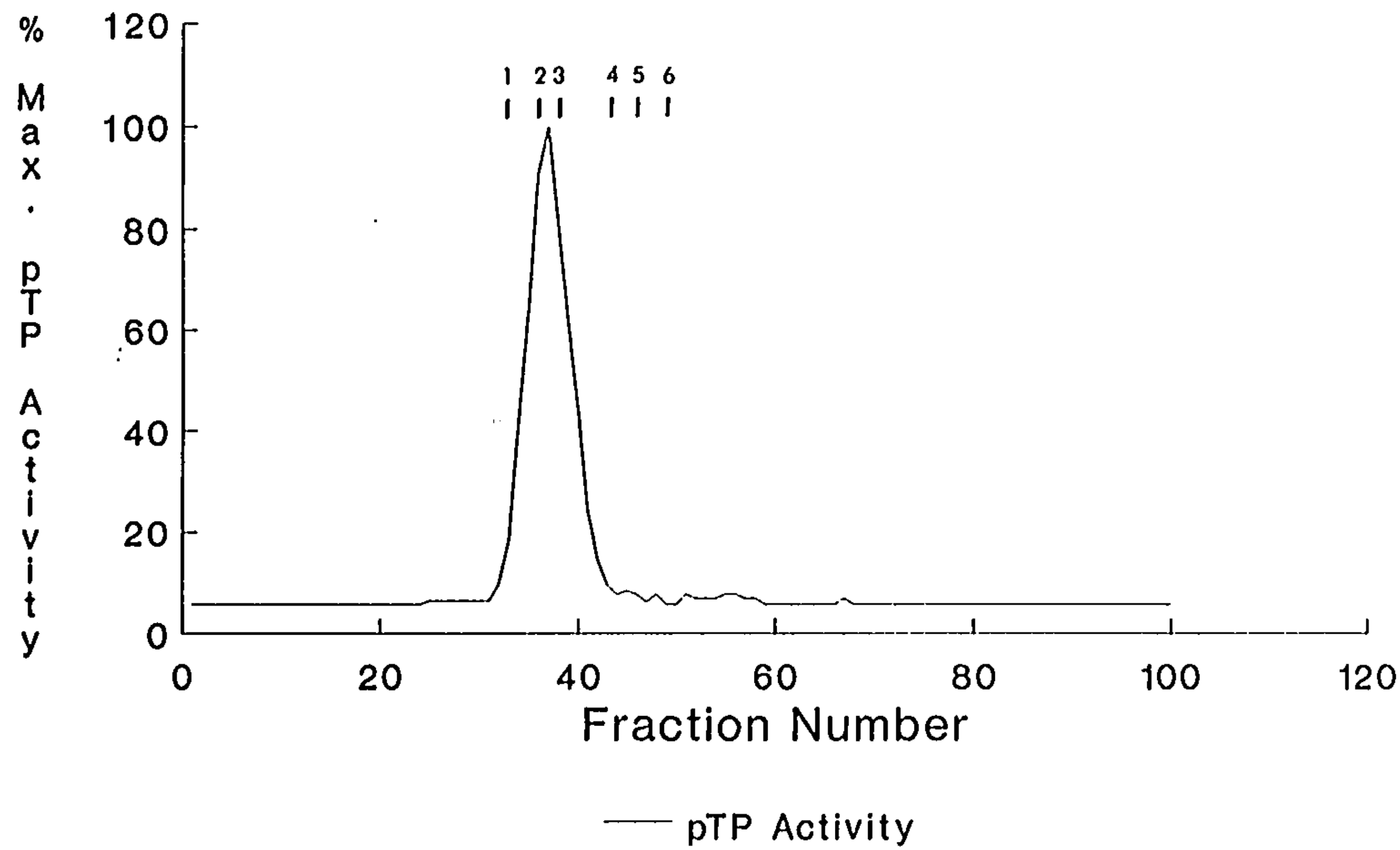
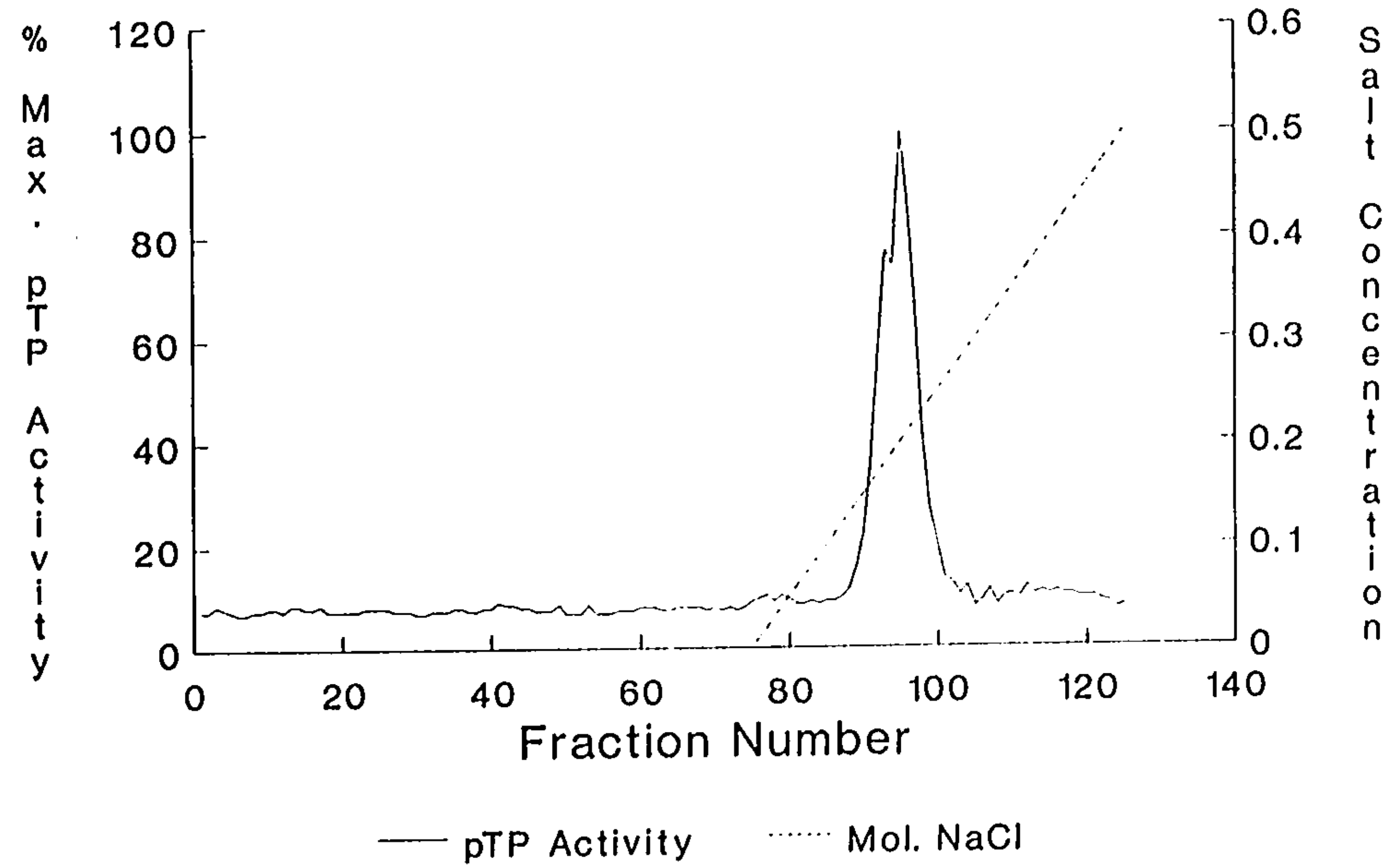


Figure 6. Chromatography of pTPIII on Poly-Lysine Sepharose.

Sephacryl S-200 purified pTPIII was subjected to Polylysine Sepharose chromatography as described in Materials and Methods. Aliquots were assayed for pTP activity using ^{32}P-labelled RCM lysozyme as substrate. Values have been expressed as a % of the highest activity observed. The solid line indicates the pTP activity. The dashed line denotes the salt concentration within the gradient.

Poly-Lysine Agarose pTP Profile



illustrates such a purification step.

Following overnight dialysis to remove salt, pTPIII was subjected to the final step in the purification protocol. The enzyme was purified by affinity chromatography on a resin prepared by coupling thio-phosphotyrosyl RCM lysozyme to CNBr activated Sepharose. Affinity chromatography resulted in a further 6-fold purification of the enzyme. pTP activity eluted at salt concentration of approximately 75 mM NaCl (see Figure 7).

A summary of the purification of pTPIII is shown in Table 2. A total purification of approximately 1900-fold was achieved. To examine the pattern of proteins present at each purification step more directly, samples were analyzed by SDS-PAGE. Figure 8 shows the results following coomassie blue staining of a 10% polyacrylamide gel on which protein samples (2.5 μg) from each purification step were analyzed. While it was clear that samples from the preliminary steps in the purification protocol contained many protein species, fewer were observed with each subsequent purification step. A single protein band at approximately 58 kd can be seen in the affinity purified pTPIII preparation (lane 7). This observation suggested the possibility that the enzyme had been purified to homogeneity. However, further analysis suggested that this was not the case. Figure 9 shows the results of silver staining of a similar SDS-PAGE gel in which 1/10th the amount of protein used in Figure 8 was analyzed. Examination of lane 7 indicated

Figure 7. Chromatography of pTPIII on Thio-phosphotyrosyl-RCM Lysozyme Sepharose.

Poly-lysine Sepharose purified pTPIII was further purified by affinity chromatography on a thio-phosphotyrosyl-RCM lysozyme Sepharose column as described in Materials and Methods. Aliquots were assayed for pTP activity using ^{32}P-labelled RCM lysozyme as substrate. Values have been expressed as a % of the highest activity observed. The solid line indicates the pTP activity. The dashed line denotes the salt concentration within the gradient.

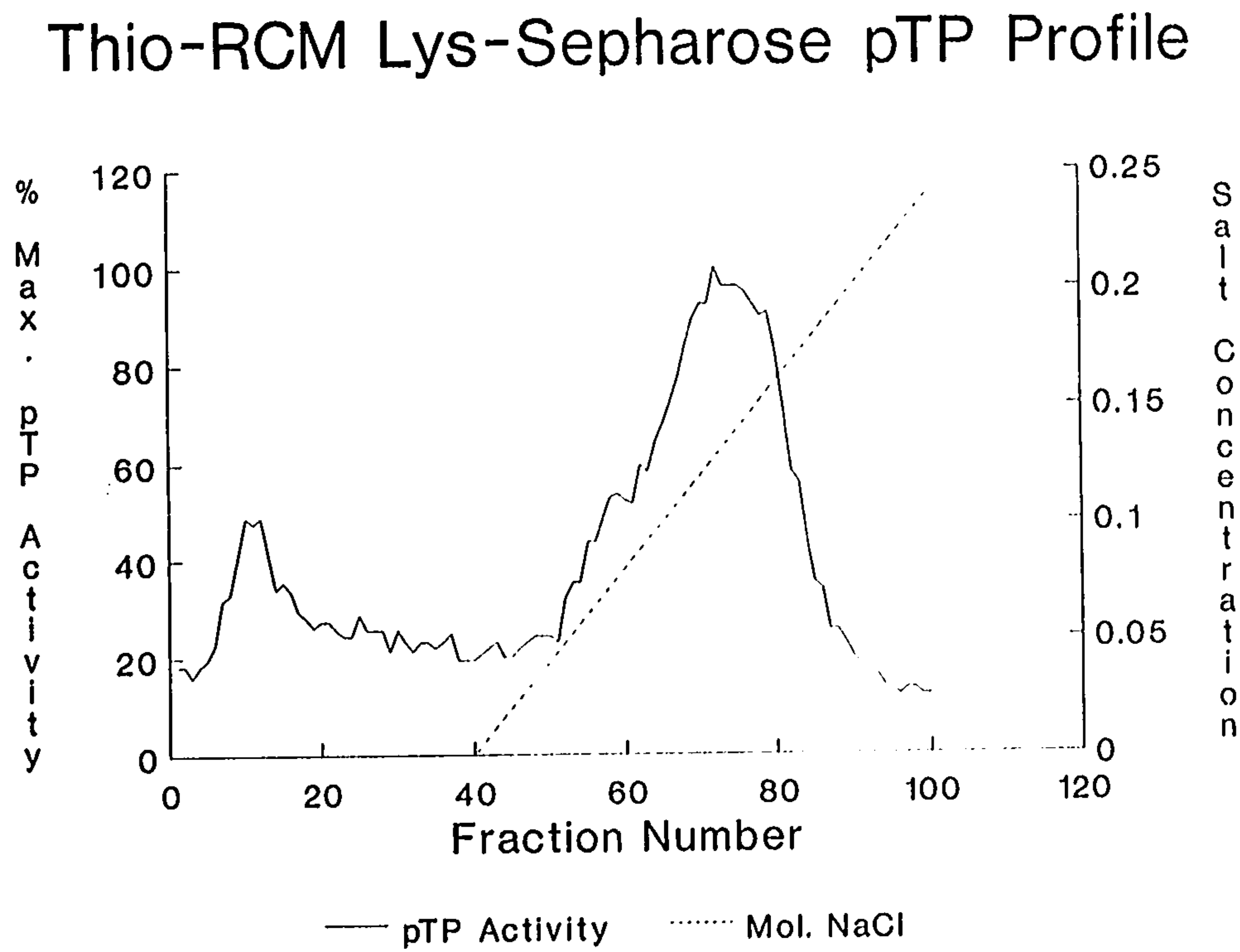

Thio-RCM Lys-Sepharose pTP Profile
% Max. pTP Activity
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Fraction Number
0.25
0.2
0.15
0.1
0.05
0
Salt Concentration
pTP Activity
Mol. NaCl

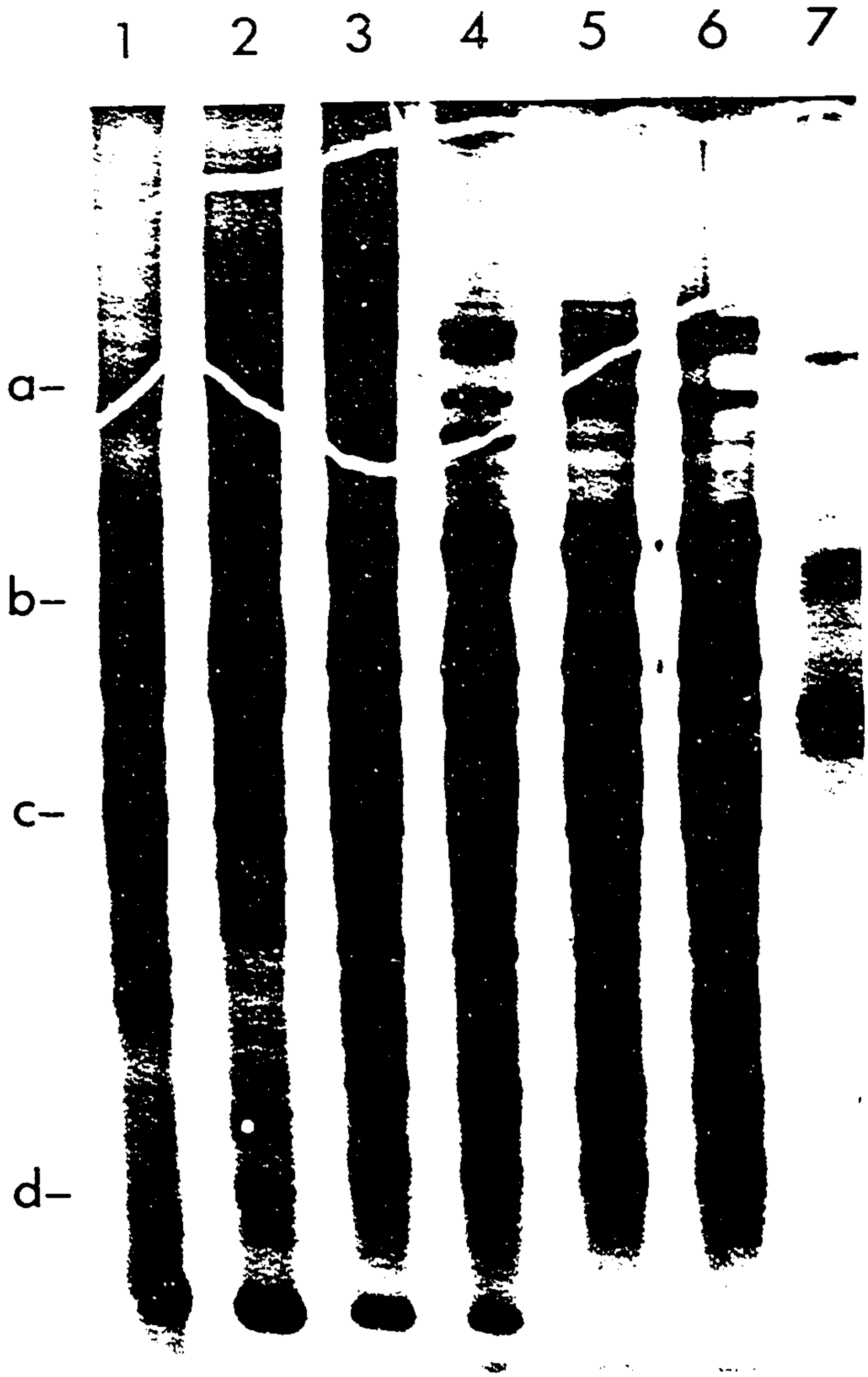
Figure 8. SDS-PAGE Analysis of Purified pTPIII I.

Aliqouts of protein (2.5 μg) from each stage of purification were separated by electrophoresis on a 10% polyacrylamide as described in Materials and Methods. Protein bands were visualized by staining the gel with coomassie brilliant blue dye. Lane 1) cell extract; lane 2) DE-52 purified pTPIII; lane 3) Cibracon Blue 3GA Agarose purified pTPIII; lane 4) Sephacryl S-200 purified pTPIII; lane 5) Poly-lysine Sepharose purified pTPIII; lane 6) Thio-phosphotyrosyl-RCM lysozyme Sepharose column wash-through protein; 7) Thio-phosphotyrosyl-RCM lysozyme Sepharose purified pTPIII. The arrows denote the positions of the marker proteins: phosphorylase b (92.5 kd, arrow a), bovine serum albumin (67 kd, arrow b), ovalbumin (45 kd, arrow c), and carbonic anhydrase (31 kd, arrow d).


pTP III
1 2 3 4 5 6 7
a-
b-
c-
d-

Figure 9. SDS-PAGE Analysis of Purified pTPIII II.

Aliqouts of protein (0.25 μg) from each stage of purification were separated by electrophoresis on a 10% polyacrylamide as described in Materials and Methods. Protein bands were visualized by silver staining. Lane 1) cell extract; lane 2) DE-52 purified pTPIII; lane 3) Cibracon Blue 3GA Agarose purified pTPIII; lane 4) Sephacryl S-200 purified pTPIII; lane 5) Poly-lysine Sepharose purified pTPIII; lane 6) Thio-phosphotyrosyl-RCM lysozyme Sepharose column wash-through protein; 7) Thio-phosphotyrosyl-RCM lysozyme Sepharose purified pTPIII. The arrows denote the positions of the marker proteins: phosphorylase b (92.5 kd, arrow a), bovine serum albumin (67 kd, arrow b), ovalbumin (45 kd, arrow c), and carbonic anhydrase (31 kd, arrow d).



1
2
3
4
5
6
7
a–
b–
c–
d–

Table 2 Purification of pTPIII

Step	Volume (ml)	Protein (mg/ml)	Activity (nm/ml/min)	Specific Activity (nm/mg/min)	Purification (fold)
Cell Extract	675	6.36	3.8	0.6	---
DE-52 Cellulose	500	1.46	11.9	8.2	14
Cibracon Blue 3GA Agarose	132	.32	59.9	18.7	31
Sephacryl S-200	45	.82	58.8	72.0	120
Poly-Lysine Sepharose	38	.32	60.7	190.0	317
Thio-pTyr RCM Lysozyme Sepharose	37	.02	18.1	1134.0	1890

Table 2

Table 2 shows the results of the purification of pTPIII from a total of 400 g chicken embryos as starting material. pTP activity was measured with ^{32}P-labelled RCM lysozyme as substrate, at a final concentration of 5 μM in the assay mixture. The results presented are the average of triplicate determinations.

the presence of some minor impurities within the affinity purified enzyme preparation. Silver staining has been shown to be approximately 100 fold more sensitive than coomassie blue staining. While as little as 1.0 ng of protein can be visualized with siver staining, approximately 100 ng of protein is required for detection using coomassie blue. Further, the intensity of staining observed with coomassie blue is roughly proportional to the amount of protein present, whereas that observed with silver is not. Thus, despite the presence of the impurities visualized by silver staining, the results presented suggested that an approximately 90% purification of pTPIII had been accomplished. This estimation of the purity was based on the fact that while a total of 2.5 μg of affinity purified pTPIII was applied to the polyacrylamide gel presented in figure 8, none of the impurities were visualized by coomassie blue staining.

3-Ie. Purification of pTPI

Following purification by chromatography on DE52 (pTPI was not retained on the DE52 column), a protocol somewhat similar to that employed with pTPIII was utilized to purify pTPI. As summarized in Table 3, a purification of only about 200-fold was obtained based on the specific pTPI enzyme activity. Figure 10 shows the results of silver staining of a

Table 3 Purification of pTPI

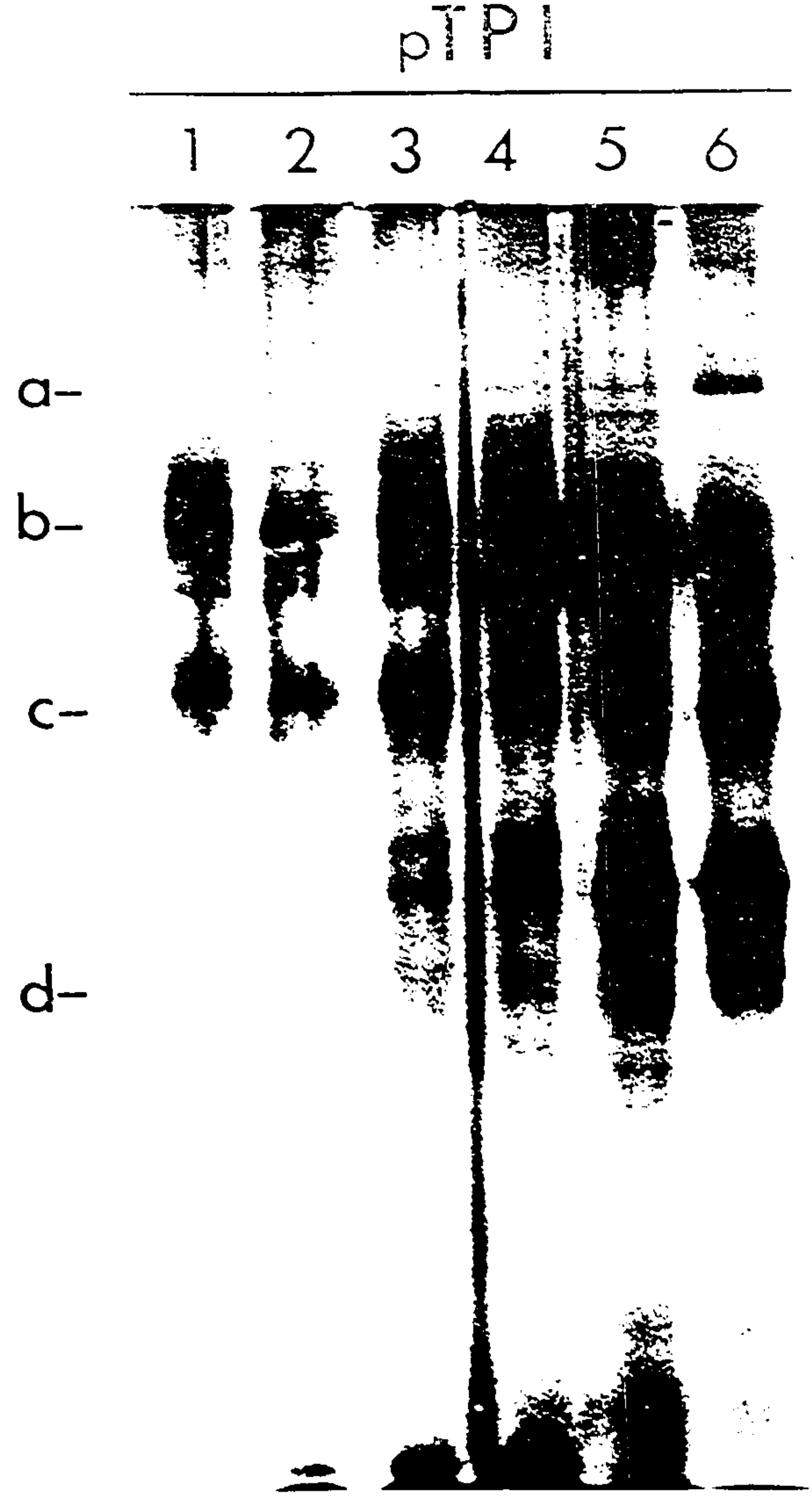
step	Volume (ml)	Protein (mg/ml)	Activity (nm/ml/min)	Specific Activity (nm/mg/min)	Purification (fold)
Cell Extract	845	8.5	6.0	0.7	-----
DE-52 Cellulose	120	13.1	33.5	2.6	4
Red A Matrix	65	5.6	55.9	10.0	14
G-75 Sephadex	40	3.2	67.1	21.0	30
CM-52 Cellulose	85	0.54	33.5	62.0	89
Thio-RCM Lysozyme Sepharose	27	0.05	6.2	124.0	177

Table 3

Table 2 shows the results of the purification of pTPI from a total of 450 g chicken embryos as starting material. pTP activity was measured with ^{32}P-labelled RCM lysozyme as substrate, at a final concentration of 5 μM in the assay mixture. The results presented are the average of triplicate determinations.

Figure 10. SDS-PAGE Analysis of Purified pTPI.

Aliqouts of protein (0.25 μg) from each stage of purification were separated by electrophoresis on a 10% polyacrylamide as described in Materials and Methods. Protein bands were visualized by silver staining. Lane 1) cell extract; lane 2) DE-52 purified pTPI, ie. flow through; lane 3) Red A Matrix purified pTPI; lane 4) Sephadex G-75 purified pTPI; lane 5) CM-52 purified pTPI, lane 6) Thio-phosphotyrosyl-RCM Lysozyme Sepharose purified pTPI. The arrows denote the positions of the marker proteins: phosphorylase b (92.5 kd, arrow a), bovine serum albumin (67 kd, arrow b), ovalbumin (45 kd, arrow c), and carbonic anhydrase (31 kd, arrow d).



pTPI
1 2 3 4 5 6
a–
b–
c–
d–

10% polyacrylamide gel in which samples of extracts (0.25 μg) from each purification step were resolved by electrophoresis. Clearly, the enzyme had only been partially purified. The most prominent protein band migrated at an approximate molecular mass of approximately 35 kd. This species could represent the pTPI protein but this possibility was clearly uncertain.

In general, pTPI activity was unstable and was often lost during the purification process. The peaks of activity eluting from chromatography columns were often quite broad, resulting in only low levels of purification. The reasons for these observations were unclear, however, because of this property, it was not possible to purify pTPI to the same levels as attained with pTPIII. Purification of pTPI will likely require the establishment of conditions that would stabilize enzyme activity.

3-II. RELATIONSHIP OF CHICKEN EMBRYO pTPs TO CALCINEURIN

It was possible that the enzymes pTPIII and pTPI isolated from chicken embryos may have represented previously identified phosphoprotein phosphatase activities. The type 2B phosphoserine specific phosphatase, also known as calcineurin, has been shown to possess some activity against phosphotyrosyl protein substrates (Pallen and Wang, 1983; Chernoff et al., 1984; Chan et al., 1986; Kincaid et al., 1986). It is unlikely that pTPI represents calcineurin activity because unlike calcineurin, it fails to bind to DEAE-cellulose. However, pTPIII shares some properties with the type 2B phosphatase. It binds to both DEAE-cellulose and Cibracon Blue 3GA-agarose, and elutes at similar, though somewhat higher, salt concentrations (Sharma et al., 1983). Although previous studies had failed to show any stimulatory effects by the addition of calcium and/or calmodulin (Nelson and Branton, 1984), it was possible either that sufficient quantities of the holoenzyme were not present in these preparations, or that the assay conditions used were not appropriate. To address this question more carefully, partially purified pTPIII was assayed against ^{32}P-labelled enolase in the presence of divalent cations and bovine calmodulin. As a positive control, purified calcineurin (a gift of Jerry Wang) was also tested using enolase as a phosphotyrosyl substrate and ^{32}P-labelled casein as a phosphoseryl substrate. Table 4 shows that divalent cations, both in the presence and absence of calmodulin, had little effect on pTPI or pTPIII activity. Under similar conditions using enolase as a substrate, bovine calcineurin was stimulated by a small amount, 1.17- to 1.43-fold by divalent cations alone, and 1.64- and 2.64-fold by calmodulin in the presence of Ca^{+2}, or Ca^{+2} plus Mg^{+2}, respectively. Using casein as substrate, calmodulin stimulated calcineurin activity by over 20-fold, indicating that the effects on calcineurin were much more pronounced against phosphoseryl substrates.

TABLE 4 BIOCHEMICAL ANALYSIS OF pTP ACTIVITIES

Conditions	pTPI (enolase)	pTPIII (enolase)	Calcineurin (enolase)	Calcineurin (casein)
standard	100	100	100	100
1 mM ATP	4	4	nd	nd
10 μM Na_2VO4	26	23	nd	nd
100 μM Zinc Acetate	27	24	nd	nd
250 μM Mg^{++}	82	90	122	172
250 μM Mn^{++}	73	84	143	nd
100 μM Ca^{++}	85	104	117	nd
100 μM Ca^{++} + calmodulin	97	86	164	2047
250 μM Mg^{++} + 100 μM Ca^{++} + calmodulin	84	90	240	2149
50 mM NaF + 1 mM EDTA	100	83	nd	nd

Table 4

Table 4 shows the results of phosphatase assays carried out under standard conditions, with modifications noted, using purified pTPI or pTPIII preparations or affinity purified bovine calcineurin (provided by Jerry Wang) with either ^{32}P-labelled enolase or ^{32}P-labelled casein as substrate. Each value represents an average of two experiments done in triplicate and has been presented as a % of the activity obtained under standard conditions. In all cases the amount of phosphate released was in the fmol range and represented about 10 to 20% of the added substrate.

To examine the relationship between pTPIII and calcineurin further, these enzymes were tested for their ability to bind to calmodulin (CAM)-agarose in the presence of calcium, a property characteristic of calcineurin (Sharma etal., 1983; Stewart et al., 1983). Column fractions were assayed using ^{32}P-labelled enolase or ^{32}P-labelled casein as substrate. Figure 11a shows the results obtained using the phosphotyrosine containing substrate, while Figure 11b depicts the results obtained using the phosphoserine substrate. The data demonstrate that while calcineurin activity was retained on the calcium-calmodulin column, partially purified pTPIlI failed to bind. These results strongly suggested that pTPIII is not a calcineurin-like enzyme. While calcineurin is undoubtedly present in chicken embryos, it presumably represents only a minor portion of the phosphotyrosyl phosphatase activity present in the extracts.

3-III. RELATIONSHIP OF CHICKEN EMBRYO pTPs TO ACID AND ALKALINE PHOSPHATASES

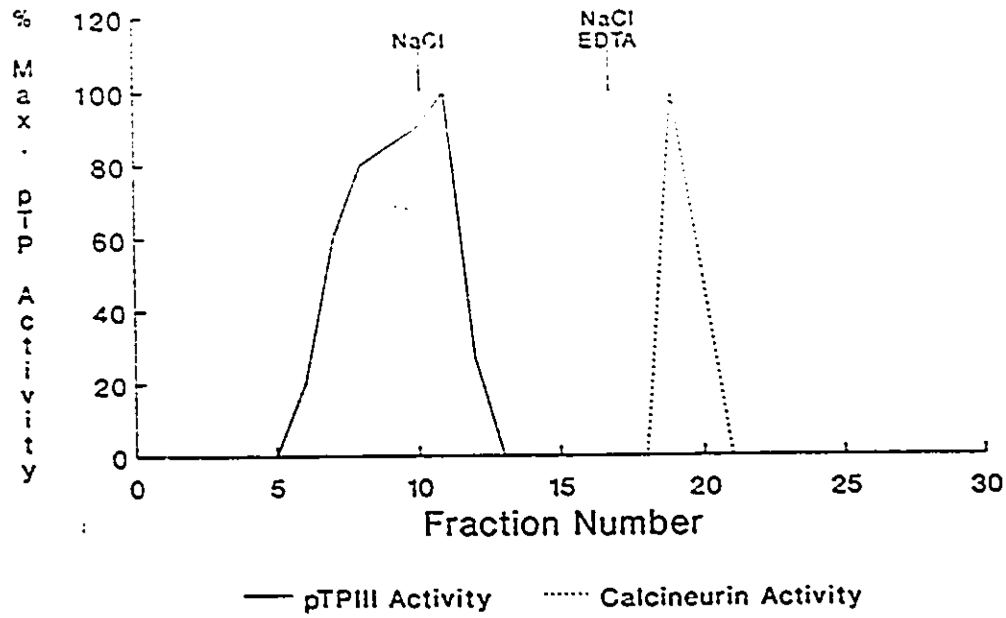
Phosphotyrosyl phosphatases isolated from human liver (Taga and Van Etten, 1982), placenta (Waheed et al., 1988), erythrocytes (Boivin and Garland, 1986), and prostate (Li. et al., 1984); bovine skeleton (Lau et al., 1985); and rabbit kidney (Shriner and Brautigan, 1984; Sparks and Brautigan,

Figure 11. Calmodulin-Agarose Chromatography of Partially Purified pTPIII.

Partially purified pTPIII was subjected to calmodulin-agarose chromatography as described in Materials and Methods. Aliquots were assayed for pTP activity using ^{32}P-labelled enolase as substrate (figure 11a), and for pSP activity using ^{32}P-labelled casein as a substrate (figure 11b). Values have been expressed as a % of the highest activity observed. The solid line indicates pTPIII activity and the dotted line represents calcineurin activity. The vertical lines indicate the points at which the 200 mM NaCl wash buffer and the 200 mM/2mM EDTA elution buffer were applied to the column.

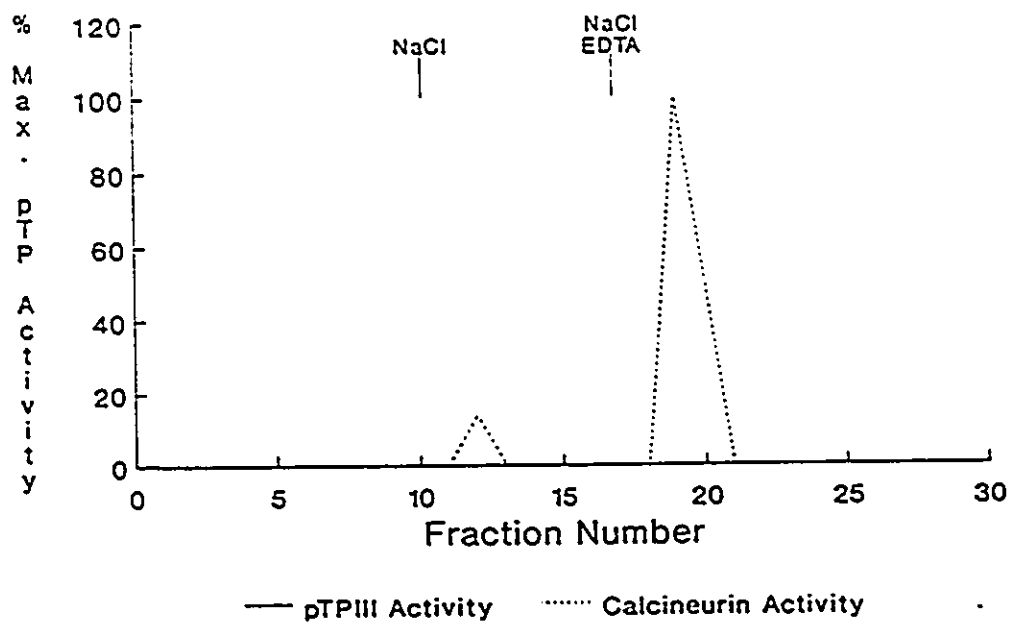
A) Calmodulin-Agarose Chromatography

pTP Activity



B) Calmodulin-Agarose Chromatography

pSP Activity



1985), all display properties of acid phosphatases. Activities purified from canine heart (Li et al., 1979), rat liver (Strout et al., 1988) and human TCRC-2 cells (Swarup et al., 1981; Swarup et al., 1982) clearly resemble alkaline phosphatase. Although previous studies on pTPs from CEFs had indicated a broad but neutral pH optimum against ^{32}P-IgG (Nelson and Branton, 1984), it was possible different results might be obtained using other substrates. The pH ranges for pTPI and pTPIII, as well as for commercial potato acid phosphatase and bovine alkaline phosphatase were examined using ^{32}P-enolase and p-nitrophenol phosphate (pNPP) as substrates. Table 5 shows that acid phosphatase demonstrated an acid optimum against both enolase and pNPP. Alkaline phosphatase, while having an evident alkaline optimum for activity against pNPP, the enzyme was inactive at pH 10.5 with enolase as substrate. Rather, alkaline phosphatase demonstrated activity against enolase at acid and neutral pH. Both pTPI and pTPIII were active against pNPP, and both enzymes displayed a pH optimum typical of acid phosphatases. With enolase as substrate, pTPI was equally active at pH 5.0 and pH 7.0 and pTPIII was active at pH 5.0 but two fold higher at pH 7.0. These data indicated that neither pTPI nor pTPIII possess properties typical of alkaline phosphatases, but both have characteristics of acid phosphatases. In separate studies potato acid phosphatase was shown to respond to divalent cations in a fashion comparable to the chicken embryo pTPs (data not shown). Differences in other

TABLE 5 THE EFFECT OF pH ON pTP ACTIVITY

	Enolase			pNPP		
Enzyme	pH 5.0	pH 7.0	pH 10.5	pH 5.0	pH 7.0	pH 10.5
pTPI	96	100	3	100	74	0
pTPII	46	100	0	100	50	0
acid phosphatase	100	14	1	100	72	0
alkaline phosphatase	77	100	0	0	38	100

Table 5

Table 5 shows the results of phosphatase assays carried out at various pH using purified pTPI or pTPIII preparations or commercial (Sigma) bovine alkaline phosphatase or potato acid phosphatase. Each value represents an average of two experiments done in triplicate and is presented as a % of the maximal activity observed at each pH. In all cases the amount of phosphate released was in the fmol range and represented about 10 to 20% of the added substrate.

properties also argued that pTPI and pTPIII are unrelated to alkaline phosphatases. These enzymes generally require Zn^{+2} and may be stimulated by Mg^{+2} (Cathala and Brunel, 1975). The pTPs from CEFs and chicken embryos are inhibited by Zn^{+2} (Nelson and Branton, 1984) and, as can be seen from the data presented in Table 4, divalent have no stimulatory effect on pTPI or pTPIII activity. These data, as well the pH optima, suggested that pTPI and pTPIII may be related to acid phosphatases.

3-IV. RELATIONSHIP OF CHICKEN EMBRYO pTPS TO PHOSPHOSERYL PHOSPHATASES

Of the classic forms of phosphoseryl-protein phosphatases (Cohen, 1989), results already presented clearly demonstrate that pTPI and pTPIII do not possess properties characteristic of the type 2B calcium-calmodulin stimulated phosphatase (Table 4 and Figure 11).

Type I and 2A phosphatases are typically inactivated by treatment with NaF in the presence of EDTA at 30°C for 30 min (Ingebritsen and Cohen, 1983; Ingebritsen et al, 1983), and as shown in Table 4, such treatment had little or no effect on either pTPI or pTPIII. This observation suggested that the chicken embryo pTPs are distinct from the type I and 2A enzymes.

The type 2C phosphatases possess a marked Mg^{+2} dependence (Cohen, 1989) and such was not observed with the chicken embryo pTPs, indicating that these enzymes are distinct from the type 2C phosphatases. Thus, phosphatases pTPIII and pTPI both appeared to represent novel enzymes which were clearly unrelated to the classic forms of protein phosphatases and likely members of the new class of phosphotyrosine specific phosphatases.

CHAPTER 4: ISOLATION OF PHOSPHOTYROSYL-PROTEIN PHOSPHATASE cDNA CLONES

During the course of this study the amino acid sequence of pTP1B, the placental pTP purified by Tonks et al. (1988a), was reported. Based upon a comparison of the sequences of pTP1B with those previously determined for CD45 and LAR, Streuli et al. (1989) designed oligonucleotide probes which were then used to screen a Drosophila cDNA library. This work resulted in the isolation of two Drosophila pTP cDNA clones. The oligonucleotide probes used corresponded to the amino acid sequence VHCSAGV. This sequence is found at amino acid residues 303-9 of pTP1B (Tonks et al., 1988a), and is highly conserved in other pTPs. A mixture of two oligonucleotides, pTP A and pTPB (for sequences see Materials and Methods, section XIX), was required in order that all possible serine codons could be represented within the hybridization mixture without including any irrelevant amino acid codons. Also, deoxy-Inosine was incorporated at the 3' and 5' ends of the oligonucleotides in order to minimize degeneracy. In light of the success achieved by Streuli et al. (1989), the oligonucleotide probes described above were prepared and used to screen a total chicken embryo cDNA library in order to isolate cDNAs encoding chicken pTP enzymes.

4-I. Isolation of Clones H3 and E4

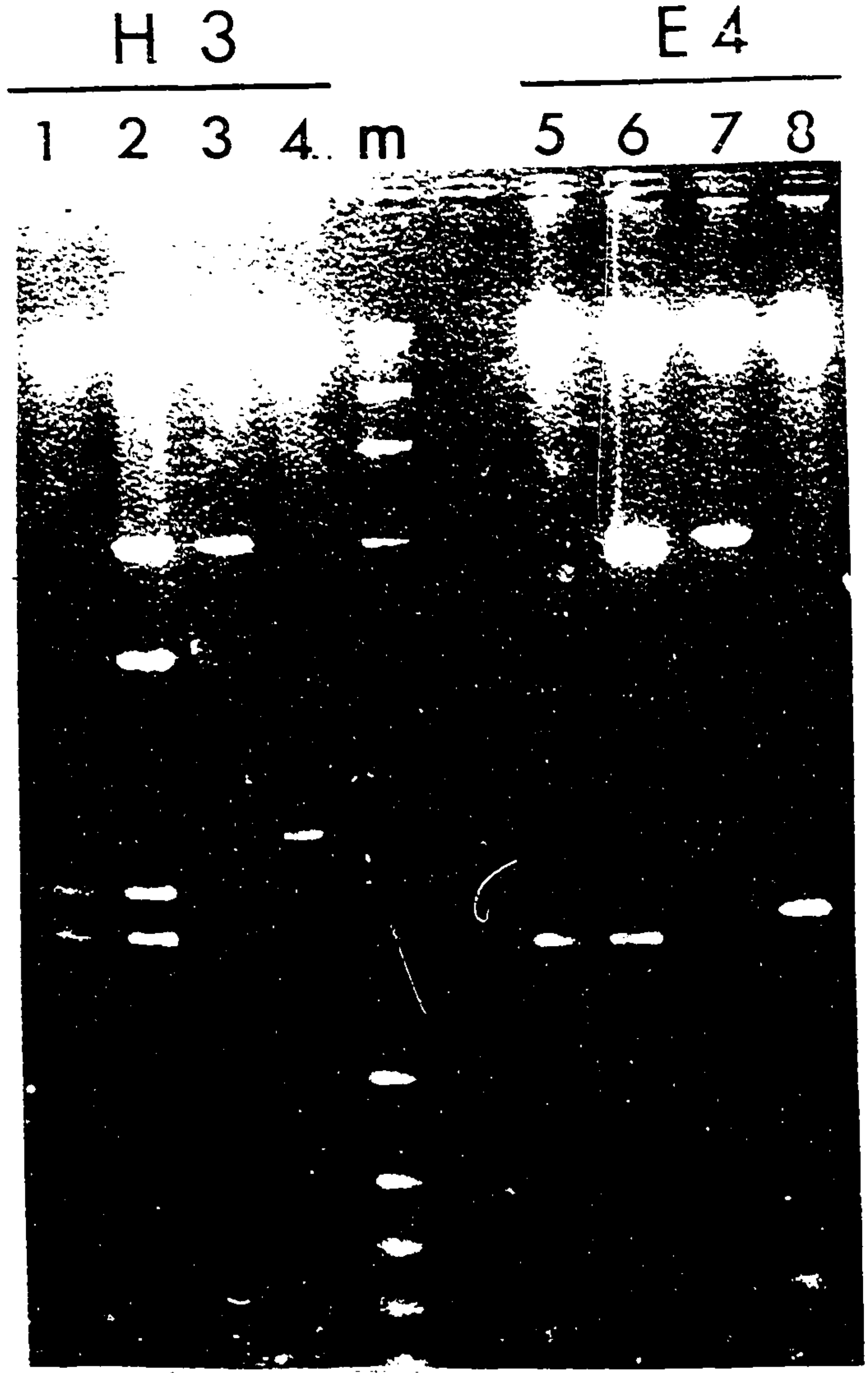
Approximately 3 x 10^5 plaques from a lambda gt11 cDNA library prepared from whole chicken embryo tissues (10-11 days old) were screened by hybridization with ^{32}P-labelled degenerate oligonucleotide probes (described above). As a result of this screen, two positive clones, designated E4 and H3, were identified and purified. Viral DNA from each clone was isolated and subjected to restriction enzyme analysis. The results indicated that the two clones likely contained inserts corresponding to two different pTP cDNAs. Figure 12 shows that digestion of E4 with EcoR I (lane 8) yielded fragments of approximately 0.6 kb and 1.7 kb, whereas a similar digest of H3 (lane 4) resulted in fragments of 0.9 kb and 1.9 kb. These results indicated that E4 and H3 contained cDNA inserts of approximately 2.3 kb and 2.8 kb, respectively.

The enzymes Kpn I and Sac I each cut lambda gt11 viral DNA twice on opposite sides of the EcoR I cloning site, including sites approximately 1 kb from the EcoR I site. Digestion of the lambda virus vector (ie. virus lacking a cDNA insert) with these enzymes yielded a 4.4 kb Sac I viral DNA specific fragment, a 1.6 kb Kpn I viral DNA specific fragment, as well as two fragments of approximately 23 kb corresponding to the lambda virus arms.

Digestion of E4 with Kpn I and Sac I (Figure 12, lane 6) resulted in the generation of fragments of the expected

Figure 12. Restriction Digest Analysis of H3 and E4 Clones.

Purified E4 and H3 lambda DNA samples were digested with _Kpn_ I (lanes 1 and 5 respectively), _Kpn_ I and _Sac_ I (lanes 2 and 6, respectively), _Sac_ I (lanes 3 and 7, respectively), and _EcoR_ I (lanes 4 and 8, respectively), and electrophoresed on a 1.5% agarose gel. Lane M shows the migration of molecular weight markers (in order of decreasing size: 23 kb, 9.5 kb, 6.5 kb, 4.5 kb, 2.3 kb, 2.0 kb, 1.0 kb, 0.77 kb, 0.6 kb, 0.5 kb, and 0.4 kb).


H 3
E 4
1 2 3 4 m 5 6 7 8

sizes, 4.4 kb and 1.6 kb, as well as an extra fragment of approximately 4.3 kb which represented the cDNA insert flanked by lambda DNA sequences. Digestion of H3 however, yielded two extra fragments of 3.2 kb and 1.5 kb (lane 2). Digestion of H3 DNA with Kpn I or Sac I alone (lanes 1 and 3, respectively) demonstrated the presence of an extra 1.5 kb fragment amongst the Kpn I digestion products, while no extra fragments were observed as a result of the Sac I digest. These results suggested the presence of a Kpn I site within the H3 cDNA insert that is absent in the E4 clone.

The fact that the two clones contained cDNAs encoding different pTPs was confirmed by sequence analysis of the EcoR I cDNA insert specific DNA fragments from each clone following subcloning into the plasmid pGEM7zf(+). Comparison of the amino acid sequence derived from translation of the nucleotide sequences obtained for the H3 clone with known pTP amino acid sequences indicated that the H3 insert likely represented a truncated cDNA encoding the chicken homolog of LAR (Streuli et al, 1988). The 5' end of the cDNA appeared to have been lost during preparation of the cDNA library, resulting in a cDNA insert encoding only amino acids present in the carboxy-terminal pTP domain of LAR, as well as some of the 3' untranslated sequences (data not shown). At present, only the 1.9 kb EcoR I fragment of H3 has been sequenced. The 0.9 kb fragment appears to contain sequences 5' to those of the 1.9 kb fragment, however this fragment was too small to encode all of the missing chicken LAR sequences. Figure 13 shows a comparison of the partial H3 amino acid sequence with the equivalent regions of the human (Streuli et al., 1988) and Drosophila (Streuli et al., 1989) LAR proteins. This comparison demonstrates that the LAR protein has been highly conserved over the course of evolution, with the chicken protein displaying approximately 94% and 90% identity to the human and Drosophila homologs, respectively. No further analysis of the H3 clone was carried out.

At the time that the E4 clone was first sequenced, it appeared that this clone encoded a previously unidentified pTP. Sequence analysis suggested that this clone encoded a member of the transmembrane class of pTPs, as the deduced amino acid sequence indicated the presence of tandem phosphatase domains. As with the H3 clone, the cDNA appeared to have been truncated at the 5' end as the amino acid sequence of E4 began midway through the amino terminal phosphatase domain (see figure 17).

4-II. Isolation of Clones Containing E4 5' Sequences

The 0.6 kb EcoR I fragment of the E4 clone was determined to contain the 5'-most insert cDNA sequences. This determination was based on upon the analysis of sequence information obtained using lambda forward and lambda reverse oligonucleotide primers. These primers bind to lambda gt11 DNA

<u>Figure 13</u>. Homology Comparison of the Amino Acid Sequences of H3, human LAR, and drosophila LAR.

Figure 13 shows a comparison of the partial amino acid sequence deduced for the H3 clone with the equivalent regions of amino acid sequence of human LAR (Streuli <u>et al.</u>, 1988) and drosophila LAR (Streuli <u>et al.</u>, 1989). Dashes indicate identical amino acids. H3 is 94% and 90% homologous to Human LAR and drosophila LAR, respectively.

Figure 13

```
H3      EFKVTDARDGQSRTVRQFQFTDWPEQGVPKSGEGFIDFIGQVHKTKEQFGQDGPIS
hLAR    --------------I---------------T------------------------T
dLAR    ----------S---------I----------------------------------T

H3      VHCSAGVGRTGVFITLSIVLERMSYEGVVDVFQTVKMLRTQRPAMVQTEDQYQFCY
hLAR    -----------------------R------M-----T----------------L--
dLAR    ---------S-------------Q----L------RI--S------------H---

H3      QAALEYGSFDHYAT*
hLAR    R--------------
dLAR    R---------N-TN-
```

sequences flanking the cDNA insert and can be used to determine the sequences present at the 5' and 3' ends of cDNA inserts. These primers were used in sequencing reactions in which a plasmid containing the E4 Kpn I/Sac I fragment (this fragment contains 1 kb of lambda DNA flanking each end of the cDNA insert, including the primer binding sites) served as the template (data not shown).

Based upon the determination that the 0.6 kb EcoR I fragment of the E4 clone contained the 5'-most insert cDNA sequences, this fragment was chosen for use as a nick translated probe in order to rescreen the lambda gt11 library in the hopes of identifying clones containing the missing E4 5' sequences. Approximately 1.5×10^5 plaques were screened using this probe, resulting in the isolation of two positive clones designated Pos1 and Pos2.

In order to determine whether either clone contained 5' sequences, purified DNA from each clone was used as template for the polymerase chain reaction (PCR) using either lambda forward or lambda reverse oligonucleotide primers in combination with an E4 specific antisense oligonucleotide (corresponding to a nucleotide sequence 200 bp from the 5' end of the 0.6 kb EcoR I fragment, ie. complementary to bases 2185-2206 in figure 17). Figure 14 shows that a band of approximately 1.8 kb was obtained using the Pos1 DNA template with the lambda reverse and E4 oligonucleotides, while a band of 2.3 kb was obtained using the lambda forward and E4

Figure 14. PCR Analysis of Pos1 and Pos2 Clones.

Purified Pos1 and Pos2 lambda DNA samples were used as templates for PCR reactions using either a lambda forward primer and an E4 specific oligonucleotides (lanes 1 and 3, respectively) or a lambda reverse primer and an E4 specific oligonucleotide (lanes 2 and 4). The reaction products were electrophoresed on a 1.5% agarose gel. Lane M shows the migration of molecular weight markers (in order of decreasing size: 23 kb, 9.5 kb, 6.5 kb, 4.5 kb, 2.3 kb, 2.0 kb, 1.0 kb, 0.77 kb, 0.6 kb, 0.5 kb, and 0.4 kb).


Pos 1
Pos 2
M
1
2
3
4

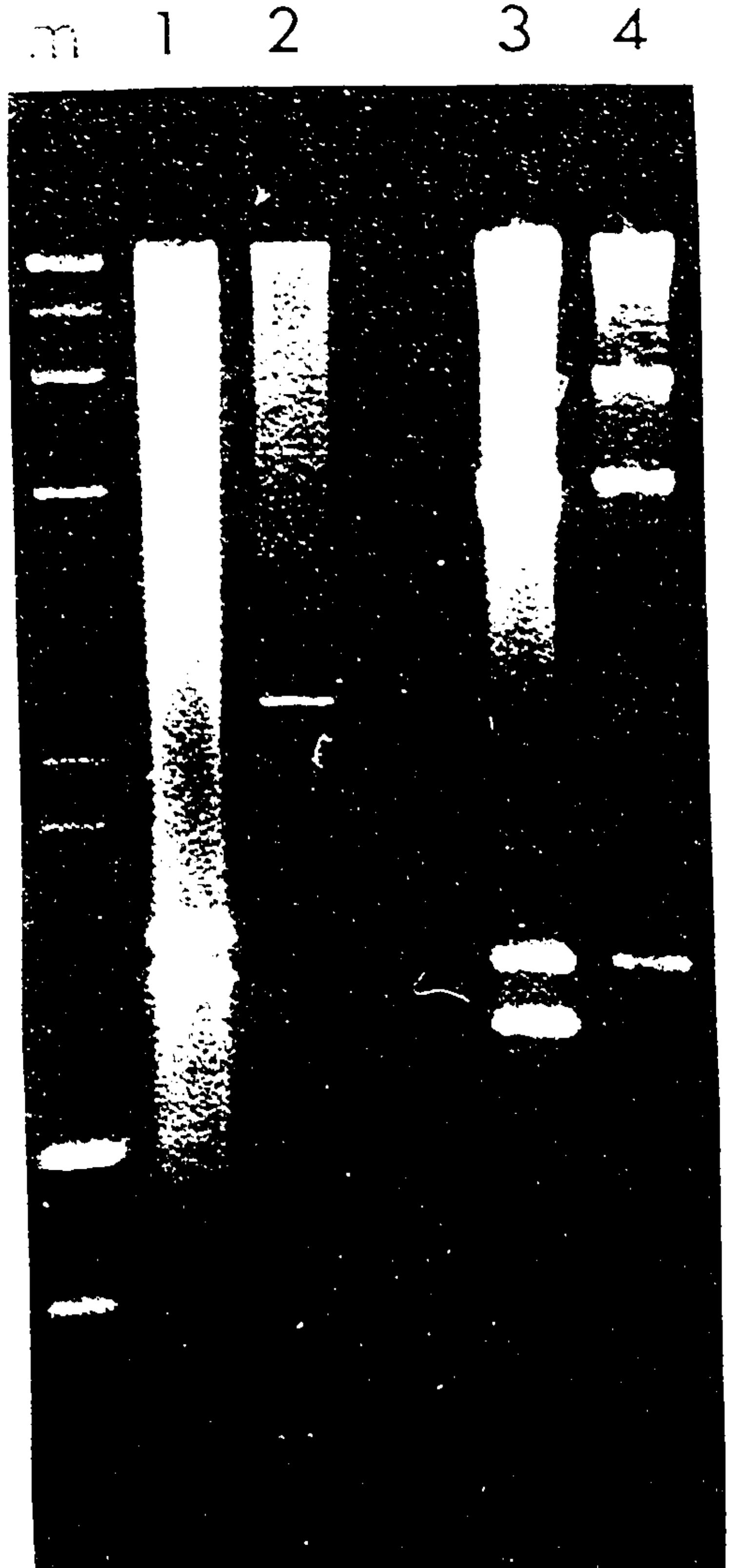
oligonucleotides in combination with the Pos2 DNA template. No bands were observed using either template and each oligonucleotide independently (data not shown). These results suggested that both clones contained cDNA sequences 5' to those present in the initial E4 clone.

Once again, restriction analysis suggested that two different clones had been isolated. Figure 15 shows that while digestion with EcoR I yielded Pos 1 insert specific fragments of approximately 1.7 kb, 1.6 kb, and 0.5 kb (lane 1), a similar digest of Pos 2 yielded a single fragment of approximately 2.6 kb (lane 2). Further, a double digest of both clones with Kpn I and Sac I resulted in insert specific fragments of 4.3 kb and 1.4 kb for Pos 1 (lane 3) and a single fragment of 6.3 kb for Pos 2 (lane 4). This observation indicated the presence of either a Kpn I or Sac I site within Pos 1 that was absent in the Pos 2 clone (later identified as a Kpn I site by sequence analysis).

It was somewhat surprising that a single insert containing fragment of 6.3 kb was obtained following Kpn I/Sac I digestion of Pos 2 lambda DNA. Given that EcoR I digestion yielded a single band of 2.6 kb, one would have expected a band of only 4.6 kb (ie 2.6 kb insert plus 2 kb flanking lambda sequences). Sequence analysis subsequently revealed the absence of an EcoR I site at the junction between the 3' end of the insert and the lambda DNA (see below). Thus, the actual size of the Pos 2 insert cDNA was approximately 4.3 kb.

Figure 15. Restriction Digest Analysis of Pos1 and Pos2 Clones.

Purified Pos1 and Pos2 lambda DNA samples were digested with *Kpn* I and *Sac* I (lanes 3 and 4, respectively), and *EcoR* I (lanes 1 and 2, respectively), and electrophoresed on a 1.5% agarose gel. Lane M shows the migration of molecular weight markers (in order of decreasing size: 23 kb, 9.5 kb, 6.5 kb, 4.5 kb, 2.3 kb, 2.0 kb, 1.0 kb, 0.77 kb, 0.6 kb, 0.5 kb, and 0.4 kb).


m 1 2 3 4

The EcoR I fragments from both clones, as well as the Kpn I/Sac I fragment from Pos 2 were subcloned into the pGem7zf(+) plasmid and sequenced.

The results of sequence analysis indicated that the 1.5 kb Pos 1b EcoR I fragment contained sequences corresponding to the 1.7 kb EcoR I fragment from the E4 clone, except that approximately 200 bases of 3' sequence, including the poly A tract, had been deleted. The 0.5 kb Pos 1 EcoR I fragment corresponded to the E4 0.6 kb EcoR I fragment, except that the 5'-most 150 bases had been replaced by 40 bases of novel sequence. The 1.7 kb Pos 1 EcoR I fragment was thereby determined to contain the 5' sequences of interest.

This analysis also demonstrated that the 2.6 kb Pos 2 EcoR I fragment contained the entire E4 0.6 kb EcoR I fragment, as well as 2 kb of upstream sequence information. The entire E4 1.7 kb EcoR I fragment, including the poly A tract, was found to be fused directly to lambda sequences at the 3' end (lambda reverse primed sequencing of the Pos 2 Kpn I/Sac I fragment; data not shown). Figure 16 presents a schematic comparison of the structures of the E4, Pos 1, and Pos 2 cDNA clones.

The nucleotide sequences of the entire Pos 1 and Pos 2 inserts, and the predicted amino acid sequences derived from them, are presented in figures 17 and 18. Aside from exhibiting different sequences at their 5' ends, the clones were found to be identical, at both the nucleotide and amino acid levels, except that the Pos 1 clone contained a small insert of 29

Figure 16. Schematic Comparison of the E4, Pos 1, and Pos 2 cDNA Clones

The structures presented in this figure have not been drawn to scale. The 5' terminus of each clone is at the left end. Nucleotide sequences common to all three clones are indicated in black. Sequences common to the Pos 1 and Pos 2 clones are displayed in white. Pos 1 and Pos 2 specific sequences, as well as the positions of EcoR I digestion sites within each clone, are indicated as shown below the structures diagramed. The dashed lines mark the boundaries between regions containing common sequences. The sizes of the fragments generated by EcoR I digestion of the cDNA inserts are indicated below each clone. The boxed A represents the position of the poly-A sequences in the E4 and Pos 2 clones.

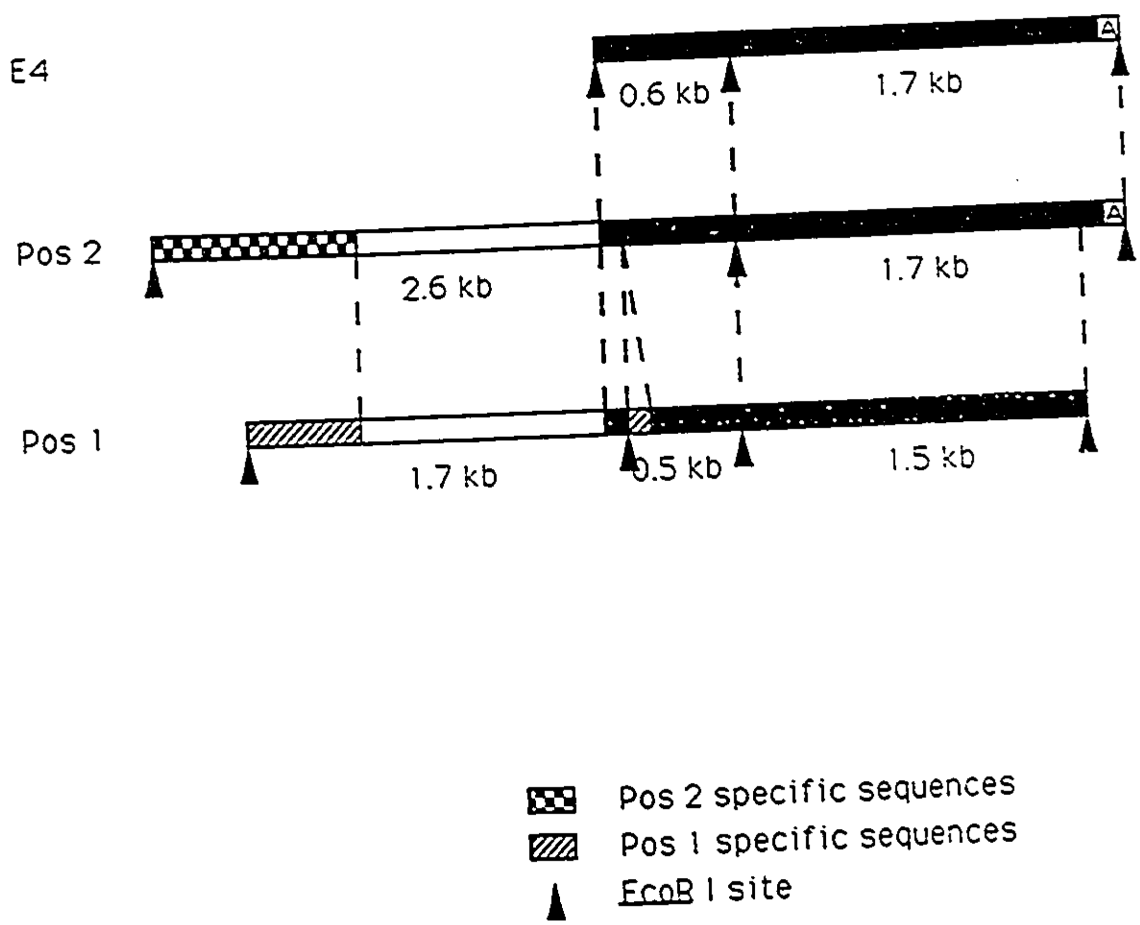

E4
0.6 kb
1.7 kb
Pos 2
2.6 kb
1.7 kb
Pos 1
1.7 kb
0.5 kb
1.5 kb
Pos 2 specific sequences
Pos 1 specific sequences
EcoR I site

Figure 17. Nucleotide and Deduced Amino Acid Sequence of Pos1.

The nucleotide and deduced amino acid sequences of the Pos1 clone are presented. The predicted amino acids are shown under the nucleotide sequence using the single letter code. The numbers on the left refer to the nucleotide positions while those on the right refer to the amino acid. This clone does not contain NH_2-terminal sequences and therefore the amino acid sequences are indicated with an apostrophe. The predicted transmembrane peptide is underlined, and potential N-glycosylation sites are indicated by the { } brackets. The [ ] brackets define the Pos1 specific insert. The * denotes a stop codon. EcoR I restriction sites are indicated by bold print.

Figure 17

```
Base                                                          a.a.'

1     GAATTCCGGATTTGTGGCCTTCTGTAGTTGAGGATGACTACGATGAGTATGAC
        I  P  D  L  W  P  S  V  V  E  D  D  Y  D  E  Y  D     17'
54    GATGGCTTCCCCATAAGTAACTGTATCTCATGCTCTTCCCATAGAGAAGAT
        D  G  F  P  I  S  N  C  I  S  C  S  S  H  R  E  D     34'
105   GAGGATATGGTAGTAGAGGAACAAAATGCAGAGGTAAACAATAACAACCTA
        E  D  M  V  V  E  E  Q  N  A  E  V  N  N  N  N  L     51'
156   ATTATAAGCTCACATTCTGAGAAACCTGAAGAAGAAGAGAGAATTTCGACT
        I  I  S  S  H  S  E  K  P  E  E  E  E  R  I  S  T     68'
207   GCTGCATCAGACAGGCAGATAAGCCATGCCACGGACAGACATACATCTGTA
        A  A  S  D  R  Q  I  S  H  A  T  D  R  H  T  S  V     85'
258   CCATCTCTGACAGCAAGTGTGATACCTGAGAAGCACAACGACCCAACATTT
        P  S  L  T  A  S  V  I  P  E  K  H  N  D  P  T  F    102'
309   TTGGAGAACCATGTTCAAACTTCTAGTGTCCCATTGCAAAACACATCAGAG
        L  E  N  H  V  Q  T  S  S  V  P  L  Q {N  T  S} E    119'
360   TCTAAGTCTTGGGCTGTTTTTACAAGTGATGAAGAAAGTGGTTCTGGCCAA
        S  K  S  W  A  V  F  T  S  D  E  E  S  G  S  G  Q    136'
411   GGTACCTCAGATAGCCTTAATGATAATGAAACGTCAACAGATTTCAGTTTT
        G  T  S  D  S  L  N  D {N  E  T} S  T  D  F  S  F    153'
462   CCTGACCTTAATGAGACAGACACTGAAGGGGCAGTTGAAGCAGGTAACTCA
        P  D  L {N  E  T} D  T  E  G  A  V  E  A  G  N  S    160'
513   GAATTAACTCCTGGATCATCACAGTCATCAGCTTCATCTGTAACTAGTGAT
        E  L  T  P  G  S  S  Q  S  S  A  S  S  V  T  S  D    177'
564   CACTCATCAGTATTCAACATCTCTGAGGCAGAGGCAAGTAATAGTAGCCAT
        H  S  S  V  F {N  I  S} E  A  E  A  S {N  S  S} H    194'
615   GAGTCTCGTATCGGTCTAGCTGAGAGTCTGGAATCAGAGAAGAAGACAGTT
        E  S  R  I  G  L  A  E  S  L  E  S  E  K  K  T  V    211'
666   ATACCACTTGTGGTCGTATCCGCCCTGACTTTTATCTGTCTAGTGATTCTT
        I  P  L  V  V  V  S  A  L  T  F  I  C  L  V  I  L    228'
717   GTGGGTATTCTCATATACTGGAGGAAATGTTTCCAGACTGCTCACTTTTAT
        V  G  I  L  I  Y  W  R  K  C  F  Q  T  A  H  F  Y    245'
768   TTAGAAGATAACACGTCACCTCGAGTGATTTCTGCTCCCCCGGCTCCAGTC
        L  E  D  N  T  S  P  R  V  I  S  A  P  P  A  P  V    262'
819   TTCCCAGTCTCAGATGATGTTGGAGCAATTCCAATAAAGCATTTTCCAAAA
        F  P  V  S  D  D  V  G  A  I  P  I  K  H  F  P  K    279'
870   CATGTTGCAGATTTACATGCAAGTAATGGTTTTTCTGAAGAATTTGAGGAA
        H  V  A  D  L  H  A  S  N  G  F  S  E  E  F  E  E    296'
921   ATCCAGAGCTGTACTGTAGATCTAGGTATTACGTCAGACAGCTCTAATCAC
        I  Q  S  C  T  V  D  L  G  I  T  S  D  S  S  N  H    313'
972   CCTGACAACAAGAATAAGAATCGATACATAAACATTGTTGCTTATGATCAT
        P  D  N  K  N  K  N  R  Y  I  N  I  V  A  Y  D  H    320'
1023  ACCAGGGTCAAACTAGCACAGCTTGCAGAAAAGGATGGAAAACTGACTGAT
        T  R  V  K  L  A  Q  L  A  E  K  D  G  K  L  T  D    337'
1074  TACATTAATGCCAACTATGTTGATGGCTACAACAAGCCAAAAGCCTACATT
        Y  I  N  A  N  Y  V  D  G  Y  N  K  P  K  A  Y  I    354'
1125  GCAGCTCAAGGGCCACTAAAATCAACAGCAGAAGATTTCTGGAGAATGATA
        A  A  Q  G  P  L  K  S  T  A  E  D  F  W  R  M  I    371'
```

```
Base                                                              a.a.'

1176  TGGGAACATAACGTAGAAGTTATTGTGATGATAACTAATCTCCTTGAGAAA
       W  E  H  N  V  E  V  I  V  M  I  T  N  L  L  E  K    388'
1227  GGAAGGAGAAAGTGCGACCAGTACTGGCCTGCTGAAGGTAGTGAAGAATAT
       G  R  R  K  C  D  Q  Y  W  P  A  E  G  S  E  E  Y    405'
1278  GGGAACTTCTTGGTTACTCAGAAGAGTGTCCACGTACTTGCTTATTACACT
       G  N  F  L  V  T  Q  K  S  V  H  V  L  A  Y  Y  T    422'
1329  GTGAGGAATTTTACTCTTAGAAACACAAAGATCAAAAAGGGTTCCCAGAAA
       V  R  N  F  T  L  R  N  T  K  I  K  K  G  S  Q  K    439'
1380  GGGAGGTCTAGTGGACGTGTAGTAACTCAGTACCATTATACCCAGTGGCCT
       G  R  S  S  G  R  V  V  T  Q  Y  H  Y  T  Q  W  P    456'
1431  GACATGGGTGTTCCAGAGTACACGCTGCCCGTGCTCACCTTCGTGCGGAAG
       D  M  G  V  P  E  Y  T  L  P  V  L  T  F  V  R  K    473'
1482  GCATCGCACGCCAAGCGCCATGCTGTCGGGCCGATTGTGGTTCATTGCAGT
       A  S  H  A  K  R  H  A  V  G  P  I  V  V  H  C  S    490'
1533  ACCTCACCTTTGATGGAACATTTGGAAAGAGAACCTCCTCATGAATTCTGT
      [T  S  P  L  M  E  H  L  E  R  E  P  P  H  E  F  C    507'
1584  CCTTCTGGAGCTGACTCTGAGGAAGCCAACAGCGCTGGTGTTGGAAGGACA
       P  S  G  A  D  S  E  E  A  N  S] A  G  V  G  R  T    524'
1635  GGCACTTACGTAGTGTTAGACAGCATGCTGCAGCAAATTCAGCATGAGGGG
       G  T  Y  V  V  L  D  S  M  L  Q  Q  I  Q  H  E  G    541'
1686  ACAGTCAACATATTTGGATTCTTAAAACACATACGTACCCAAAGGAACTAT
       T  V  N  I  F  G  F  L  K  H  I  R  T  Q  R  N  Y    558'
1737  TTGGTGCAGACTGAGGAGCAATACATCTTCATTCATGATGCACTGGTTGAA
       L  V  Q  T  E  E  Q  Y  I  F  I  H  D  A  L  V  E    575'
1788  GCAATTCTCAGTAAAGAAACAGAAGTCCTTGAGACTCATATTCATGCCTAT
       A  I  L  S  K  E  T  E  V  L  E  T  H  I  H  A  Y    592'
1839  GTTAATGCCCTCTTGATACCTGGACCAACAGGAAAAACCCGGCTGGAGAAG
       V  N  A  L  L  I  P  G  P  T  G  K  T  R  L  E  K    609'
1890  CAATTCAAGTTACTGAGCCAGTCTAACACACAGCAGTGCGATTACTCAACT
       Q  F  K  L  L  S  Q  S  N  T  Q  Q  C  D  Y  S  T    626'
1941  GCACTCAAACAGTGTAACAGGGAAAAGAATCGAACTTCATCCATCATTCCT
       A  L  K  Q  C  N  R  E  K  N  R  T  S  S  I  I  P    643'
1992  GTGGAAAGATCTAGAGTGGGTATCTCATCACTGTCTGGTGAAGGCACAGAC
       V  E  R  S  R  V  G  I  S  S  L  S  G  E  G  T  D    660'
2043  TACATCAATGCTTCCTATATTATGGGTTATTATCAAAGTAATGAATTCATT
       Y  I  N  A  S  Y  I  M  G  Y  Y  Q  S  N  E  F  I    677'
2094  ATTACCCAGCATCCCTTACTGCACACTATCAAGGATTTCTGGAGAATGATA
       I  T  Q  H  P  L  L  H  T  I  K  D  F  W  R  M  I    694'
2145  TGGGACCATAATGCCCAGCTAATAGTCATGCTCCCTGATAGCCAGAATATG
       W  D  H  N  A  Q  L  I  V  M  L  P  D  S  Q  N  M    711'
2196  GCTGAAGATGAATTTGTGTACTGGCCAAACAAAGACGAGCCTATAAACTGT
       A  E  D  E  F  V  Y  W  P  N  K  D  E  P  I  N  C    728'
2247  GAGACTTTCAAAGTTACTATGATTGCTGAAGAACACAAGTGTCTGTCTAAG
       E  T  F  K  V  T  M  I  A  E  E  H  K  C  L  S  K    745'
2298  GAGGAAAAGCTCATAATCCAAGACTTTATTTTAGAAGCTACACAGGATGAC
       E  E  K  L  I  I  Q  D  F  I  L  E  A  T  Q  D  D    762'
2349  TATGTACTCGAAGTGAGGCATTTTCAGTGTCCAAAATGGCCAAATCCTGAT
       Y  V  L  E  V  R  H  F  Q  C  P  K  W  P  N  P  D    779'
2400  AGTCCTATTAGCAAAACCTTTGAACTGATCAGCATCATCAAAGAGGAGACG
       S  P  I  S  K  T  F  E  L  I  S  I  I  K  E  E  T    796'
```

Base a.a.'

2451 TCCAACCGTGACGGACCCATGATTGTACATGATGAGCACGGAGGGGTGACA
S R N D G P M I V H D E H G G V T 813'
2502 GCAGGCACTTTCTGTGCTCTGACAACACTCATGCATCAGCTGGAAAATGAG
A G T F C A L T T L M H Q L E N E 830'
2553 AACTCGGTGGATGTTTACCAGGTAGCAAAGATGATAAATTTGATGAGGCCT
N S V D V Y Q V A K M I N L M R P 847'
2604 GGAGTCTTTACAGACATCGAACAGTACCAGTTTCTGTACAAAGCGATTCTC
G V F T D I E Q Y Q F L Y K A I L 864'
2655 AGCCTTGTCAGCACAAGGCAAGAAGAAAACCCTTCAGCATCTATGGACAGT
S L V S T R Q E E N P S A S M D S 881'
2706 AATGGCTCAGCTTTACCAGATGGAAACGCAGCTGAAAGTTTAGAATCTTTA
N G S A L P D G N A A E S L E S L 898'
2757 GTTTAAGTAACGAAGCAAATGGAACACACAAACACTTGCACCACATCGGTC
V * 899'
2808 ACATCTGGAGTTTACCATTATTCTTTTCAGTTTTATACTCAGTGGGGGAA

2859 ATCTGTCACTCTATATATAATCAGTCCCCAACCTTTGTTCCAGCAAAGTCA

2910 TTTCTGTGACATATGACTTTACTGAGGAACTTTTATCATTAACAATGTGTG

2961 CCTTTTCTTGAACGATTTCTTGTAATACGTTGTTCTGTCTGGACTAACTTG

3012 ATTGGCTTTTACAGTGTTTCTAAG[illegible]TGAATTGTGGTACGTTTTTTCAGT

3063 ATTAGTTTTTGATTTATCTGGCTTTACTTTAACTTAAAATTATCATATTTA

3114 TAGGTGTTAGGGGATTCTCAATTACAAACAAACGTCATGGTTTTAGTATTT

3165 GATTTATTTGCTGTATTTATAACAATCTACATTTGCTAGAAATATAACTTT

3216 TAATATAGTAGAATGTAAATAAAATACTGTTCTCCATAACAGTTCCGGCAT

3267 TTTAAGACTTCAATTAGACATTTAGCATAGTAATCTGATACTTACTGTAAA

3318 TACTGCTACTTCTATAGTGATTCCATGGACCAAATTTATATTTATAATTGT

3369 AGATTTTTATATTTTACCTACAGAGTCAGTTTTCTAGTTCTGTGTAATTGC

3420 CTTGTTAAAATGATGTAGTCCAGTATGTTTTTATTTTAACAAAGTCTTCTG

3471 ATATATAACTGTGCATATTAAGCAATTAACCTTCATATAGCTGCATGTGAT

3522 TTTAACCTTTTGTGGAATTC

Figure 18. Nucleotide and Deduced Amino Acid Sequence of Pos2.

Nucleotide and deduced amino acid sequences of the Pos2 clone are presented. The predicted amino acids are shown under the nucleotide sequence using the single letter code. The numbers on the left refer to the nucleotide positions while those on the right refer to the amino acid. It has not been established whether this clone contains the proper NH_2-terminal sequences and therefore the amino acid sequences are indicated with an apostrophe. The predicted transmembrane peptide is underlined, and potential N-glycosylation sites are indicated by the { } brackets. The * denotes a stop codon. ↑ denotes the 5' end of the E4 clone. underlines the polyadenylylation signal. EcoR I restriction sites are indicated by bold print.

Figure 18

```
Base                                                                  a.a.'

1       GAATTCCGATCAGGACTTGGGAGCTATTCTAAATAATCTGCTGCCCAATAC
                                          *
52      AAGTTATGTTCTTCAGATAGTTGCTGTTTGCTCTAATGGTCTGCACGGAAA
                                          *
103     ATACAGTGACCAAGTCATTGTTGATATGCCTTTAGAGGACCAAGAGACTGA
               *              *                          *
154     CCTCATTCCTGAACTGAATGAAACTGAAGATTATGAGGATGAAGCTGATGA
                  *        *     *        *     *     *  *
205     AAAAGAAACAGAGGTGGATGAGGAAACTGCAGTGATTCCTAGCACTGACAG
                           *                    *     *
256     TGCAATAAAACAAATAAAGAGGAAAGATTTCCAGATGACTACATCAAGCTA

307     CAGCCAAGAAAACATGGGGACAAAACC[illegible]ATGAAGCTAAAATAAATAGATA
                                    *  *     *        *
358     CTTAACAAGAGAAGATTGCCTGGTAAGGATGAGGTTTTCAAAGCAGTTTAT

409     TATCCTACTTCTCCACCTGTCAGTGAAACCTCTGAAGAAGAAGATGCTTTT

460     TTGGAATCCCAAACGATTACTGAAATTCCTGCTCAATTCACTGACTCCACC

511     AAAGGCATAGAAGAAGAATACATATACCTTTCTTCAGGCACTGAACCAACC

562     AGGATAACTACTACTACAGGCCATAGTGGTGAAGATTTCTTATTGTCTCCT

613     TTTAAACCTGATCAGGAATCTGATGACTTTTGGAGTTCAGTTCTCACCACT
            :
664     TCTTCAGCACATCTGGTTACAGAGGAGACCTCCCAAGAAGACATCCTTCCT

715     TCCGGAAGCCCAGATATAAGCATGCAGGATGTCCTTTACCAAGGCTCTGTT
                              M  Q  D  V  L  Y  Q  G  S  V      10'
766     ACACATGTTTCAGAAGGTGCAGCACCCCCAAGCTCTGATAAGCCTCCAAAG
         T  H  V  S  E  G  A  A  P  P  S  S  D  K  P  P  K      27'
817     CATACATCTTCCACTGATGGAAATTTGTGGTTTCGGAATGCTACGGAGCTG
         H  T  S  S  T  D  G  N  L  W  F  R {N  A  T} E  L      44'
868     ACAAGTCAGCCTGTTGGTACAATGGATAGCAGTCATTTGTCTCAGACCAGT
         T  S  Q  P  V  G  T  M  D  S  S  H  L  S  Q  T  S      63'
919     TACACTGAGGATTACGTAGAGGGATTAAAGCCAGCTACAGTGCCCTATTCC
         Y  T  E  D  Y  V  E  G  L  K  P  A  T  V  P  Y  S      80'
970     GGCAGCCCAGTGGTTTCACAAGACACGTCAGATGTGGATTTAGAATTGCCA
         G  S  P  V  V  S  Q  D  T  S  D  V  D  L  F  L  P      97'
1021    CATTATTCTACCTTTGCTTTCTCCTCTGCTGAATTACCACCTCACTCTCTC
         H  Y  S  T  F  A  F  S  S  A  E  L  P  P  H  S  L     114'
1072    TCTTCAAGCTCTGGAGAATATGGTTCTGCTTCTGCTGCCAGTGAGGTACTC
         S  S  S  S  G  E  Y  G  S  A  S  A  A  S  E  V  L     131'
1123    TCTCAGACAACTCAACCAATCTATAATGAGGCAAGTAATAGTAGCCATGAG
         S  Q  T  T  Q  P  I  Y  N  E  A  S {N  S  S} H  E     148'
```

Base a.a.'

1174 TCTCGTATCGGTCTAGCTGAGAGTCTGGAATCAGAGAAGAAGACAGTTATA
S R I G L A E S L E S E K K T V V 165'
1225 CCACTTGTGGTCGTATCCGCCCTGACTTTTATCTGTCTAGTGATTCTTGTG
P L V V V S A L T F I C L V I L V 182'
1276 GGTATTCTCATATACTGGAGGAAATGTTTCCAGACTGCTCACTTTTATTTA
G I L I Y W R K C F Q T A H F Y L 199'
1327 GAAGATAACACGTCACCTCGAGTGATTTCTGCTCCCCCGGCTCCAGTCTTC
E D N T S P R V I S A P P A P V F 216'
1378 CCAGTCTCAGATGATGTTGGAGCAATTCCAATAAAGCATTTTCCAAAACAT
P V S D D V G A I P I K H F P K H 233'
1429 GTTGCAGATTTACATGCAAGTAATGGTTTTTCTGAAGAATTTGAGGAAATC
V A D L H A S N G F S E E F E E I 250'
1480 CAGAGCTGTACTGTAGATCTAGGTATTACGTCAGACAGCTCTAATCACCCT
Q S C T V D L G I T S D S S N H P 267'
1531 GACAACAAGAATAAGAATCGATACATAAACATTGTTGCTTATGATCATACC
D N K N K N R Y I N I V A Y D H T 284'
1582 AGGGTCAAACTAGCACAGCTTGCAGAAAAGGATGGAAAACTGACTGATTAC
R V K L A Q L A E K D G K L T D Y 301'
1633 ATTAATGCCAACTATGTTGATGGCTACAACAAGCCAAAAGCCTACATTGCA
I N A N Y V D G Y N K P K A Y I A 318'
1684 GCTCAAGGGCCACTAAAATCAACAGCAGAAGATTTCTGGAGAATGATATGG
A Q G P L K S T A E D F W R M I W 335'
1735 GAACATAACGTAGAAGTTATTGTGATGATAACTAATCTCCTTGAGAAAGGA
E H N V E V I V M I T N L L E K G 352'
1786 AGGAGAAAGTGCGACCAGTACTGGCCTGCTGAAGGTAGTGAAGAATATGGG
R R K C D Q Y W P A E G S E E Y G 369'
1837 AACTTCTTGGTTACTCAGAAGAGTGTCCACGTACTTGCTTATTACACTGTG
N F L V T Q K S V H V L A Y Y T V 386'
1888 AGGAATTTTACTCTTAGAAACACAAAGATCAAAAAGGGTTCCCAGAAAGGG
R N F T L R N T K I K K G S Q K G 403'
1939 AGGTCTAGTGGACGTGTAGTAACTCAGTACCATTATACCCAGTGGCCTGAC
R S S G R V V T Q Y H Y T Q W P D 420'
1990 ATGGGTGTTCCAGAGTACACGCTGCCCGTGCTCACCTTCGTGCGGAAGGCA
M G V ↑P E Y T L P V L T F V R K A 437'
2041 TCGCACGCCAAGCGCCATGCTGTCGGGCCGATTGTGGTTCATTGCAGCGCT
S H A K R H A V G P I V V H C S A 454'
2092 GGTGTTGGAAGGACAGGCACTTACGTAGTGTTAGACAGCATGCTGCAGCAA
G V G R T G T Y V V L D S M L Q Q 471'
2143 ATTCAGCATGAGGGGACAGTCAACATATTTGGATTCTTAAAACACATACGT
I Q H E G T V N I F G F L K H I R 488'
2194 ACCCAAAGGAACTATTTGGTGCAGACTGAGGAGCAATACATCTTCATTCAT
T Q R N Y L V Q T E E Q Y I F I H 505'
2245 GATGCACTGGTTGAAGCAATTCTCAGTAAAGAAACAGAAGTCCTTGAGACT
D A L V E A I L S K E T E V L E T 522'
2296 CATATTCATGCCTATGTTAATGCCCTCTTGATACCTGGACCAACAGGAAAA
H I H A Y V N A L L I P G P T G K 539'
2347 ACCCGGCTGGAGAAGCAATTCAAGTTACTGAGCCAGTCTAACACACAGCAG
T R L E K Q F K L L S Q S N T Q Q 556'

Base a.a.'

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2398 TGCGATTACTCAACTGCACTCAAACAGTGTAACAGGGAAAAGAATCGAACT
      C  D  Y  S  T  A  L  K  Q  C  N  R  E  K  N  R  T   573'
2449 TCATCCATCATTCCTGTGGAAAGATCTAGAGTGGGTATCTCATCACTGTCT
      S  S  I  I  P  V  E  R  S  R  V  G  I  S  S  L  S   590'
2500 GGTGAAGGCACAGACTACATCAATGCTTCCTATATTATGGGTTATTATCAA
      G  E  G  T  D  Y  I  N  A  S  Y  I  M  G  Y  Y  Q   607'
2551 AGTAATGAATTCATTATTACCCAGCATCCCTTACTGCACACTATCAAGGAT
      S  N  E  F  I  I  T  Q  H  P  L  L  H  T  I  K  D   624'
2602 TTCTGGAGAATGATATGGGACCATAATGCCCAGCTAATAGTCATGCTCCCT
      F  W  R  M  I  W  D  H  N  A  Q  L  I  V  M  L  P   641'
2653 GATAGCCAGAATATGGCTGAAGATGAATTTGTGTACTGGCCAAACAAAGAC
      D  S  Q  N  M  A  E  D  E  F  V  Y  W  P  N  K  D   658'
2704 GAGCCTATAAACTGTGAGACTTTCAAAGTTACTATGATTGCTGAAGAACAC
      E  P  I  N  C  E  T  F  K  V  T  M  I  A  E  E  H   675'
2755 AAGTGTCTGTCTAAGGAGGAAAAGCTCATAATCCAAGACTTTATTTTAGAA
      K  C  L  S  K  E  E  K  L  I  I  Q  D  F  I  L  E   692'
2806 GCTACACAGGATGACTATGTACTCGAAGTGAGGCATTTTCAGTGTCCAAAA
      A  T  Q  D  D  Y  V  L  E  V  R  H  F  Q  C  P  K   709'
2857 TGGCCAAATCCTGATAGTCCTATTAGCAAAACCTTTGAACTGATCAGCATC
      W  P  N  P  D  S  P  I  S  K  T  F  E  L  I  S  I   726'
2908 ATCAAAGAGGAGACGTCCAACCGTGACGGACCCATGATTGTACATGATGAG
      I  K  E  E  T  S  N  R  D  G  P  M  I  V  H  D  E   743'
2959 CACGGAGGGGTGACAGCAGGCACTTTCTGTGCTCTGACAACACTCATGCAT
      H  G  G  V  T  A  G  T  F  C  A  L  T  T  L  M  H   760'
3010 CAGCTGGAAAATGAGAACTCGGTGGATGTTTACCAGGTAGCAAAGATGATA
      Q  L  E  N  E  N  S  V  D  V  Y  Q  V  A  K  M  I   777'
3061 AATTTGATGAGGCCTGGAGTCTTTACAGACATCGAACAGTACCAGTTTCTG
      N  L  M  R  P  G  V  F  T  D  I  E  Q  Y  Q  F  L   794'
3112 TACAAAGCGATTCTCAGCCTTGTCAGCACAAGGCAAGAAGAAAACCCTTCA
      Y  K  A  I  L  S  L  V  S  T  R  Q  E  E  N  P  S   811'
3163 GCATCTATGGACAGTAATGGCTCAGCTTTACCAGATGGAAACGCAGCTGAA
      A  S  M  D  S  N  G  S  A  L  P  D  G  N  A  A  E   828'
3214 AGTTTAGAATCTTTAGTTTAAGTAACGAAGCAAATGGAACACACAAACACT
      S  L  E  S  L  V  *                                 834'
3265 TGCACCACATCGGTCACATCTGGAGTTTACCATTATTCTTTTCAGTTTTAT

3316 ACTCAGTGGGGGAAATCTGTCACTCTATATATAATCAGTCCCCAACCTTT

3367 GTTCCAGCAAAGTCATTTCTGTGACATATGACTTTACTGAGGAACTTTTAT

3418 CATTAACAATGTGTGCCTTTTCTTGAACGATTTCTTGTAATACGTTGTTCT

3469 GTCTGGACTAACTTGATTGGCTTTTACAGTGTTTCTAAGAATTGAATTGTG

3520 GTACGTTTTTTCAGTATTAGTTTTTGATTTATCTGGCTTTACTTTAACTTA

3571 AAATTATCATATTTATAGGTGTTAGGGGATTCTCAATTACAAACAAACGTC

3622 ATGGTTTTAGTATTTGATTTATTTGCTGTATTTATAACAATCTACATTTGC
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Base

3673 TAGAAATATAACTTTTAATATAGTAGAATGTAAATAAAATACTGTTCTCCA

3724 TAACAGTTCCGGCATTTTAAGACTTCAATTAGACATTTAGCATAGTAATCT

3775 GATACTTACTGTAAATACTGCTACTTCTATAGTGATTCCATGGACCAAATT

3826 TATATTTATAATTGTAGATTTTTATATTTTACCTACAGAGTCAGTTTTCTA

3877 GTTCTGTGTAATTGCCTTGTTAAAATGATGTAGTCCAGTATGTTTTTATTT

3928 TAACAAAGTCTTCTGATATATAACTGTGCATATTAAGCAATTAACCTTCAT

3979 ATAGCTGCATGTGATTTTAACCTTTTGTGAGGAATAGAAATCATTTGTTTT

4030 GAAATGAGAAGTTTTTATGAGAATAACACCAGTACTTGGCATTGTTAAATT

4081 GTTTTTACCTATGGCATTGCAAAAATAAATATAAATATTCCAAAAAAAAAA

4132 AAAAAAAAAAAAAAAAAAAAACGGAATTC

amino acids (87 bases) not found in the Pos 2 clone. the significance of this insert was and still is unclear. The fact that the insert lies within the VHCSAGV sequence was extremely surprising, given that this region has been found to be a functionally important and highly conserved pTP domain (Tonks N. et al., 1988b, Strueli M. et al., 1989, 1990). While the nucleic acid sequence of the insert contained the 5' and 3' consensus RNA splice sites (GT and AG respectively), removal of this sequence through splicing would result in a shift in the amino acid reading frame of the cDNA. It therefore seemed possible that the insert resulted from a cloning artifact.

During the course of isolation of the Pos 1 and Pos 2 clones, three reports appeared in the literature which described the identification of cDNAs encoding novel pTPs related to CD45, the first transmembrane class pTP identified (Kaplan, R. et al., 1990, Krueger, N. X. et al., 1990, Guan and Dixon, 1990; Charbonneau et al., 1988; Tonks et al., 1988c). Figure 19, which presents a comparison of the amino acid sequences of Pos 1 and Pos 2 with the partial amino acid sequences reported for human PTP zeta (HPTP zeta) indicated that these clones likely encode the chicken homolog of this protein. The HPTP zeta sequences presented in this figure represented a combination of both the LCA beta sequences of Kaplan et al. (1990), and the HPTP zeta sequences of Krueger et al. (1990). Neither group isolated a full length cDNA, however, it appeared that both cDNAs encoded the same protein. Since the

Figure 19. Homology Comparison of the Amino Acid Sequences of Pos1, Pos2, and HPTP zeta.

Figure 19 shows a comparison of the predicted amino acid sequences of human HPTP zeta, a combination of the sequences of Krueger et al. (1990) and Kaplan et al. (1990), and those deduced for the Pos1 and Pos2 clones. Dashes indicate identical amino acids. Spaces in either sequence indicate gaps. Pos1 and Pos2 are 75% and 76% homologous to Human HPTP zeta, respectively.

Figure 19

```
HPTPz
Pos1    IPDLWPSVVEDDYDEYDDGFPISNCISCSSHREDEDMVVEEQNAEVNNNNLIISSH
Pos2

HPTPz         NSEEDNRVTSVSSDSQTGMDRSPGKSPSANGLSQKHNDGKEENDIQTGSA
Pos1    SEKPEEEERISTAASDRQISHA-DRHT-VPSLTASVIPEKHNDPTFL-NHV--S-V
Pos2       MQDVLYQGSVTHVSEGAAPPSS-KP-KHTS-TDGNLWFR-ATELTSQPVGTMD

HPTPz   LLPLSP--KAWAVLTSDEESGSGQGTSDSLNENETSTDFSFADTNEKDADGILAAG
Pos1    P-QNTSES-S---F------------------D---------P-L--T-TE-AVE--
Pos2    SSH--QTSYTEDYVEGLKPATVPYSG-PVVSQDTSDV-LELPHYSTFAFSSAELPP

HPTPz   DSEITPGFPQSPTSSVTSENSEVFHVSEAEASNSSHESRIGLAEGLESEKKAVIPL
Pos1    N--L---SS--SA-----DH-S--NI------------------S------T----
Pos2    H-LSSSSGEYGSA-AASEVL-QTTQPIYN---------------S------T----

HPTPz   VIVSALTFICLVVLVGILIYWRKCFQTAHFYLEDSTSPRVISTPPTPIFPISDDVG
Pos1    -V----------I---------------------N-------A--A-V--V-----
Pos2    -V----------I---------------------N-------A--A-V--V-----

HPTPz   AIPIKHFPKHVADLHASSGFTEEFETLKEFYQEVQSCTVDLGITADSSNHPDNKHK
Pos1    ----------------N--S-----       -I----------S---------N-
Pos2    ----------------N--S-----       -I----------S---------N-

HPTPz   NRYINIVAYDHSRVKLAQLAEKDGKLTDYINANYVDGYNRPKAYIAAQGPLKSTAE
Pos1    -----------T---------------------------K----------------
Pos2    -----------T---------------------------K----------------

HPTPz   DFWRMIWEHNVEVIVMITNLVEKGRRKCDQYWPADGSEEYGNFLVTQKSVQVLAYY
Pos1    --------------------L-------------E---------------H-----
Pos2    --------------------L-------------E---------------H-----

HPTPz   TVRNFTLRNTKIKKGSQKGRPSGRVVTQYHYTAAYAKRHAVGPVVVHC
Pos1    --------------------S------------SH--------I----STSPLMEH
Pos2    --------------------S------------SH--------I----

HPTPz                       SAGVGRTGTYIVLDSMLQQIQHEGTVNIFGFLKHIR
Pos1    LEREPPHEFCPSGADSEEAN----------V-------------------------
Pos2                        ----------V-------------------------

HPTPz   SQRNYLVQTEEQYVFIHDTLVEAILSKETEVLDSHIHAYVNALLIPGPAGKTKLEK
Pos1    T------------I-------------------ET-------------T---R---
Pos2    T------------I-------------------ET-------------T---R---
```

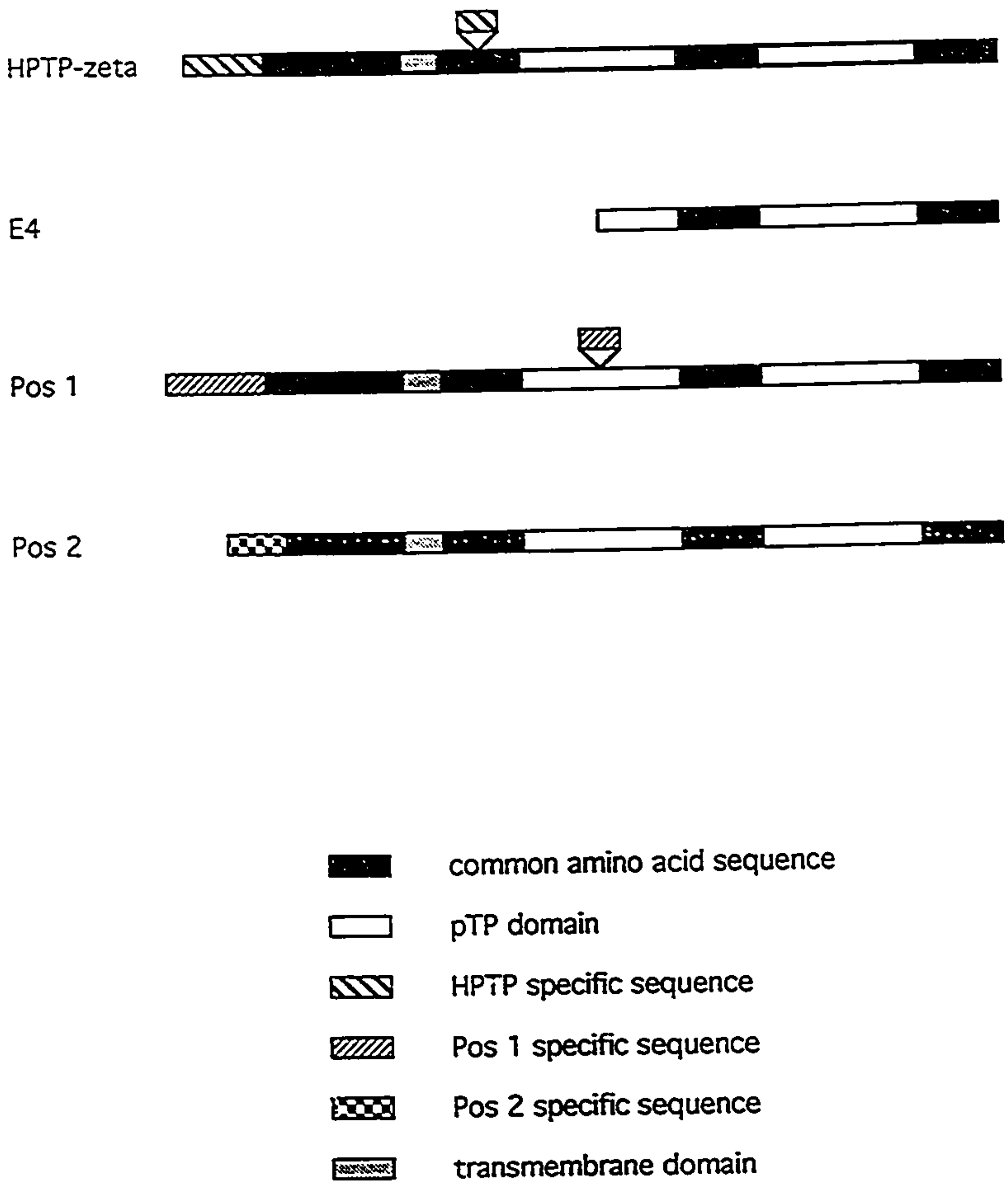
```
HPTPz   QFQLLSQSNIQQSDYSTAALKQCNREKNRTSSIIPVERSRVGISSLSGEGTDYINA
Pos1    --K------T--C-------------------------------------------
Pos2    --K------T--C-------------------------------------------

HPTPz   SYIMGYYQSNEFIITQHPLLHTIKDFWRMIWDHNAQLVVMIPDGQNMAEDEFVYWP
Pos1    -------------------------------------I--L--S------------
Pos2    -------------------------------------I--L--S------------

HPTPz   NKDEPINCESFKVTIMAEEHKCLSNEEKLIIQDFILEATQDDYVLEVRHFQCPKWP
Pos1    ---------T----MI--------K-------------------------------
Pos2    ---------T----MI--------K-------------------------------

HPTPz   NPDSPISKTFELISVIKEEAANRDGPMIVHDEHGGVTAGTFCALTTLMHQLEKENS
Pos1    --------------I----TS-------------------------------N---
Pos2    --------------I----TS-------------------------------N---

HPTPz   VDVYQVAKMINLMRPGVFTDIEQYQFLYKVILS
Pos1    -----------------------------A---LVSTRQEENPSASMDSNGSALPD
Pos2    -----------------------------A---LVSTRQEENPSASMDSNGSALPD

HPTPz
Pos1    GNAAESLESLV
Pos2    GNAAESLESLV
```

two cDNAs contained different but overlapping sequences, the sequences presented in the two reports were combined to generate the HPTP zeta sequence presented in Figure 19. The sequence of the rat brain homolog identified by Guan and Dixon (1990) have not been included as these sequences were incomplete, only the sequences of the two catalytic domains having been reported. The three sequences shown in Figure 19 displayed three significant differences: 1) the presence of different sequences at their respective amino termini; b) the presence of the insert in the Pos 1 clone; and 3) the presence of an insert in the HPTP zeta cDNA clone located within the region between the transmembrane domain and the first pTP domain, but absent in both chicken cDNA clones. The functional importance of these inserts remains unclear. Figure 20 presents a schematic comparison of the predicted structures of the HPTP, E4, Pos 1, and Pos 2 proteins.

There were several in-frame stop codons located upstream of the first methionine codon in the amino acid sequence deduced by translation of the Pos2 clone nucleic acid sequence (figure 18). It was possible that this ATG codon could be the translation initiation site for an alternatively spliced form of chicken PTP zeta RNA. The nucleotide sequence surrounding this ATG did not correspond exactly with the CCACCATGG consensus translation initiation signal (Kozak, 1986), but the presence of an A at position -3 suggested that this ATG could function as a reasonably efficient initiation

<u>Figure 20</u>. Schematic Comparison of the HPTP, E4, Pos 1 and Pos 2 Proteins

The structures presented have not been drawn to scale. The NH_2-terminus of each protein is at the left end. Amino acid sequences common to all the proteins are indicated in black. The phosphatase domains, which also contain common sequences, are displayed in white. HPTP, Pos 1, and Pos 2 specific sequences, as well as the transmembrane sequences common to HPTP, Pos 1 and Pos 2, are indicated as shown below the structures diagramed. Cytoplasmic amino acid sequences are located to the right of the transmembrane domain, while those corresponding to extracellular sequences are located to the left. The positions of the 7 amino acid HPTP and 29 amino acid Pos 1 specific inserts are also indicated.



HPTP-zeta
E4
Pos 1
Pos 2
common amino acid sequence
pTP domain
HPTP specific sequence
Pos 1 specific sequence
Pos 2 specific sequence
transmembrane domain

site. The sequence surrounding the second and only other methionine codon before the transmembrane domain was in closer agreement with the consensus site sequence and might provide an even more efficient site for initiation of translation.

While the first 21 amino acids following both possible translation initiation sites were relatively hydrophobic (see Figure 18), neither contained sequences characteristic of a signal peptide, the presence of which is required for proper post translational processing and subcellular distribution of transmembrane proteins (von Heijne 1985; von Heijne, 1986). Initiation at the first methionine would yield the better of the two possible signal peptides, however, computer analysis using the method of von Heijne (1986), suggested that the peptide might not be efficiently cleaved. The analysis indicated that if cleavage were to take place, the most probable cleavage site would be between the two alanine residues at positions 16 and 17.

The lack of a consensus signal peptide suggested the possibility that the Pos2 clone may not contain an authentic alternatively spliced form of chicken PTP zeta cDNA, but rather may represent a cDNA insert derived from an incompletely processed nuclear RNA. It is also possible that Pos1 cDNA insert arose from an incompletely processed RNA. The NH_2-terminal most amino acid sequences deduced from translation of this clone differed greatly from the NH_2-terminal sequences reported by Krueger et al. (1990). The suggestion that the two cDNA inserts were derived from incompletely processed RNA was supported by the observation that the nucleotide sequences at the points where the amino acid sequences of the two clones diverged from those of HPTP zeta, contained the 3' AG consensus splice acceptor signal. It still remains possible, however, that both the Pos 1 and Pos 2 clones represented alternatively spliced forms of chicken PTP zeta cDNA, the Pos 1 clone having been truncated, resulting in the loss of the 5' end of the full length cDNA.

While both clones appeared to contain the entire transmembrane and cytoplasmic domains of chicken PTP zeta, neither appeared to contain the complete extracellular domain. A full length amino acid sequence of PTP zeta has not been reported, making it difficult to assess the amount of PTP zeta sequence information missing in the two clones. The Pos 1 and Pos 2 cDNAs encode products of 899 and 834 amino acid residues, respectively.

CHAPTER 5: DISCUSSION

Several years ago our group reported the partial purification of chicken pTP enzymes from the soluble fraction of CEF extracts (Nelson and Branton, 1984). In order to gain a better general understanding of pTPs, those investigations have been extended. The original goal of the studies presented herein was to purify a tyrosine phosphatase to homogeneity, and to clone the corresponding cDNA encoding the enzyme.

5-I. Purification and Characterization of a Nonreceptor Phosphotyrosyl-protein Phosphatase

Using a method very similar to that used by Tonks et al. (1988a), we have managed to purify a low molecular weight nonreceptor pTP to near homogeneity from the soluble fraction of extracts prepared from chicken embryo bodies. The key step in this protocol was affinity chromatography on thiophosphorylated RCM lysozyme-Sepharose, a procedure only developed by Tonks et al. (1988a) during the course of the present studies. As judged by SDS-PAGE (figure 8), this final step in the protocol resulted in the separation of pTPIII from the majority of other proteins that copurified through the previous purification steps.

The biochemical properties exhibited by pTPIII (table 4) were very similar to those displayed by the pTP1B enzyme purified by Tonks et al. (Tonks et al., 1988a; Tonks et al., 1988b; Pallen et al., 1991) from the soluble portion human placental extracts. Both enzymes eluted from the various chromatography columns at similar positions in the respective activity elution profiles, and both displayed a requirement for the inclusion of reducing agents (dithiothreitol, or beta-mercaptoethanol) in the purification and assay buffers (data not shown). Neither enzyme demonstrated a dependence upon divalent cations for full activity. In fact both were generally mildly inhibited by the inclusion of metal ions in the assay buffer (Pallen et al., 1991). Both enzymes also displayed similar susceptibilities to inhibition by Na_2VO4 and Zn^{+2} (Tonks et al., 1988b; Nelson and Branton, 1984). Together, these observations suggested that pTPIII might represent the chicken homolog of pTP1B, or a PTP with similar properties.

The difference in molecular weights determined for the two enzymes could be due to partial proteolysis of pTP1B during purification. The molecular weight of pTP1B was determined to be approximately 37 kd by SDS-PAGE analysis (Tonks et al., 1988a). However, bacterial expression of the cDNA encoding human placental pTP1B or the rat brain homolog of pTP1B resulted in the identification of protein bands with molecular weights of 56 kd and 50 kd, respectively, that possessed pTP activity (Guan et al., 1990; Chernoff et al., 1990). Comparison of the amino acid sequences of pTP1B predicted from translation of the nucleotide sequences of the human and rat cDNAs with the amino acid sequence determined from peptide sequencing of the purified 37 kd pTP1B protein revealed that approximately 11 kd of protein at the COOH-terminus were lost during purification of the enzyme. Recently, a pTP with a molecular weight of approximately 50 kd has been purified from solubilized membrane extracts prepared from human placenta (Pallen et al., 1991). Analysis of the amino acid composition of this enzyme suggested that it represents a larger, perhaps unproteolyzed, version of pTP1B. In light of these results, it is possible, despite the discrepancy in molecular weights, that pTPIII represents the chicken homolog of pTP1B.

The differences in molecular weight between pTPIII and the human enzyme purified by Pallen et al. (1991) may be due to differences in post-translational modifications. Possible modifications such as isoprenylation and/or myristoylation have been suggested (Guan et al., 1990). Such modifications could serve to localize pTP1B to the membrane. Phosphorylation is another possible modification that could alter mobility of the protein on SDS-PAGE. However, no post-translational modifications have been positively identified in pTPs.

Tonks et al. (1988a) attained an approximately 11,000-fold purification of pTP1B, while chicken pTPIII was purified only approximately 1900-fold. However, the degree of purification of pTPIII was similar to that achieved by Pallen et al. (1991) who purified a larger molecular weight version of pTP1B. As discussed above, the differences in molecular weight between the two forms of human placental pTP1B appears to be due the absence of approximately 11 kd at the COOH-terminus in the form isolated by Tonks et al. (1988a). Experiments involving in vivo expression of full length and truncated forms of a T cell pTP suggested that the COOH-terminal sequences of these proteins may play a very important role in regulating phosphatase activity (Cool et al., 1990,; Zander et al., 1991). Further, truncation of the T cell protein by partial proteolysis resulted in increased phosphatase activity. Thus, the differences in specific activity and purification achieved may have reflected the presence or absence of an intact COOH-terminus.

Clarification of the relationship of pTPIII to pTP1B, and any of the other pTPs that have been identified to date, will likely not be possible until the amino acid sequence of pTPIII has been determined either by peptide sequencing, or through the isolation and sequencing of the cDNA encoding this enzyme. Similarly, the relationship of pTPI to other known tyrosine phosphatases will have to await further purification and biochemical characterization of this enzyme.

The present study has shown that although as many as six pTPs can be detected in soluble extracts prepared from CEFs, or from 11 day old chicken embryo bodies, only two major activities may actually exist, pTPI and pTPIII. The other species appeared to represent degradation products of the two major forms, probably resulting from the action of calcium-dependent proteases. While three different substrates were used in this study, these results did not rule out the possibility that other pTPs might be identified through the use of other substrates (See Appendix). Each of the tyrosine phosphorylated substrate proteins used in this study represented an artificial nonphysiologically relevant substrate. The recent finding that $p80^{cdc25}$ is in fact a very specific tyrosine phosphatase, coupled with the demonstration that both domains of HPTP alpha possess pTP activity with differing substrate specificities, underscore the importance of using the appropriate substrates in the detection and identification of pTPs (Dunphy and Kumagai, 1991; Gautier et al., 1991; Yang and Pallen, 1991). Through the use of novel substrates, such as the phosphotyrosyl-proteins that have been demonstrated to be authentic pTK substrates, or phosphotyrosyl-peptides corresponding to the phosphorylation sites of these pTK substrate proteins, it may be possible to isolate previously unidentified and highly specific pTP activities.

Several reports had demonstrated that calcineurin, alkaline phosphatases and acid phosphatases are active against phosphotyrosyl proteins. The results of the present study strongly suggested that the type 2B pSP, or calcineurin, was not responsible for a significant proportion of either pTPI or pTPIII activity. Both of these enzymes displayed characteristics inconsistent with the properties of the type 2B pSP.

Using both enolase and pNPP as substrates, pTPI and pTPIII exhibited pH optima and other properties comparable to acid phosphatases. Both enzymes demonstrated a clear acid optimum against pNPP and a broad acid to neutral range with enolase. Several reports have described similar results with pTPs from other sources (Taga and Van Etten, 1982; Shriner and Brautigan, 1984; Li et al., 1984; Lau et al., 1985; Sparks and Brautigan, 1985; Boivin and Galand, 1986; Waheed et al., 1988). It is possible that a major portion of pTP activity in the cell can be attributed to acid phosphatases, although this conclusion must be regarded with caution as it is becoming clear that the type of pTP detected may be highly dependent upon the nature of the substrate used. As reviewed by Sparks and Brautigan (1986), activity observed against pNPP is often misleading, especially when the assay is carried out in the presence of Mg^{2+}. However, both pTPI and pTPIII displayed considerable activity against pNPP both in the absence and presence of divalent cations. Most pTPs can employ pNPP as a substrate (Sparks and Brautigan, 1986), and such activity seems not unreasonable as the molecular structure of pNPP resembles that of phosphotyrosine. It should be noted that pTP1B will also utilize pNPP as a substrate, however, unlike pTPIII, this enzyme exhibits a pH optimum in the 6.4-7.0 range (Pallen et al., 1991).

It is still not clear if acid phosphatases are physiologically important in the regulation of tyrosine phosphorylation in cells. Acid phosphatases act on a number of classes of substrates and, although they frequently prefer phosphotyrosyl to phosphoseryl proteins, they are also active against an extremely broad spectrum of phosphoesters. As discussed above, it is possible that the choice of substrates used to identify pTPs may have favored the detection of acid phosphatases which are quite abundant in most tissues. Thus highly specific pTPs may exist which have escaped detection because of their low abundance and high specificity. It is also possible that acid phosphatases are the physiologically important enzymes. Their relative preference for phosphotyrosyl over phosphoseryl substrates would provide some specificity as would the fact that they display a more neutral pH optimum against phosphotyrosyl proteins than against low molecular weight substrates such as pNPP. Clearly it will be necessary to use DNA cloning techniques in order to allow for a careful analysis of the identity of different pTP species, their substrate specificities, and the physiological role in regulation of biological processes.

The results of biochemical characterization of the phosphatase activities of pTPI and pTPIII were presented in Table 4. These results showed that both pTPI and pTPIII possess properties that clearly distinguish these enzymes from the classical pSPs. Type 1 and type 2A pSPs can be totally inactivated by treatment with NaF and EDTA at 30°C for 30 minutes (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1983). This treatment had virtually no effect on the activities of pTPI and pTPIII. Further, neither enzyme exhibited a requirement for Mg^{+2} or Mn^{+2}, a property that distinguishes these enzymes from the type 2C phosphatases that require divalent cations for full activity. Finally, while pTPIII activity eluted from the ion exchange resin DE-52 at a position in the salt gradient just prior to the elution of the major forms of pSP activity that are also bound on this column (Nelson and Branton, 1984), no pSP activity was detected in pTPIII preparations following Cibracon Blue 3GA chromatography (data not shown). Similarly, no pSP activity was associated with pTPI following CM-52 chromatography. Together, all of these results left little doubt that the pTPI and pTPIII phosphatases are distinct from the classical pSP enzymes.

Attempts were made to develop polyclonal rabbit antisera and monoclonal antibodies to pTPIII in the hopes of using such antibodies to isolate the cDNA encoding this enzyme. Unfortunately, these attempts were not successful because the screening method used, the immunoprecipitation of pTP activity from partially purified pTPIII preparations, yielded very erratic results. While one of the rabbit immune sera tested specifically immunoprecipitated a 58 kd protein band from [^{35}S] methionine labelled CEF extracts (data not shown), direct correlation of this protein band with pTPIII was not possible.

Attempts to use this antiserum to screen western blots of purified pTPIII yielded negative results. As a result of the failure of these experiments, it was decided to abandon further characterization of these immune sera. Instead, a better strategy would be to generate anti-sera against synthetic peptides designed on the basis of regions of homology between the rat and human pTP1B proteins. Through the use of such antibodies, it should be possible to establish whether pTPIII is in fact related to pTP1B. Further, should such tools become available, it will make future experiments designed to investigate and characterize the role of pTP1B and/or pTPIII in regulating in CEFs much more feasible.

5-II. Isolation of Phosphotyrosyl-protein Phosphatase cDNAs

One of the goals of this study was to isolate cDNAs encoding chicken pTP enzymes. Through the use of degenerate oligonucleotide probes, cDNAs encoding two different chicken pTPs have been isolated. The probes were designed to hybridize to cDNAs encoding the VHCSAGV amino acid sequence, a functionally important and highly conserved pTP domain. Unfortunately, while this study resulted in the identification of two pTP cDNA clones, neither was likely to encode the pTPIII enzyme purified from soluble chicken embryo body extracts.

Analysis of the H3 clone indicated that the cDNA insert corresponded to a truncated cDNA encoding a portion of the chicken homolog of LAR, a transmembrane pTP that has been proposed to play an important role in cell adhesion. The cDNA insert contained sequences corresponding to the COOH-terminal pTP domain of LAR as well as some 3' untranslated sequences. A comparison of the predicted amino acid sequence of H3 and the equivalent regions of the human (Streuli et al., 1988) and Drosophila (Streuli et al., 1989) LAR proteins (Figure 13) showed that the chicken LAR amino acid sequence is 94% and 90% identical its human and Drosophila counterparts, respectively. Given the widely divergent nature of the species from which these sequences were determined, the high level of homology observed suggests that LAR must play a very important functional role within the cell. However, the nature of this role remains to be defined.

Analysis of the second pTP clone indicated that the cDNA insert contained sequences encoding the chicken homolog of HPTP zeta, another member of the transmembrane class of pTPs. Partial cDNAs encoding HPTP zeta have been independently isolated by two different laboratories (Kaplan et al., 1990; Kreuger et al., 1990). In an attempt to isolate cDNA inserts containing the missing 5' sequences of the chicken homolog of PTP zeta, two clones designated Pos 1 and Pos 2, were isolated. PCR analysis demonstrated that each contained 5' sequences which were absent in the original clone (Figure 14).

Sequence analysis of Pos 1 and Pos 2 has shown that the two clones contain different nucleotide sequences at the 5' ends, and that sequences present at the 3' end of Pos 2 were absent in the Pos 1 clone. Further, the Pos 1 clone was found to contain an insertion of 87 nucleotides (29 amino acids) in the middle of the first or most NH_2-terminal pTP domain that is absent in Pos 2. These observations suggested that cDNA inserts arising from alternatively spliced RNA transcripts had been isolated.

In the case of the Pos 1 insert within the Pos 1 catalytic domain, this possibility seemed doubtful. While each end of this possibly alternatively spliced exon contained sequences compatible with RNA splicing, such an event would have resulted in a shift in the open reading frame of the RNA. It was possible that the insertion represented an intron, and that during reverse translation of the RNA used to prepare the cDNA library, or perhaps over the course of virus replication, a single nucleotide has been lost from the terminus of either the 5' or 3' flanking sequences. The insertion of a single nucleotide on either side of the proposed splice junction would allow for removal of the insertion sequences by RNA splicing while still maintaining the open reading frame.

Alternative splicing of pTP RNAs has been reported to occur in regions of the RNA encoding the extracellular (Streuli et al., 1987), as well as catalytic domain (Matthews et al., 1990). However, the presence of an insert within the VHCSAGV sequence has never been observed to date. The fact that this sequence is a highly conserved and important catalytically suggested that the Pos1 insert likely represented a cloning artifact. A computer analysis designed to identify proteins containing domains homologous to the Pos 1 insert was performed, however, no such proteins were identified. Thus, if this insert does in fact represent a legitimate pTP domain, it is difficult to speculate as to its possible functional significance.

Analysis of the 5' sequences of the Pos 1 and Pos 2 clones indicated that each clone encodes a protein with a different NH_2-terminus. Further, in both cases the NH_2-terminal amino acid sequences differ from those predicted by Kreuger et al. (1990) for HPTP zeta. Once again, this observation suggested the possibility of alternative splicing.

To date a full length clone containing all of the 5' PTP zeta sequences has not been identified in our laboratory or by other workers. Such a clone would provide valuable information that would help to determine which, if any, of the cDNAs isolated to date encode a legitimate NH_2-terminus of PTP zeta. So far, Pos 2 is the only clone isolated that contains an entire open reading frame. For reasons outlined above, there is some doubt as to whether the Pos 2 clone actually represents an alternatively spliced form of chicken PTP zeta or a truncated form of an incompletely processed chicken PTP zeta cDNA. Primer extension analysis using antisense oligonucleotides corresponding to Pos 2 specific 5' sequences, or RNAse protection assays using a Pos 2 specific probe would help to address this problem. However, such experiments have not yet been attempted.

Likewise, it has not yet been determined whether the Pos 1 cDNA clone represents a truncated alternatively spliced form of chicken HPTP zeta homolog, or if it simply represents a truncated form of an incompletely processed RNA. Experiments similar to those proposed above for analysis of the Pos 2 clone would also be of value in resolving questions concerning the authenticity of the Pos 1 clone.

An approach that could be used to isolate NH_2-terminal sequences of the chicken PTP zeta homolog is the "RACE" (Rapid Amplification of cDNA Ends) protocol developed by Frohman et al. (1988). Oligonucleotides would be designed to hybridize to the 5'-most region of the chicken PTP zeta RNA that corresponds to amino acid sequences conserved in Pos 1, Pos 2, and the partial PTP zeta clone of Kreuger et al. (1990).

The total amino acid sequences of the Pos 1 and Pos 2 clones were 75% and 76% homologous, respectively, to the HPTP zeta sequences predicted by Kreuger et al. (1990). If this comparison is restricted to only those sequences COOH-terminal to the points at which the NH_2-termini of the proteins diverge, the homology increased to 90%. As with LAR, this high degree of conservation between very divergent species suggests that the PTP zeta protein must play an important role within the cell.

5-III. Conclusion

Over the course of this study, a phosphotyrosyl-protein phosphatase, designated pTPIII, was purified to near homogeneity from soluble extracts prepared from 11 day-old chicken embryo bodies. SDS-PAGE analysis suggested that this pTP activity correlated with the presence of a 58 kd protein band in affinity purified enzyme preparations. The purified protein exhibited biochemical characteristics in common with the pTP1B enzyme purified from human placental protein extracts and thus may represent the chicken homolog of this protein.

Two partial cDNA clones encoding phosphotyrosyl-protein phosphatases were also isolated. Unfortunately, neither cDNA is likely to encode the pTPIII enzyme. Both code for members of the transmembrane class of tyrosine specific protein phosphatases, and thus probably bear no relation to the pTPIII and pTPI activities purified from soluble protein extracts. One cDNA seemed to encode the COOH-terminus of the chicken homolog of LAR, while the second coded for the chicken homolog of PTP-zeta. Two cDNAs encoding PTP-zeta were isolated, however the evidence presented suggested that both clones were likely derived from incompletely processed nuclear RNAs.

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APPENDIX

IDENTIFICATION AND PURIFICATION OF MULTIPLE SPECIES OF CHICKEN pTP ACTIVITIES

Introduction

In an attempt to obtain larger quantities of highly purified pTPIII enzyme, the entire purification protocol was scaled up threefold, such that approximately 1 to 1.2 kg of chicken embryo bodies were used as starting material. It was hoped that the availability of larger quantities of enzyme would provide sufficient material to allow for peptide sequencing of the purified pTPIII enzyme. Further, it was hoped that the quantities of highly purified enzyme would be sufficient for the inoculation and screening processes involved in the generation of monoclonal or polyclonal antibodies.

To achieve this goal, it was necessary to slightly modify the purification protocol used previously to make processing of the larger amounts of starting material feasible. The first change was that 12 day-old rather than 11 day-old chicken embryos were used to increase the amount of starting material while reducing the labour involved in the processing of the fertilized eggs. Second, the 100,000 x g centrifugation step was eliminated from the protocol. Instead, cell extract was prepared by filtration of the 40,000 x g supernatant through Whatman 114 paper in a Buchner funnel in order to remove any fatty material. Finally, batch elution with buffer containing 0.3 M NaCl, rather than gradient elution, of pTPIII from DE52 was employed as the first step in the purification protocol. A second DE52 chromatography step in which pTPIII was eluted in a salt gradient was inserted into the protocol following the Cibracon Blue 3GA chromatography step. These seemingly simple manipulations of the protocol led to some surprising results.

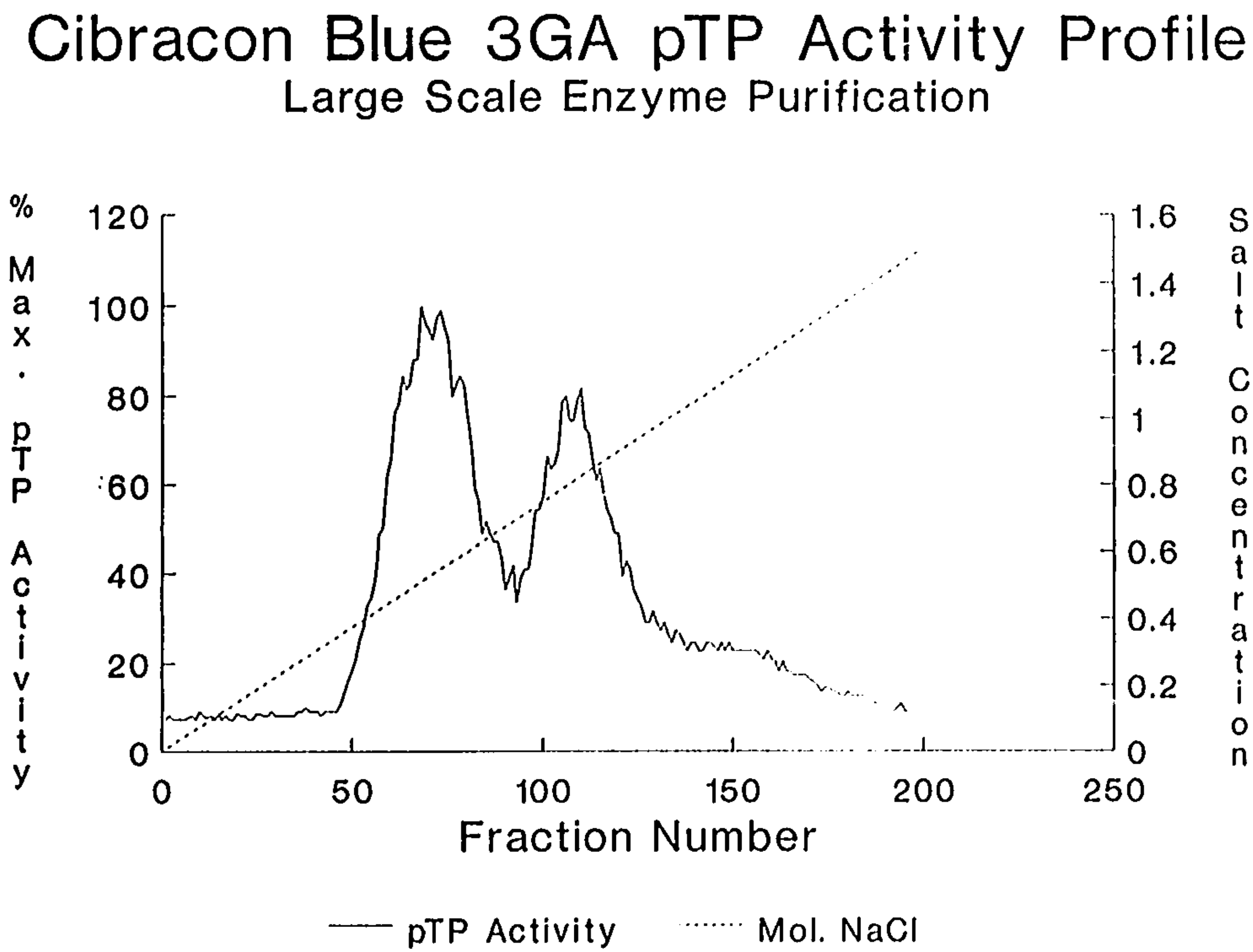
Results and Discussion

Two peaks of activity were resolved following Cibracon Blue 3GA agarose chromatography, rather than one as previously observed (compare Figures 4 and A1). The first peak, pTPIIIa, eluted at approximately 0.4 M NaCl, and in subsequent purification steps demonstrated characteristics consistent with the pTPIII activity purified from the smaller scale preparations. The second peak of activity, pTPIIIb, which eluted at approximately 0.7 M NaCl, showed different characteristics. Each peak contained approximately 50% of the pTP activity that had been initially applied to the Cibracon Blue 3GA column.

When purified by molecular sieving chromatography, pTPIIIb eluted from the column at a position corresponding to a

<u>Figure A1</u>. Chromatography of DE-52 Batch Purified pTPIII on Cibracon Blue 3GA Agarose (Large Scale).

DE-52 batch purified pTPIII was subjected to cibracon blue 3GA agarose chromatography as described in the text. Aliquots from each fraction were assayed using ^{32}P-labelled RCM-lysozyme as substrate. Values have been expressed as a % of the highest activity observed. The solid line represents pTP activity, while the dashed line indicates the salt concentration within the gradient. pTPIIIa eluted in the salt gradient at approximately 0.4 M NaCL, while pTIIIb eluted at approximately 0.7 M NaCl.


Cibracon Blue 3GA pTP Activity Profile
Large Scale Enzyme Purification
% Max. pTP Activity
120
100
80
60
40
20
0
0
50
100
150
200
250
Fraction Number
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Salt Concentration
pTP Activity
Mol. NaCl

protein with a molecular weight of approximately 100 kd, whereas pTPIII and pTPIIIa both eluted at a position corresponding to a molecular weight of 60 kd (Figure 5 and data not shown). pTPIIIb also behaved differently during gradient elution from Whatman DE-52 cellulose, eluting at approximately 0.23 M NaCl, rather than 0.15 M NaCl as observed for pTIII and pTPIIIa (data not shown).

pTPIIIb activity was further resolved into 2 peaks of activity by poly-lysine Sepharose chromatography, one peak eluting at approximately 0.15 M NaCl, and the second at approximately 0.2 M NaCl (Figure A2). Only the first peak eluting from this column, pTPIIIb, could be further purified by affinity chromatography on thio-RCM lysozyme Sepharose. The second peak, pTPIIIb' was not retained by this resin for reasons which were unclear.

Tables A1 and A2 show the results of the purification of pTPIIIa and pTPIIIb. Figures A3 and A4 show the results of coomassie blue staining of 10% SDS-PAGE gels on which aliqouts of protein from each step of purification were analyzed. pTPIIIa, while purified to a lesser extent, appeared to correspond to pTPIII, the protein previously isolated using the small scale purification procedure. As observed with pTPIII, pTPIIIa activity appeared to correlate with the presence of a major protein band in the affinity purified enzyme preparation that migrated at an apparent molecular mass of approximately 58 kd (Figure A3, lane 6). It would appear that pTPIII and pTPIIIa

Figure A2. Chromatography of pTPIIIb on Poly-Lysine Sepharose.

pTPIIIb was subjected to poly-lysine sepharose chromatography as described the Appendix Methods Section. Aliquots were assayed for pTP activity using ^{32}P-labelled RCM Lysozyme as substrate. Values have been expressed as a % of the highest activity observed. The solid line indicates the pTP activity. The dashed line denotes the salt concentration within the gradient. pTPIIIb eluted at approximately 0.15 M NaCl, while pTPIIIb' eluted at approximately 0.2 M NaCl.

Poly-Lysine Sepharose pTP Profile

Large Scale pTP Purification

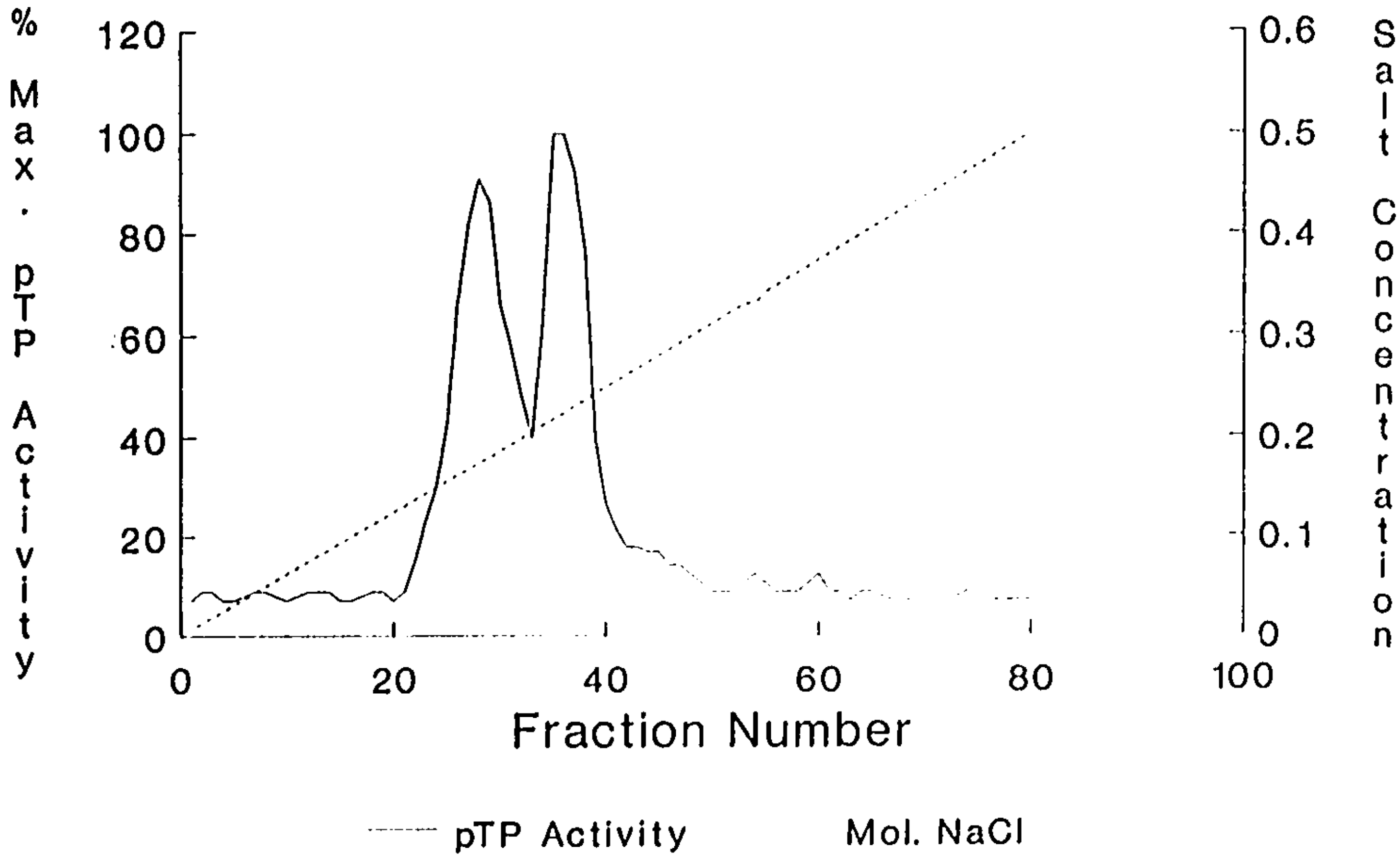


Table A1 Purification of pTPIIIa

Step	Volume (ml)	Protein (mg/ml)	Activity (nm/ml/min)	Specific Activity (nm/mg/min)	Purification (fold)
Cell Extract	2400	11.21	9.76	0.87	----
DE-52 Cellulose (Batch)	2950	2.68	8.71	3.25	4
Cibracon Blue 3GA Agarose	174	2.71	73.80	27.2	31
DE-52 Cellulose (Gradient)	150	0.46	37.71	82.0	94
Sephacryl S-200	35	0.97	256.7	264.7	304
Poly-Lysine Sepharose	47	0.65	475.0	730.8	840
Thio-RCM Lysozyme Sepharose	27	0.06	72.3	1148	1320

Table A1

Table A1 shows the results of the purification of pTPIIIa from a total of 1200 g chicken embryos as starting material. pTP activity was measured with ^{32}P-labelled RCM lysozyme as substrate, at a final concentration of 5 μM in the assay mixture. The results presented are the average of triplicate determinations.

Table A2 Purification of pTPIIIb

step	Volume (ml)	Protein (mg/ml)	Activity (nm/ml/min)	Specific Activity (nm/mg/min)	Purification (fold)
Cell Extract	2400	11.21	9.76	0.87	----
DE-52 cellulose (Batch)	2950	2.68	8.71	3.25	4
Cibracon Blue 3GA Agarose	146	2.23	76.70	34.4	40
Sephacryl S-200	50	1.47	145.1	98.7	113
DE-52 Cellulose (Gradient)	90	0.36	72.0	200	230
Poly-Lysine Sepharose					
Peak 1*	32	0.12	52.8	456	523
Peak 2	20	0.36	166.5	481	553
Thio-RCM Lysozyme Sepharose*	25	0.073	70.6	967	1112

Table A2

Table A2 shows the results of the purification of pTPIIIb from a total of 1200 g chicken embryos as starting material. pTP activity was measured with ^{32}P-labelled RCM lysozyme as substrate, at a final concentration of 5 μM in the assay mixture. The results presented are the average of triplicate determinations.

* Only the first peak of activity that eluted from the poly-lysine was retained by, and subsequently eluted from the thio-RCM lysozyme affinity column.

<u>Figure A3</u>. SDS-PAGE Analysis of Purified pTPIIIa.

Aliqouts of protein (2.5 μg) from each stage of purification were separated by electrophoresis on a 10% polyacrylamide as described in Materials and Methods. Protein bands were visualized by staining the gel with coomassie brilliant blue dye. Lane 1) DE-52 batch purified pTPIIIa; lane 2) Cibracon Blue 3GA agarose purified pTPIIIa; lane 3) Sephacryl S-200 purified pTPIII; lane 4) DE-52 gradient purified pTPIIIa lane 5) Poly-lysine Sepharose purified pTPIIIa; lane 6) thio-phosphotyrosyl-RCM lysozyme Sepharose purified pTPIIIa. The arrows denote the positions of the marker proteins: phosphorylase b (92.5 kd, arrow a), bovine serum albumin (67 kd, arrow b), ovalbumin (45 kd, arrow c), and carbonic anhydrase (31 kd, arrow d).

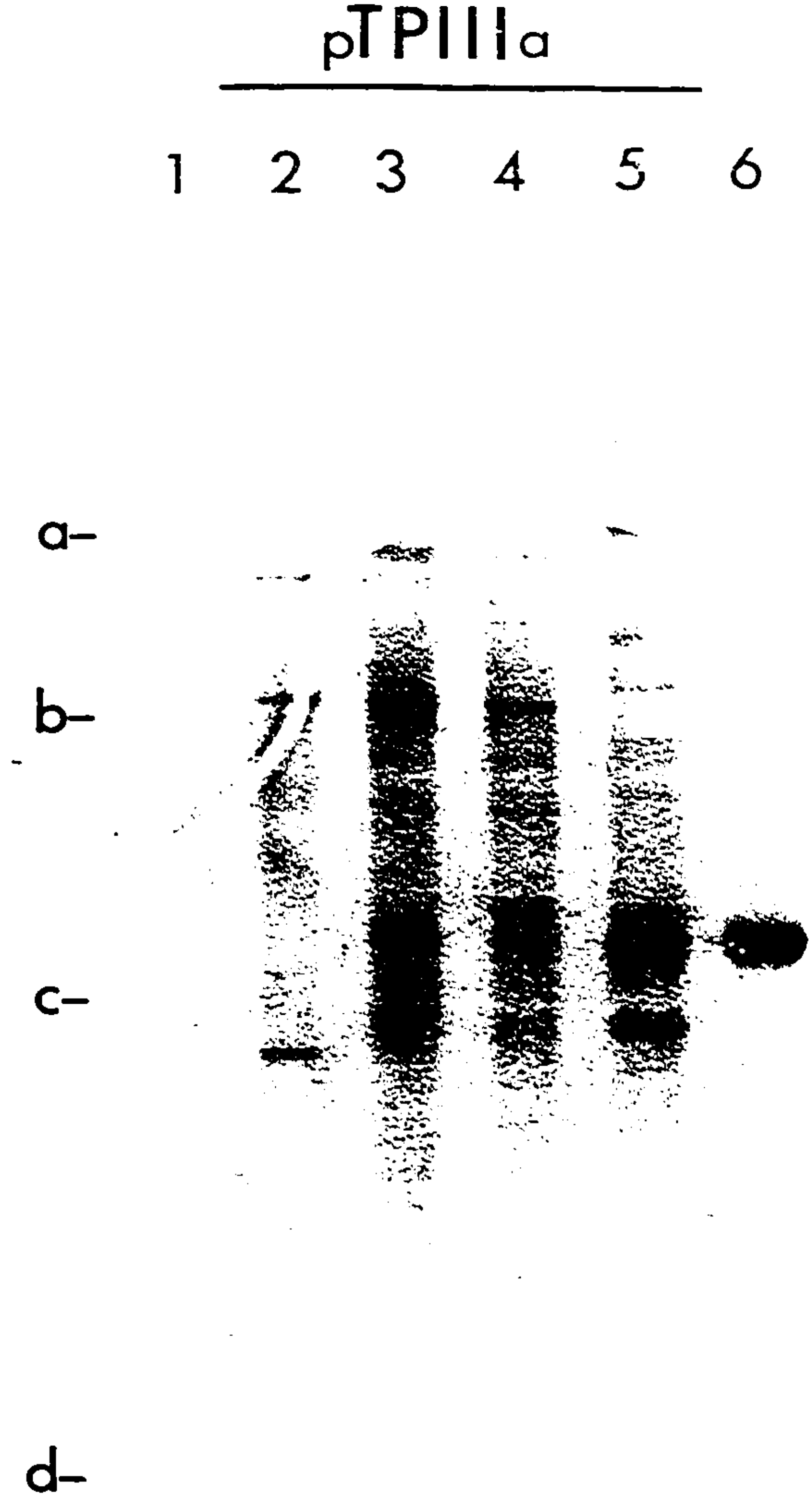
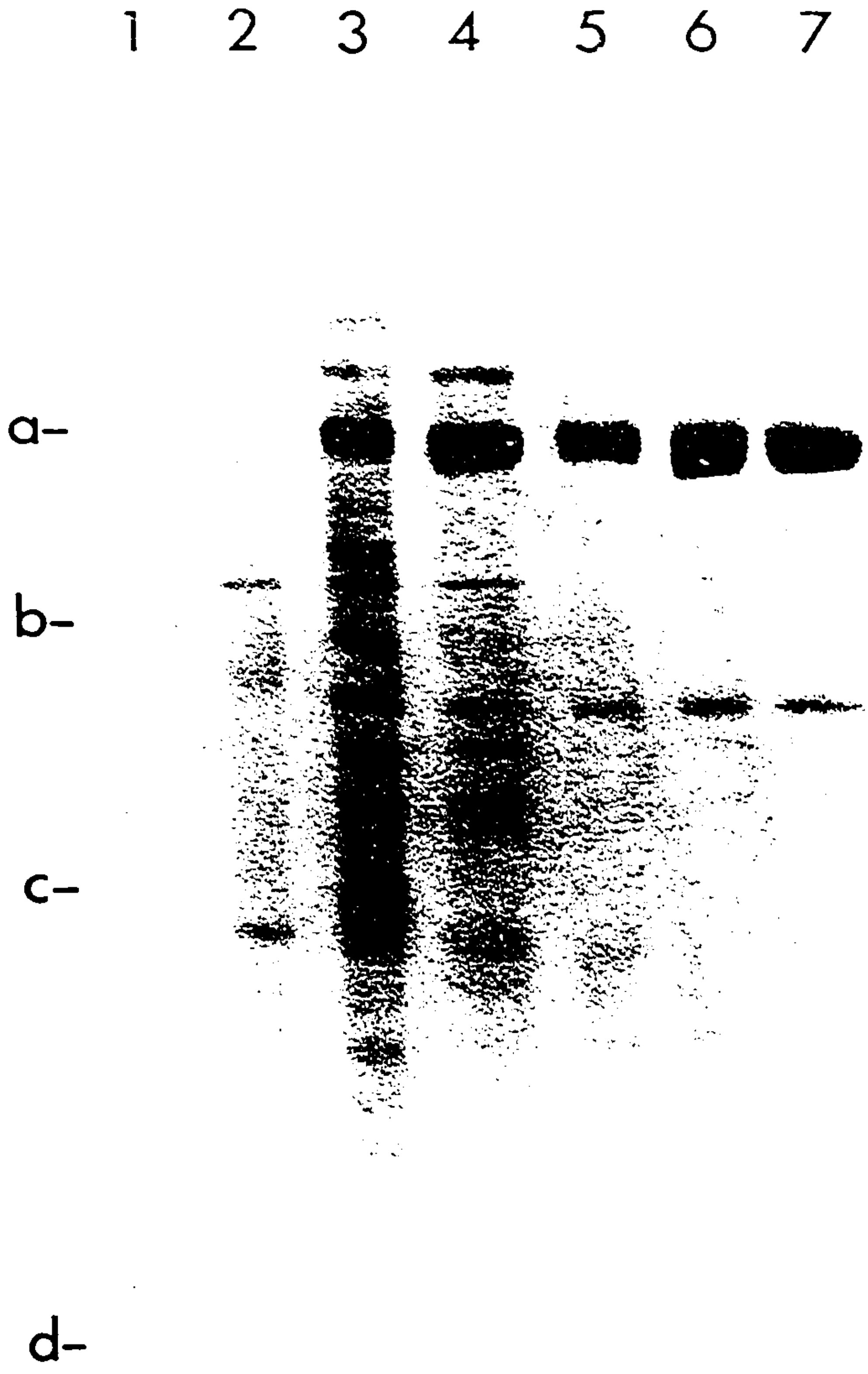

pTPIIIa
1
2
3
4
5
6
a–
b–
c–
d–

Figure A4. SDS-PAGE Analysis of Purified pTPIIIb.

Aliqouts of protein (2.5 μg) from each stage of purification were separated by electrophoresis on a 10% polyacrylamide as described in Materials and Methods. Protein bands were visualized by staining the gel with coomassie brilliant blue dye. Lane 1) cell extract; lane 2) DE-52 batch purified pTPIIIb; lane 3) Cibracon Blue 3GA agarose purified pTPIII; lane 4) Sephacryl S-200 purified pTPIII; lane 5) DE-52 gradient purified pTPIIIb; lane 6) Poly-lysine Sepharose purified pTPIIIb; lane 7) thio-phosphotyrosyl-RCM lysozyme Sepharose purified pTPIIIb. The arrows denote the positions of the marker proteins: phosphorylase b (92.5 kd, arrow a), bovine serum albumin (67 kd, arrow b), ovalbumin (45 kd, arrow c), and carbonic anhydrase (31 kd, arrow d).


1 2 3 4 5 6 7
a-
b-
c-
d-

represented the same enzyme but this remains to be verified. Microsequencing analysis of tryptic peptides derived form pTPIII and pTPIIIa will likely be required to resolve this question.

The major protein band visualized following SDS-PAGE analysis of affinity purified pTPIIIb preparations migrated at an apparent molecular mass of approximately 85 kd (Figure A4, lane 7). Boiling the samples for prolonged periods had no effect on the pattern of protein bands observed on SDS-PAGE, suggesting that dimerization, or aggregation, of the pTPIII protein did not explain the presence of an 85 kd species.

The identity of the pTPIIIb species remains to be established. It did appear to be different from the 85 kd enzyme isolated by Singh (1990), as pTPIIIb does not require divalent cations for activity. Once again, peptide sequencing of pTPIIIb will help to resolve this question. Recently, cDNAs defining two new subtypes of nonreceptor pTP have been identified. One of the new subtypes was defined by the presence of an SH2 domain, a domain conserved amongst proteins involved in signal transduction (Shen et al., 1991), while the second subtype was shown to contain a domain that is conserved amongst cytoskeletal proteins involved in regulating cell shape (Yang and Tonks, 1991; Gu et al., 1991). The molecular weights predicted for these pTP subtypes were found to fall in the range of 70-105 kd. It was possible that the pTPIIIb enzyme is related to one of these recently identified nonreceptor pTPs.

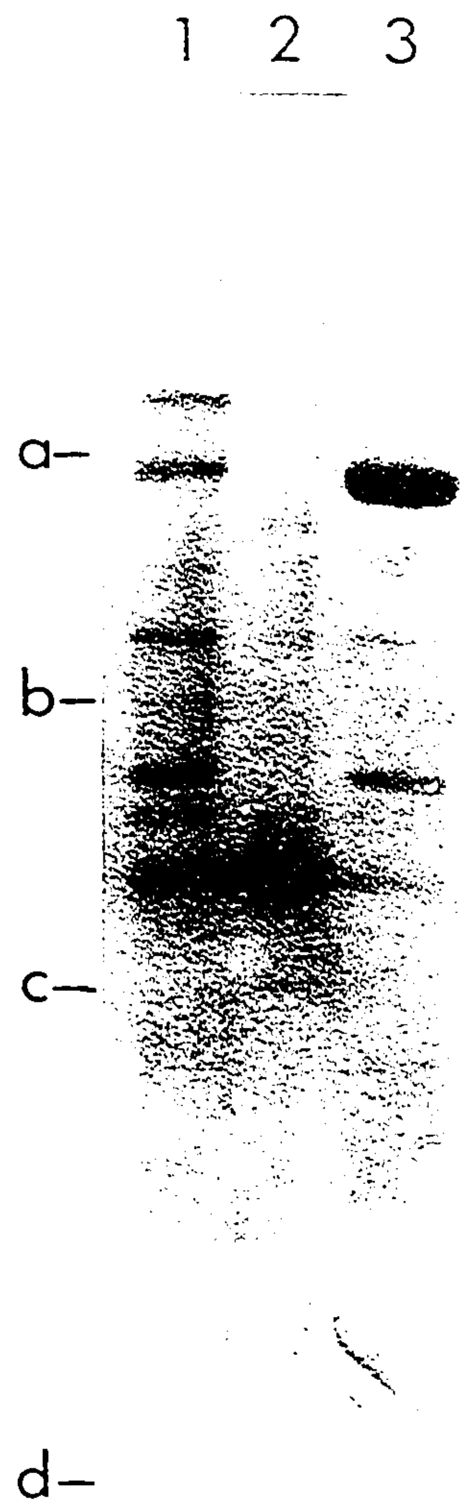
Protein samples of Poly-lysine Sepharose purified pTPIIIa, pTPIIIb, and pTPIIIb' were also examined by SDS-PAGE. Figure A5 shows the results of coomassie blue staining of a 10% SDS-PAGE gel on which these protein samples were analyzed. It appeared that pTPIIIb' may be similar to pTPIIIa and pTPIII. One of the major protein band present in the pTPIIIb' preparation migrated at approximately 58 kd. This band appeared to comigrate with the 58 kd band in the Poly-lysine Sepharose purified pTPIIIa sample. While there were protein bands of higher molecular masses present in the pTPIIIb' preparation, none of the bands appeared to comigrate with the 85 kd pTPIIIb protein band. Multiple protein bands were observed within SDS-PAGE analyzed samples of pTPIIIb' and thus the protein species corresponding to pTPIIIb' remains unclear.

The true nature of the pTPIII, pTPIIIa, pTPIIIb, pTPIIIb' enzymes, and their relationship to each other will likely only be clarified by determining their individual amino acid sequences or by molecular cloning of the cDNAs encoding these enzymes. If the enzymes are in fact distinct, it should be possible to generate specific antibodies against each enzyme which could then be used to clone the respective cDNAs and provide useful tools to investigate their functional.

The reasons for the differences in activity profiles observed during large scale pTP purification have not been resolved. This procedure has been performed twice and similar results were obtained during both experiments. The resolution

<u>Figure A5</u>. SDS-PAGE Analysis of Poly-Lysine Sepharose Purified pTPIIIa, pTPIIIb, and pTPIIIb'.

Aliqouts of each partially purified protein (2.5 μg) were separated by electrophoresis on a 10% polyacrylamide as described in Materials and Methods. Protein bands were visualized by staining the gel with coomassie brilliant blue dye. Lane 1) Poly-lysine Sepharose purified pTPIIIb'; lane 2) Poly-lysine Sepharose purified pTPIIIa; lane 3) Poly-lysine Sepharose purified pTPIIIb. The arrows denote the positions of the marker proteins: phosphorylase b (92.5 kd, arrow a), bovine serum albumin (67 kd, arrow b), ovalbumin (45 kd, arrow c), and carbonic anhydrase (31 kd, arrow d).


1 2 3
a–
b–
c–
d–

of two pTP activities during the first experiment suggested the possibility that proteolysis of pTPIII was contributing to the change in profiles. During the second attempt, minor modifications were made to the protocol in order to minimize the possibility proteolysis over the course pTPIII purification, particularly at the earlier stages. The results of the second purification attempt indicated that proteolysis of pTPIII was not a major contributing factor to the observation of multiple peaks of pTP activity during large scale pTPIII purification.

It is possible that the columns used during small scale preparations were overloaded, but this does not seem likely. The volumes of resin, and salt gradients, used at each step of the large scale purification were proportionally increased in relation to those used in the small scale procedure, so as to maintain the same ratio of volume of resin to protein chromatographed. Thus, the columns used in the large scale protocol should have been similarly overloaded, leading to the same level of resolution.

In the original small scale protocol, the cell extract was prepared by subjecting the homogenate to centrifugation at 40,000 x g, followed by a second centrifugation step at 100,000 x g. The second centrifugation step had to be eliminated in the large scale protocol due to the large volume of homogenate generated from 1200 g of chicken embryo bodies. Instead, fatty material was removed by filtration through Whatman 114 filter paper. The different methods of preparing the cell extracts could have contributed to the resolution of multiple peaks of pTP activity. The 100,000 x g centrifugation step could have resulted in the removal of certain pTP species from the cell extracts used in small scale pTP purifications.

Finally, it is possible that a major factor contributing to the different profiles was the use of 12 day-old chicken embryos, instead of the previously used 11 day-old embryos. It is possible that there are differences in the number, and/or level, of pTP activities expressed in the later stage embryos. Further experiments will be required to resolve this question. It is possible that chicken embryo bodies at different stages of development may serve as suitable starting material for the purification of a variety of pTP activities.