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Human Deoxycytidine Kinase: Kinetic Mechanism and End Product Regulation†

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ABSTRACT: The kinetic properties of the monomeric deoxycytidine kinase (EC 2.7.1.74) from leukemic human T-lymphoblasts have been investigated. The results of steady-state initial-rate kinetic analysis and product inhibition studies at pH 7.5 and 37 °C indicate that substrate binding follows an ordered sequential pathway, with the magnesium salt of ATP being the first substrate to bind and dCMP the last product to dissociate. At subsaturating substrate concentrations, dCMP produced competitve inhibition against ATP, while against varied deoxycytidine concentrations dCMP exhibited mixed-type inhibition. ADP produced noncompetitive inhibition against either substrate. The limiting K_m values for deoxycytidine and MgATP were 0.94 and 30 μM, respectively. The end product inhibitor dCTP exhibited competitive inhibition against varied ATP concentration, with a dissociation constant estimated to be 0.7 μM when extrapolated to zero ATP concentration. dCTP was purely noncompetitive against varied deoxycytidine concentration. On the basis of these kinetic results, and on the strong and specific inhibition by dCTP, it is proposed that this end product functions as a multisubstrate analogue, with its triphosphate group binding to the phosphate donor site of the enzyme and its deoxycytidine moiety overlapping and binding to the deoxynucleoside site in a highly specific manner.

Recent work in this laboratory has suggested a simple but very effective nonallosteric mechanism by which accumulating deoxynucleoside triphosphate end products may regulate at least some deoxynucleoside kinases in bacteria (Ikeda et al., 1986). It was found that these triphosphates behave kinetically very much like synthetic bisubstrate analogues in which are joined the 5′-hydroxyl of the deoxynucleoside, and of the adenosine moiety of ATP by tetrapolyphosphate. It was postulated that the nucleoside moiety of the end product deoxynucleotide binds, with great specificity, to the deoxynucleoside binding site on the enzyme and its phosphates overlap and bind to the ATP subsite. This combination of binding forces causes the triphosphate to be bound more tightly than either substrate.

Such a mechanism is possible only when substrate binding leads to formation of a ternary complex; i.e., both subsites must exist at the same time. However, the pattern of inhibition by end product, acting as a bisubstrate analogue, would depend on whether the kinetic mechanism were a random or an ordered sequential process (Fromm, 1977). This inhibitor should compete with both substrates in cases of random substrate binding, but only with the leading substrate in an ordered mechanism. Unfortunately, the kinetic mechanisms have been worked out for very few mammalian deoxynucleoside kinases. This can be explained, in part at least, by the nonlinear kinetic patterns observed in a number of instances. Mammalian cytosol thymidine kinase (EC 2.7.1.21) fractions have exhibited the sigmoidal ATP saturation curves and end product modulation characterisitic of cooperative binding in heterotropically regulated allosteric enzymes, although there is no direct proof of a regulatory site (Lee & Cheng, 1976; Munch-Petersen & Tyrsted, 1985). While classical cooperative behavior has not been observed with cytosol deoxycytidine kinase, both calf thymus (Ives & Durham, 1970; Kozai et al., 1972) and human deoxycytidine kinase preparations (Hershfield et al., 1982; Sarup & Fridland, 1987; Bohman & Eriksson, 1988) have yielded bimodal double-reciprocal plots. Kozai et al. (1972)

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recognized that this could be due to the existence of different forms of the enzyme, and we have demonstrated that this kinetic phenomenon can be produced experimentally with a heterogeneous mixture containing native and proteolytically degraded enzyme (Kim et al., 1988).

In this report, homogeneous monomeric human deoxycytidine kinase prepared by affinity chromatography is shown to yield simple linear kinetics, and a nonallosteric mechanism of end product regulation is proposed.

Experimental Procedures

Materials. [5-^{3}H]Deoxycytidine was supplied by ICN. Unlabeled dCyd and nucleotides were purchased from Boehringer-Mannheim, Calbiochem, P-L Biochemicals, or Sigma. Nucleotides were checked for purity by FPLC anion-exchange chromatography, and concentrations were determined by UV extinction. Deoxycytidine kinase was purified by affinity chromatography from an extract of human acute lymphoblastic leukemic T-cells collected by leukophoresis, and supplied by the Tissue Procurement Service of the Ohio State University Comprehensive Cancer Center. The affinity medium was prepared from the bisubstrate analogue dCp_4A (Ikeda & Ives, 1985), and homogeneous enzyme was isolated as described previously (Kim et al., 1988).

Enzyme activity (0.4 μg of enzyme per assay) was measured by a fixed-time radiometric assay (Ives, 1984) in 0.1 M Tris-HCl at pH 7.5 and 37 °C, and reaction mixtures also contained 0.5 μCi of [5-^{3}H]dCyd, 2 mM dithioerythritol, and 5% glycerol, in a final volume of 0.08 mL. Substrate concentrations were varied as detailed below. Each data point is the average of duplicate assays, and experiments were performed at least twice. The concentration of MgATP in each assay was calculated from the total ATP and $MgCl_2$ concentrations and appropriate equilibrium constants (Morrison, 1979). In experiments involving product inhibition by ADP, sufficient $MgCl_2$ was added to maintain the stated molarity of free Mg^{2+} and a 1:2 ratio of MgADP to total ADP. (Since large concentrations of free magnesium are somewhat inhibitory, and since ADP binds it relatively weakly, it was not practical to add the metal ion in sufficient quantity to convert all of the ADP to MgADP.) The enzyme was dialyzed before use to remove dCTP contained in the eluate. Reactions were terminated with 0.2 mL of 0.1 N formic acid. Double-reciprocal kinetic plot lines were fitted by first-order linear regression, using SigmaPlot (Jandel Scientific), and standard errors of kinetic constants were determined by means of the Enzfitter program (Elsevier–Biosoft).

Results

The results of initial velocity measurements made in the presence or absence of product or end product inhibitors are summarized in Table I. Initial velocities were determined at substrate concentrations ranging from 0.5 to 2.5 μM dCyd and from 0.042 to 0.9 mM MgATP, yielding two sets of completely linear reciprocal plots which converged in the upper left quadrant (not shown). Such converging patterns are consistent with a sequential kinetic mechanism leading to a ternary complex. Since neither family of lines coverged on the abscissa, it was apparent that the limiting K_m values are not identical with the respective dissociation constants of the individual substrates. Slope and intercept replots, which generally appeared to be linear, yielded the kinetic constants listed for the two substrates.

Product Inhibition. Initial velocity measurements in the presence of products were then conducted to determine whether substrate binding is an ordered process, or not. As may be seen in Figure 1, dCMP proved to be a fairly strong competitive inhibitor against varied [ATP] when the dCyd concentration was fixed at 2.2 μM. However, when [ATP] was fixed at 1.0 mM and [dCyd] was varied, mixed-type inhibition was observed.

Inhibition experiments with ADP were more difficult due to relatively weak inhibition by this product. Also, several ADP preparations were found to contain significant concentrations of ATP, causing nonlinear kinetics when large concentrations of ADP were added and ATP was the varied substrate. Fortunately, a fresh ADP preparation from Boehringer-Mannheim was found to be chromatographically free of detectable ATP. ADP appears to be a noncompetitive inhibitor vs either dCyd or ATP (Figure 2). Thus, we see only one competitive relationship and noncompetitive or noncompetitive–mixed-inhibition patterns with the other three

Table I: Summary of Kinetic Constants and Patterns of Product Inhibition for Human Deoxycytidine Kinase

kinetic constant	value (μM)	varied substrate	concn of fixed S	pattern
From Slope and Intercept Replots of Primary Kinetic Plots				
K_{mA}(ATP)	30 ± 1.5	dCyd, MgATP		converging
K_{ia}(ATP)	73 ± 20	dCyd, MgATP		
K_{mB}(dCyd)	0.94 ± 0.15	dCyd, MgATP		
Apparent Product Inhibition Constants				
K_{iq}(dCMP)	30 ± 3.1	MgATP	2.2 μM	comp
K_{iq}(dCMP)	49 ± 4.8	dCyd	1.0 mM	mixed
K_{ip}(ADP)	7.9 ± 1.9 (mM)	MgATP	2.2 μM	noncomp
K_{ip}(ADP)	19.8 ± 6.7 (mM)	dCyd	2.1 mM	noncomp
Apparent End Product Inhibition Constants				
K_i(dCTP)	2.1 ± 0.2	dCyd	120 μM	noncomp
K_i(dCTP)	4.6 ± 0.7	MgATP	2.2 μM	comp
True End Product Dissociation Constant				
K_{ia}(dCTP) = 0.7 μM (extrapolated to zero [MgATP])[a]				

[a] The dissociation constant was obtained by interpolation on a plot of apparent K_i vs [MgATP]. A straight line drawn between two known points yields the value of K_i at zero [MgATP] lying at the intersection with the ordinate. The two known points: the apparent K_i at 0.12 mM ATP and the value of $-K_{ia}$(ATP) plotted on an extension of the abscissa into the left-hand quadrant.

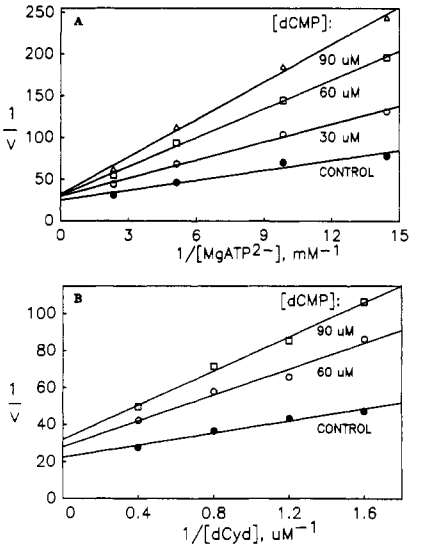


FIGURE 1: Product inhibition by dCMP. (A) Effect of varying MgATP concentration; [dCyd] fixed at 2.2 μM. (B) Effect of varying dCyd concentration; [MgATP] fixed at 1.0 mM.

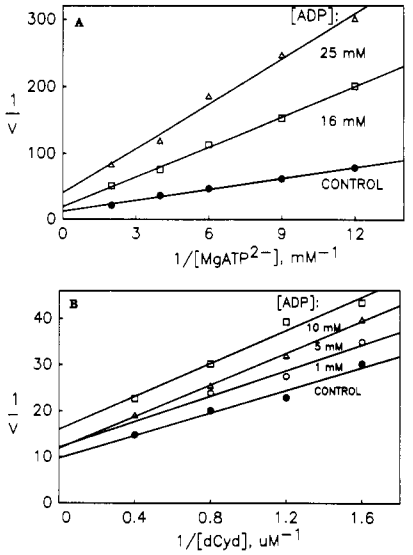
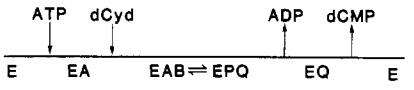


FIGURE 2: Product inhibition by ADP. (A) Effect of varying MgATP concentration; [dCyd] fixed at 2.2 μM and unbound [Mg^{2+}] at 0.5 mM. A minimum of 97% of the total ATP is in the form of MgATP. (B) Effect of varying dCyd concentration; total [ATP] fixed at 2.1 mM and unbound [Mg^{2+}] at 0.5 mM. A minimum of 92% of the total ATP is in the form of MgATP.

Scheme I



combinations (at subsaturating fixed substrate concentrations). Such an inhibition pattern clearly rules out a rapid-equilibrium random kinetic mechanism but is fully consistent with a mechanism in which the substrates bind in an obligatory order in the steady state, with ATP being the first substrate to bind and dCMP being the last product to dissociate (Segel, 1975). The proposed kinetic mechanism is illustrated in Scheme I. Inhibitor dissociation constants were obtained from plots of slope vs inhibitor concentration, all of which were linear. The individual inhibitor constants are also listed in Table I, using the conventional notation for the steady-state assumption. K_{ia} is the dissociation constant for ATP binding to the free enzyme, while K_{mA} is its limiting K_m. Since dCyd binds only after ATP does, it has only a limiting K_m, K_{mB}.

End Product Inhibition. The kinetics we observed were straightforward linear patterns, with no hint of cooperative or allosteric behavior. If deoxycytidine kinase has no allosteric site, how then does dCTP serve to regulate this enzyme so effectively? Several cases have been documented in this laboratory supporting the view that the end product nucleoside triphosphates may function as bisubstrate inhibitors of the deoxynucleoside kinases, bridging the subsites for both ATP and deoxynucleoside (Ikeda et al., 1986). For an ordered kinetic pathway, a multisubstrate analogue should compete only with the leading substrate. As can be seen in Figure 3, dCTP is competitive versus ATP, but exhibits purely noncompetitive inhibition with dCyd as the varied substrate, fully in accord with the view that it behaves as a multisubstrate analogue in its interaction with the enzyme. The apparent K_i value obtained is not the true dissociation constant for this ligand, since the concentration of competing substrate affects this result. However, a method of extrapolation to zero concentration of leading substrate has been devised (Ikeda et al., 1986), enabling us to estimate the K_{is} for dCTP to be about 0.7 μM.

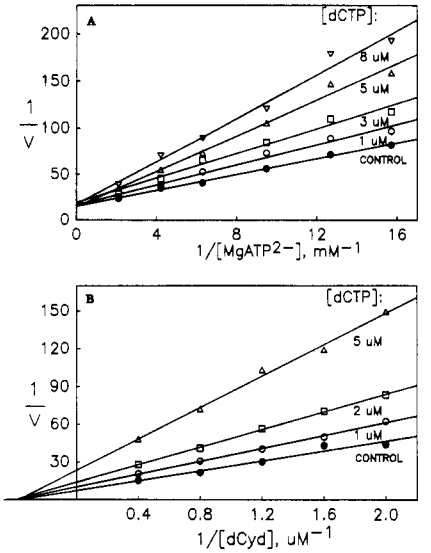


FIGURE 3: End product inhibition by dCTP. (A) Effect of varying MgATP concentration; [dCyd] fixed at 2.2 μM and unbound [Mg^{2+}] at 0.64 mM. A minimum of 95% of the total ATP is in the form of MgATP. (B) Effect of varying dCyd concentration; [MgATP] fixed at 0.12 mM and [$MgCl_2$] at 0.24 mM.

Discussion

There have been very few reports of attempts to determine the kinetic mechanisms followed by any of the mammalian deoxynucleoside kinases, and of these, two present conflicting results with human mitochondrial thymidine kinase, one reporting ping-pong kinetics (Lee & Cheng, 1976) and the other citing evidence for an ordered sequential pathway (Ellims & Van Der Weyden, 1981). A report from this laboratory provides a new impetus for taking time to analyze kinetic pathways, because such information has been found to be very useful in deciphering the mechanism of end product inhibition (Ikeda et al., 1986). With kinases isolated from *Lactobacillus acidophilus* R-26, the triphosphate end products appeared to behave as multisubstrate analogues. Thus, for those kinases following a random kinetic pathway, the triphosphate competed with both substrates, while in the case of a dAdo kinase (EC 2.7.1.76) shown to follow an ordered mechanism, dATP was found to compete only with the leading substrate (Ikeda et al., 1986). These patterns of end product inhibition were exactly those predicted for multisubstrate analogues, where the respective kinetic pathways are random or ordered (Fromm, 1977).

The kinetic data we have obtained with homogeneous human T-lymphoblast deoxycytidine kinase suggest an ordered kinetic pathway, involving a ternary complex of the enzyme with its two substrates. The leading substrate is MgATP, and the last product to leave is dCMP. Accordingly, dCTP competes with ATP but is noncompetitive toward dCyd, consistent with this inhibitor functioning as a multisubstrate analogue. An identical pattern of inhibition by dCTP was reported for two kinase activities, dCK and TSK, from a human T-cell cultures by Yamada et al. (1985). However, while their apparent K_i value of 1.9 μM for the dCK fraction is comparable to ours, dCTP appeared to inhibit a "T-lymphoblast-specific kinase" (TSK) much more weakly, with an apparent K_i of 380 μM. The molecular properties (M_r, p*I*, and electrophoretic R_f) of our T-cell deoxycytidine kinase (Kim et al., 1988) resemble those of Yamada's dCK preparation much more closely than they do the TSK fraction. Only the dCK type of activity was retained by our affinity columns. A recent report describing purification of homogeneous dCyd kinase

from human leukemic spleen also states that dCTP is competitive vs MgATP and noncompetitive–mixed toward varied [dCyd]; however, nonlinear reciprocal plots were obtained with varied [MgATP] (Bohman & Eriksson, 1988).

In a report just received, Datta et al. (1989b) conclude that the dCyd kinase from cultured (Molt 4) human T-lymphoblasts follows a rapid-equilibrium random Bi-Bi pathway, with a dead-end branch leading to enzyme complexed to dCyd and ADP. This model, in contradistinction to ours, arises from their interpretation of product inhibition experiments which are complicated by curvature of primary or secondary plots. For example, a plot of ADP vs ATP curves profoundly below 3.5 μM ATP, tending toward a velocity plateau; this effect could be caused by contaminating ATP in the ADP preparation. The other plot in which their interpretation differs from ours is that of dCMP vs dCyd where there seems to be uncertainty as to whether the lines converge at the ordinate of their plot. Moreover, the slope replot is hyperbolic. We found unambiguous mixed inhibition for the dCMP vs dCyd relationship, with a linear replot of slope vs dCMP concentration. We also differ with Datta et al. (1989b) on the pattern of inhibition by dCTP; e.g., they interpret the reciprocal plot of dCTP vs dCyd as competitive, even though the cluster of points near the ordinate do not seem clearly to converge there.

We have previously demonstrated that an apparent proteolytic cleavage of the protein can result in nonlinear kinetics and altered kinetic constants (Kim et al., 1988). It seems quite likely that such proteolytic modification may have contributed to differences in the kinetic results reported for dCyd kinase by various laboratories. Our present kinetic studies have been carried out with monomeric, presumably unproteolyzed, enzyme protein, and linear plots were obtained in each experiment. It is unclear whether the recent reports of dimeric dCyd kinase (Yamada et al., 1985; Bohman & Eriksson, 1988; Datta et al., 1989a) are describing a genetically distinct oligomeric enzyme, an enzyme protein subjected to altered intracellular posttranslational processing, or an enzyme protein partially proteolyzed during isolation.

Assuming that we have diagnosed the kinetic mechanism correctly, and that no allosteric site exists for dCTP, the competition between dCTP and ATP could be explained either by simple competition for the site normally occupied by the phosphate donor nucleotide or by binding of dCTP in the reversed orientation by which it can overlap both the nucleoside and the nucleotide binding sites, enabling it to block the ordered binding of both substrates as a multisubstrate analogue. The latter mechanism has been proposed for the regulation of several bacterial deoxynucleoside kinases (Ikeda et al., 1986). Were dCTP to bind only to the ATP site to produce this pattern of inhibition, it should be able to serve as a phosphate donor; it does not (Kim et al., 1988). Moreover, such a mechanism would not readily explain the fact that dCTP binds to the enzyme 100 times more tightly than ATP. (The estimated true dissociation constant of dCTP is about 0.7 μM, compared with the K_{ia} of 73 μM for ATP and the K_{mB} of 0.94 μM for dCyd.) It should also be noted that, unlike dCTP, the phosphorylated product, dCMP, produces mixed inhibition, not pure noncompetitive inhibition. Thus, dCTP does not appear to be acting merely as a more highly phosphorylated version of one of the products; the multisubstrate binding model seems to provide a simpler and more plausible explanation of its effect on the human deoxycytidine kinase, as well as for the bacterial enzymes. Preliminary experiments reveal that the dAdo and dGuo kinase activities inherent in human dCyd kinase are each inhibited by their respective end product triphosphates (as well by dCTP) and that the enzyme is also retained on a dAp_4A–Sepharose column. If a low-K_m version of dAdo kinase is present in human lymphoblasts, we expect that it too should be retained on such a column.

Interaction of the deoxycytidine moiety of dCTP with the deoxycytidine enzymatic site would account both for the specificity of the inhibition and for the inability of dCTP to serve as a phosphate donor. By overlapping the triphosphate site, reinforcement of this binding by electrostatic forces can take place, resulting in a dissociation constant which is smaller than the K_{ia} or K_m of either substrate. It is well established that kinases and other nucleotide binding proteins have highly conserved nucleotide binding loops containing a lysine residue which is a primary contributor to nucleotide binding due to its interaction with the phosphate residues (Reinstein et al., 1988); the consensus Gly-X-X-Gly-X-Gly-Lys sequence has been found in a bacterial dCyd kinase (Ikeda et al., 1988). Given the ordered pattern of substrate binding, it must be assumed that there is sufficient initial interaction of the triphosphate portion of dCTP with the phosphate donor site to induce the conformational changes necessary for the creation of deoxycytidine binding site, followed, in turn, by the binding of the deoxycytidine moiety of dCTP to the latter. As with the bacterial dCyd kinase, it is significant that human deoxycytidine kinase binds even more strongly to deoxycytidine 5′-tetraphosphate–Sepharose than to dCp_4A–Sepharose, suggesting that the adenosine moiety of the bisubstrate analogue does not participate in the binding of the enzyme (M.-Y. Kim, unpublished work). Affinity labeling with analogues of dCTP, dCyd, and ATP may serve to confirm the regulatory mechanism we have proposed.

Registry No. MgATP, 1476-84-2; dCyd, 951-77-9; dCMP, 1032-65-1; ADP, 58-64-0; dCTP, 2056-98-6; deoxycytidine kinase, 9039-45-6.

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Purification of Recombinant pp60$^{v\text{-}src}$ Protein Tyrosine Kinase and Phosphorylation of Peptides with Different Secondary Structure Preference†

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ABSTRACT: The expression of the transforming gene product of Rous sarcoma virus (pp60$^{v\text{-}src}$) in *Saccharomyces cerevisiae* has recently been reported (Kornbluth et al., 1987; Brugge et al., 1987). To carry out biochemical and structural studies of this enzyme, a facile purification was developed. The purification was accomplished in four chromatographic steps: Q–Sepharose, Affi-Gel Blue, phosphoagarose, and hydroxylapatite chromatography. The tyrosine kinase was isolated in milligram quantities as two highly active proteolytic fragments (52 and 54 kDa). Three model tyrosine kinase substrates with propensities to adopt helical or Ω-loop conformations were synthesized and characterized. The peptides were based on the sites of phosphorylation of pp60$^{v\text{-}src}$, lipocortin I, and lipocortin II. Circular dichroism spectroscopy was used to study the conformation of the helix-forming peptides in 50 mM Tris and in 50% trifluoroethanol/Tris. Peptide 1, which was designed to form an amphiphilic α-helix, displayed 24.2% helicity in buffer and 40.2% helicity in 50% TFE/buffer. Similar experiments for peptide 3, the other helix former, showed a lower helicity (8.1% helical and 26.0% helical in buffer and in 50% TFE/buffer, respectively). All three peptides were shown to be substrates for the recombinant tyrosine kinase. Kinetic measurements using high-voltage paper electrophoresis indicated that the helix-forming peptides exhibited low K_M values (~450 μM) for the purified *src* gene product, consistent with the notion that elements of secondary structure may be important in substrate recognition by tyrosine kinases.

The finding that protein tyrosine kinase activity is associated with the transforming gene product of the Rous sarcoma virus (Hunter & Sefton, 1986) was a major step in our understanding of neoplastic transformation at the molecular level. The oncogene-encoded protein tyrosine kinases have been the subject of intense research efforts focused predominantly on the elucidation of the intracellular modes of action of tyrosine kinases that lead to cell transformation (Jove & Hanafusa, 1987). Despite numerous efforts to identify crucial substrates for these enzymes, no single cellular phosphoprotein has surfaced as the mediator of transformation. Though protein phosphorylation plays a prominent role in the regulation of cellular metabolism, the details of the enzymatic mechanisms by which protein kinases catalyze phosphate transfer are not well understood. In the case of the tyrosine-specific protein kinases, essentially no data exist regarding the identity and the spatial arrangement of residues comprising the active site of these enzymes.

The transforming gene product of the Rous sarcoma virus pp60$^{v\text{-}src}$ was the first recognized tyrosine-specific protein kinase. Though it is the most thoroughly characterized member of this family of enzymes, analysis of its tertiary structure has been hindered by the lack of adequate quantities of purified pp60$^{v\text{-}src}$. Such studies usually require milligram quantities of active enzyme. Available lines of transformed cells produce only very limited amounts of pp60$^{v\text{-}src}$. To create an abundant source of v-*src* kinase, we inserted 5′ truncated *src* cDNA into a bacterial secretion vector. Unfortunately, our efforts resulted in the production of a polypeptide that lacked kinase activity. Brugge et al. (1987) and Kornbluth et al. (1987) recently reported the expression of enzymatically active Rous sarcoma virus gene product in *Saccharomyces cerevisiae*. The recombinant kinase produced in yeast was myristylated at its amino terminus and phosphorylated at tyrosine-416. It was found to have approximately the same in vitro activity as its wild-type counterpart expressed in Rous sarcoma virus transformed cells (Kornbluth et al., 1987).

Because of the facility with which yeast can be grown in

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Deceased. This paper is dedicated to the memory of Dr. Emil T. Kaiser for his friendship, leadership, and inspiration as a scientist and teacher.

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