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Biochemistry

Lysine residue 121 in the proposed ATP-binding site of the v-mos protein is required for transformation

(protein kinases/oligonucleotide site-directed mutagenesis)

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ABSTRACT **The transforming gene product encoded by Moloney murine sarcoma virus clone 124, $p37^{mos}$, contains a lysine residue (lysine-121) that is conserved among all members of the protein kinase family. This lysine has been shown to be part of a conserved ATP-binding site in both the catalytic subunit of the cAMP-dependent protein kinase and $p60^{v\text{-}src}$. We wished to determine whether this lysine is required for the transforming activity of $p37^{mos}$. Two site-specific mutations were therefore constructed, which result in the substitution of an aspartic acid or arginine codon in place of the codon for lysine-121. Both mutations abolished the ability of the *mos* gene to transform cells. These results show that lysine-121 is required for the ability of $p37^{mos}$ to transform cells and provide evidence for an ATP-binding site in $p37^{mos}$. Furthermore, these results suggest that the conserved lysine residue is specifically involved in the catalytic activity of protein kinases in general.**

Moloney murine sarcoma virus (Mo-MSV) is a replication-defective retrovirus that can induce neoplastic transformation (1). This virus arose by recombination between the genome of Moloney murine leukemia virus (Mo-MLV) and normal mouse genetic information (2–5). Nucleotide sequencing of the Mo-MSV genome revealed the presence of an open reading frame of 1125 nucleotides, designated v-*mos*, that transforms fibroblasts in tissue culture when expressed under the control of a viral long terminal repeat (6–9). The v-*mos* gene product was first identified by *in vitro* translation of Mo-MSV virion RNA as a 37-kDa protein ($p37^{mos}$) (10, 11). This protein has also been identified in cells transformed by Mo-MSV (12) and has been localized to the cytoplasm by indirect immunofluorescence, using antiserum specific for the predicted C terminus of $p37^{mos}$ (13).

Comparison of the predicted amino acid sequence of $p37^{mos}$ with those of the catalytic subunit of the cAMP-dependent protein kinase and of $p60^{v\text{-}src}$ has revealed the existence of limited regions of sequence homology (14). A region of particular interest is that surrounding lysine-121 of $p37^{mos}$; in both the catalytic subunit of the cAMP-dependent protein kinase (15), and in $p60^{v\text{-}src}$ (16), the homologous lysine specifically binds the ATP analog, *p*-fluorosulfonylbenzoyladenosine (FSBA) with concomitant loss of kinase activity. This suggests the existence of a conserved ATP-binding site among members of the protein kinase family, containing a Gly-Xaa-Gly-Xaa-Xaa-Gly sequence that is also found in other nucleotide-binding proteins (16).

To test the importance of lysine-121 of $p37^{mos}$ in transformation, we have constructed two mutants of the v-*mos* gene. These mutations result in the substitution of a codon for aspartate or for arginine in place of the codon for lysine-121. Both of these mutations abolish the ability of the v-*mos* gene to transform NIH 3T3 cells. In transient expression assays in COS-1 cells, $p37^{mos}$ and $p37^{mos(R121)}$ are expressed at equal levels and have comparable half-lives. However, no $p37^{mos(D121)}$ was detected *in vivo*. These results indicate that lysine-121 of $p37^{mos}$ is required for its biological activity and by analogy suggest that $p37^{mos}$, like the cAMP-dependent protein kinase and $p60^{v\text{-}src}$, possesses an ATP-binding site.

MATERIALS AND METHODS

Oligonucleotide Site-Directed Mutagenesis. The following synthetic oligonucleotides were used to construct the site-directed mutants: (*i*) a 16-mer, TACTTGATCGATGGCC, used for the aspartate mutation, and (*ii*) a 20-mer, CCATCCGGCAAGTAAACAAG, used for the arginine mutation. Gapped circular heteroduplex molecules, constructed from restriction fragments of plasmid subclones of the v-*mos* gene, were used as substrates for the mutagenic oligonucleotides. The plasmid subclones were derivatives of pDDO, which was constructed from a cDNA clone of Mo-MSV^{124} (8). The heteroduplex molecules were formed by using NaOH to denature the DNA and formamide to control the reannealing of the strands (17). Mutagenic oligonucleotides were hybridized to the gapped heteroduplex molecules at room temperature for 30 min in H_2O, using a 50- to 100-fold molar excess of oligonucleotide. The solution was then made 50 mM Tris·H_2Cl 7.6/10 mM $MgCl_2$/10 mM dithiothreitol/100 μM dATP/dCTP/dGTP/dTTP/200 μM ATP. Two units of the Klenow fragment of *Escherichia coli* DNA polymerase I and 10 units of T4 DNA ligase were added, and the mixture was incubated at room temperature for 45 min and then transfected into competent *E. coli* C600. The resultant ampicillin-resistant colonies were transferred to Whatman 540 paper (18) and subjected to colony hybridization essentially as described (19) using at least 5×10^6 cpm of the 5′-^{32}P-labeled oligonucleotide. The hybridization was carried out overnight at room temperature. Colonies that specifically hybridized to the 5′-^{32}P-labeled oligonucleotide were selected and their plasmid DNA was used in a second round of transfection and hybridization. Maxam and Gilbert sequencing (20) was used to confirm the sequence of the mutations. Reconstruction experiments showed that no other alterations of the v-*mos* gene occurred during the mutagenesis.

DNA Transfections and Immunoprecipitations. For examination of the biological activities of the mutant v-*mos* genes, the plasmid DNAs were transfected into NIH 3T3 cells by using the calcium phosphate coprecipitation technique (21, 22). Focus assays and viral titers were determined as described (22, 23). For examination of protein expression in COS-1 cells, semiconfluent monolayers of COS-1 cells on 60-mm plates were transfected with 5 μg of plasmid DNA. The cells were labeled for 2 hr using 200 μCi of [^{35}S]cysteine

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Abbreviations: Mo-MSV, Moloney murine sarcoma virus; Mo-MLV, Moloney murine leukemia virus; FBSA, *p*-fluorosulfonylbenzoyladenosine; SV40, simian virus 40.

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(1 Ci = 37 GBq) in 400 μl of serum-free cysteine-free medium. For ^{32}P labeling, 330 μCi of [^{32}P]orthophosphate in phosphate-free medium was used. Cell lysates were prepared and immunoprecipitations with anti-C-terminal serum (C3) were carried out as described (12).

RESULTS

***In Vitro* Mutagenesis of Lysine Codon 121 of the v-*mos* Gene Results in Loss of Biological Activity.** We have used the technique of oligonucleotide-directed mutagenesis to construct two mutants of the v-*mos* gene. These mutants contain either an aspartate codon or an arginine codon in place of lysine codon 121 of the v-*mos* gene. The effect of these mutations on the biological activity of the v-*mos* gene was assayed using a Mo-MLV-derived vector to provide for expression of the v-*mos* gene in NIH 3T3 cells (23, 24). This vector, pDD102, is derived from a DNA clone of Mo-MLV (26) and contains a unique *Xho* I site for the insertion of foreign genes in place of the envelope (*env*) gene of Mo-MLV. The 5′ and 3′ splice sites for expression of the *env* gene are retained, although the foreign gene must provide its own signals for initiation and termination of translation. The parental and mutant v-*mos* genes were inserted into pDD102 in the correct orientation for expression (Fig. 1). The biological activities of the respective clones were assayed by introducing the DNAs into NIH 3T3 cells by the calcium phosphate coprecipitation technique. Focus assays were performed in which the cells were incubated for 10–14 days and scored for the presence of foci. When pMOS, containing the parental v-*mos* gene, was transfected into NIH 3T3 cells in the presence of a DNA clone of Mo-MLV, it transformed cells with an efficiency of 1×10^3 foci per microgram of DNA. Neither pMOS-asp nor pMOS-arg, when transfected in an identical manner, induced the formation of foci. In addition, the conditioned media from the focus assays were collected and assayed on fresh monolayers of NIH 3T3 cells for the presence of focus-forming virus. The conditioned medium from cells transfected with pMOS was found to contain 1×10^5 focus-forming virus per ml. The conditioned media from cells transfected with either pMOS-asp or pMOS-arg contained no detectable focus-forming virus. These experiments show that mutations that alter lysine codon 121 of the v-*mos* gene abolish its ability to transform NIH 3T3 cells.

Expression of *mos* Genes in COS-1 Cells. In cells stably transformed by Mo-MSV, $p37^{mos}$ represents ≈0.0005% of total cellular protein. To achieve greater synthesis of $p37^{mos}$, the v-*mos* gene was expressed under transcriptional control of the SV40 late promoter. For these experiments we used pJC119, an expression vector containing a SV40 origin of replication and a unique *Xho* I site in the late coding region of SV40, replacing the *VP1* gene (25). The various v-*mos* genes were inserted into the *Xho* I site of this vector in the correct orientation for expression, and the resultant plasmids were introduced into COS-1 cells (27). Cell lysates were prepared from [^{35}S]cysteine-labeled cells and immunoprecipitation was performed with antiserum raised against a synthetic peptide corresponding to the predicted carboxyl terminus of $p37^{mos}$ (12). A protein of approximately 37 kDa was immunoprecipitated with the anti-C-terminal serum from cells transfected with the parental v-*mos* gene (Fig. 2*A*, lane 4). This protein comigrated with the $p37^{mos}$ synthesized by *in vitro* translation of Mo-MSV^{124} RNA and immunoprecipitation was specifically blocked by prior incubation of the antiserum with the peptide. Quantification of the $p37^{mos}$ present revealed that up to 0.03% of the protein in a total cell lysate was $p37^{mos}$ (data not shown). $p37^{mos(R121)}$ was expressed at the same level as wild-type $p37^{mos}$ (Fig. 2*A*, lane 3). In contrast, immunoprecipitates from cells transfected with the pMOS-asp gene contained no detectable $p37^{mos(D121)}$

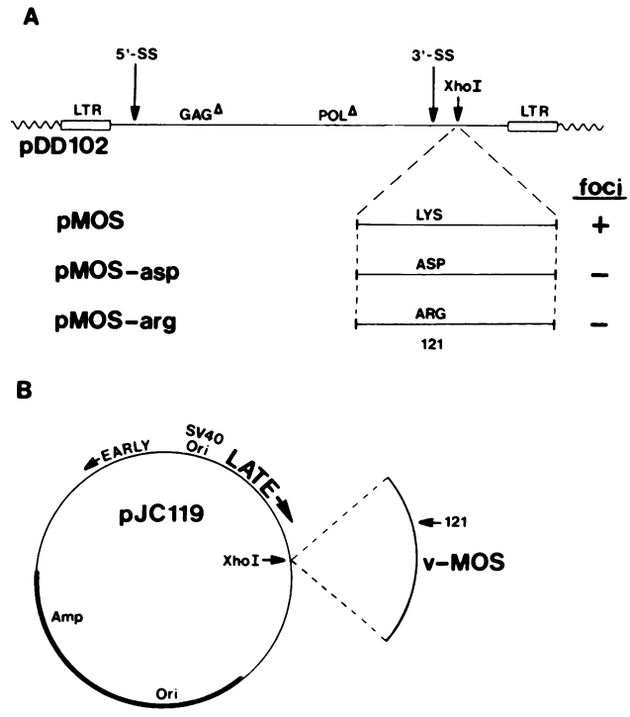

FIG. 1. (*A*) Expression of the v-*mos* genes in a retroviral vector. A Mo-MLV-derived vector, pDD102 (23, 24), was used for expression of the v-*mos* genes in NIH 3T3 cells. The retroviral signals for transcription and splicing are indicated. Plasmids pMOS, pMOS-asp, and pMOS-arg contain codons for lysine, aspartate, and arginine, respectively, at codon position 121. The results of focus assays (22, 23) are indicated. A positive result indicates that a value of $>1 \times 10^3$ foci per microgram of plasmid DNA was obtained in duplicate experiments and that the conditioned medium had $>1 \times 10^5$ focus-forming virus per ml (ffu/ml). A negative result indicates that no foci were detected above a background of 1 to 5 foci obtained with transfection of calf thymus DNA alone. All of the background foci were found to be morphologically distinct from foci induced by the parental v-*mos* gene. Less than 5 ffu/ml were detected in the titration of focus-forming virus in the conditioned media from cells transfected with the mutant v-*mos* genes. LTR, long terminal repeat. (*B*) Expression of the v-*mos* genes from the simian virus 40 (SV40) late promoter. The respective v-*mos* genes, containing a lysine, an aspartate, or an arginine codon at position 121, were inserted into pJC119 (25), a SV40-derived expression vector, in the correct orientation for expression from the SV40 late promoter. The resulting plasmids were transfected into COS-1 cells and then used for transient expression assays.

(Fig. 2*A*, lane 2), even for labeling periods as short as 10 min (data not shown).

Although $p37^{mos}$ migrated as a doublet in this experiment, $p37^{mos(R121)}$ yielded a single band that comigrated with the lower band of the doublet. $p37^{mos}$ migrates as a doublet during $NaDodSO_4$/polyacrylamide gel electrophoresis as a result of phosphorylation of some of the $p37^{mos}$ molecules (12). To determine whether $p37^{mos(R121)}$ is phosphorylated *in vivo*, COS-1 cells transfected with the appropriate DNAs were metabolically labeled with [^{32}P]orthophosphate. $p37^{mos}$ was readily labeled (Fig. 2*B*, lane 3), while $p37^{mos(R121)}$ was weakly phosphorylated (Fig. 2*B*, lane 2). We estimate that $p37^{mos(R121)}$ contains <10% of the phosphate found in $p37^{mos}$.

Although approximately equal amounts of $p37^{mos}$ and $p37^{mos(R121)}$ were detected after a 2-hr labeling period, it is possible that $p37^{mos(R121)}$ is less stable than $p37^{mos}$ and that a more rapid turnover is responsible for the loss of transforming ability in NIH 3T3 cells. A pulse–chase experiment carried out 40 hr after transfection of COS-1 cells with the various DNAs, however, showed that $p37^{mos}$ and $p37^{mos(R121)}$

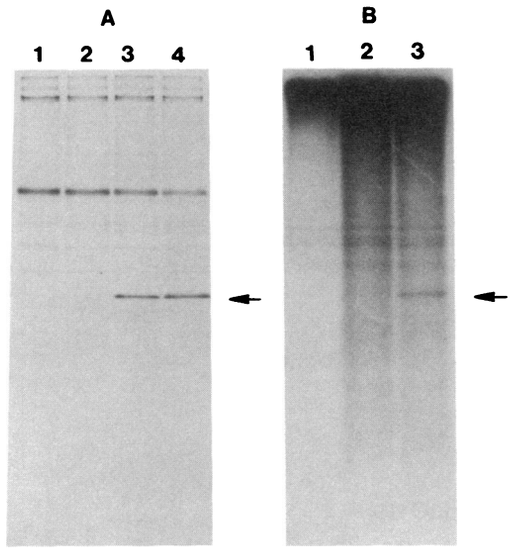

FIG. 2. Expression of the v-*mos* genes in COS-1 cells. The v-*mos* genes were inserted into pJC119 (25) (Fig. 1) and introduced into COS-1 cells by using the calcium phosphate coprecipitation technique. The transfected COS-1 cells were labeled 48 hr after transfection for 2 hr. Total cell lysates were prepared and then subjected to immunoprecipitation with C3 antiserum and 15% $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography to visualize [^{35}S]cysteine-labeled proteins or autoradiography to visualize ^{32}P-labeled proteins. The following marker proteins were used: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; soybean trypsin inhibitor, 20.1 kDa; lysozyme, 14.3 kDa. (*A*) Immunoprecipitates of COS-1 cells were transfected with various DNAs and labeled with [^{35}S]cysteine. Lane 1, pJC119; lane 2, the aspartate-encoding v-*mos* gene; lane 3, the arginine-encoding v-*mos* gene; lane 4, the parental v-*mos* gene. The arrow indicates $p37^{mos(R121)}$ and $p37^{mos}$ in lanes 3 and 4, respectively. (*B*) Immunoprecipitates of COS-1 cells were transfected with various DNAs and labeled with [^{32}P]orthophosphate. Lane 1, pJC119; lane 2, the arginine-encoding v-*mos* gene; lane 3, the parental v-*mos* gene. The arrow indicates $p37^{mos}$ in lane 3.

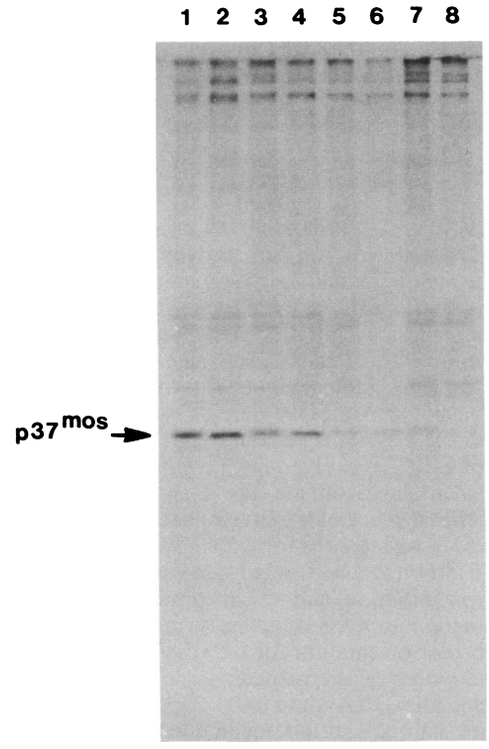

FIG. 3. Pulse–chase analysis. COS-1 cells were transfected with the parental (lanes 1, 3, 5, and 7) or with the arginine-encoding mutant v-*mos* (lanes 2, 4, 6, and 8) gene. Forty hours after transfection, the cells were labeled for 2 hr with [^{35}S]cysteine and this was followed by chasing with fresh medium containing unlabeled cysteine. Total cell lysates were prepared at each time point and analyzed by immunoprecipitation with C3 antiserum and 15% $NaDodSO_4$/polyacrylamide gel electrophoresis. Chase periods were as follows: lanes 1 and 2, 0 hr; lanes 3 and 4, 1.5 hr; lanes 5 and 6, 3 hr; lanes 7 and 8, 4.5 hr.

had similar half-lives (Fig. 3). Our estimate of 3 hr is somewhat less than the half-life found for $p37^{mos}$ in cells stably transformed by MSV (12) and may be a result of the different host cells or of stable vs. transient expression of $p37^{mos}$.

DISCUSSION

Substitution of another amino acid for lysine-121 has a profound effect on the biological activity of $p37^{mos}$. The importance of the homologous lysine in the cAMP-dependent protein kinase was first identified by studies using the ATP analog FSBA (15) in which it was found that FSBA inhibits the kinase activity of the catalytic subunit by covalent modification of lysine-71. Recently, FSBA has been used to demonstrate the existence of an ATP-binding site in $p60^{v\text{-}src}$ (16). Inactivation by FSBA of the tyrosine-specific kinase activity of $pp60^{v\text{-}src}$ resulted from the covalent modification of lysine-295 of $pp60^{v\text{-}src}$. These studies have allowed the identification of a conserved ATP-binding site in the respective proteins (see Table 1). This binding site includes the Gly-Xaa-Gly-Xaa-Xaa-Gly sequence located 7–16 residues from the conserved lysine; this glycine-rich region is present in $p37^{mos}$ as Gly-Ser-Gly-Gly-Phe-Gly. Our results suggest that $p37^{mos}$ likewise has an ATP-binding site that is required for its biological activity and are supported by the recent finding that $p35^{mos\text{-}HT1}$, the gene product of the MSV variant Mo-MSV^{HT1} (28), possesses an ATPase activity when expressed in *E. coli* (29).

The reason for the failure to detect $p37^{mos(D121)}$ expression *in vivo* is not clear, for it differs only in the aspartate-for-lysine substitution. Furthermore, a full-length gene product can be synthesized from this mutant v-*mos* gene by using an *in vitro* transcription and translation system (unpublished results). The alteration from a positively charged lysine to a negatively charged aspartate could result in improper folding such that the protein is rapidly degraded *in vivo*. Our results suggest the presence of a potentially important interaction in $p37^{mos}$ between lysine-121 and a negatively charged residue elsewhere in the molecule. It will be of interest to determine the cause of the instability of $p37^{mos(D121)}$.

The most likely explanation for the loss of biological activity resulting from the aspartate-for-lysine substitution is the instability of $p37^{mos(D121)}$. However, in the case of $p37^{mos(R121)}$, which has a conservative amino acid substitution, the loss of transforming ability is clearly a direct effect of the arginine-for-lysine substitution. Although conformational differences may be responsible for the loss of biological activity of $p37^{mos(R121)}$, the conservative nature of the substitution and the similar stabilities of the respective proteins argue against this possibility. The only detectable difference between $p37^{mos}$ and $p37^{mos(R121)}$ is the lower level of phosphorylation of $p37^{mos(R121)}$. However, $p35^{mos\text{-}HT1}$ is also significantly less phosphorylated than $p37^{mos}$ (12), yet it retains full biological activity. This suggests that the lower level of phosphorylation in $p37^{mos(R121)}$ is irrelevant to its lack of biological activity.

Our conclusions are in agreement with the recent finding of protein kinase activity associated with $p37^{mos}$ when it is assayed as an immune complex after immunoprecipitation

Table 1. Alignment of the ATP-binding site in members of the protein kinase family

		Sequence at ATP-binding site				Ref.
Serine/threonine				*		
cAMP-PK	(48)	T L G T G S F G R V M	-10-	Y A M K I L D	(75)	32
cGMP-PK	(364)	T L G V G G F G R V E	-11-	F A M K I L K	(392)	33
Phos b kinase	(24)	I L G R G V S S V V R	-10-	Y A V K I I D	(51)	34
p100$^{gag-mil/raf}$	(601)	R I G S G S F G T V Y	-7-	V A V K I L K	(625)	35
p37^{mos}	(99)	R L G S G G F G S V Y	-8-	V A I K Q V N	(124)	6
Tyrosine						
p60^{src}	(272)	K L G Q G C F G E V W	-9-	V A I K T L K	(298)	36
p67^{erbB}	(137)	V L G S G A F G T I Y	-14-	V A I K E L R	(168)	37
p120$^{gag-abl}$	(368)	K L G G G Q Y G E V Y	-10-	V A V K T L K	(395)	38
p130$^{gag-fps}$	(926)	R I G R G N F G E V F	-10-	V A V K S C R	(953)	39
p90$^{gag-yes}$	(546)	K L G Q G C F G E V W	-9-	V A I K T L K	(572)	40
p70$^{gag-fgr}$	(409)	R L G T G C F G D V W	-9-	V A V K T L K	(435)	41
p62^{ros}	(253)	L L G S G A F G E V Y	-16-	V A V K T L K	(286)	42
Unknown						
p180$^{gag-fms}$	(1077)	T L G T G A F G K V V	-15-	V A V K M L K	(1109)	43
cdc28	(13)	K V G E G T Y G V V Y	-13-	V A L K K I R	(43)	44
Growth factor receptor						
EGF-r	(693)	V L G S G A F G T V Y	-14-	V A I K E L R	(724)	45
Insulin-r	(989)	E L G Q G S F G M V Y	-15-	V A V K T V N	(1021)	46
Consensus		X L/I G X G X F/Y G X V Y/W	.....	V A V/I K X L/I K/R		

Amino acid sequences of the various proteins were aligned to give maximum homology in the area surrounding the conserved lysine. The standard one-letter code is used. The consensus sequence for the ATP-binding site is shown, containing a Gly-Xaa-Gly-Xaa-Xaa-Gly stretch of amino acids followed by a lysine. The conserved lysine is indicated by an asterisk. The proteins are grouped according to substrate specificity. Also included are the amino acid sequences of two growth factor receptors. Numbers in parentheses indicate locations of ATP-binding sites. cAMP-PK, catalytic subunit of cAMP-dependent protein kinase; cGMP-PK, cGMP-dependent protein kinase; Phos b kinase, the γ subunit of phosphorylase b kinase; EGF-r, receptor for epidermal growth factor; insulin-r, insulin receptor.

with anti-N-terminal serum (30). Using the anti-C-terminal serum C3, we have been unable to identify a protein kinase activity associated with immunoprecipitates of p37^{mos} (unpublished data). However, this discrepancy may be a result of the different antisera used, since the C-terminal 23 amino acids of p37^{mos} are required for biological activity (24) and antiserum binding to the C terminus of p37^{mos} may inhibit the associated kinase activity found with the anti-N-terminal serum.

Our results have general implications for the family of protein kinases defined by limited amino acid homology. As shown in Table 1, the region surrounding lysine-121 of p37^{mos} is highly conserved in all members of this family. The importance of the conserved lysine residue in several other members of this family for transformation and protein kinase activity has recently been demonstrated. Substitution for lysine-295 in p60^{v-src} of a glutamate, arginine, or histidine (M. Kamps and B. Sefton, personal communication) or of a methionine residue (31), or for lysine-950 in p130$^{gag-fps}$ of an arginine or glycine (G. Weinmaster and T. Pawson, personal communication), results in loss of transforming potential and tyrosine-specific protein kinase activity. The inactivation of several of these proteins by an arginine-for-lysine substitution indicates that the presence of a positive charge at this position in the ATP-binding site is not sufficient to account for the enzymatic or biological activity of these proteins. Instead, the lysine residue must play a more specific role. One significant difference between arginine and lysine is the pKa of their respective side chains: 12.5 for arginine vs. 10.5 for lysine. If the neutral form of the lysine side chain contributes to the enzymatic activity, perhaps by abstraction of a proton from the phosphate acceptor molecule (16), then substitution of an arginine would be expected to reduce or eliminate the kinase activity by virtue of its greater pKa. Alternatively, the additional length of the arginine side chain or the steric bulk of its guanidinyl group may disrupt the ATP-binding site as a result of steric interference with the side chains of nearby amino acids or the ATP molecule. In this case, all the protein kinases would be expected to have a strict spatial arrangement between the lysine and the other residues that comprise the ATP-binding site. However, the primary sequence requirements of the ATP-binding site are quite variable, as illustrated in Table 1. The resolution of this issue must await crystallographic studies on the protein kinases. In either case, the conserved lysine residue specifically participates in the enzymatic activity of these proteins, and substitution of this residue results in loss of activity.

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