Biologically Active Mutants with Deletions in the *v-mos* Oncogene Assayed with Retroviral Vectors

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We have constructed retroviral expression vectors by manipulation of the Moloney murine leukemia virus genome such that an exogenous DNA sequence may be inserted and subsequently expressed when introduced into mammalian cells. A series of N-terminal deletions of the *v-mos* oncogene was constructed and assayed for biological activity with these retroviral expression vectors. The results of the deletion analysis demonstrate that the region of p37^{mos} coding region upstream of the third methionine codon is dispensable with respect to transformation. However, deletion mutants of *v-mos* which allow initiation of translation at the fourth methionine codon have lost the biological activity of the parental *v-mos* gene. Furthermore, experiments were also carried out to define the C-terminal limit of the active region of p37^{mos} by the construction of premature termination mutants by the insertion of a termination oligonucleotide. Insertion of the oligonucleotide just 69 base pairs upstream from the wild-type termination site abolished the focus-forming ability of *v-mos*. Thus, we have shown the N-terminal limit of the active region of p37^{mos} to be between the third and fourth methionines, while the C-terminal limit is within the last 23 amino acids of the protein.

Moloney murine sarcoma virus (M-MSV) was originally isolated from a sarcoma which appeared after injection of Moloney murine leukemia virus (M-MLV) into BALB/c mice (32). M-MSV arose by recombination between nondefective M-MLV and normal mouse cellular DNA (4, 13-15). The acquisition of the cellular DNA occurred concomitantly with significant deletions of the parental M-MLV genome. Thus, M-MSV is able to transform fibroblasts but is defective for viral replication (1, 4, 35-37).

The acquired mouse cellular DNA of M-MSV has been mapped to an uninterrupted sequence of approximately 1,200 base pairs (bp) near the 3' terminus of the M-MSV genome (14, 23, 47) and is referred to as *v-mos*. The nucleotide sequence of *v-mos* reveals that the 1,125-bp open reading frame is an *env-mos* fusion such that the first five codons of *v-mos* are derived from the M-MLV *env* gene and the remainder are derived from *c-mos* (11, 45, 46).

A 37,000-dalton protein was first identified as the *v-mos* gene product by in vitro transcription of M-MSV viral RNA (34). Subsequently, a 37,000-dalton protein was immunoprecipitated from M-MSV-transformed cells with an antibody directed against a peptide corresponding to the predicted C terminus of the *v-mos* gene product (36, 37). This protein, referred to as p37^{mos}, is presumably responsible for transformation by M-MSV. p37^{mos} exhibits limited regions of amino acid sequence homology with the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase (3). While other oncogenic proteins which show a similar homology with the cAMP-dependent protein kinase possess protein kinase activity, such as p60^{src}, no enzymatic activity has been unequivocally demonstrated for p37^{mos} (27).

We have constructed a series of deletions in the N-terminal coding region of *v-mos*. In addition, premature termination mutants of the *v-mos* gene were constructed. These mutations define the region of the gene requisite for transformation. The mutants of *v-mos* were expressed in eucaryotic cells with retroviral expression vectors. The retroviral vectors described here represent deleted deriva-

tives of M-MLV constructed such that the inserted DNA fragment replaces the retroviral *env* gene. Our work allows us to define the limits of the *v-mos* gene which are required to encode a transforming gene product. Mutants of *v-mos* with N-terminal deletions which allow initiation of translation at the third in-frame ATG of *v-mos* still retain biological activity, whereas mutants with more extensive N-terminal deletions are biologically inactive. Premature termination mutants have demonstrated that some portion of the C-terminal 69 nucleotides is necessary to maintain biological activity.

MATERIALS AND METHODS

Construction of retroviral expression vectors. Many of the details of the retroviral vectors are summarized in Fig. 1. The proviral clone of M-MLV, p836, was initially described by Hoffman et al. (22) and served as the parental plasmid for the vectors described in this work. We desired a unique *Xho*I restriction site in our vectors, which was achieved by the following steps. First, the *Xho*I-*Hind*III fragment (nucleotides [nt] 1560 to 4894 in the M-MLV sequence, reference 39), which encompasses the *gag-pol* region of M-MLV, was deleted. This was accomplished by blunt-end ligation of the *Xho*I terminus to the *Hind*III terminus after treatment with the Klenow fragment of DNA polymerase I. This protocol destroyed the *Xho*I site but restored the *Hind*III site. Second, the *Xho*I site downstream of the 3' long terminal repeat (LTR) was removed by digestion with *Xho*I, followed by treatment with Klenow fragment, and religation. Third, the *Cl*aI site in the *env* gene (nt 7674) was converted to an *Xho*I site by the insertion of an *Xho*I linker (CCTCGAGG). It should be noted that there is also a second *Cl*aI site in M-MLV, at nt 4980, but this site is methylated in DNA grown in *dam*⁺ strains of *Escherichia coli* and is refractory to cleavage by *Cl*aI.

To position the *Xho*I restriction site at the correct location in the vectors, the following sequence of steps was undertaken. First, the *Hind*III-*H*haI fragment of M-MLV (nt 4894 to 5780 of M-MLV), which contains the 3' splice acceptor site of the *env* gene and also the initiator ATG for the *env* gene product, was subcloned as an *Hind*III-*E*coRI fragment

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to yield the plasmid pDD90. Furthermore, the *EcoRI* site was converted to an *XhoI* site by linker insertion (CCTCGAGG) to yield the clone pDD92. Second, the subclone pDD90 was linearized at the *EcoRI* site, which

FIG. 1. (A) Construction of retroviral expression vectors. Shown is the genome of M-MLV with the splice sites and the retroviral genes denoted. Restriction sites used in the construction of the expression vectors are also shown as well as the corresponding nucleotide positions (39). Details of the construction of pDD102 and pDD103 are given in Materials and Methods. Each vector contains a unique *XhoI* site for insertion of a DNA fragment in place of the retroviral *env* gene. In the vector pDD102, the *env* gene initiation codon has been removed, so a DNA insert must provide its own ATG codon for the initiation of translation. In the vector pDD103, the *env* gene initiation codon remains and will be fused to

replaced the former *HhaI* site (at nt 5780), and treated with BAL 31 nuclease (28). After BAL 31 digestion, *XhoI* linkers (CCTCGAGG) were ligated onto the digested DNA. Third, several of the resulting plasmids were isolated, and the position of the *XhoI* linker was determined in each by nucleotide sequencing (30). One such clone, pDD94, had 29 bp removed by BAL 31, including the *env* gene ATG. In this clone, the *XhoI* site was located at nt 5751 of M-MLV, whereas the *env* gene ATG was located at nt 5777. The final step in the construction of the vectors was to replace the *HindIII-XhoI* fragment of the M-MLV derivative pDD99 with the *HindIII-XhoI* fragment of each of the subclones pDD94 and pDD92 described above. Insertion of the *HindIII-XhoI* fragment of pDD94 gave rise to the vector pDD102, while insertion of the *HindIII-XhoI* fragment of pDD92 gave rise to the vector pDD103.

The vectors pDD102 and pDD103 are similar in the following details. (i) The *XhoI-HindIII* region of M-MLV (nt 1560 to 4894) is deleted. (ii) Most of the *env* gene, from *HhaI* to *ClaI* (nt 5780 to 7674), is deleted. (iii) The 5' splice donor site and the 3' splice acceptor site for the *env* gene are retained. The two vectors differ in that pDD102 lacks the *env* gene ATG codon, whereas pDD103 retains the *env* gene ATG codon.

The clone pDD98 is similar to the clone pRB15, shown in Fig. 2, which contains the wild-type *v-mos* gene inserted into the vector pDD102. pDD98 differs from pRB15, however, by the retention of the *gag-pol* region of M-MLV, contained on the *XhoI-HindIII* fragment (nt 1560 to 4894 in the M-MLV sequence, reference 39), as shown in Fig. 1B.

Construction of deletion mutants in the *mos* gene. The *v-mos* gene which served as the starting material for the isolation of deletion mutants was the *XbaI* to *HindIII* fragment of the 124-MSV strain contained in the plasmid pDD0 (11). The long open reading frame of the *v-mos* gene which encodes p37^{mos} is conveniently flanked by a unique *XbaI* site upstream and a unique *HindIII* site downstream. The *HindIII* restriction site downstream of the coding region was first converted to an *XhoI* site by insertion of an *XhoI* linker (CCTCGAGG). The resulting plasmid was then linearized at the *XbaI* site upstream of the coding region and treated with BAL 31 exonuclease (28) for varying lengths of time. *XhoI* linkers were then ligated to the DNA, yielding *mos* genes deleted for various distances and flanked by *XhoI* sites. The exact length of deletion was determined by Maxam-Gilbert sequencing (30) with 3'-end-labeled restriction fragments. The endpoint of each deletion, as determined by sequencing, is indicated in Fig. 2.

Construction of C-terminal premature termination mutants. The *v-mos* gene was linearized at one of three unique restriction sites, *KpnI*, *HpaI*, or *SstII*, in the long open reading frame (see Fig. 3). The linear fragments were then

any DNA fragment inserted at the *XhoI* site. The lower portion of the figure shows the entire plasmids with the splice sites, LTR sequences, and important restriction sites denoted. Both pDD102 and pDD103 are approximately 15.8 kilobases in size. (B) Comparison of two vectors which express *v-mos*. The genome structures of pDD98 and pRB15 are compared with the parental retroviral genome of M-MLV. pDD98 retains the complete *gag-pol* region of M-MLV, whereas most of the *gag-pol* region has been deleted from pRB15. Given that pRB15 demonstrated greater biological activity than pDD98 (see text and Table 1), a vector with this same deletion (pDD102) was used for the assay of all *v-mos* deletion mutants.

treated with the Klenow fragment of DNA polymerase in the presence of all four deoxyribonucleoside triphosphates to yield blunt ends. The termination oligonucleotide (TCAATCAGTCAAGCTTGACTGATTGA) was then ligated onto the DNA. This oligonucleotide is self-complementary and contains termination codons in all three reading frames. The presence of the oligonucleotide in the resulting plasmids was monitored by the acquisition of an *Hind*III site contained within the oligonucleotide.

Biological assay. The biological activity of the various mutants of the *v-mos* gene was assayed with the expression vector pDD102. The *Xho*I-*Xho*I fragment of each mutated gene was inserted into the unique *Xho*I site of pDD102. Colony hybridization was used to ensure the presence of an insert, and restriction mapping was used to define the correct orientation of the insert (20).

FIG. 2. N-terminal deletion analysis of the *v-mos* gene. The structure of the M-MLV-derived expression vector pDD102 is shown with the full-length wild-type *v-mos* gene inserted as an *Xho*I-*Xho*I fragment. The long open reading frame of *v-mos* has been expanded to show the five ATG codons in the 1125-bp coding sequence. The ATG codons are represented by solid circles, and the opal terminator codon is also shown. Nucleotide numbers associated with the ATG codons and the terminator codon are from the published sequence of 124-MSV (46). The nucleotide position at each deletion endpoint is indicated, as determined by Maxam-Gilbert sequencing; the numbers refer to the first remaining base pair of *v-mos* after the *Xho*I linker. The biological activity of each deletion mutant was assayed by its ability to induce foci when introduced into NIH 3T3 cells. A plus indicates a minimum value of 10^3 FFU/pmol of cloned DNA. A minus indicates a maximum value of 8.3×10^1 FFU/pmol.

TABLE 1. Transforming activities of deletion mutants^a

Plasmid	First ATG remaining	Site of termination linker	Biological activity (FFU/pmol)	Rescued virus titer (FFU/ml)
pDD98	1		7.4×10^2	1.7×10^3
pRB15 (wild type)	1		1.3×10^4	3.2×10^5
pRB16	2		1.2×10^3	6.0×10^4
pRB6	2		1.1×10^3	9.5×10^4
pRB3	2		1.0×10^3	9.0×10^4
pRB1	2		1.0×10^3	9.7×10^4
pRB9	3		1.1×10^3	1.1×10^5
pRB19	3		1.1×10^3	1.2×10^5
pRB5	4		$<6.3 \times 10^1$	$<5.0 \times 10^0$
pRB18	4		$<8.3 \times 10^1$	$<5.0 \times 10^0$
pRB8	4		$<6.0 \times 10^1$	$<5.0 \times 10^0$
pRB27		<i>Kpn</i> I	9.7×10^1	4.0×10^1
pRB28		<i>Hpa</i> I	1.3×10^1	$<1.0 \times 10^1$
pRB26		<i>Sst</i> II	4.1×10^1	$<2.5 \times 10^1$

^a The upper portion of the table indicates the biological activity of the N-terminal deletion mutants. The table also denotes the first in-frame ATG codon available for initiating translation in each of the deletion mutants. The biological activity of each plasmid was assayed as described in Materials and Methods. Values shown are calculated as focus-forming units per picomole (FFU/pmol) of transfected DNA. The lower portion of the table shows the biological activity of the premature termination mutants. The restriction sites at which the termination linker was inserted are denoted. The virus titer of collected culture fluid from the transfection plates was determined as described in Materials and Methods. The titers shown are calculated as focus-forming units per milliliter of culture fluid (FFU/ml) which was collected approximately 7 days after transfection.

The general protocol used for the focus assay was that described by Graham and Van der Eb (19). A 1- μ g portion of each DNA, together with 0.5 μ g of DNA of the replication-competent clone of M-MLV, p836 (22), was transfected in the presence of 15 μ g of sheared calf thymus DNA. Each sample was applied to a 5-cm plate of approximately 50% confluent NIH 3T3 cells as a calcium phosphate coprecipitate. After approximately 12 to 18 h, the plates were trypsinized, and the cells were distributed to four 10-cm plates. The focus assays were then scored 12 to 14 days after transfection. Results of the focus assays are shown in Table 1. These results were also confirmed by collecting the culture fluid from the transfection plates and determining titers for this culture fluid on fresh monolayers of NIH 3T3 cells for focus-forming virus. The results of the transforming virus titers are shown in Table 1 and were always consistent with the results of the primary transfection assays.

RESULTS

Construction of retroviral expression vectors. We constructed retroviral vectors which permit the expression in eucaryotic cells of an inserted gene (2, 8, 10, 17, 18, 21, 24, 33, 43). The vectors are constructed in such a way that the inserted DNA fragment is substituted for the *env* gene of M-MLV (21, 39). Consequently, the mRNA of the inserted gene will be transcribed and spliced in a fashion identical to that of the M-MLV *env* gene. Furthermore, the vectors provide LTRs both 5' and 3' to the insert which are required for the enhancement of transforming activity of *v-mos* as well as recovery of infectious virus (4, 44, 49). The two vectors described here, pDD102 and pDD103, contain 5' and 3' LTRs, 5' and 3' splice sites for the *env* gene, similar deletions, and a unique *Xho*I site for insertion of a DNA fragment (Fig. 1). However, pDD102 and pDD103 differ by the absence or presence of the *env* gene ATG codon.

In the vector pDD103, a unique *Xho*I site was inserted

directly downstream of the *env* gene ATG codon so that an inserted gene need not provide its own ATG for initiation of translation when expressed. In contrast, pDD102 lacks the *env* gene initiation codon. Treatment with BAL 31 nuclease was used to remove the *env* gene ATG and a short region upstream (Fig. 1). The expression of a gene inserted at the unique *Xho*I site of pDD102 requires that the inserted gene provide its own initiation codon for translation.

The parental *v-mos* gene was inserted into the vector pDD102 to yield the plasmid pRB15. This plasmid was biologically active when transfected into NIH 3T3 cells and scored in a conventional focus assay (Table 1). When the transfection was carried out in the presence of DNA of a clone of infectious M-MLV, it was possible to demonstrate transmissible transforming virus in the culture fluid from the transfected cells. We also constructed another retroviral vector, pDD98, which contains the wild-type *v-mos* gene. This construct is similar to pRB15 but retains the complete *gag-pol* region from M-MLV. The genome structures of M-MLV, pRB15, and pDD98 are summarized in Fig. 1B. pDD98 consistently yielded fewer foci (7.4×10^2 foci per pmol) compared with pRB15 (1.3×10^4 foci per pmol) (Table 1). Also, the titer of recovered transforming virus was lower for pDD98 (1.7×10^3 focus-forming units [FFU]/ml) compared with pRB15 (3.2×10^5 FFU/ml). The low transformation frequency of pDD98 was shown not to be due to the reported cytotoxicity of *v-mos* (37), as the transformation efficiency of the herpesvirus thymidine kinase gene was not lowered by cotransfection with pDD98 (data not shown). Due to the higher biological activity of the clone pRB15 compared with the clone pDD98, we decided to use the vector pDD102 for subsequent analysis of the *v-mos* deletion mutants described below.

N-terminal deletions of *v-mos*. To delimit the region of the *v-mos* gene necessary for neoplastic transformation, we constructed a series of deletions of the N-terminal region. The N-terminal region of *v-mos* was treated with BAL 31 nuclease for varied amounts of time to yield deletions in this region. The position of the deletion endpoints varied from just beyond the first ATG (pRB16), a deletion of 18 bp, to just before the fourth ATG (pRB8), which has 510 bp of *v-mos* removed (Fig. 2). The deleted *v-mos* genes, after insertion into the expression vector pDD102, were introduced into NIH 3T3 cells by the calcium phosphate coprecipitation technique to test the effect of each deletion on the transforming activity (19).

The biological activity of these deletions, as determined by standard focus assay, is indicated by + or – in Fig. 2, and quantitation is presented in Table 1. Deletion mutants with transforming activities denoted as – did not possess any transforming activity above the level of detection. This was confirmed by assaying the culture fluids for the presence of transmissible focus-forming virus, as described in Table 1. Mutants with deletions of only the first ATG codon of the open reading frame, e.g., pRB1, as well as mutants with deletions of both the first and second ATG codons, e.g., pRB9, were still biologically active. However, deletions extending beyond the third ATG codon abolished biological activity. Therefore, *v-mos* deletion clones such as pRB9 and pRB19, which presumably initiate translation at the third ATG, produce polypeptides capable of transforming NIH 3T3 cells. This is in contrast to the *v-mos* deletion clones, such as pRB8, which must initiate translation at the fourth ATG and are incapable of transformation. Thus, the region of *v-mos* upstream of the third ATG codon is unnecessary for neoplastic transformation activity.

It must be noted that the transforming ability of the deletions is below the level of wild-type *v-mos* activity. As observed in Table 1, there is approximately a 10-fold reduction in the transforming activity of the *v-mos* deletions below that of the wild-type gene. This might result from initiation at out-of-frame ATG codons which are upstream from the correct, in-frame ATG (11). However, this seems unlikely as all the transforming deletions exhibit the same reduction in transforming activity, whereas they do not all possess out-of-frame ATG codons upstream of the first remaining in-frame initiation codon. Another possible reason for the decrease in activity involves protein structure. While the N-terminal region upstream of the third ATG may not be absolutely required for transforming activity, it may play a significant role in the stability or the correct tertiary structure of the protein. The important point to note, though, is that the mutants with deletions up to but not including the third ATG codon still possess the ability to transform cells, albeit not as efficiently as the wild-type parent.

A cytotoxic effect has been reported during acute infection of NIH 3T3 cells by 124-MSV (37). However, periodic examination of the transfected cells by phase-contrast microscopy revealed no evidence of extensive cell death, as would be expected if any of these constructs resulted in cytotoxicity.

Premature termination of *v-mos* by oligonucleotide insertion. We also constructed mutations of the *v-mos* gene specifying premature termination of translation. A self-complementary oligonucleotide (Fig. 3) was synthesized which contains opal termination codons in all three reading frames. This oligonucleotide was then inserted at three

FIG. 3. Premature termination of the *v-mos* gene by oligonucleotide insertion. The full-length coding region of the *v-mos* gene is shown from the initiation codon (ATG) to the termination codon (TGA). The nucleotide positions of these codons and of the restriction sites indicated refer to the sequence of 124-MSV (46). The synthetic oligonucleotide as shown was inserted at each of three restriction sites so as to cause premature termination. Upon insertion of this linker at any restriction site, regardless of reading frame, translation will be terminated due to the presence of termination codons in all three possible reading frames. The biological activity of the premature termination mutants was assayed by their ability to induce focus formation in NIH 3T3 cells. All termination mutants possessed no biological activity and had a maximum value of 9.7×10^1 FFU/pmol.

unique restriction sites in *v-mos* (Fig. 3). These mutants were assayed for focus-forming activity in the vector pDD102, and all were biologically inactive. Thus, the C-terminal limit of the gene required for the transforming ability of *v-mos* is beyond the *Sst*II site at the 352nd amino acid of the protein, since termination at this site destroys biological activity. This indicates that some portion of the 23 amino acids of the C terminus is essential for *v-mos* to transform NIH 3T3 cells.

DISCUSSION

Retroviral expression vectors have become the method of choice for the introduction of foreign genetic material into eucaryotic cells (8, 10, 21, 24, 26, 31, 33, 38, 41, 43, 48). The major characteristics which can be used to differentiate the various retroviral expression vectors are: (i) the retroviral gene which is replaced with the exogenous gene, (ii) the presence or absence of a selectable marker within the expression vector, and (iii) whether the expression vector provides a promoter for the inserted gene.

Our expression vectors, derived by deletion of M-MLV, allow for the replacement of the viral *env* gene sequence by the desired foreign gene while maintaining both LTR sequences, splicing signals, and packaging signals. The expression vectors are replication defective and therefore must be accompanied by coinfection by a helper virus if a transmissible virus stock is desired (29). While the vectors we constructed do not have a selectable marker other than the gene insert, there exists a unique *Hind*III restriction site in the deleted *gag-pol* region for the insertion of such a selection marker. Two other groups have constructed retroviral expression vectors which are similar to those described in this work and deserve special note. Cepko et al. (8) have constructed an expression vector derived from M-MLV such that a foreign gene may be inserted so as to replace the retroviral *env* gene. A special feature of this vector is the presence of the Tn5 transposon-derived neomycin resistance gene which replaces the *gag-pol* region of the parental retrovirus. Hwang and Gilboa (24) constructed M-MLV-derived vectors which replace the *env* gene of the retrovirus with the neomycin resistance gene derived from Tn5. Our vectors, however, contain deletions within the *gag-pol* region, whereas the vectors of Hwang and Gilboa retain intact the *gag-pol* region.

In this work, we described two very similar retroviral constructs, pDD98 and pRB15, which express the *v-mos* oncogene but which differ by the presence or absence of the *gag-pol* region. Hwang et al. (25) have previously reported the existence of two regions in the *gag-pol* region which are required for efficient *env* gene expression. However, when the *v-mos* oncogene is inserted into the deleted vector, expression is more efficient than when inserted in the nondeleted vector. This increased expression in the deleted vector is reflected both in higher numbers of foci per picomole of transfected DNA and higher titers of transmissible transforming virus which is recovered from transfected cells (Table 1). These results suggest that the presence of the *gag-pol* intron is not essential, when using the retroviral vectors described here, for efficient expression of genes substituted in place of the retroviral *env* gene.

Through the use of retroviral expression vectors which we have constructed, we have been able to assay the biological activity of N-terminal deletions of *v-mos*. The results of this work show that some region between the third and fourth initiation codons is absolutely required for induction of focus formation by *v-mos*. Thus, the remainder of the gene down-

stream from the third ATG codon possesses all regions requisite for biological activity. This defines the N-terminal limit of the *v-mos* gene necessary for focus formation.

Previous delimitation of the *v-mos* gene required for biological activity has relied on altered recombinational events of the *mos* gene (6, 13, 14, 16, 40, 42). Myeloproliferative sarcoma virus is a member of the MSV family originating upon serial transplantation of a tumor induced in a newborn mouse by uncloned MSV with M-MLV as a helper (42). In the myeloproliferative sarcoma virus genome, a frameshift mutation in the N-terminal coding region produces a truncated gene product of 19 amino acids upon initiation of translation at the first ATG of the *v-mos* gene. However, in myeloproliferative sarcoma virus, initiation can begin at the second ATG of *mos* and continue through the proper C terminus, presumably generating the gene product responsible for transformation. This work with myeloproliferative sarcoma virus places the active region of the *v-mos* gene beyond the second ATG.

Another altered recombinational event involving the *mos* gene was discovered by Canaani et al. when a rearranged *c-mos* gene (*rc-mos*) was isolated from a mouse myeloma tumor (7, 9, 40). The rearrangement involved the deletion of 263 nucleotides from the 5' coding sequence of *c-mos* and the substitution of this information with a new cellular DNA sequence. Thus the gene product from *rc-mos* has an N terminus of 28 amino acids donated by the substituted cellular sequence while the remainder of the protein is from the *c-mos* gene. The point of recombination is at the 73rd amino acid of *v-mos*, which lies between the second and third ATG codons of the 124-MSV *v-mos* gene. This work demonstrates that the transforming potential of *c-mos* lies downstream of the 73rd amino acid of the full-length gene product (7, 9, 40). Our work is consistent with these earlier observations by placing the N-terminal limit of the biologically active region of the *v-mos* gene product at the third ATG, which codes for the 97th amino acid of the *v-mos* protein.

Using a termination oligonucleotide, we have shown that some portion of the 23 amino acids at the C terminus of p37^{mos} is necessary for biological activity. The sites of recombination among different variants of M-MSV have provided the only previous delimitation of the C-terminal active region of *v-mos* (13, 14). The m1-MSV strain has a small internal deletion at the C terminus which results in an altered amino acid sequence beyond the site of deletion (6). Figure 4 shows the point of divergence between m1-MSV and 124-MSV. However, of the C-terminal 23 amino acids which we have shown to contain a region necessary for transformation, 17 are conserved in m1-MSV. Since only those variants of MSV which still possess the transforming capabilities of *v-mos* will be isolated, the recombinational site at the C terminus can be used to delimit the region necessary for biological activity (6, 13, 14). Our work, as well as the absence of MSV variants with C-terminal recombinational sites upstream of these terminal 23 amino acids, demonstrates that some portion of this sequence is required for biological activity of *v-mos*.

The *v-mos* gene product is one of several oncogenic proteins which show homology to the bovine cAMP-dependent protein kinase but possess no specific tyrosine kinase activity (3, 27). There are several specific regions within the *v-mos* gene product which show conservation of residues with the protein kinase (Fig. 4). Directly downstream of the third ATG codon is a sequence of nine amino acids, of which six are conserved with the cAMP-dependent

FIG. 4. Location of *v-mos* mutants and relationship to homology with cAMP-dependent protein kinase. The amino acid sequence of p37^{mos} is shown (in one-letter code) in alignment with the catalytic subunit of cAMP-dependent protein kinase (cat) (3). Asterisks above the *mos* sequence denote the five methionine residues within the protein. The endpoint of deletion mutant pRB5 represents the smallest deletion which destroys biological activity. The site of termination linker insertion in pRB26 is also shown. This leads to truncation of 23 amino acids from the C terminus of p37^{mos} and the loss of biological activity. The point of divergence between 124-MSV and m1-MSV is also shown. The closely related strain m1-MSV encodes a gene product with a slightly altered C terminus, due to a shift in the reading frame near the C terminus. As a result, the gene product of m1-MSV retains only the first 17 residues of the 23 C-terminal residues of the 124-MSV gene product.

protein kinase (5). Figure 4 shows the N-terminal endpoint of the smallest deletion mutant, pRB5, which is incapable of transformation. This mutant initiates translation at the fourth in-frame ATG when expressed in the vector pDD102.

It should be noted that a presumptive ATP-binding site in p37^{mos} lies within the region which is required for transformation, as shown by the deletion analysis presented here. This presumptive ATP-binding site, which includes lysine residue 121 of p37^{mos}, occurs just downstream of the third methionine. A similar ATP-binding site occurs in the catalytic subunit of the cAMP-dependent protein kinase and also in p60^{src}, and site-directed mutagenesis of lysine 121 of

p37^{mos} indicates that this residue is functionally important for transformation by *v-mos* (M. Hannink and Daniel J. Donoghue, manuscript submitted for publication).

Figure 4 also shows the site at which the biologically inactive C-terminal mutant pRB26 terminates translation because of the termination linker insertion. Beyond the site of premature termination, another region of *v-mos* is highly conserved with the protein kinase in which five of six residues are identical (reference 3; Fig. 4). This sequence homology lies within the C-terminal region which is required for transformation by *v-mos*. Although the *v-mos* gene product function has not been identified, it appears to

require regions which have conserved amino acid sequences with cAMP-dependent protein kinase.

The normally quiescent *c-mos* gene, when acquired and expressed by the M-MSV retrovirus, becomes acutely transforming (5, 7, 9, 16). Since the function of the *v-mos* gene product has yet to be determined, neither its original role as a *c-mos* gene product nor its method of transformation when expressed by a retrovirus is known (12, 27, 35). Our work delineates the N- and C-terminal regions of the *mos* gene requisite for biological activity and consequently the sequences which are indispensable for an active *v-mos* gene product.

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