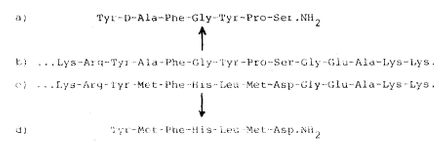

Fig. 3. Sequences of end products and of the corresponding segments in the precursors. (a) Sequence of dermorphin; (b) dermorphin and flanking sequences deduced from the cloned cDNAs; (c) homologous region from insert of clone D-1/2 (see Fig. 1); (d) sequence of predicted peptide, assuming the same processing cascade as for the release of dermorphin. Arrows indicate post-translational processing.

only dermorphin with a D-alanine has been detected in *Phyllomedusa* skin (*4*). The reaction may thus require energy.

The presence of a D-amino acid in other animal peptides has not been considered in recent decades. Minute quantities of D-aspartic acid have been detected in proteins of aging erythrocytes (*19*), but these apparently arise at random via chemical isomerization. The postulated existence of a mechanism in an amphibian species which converts a particular L-alanine to its D-isomer raises the question whether this may also occur elsewhere. It will now be possible to use the cDNAs cloned from skin of *Ph. sauvagei* to search for dermorphin-like sequences in genomic libraries from other sources. The detection of such sequences in a more accessible species is a prerequisite to the study of epimerization of an amino acid during processing of a peptide precursor.

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14 April 1987; accepted 1 July 1987

Transformation by Oncogenes Encoding Protein Kinases Induces the Metastatic Phenotype

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Oncogenes encoding serine/threonine or tyrosine kinases were introduced into the established rodent fibroblast cell line NIH 3T3 and tested for tumorigenic and metastatic behavior in T cell–deficient nude mice. Transforming oncogenes of the *ras* family were capable of converting fibroblast cell lines to fully metastatic tumors. Cell lines transformed by the kinase oncogenes *mos, raf, src, fes*, and *fms* formed experimental metastases and (in some cases) these genes were more efficient at metastatic conversion than a mutant *ras* gene. In contrast, cells transformed by either of two nuclear oncogenes, *myc* or p53, were tumorigenic when injected subcutaneously but were virtually nonmetastatic after intravenous injection. These data demonstrate that, in addition to *ras*, a structurally divergent group of kinase oncogenes can induce the metastatic phenotype.

METASTASIS IS THE PROCESS BY which tumor cells spread and colonize secondary sites throughout an organism. For a cell to be able to metastasize, many specialized characteristics are required, including the ability to invade through host barriers into the vasculature, survive in the circulation, extravasate, and subsequently establish and grow. Recent work has firmly established that NIH 3T3 and 10T1/2 cells transformed by *ras* genes are capable of forming metastases (*1, 2*), and this appears to be a direct result of *ras* function (*2*). Also, transfection of activated *ras* into poorly metastatic murine adenocarcinoma cells significantly enhances metastatic potential (*3*). However, it is unlikely that aberrant *ras* function is essential for all tumor dissemination since not all metastases contain activated *ras* sequences (*4*), and correlative studies have implicated amplification of non–*ras* oncogenes in progression of specific tumors (*5, 6*). To directly test the hypothesis that other oncogenes may be involved in regulating or inducing metastatic activity, we have assessed the ability of NIH 3T3 (clone 7) cells transformed by a wide variety of oncogenes to form experimental and spontaneous metastases. We report here that transformation by all kinase-encoding oncogenes tested results in the metastatic conversion of this fibroblast line.

Representative oncogenes of the cytoplasmic serine/threonine kinases were evaluated for their ability to induce experimental metastases. Three lines of NIH 3T3 cells transformed by v-*mos* were isolated and cloned from transformed foci after Moloney murine sarcoma virus infection (*7*). Southern blot analysis revealed the presence of v-*mos* sequences in each clone (*7*). These freshly transformed cell lines (which were not releasing

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virus) were injected intravenously into BALB/c nu/nu mice for assessment of lung colonizing ability. All three *mos*-transformed clones produced lung tumors, although frequencies varied significantly (Table 1). Two of the lines were equal in metastatic ability or more metastatic than the *ras*-transformed positive control. The relatively low metastatic potential of Mos 3 was probably due to a low level of *mos* expression (which was observed on Northern blots), since the level of *mos* expression correlated with metastatic efficiency of the three clones (*7*). NIH 3T3 cells transformed by the *raf* oncogene, which also encodes a serine/threonine kinase, were next examined. Cells transformed by v-*raf* and A-*raf* were cloned from foci after transfection of cloned viral DNA, and have been shown to contain and express their respective transforming sequences (*8, 9*). Both *raf*-transformed cell lines were also capable of forming experimental metastases in nude mice (Table 1).

We next tested oncogenes that code for tyrosine kinase products: membrane-associated v-*src* and v-*fes* as well as the CSF-1 receptor–related v-*fms*. Src 1 was cloned from a transformed NIH 3T3 focus after infection with a murine retrovirus containing the v-*src* from Rous sarcoma virus (*10*). Src 2, on the other hand, was isolated as a G418-resistant colony obtained by infection with a retroviral vector containing v-*src* and the neomycin resistance gene (*11*). The v-*fes*– and v-*fms*–transformed cells were isolated from foci after transfection with plasmids containing cloned Gardner-Arnstein and McDonough strains of feline sarcoma virus, respectively (*12, 13*). Cell lines not obtained through selection in G418 have been shown to contain and express their oncogene sequences as well as produce transforming virus upon rescue through transfection of helper virus (*10, 12, 13*). All four cell lines transformed by tyrosine kinase–encoding oncogenes were very potent in the experimental metastasis assay (Table 1) as were the serine/threonine class of oncogenes. Although we have not formally excluded the possibility that the cell lines transformed by these kinase-encoding oncogenes contain activated *ras*, it seems highly unlikely that all of the transfectants would have acquired this mutation and consequently express metastatic ability.

Histological examination of lungs revealed that the experimental metastases from cells transformed by either serine/threonine– or tyrosine kinase–encoding oncogenes were typical fibrosarcomas, similar to those formed after the injection of N(Z61.8) cells, which are transformed by a mutant c-H-*ras* gene (*14*) (Fig. 1).

Lung-colonizing ability after intravenous injection requires most of the characteristics essential for metastasis from a primary tumor except invasion and entry into the vasculature. The ability to form experimental metastases by H-*ras*–transformed cells (*2*) and other tumors (*15*) have been found to correlate very well with their ability to induce spontaneous metastasis formation from a subcutaneous site. NIH 3T3 cell lines transformed by *mos, raf, src, fes, fms*, and *ras* oncogenes were all capable of forming metastases from a subcutaneous tumor and are, therefore, fully metastatic in nude T cell–deficient mice (*16*).

All of the kinase-oncogene–transformed cell lines reported above (except Src 2) were obtained through transfection of cloned viral DNA or viral infection of NIH 3T3 clone 7. This 3T3 subclone, which was selected for its flat morphology and contact-inhibited growth properties (*17*), was found to be completely nonmetastatic (Table 1). These cells were nontumorigenic after subcutaneous injection of 10^7 cells (*16*). We have also tested ψ2 cells, which were derived by transfection of defective cloned Moloney murine leukemia virus (MoMuLV) into NIH 3T3 cells (*11*). This retroviral packaging cell line as well as clone 7 infected with MoMuLV were controls, representing 3T3 cells transfected or infected with viral sequences that lack transforming oncogenes. Both ψ2 and MoMuLV-infected clone 7 were completely negative in the experimental metastasis assay (Table 1). However, uncloned NIH 3T3 cells produced a very low but detectable level of lung colonies when injected intravenously, suggesting the existence of a small subpopulation of transformed and metastatic cells in this parental 3T3 line, as previously reported (*1*).

In contrast to the ability of kinase-encoding oncogenes to induce the metastatic phenotype, the most tumorigenic clones transformed by the nuclear oncogenes c-*myc* and p53 (*18*) were not significantly more metastatic than NIH 3T3 (Table 1). No gross spontaneous metastases were detected after subcutaneous injection of these two lines. Furthermore, lung tissue was free of micrometastases when cultured in G418, a sensitive method for detecting tumors bearing the neomycin-resistance gene (*16*). Both of these cell lines have been previously shown to contain and express their transfected oncogene (*18*). In addition, three cell lines were isolated in G418 through infection of

Table 1. Metastasis formation by NIH 3T3 cells transformed by oncogenes encoding tyrosine and serine/threonine kinases. Subconfluent and exponentially growing monolayer cultures were lightly trypsinized (0.05% trypsin in 0.5 m*M* EDTA), washed, and resuspended in Hanks balanced salt solution. Aliquots of 3×10^5 cells in 0.1 ml were each injected into the lateral tail vein of 5- to 8-week-old BALB/c female nu/nu mice for the experimental metastasis assay. Twenty-one days later, mice were killed by ether anesthesia and Bouin's solution instilled directly in the trachea with a syringe. Lungs were removed and metastases counted under a dissecting microscope. Tumorigenicity of the lines was evaluated by monitoring tumor formation after subcutaneous injection of 3×10^5 cells into BALB/c nu/nu mice. All transformed cell lines produced large rapidly growing tumors. Latency was scored as the day at which a 2-mm tumor was detectable. NT, not tested.

Cell line*	Transforming gene	Experimental metastasis		Tumorigenicity	
		Frequency	Lung nodules ($\bar{x}$ ± SE)	Frequency	Latency ($\bar{x}$ days ± SE)
N(Z61.8)	c-H-*ras* (61-Leu)	6/6	32 ± 21	5/5	7.6 ± 0.2
Mos 1	v-*mos*	6/6	29 ± 11	NT	NT
Mos 2	v-*mos*	5/5	62 ± 27	5/5	7.4 ± 0.6
Mos 3	v-*mos*	4/6	1.8 ± 0.8	NT	NT
NIH/F4-3611	v-*raf*	4/6	4.7 ± 2.3	5/5	5.0 ± 0.0
NIH/9IV#5	A-*raf*	5/6	38 ± 14	5/5	6.2 ± 0.8
Src 1	v-*src*	6/6	59 ± 25	6/6	7.3 ± 0.5
Src 2	v-*src*	5/5	6.8 ± 1.3	NT	NT
Fes 1	v-*fes*	6/6	178 ± 38	5/5	6.4 ± 0.7
Fms 1	v-*fms*	6/6	110 ± 45	5/5	6.2 ± 0.7
NIH 3T3 clone 7 (MoMuLV)		0/5	0.0 ± 0.0	NT	NT
NIH 3T3 clone 7		0/10	0.0 ± 0.0	0/3	>50
ψ2 (MoMuLV)		0/5	0.0 ± 0.0	NT	NT
NIH 3T3		3/10	0.5 ± 0.3	NT	NT
NIH/hmyc1	c-*myc*	2/5	1.0 ± 0.6	6/6	13.7 ± 0.3
NIH/p53.3	p53	1/5	0.2 ± 0.2	5/5	8.0 ± 0.6
VM1	v-*myc*	0/5	0.0 ± 0.0	NT	NT
VM4	v-*myc*	0/5	0.0 ± 0.0	NT	NT
VM5	v-*myc*	0/20	0.0 ± 0.0	NT	NT
VM5†	v-*myc*	0/5	0.0 ± 0.0	NT	NT

*Cell lines are as described (*30*). †Experimental metastasis assay terminated at 46 days.

clone 7 with a retroviral vector containing v-*myc* and the neomycin resistance gene (*19*). These three cell lines were completely nonmetastatic in the standard experimental metastasis assay of 21 days (Table 1). VM5 was also completely negative in a 6-week assay; therefore, decreased latency is probably not responsible for the negative result. Although expression of *myc* or p53 did not significantly induce metastatic behavior, these genes may still be important in tumor progression through complementation with other oncogenes. For example, N-*myc* amplification has been linked to tumor progression of neuroblastoma (*5*), and Bernards *et al.* recently demonstrated that N-*myc* overexpression in the B104 rat neuroblastoma line profoundly affected expression of the MHC class 1 gene and metastatic behavior (*20*). In the B104 tumor, which was obtained by ethylnitrosourea treatment of perinatal BDIX rats, activation of the tyrosine kinase *neu*-oncogene had occurred (*21*). Therefore, N-*myc* may contribute to metastatic progression by complementation with *neu*. A similar type of complementation has been suggested for *myc* and *ras* (*22*). It is also possible that overexpression or complementation of p53 will contribute to metastatic progression in the same way.

With the observation that the kinase group of oncogenes described in this study, as well as the *ras* family (*1, 2*), can produce metastatic conversion of NIH 3T3 cells, it is tempting to speculate that a common pathway may be involved in this process. In that regard, *ras*-mediated transformation can result in alterations of inositol phospholipid metabolism (*23, 24*) including increased steady-state levels of diacylglycerol (*24*). Recent work suggests that tyrosine kinase oncogenes also stimulate elevated phosphatidylinositol turnover, possibly through modification of cellular phosphoinositide kinases (*25, 26*). Furthermore, Smith *et al.* have shown that transformation by *src*, *fes*, and *fms* can be blocked by microinjection of antibodies to p21 (*27*) indicating that these genes act via a *ras*-dependent mechanism. While *mos*- and *raf*-mediated transformation are not dependent on *ras* p21 (*9, 27*), these serine/threonine kinase oncogenes may act on the same metabolic pathway (*28*), although downstream of *ras*. This pathway may also utilize inositol phospholipid–derived second messengers that regulate Ca^{2+} mobilization and protein kinase C activity (*26*). Both *ras* and *mos*, for example, suppress the transcription of α_2 type I procollagen, which is also downregulated by the protein kinase C agonist PMA (phorbol myristate acetate) (*29*). These observations suggest that unregulated alterations in the membrane-associated inositol second messenger system resulting from the action of the *ras* or kinase group of oncogenes are an important, if not critical, event in the induction of metastatic behavior.

In conclusion, we have shown that a single step conversion of NIH 3T3 cells by oncogenes encoding either serine/threonine or tyrosine kinases can induce the metastatic phenotype. Thus, mutation or aberrant regulation of structurally diverse oncogenes can effect metastatic dissemination.

Note added in proof: Since submission of this manuscript, several additional freshly isolated clones of NIH 3T3 clone 7 transformed by v-*src* (*11*), v-*fms*, or v-*fes* have been evaluated and found to be metastatic.

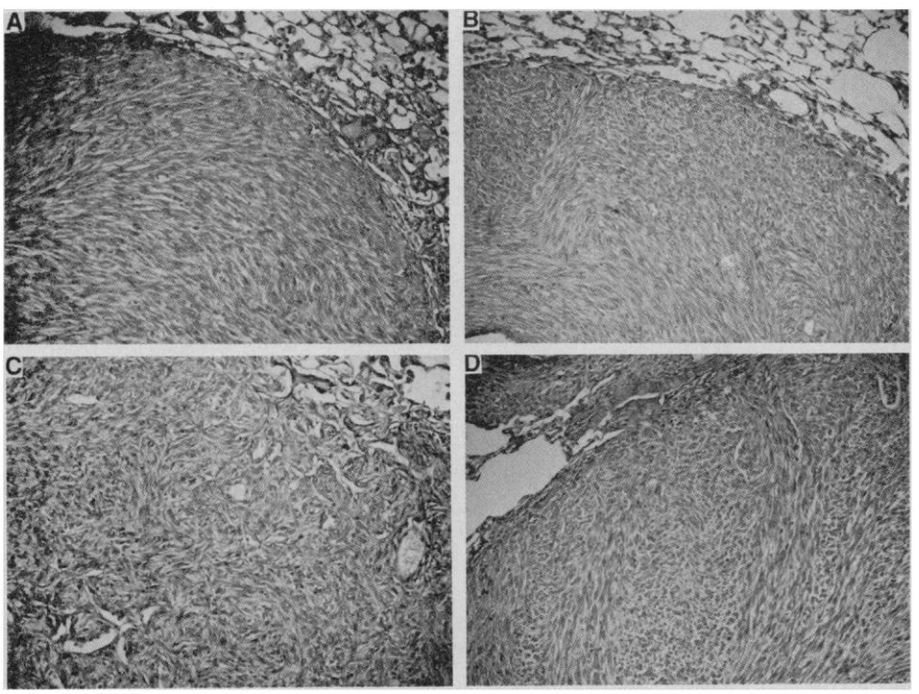

Fig. 1. Histopathology of lung metastases of NIH 3T3 transformed by (**A**) v-*mos*, line Mos 2; (**B**) v-*fms*, line Fms 1; (**C**) v-*src*, line Src 1; and (**D**) c-H-*ras* (61-Leu), line N (Ż61-8). All lines produced typical fibrosarcomas.

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31. We wish to thank G. M. Cooper (NZ61.8 cells), U. R. Rapp (NIH/F4-3611, NIH/91V#5, NIH 3T3 clone 7), J. E. Dick (ψ2), S. M. Anderson (Src 1), J. Even (Fes 1), C. J. Sherr (Fms 1), and M. D. Cole (NIH/hmyc1 and NIH p53.3) for the generous gift of cell lines. We are also very grateful for the use of MX2122-B31/NEO supplied to us by M. Verderame together with M. Scott and H. E. Varmus, as well as G. P. Dotto and R. A. Weinberg, for the use of VM retrovirus and valuable advice. Supported by the National Cancer Institute of Canada, The Manitoba Health Research Council, and The Winnipeg Children's Hospital Research Foundation. S.E.E. is supported by a Steve Fonyo National Cancer Institute of Canada Studentship. A.H.G. and J.A.W. are Terry Fox Cancer Research Scientists.

An Amylose Antiparallel Double Helix at Atomic Resolution

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In the crystal structure of the polyiodide complex (*p*-nitrophenyl-α-maltohexaose$_2$) · $Ba(I_3)_2$ · $22H_2O$, the maltohexaose units form an antiparallel, left-handed double helix with O-2 ··· O-3 and O-6 ··· O-6 hydrogen bonding and a central cavity that encloses two triiodide units. This structure contrasts with the parallel, left-handed double helix with no central cavity proposed for the A- and B-starch helix and the left-handed single helix in V-amylose and may be relevant for the stabilization of glycogen structure.

STARCH, THE STORAGE POLYSACCHARIDE for glucose in plants, is composed of D-glucopyranose units. It can be separated into the branched amylopectin, which has interglucose bonds of the α(1→4) and α(1→6) type, and the linear amylose, which has exclusively α(1→4) links. Although there is no detailed information on the three-dimensional structure of amylopectin, a series of models have been proposed that suggest that the native crystalline amylose that is deposited in the granules of the cereals (A form) and the tubers (B form) consists of a double helix with intertwined, parallel chains (*1*). On the basis of x-ray fiber diagrams and model building, this double helix was originally proposed to be right-handed (*2*). However, a recent study that combined electron diffraction on microcrystals and single crystal data on methyl-α-maltotrioside (*3*) indicated that it should be left-handed (*4*). In addition, various left-handed single-stranded helices are formed by chemical derivatives of amylose and by complexation of amylose with ionic or molecular guest molecules into the central cavity of the helix (Table 1).

Because these previous structural studies relied mainly on the combination of x-ray fiber diffraction and model building and thus are somewhat controversial (*2–6*), it is necessary to obtain more detailed data from single crystals of oligomers larger than the di- (*7, 8*) and trisaccharides (*3*) that have not been available. In only one case, the complex of maltoheptaose with the enzyme phosphorylase a, was the structure of a larger amylose fragment elucidated (*9*) from single crystal data, albeit at medium resolution (2.5 Å). We report the detailed crystal structure of a longer oligomer of amylose, a maltohexaose that is blocked at the reducing end with a *p*-nitrophenyl group and cocrystallized with barium triiodide. Our study provides information on the structure of a left-handed antiparallel double helix of amylose complexed with polyiodide.

We were unable to crystallize maltooligomers in a form suitable for x-ray analysis [see also (*10*)], and resorted to derivatives in which the reducing end was blocked by a *p*-nitrophenyl group in the α-position. Because α-cyclodextrin polyiodide complexes crystallize in a variety of space groups depending on the cation (*11*), we mixed aqueous solutions of the commercially available (Boehringer Mannheim) maltooligomer derivatives with a series of metal iodides and iodine. Addition of BaI_2 and iodine to *p*-nitrophenyl-α-maltohexaose immediately yielded a fine, brown powder. Brown plates from this material suitable for single crystal

Table 1. A selection of parameters for helical structures formed by amylose and its derivatives (*1, 9*).

Structure of amylose or derivative	Helix					
	Type*	Handedness	Pitch height (Å)	Number of residues per turn	Rise per residue (Å)	Helix diameter‡ (Å)
A	DP	Right†	21.04	6	3.51	10.66
B	DP	Right†	20.80	6	3.47	10.68
V_h	S	Left	8.05	6	1.34	13.70
V_h-iodine	S	Left	8.17	6	1.36	13.54
KOH	S	Left	22.41	6	3.74	7.58
KBr	S	Left	16.52	4	4.13	7.21
Amylose triacetate	S	Left	52.53	14	3.75	10.87
Trimethyl amylose	S	Left	15.64	4	3.91	9.66
Heptaamylose in complex with phosphorylase a	S	Left	15.8	6.6	2.3	
Idealized helix (this work)	DAP	Left	18.6	8.0	2.33	14.8

*Abbreviations: S, single; DP, double, parallel; DAP, double, antiparallel. †In a recent electron diffraction study (*4*), a left-handed double helix was proposed. ‡Taken as the interchain spacings in (*1*) for amylose helices and as van der Waals diameters for the double helix derived from *p*-nitrophenyl-α-maltohexaose (this work).

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