cose (pH 7.25)]. The mixture was centrifuged at 10,000*g* for 10 min, and the supernatant was diluted 1:10 with Tyrode's solution. Oligonucleotides were added to a final concentration of 80 μM. Embryos were incubated with the following antisense oligonucleotides: dHAND (1), CCCGTTCTGGTCGTCCTTG; eHAND (1), CTCCGTTCTCCTCCTCTCC; dHAND (2), ACTCGGGGCTGTAGGAC; or eHAND (2), TCCTCCCGAACCGAGCTC. Random oligonucleotides were also synthesized with a base composition similar to that of the antisense oligonucleotides.

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37. We thank M. Chase for assistance with DNA sequencing, K. Tucker for editorial assistance, and A. Tizenor for assistance with graphics. Supported by grants from NIH, the Muscular Dystrophy Association, and the Robert A. Welch Foundation to E.N.O. D.S. was supported by a fellowship from the NIH Pediatric Scientist Development Program.

28 June 1995; accepted 28 September 1995

The ARF1 GTPase-Activating Protein: Zinc Finger Motif and Golgi Complex Localization

Edna Cukierman, Irit Huber, Miriam Rotman, Dan Cassel*

Hydrolysis of guanosine triphosphate (GTP) by the small guanosine triphosphatase (GTPase) adenosine diphosphate ribosylation factor-1 (ARF1) depends on a GTPase-activating protein (GAP). A complementary DNA encoding the ARF1 GAP was cloned from rat liver and predicts a protein with a zinc finger motif near the amino terminus. The GAP function required an intact zinc finger and additional amino-terminal residues. The ARF1 GAP was localized to the Golgi complex and was redistributed into a cytosolic pattern when cells were treated with brefeldin A, a drug that prevents ARF1-dependent association of coat proteins with the Golgi. Thus, the GAP is likely to be recruited to the Golgi by an ARF1-dependent mechanism.

The budding of transport vesicles from the Golgi compartment requires the association of cytoplasmic coat proteins with the organelle membrane. The small GTP-binding protein ARF1 acts as a key regulator of the interactions of nonclathrin coat protein (coatomer) with Golgi stacks (*1*) and of clathrin adaptor particles with the trans-Golgi network (*2*). Like other GTP-binding proteins, ARF1 exerts its regulatory effect by virtue of its GTPase cycle (*3*). In its GTP-bound form, ARF1 triggers the association of coat protein with the Golgi membrane. The subsequent hydrolysis of ARF1-bound GTP is required for the dissociation of coat protein from Golgi membranes and vesicles (*4*). The ARF1 protein also functions as a regulator of the enzyme phospholipase D (*5*), and a possible relation between this role of ARF1 and its function in membrane traffic has been proposed (*6*). The fact that pure ARF1 is unable to hydrolyze GTP (*7*) suggests the existence of an ARF1-directed GAP. Because GTP hydrolysis on ARF1 is required for coat protein dissociation, an ARF1 GAP is likely to function in the uncoating of Golgi-derived vesicles that must take place before their fusion with the target membrane.

We recently purified a 49-kD ARF1 GAP from rat liver cytosol (*8*). Polymerase chain reaction (PCR) amplification of complementary DNA (cDNA) with degenerate primers based on amino acid sequences of this protein generated a 0.5-kb fragment. Screening of a rat liver cDNA library with this fragment as a probe yielded several positive clones. Alignment of the sequences of two overlapping clones (Z6 and G11) revealed an entire open reading frame flanked by 5′ and 3′ untranslated regions (Fig. 1A). The coding sequence predicts a protein of 415 amino acids (45,448 daltons) that includes all peptides that we have sequenced from the tissue-purified protein. The initiating methionine conforms with the Kozak rules for the initiation of translation (*9*) and is preceded 267 nucleotides upstream by an in-frame stop codon.

Department of Biology, Technion–Israel Institute of Technology, Haifa 32000, Israel.

*To whom correspondence should be addressed. E-mail: bir06dc@technion.technion.ac.il

Analysis of the primary structure of the GAP revealed a hydrophilic protein with multiple potential phosphorylation sites of protein kinase C. Although the ARF1 GAP does not show similarity to other GAPs, it shows a high degree of similarity to *Saccharomyces cerevisiae* proteins (*10*) designated Gcs1p, Glo3p, and Sps18p (48, 46, and 33% identity, and 70, 70, and 60% similarity, including evolutionarily conserved substitutions, respectively). An even higher similarity exists between the NH_2-terminal parts of the proteins (Fig. 1B). A common feature of the GAP and the yeast proteins is the presence near their NH_2-termini of a conserved $CXXCX_{16}CXXC$ motif (where X is any amino acid), which apparently represents a zinc finger structure (*10*). Additional proteins that contain some of the conserved sequences shown in Fig. 1B, including the zinc finger domain, are presented in databank entries from humans, nematodes, and plants.

In addition to clone Z6, which appears to encode the tissue-purified protein, we isolated from the rat liver library two clones that are likely to represent alternative splice variants (Fig. 2A). One variant (W15) had a deletion of 110 base pairs (bp) near the 5′ end of the coding region, including the putative zinc finger domain. Although the initiation codon is not removed by the deletion, this codon cannot be used for translation in clone W15 because of a frame shift that generates an early stop codon. However, the W15 variant may be translated from a second in-frame methionine.

A second variant (Z5) contained a 0.6-kb insert within the codon for amino acid 278 (Fig. 2A). Only five insert-derived amino acids are added before a new stop codon is encountered, predicting a truncated protein of 31 kD. The presence of the Z5 variant in rat liver cDNA preparations was demonstrated by PCR amplification with a set of primers, each derived from the Z5 insert and from flanking sequences (*11*).

PCR amplification of genomic DNA with primers flanking the 3′ and 5′ junctions of the deletion found in the W15 clone revealed the presence of introns on both sides of the deletion (Fig. 2B). Thus, clone W15 was generated by alternative splicing and the zinc finger domain of the ARF1 GAP is encoded by a distinct exon. In addition to four cysteines, two conserved histidines that are encoded by this exon are also likely to participate in the formation of the zinc finger.

Coupled in vitro transcription and translation of the Z6 clone in a reticulocyte lysate (Fig. 3A, left panel) resulted in two ^{35}S-labeled bands. The upper band of 49 kD comigrated with the tissue-purified GAP (*11*), whereas the lower band of 43 kD appears to represent a product of initiation

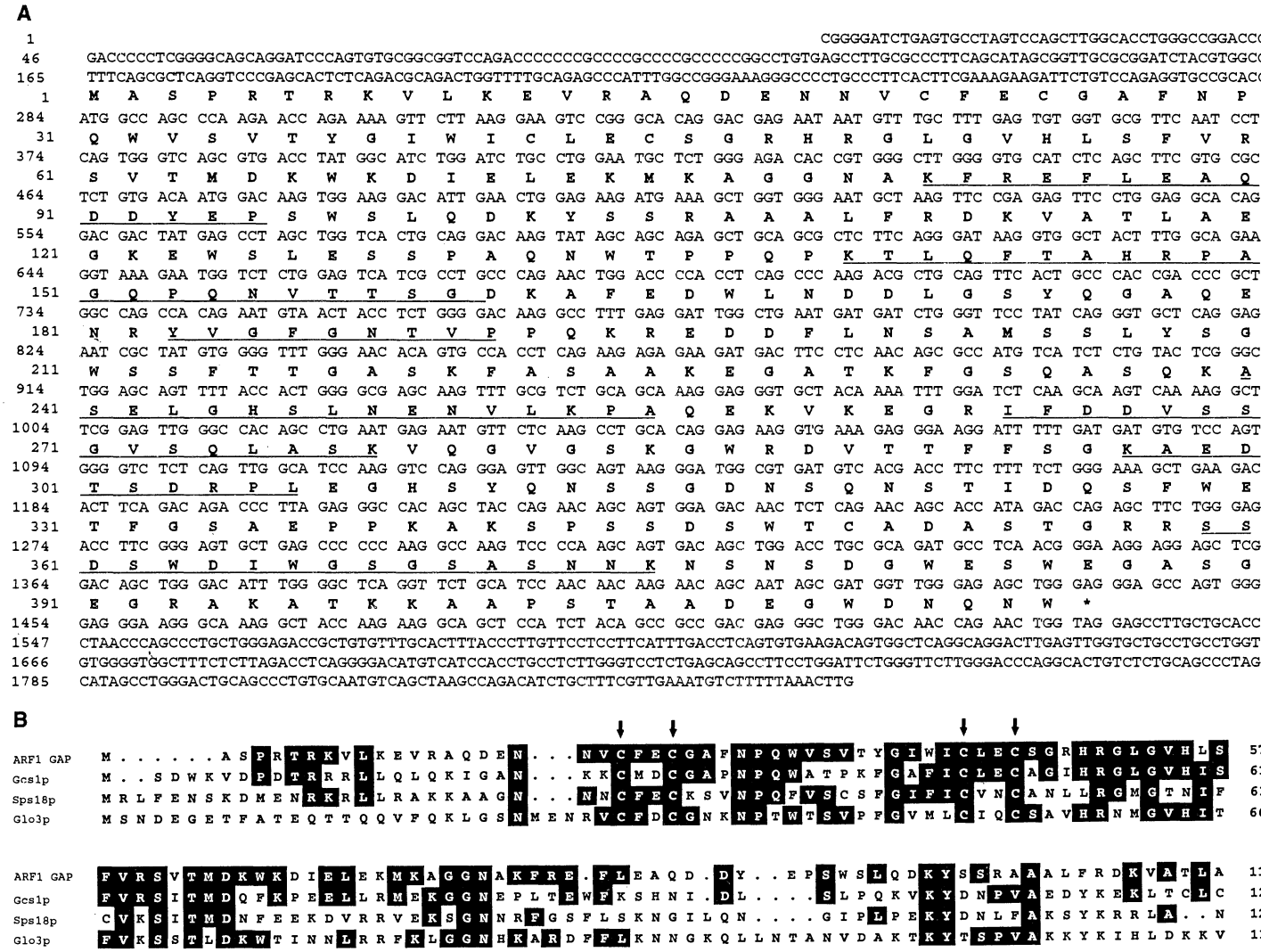

Fig. 1. (**A**) Nucleotide and deduced amino acid sequence of ARF1 GAP cDNA (*19*). Peptide sequences derived from tissue-purified ARF1 GAP are underlined; asterisks mark a stop codon. Nucleotide and amino acid numbers are shown on the left. The sequence has been deposited in SwissProt (accession number U35776). (**B**) Comparison of NH_2-terminal sequences of the ARF1 GAP and *S. cerevisiae* proteins. Sequences were aligned with the Blast program; only identities are presented. The position of the four conserved cysteines is marked by arrows. Residue numbers are shown on the right. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

from the second in-frame ATG (Met^{64}). Translation of clone W15 generated only the 43-kD band, as expected from the frame shift in Met^{1}. The translation product of clone Z6, but not that of the control vector, stimulated GTP hydrolysis on ARF1, demonstrating that we have indeed cloned an ARF1 GAP (Fig. 3A, center panel, lanes 1 and 3). On the other hand, the translation product of clone W15 was devoid of GAP activity (Fig. 3A, center panel, lane 2). In vitro translation of clone Z5 was inefficient; however, a protein with the expected size of 32 kD was expressed in *Escherichia coli* (*11*) and was found to possess GAP activity (Fig. 3A, right panel).

The results obtained with the variant clones suggested that the "catalytic" domain of the GAP resides within the NH_2-terminal part of the protein. To localize the GAP domain further, we carried out systematic truncations (Fig. 3B). COOH-terminal truncations showed that GAP activity is essentially retained in a polypeptide encoded by the first 146 amino acids. A polypeptide containing the first 121 residues still retained GAP activity but its apparent affinity was significantly reduced, whereas polypeptides with 99 residues or fewer were inactive. Comparison of a series of proteins that terminate in amino acid 257 (*12*) showed that the first 16 amino acids were not essential for activity, whereas a protein whose translation is initiated at position 43 was devoid of GAP activity. These results suggested a possible role of the zinc finger, whose four cysteines are located between residues 22 and 45. Such a role was further supported by the finding that substitutions of Cys^{22} or Cys^{25} with alanine, which are both expected to prevent the formation of the zinc finger (*10*), resulted in a complete loss of GAP activity (Fig. 3B).

Antibodies raised against recombinant GAP were used to determine the tissue and subcellular distribution of the protein. Protein immunoblot analysis revealed a major 49-kD protein in all rat tissues examined, being most abundant in brain, then in liver. In brain, the antigen appeared in a cytosolic as well as a membrane-bound pool, from which it could be released by treatment with 0.8 M NaCl (*11*). Double-label immunofluorescence experiments in NRK cells (Fig. 4) showed that the GAP was localized at the Golgi complex and its distribution closely resembled that of the coatomer component β-COP. Treatment of cells with the drug brefeldin A (BFA) is known to cause the rapid release of coat proteins from

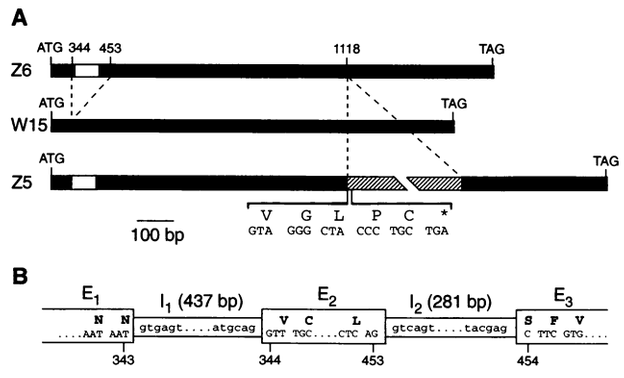

Fig. 2. (**A**) Schematic presentation of variant clones. In comparison with clone Z6, clone W15 has a deletion that encompasses nucleotides 344 through 453, whereas clone Z5 has a 0.6-kb insert (striped box) at position 1118 (numbering as in Fig. 1A). An open box denotes the domain defined by the four zinc finger cysteines (encoded by bases 347 to 418). Nucleotide and deduced amino acid sequences of the Z5 insert between the insert junction and the nearest stop codon are shown. (**B**) Exon-intron structure around the deletion found in clone W15 (*20*). Exons (E) and introns (I) are schematically presented, and nucleotides flanking the junctions are shown (intron sequences in lowercase). Amino acids represent contiguous translation from the three exons. Exon E_2 is identical to the deletion found in clone W15.

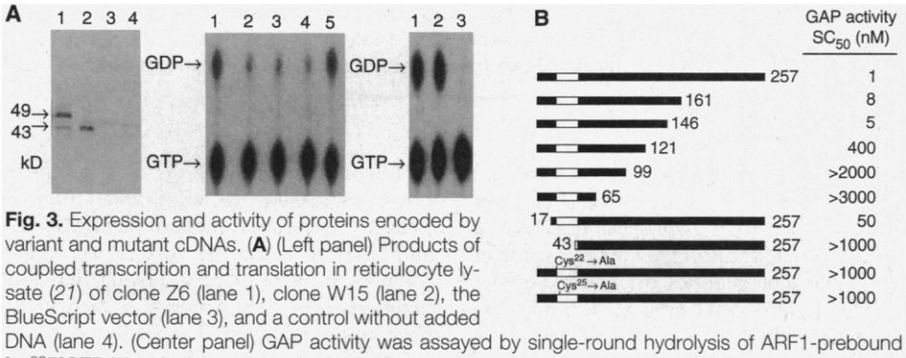

Fig. 3. Expression and activity of proteins encoded by variant and mutant cDNAs. (**A**) (Left panel) Products of coupled transcription and translation in reticulocyte lysate (*21*) of clone Z6 (lane 1), clone W15 (lane 2), the BlueScript vector (lane 3), and a control without added DNA (lane 4). (Center panel) GAP activity was assayed by single-round hydrolysis of ARF1-prebound [α-^{32}P]GTP (*8*) with 1-μl portions from each lysate. Lanes 1 to 4 are as in the left panel; lane 5, 10 ng of tissue-purified GAP. (Right panel) GAP activity in 10 ng of inclusion body extract from bacterially expressed (*22*) clone Z5 (lane 1), 10 ng of tissue-purified GAP (lane 2) (*8*), and the buffer control (lane 3). Center and right panels represent results from different experiments, in which the efficiency of preloading of ARF1 with [α-^{32}P]GTP was 20 and 40%, respectively. (**B**) Polypeptides encoded by a series of truncated cDNAs (*22*) are schematically presented. In the last two polypeptides, point mutations were introduced that converted Cys^{22} or Cys^{25} into an alanine. Amino acids are numbered as in Fig. 1A. Open boxes designate the four zinc finger cysteines (amino acids 22 to 45). GAP activity of the mutant proteins is expressed as the concentration (SC_{50}) that was required for half-maximal stimulation of GTP hydrolysis on ARF1.

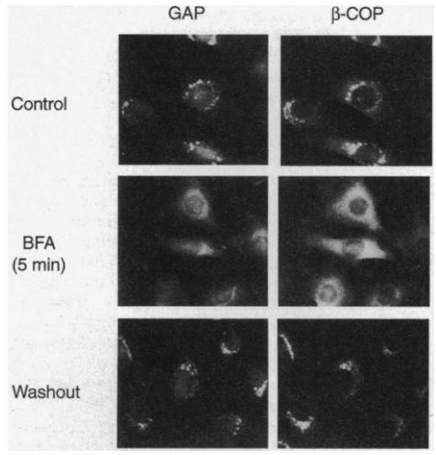

Fig. 4. Golgi association of ARF1 GAP and its disruption in BFA-treated cells. NRK cells were treated with 7 μM BFA for 5 min at 37°C. In the washout experiment, cells were treated with BFA as above, then allowed to recover for 30 min in the absence of the drug. Cells were fixed with 2% formaldehyde, treated with cold methanol for 1 min, rehydrated in phosphate-buffered saline, and permeabilized with 0.2% saponin. Cells were stained with affinity-purified GAP antibodies (*23*) or with β-COP monoclonal antibody M3A5, followed by fluorescein isothiocyanate–conjugated and rhodamine-conjugated secondary antibodies, respectively. Magnification, ×330.

the Golgi (*3*). Treatment with BFA for 5 min caused the redistribution of the GAP as well as β-COP into an apparently cytosolic pattern (Fig. 4), whereas the Golgi marker mannosidase II remained essentially Golgi-localized (*11*). Upon washout of the drug followed by a 30-min recovery period, both GAP and β-COP reappeared in a Golgi-like pattern.

Thus, the ARF1 GAP represents a new type of GTPase activating protein, with a distinct zinc finger structure near the NH_2-terminus. Determinants that are segregated within the NH_2-terminal one-third of the protein, including the zinc finger domain, are required for GAP activity. The catalytic activity of previously identified GTPase activating proteins is also segregated in distinct domains, usually near the COOH-terminus (*13*). The catalytic domain of the ARF1 GAP shows similarity to about a dozen proteins in the databank, which suggests that at least some of these proteins may have an ARF GAP function. The closest homolog of the protein that we have cloned, the yeast Gcs1 protein, was recently found to possess GAP activity for both bovine and yeast ARF1 (*14*).

Because the ARF1 GAP is associated with the Golgi complex and because BFA affects its cellular distribution, it is likely that this protein plays a role in membrane traffic. The drug BFA affects the Golgi system by inhibiting guanosine diphosphate (GDP) to GTP exchange on ARF1, thereby preventing the ARF1-dependent recruitment of cytosolic coat proteins (*15*). The cytosolic redistribution of the ARF1 GAP after BFA treatment suggests that the GAP is recruited to the Golgi by an ARF1-dependent mechanism. A mechanistically similar process takes place during the assembly of the coat protein COPII onto the endoplasmic reticulum in *S. cerevisiae* (*16*).

Thus, it appears that recruitment of a GAP is an early event in the vesicle budding process. Because GTP hydrolysis is necessary for coat protein dissociation, it is unclear how recruitment of a GAP does not result in the shedding of the coat before the completion of vesicle budding. If GTP hydrolysis is the rate-limiting factor in coat shedding, it is possible that the GAP is regulated such that it becomes active only after vesicle formation is complete or upon the encounter of the vesicle with the target membrane. The molecular characterization of the ARF1 GAP will contribute to the understanding of mechanisms underlying the cycling of coat protein in the Golgi complex.

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12. Attempts to express in *E. coli* GAP proteins containing the entire COOH-terminal part were unsuccessful. However, a large amount of expression was obtained from all constructs that encoded the first 257 amino acids or less. We therefore studied the effect of variations in the NH_2-terminal region using constructs that ended in amino acid 257. The activity of GAP(1-257) (Fig. 3B) was similar to that of the tissue-purified protein (8). In view of the segregation of the GAP domain in the first 146 residues, the absence of residues 258 to 415 is unlikely to affect the results.
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19. Fully degenerate oligonucleotide primers were prepared on the basis of the amino acid sequences KFREFL (sense orientation) and NENVLKP (antisense orientation). "Touchdown" PCR (*17*) was carried out using rat liver cDNA, and the 0.5-kb product was radiolabeled and used as a probe to screen a rat liver Uni-Zap cDNA library (Stratagene). The Blue-Script vector from positive clones was prepared after in vivo excision, and inserts were sequenced by the dideoxy chain termination method with an Applied Biosystems automated sequencer and "walking" primers.
20. PCR was carried out with rat genomic DNA using the following sense-antisense primer combinations: (i) 288 to 305 and 397 to 418 and (ii) 398 to 419 and 486 to 509 (numbering as in Fig. 1A). The exon-intron structure was determined by comparing the sequences of the products with that found in cDNA clone Z6.
21. Coupled in vitro transcription and translation was carried out with the TNT system (Promega) according to the manufacturer's instructions.
22. Truncated proteins (as well as clone Z5) were expressed in *E. coli* under the control of the T7 promoter (*18*). Different fragments from the coding region of clone Z6 were generated by PCR catalyzed by the Vent DNA polymerase (New England Biolabs) with primers containing sites for Nco I (sense orientation) or Bam HI (antisense orientation). The fragments were cloned into the pKM260 vector. Proteins were expressed in λDE3 lysogens of strain BL21 harboring pLysS by induction for 2.5 hours at 37°C in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside. Recombinant proteins were exclusively localized in inclusion bodies, which were purified by washing with 1% each of sodium cholate and NP-40 and were extracted with 5 M guanidine hydrochloride in 0.1 M phosphate buffer (pH 8). The guanidine extracts were diluted 15-fold with 25 mM MOPS (pH 7.5), which contained 0.1% hydrogenated Triton X-100. Insoluble material was removed by centrifugation, and 2-μl portions of the supernatant and serial dilutions thereof were assayed for protein concentration and GAP activity. Similar results were obtained when inclusion bodies were extracted with the detergent laurylsarcosine in place of guanidine.
23. Antibodies were raised by injecting rabbits with recombinant GAP (residues 1 to 257) in the form of purified inclusion bodies in complete Freund's adjuvant. For booster injections, the inclusion bodies were solubilized at a concentration of 12 mg/ml in 4 M guanidine-HCl, which contained 0.6% hydrogenated Triton X-100, and dialyzed overnight against phosphate-buffered saline. Serum was prepared after four injections in incomplete Freund's adjuvant at 3-week intervals. Antibodies were affinity-purified by adsorption to recombinant protein that had been resolved by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose paper, and by elution with 0.1 M triethylamine (pH 11.4).
24. We thank K. Leuther for providing the pKM260 vector, and D. Ron and G. Neufeld for critical review of the manuscript. Supported by grants from the Israel Academy of Sciences and from the Fund for the Promotion of Research at the Technion.

13 April 1995; accepted 25 October 1995

Gene Transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* Transposable Element

Thanasis G. Loukeris, Ioannis Livadaras, Bruno Arcà, Sophia Zabalou, Charalambos Savakis*

Exogenous functional DNA was introduced into the germline chromosomes of the Mediterranean fruit fly (medfly) *Ceratitis capitata* with a germline transformation system based on the transposable element *Minos* from *Drosophila hydei*. Transformants were identified as phenotypic revertants of a white-eyed mutation carried by the recipient strain. Clusters of transformants were detected among the progeny of 390 individuals screened for germline transformation. Five independent and phenotypically active integration events were identified, in each of which a single copy of the transposon was inserted into a different site of the medfly genome. Molecular analysis indicates that they represent transposase-mediated insertions of the transposon into medfly chromosomes.

The absence of methodology for introduction of exogenous DNA into the genome of insects other than *Drosophila* has been a serious obstacle to progress in the study of the molecular genetics of multiple insect species. In *Drosophila melanogaster* and related species, the *P* element has been used as the basis of a transformation system (*1*). This system does not, however, function in non-*Drosophila* species. Attempts to introduce *P* in *Anopheles* and *Aedes* mosquitoes have yielded only rare germline transformation events that represent random integrations of DNA segments including plasmid sequences, instead of transposase-mediated insertions of the transposable element alone (*2*).

Three transposable elements, unrelated to *P*, have been used for germline transformation of *D. melanogaster*: *hobo* from *D. melanogaster*, *Mariner* from *D. mauritiana*, and *Minos* from *D. hydei* (*3*, *4*). Moreover, mobilization of *hobo* transposons from plasmids injected into preblastoderm embryos has been documented in non-*Drosophila* species (*5*). These results, in combination with evidence that horizontal transmission of mobile elements across insect orders may be widespread (*6*), suggest that transposons other than *P* may be useful for germline transformation of non-*Drosophila* species.

T. G. Loukeris, I. Livadaras, B. Arcà, S. Zabalou, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology–Hellas, Heraklion 71110, Crete, Greece.
C. Savakis, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology–Hellas, Heraklion 71110, Crete, Greece, and Division of Basic Medical Sciences, Medical School, University of Crete, Heraklion, Crete, Greece.
*To whom correspondence should be addressed.

Minos, a member of the *Tc1* family of transposable elements, was isolated from *D. hydei* and is absent from *D. melanogaster* (*7*) and *Ceratitis capitata* (*8*). Like *P*, *hobo*, and *Mariner*, it belongs to a phylogenetically heterogeneous class of mobile elements that have terminal inverted repeats and transpose by means of a DNA intermediate (*9*). The transposase encoded by *Minos* has been expressed in *D. melanogaster* (*10*) and can catalyze precise insertion of a *Minos* transposon into *D. melanogaster* chromosomes in a manner that can be used for germline transformation (*11*).

The Mediterranean fruit fly *C. capitata* (Diptera: Tephritidae) is a widespread agricultural pest of many fruit species. The medfly has been introduced recently into the New World and is spreading rapidly, threatening fruit production in North America (*12*). The sterile insect technique (SIT) has been used successfully for medfly eradication or control. This method relies on the decrease in or collapse of fly populations caused by the release of large numbers of sterile insects (*13*). The ability to produce transgenic medflies is needed to improve the SIT (*14*) and also to advance knowledge about the molecular genetics of this insect pest.

In addition to a vector, a second requirement for transforming the medfly is an effective genetic marker for transformation. Transformation of the medfly with *Minos* and other transposable element vectors by means of a neomycin resistance marker was unsuccessful (*15*). In *D. melanogaster*, dominant eye color genetic markers have been effective in monitoring transformation. The medfly homolog of the *white* gene of *Dro-*