that has been identified in yeast as playing a crucial role in the control of growth (*26*, *32*).

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16. Yeast chromosomes were isolated and separated as described (*12*) to purify YAC DNA from agarose slices by electroelution (*13*). After phenol extraction and partial Hind III restriction, the purified YAC DNA was cloned into the Hind III cloning site of the cosmid pBIC 20, and the DNA was packaged. The plasmid pBIC20 (*15*) is a 28-kb binary vector derived from the plasmid pLAFR3 [B. Staskawicz, D. Dahlbeck, N. Keen, C. Napoli, *J. Bacteriol.* **169**, 5789 (1987)]. After transfection of *E. coli* NM554 (Stratagene), 288 cosmid clones were analyzed by colony hybridization with purified YAC DNA as a probe, which was prehybridized [S. Baxendale, G. P. Bates, M. E. MacDonald, J. F. Gusella, H. Lehrach, *Nucleic Acids Res.* **19**, 6651 (1991)] in the presence of total wild-type yeast DNA (5 mg). Positive cosmids were selected, and the cloned fragments were assembled into a contig on the basis of their Hind III restriction map and cross-hybridization (*13*). Additional clones were identified from an *abi1* genomic library constructed in pBIC20 to fill in regions of poor overlap in the contig (*15*).
17. Cells of *A. tumefaciens* strain C58 pGV3850 [P. Zambryski *et al.*, *EMBO J.* **2**, 2143 (1983)] were electroporated according to the protocol of E. Grill and C. Somerville (unpublished results). Cells were grown to optical density at 600 nm (OD_{600}) = 1.5 in Luria Broth (Difco, Detroit, MI) medium (*13*) at 30°C and harvested by centrifugation (4000*g*, 10 min) at 4°C. The cells were washed three times in half the original volume by their gentle resuspension in ice-cold water and their subjection to pelleting as mentioned above. Finally, 0.4 ml of cell suspension adjusted to OD_{600} = 20 was mixed with 5 μl of DNA solution [0.1 μg of DNA in 10 mM tris-Cl, 1 mM ethylenediaminetetraacetic acid (pH 8.0)], electroporated (Bio-Rad electroporator; settings: 10 kV/cm, 1 kilohm, 25 μF), and immediately diluted with 1 ml of Luria Broth medium. After 3 hours of phenotypic expression at 30°C, the cells were transferred to selective media.
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22. Nucleotide sequences were determined on both strands with the use of the chain termination method and standard strategies (*13*). The genomic sequence was submitted to the European Molecular Biology Laboratory (EMBL), accession number X78886. The sequence of the 2.0-kb cDNA insert from clone λabi1-7 was determined and, in addition, the corresponding nucleotide sequence of the *abi1* mutant gene (including three introns of 70, 92, and 131 bp). The nucleotide sequence of the introns and the difference at the mutation site were verified by the sequencing of a genomic fragment (1.39 kb) of the wild-type *ABI1* allele, which was amplified by PCR [R. K. Saiki *et al.*, *Science* **230**, 1350 (1985)].
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Estrogen Receptor–Associated Proteins: Possible Mediators of Hormone-Induced Transcription

Shlomit Halachmi, Emily Marden, Glover Martin, Heather MacKay, Ciro Abbondanza, Myles Brown*

The estrogen receptor is a transcription factor which, when bound to estradiol, binds DNA and regulates expression of estrogen-responsive genes. A 160-kilodalton estrogen receptor–associated protein, ERAP160, was identified that exhibits estradiol-dependent binding to the receptor. Mutational analysis of the receptor shows that its ability to activate transcription parallels its ability to bind ERAP160. Antiestrogens are unable to promote ERAP160 binding and can block the estrogen-dependent interaction of the receptor and ERAP160 in a dose-dependent manner. This evidence suggests that ERAP160 may mediate estradiol-dependent transcriptional activation by the estrogen receptor. Furthermore, the ability of antiestrogens to block estrogen receptor–ERAP160 complex formation could account for their therapeutic effects in breast cancer.

The estrogen receptor (ER) is a member of a superfamily of nuclear receptors for small hydrophobic ligands including the steroid hormones, thyroid hormone, vitamin D, and the retinoids (*1*). As a class, these receptors are transcription factors that are regulated allosterically by ligand binding. Extracellular estradiol freely diffuses across the cell membrane and binds ER, leading to ER dimerization and to tight binding of ER to its specific DNA target, the estrogen responsive element (ERE). After ERE binding, the liganded ER activates transcription by as yet unknown mechanisms. Although the targets of ER activation in breast cancer are unknown, they are believed to be critical for cellular proliferation because a large

percentage of tumors that express ER depend on estrogen for growth.

Amino acid sequence comparison of the ER with other nuclear receptors has shown that the receptor is composed of six regions (Fig. 1A, regions A through F) (*2*). The central C region encodes a zinc-nucleated DNA binding domain (DBD) (*3*) and is flanked on either side by two independent nonacidic activation domains (AF-1 and AF-2) (*2*). The AF-1 domain in the NH_2-terminal region can activate transcription constitutively when bound to DNA through the DBD of ER or through a heterologous DBD (*4–10*). In contrast, the AF-2 domain in the E region of ER overlaps the hormone binding domain (HBD) and activates transcription only when it is bound by estradiol or another estrogen agonist (*4, 5, 7, 9, 11*). The individual transactivating abilities of AF-1 and AF-2 depend on the promoter and cell context (*7, 12*), suggesting that the different activities of AF-1 and AF-2 may be mediated by adaptors or coactivators that are specific to one or the other of the domains (*13*). Tamoxifen acts as an agonist of ER in some tissues, such as uterus, and as an antagonist in other tissues, notably breast. Tamoxifen's agonistic character has been attributed to its ability to stabilize DNA binding and to activate AF-1; its antagonist activity is due to competitive inhibition of the estradiol-dependent activation of AF-2 (*7, 12*). Various studies have shown that estradiol and tamoxifen induce different conformations of the HBD/AF-2 region of ER (*8, 14–16*). This may account for the differential ability of estrogens and antiestrogens to activate the AF-2 transactivation function.

We hypothesized that the ligand-induced alterations in the conformation of the HBD/AF-2 domain might be sensed by cellular factors that could mediate the activation function of AF-2. This was tested in a protein-protein interaction assay. We constructed a bacterial fusion protein expression vector in which the HBD/AF-2 region of the wild-type human ER complementary DNA (cDNA) was cloned downstream of the glutathione-S-transferase (GST) gene (*17, 18*) (Fig. 1A). The resulting GST-HBD fusion proteins were expressed in *Escherichia coli* and purified by adsorption onto glutathione Sepharose (*19*). The affinity of the GST-HBD fusion proteins for estradiol [dissociation constant (K_d) = 0.1 to 0.3 nM] was comparable with previously reported values measured for wild-type MCF-7 ER expressed in vivo (*9, 20*). The GST-HBD protein was immobilized on glutathione Sepharose for use as an affinity reagent. Whole cell extracts of metabolically ^{35}S-labeled MCF-7 cells were incubated with the HBD column in either the absence or presence of 1 μM 17β-estradiol (*21*). Specifically bound proteins were eluted and resolved by electrophoresis.

An ER-associated protein of ~160 kD, ERAP160, bound specifically to the HBD/AF-2 region of the ER fusion protein in the presence of estradiol, but not in its absence, and did not bind control GST under any conditions (Fig. 1B). Binding to GST-HBD was observed at estradiol concentrations as low as 1 nM. ERAP160 was detectable in a variety of cell lines including the ER-negative breast line MDA-MB-231 and non-breast cell lines. A protein of 140 kD was also found to bind GST-HBD, and the abundance of this protein varied widely among different cell lines. Pulse-chase experiments and partial proteolytic digestion revealed that the 140-kD protein is not a

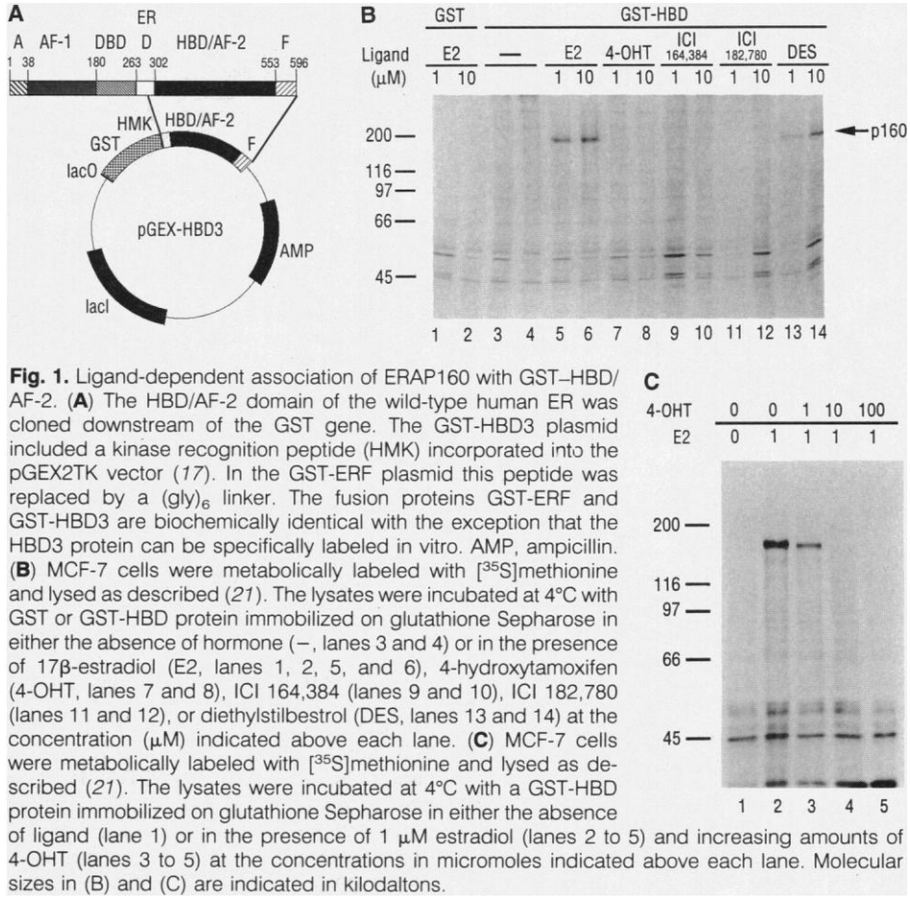

Fig. 1. Ligand-dependent association of ERAP160 with GST–HBD/AF-2. (**A**) The HBD/AF-2 domain of the wild-type human ER was cloned downstream of the GST gene. The GST-HBD3 plasmid included a kinase recognition peptide (HMK) incorporated into the pGEX2TK vector (*17*). In the GST-ERF plasmid this peptide was replaced by a $(gly)_6$ linker. The fusion proteins GST-ERF and GST-HBD3 are biochemically identical with the exception that the HBD3 protein can be specifically labeled in vitro. AMP, ampicillin. (**B**) MCF-7 cells were metabolically labeled with [^{35}S]methionine and lysed as described (*21*). The lysates were incubated at 4°C with GST or GST-HBD protein immobilized on glutathione Sepharose in either the absence of hormone (−, lanes 3 and 4) or in the presence of 17β-estradiol (E2, lanes 1, 2, 5, and 6), 4-hydroxytamoxifen (4-OHT, lanes 7 and 8), ICI 164,384 (lanes 9 and 10), ICI 182,780 (lanes 11 and 12), or diethylstilbestrol (DES, lanes 13 and 14) at the concentration (μM) indicated above each lane. (**C**) MCF-7 cells were metabolically labeled with [^{35}S]methionine and lysed as described (*21*). The lysates were incubated at 4°C with a GST-HBD protein immobilized on glutathione Sepharose in either the absence of ligand (lane 1) or in the presence of 1 μM estradiol (lanes 2 to 5) and increasing amounts of 4-OHT (lanes 3 to 5) at the concentrations in micromoles indicated above each lane. Molecular sizes in (B) and (C) are indicated in kilodaltons.

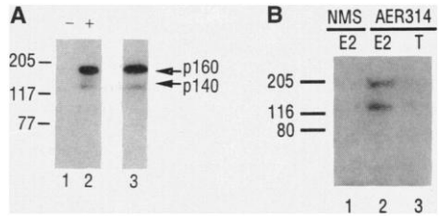

Fig. 2. Direct interaction of ER with ERAP160 and ERAP140. (**A**) Unlabeled MCF-7 cells were lysed as described (*21*) and incubated at 4°C with GST-HBD protein immobilized on glutathione Sepharose in either the absence (−, lane 1) or presence (+, lane 2) of 1 μM estradiol. Specifically bound proteins were eluted in sample buffer, resolved by electrophoresis, and transferred to nitrocellulose by electroblotting. Unfractionated HeLa nuclear extract (*30*) was also tested (lane 3). The GST-HBD3 protein was ^{32}P-labeled in vitro and used as a probe (*25*). The probe was incubated with 1 μM estradiol throughout the procedure. (**B**) Lysates of MCF-7 cells grown for 3 hours in the presence of 1 μM estradiol (E2) (lanes 1 and 2) or 1 μM 4-OHT (T) (lane 3) were immunoprecipitated with normal mouse serum (NMS) or monoclonal antibody to ER (AER314) (*31*) and analyzed with the HBD3 probe. Molecular sizes are indicated in kilodaltons.

S. Halachmi, Program in Biological and Biomedical Sciences, Harvard Medical School, and Division of Neoplastic Disease Mechanisms, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA.
E. Marden, G. Martin, H. MacKay, Division of Neoplastic Disease Mechanisms, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA.
C. Abbondanza, Instituto di Patologia Generale e Oncologia, Facolta di Medicina e Chirurgia, Via S. Andrea delle Dame 2, I-80138 Naples, Italy.
M. Brown, Department of Medicine, Harvard Medical School, and Division of Neoplastic Disease Mechanisms, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA.
*To whom correspondence should be addressed.

degradative product of ERAP160 (*22*), suggesting that there may be multiple ERAPs. Thus, cellular factors can bind ER in a ligand-dependent manner, presumably by recognition of a particular conformational state.

We next tested a variety of estrogenic and antiestrogenic compounds for their ability to affect the formation of the ER-ERAP160 complex. Both 17β-estradiol, the natural ligand of ER, and the synthetic estrogen diethylstilbestrol (DES) were able to promote this association (Fig. 1B). In contrast, the pure antiestrogens ICI 164,384 and ICI 182,780 were unable to promote ER-ERAP160 complex formation (Fig. 1B). These compounds have been proposed to block receptor activation by preventing its dimerization (*23*) or by promoting its degradation (*24*). The tamoxifen derivative 4-hydroxytamoxifen (4-OHT) also failed to induce the association of ERAP160 and the HBD/AF-2 region of ER (Fig. 1B). Furthermore, 4-OHT was able to interfere with the estradiol-dependent interaction of the HBD/AF-2 domain and ERAP160 in a dose-dependent manner (Fig. 1C). Because tamoxifen induces ER dimerization (*15*), receptor dimerization is not sufficient for ERAP160 binding. Together, these results suggest that the antiestrogenic activity of tamoxifen is due at least in part to its ability to block the association of ER with ERAP160.

To test whether the interaction between the HBD/AF-2 domain and ERAP160 is direct, we labeled the GST-HBD3 protein with ^{32}P in vitro and used it as a probe in far Western blot analysis (*25*). The GST-HBD3 probe failed to detect any protein when the incubation was carried out in the absence of estradiol (*22*). When the incubation was carried out in the presence of estradiol, the GST-HBD3 probe recognized a prominent protein of 160 kD as well as a protein of 140 kD in HeLa cell nuclear extract (Fig. 2A, lane 3), demonstrating that the interaction between the HBD/AF-2 domain and these proteins was direct. Furthermore, the detection of these proteins in HeLa nuclear extracts reveals that these ERAPs are present in the nucleus and confirms that their expression is not limited to ER-expressing cell lines or to breast-derived cell lines. In parallel experiments, unlabeled MCF-7 whole-cell extracts were first bound to GST-HBD in either the absence or presence of 1 μM estradiol; specifically bound proteins were eluted, resolved by electrophoresis, and visualized with the ^{32}P-labeled GST-HBD3 probe. The probe detected a protein of 160 kD and a protein of 140 kD that comigrated with the proteins seen in ^{35}S-labeled extracts. Both proteins were present only in samples eluted from the estradiol-containing GST-HBD affinity column (Fig. 2A, lanes 1 and 2). The HBD probe also recognized ERAP160 and ERAP140 which were coimmunoprecipitated with ER from MCF-7 cells treated with estradiol but not from cells treated with tamoxifen (Fig. 2B), suggesting that these proteins can associate with the endogenous ER in vivo in a ligand-dependent manner.

To define further the requirements for ERAP160 binding to HBD/AF-2, we produced several mutant ER HBDs as GST fusion proteins. A glycine to valine substitution at amino acid 400, which is temperature-sensitive for hormone binding (*20*), exhibited reduced binding to ERAP160 at the nonpermissive temperature (Fig. 3A, lanes 5 and 6). In contrast, cysteine to alanine mutations at either position 447 or 530 in the HBD, which have only minor effects on ER function in vivo (*26*), had little or no effect on ERAP160 binding (Fig. 3A, lanes 7 to 10). Thus, the ability of ER to bind ERAP160 correlates with its ability to bind estradiol and to transactivate.

An important activation domain, from amino acid 535 to 550 of the human ER, is conserved among many members of the nuclear receptor superfamily. In ER this domain is necessary for activation but not for hormone binding (*9*, *27*). A COOH-terminal truncation mutant (Δ534) lacking this domain has wild-type affinity for estradiol but is defective for activation in vivo (*9*). This finding reveals that HBD and AF-2 are overlapping yet distinct functional domains. We produced a GST–HBD/AF-2 fusion protein with the Δ534 truncation and confirmed that it binds estradiol with wild-type affinity (K_d < 1 nM). This mutant failed to bind ERAP160 (Fig. 3B). Thus, hormone binding is necessary but not sufficient to induce ERAP160 binding. Furthermore, the Δ534 truncation mutant retains an intact dimerization domain, again demonstrating that dimerization is not sufficient for ERAP binding. We have recently found that ERAP160 binds to two other members of the nuclear receptor family that contain this activation domain, RARβ and RXRα (*28*). Potential competition for ERAP160 may explain the cross-inhibition between nuclear receptors and the augmentation by retinoic acid of the response of breast tumors to tamoxifen (*29*). ERAP160 does not bind to two unrelated transcriptional regulators, Rb and Pit-1 (*22*), supporting the possibility that ERAP160 may be an adaptor specific to nuclear receptors.

It appears that ERAP160 recognizes only an activated conformation of ER. Furthermore, ERAP160 binding correlates with the ability of ER to activate transcription in vivo. These findings suggest that ERAP160 may be a mediator of the estrogen-dependent AF-2 transactivation by ER. Our results support a model in which tamoxifen and the pure antiestrogens do not activate through AF-2 because the conformation they induce does not allow binding of ERAP160. Furthermore, tamoxifen's competitive inhibition of estradiol-induced ERAP160 binding suggests that its growth-inhibitory effects in breast cancer may be explained at least in part by its ability to disrupt a complex between ER, ERAP160, and other factors necessary for transactivation.

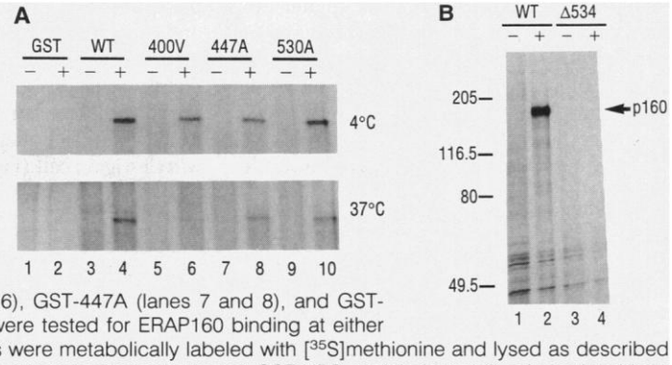

Fig. 3. Disruption of ERAP160 binding by mutations in the hormone binding domain. (**A**) Point mutations were introduced into the GST–HBD/AF-2 domain (*18*). Unmodified GST protein (lanes 1 and 2), wild-type GST-HBD (lanes 3 and 4), and mutant proteins GST-400V (lanes 5 and 6), GST-447A (lanes 7 and 8), and GST-530A (lanes 9 and 10) were tested for ERAP160 binding at either 4°C or 37°C. MCF-7 cells were metabolically labeled with [^{35}S]methionine and lysed as described (*21*). The lysates were incubated with the indicated GST-HBD protein immobilized on glutathione Sepharose in either the absence of ligand (−) or in the presence of 1 μM estradiol (+). (**B**) The wild-type human HBD/AF-2 domain was truncated at residue 534 by site-directed mutagenesis of the GST-ERF plasmid to yield GST-Δ534 (*18*). MCF-7 cells were metabolically labeled with [^{35}S]methionine and lysed as described (*21*). The lysates were incubated at 4°C with wild-type GST-ERF (lanes 1 and 2) or Δ534 (lanes 3 and 4) protein immobilized on glutathione Sepharose in either the absence of hormone (−) or in the presence of 10 μM estradiol (+). Molecular sizes are indicated in kilodaltons.

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31. MCF-7 cells were incubated with 1 μM estradiol or 4-OHT, lysed as described (*21*), and immunoprecipitated with monoclonal antibody AER314 to the NH_2-terminus of human ER as described in C. Abbondanza *et al.*, *Steroids* **58**, 4 (1993).
32. We thank M. Lazar, J. Lees, D. Livingston, R. Meyers, B. Rutledge, and J. Settleman for helpful discussions and critical reading of the manuscript, W. Kaelin for the gift of pGEX2TK and for invaluable advice, A. Wakeling for ICI 182,780, ICI 164,384, and 4-hydroxytamoxifen, and B. Katzenellenbogen for ER mutants C447A and C530A. Supported by an American Cancer Society grant BE-154 to M.B. and by a Claudia Adams Barr Research Award to M.B.

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Tetrad Analysis Possible in *Arabidopsis* with Mutation of the *QUARTET* (*QRT*) Genes

Daphne Preuss,* Seung Y. Rhee, Ronald W. Davis

Two *Arabidopsis thaliana* genes, *QRT1* and *QRT2*, are required for pollen separation during normal development. In *qrt* mutants, the outer walls of the four meiotic products of the pollen mother cell are fused, and pollen grains are released in tetrads. Pollen is viable and fertile, and the cytoplasmic pollen contents are discrete. Pollination with a single tetrad usually yields four seeds, and genetic analysis confirmed that marker loci segregate in a 2:2 ratio within these tetrads. These mutations allow tetrad analysis to be performed in *Arabidopsis* and define steps in pollen cell wall development.

In most higher fungi and in many algae and bryophytes, all four products of each meiosis are viable and are either encased in a single structure or remain adherent so that they can be recovered as a group (a tetrad). This feature provides the means to analyze allele segregation through individual meioses and is tremendously advantageous for genetic studies. Only with tetrad analysis is it possible to map centromeres genetically with high precision, to measure chromosomal gene conversion frequencies, and to detect every genetic exchange between chromatids (*1*). In addition, tetrad analysis expedites the construction of multiply marked strains, simplifies the creation of genetic maps, provides a simple method for distinguishing between nuclear and cytoplasmic forms of inheritance, and allows rapid identification of strains with lethal mutations or chromosomal rearrangements, such as reciprocal translocations, inversions, deletions, or duplications (*2*). Tetrad analysis is not possible in most higher eukaryotes; only one of the four products of female meioses typically survives, and although all four products of male meioses usually reach maturity, they are rarely packaged together. This report describes two *A. thaliana* mutations (*qrt1* and *qrt2*) that result in the release of viable pollen tetrads, permitting tetrad analysis to be performed in this flowering plant.

A visual screen of mutagenized plants of the Landsberg ecotype identified two mutations that dramatically affect pollen development (*3*). Unlike wild-type plants, which release single pollen grains, these *quartet* (*qrt*) mutants produce pollen that is arranged in a tetrahedron of four grains (Fig. 1, A and B). Examination of the fusion junction (Fig. 1C) indicated that portions of the outermost layer (exine) of the pollen wall are aberrant, extending between the pollen grains within each tetrad.

Although the *qrt* mutations result in fusion of the outer pollen wall, the cytoplasmic contents of each pollen grain remain separated. Sections of mature pollen grains showed no evidence of fusion of the cytoplasm or of the inner pollen wall (intine), and mechanical separation of the members of a tetrad typically did not affect pollen viability (*4*). When a single *qrt1* pollen tetrad was placed on the stigma surface such that only one pollen grain was in contact with a stigma cell (Fig. 1, D and E), only the

Table 1. Penetrance of the *qrt* phenotypes. Pollen from open flowers was dispersed on a glass slide, and the number of pollen tetrahedrons (tetrads), clusters of three (triads), two (dyads), or single (monads) pollen grains was determined. Percentages (in parentheses) represent the number of pollen grains in each class per total number of pollen grains.

Genotype	Number of tetrads	Number of triads	Number of dyads	Number of monads
+/+	0 (0)	0 (0)	38 (12.2)	547 (87.8)
qrt1/qrt1	1007 (98.7)	3 (0.22)	13 (0.64)	18 (0.44)
qrt2/qrt2	512 (97.5)	6 (0.86)	6 (0.57)	22 (1.05)
qrt1/+	0 (0)	0 (0)	27 (6.8)	738 (93.2)
qrt2/+	0 (0)	4 (1.3)	52 (11.6)	792 (88.0)

D. Preuss and R. W. Davis, Department of Biochemistry, Stanford University, Stanford, CA 94305–5307, USA.
S. Y. Rhee, Department of Biological Sciences, Stanford University, Stanford, CA 94305–5020, USA.
*To whom correspondence should be addressed.