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Meeting Report

Eighth International Workshop on Ataxia-Telangiectasia (ATW8)[1]

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Abstract

ATW8 was a unique opportunity to review the complex and growing field of ataxia-telangiectasia (A-T) research and to cross-fertilize ideas for new experimental designs. A-T biology now encompasses human and mouse neurology, neurobiology, immunology, radiobiology, cell signalling, cell cycle checkpoints, gametogenesis, and oncogenesis, as well as radiotherapy, cancer epidemiology, premature aging, cytogenetics, and DNA repair mechanisms. By an as yet undetermined mechanism, the ATM protein appears to sense double strand breaks (DSB) during meiosis or mitosis, or breaks consequent to the damage of free radicals which are generated during the metabolism of food. As a protein kinase, ATM then directly phosphorylates p53 and interacts with many other molecules involved in homologous and nonhomologous DSB repair, as well as in cell signalling. Some of these molecule targets include: c-ab1, ATR, chk-1, chk-2, RPA, BRCA1, BRCA2, NFκB/IκBα, β-adaptin, and perhaps ATM itself. Thus, ATM is a "hierarchical kinase," initiating many pathways simultaneously. Parallel sessions or longer meetings will clearly be necessary for future A-T workshops.

Introduction

A-T[3] was first described as a separate disease entity by Boder and Sedgwick (1), although the disorder had been recognized as early as 1926. The syndrome is characterized by neurodegeneration, immunodeficiency, sensitivity to ionizing radiation, defective cell cycle checkpoints, and an increased incidence of cancer, primarily lymphoid tumors (2). With a broad phenotype like this, it is not surprising that the most recent gathering of A-T researchers, ATW8, drew together individuals with a myriad of backgrounds in laboratory-based and clinical science.

An attendance of ~200 scientists from all corners of the world provides evidence for the remarkable increase in interest in A-T since the gene was isolated by positional cloning in 1995 (3, 4). *ATM* is a member of the phosphatidylinositol 3-kinase family of genes capable of sensing double strand breaks in DNA and activating a number of cell cycle checkpoints. *ATM* also plays an important role in meiosis and DNA recombination. Recent data localize variable amounts of the protein to vesicles in the cytoplasm and support a role for this protein in more general intracellular signaling. Parallels were drawn between A-T and NBS, which was originally classified as one of the A-T complementation groups and was shown recently to arise from mutations in a separate gene, NBS1.

Genotype/Phenotype

The workshop first addressed the spectrum of over 300 mutations found in A-T patients.[4] These mutations are distributed across the full-length (150 kb) of the *ATM* gene. Gatti described a rapid optimized-SSCP method to screen all 66 exons of ATM cDNA for mutations, using sequential loading of three gels for testing two individuals. However, it was also suggested that laboratory confirmation of A-T might more readily be achieved by immunoblotting for the presence of ATM protein (absent in 85% of patients) and assays for ATM kinase activity, with defined substrates; in the 15% of patients who have ATM protein (poster by Sara Becker-Catania *et al.*). Phillip Byrd (Birmingham, UK) focused on two mutations (DNA: IVS40+1126A>G/RNA:5762ins137, and 7271T>G) that are associated with milder phenotypes in the United Kingdom population, i.e., slower progression of cerebellar ataxia and intermediate radiosensitivity. In the former mutation, a small amount of ATM protein was detectable by immunoprecipitation, and there was an absence of early onset tumors among patients. In the latter mutation, a normal level of mutant protein was expressed, and there was historical evidence for increased cancer risk. Patients homozygous for the 7271T>G-missense mutation displayed an atypical clinical course for A-T, remaining ambulatory with increased longevity and, in one case, bearing a child. Gatti proposed a model for the phenotypic effects of *ATM* mutations in which truncating mutations, as are found in most A-T patients, would predispose to "classical" A-T with full neurological features, whereas missense mutations, such as 7271T>G, might predispose to malignancy but not to the full neurological aspects of the classical A-T syndrome.

atm$^{-/-}$ Mouse Models

The debate over the importance of the atm$^{-/-}$ mouse as a model for A-T continued. Although it was recognized that the phenotype in atm$^{-/-}$ mice reflected accurately that of the human syndrome in most respects, the most debilitating aspects of the A-T phenotype (*i.e.*, the progressive cerebellar degeneration and the accompanying ataxia) are not seen in the mouse. A marked increase in the number of lysosomes was observed in Purkinje cells from one atm$^{-/-}$ mouse model (Carrolee Barlow, La Jolla, CA). In another knockout model with a targeted mutation in the *atm* gene, abnormal dendritic arborization and lamination was apparent in Purkinje cells (Yansong Gu, Boston, MA). These defects were not observed in the Barlow/Wynshaw-Boris atm$^{-/-}$ mouse. Raya Eilam (Rehovot, Israel) described the very significant loss with time of tyrosine hydroxylase-positive, dopaminergic nigro-striatal neurons (a Parkinson's disease-type lesion) in atm$^{-/-}$ mice. It seems that tyrosine hydroxylase-positive cells are

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[3] The abbreviations used are: A-T, ataxia-telangiectasia; ATM, A-T mutated; NBS, nijmegen breakage syndrome; RPA, replication protein A; ATR, A-T and rad 3- related; T-PLL, T-cell prolymphocytic leukemia; SSCP, single-strand confirmational polymorphism; LOH, loss of heterozygosity.

[4] http://www.vmmc.org/vmrc/atm.htm.

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found normally in atm$^{-/-}$ mice, but disappear between 1 and 4 months of age. Furthermore, the locomotor abnormalities (much less severe than that observed in A-T patients) were corrected by peripheral application of L-DOPA, offering some hope for this approach in a treatment protocol for patients (discussed below). Contrary to an expected widespread sensitivity to radiation in the atm$^{-/-}$ mouse, Peter McKinnon (Memphis, TN) described a pronounced resistance to *in vivo* radiation-induced apoptosis in the central nervous system of these mice, especially in the external granular layer of the cerebellum, and proposed a model in which Atm functions as a developmental survival checkpoint to eliminate damaged neurons. It has been suggested that the neurodegeneration in A-T is due to loss of neuronal cells as a consequence of accumulating damage in DNA. However, it has been unclear why this loss is selective for only some neuronal cell types. Some insight into this mystery was provided by Alevia Oka (Tottori, Japan), who showed that Purkinje cells exhibited marked immunoreactivity for ATM protein during late prenatal and early postnatal periods and that this immunoreactivity was evident primarily in the cytoplasm. Thus, ATM is largely present in the nucleus in proliferating cells, but predominantly in the cytoplasm (presumably in vesicles) in differentiated cells, such as neurons. This would provide an alternate explanation for the premature death of neurons in A-T patients and atm$^{-/-}$ mice. These cells depend heavily on signaling from adjacent cells for survival, suggesting that extranuclear ATM may participate in this process to prevent cell death.

All studies, to date, reveal that the majority of atm$^{-/-}$ mice develop malignant thymic lymphomas between 1 and 4 months of age. When these mice are crossed with p53$^{-/-}$ mice, the onset of lymphomas is earlier. On the other hand, atm$^{-/-}$ p21$^{-/-}$ mice are radiosensitive and severely defective in G_1 arrest, but do not show the increased rate of onset of tumors. A new addition to these crosses was provided by Yang Xu (La Jolla, CA) with an atm$^{-/-}$ RAG-2$^{-/-}$ mouse, suggesting that aberrant V(D)J recombination promotes lymphoid tumorigenesis in atm$^{-/-}$ mice. Of interest also were the observations that atm$^{-/-}$ mice exposed to 4-gray X-rays showed rapid greying of their coats and accelerated death (72 weeks *versus* 99 weeks), but they did not get more cancers than wild-type mice.

Radiosensitivity, Double Strand Break Repair, and Recombination

Hypersensitivity to ionizing radiation and radiomimetic agents is one of the hallmarks of classical A-T. This can be explained by less efficient detection and repair of double strand breaks. Inability to efficiently ligate breaks in DNA may also account for abnormalities in recombination observed in this syndrome. Using a "sleeping beauty-like element" (mobile element), Stephen Meyn (Toronto, Ontario, Canada) reported more transposition-mediated events in A-T cells than in controls, confirming earlier observations of enhanced intrachromosomal recombination in these cells. Double strand breaks in meiotic chromosomes also attract the ATM protein; this was elegantly demonstrated by Terry Ashley (New Haven, CT) when she localized ATM to paired synapses in the synaptonemal complex, colocalizing with RPA in zygotene and pachytene. These results suggest that ATM tracks RPA to sites where interhomologous-DNA interactions occur during meiotic prophase, and together the two molecules play a joint role in meiotic recombination after synapsis. Interaction of ATM and RPA is of further interest because RPA has been shown to be a substrate for ATM kinase and it has been implicated in the inhibition of DNA synthesis. However, defective phosphorylation of RPA in A-T cells seems not to be an explanation for radioresistant DNA synthesis. In a similar study, Peter Moens (Toronto, Canada) could not localize ATM to the synaptonemal complex; instead, ATM seemed to be required at an earlier stage of meiosis (leptonema).

Eric Hendrickson (Providence, RI) described the induction of Karp1 from a bifunctional locus, also coding for Ku86, by ionizing radiation. Induction of this gene is ATM- and p53-dependent and a dominant negative construct enhanced radiosensitivity. A link between the G_2-M checkpoint and double strand break repair was suggested by Eva Lee (San Antonio, TX). This lesion in DNA failed to cause activation of chk1 in either A-T or NBS cells, both of which are defective in repairing double strand breaks.

Cell Cycle Checkpoints

A-T cells are defective in activating checkpoints at G_1-S and G_2-M in response to radiation exposure; they also exhibit radioresistant DNA synthesis. An overview of these cell cycle defects with reference to p53 phosphorylation and the radiation signal transduction pathway leading to G_1 arrest was provided by Lavin. The presence of ATM, predominantly in the nucleus of proliferating cells, is compatible with a role for ATM in DNA damage recognition and cell cycle control. Although ATM rapidly phosphorylates p53 on serine 15 in response to radiation, a second member of the PI3-kinase family, ATR, also phosphorylates p53 on serine 15, and, in addition, it phosphorylates serine 37, but this phosphorylation is delayed in normal cells as compared with that with ATM cells (Robert Abraham, Durham, NC). Overexpression of a kinase-dead form of ATR inhibited the phosphorylation of p53. Karlene Cimprich (Stanford, CA) also used a dominant negative approach with ATR to induce a radiosensitive phenotype in transfected cells. Dominant negative ATR also abrogated the G_2-M checkpoint and sensitized cells to hydroxyurea, implicating ATR in both the S phase and G_2-M checkpoints. Another aspect of G_2-M cell cycle control, involvement of chk1 kinase, was described by both Kum Kum Khanna (Brisbane, Australia) and Lee. Chk1 was present at normal levels in A-T cells, but failed to show the reduction in electrophoretic mobility that is characteristic of phosphorylation in response to radiation, as observed in control cells. Thus, radiation-induced phosphorylation of chk1 is ATM dependent. Introduction of double strand breaks into DNA from NBS cells also failed to cause activation of Chk1, indicating that activation of the ATM-dependent kinase cascade requires nibrin/p95 (Lee).

A second unrelated kinase, chk2, participates in the G_2-M checkpoint by phosphorylating Cdc25C on serine 216, preventing it from blocking the passage of cells into mitosis. Tamar Uziel (Ramat Aviv, Israel) failed to detect accumulation of tyrosine-phosphorylated cdc2 in irradiated A-T cells or early inhibition of cyclinB-cdc2 kinase activity, which could be explained by a delayed modification in chk2. All of these data point to a role for ATM in the initial stages of checkpoint activation.

ATM Protein

ATM is a high molecular weight phosphoprotein of approximately 370 kDa containing a PI3-kinase domain, a putative leucine zipper, and a proline-rich region that has been shown to bind the nonreceptor tyrosine kinase c-Abl (5, 6). An ever-growing number of *in vivo* and *in vitro* substrates for ATM were reported, including p53, c-Abl, PHAS-1, chk2, RPA, and mdm2. Some of these data were derived using immunoprecipitated ATM kinase and were supported by results with A-T cell lines that lack ATM and in A-T cell lines in which ATM expression has been restored after transfection of a full-length ATM cDNA expression construct. The frequently modified tumor suppressor protein p53 attracted considerable attention (in the form of several presentations) as a substrate for ATM. These data were largely drawn from a series of studies published in *Science* and *Nature Genetics*

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toward the latter part of 1998 (7–9). These studies reported that p53 was phosphorylated by ATM at serine 15 in response to DNA damage, and this was further substantiated by *in vitro* kinase assays. Of some interest was the consistent observation that ATM had basal kinase activity in extracts from untreated cells, and this only increased 2–3-fold after irradiation. Binding of ATM to p53 occurred through the NH_2 terminus and the P13-kinase domain. A description of possible modification to ATM in response to radiation represents a future challenge in understanding the mechanism of activation of ATM. As mentioned previously, ATR was also capable of phosphorylating p53 on serine 15, but the activity was 20-fold lower than with ATM (Christin Canman, Memphis, TN; Abraham). Thanos Halazonetis (Philadelphia, PA) shed further light on the importance of specific modifications in p53 in altering its stability or contributing to its activation. Substitution of serine 15 on p53 did not alter its stabilization by radiation, suggesting that modification at this site contributes to its activation as a transcription factor. Binding to mdm2 results in destabilization of p53. Although serine 15 phosphorylation did not affect stability, phosphorylation on serine 20 disrupted the mdm2/p53 complex, as predicted from the three-dimensional structure, and stabilized p53. The kinase responsible for this phosphorylation remains unknown but is dependent on ATM for its activity. Dephosphorylation of serine 376 in p53 was also somehow ATM-dependent and contributed to the activation of p53. Furthermore, rapid ATM-dependent phosphorylation of mdm2 preceded the accumulation of p53 after exposure to ionizing radiation but not to UV (Shiloh, Ramat Aviv, Israel). Phosphorylation of mdm2 was p53 independent, indicating that ATM was modulating more than one protein in the same pathway.

A well-worn approach to studying the function of enzymes and pathways is to purify the molecules and examine their properties *in vitro*. Two groups, Stephen Jackson *et al.* (Cambridge, UK) and Susan Lees-Miller *et al.* (Calgary, Canada), described the purification of ATM, which was achieved based on prior experience with an equally demanding enzyme, DNA-PKcs. Although there was agreement on the ability of highly purified ATM preparations to phosphorylate p53 on serine 15 in the presence of Mn^{+2}, there was dispute over the possible requirement for DNA in the reaction. The use of atomic force microscopy revealed that ATM was bound primarily to the ends of DNA fragments (Jackson). Purified ATR also phosphorylated p53 on serine 15, in agreement with the immunoprecipitated kinase data and *in vivo* observations (Abraham and Jackson).

Another contentious issue related to a previous study by Woo *et al.* (10), who showed that p53 was not active as a transcription factor in a scid cell line, suggesting that DNA-PK was upstream of p53 in DNA damage response. This would agree with the *in vitro* phosphorylation of p53 on serine 15 by DNA-PK. Gretchen Jiminez (La Jolla, CA) dealt a telling blow to this contention by revealing that p53 was mutated in its transactivation domain in the scid cell line. She and others (Sandeep Burma, Los Alamos, NM; Yosef Shiloh, Ramat Aviv, Israel) demonstrated that p53 accumulates and is phosphorylated on serine 15 to the same or to a greater extent in DNA-PKcs$^{-/-}$ mouse and human cells, supporting ATM and other kinases as the candidate enzymes rather than DNA-PK.

ATM Mutations and Cancer Risk

The incidence of leukemias and lymphomas is markedly elevated in A-T patients, with as many as 30% developing tumors (11). A number of the malignancies seen in A-T also occur at lower frequencies in the non-AT population, which led to a search for ATM mutations in sporadic leukemias. Martin Yuille (Sutton, UK) reported frequent ATM mutations in sporadic T-PLL varying from point mutations to complex rearrangements detected by DNA fiber hybridization with a cosmid contig. These observations, together with other mutation data, LOH analysis, and immunoblotting for ATM suggest that ATM acts as a tumor suppressor. Mutations in the *ATM* gene were also detected in 6 of 32 (18%) of B-cell chronic lymphocytic leukemia patients (Tatjana Stankovic, Birmingham, UK), and ATM protein expression was reduced or absent in 8 of 20 (40%) of such tumors. Complex rearrangements involving the *ATM* gene were described in T-cell acute lymphoblastic leukemia (Yuille), but other studies have not detected ATM mutations where LOH was observed.

The breakpoints at 14q32 in T-PLL in A-T and non-A-T patients occur in the vicinity of the *TCL-1* (T-cell leukemia) locus, and this gene is expressed at high levels in cells with rearrangements of chromosome 14. Transcriptional activation of this proto-oncogene in transgenic mice led to the development of leukemias with a long latency period. Carlo Croce (Philadelphia, PA) described the cloning of *TCL-1* and *TCL-2* from the same locus on 14q32.1; these code for transporter proteins that move small molecules between the cytoplasm and nucleus. A more aggressive form of B-cell chronic lymphocyte leukemia was reported in patients showing LOH at 11q22-23; sequence analysis of all 66 ATM exons revealed genomic mutations predicted to give rise to splice abnormalities. A germ-line mutation in ATM was also observed in one patient.

Cancer Risk in A-T Heterozygotes

Some penetrance of A-T appears in heterozygotes; this includes intermediate radiosensitivity and increased risk of cancer, particularly breast cancer. Yun Su (Hawthorne, NY), studying A-T grandparents, observed that the median age at death was earlier for A-T carriers than for noncarriers—a striking corollary to Barlow's recent observations on irradiated atm$^{+/-}$ mice (as discussed above). Significant differences between carriers and noncarriers in these analyses could be seen for a variety of different causes of death, including heart disease and cancer. Michael Swift (Hawthorne, NY) summarized his laboratory's results from earlier retrospective and prospective studies of A-T relatives, as well as more recent haplotyping studies in A-T families, all of which revealed a significantly increased incidence of breast cancer among female A-T carriers. A number of posters and presentations at the meeting took on the challenge of assessing the public health ramifications of these results by screening for ATM carriers among cohorts of breast cancer patients. A prior study by FitzGerald *et al.* (12), using the protein truncation test that detects 70% of mutations in A-T homogotes/patients, failed to find heterozygosity for ATM truncation mutations in 401 early onset breast cancer patients. Swift pointed out that the breast cancers in the study of FitzGerald *et al.* (12) were confined to early onset cancers and that larger studies were required to reduce the influence of other confounders. Annegiem Broeks (Amsterdam, the Netherlands) provided evidence of increased ATM germ-line mutations among breast cancer patients from the Netherlands. Seven ATM germ-line mutations (8.5%) were detected in 82 breast cancer patients, and three were found in 115 women who were *BRCA1*- or *BRCA2*-negative. They concluded that ATM heterozygotes in their cohort had a 9-fold increased risk of developing breast cancer, characterized by frequent bilateral occurrence, early age of onset, and long-term survival. These results were obtained using the protein truncation test (PTT) assay and DNA sequencing for confirmation. This raises an important issue, namely, the significance of the approximately 30% of mutations that are not detected by PTT. Whereas only 8% of ATM mutations in A-T patients are missense mutations, up to 80% of mutations in T-PLL patients are missense mutations, suggesting that missense and nonsense mutations in ATM may

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differ in their penetrance for cancer predisposition. Indeed, Gatti pointed out that there seems to be two populations of A-T carriers, those heterozygous for a truncating allele and a second group heterozygous for a missense mutation, and that the latter group might include predominantly those individuals who are predisposed to developing breast cancer. In keeping with this, Magdalena Rozyka (Sutton, UK) described 15 (2.5%) rare in-frame sequence variants in 600 breast cancer cases. These included nine missense variants, changing the amino acid sequence, and five silent variants. Sharon Teraoka (Seattle, WA) reported finding 11 putative ATM missense mutations among 142 breast cancer patients *versus* only 1 in 80 controls. All of Teraoka's mutations were observed in a subset of patients with a first-degree family history of breast cancer. Rare DNA variants in ATM, initially described by Igor Vorechovsky (Stockholm, Sweden), were detected in a Norwegian cohort by Anne-Lise Borresen-Dale (Oslo, Norway) and by Elizabeth Schubert (Seattle, WA), who identified six DNA variants among 140 Caucasian and African-American breast cancer patients. A poster by Sergi Castellvi-Bel *et al.* (Los Angeles, CA) listed 34 new intragenic polymorphisms, 24 of which were rare DNA variants, from an SSCP mutation detection study of 92 A-T patients. The significance of these rare DNA variants in breast cancer is currently being investigated by introducing these changes into full-length ATM cDNA for transfection studies to determine whether they alter the phenotype of A-T or control cells. Finally, Doerk (Hannover, Germany) described a population-based study of 192 breast cancer patients in which 22 different ATM sequence changes were identified, one of which was truncating, 17 missense substitutions, and 4 synonymous nucleotide (silent) substitutions. Several groups [Joseph Hacia *et al.* (Bethesda, MD); Yvonne Thorstenson *et al.* (Stanford, CA); Carolyn Buzin *et al.* (Duarte, CA); and Teraoka *et al.*] reported on the application of promising new mutation screening methods to ATM, including DNA "chip"-based detection, multiconditional SSCP, and denaturing high performance liquid chromatography.

Nijmegen Breakage Syndrome

NBS overlaps significantly with A-T in clinical and cellular features including: immunodeficiency, 7:14 translocations, increased incidence of cancer (primarily of lymphoid origin), radiation sensitivity, and cell cycle checkpoint defects. As a result, it was long considered a clinical variant of A-T and has been included in a number of prior workshops. Recently, its relationship to A-T has been clarified, first with the mapping of a gene for NBS to chromosome 8q21, and subsequently with the cloning of the gene, *NBS1*, that encodes the protein nibrin (13, 14). All NBS patients studied to date have truncating mutations in the *NBS1* gene and are presumed to make no nibrin protein, although this has not yet been rigorously established. Karl Sperling (Berlin, Germany) summarized the positional cloning of the *NBS1* gene based on linkage and haplotyping studies in a collection of families of largely eastern European ancestry. Kenshi Komatsu (Hiroshima, Japan) reviewed the independent identification of NBS1 by functional complementation. Concannon raised the possibility of additional genes related to NBS or A-T by describing several variant families that met the clinical criteria for NBS, but produced normal amounts of nibrin and lacked detectable mutations in the *NBS1* gene. An additional family of this type was described by Dominique Smeets (Nijmegen, the Netherlands). Krystyna Chrzanowska (Warsaw, Poland) reported on tissue-specific expression of *NBS1* in the mouse, as assessed by *in situ* RNA hybridization. High-level expression was observed in all parts of the brain during embryonic development, but was more modest in the adult brain, where it was largely confined to the hippocampus and cerebellum. Richard Maser (Madison, WI) summarized current understanding of the function of nibrin and provided a preliminary report of the complementation of γ-radiation sensitivity by transfection of an *NBS1* expression construct into an embryonic stem cell line, in which both copies of the endogenous *NBS1* gene had been inactivated by homologous recombination.

Potential Therapeutic Approaches

Two A-T parents cochaired the session on therapeutic approaches. Gerard Berry (Philadelphia, PA) and Howard Lederman (Baltimore, MD) reported that ongoing clinical trials for myoinositol and L-DOPA were not promising any breakthroughs. Edward Clarkson (Denver, CO) reviewed progress in the therapy of over 300 Parkinson's disease patients with human fetal tissue, and Richard Wade-Martins (Oxford) described episomal genomic transgene constructs that attempt to correct the A-T phenotype in cell cultures. The need for a cooperative experience in the treatment of A-T patients who develop cancer is finally being addressed by John Sandlund and Michael Kastan (Memphis, TN), who are establishing a referral center at St. Jude Children's Research Hospital.

Conclusions

Although much progress has been made since the cloning of the *ATM* gene, several major obstacles remain. First, an animal model for the progressive severe ataxia seen in A-T patients is lacking, making therapeutic animal trials impossible. Second, the size of the gene has thwarted all efforts to isolate substantial amounts of the protein, such as might be used for structural studies. Third, the degree to which cancer risk is increased for A-T heterozygotes (mouse or man) will require further study, although the evidence presented at ATW8 certainly seems to support a preponderance of *ATM* mutations in patients with various types of malignancies. The A-T research community has had to learn how to work with a very large gene, whether for cloning, for expression studies, or for detection of mutations. However, this community of investigators works especially well together, and this insures further progress.

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