becco's minimum essential medium (DMEM) in the presence of polybrene (8 μg/ml) as described (18). Cells were washed twice in phosphate-buffered saline (PBS), resuspended in fresh medium, and cultured for 3 to 4 days. Transduced cells were tested for the presence of helper virus and cryopreserved until use. 36. BM mononuclear cells were obtained as a Ficoll fraction and grown for 2 to 3 days in complete DMEM at a density of 6 × 10^5 to 8 × 10^5 cells/cm² (33). T cell depletion and progenitor cell enrichment were obtained as described (3, 35). Gene transfer was carried out by multiple infection cycles with cell-free, helper virus-tested viral supernatants in the presence of polybrene (8 μg/ml) (35). BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and infected during the first 3 days of culture. Transduced cells were tested for the presence of helper virus and cryopreserved until use. At that time, the transduced cells were washed, resuspended in normal saline containing 4% human albumin, and reinjected into the patient. 37. C. Bordignon et al., Proc. Natl. Acad. Sci. U.S.A. 86, 6748 (1989).

T Lymphocyte–Directed Gene Therapy for ADA− SCID: Initial Trial Results After 4 Years

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In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA− SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal. The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (1, 2), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (3).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosine deaminase [ADA− SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; deoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune system (4).

Because this disease is curable by allogeneic bone marrow transplantation given without pretransplantation cytokoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients “cured” by allogeneic bone marrow transplantation was their T cells—the others remaining ADA-deficient (5)—raised the possibility that T cell–directed gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (6) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (7) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA− SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell function as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistent immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (8–10). Nearly all PEG-ADA–treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to test an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (11) is 1.5 kb and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadenosine (12, 13). Next, studies in mice, rabbits, and nonhuman primates using T cell lines modified with retroviral vectors showed normal cell survival and function after their reintroduction into recipient animals (14). Finally, Bordignon and colleagues (15) showed that ADA gene–corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into immunodeficient, but ADA-
normal BNX recipient mice.

The clinical protocol used here has been described elsewhere (16). Patients with documented ADA− SCID were eligible if they did not have a human lymphocyte antigen–matched sibling as a potential donor for marrow transplantation and if they had been treated with PEG-ADA for at least 9 months without full immune reconstitution. T cells were obtained from their blood by apheresis, induced to proliferate in culture, transduced with the ADA retroviral vector LASN, culture-expanded, and then reinjected into the patient after 9 to 12 days (17). No selection procedure was used to enrich for gene-transduced cells.

The clinical histories and ADA gene mutations of each patient have been reported (18, 19). Patient 1 presented with infection at 2 days of age and had recurrent infections and very poor growth until 26 months of age, when the diagnosis of ADA deficiency was established and she was started on PEG-ADA (30 U per kilogram of body weight per week (30 U/kg/week)). Treatment with PEG-ADA enzyme for approximately 2 years had resulted in significant, but incomplete, benefit. With PEG-ADA she gained weight, had fewer infections, and transiently developed a normal peripheral blood T cell count (Fig. 1A), and her T cells had acquired the ability to respond to mitogens in vitro. However, significant immune deficiency persisted, including recurrence of her T lymphopenia (Fig. 1A), DTH skin test anergy (Table 1), depressed in vitro immune reactivity to specific antigens such as tetanus toxoid, failure to generate normal cytotoxic T cells to viral antigens or allogeneic cells, defective immunoglobulin production and absent or weak antibody responses to several vaccine antigens, and borderline isohemagglutinin titers (Table 1). At 4 years of age, she was enrolled in this trial.

The course of disease in patient 2 (who was 9 years old when enrolled in the trial) was milder than that seen in classic SCID (19). She had her first serious infection at age 3, and septic arthritis at age 5; the diagnosis was finally established at age 6 when significant lymphopenia with ADA deficiency was confirmed. This patient had an excellent initial improvement in peripheral T cell numbers after the start of PEG-ADA therapy (30 U/kg/week) at age 5, but lymphopenia recurred in the third and fourth years of enzyme treatment (Fig. 1B). During the year before gene therapy, repeated evaluation of her immune system showed persisting immunodeficiency, but less severe than that in patient 1. Despite 4 years of enzyme treatment, DTH skin test reactivity was absent (Table 1), cytotoxic T cells to viral antigens and allogeneic cells were deficient, and isohemagglutinins were barely detectable. However, illustrating the variability seen in the responses of patient 2 over time, blood lymphocytes that were cryopreserved from the day the clinical trial began and tested later showed normal cytotoxic activity to allogeneic cells. Within 5 to 6 months of beginning gene therapy, the peripheral blood T cell counts for patient 1 (Fig. 1A) rapidly increased in number and stabilized in the normal range and have remained normal since that time (20). ADA enzyme activity, nearly undetectable in her blood lymphocytes initially, progressively increased in concentration during the first 2 years of treatment to reach a level roughly half the concentration found in heterozygous carriers (expressing only one intact ADA allele) and has re-

**Table 1.** DTH skin test reactivity and isohemagglutinin titers in sera of each patient at various times during the treatment protocol. Skin tests were applied as Multitest (Pasteur Merieux, Lyon, France) and scored according to the manufacturer's instructions 48 to 72 hours after being placed. Seven antigens were placed on the dates indicated, although only five were technically satisfactory on day 1252 for patient 1 and on day 1118 for patient 2. Isohemagglutinin titers were determined by standard blood bank techniques (34). Ninety-five percent of normal children over the age of 2 years will have a titer of ≈1:16 and 92% will have a titer of ≈1:32 (35). ND, not done. For the DTH skin tests, positive tests were elicited: T, tetanus toxoid; D, diphtheria toxoid; C, Candida albicans; P, Proteus antigen; S, streptococcal antigen; OT, old tuberculin.

![Fig. 1.](image-url) Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, dates of treatments, and the total number of cells infused for each patient. ADA level is measured in nanomoles of adenosine deaminated per minute per 10⁶ cells. Vertical bars indicate the dates of cell infusion, and their height represents the total number of nonselected cells infused at each treatment. The T cell numbers represent total CD3-bearing T cells determined by standard flow cytometric analysis. (A) Patient 1 began gene therapy on 14 September 1990 (protocol day 0) and received a total of 11 infusions. Cellular ADA enzyme level is indicated by the dashed line. ADA activity was determined as described (13, 25). Values shown are the mean of duplicate samples and represent EHE-sensitive ADA enzyme activity. (B) Patient 2 began gene therapy on 31 January 1991 (protocol day 0) and received a total of 12 infusions.
mained at that level since (Fig. 1A). Thus, both the reconstituted number of peripheral blood T cells and the elevated T cell ADA enzyme concentration have persisted since the patient's last treatment, indicating that peripheral T cells can have an unexpectedly long life-span and that gene expression from the retroviral vector has not been silenced over this period.

Patient 2, who had variable immune reactivity before enrollment, responded to the instillation of lymphocyte infusions, with her peripheral T cell count rapidly increasing to levels in the high normal range (Fig. 1B). Beginning with infusion 5, which included protocol modifications to partially deplete CD8 cells from the initially cultured cell population (21), her T cell count fell into the mid-normal range, where it persisted throughout the treatment period and for a year after the last cell infusion. In contrast to those in patient 1, ADA enzyme levels in the circulating T cells of patient 2 did not rise significantly above the small amounts seen before gene therapy treatment (~1.5 nmol/l/10^6 cells per minute).

The differences in final lymphocyte ADA concentration are consistent with the levels of gene transfer reached in these patients. For several months in the second protocol year during which cell infusions were not given, LASN vector sequences detected by polymerase chain reaction (PCR) maintained a stable frequency in the peripheral blood of patient 1 at a level greater than the PCR-positive control standard containing the equivalent of 0.3 vector copies/cell (Fig. 2). By contrast, although vector-containing cells were also stably detected throughout a similar period in patient 2, their level reached only a value equivalent to 0.1 to 1.0% of her circulating cells carrying the inserted ADA vector.

The principal contributor to the difference in the final frequency of LASN vector-modified T cells in patients 1 and 2 was the low gene transfer efficiency in the cells of patient 2; this was consistently only a tenth or less of what was routinely achieved in the cells from patient 1. Despite the gross differences in the final proportion of vector-containing cells reached in these two patients, both CD4 and CD8 T cell populations from each have remained consistently positive for integrated vector sequences since the first infusion through protocol day 1480 for patient 1 and through protocol day 1198 for patient 2 (Fig. 2).

To more accurately measure the proportion of vector-containing cells in patient 1, we performed quantitative Southern (DNA) hybridization analysis for vector sequence on DNA isolated from her peripheral blood T cells at different days during the course of this protocol. On protocol days 816 and 1252, which represent samples taken 109 and 545 days after the last treatment, the vector concentration was at the level of approximately one vector copy per cell (Fig. 3). Longitudinal studies of samples obtained throughout the study show that this large amount of integrated vector was reached by infusion 8 (D707) and that it has remained in this range since that time (22).

The use of a restriction endonuclease that cuts only once within the vector sequence does not give detectable bands (Fig. 3), indicating that the population of blood T cells at these dates is not oligoclonal with respect to integrated vector. Vector-derived mRNA was readily detected by reverse transcription (RT)–PCR at these same times (Fig. 3), confirming that vector expression persisted and was correlated with the presence of ADA enzyme activity in her circulating T cells.

To evaluate the effect of gene therapy on the immune function of these two patients in addition to its beneficial effect on T cell numbers, we performed a panel of immunologic studies both before and, at various times after, treatment. DTH skin test reactivity to common environmental and vaccine antigens tests the overall competence of the cellular immune system because a response depends on the full complement of cellular functions, not just cell proliferation or secretion of a single cytokine (Table 1). Patient 1 was anergic before our protocol treatment despite nearly 2 years of PEG-ADA treatment. Eight months after the initiation of gene therapy (protocol day 251), she had a brisk DTH response to a single intradermal skin test with tetanus toxoid. By protocol day 455, DTH responses to five of seven antigens were present, and this increased responsiveness has persisted, through day 1522.

Before the protocol, patient 2 had no positive DTH skin test (Table 1). At protocol day 501, five positive DTH skin tests were elicited, and this increased DTH reactivity had persisted when she was last tested on day 1118. She also acquired palpable lymph nodes and visible tonsils during the period of protocol treatment.

To corroborate the improved immune function indicated by these DTH tests, we evaluated the capacity of peripheral T cells from our patients to produce interleukin-2 (II-2) or to kill antigen target cells in vitro. In several patients treated with PEG-ADA, in vitro T cell proliferative responses to mitogens may normalize, whereas responses to specific antigens are less improved (7–10). During PEG-ADA treatment before gene therapy, T cells from patient 1 produced IL-2 in response to stimulation with

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**Fig. 2.** PCR evaluation of the frequency of LASN vector–positive cells in the blood of patients 1 and 2 at various protocol days. (A) Cells from patient 1 for protocol days (D) 304 to 591 (see Fig. 1A). PCR analysis was performed as described (26) in an ethidium-stained gel. (B) Cells from patient 2 for protocol days (D) 333 to 501 (see Fig. 1B). PCR products were probed with 32P-labeled neo gene as described (26). (C) Purified CD4+ and CD8+ subpopulations of patient 1 (D1480) and patient 2 (D1198) prepared by separation of peripheral blood mononuclear cells (PMBCs) by fluorescence-activated cell sorting (FACS). The purity of the separated T cell subpopulations from which DNA was extracted exceeded 98%, as confirmed by FACS analysis. Direct PCR with [32P]dideoxy-cytidine triphosphate was performed as described (27). Standards (STD) were prepared from DNA obtained from cell mixtures of a known proportion of LASN-transduced cells containing a single vector insert mixed with vector-negative cells. C, vector-negative control cells.

**Fig. 3.** Quantitative Southern hybridization analysis of DNA prepared from the blood mononuclear cells of patient 1 on protocol days (D) 816 and 1252 (28). DNA digested with Sst I should yield a single restriction fragment of 3.1 kb containing both the vector neo and ADA genes. Eco RI cuts only once within the vector sequence, and therefore a detectable band would indicate that a predominant clone with a single unique vector integration site was present in that blood sample. None was detected. Polyadenylated mRNA was extracted from the patient cells on days 0, 816, and 1252 and analyzed for vector message by RT-PCR (29). The primer locations used are indicated as short solid lines above the vector diagram. SV, SV40 early promoter; (A), polyadenylation site; Ψ, extended retrovirus packaging signal. Hatched regions indicate protein coding regions.
the mitogen phytohemagglutinin (PHA) (Fig. 4A) but were unable to produce IL-2 in response to stimulation with influenza A virus or tetanus toxoid, despite repeated immunization with these antigens. Over the first months of gene therapy, IL-2 production improved and became normal after 1 year (Fig. 4A). Again before gene therapy, patient 1's T cells failed to show significant cytolytic reactivity against either allogeneic cells or influenza A–infected target cells. Almost mirroring the steady increase in IL-2 production, she acquired normal in vitro cytolytic T cell responses to these antigens, reaching normal values in her second year of treatment. (Fig. 4B).

The results of these cytolytic assays for patient 2 are shown in Fig. 4C. Tests done 120 days before the beginning of gene therapy also showed impaired responses. However, cells that were obtained at the time of the first gene therapy infusion, cryopreserved, and subsequently tested some months later showed a normal cytolytic response to allogeneic cells. After a year on gene therapy, cytolytic T cell activity against influenza also became normal.

To evaluate the effects of our treatment on humoral immune function in these patients, we measured antibody responses to several antigens. Despite their PEG-ADA treatment, both patients 1 and 2 had only low or borderline titers of isohemagglutinins on repeated testing before gene therapy. Each patient showed significant elevations in the levels of these antibodies within 90 to 115 days of beginning treatment with gene-modified cells (Table 1). Isohemagglutinins are antibodies that react with group A and B red blood cell antigens and occur spontaneously as a result of environ-mental exposure to cross-reacting antigens. Isohemagglutinin responses are, therefore, less dependent on the timing of previous immunizations than are responses to common vaccine antigens. After gene therapy, each patient also had improvement in antibody responses to vaccines to *Hemophilus influenzae* B (HIB) and tetanus toxoid (Fig. 5). With enzyme therapy alone, peripheral lymphocytes from each patient were unable to produce immunoglobulin M (IgM) in vitro after stimulation with pokeweed mitogen (PWM), but made robust responses after a year on the gene therapy protocol (Fig. 5A). Immunoglobulin production to PWM depends on T cells; these results further confirm the reconstitution of T cell function associated with gene therapy.

The effects of this treatment on the clinical well-being of these patients is more difficult to quantitate. Patient 1, who had been kept in relative isolation in her home for her first 4 years, was enrolled in public kindergarten after 1 year on the protocol and has missed no more school because of infectious disease than her classmates or siblings. She has grown normally in height and weight and is considered to be normal by her parents. Patient 2 was regularly attending public school while receiving PEG-ADA treatment alone and has continued to do well clinically. Chronic sinusitis and headaches, which had been a recurring problem for several years, cleared completely a few months after initiation of the protocol.

This trial of retroviral-mediated gene transfer shows that the survival of reinforced transduced peripheral blood T cells is prolonged in vivo; the erroneous assumption that T cells would not have such long-term survival was often cited as a potential problem with this treatment strategy. Patient 1 has had a normal total peripheral T cell count since the last cell infusion, and the proportion of her circulating T cells carrying vector DNA has remained stable over that period. Further, expression of the ADA transgene under the influence of the retroviral long terminal repeat (LTR) promoter has persisted for a long period in vivo without obvious extinction. There have been swings in the level of ADA enzyme in her peripheral lymphocytes throughout the period of observation, but the level of blood ADA enzyme activity at 4 years (protocol day 1480) is equivalent to that found immediately after the last cell infusion 2 years earlier (Fig. 1A). Although the data have not yet been completely analyzed, blood obtained after 5 years showed continuation of this trend with, again, a normal T lymphocyte count and an equivalent ADA level.

The mechanism by which our treatment aided immune reconstitution in patient 2 is less clear. The responses of patient 2 to some in vitro immunologic tests were variable before beginning our treatment protocol, ranging from little or no detectable response to nearly normal responses on the blood sample from the day gene therapy began. This patient produced a normal antibody response to immunization with bacteriophage φX174 about a year before beginning gene therapy (8). Although we have shown several examples of depressed cellular and humoral immune responses that strongly improved after gene therapy, this highly variable immune reactivity while patient 2 was on PEG-ADA therapy alone complicates interpretation of the contribution of our therapy. There was a temporal relation between initiation of gene therapy and a normalized peripheral T cell count.
improved DTH, appearance of tonsils and palpable lymph nodes, normalized isohemagglutinin response, and improved PWM response, as well as other factors. In view of the relatively low level of ADA gene transfer achieved in this patient, the potential contribution of the infusions of the culture-activated T cells to the patient's response must also be considered. Perhaps ex vivo T cell activation somehow bypassed a differentiation block that PEG-ADA alone was unable to relieve. Despite the low final percentage gene transfer achieved, a 1% level of ADA gene–corrected cells could represent 10^10 to 10^11 ADA-expressing T cells distributed throughout the body that could readily contribute to immune improvement.

Since the beginning of the trial, the dose of PEG-ADA enzyme given to each of our patients has been decreased by more than half (patient 1, 14 U/kg/week; patient 2, 10 U/kg/week), during which time their immune function has improved. By contrast, worsened immune function has been seen in other ADA− SCID patients when their dose of enzyme has been similarly reduced (10, 23). We do not want to expose these patients to the potential risk of recurrent immunodeficiency by completely stopping PEG-ADA enzyme treatment until we have better information about the quality and duration of the immune improvement achieved by this first-generation gene therapy trial. The role of continued exogenous enzyme treatment will be clarified here or in companion studies attempting stem cell gene correction (24).

The safety of retroviral-mediated gene transfer has been a central concern. At least in the short and intermediate term, no problems have appeared in any clinical trial using these vectors. In the longer term, the theoretical potential for retroviral vectors to cause insertional mutagenesis remains the primary concern. To date, there has been no indication that malignancy associated with this process will be a complication of retroviral-mediated gene transfer.

Our trial here has demonstrated the potential efficacy of using gene-corrected autologous cells for treatment of children with ADA− SCID. Eleven children with this disease have been enrolled in various gene therapy protocols, each using different strategies and retroviral vector designs and focusing on different target cell populations. The experience gained from these approaches should provide guidance for gene therapy as a treatment for this disorder as well as for a larger array of inherited and acquired diseases.

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20. The protocol was reviewed and approved by the Clinical Research Subpanels of the NCI and NHLBI, the NCI Cancer Treatment and Evaluation Program (CTEP), the Food and Drug Administration, the Human Genetic Therapy Subcommittee, the Recombinant DNA Advisory Committee, the Director of NIH, and the U.S. Food and Drug Administration. Informed consent was obtained from the parents of each patient.

21. Beginning with culture 9 for patient 1 and culture 5 for patient 2, the patients’ lymphocyte populations obtained by apheresis were fractionated by adherence to flasks coated with CD8 monoclonal antibodies (Applied Immune Sciences) following the manufacturer’s instructions. This protocol modification for CD8 depletion was introduced because both patients were developing a progressively inveterate CD8+ CD3- ratio. This effect was apparently the result of preferential growth of CD8+ cells during the last 4 to 5 days of culture and the subsequent persistence of these infused CD8+ cells in the circulation. Consequently, each subsequent apheresis sample was digested and plated in culture to determine the ratio of CD4+ to CD8+ cells and to prepare a positive selection method. By partially depleting the apheresis sample of CD8+ cells by an immunofluorescence separation step, the latter treatments for each patient consisted of cells with a more defined phenotype. The perturbation in normal CD4+ CD3- cell proportions did not have detectable untoward effects for either patient.

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25. The ADA enzyme activity assay was performed in duplicate as described (13). Positive control cells were obtained from healthy normal donors and had a mean of 82 U (normal range, 66 to 102 U). Duplicate samples were run in the presence of the ADA enzyme inhibitor EHNA (30 μM), Specific ADA activity was calculated as total adenosine deaminating activity minus EHNA-resistant activity. EHNA-resistant activity represents metabolic activity of a nonspecific adenosine hydrolase present in human cells.

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28. Southern hybridization analysis for LASN vector consisted of the following: 10 μg of DNA was digested with SalI and hybridized with a 278-bp Neo fragment from LASN corresponding to the SV40 promoter and neo gene. DNA from K562-LASN cells served as a positive control.

29. RT-PCR analysis for LASN vector transcripts was as follows: 3 μg of polyadenylated RNA was treated with deoxyribonuclease and reverse-transcribed. The cDNA (0.3 μg) was amplified with LASN vector-specific primers in a 30-cycle PCR reaction. The oligonucleotides 5'-CAGCCTCTGGACGGGCAA-3' (corresponding to the 3' end of the ADA gene in LASN) and 5'-GGCACCGTGGGCGGTAAGA-3' (complementary to the 5' end of the neo gene in LASN) were used as primers. After electrophoresis and blotting, the sequences were hybridized with a 572-bp probe corresponding to the entire length of the predicted PCR product.

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Physical Map and Organization of Arabidopsis thaliana Chromosome 4

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A physical map of Arabidopsis thaliana chromosome 4 was constructed in yeast artificial chromosome clones and used to analyze the organization of the chromosome. Mapping of the nucleolar organizing region and the centromere integrated the physical and cytogenetic maps. Detailed comparison of physical with genetic distances showed that the frequency of recombination varied substantially, with relative hot and cold spots occurring along the whole chromosome. Eight repeated DNA sequence families were found in a complex arrangement across the centromeric region and nowhere else on the chromosome.

Arabidopsis thaliana has been adopted as a model organism for the analysis of complex plant processes by means of molecular genetic techniques (1). The increase in map-based cloning experiments makes the generation of a complete physical map of the Arabidopsis genome a high priority. In addition, the availability of such a map would enable the organization of the chromosome to be studied in more detail. Little is known about the organization of plant chromosomes, but the general picture is that of chromosomes carrying large numbers of dispersed [often retrotransposons (2)] and tandemly repeated DNA sequences (3). The relatively small (100 Mb) Arabidopsis genome has a much smaller number of repeated DNA sequences than do most other plant species; its five chromosomes contain <10% highly repetitive and ~10% moderately repetitive DNA (4). The dispersion of most of these sequences among the low-copy DNA is unknown.

We discuss here a physical map, which we have presented on the World Wide Web (WWW) at URL: http://nasc.nott.ac.uk/JIC-contigs/YAC-contigs.html, of Arabidopsis chromosome 4, one of the five repeats carrying nucleolar organizing regions. The construction of this map allowed us to analyze the frequency of recombination along the whole chromosome, the integration of the physical with the cytogenetic map, the interspersion pattern of repeated and low-copy DNA sequences over the whole chromosome, and the arrangement of repeated DNA sequences over the centromeric region.

We generated the physical map by hybridizing probes to four yeast artificial chromosome (YAC) libraries (5), using colony hybridization experiments (6). The probes consisted of 112 markers genetically mapped to chromosome 4, 20 previously unmapped genes, random genomic fragments and sequences flanking transposable elements, and the 180-base pair (bp) repetitive element carried in pAL1 (7). Southern (DNA) blot analysis of YAC clones confirmed the colony hybridization results and revealed common restriction fragments in the different YAC clones hybridizing to a given marker. This demonstrated overlap between the inserts of the YAC clones. On the basis of these results, the YAC clones could be placed into 14 YAC contigs with a high degree of redundant YAC cover, ensuring an accurate map despite the presence of chimeric clones in the YAC libraries.

We generated YAC end fragments, using either inverse polymerase chain reaction (IPCR) or plasmid rescue (8), from YAC clones lying near the ends of each of the 14 contigs. The fragments were hybridized to Southern blots of YAC clones from adjacent contigs. In addition, YACs, as well as some of the end fragments generated by IPCR, were used to identify clones from a cosmid library of the Columbia ecotype (9). The cosmids were then used as new markers on the YAC libraries. These experiments reduced the number of contigs to four. In all but two instances, the end fragments revealed that the contigs were already overlapping. Experiments aimed at closing the last three gaps have been attempt-