min in 1× SSC at 50°C, treated with 500 μg of ribonuclease A per milliliter in 4× SSC at 37°C for 30 min, washed twice for 40 min and 5 min in 1× SSC at 50°C, rinsed twice for 5 min in 2× SSC at room temperature, and dipped five times in water. After drying, sections were dipped in Kodak NTB-2 emulsion, exposed for 1 (total APP mRNA probe) or 4 (HL124i-containing APP mRNA) days, developed for 2 min with Kodak D-19, and fixed in Kodak Rapid Fix for 1.5 min. After copious rinsing in distilled water, sections were stained with cresyl violet or hematoxylin and Congo red, dehydrated, and mounted. To quantitate the hybridization of the APP probes to mRNA within perikarya in the various brain regions examined, we focused on the plane of the cells, identified the perikaryon to be assessed at ×1000 magnification, and superimposed a grid of a size chosen so that it just fit within that perikaryon. We then adjusted the focus so that the grains within the grid (which are not clearly visible when one focuses on cells) could all be counted. In each tissue block, the average number of grains produced over perikarya by antisense probe was typically obtained by analyzing 30 randomly chosen neurons on each of two or three sections, and the number produced by noncomplementary probe was obtained by analyzing 30 randomly chosen neurons on one section. The number of grains produced by the specific binding of antisense probe was then calculated by taking the difference between the antisense and noncomplementary grain counts. The antisense/sense ratio ranged from 5:1 to 10:1 in the various cell types examined. To illustrate the relative amount of APP mRNA in soma of the various regions that we examined, we have expressed specific grain counts per average somal area. Average somal sizes, which were not appreciably different in the AD and control cases, were determined by averaging morphometric measurements made on three AD and three control cases with the Bioquant II system (R&M Biometrics) (nbM 700, LC 809, basis pontis 264, and subiculum 262 μm^2).

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22. The increased KPI-free APP mRNA observed in this study occurred in two nuclei in which there was a marked loss of neurons (*11*). This neuronal loss does not, however, obviate the potential significance of the increased KPI-free APP mRNA that we observed. The amount of amyloid deposited would, according to our working hypothesis, depend on (i) the duration of the increase in the affected neurons, (ii) the size of the neuronal population showing increased KPI-free APP, and (iii) the percentage increase in the KPI-free form in the affected neurons. We do not know when elevated levels of KPI-free APP mRNA develop during the protracted course of AD. The effect of elevated KPI-free APP mRNA will be greatest if this increase is an early, persistent change that develops prior to the loss of nbM or LC neurons and least if it is a brief end-stage phenomenon that occurs after the depletion of the nbM and LC populations. We have no direct evidence regarding the duration of the change in APP gene expression, but there was no statistically significant correlation between the degree of cell loss in the nbM or LC and the increase in total APP mRNA. Neurons tended, if anything, to show progressively greater increases in total APP mRNA as the number of surviving neurons increased, and cases with many surviving neurons showed marked increases in total APP mRNA. In the AD case with the least loss of nbM neurons (26%), for example, the probe for total APP mRNA produced 2.8 times more specific grains over nbM perikarya than in the mean control case. It should also be emphasized that it is the change in KPI-free APP mRNA rather than the increase in total APP mRNA that is important for amyloid deposition according to our hypothesis. Although we did not directly evaluate KPI-free APP mRNA in this study, analysis of our data suggests that this form is a small fraction of total APP mRNA in control nbM and LC neurons. Thus, in AD, the twofold increase in total APP mRNA (which occurred exclusively in APP mRNA lacking the KPI domain) appears to occur because of a dramatic percentage increase in the relevant KPI-free transcript.
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Conferring Operator Specificity on Restriction Endonucleases

MICHAEL KOOB, ERIC GRIMES, WACLAW SZYBALSKI*

Mapping and manipulation of very large genomes, including the human genome, would be facilitated by the availability of a DNA cleavage method with very high site specificity. Therefore, a general method was devised that extends the effective recognition sequences well beyond the present 8–base pair limit by combining the specificity of the restriction endonuclease with that of another sequence-specific protein that binds tightly to DNA. It was shown that the tightly binding *lac* or λ repressor protects a restriction site within the operator from specific modification methylases, M·Hha I or M·Hph I, while all other similar sites are methylated and thus rendered uncleavable. A plasmid containing a symmetric *lac* operator was specifically cleaved by Hha I, only at the site within the operator, after M·Hha I methylation in the presence of the *lac* repressor, whereas the remaining 31 Hha I sites on this plasmid were methylated and thus not cleaved. Analogous results were obtained with the Hae II site within the *lac* operator, which was similarly protected by the *lac* repressor, and with the Hph I site within the phage λ o_L operator, which was protected by λ repressor from M·Hph I methylation.

THE PRESENTLY AVAILABLE RESTRICTION enzymes cleave DNA at recognition sites of 4 to 8 bp. Statistically, 8-bp recognition leads to one cut per 65.5 kb. For mapping, manipulation, and sequencing of very large genomes (*1*) restriction systems with higher specificity are required. One of these systems is based on the natural methylation of many genomes, but such an adventitious modification process is not easy to control. Another, which has been shown to stretch effective recognition sites to 10 bp, is based on the joint use of modification methylases and restriction endonucleases for a few specific combinations of restriction sites (*2–4*). Finally, a number of laboratories have recently sought to create synthetic DNA-cleaving reagents by combining a sequence-specific DNA-binding domain with a DNA-cleaving moiety (*5–7*). However, the practical applications of these reagents are severely limited by their very low cleavage efficiency and inability to generate well-defined ends.

We have devised a general method (Fig. 1A) for efficiently extending the effective recognition sequence of a restriction endonuclease well beyond previous limits. Our method combined the activities of three proteins: (i) a repressor, which specifically binds to only very rare sites (operators) of about 20 bp in length; (ii) a modification methylase, which methylates all corresponding restriction sites with the exception of those protected by the repressor; and (iii) a restriction endonuclease with the same specificity as the modification methylase (Fig. 1A). The two specific examples we describe are operator-repressor systems of (i) the *lac* operon, which creates an effective recognition sequence of 20 bp, and (ii) the phage λ o_L operator, which gives an effective

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706.

*To whom correspondence should be addressed.

recognition sequence of 17 bp (Fig. 1, B and C, respectively).

For the *lac* system we used plasmid pOE310 (Fig. 1B), which contains a symmetric *lacO* operator (*8*). This plasmid is cleaved by Hae II into 12 fragments (Fig. 2, lane 6) and by Hha I into 32 fragments (lane 13). Both the Hae II and Hha I sites were rendered uncleavable by methylation with M·Hha I, since, in Fig. 2, lanes 4, 8, and 11, all show only one band corresponding to the entire plasmid previously linearized with Pst I or Pvu II. Creation of a "unique" Hae II site within *lacO* is demonstrated by the presence of only two bands in Fig. 2, lanes 5 and 9. All other Hae II sites were inactivated by the M·Hha I methylase used in the presence of *lac* repressor. An analogous experiment, which creates a unique Hha I site, is shown in lane 12. (The Hae II and Hha I sites within the *lacO* overlap; see Fig. 1B.) As a control for these experiments we used digestion with Pst I + Eco RI and Pvu II + Eco RI (lanes 3 and 7, respectively), because the Eco RI sites, which flank the *lac* operator, mimic the unique Hae II/Hha I site within the *lac* operator. These experiments (see legend to Fig. 2) were done in three steps, all in the same test tube: (i) addition of the *lac* repressor followed by the modification methylase, (ii) thermal inactivation of both proteins, and (iii) cleavage with the corresponding restriction endonuclease.

For the phage λ o_L operator system, we used plasmid pNH55B-1 (Fig. 1C), which was constructed by deletion of a Bam HI fragment from plasmid pNH55B (*9*). This plasmid is cleaved by Hph I into nine fragments (Fig. 3, lane 5). The Hph I sites were rendered uncleavable by methylation with M·Hph I. This is evident because lanes 3 and 7 show only one band corresponding to the

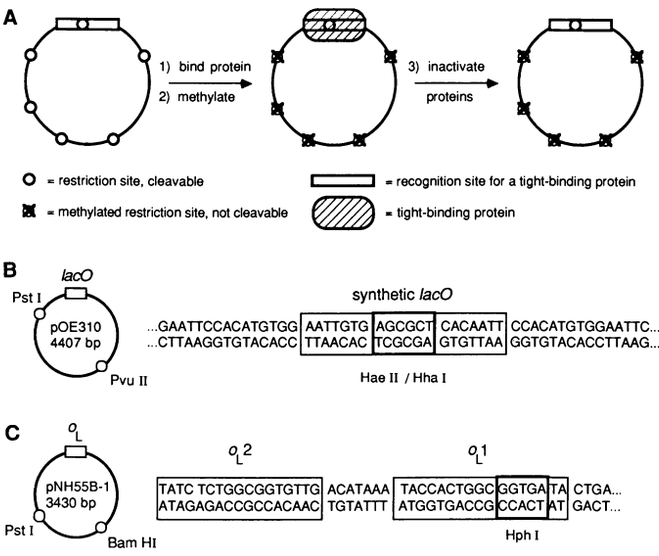

Fig. 1. A method for increasing the site specificity of restriction endonucleases. (**A**) Creation of rare restriction sites by combining the specificity of a DNA-binding protein (for example, a repressor) with that of a restriction enzyme. (**B**) Symmetric *lac* operator sequence (right) inserted at the Eco RI site of pBR322 to form pOE310 (left). Methylation of this plasmid with M·Hha I in the presence of *lac* repressor leaves unmethylated Hha I (GCGC) and Hae II (AGCGCT) sites at the position shown, while methylating and thus eliminating 31 Hha I sites and 11 Hae II sites. Cuts at *lacO* and at the Pst I site release 3633- and 774-bp fragments, whereas cuts at *lacO* and at the Pvu II site release 2319- and 2088-bp fragments. (**C**) Phage λ o_L1/o_L2 sequence (right) of plasmid pNH55B-1 (left). Methylation of this plasmid with M·Hph I in the presence of λ repressor leaves an unmethylated Hph I (GGTGA) site at the position shown, while methylating, and thus eliminating, eight Hph I sites. Cuts at o_L1 and at the Bam HI site release 2344- and 1086-bp fragments, whereas cuts at o_L1 and at the Pst I site release 2398- and 1032-bp fragments.

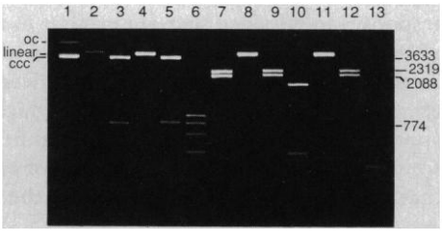

Fig. 2. Operator-specific cleavage in plasmid pOE310 (*lacO* operator, Fig. 1B) as analyzed by agarose gel electrophoresis. Lane 1, untreated pOE310. Open circular (oc) and covalently closed circular (ccc) forms are seen. Lane 2, Pst I–linearized pBR322 (operatorless control) methylated with M·Hha I in the presence of *lac* repressor followed by Hae II digestion. Lanes 3 to 6, Pst I–linearized pOE310 (4407 bp) treated as follows: lane 3, Eco RI digestion (a control showing the two fragments that resulted from cuts in *lacO* and Pst I sites; see legend to Fig. 1); lane 4, methylation with M·Hha I followed by Hae II digestion; lane 5, as in lane 4 but methylated in the presence of *lac* repressor (two bands only, similar to the lane 3 control); lane 6, Hae II digestion (unmethylated control). Lanes 7 to 13, Pvu II–linearized pOE310 treated as follows: lanes 7 to 10, as in lanes 3 to 6; lanes 11 to 13, as in lanes 4 to 6 but in each case digested with Hha I rather than Hae II. Lanes 5, 9, and 12 represent actual protection experiments (Fig. 1B); other lanes represent various controls. Sizes (in base pairs) given are for lane 5 (3633 bp, 774 bp), and lanes 9 and 12 (2319 bp, 2088 bp). Methylation was carried out in a buffer of 50 m*M* tris·HCl (*p*H 7.5), 10 m*M* EDTA, 5 m*M* 2-mercaptoethanol, 80 μ*M* *S*-adenosylmethionine (SAM), 50 μg/ml of bovine serum albumin (BSA), 2 U of M·Hha I [New England Biolabs (NEB)] and 0.5 μg of plasmid DNA in a total volume of 10 μl, and incubated for 1 hour at 37°C. For the methylation done with *lac* repressor present, 8×10^{-12} mol (tetramer) of repressor was preincubated with the DNA at 37°C for 5 min before the addition of SAM or methylase. After heat inactivation of repressor and methylase at 75°C for 15 min, the reaction volumes were raised to 30 μl with the above buffer adjusted to 5 m*M* $MgCl_2$, 100 μg of BSA per milliliter, and 10 U of Hha I (NEB) or to 5 m*M* $MgCl_2$, 100 μg of BSA per milliliter, 50 m*M* NaCl, and 10 U of Hae II (NEB), and incubated at 37°C for 1 hour. The digest (10 μl) was run on a 1.2% agarose gel containing ethidium bromide.

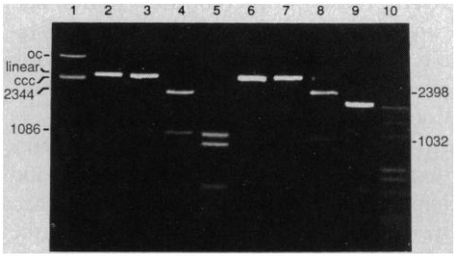

Fig. 3. Operator-specific cleavage of plasmid pNH55B-1 (phage λ o_L operator; see Fig. 1C) as analyzed by agarose gel electrophoresis. Lane 1, untreated pNH55B-1 (oc and ccc controls analogous to lane 1, Fig. 2). Lanes 2 to 5, Bam HI–linearized pNH55B-1 (3430 bp) treated as follows: lane 2, untreated; lane 3, methylation with M·Hph I followed by Hph I digestion; lane 4, as in lane 3 but methylated in the presence of λ repressor; lane 5, Hph I digestion. Lanes 6 to 9, Pst I-linearized pNH55B-1 treated as follows: lane 6, untreated; lane 7, methylation with M·Hph I followed by Hph I digestion; lane 8, as in lane 7 but methylated in the presence of λ repressor; lane 9, Hph I digestion. Lane 10, a mixture of pBR322 digested with Msp I and pBR322 doubly digested with Pvu II and Hinc II (marker lane). Lanes 4 and 8 represent the actual protection experiments (Fig. 1C); other lanes represent various controls. Sizes (in base pairs) given at the left of the figure are for lane 4 and those at the right for lane 8. Methylation was carried out in a solution of 20 m*M* tris-HCl (*p*H 7.5), 1 m*M* EDTA, 5 m*M* 2-mercaptoethanol, 80 μ*M* SAM, 1 U of M·Hph I (NEB), and 0.5 μg of plasmid DNA in a total volume of 25 μl. For reactions containing λ repressor, 2×10^{-10} mol (monomer) of repressor was added to the buffer and DNA before addition of methylase. After incubation at 37°C for 1 hour, the methylase was inactivated by heating to 75°C for 15 min, and the reactions were adjusted to 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 3 m*M* dATP, 8 μg of Sal I–linearized M13mp7 single-stranded DNA per milliliter, and 1.3×10^{-10} mol of RecA in a total volume of 50 μl. The reactions were incubated at 37°C for 6 hours, extracted with phenol/chloroform (50%/50%, v/v), precipitated with ethanol, lyophilized, and resuspended in 6 m*M* KCl, 10 m*M* tris-HCl (*p*H 7.5), 10 m*M* $MgCl_2$, 100 μg of BSA per milliliter, and 2 U of Hph I (NEB). The digests were incubated at 37°C for 2 hours and run on a 1.2% agarose gel containing ethidium bromide. Abbreviations are the same as in Fig. 2.

entire plasmid previously linearized with Bam HI or Pst I (Fig. 3). Creation of a unique Hph I site within the o_L1 operator site is demonstrated by the presence of only two bands in lanes 4 and 8. All other Hph I sites were inactivated by the M·Hph I used in the presence of λ repressor.

These experiments (see legend to Fig. 3) consisted of the following five steps: (i) addition of λ repressor followed by M·Hph I, (ii) thermal inactivation of M·Hph I, (iii) RecA-mediated cleavage of λ repressor (*10*), (iv) phenol extraction and ethanol precipitation, and (v) cleavage with Hph I. The additional steps are necessary because λ repressor, unlike *lac* repressor, is not irreversibly inactivated by heating to 75°C. The repressor protein must be cleaved by the RecA protease to prevent dimerization and cooperative binding to DNA, which is stable enough to carry the DNA into the phenol phase during the subsequent extraction. Such phenol extraction is necessary to remove NH_2-terminal repressor fragments that bind specifically to the operator and inhibit Hph I cleavage. As an alternative to treatment with RecA, the reactions can be adjusted to *p*H 10, under which conditions λ repressor undergoes autodigestion (*11*).

We have demonstrated efficient operator-specific cleavage in the *lacO* and λ o_L systems. The approach described, however, is not limited to these two systems. We anticipate that it will be applicable to many other methylases in combination with repressors or any of a wide range of other proteins that form stable, sequence-specific complexes with DNA. The particular choice of methylase and "blocking protein" would be dictated by the specificity and frequency of cleavage desired. For example, *lac* repressor mutants with altered binding specificity or affinity (*12, 13*) would generate modified cleavage patterns.

The sizes of fragments that would be generated from a digest of genomic DNA by means of this method would depend on the blocking protein used to protect restriction sites from methylation. For example, protection of restriction sites in mammalian DNA by *lac* repressor should result in very little cleavage, if any, because statistically a 20-bp recognition sequence would be expected to occur only once in every 1.1×10^9 kb. However, if eukaryotic transcription factors could be used to block methylation, the DNA should be cleaved near the genes under the control of the particular transcription factor used. Other factors that would influence the fragment size include the stability of protein binding to variant sites and the frequency of overlap between restriction sites and such variant binding sites.

The types of blocking proteins that will prove useful for this technique remain to be seen. We anticipate that many proteins involved in the regulation of transcription (for example, repressors and transcription factors) will form DNA-protein complexes stable enough to exclude a modification methylase. In addition, the use of restriction enzymes to block methylation of overlapping sequences is being investigated.

This approach, which we would like to designate "Achilles' heel cleavage" (AC), should facilitate the mapping and manipulation of large DNA molecules. A specific gene, for instance, could be tagged by integration of a mobile genetic element containing a cleavable operator, thus allowing physical localization and isolation by a variety of methods, including the use of an affinity (repressor-coated) column. Furthermore, these rare sites are ideal for use in large cloning vehicles requiring unique restriction sites, such as yeast artificial chromosome vectors (*14*). Our methods create the equivalent of new restriction enzymes of extremely high specificity and provide a powerful new tool for genetic manipulation.

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Insensitivity to Ethylene Conferred by a Dominant Mutation in *Arabidopsis thaliana*

ANTHONY B. BLEECKER,* MARK A. ESTELLE,† CHRIS SOMERVILLE, HANS KENDE

Ethylene influences a number of developmental processes and responses to stress in higher plants. The molecular basis for the action of ethylene was investigated in mutants of *Arabidopsis thaliana* that have altered responses to ethylene. One mutant line, which has a dominant mutation at a locus designated *etr*, lacks a number of responses to ethylene that are present in the wild-type plant. These include inhibition of cell elongation, promotion of seed germination, enhancement of peroxidase activity, acceleration of leaf senescence, and feedback suppression of ethylene synthesis by ethylene. These diverse responses, which occur in different tissues of *Arabidopsis*, appear to share some common element in their transduction pathways—for example, a single receptor for ethylene. Results of ethylene binding experiments in vivo indicate that this receptor may be affected by the *etr* mutation.

THE GASEOUS COMPOUND ETHYLENE is an endogenous regulator of growth and development in higher plants. Increases in the level of ethylene influence many developmental processes, from seed germination and seedling growth (*1*) to leaf abscission, organ senescence, and fruit ripening (*2*). A number of environmental stresses including oxygen deficiency, wounding, and pathogen invasion enhance ethylene synthesis (*3*). Stress-induced ethylene elicits adaptive changes in plant development—for example, the formation of adventitious roots and air-conducting channels in water-logged terrestrial plants and rapid stem elongation in submerged semiaquatic plants (*4*). Wounding and pathogen invasion may result in ethylene-mediated acceleration of senescence and abscission of infected organs and in the induction of specific defense proteins such as chitinase, glucan-

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

*Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.
†Present address: Biology Department, Indiana University, Bloomington, IN 47405.