AI-3 and *AI-4* intron could render other target genes cold-sensitive as well. We have inserted these cold-sensitive introns into the Stu I site of the yeast *URA3* gene (*URA3-AI3*, *URA3-AI4*). As expected, the yeast strain having either the *URA3-AI3* or the *URA3-AI4* gene on a 2μ plasmid showed a cold-sensitive URA phenotype (Fig. 2C). It is not likely that cold sensitivity is due to the genetic background, since experiments with another nonisogenic strain gave the same results (*14*).

In this report we have described an approach to the control of gene expression by an artificial cold-sensitive intron. This approach can be applied to foreign gene expression driven by a strong promoter whose expression is difficult to control. Further modification of the artificial intron will be necessary, however, to obtain optimal expression in the induced state and no expression at the low temperature.

This intron makes it possible for investigators to disrupt gene function conditionally, which will be helpful in studying the function of essential genes and in isolating mutants. For mutagenesis, it may be necessary to mark the cold-sensitive intron with a genetic marker.

In addition, the artificial intron can be modified in many other ways. For example, an operator sequence could be inserted to control transcription of a target gene by binding of a repressor. A native intron that has self-regulated splicing can also be used. Such a native intron cassette can be easily obtained by amplifying only the intron sequence by polymerase chain reaction (PCR) methods (*22*). This type of native intron cassette as well as artificial introns would allow us to control any gene in yeast in a variety of ways.

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13. The 2μ plasmids were constructed as follows: An artificial intron was inserted into *URA3* on plasmid YIp5. An Sph I–Pvu II fragment containing *URA3* was released from the plasmid, then cloned into the Sph I–Pvu II site of YEp13, which contains the 2μ origin and *LEU2*.
14. T. Yoshimatsu and F. Nagawa, unpublished observations.
15. The Mbo II cleavage site at the initiation codon ATG of yeast *PGK* gene [S. M. Kingsman, *Nucleic Acids Res.* **10**, 2625 (1982); R. A. Hitzeman *et al.*, *ibid.*, p. 7791] was converted to a blunt end with T4 polymerase, then ligated with the Nsi I linker (TGCATGCATGCA) in order to generate the Sph I site that overlaps with the initiation codon. The resulting plasmid was digested by Sph I and treated with T4 polymerase to generate the blunt end just after the ATG. A Bgl II linker (GCAGATCTGC) or the artificial intron fragment (previously ligated with the Bgl II linker at the 3′ end) was ligated to the blunt end. The fragments (1.7 kb and 1.6 kb) containing the *PGK* promoter and ATG with or without the intron were released by Hind III and Bgl II, then ligated into the Hind III–Bam HI site of plasmid pFN8 (*25*), which contains the *Escherichia coli lacZ* gene (from the eighth codon to the end) and *URA3* as a selectable marker. The resulting plasmids were used to transform the yeast strain NY6A (*MAT***a**, *ura3-52*, *leu2-3*, *leu2-112*, *his4-519*).
16. A 33-bp Pst I–Sna BI fragment containing the 5′ consensus sequence 5′ GTATGT 3′ was released from the pUC-AI plasmid (Fig. 1). This fragment was inserted between Pst I and Klenow-treated Xba I sites of the pUC-AI plasmid. The resulting plasmid is called pUC AI-1.
17. A 38-bp Sac I–Pvu II fragment containing the TACTAAC box was released from the pUC-AI plasmid (Fig. 1). This fragment was inserted between Sac I and Klenow-treated Xba I sites of the pUC-AI plasmid. The resulting plasmid is called pUC AI-2.
18. Plasmid pUC AI-1 (*16*) was digested with Pst I and then treated with "Slow" Bal 31 nuclease (Takara shuzo) for 5 to 120 s. Xba I linkers were added to the ends, and the fragment containing the 3′ half of the intron was released by Xba I and Sca I, then ligated with the Xba I–Sca I fragment of pUC-AI (which contains the 5′ half of the intron).
19. Plasmid pUC AI-2 (*17*) was digested with Sal I and then treated with "Slow" Bal 31 nuclease as described (*18*). Xba I linkers were added, and the fragment containing the 5′ half of the intron was released by Xba I and Sca I, then ligated with the Xba I–Sca I fragment of pUC-AI, which contains the 3′ half of the intron.
20. Each plasmid containing a deleted intron was used to transform the yeast strain NY6A. Transformants were transferred to X-gal plates (*24*). Plates were then incubated either at 36°C or at 16°C for 3 days.
21. *AI-3* retains a 13-bp inverted repeat. *AI-1* derivatives retaining 17-bp and longer or 10-bp and shorter inverted repeats were not cold-sensitive (*14*). *AI-4* retains a 17-bp inverted repeat. *AI-2* derivatives retaining 21-bp and longer or 13-bp and shorter inverted repeats were not cold sensitive (*14*).
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26. We thank K. Miyoshi and T. Kawazoe for providing oligonucleotides, and Y. Shimura, D. A. Shub, and S. Ohno for critical reading of the manuscript.

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Isolation of Single-Copy Human Genes from a Library of Yeast Artificial Chromosome Clones

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A recently developed cloning system based on the propagation of large DNA molecules as linear, artificial chromosomes in the yeast *Saccharomyces cerevisiae* provides a potential method of cloning the entire human genome in segments of several hundred kilobase pairs. Most applications of this system will require the ability to recover specific sequences from libraries of yeast artificial chromosome clones and to propagate these sequences in yeast without alterations. Two single-copy genes have now been cloned from a library of yeast artificial chromosome clones that was prepared from total human DNA. Multiple, independent isolates were obtained of the genes encoding factor IX and plasminogen activator inhibitor type 2. The clones, which ranged in size from 60 to 650 kilobases, were stable on prolonged propagation in yeast and appear to contain faithful replicas of human DNA.

IN APPLICATIONS THAT REQUIRE THE analysis of large tracts of genomic DNA, the yeast artificial chromosome (YAC) cloning system has a number of potential advantages over conventional cloning methods (*1*). Its open-ended capacity for large inserts has allowed the cloning of segments of human DNA that are ten times larger than those that can be cloned in cosmids (*1, 2*). As a eukaryotic host, yeast provides a substantially different environment than *Escherichia coli* in which to propagate the DNA of higher organisms. The ease

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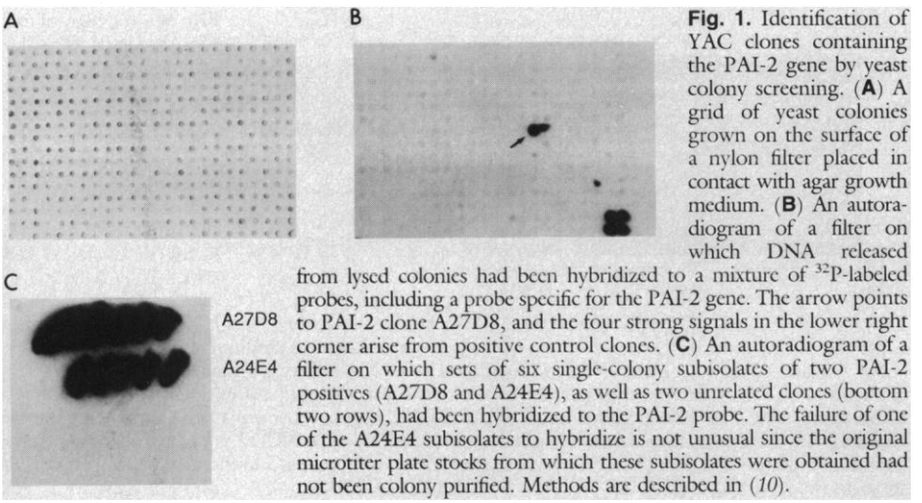

Fig. 1. Identification of YAC clones containing the PAI-2 gene by yeast colony screening. (**A**) A grid of yeast colonies grown on the surface of a nylon filter placed in contact with agar growth medium. (**B**) An autoradiogram of a filter on which DNA released from lysed colonies had been hybridized to a mixture of ^{32}P-labeled probes, including a probe specific for the PAI-2 gene. The arrow points to PAI-2 clone A27D8, and the four strong signals in the lower right corner arise from positive control clones. (**C**) An autoradiogram of a filter on which sets of six single-colony subisolates of two PAI-2 positives (A27D8 and A24E4), as well as two unrelated clones (bottom two rows), had been hybridized to the PAI-2 probe. The failure of one of the A24E4 subisolates to hybridize is not unusual since the original microtiter plate stocks from which these subisolates were obtained had not been colony purified. Methods are described in (*10*).

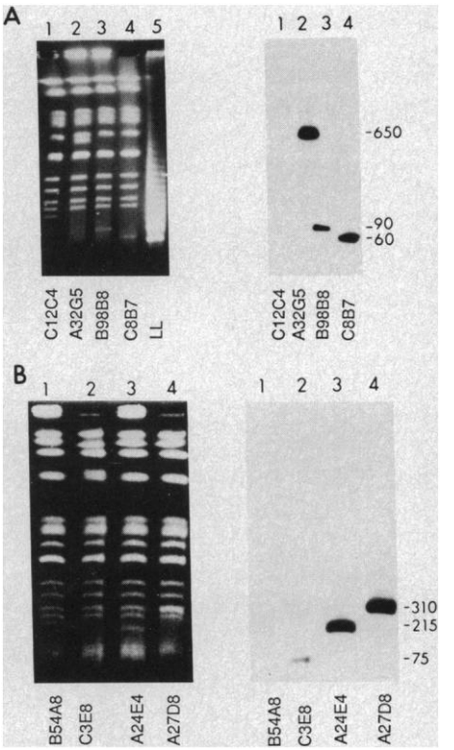

Fig. 2. Analysis of factor IX and PAI-2 YAC clones by pulsed-field gel electrophoresis. (**A**) A pulsed-field gel showing the separation of natural and artificial chromosomes present in three clones that showed strong hybridization to the factor IX probe (lanes 2 to 4) and an unrelated YAC clone (lane 1). In the left panel, the DNA molecules are visualized by fluorescent staining with ethidium bromide; in the right panel, an autoradiogram is shown of the results obtained when the DNA was transferred to a nitrocellulose membrane and hybridized to the factor IX probe. LL, lambda ladder. (**B**) Analysis of the three clones that showed strong hybridization to the PAI-2 probe (lanes 2 to 4) and an unrelated YAC clone (lane 1) on a pulsed-field gel. Sizes are in kilobases. Methods are described in (*11*).

with which chromosomal DNA can be manipulated by homology-directed recombination in yeast (*3*) offers the possibility of making specific changes in large DNA molecules cloned as YACs.

These and other applications of YAC cloning will require the ability to recover specific cloned sequences from diverse libraries of YAC clones. We describe here the cloning of single-copy genes from a YAC library prepared from total human DNA. Two independent clones are described for the factor IX gene (*4*) (mutations in which cause hemophilia B), and three independent clones are described for the gene encoding plasminogen activator inhibitor type 2 (PAI-2) (*5*). Evidence is presented that these clones, which range in size up to 650 kb, are authentic replicas of human genomic DNA. It is also shown that they can be propagated extensively in yeast without detectable changes. Together with recent reports of YAC cloning of DNA from the nematode *Caenorhabditis elegans* (*6*) and from a rodent-human hybrid cell line (*7*), these results demonstrate the basic practicality of using YACs to subdivide complex genomes.

The human genomic library was prepared by ligating human DNA that had been partially digested with Eco RI to vector arms prepared from pYAC4 (*1, 8*). This procedure produces acrocentric chromosomes that terminate in short telomere-proximal vector sequences. All but 9 kb of the length of an artificial chromosome is insert DNA. The clones described here were identified in an expanding library that contained 13,000 clones at the time of the PAI-2 screen and 14,000 at the time of the factor IX screen. The average insert size in the library was approximately 225 kb. The clones had been picked individually from transformation plates, grown in small liquid cultures, and stored in the wells of 96-well microtiter plates.

The clones were screened with ^{32}P-labeled DNA-DNA hybridization probes by colony-screening procedures adapted for yeast. Colonies were grown on nylon filters placed on the surface of an agar growth medium. The filters were inoculated by means of a 96-prong replicating device, which allowed all the clones from one microtiter plate to be stamped onto a filter in a single step. Four offset stampings were carried out to produce microtiter plate–sized filters, each containing 384 clones (Fig. 1A). After cell lysis and binding of the released DNA to the filter, hybridization was carried out with pools of up to five ^{32}P-labeled probes. An autoradiogram of a filter containing a clone that was positive for the PAI-2 probe is shown in Fig. 1B. Each potentially positive clone was colony purified on a medium that selects for maintenance of the YAC, and six subisolates were retested with all the probes used in the primary screening. Two clones in which most or all of the colonies picked for secondary screening were positive with the PAI-2 probe are shown in Fig. 1C. Similar signals were obtained for a third PAI-2 clone and three factor IX clones.

The intact artificial chromosomes in clones that continued to give strong signals through the secondary colony screening were analyzed by pulsed-field gel electrophoresis. When high molecular weight DNA from these strains is separated on pulsed-field gels, the artificial chromosomes can usually be visualized against the background of natural yeast chromosomes by ethidium bromide staining. After the stained gels were photographed, the DNA was transferred to nylon, and DNA-DNA hybridization was carried out with the appropriate gene-specific probe. Data are shown in Fig. 2 for the three factor IX and the three PAI-2 clones that were positive through the primary and secondary screening.

The next level of analysis involved comparing the sequence organization of YAC DNA with that of human genomic DNA by means of restriction enzymes with 4- or 6-bp recognition sites. Gene-specific probes derived from factor IX and PAI-2 cDNA clones were used to analyze the regions of the YACs containing the exons for these genes. In a typical analysis (Fig. 3A), the factor IX probe was used to detect hybridizing Eco RI restriction fragments in digests of human genomic DNA and yeast DNA from the 60- and 650-kb factor IX YAC clones. In addition to the pure human and yeast samples, mixtures containing human DNA and a molar excess of yeast DNA were also analyzed to demonstrate that small differences in the mobilities of corresponding fragments in the human and yeast lanes were due to differences in the amounts of DNA loaded (*9*). All four of the fragments detected in human genomic DNA were also found

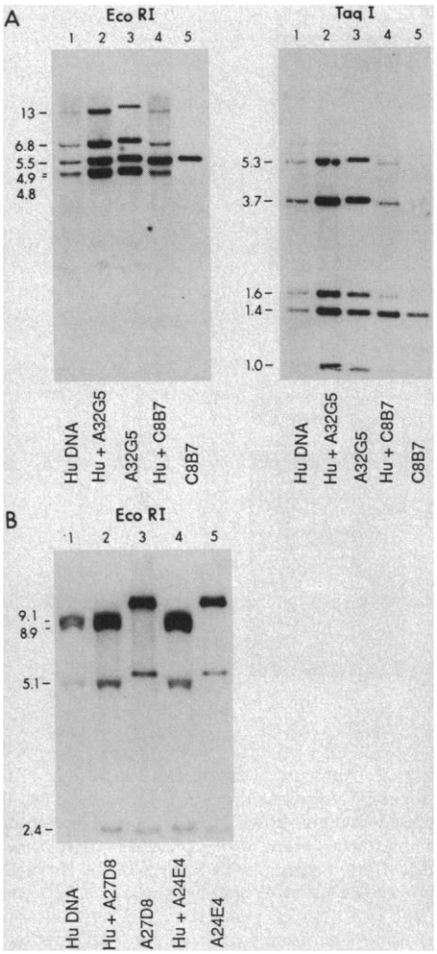

Fig. 3. Comparison of the sizes, in kilobases, of the exon-containing restriction fragments present in total human DNA and the factor IX and PAI-2 YAC clones. (**A**) An autoradiogram showing the hybridization of Eco RI and Taq I digests of human and yeast DNA with the factor IX cDNA probe. The fragments in these digests were separated by size on an agarose gel (*9*) and transferred to a nylon membrane for hybridization. In both panels, lane 1 contains digests of total human DNA prepared from the lymphoblastoid cell line CGM-1, which was derived from the same individual whose DNA was used to prepare the YAC library. The remaining lanes contain either yeast DNA from a factor IX YAC clone (lanes 3 and 5) or mixtures of total human DNA, in the same amount used in lane 1, with an approximately threefold molar excess of DNA from a factor IX YAC clone (lanes 2 and 4). Clone A32G5, 650 kb; clone C8B7, 60 kb. (**B**) Eco RI digests hybridized with the PAI-2 cDNA probe. The layout of the gel is the same as in (A).

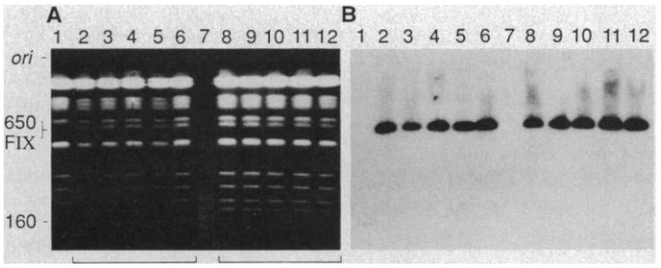

Fig. 4. Stability of the 650-kb factor IX clone during prolonged propagation in yeast. (**A**) Fluorescent staining pattern of a pulsed-field gel showing the separated natural and artificial chromosomes from ten subisolates of the 650-kb factor IX clone A32G5 (lanes 2 to 6 and 8 to 12) and an unrelated YAC (lane 1, YY212). The subisolates of A32G5 had been separately cultured for 60 generations in either the rich medium YPD (lanes 2 to 6) or the ura$^-$ trp$^-$–selective medium AHC$^-$ (lanes 8 to 12). (Lane 7 is lambda ladder.) Electrophoretic conditions were as described for Fig. 3A. (**B**) Hybridization of the 650-kb YAC in the ten subisolates of A32G5 to the factor IX probe after transfer of the DNA to a nylon membrane. The weak hybridization of the YY212 negative control to the factor IX probe is probably due to minor contamination of the factor IX probe with pBR322 sequences that are present both in the factor IX cDNA clone from which the probe was prepared and the YAC vectors. Methods are as those described for Fig. 2.

in the 650-kb YAC. In contrast, the 60-kb YAC contained only the 5.5-kb Eco RI fragment, to which the last two exons at the 3′ end of the factor IX gene have been mapped (*4*).

Comparable analyses of the factor IX clones with Taq I again show that the 650-kb YAC contains all the hybridizing fragments present in human genomic DNA, whereas the 60-kb YAC contains only one of the fragments (Fig. 3A). In contrast, the 90-kb YAC that hybridized to the factor IX probe (Fig. 2A) gave grossly discordant results in this test. The origins of this clone and the source of its hybridization to the factor IX probe remain uncertain, but preliminary data with other enzymes and probes indicate that its restriction map has little in common with the 60- and 650-kb clones.

The three PAI-2 clones were also analyzed with Eco RI, Hind III, and Bam HI. Probings of Eco RI digests of the 215- and 310-kb PAI-2 clones, as well as of human genomic DNA, with a PAI-2 cDNA probe are shown in Fig. 3B. Both clones contain all the Eco RI fragments that the probe recognizes in human genomic DNA. The results for these two clones with Hind III and Bam HI also indicate that both clones contain complete copies of the PAI-2 gene. The results for the 75-kb PAI-2 YAC showed that, like the 60-kb factor IX clone, it breaks in the middle of the gene. Taken together, these data indicate that two of the factor IX YACs and all three PAI-2 YACs are free of detectable rearrangements in the regions of the exons. Comparable analysis on a coarser scale with restriction enzymes that cleave human DNA infrequently is difficult because most or all of these enzymes are sensitive to the methylation of their recognition sites. All the sites for these enzymes are expected to be cleavable in yeast, which has no known cytosine methylation, whereas only a subset of the sites are cleavable in mammalian genomic DNA.

To test another aspect of YAC fidelity, we evaluated the stability of the clones during prolonged propagation in yeast. Sets of subclones of the 60- and 650-kb factor IX clones were grown independently for 60 generations. The experiment started with ten single-colony isolates of each clone. These subclones were grown en masse for 35 generations, taken through a second single-cell isolation step, and then grown for another 25 generations. High molecular weight DNA prepared from the cells produced during the second growth phase was analyzed by pulsed-field gel electrophoresis. As shown for the 650-kb factor IX clone (Fig. 4), none of the subclones changed detectably during this procedure. Identical results were obtained for the 60-kb factor IX clone.

The cloning of single-copy genes that can be stably propagated in yeast from a YAC library prepared from total human DNA establishes the basic feasibility of using YACs for the initial partitioning of the human genome. Only further experience will indicate what fraction of the human genome can be cloned in this way. However, a preliminary comparison of the coverage of the nematode genome obtained with cosmids and YACs suggests that many systematic absences in the cosmid library involved sequences that could be cloned in YACs (*6*).

We detected the PAI-2 and factor IX clones in a library of 13,000 to 14,000 clones with inserts averaging 225 kb in size, prepared from male DNA. The expected representation of an autosomal gene in this library is 1.0, whereas that for an X-linked gene is 0.5. The probability of finding a particular autosomal gene triply represented (as was observed for PAI-2) is calculated from the Poisson distribution to be 0.06; the probability of finding a specific X-linked gene doubly represented (as was observed for factor IX) is calculated to be 0.08. Because we chose the PAI-2 and factor IX clones for initial characterization after screening the library with a total of 11 probes to single-copy genes, the detection of two multiply represented genes is not unexpected. Most other probes appeared to give one or zero positives, but the data are too

preliminary to allow conclusive statistical analysis.

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9. The digests were fractionated on 0.8% agarose gels and transferred to nylon membranes for hybridization. The pure human samples contained 10 to 15 μg of DNA, whereas the pure yeast samples contained 0.1 to 0.2 μg; the mixed samples contained 10 to 15 μg of human and 0.1 to 0.2 μg of yeast DNA. Small differences in the electrophoretic mobilities of corresponding fragments detected in the human and yeast lanes of Fig. 3 are due to differences in the amounts of DNA loaded in the two cases. Because of the 200-fold disparity in the sizes of the yeast and human genomes, 50 ng of YAC-containing yeast DNA gives a hybridization signal comparable to that obtained from 10 μg of human DNA. When 10 to 15 μg of human DNA are mixed with 0.1 to 0.2 μg of yeast DNA the electrophoretic mobilities of the hybridizing bands are identical to those observed in the pure human samples, but the intensities of bands that contain YAC-derived fragments are enhanced (lanes 2 and 4 of all panels).
10. Methods of producing YAC clones from Eco RI partial digests of high molecular weight human DNA are described elsewhere (*1, 8*). Clones that were ura^+, trp^+, and lacked *ochre* suppression were grown to saturation in 1-ml cultures in the rich medium YPD [F. Sherman, G. R. Fink, J. B. Hicks, *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), p. 163]. Samples were adjusted to a glycerol concentration of 20% and stored at −80°C in microtiter plates. A custom-fabricated, multiprong replicator was used to stamp the colony grids onto SUREBLOT nylon membranes (Oncor), which were then placed in contact with the ura^- trp^- medium $AHC^=$ [per liter: 1.7 g of yeast nitrogen base (Difco 0335-15), 5 g of $(NH_4)_2SO_4$, 10 g of casein hydrolysate (U.S. Biochemicals 12852), 20 g of glucose, and 20 mg of adenine (*p*H 5.8)] and incubated at 30°C. Cells were converted to spheroplasts and lysed by sequentially placing the nylon filters in contact with a series of reagent-saturated paper filters. In the order of use, the reagents and treatment times were as follows: lyticase mixture CDY [yeast lytic enzyme (2 mg/ml) (ICN 152270, >70,000 U/g), 1.0*M* sorbitol, 0.1*M* sodium citrate, 0.05*M* EDTA, and 0.015*M* dithiothreitol (*p*H 7)], overnight; 10% SDS, 5 min; 0.5*M* NaOH, 10 min; 2× saline sodium citrate, *p*H 7, and 0.2*M* tris HCl, *p*H 7.5, three times for 5 min each. The lyticase reaction was carried out at 30°C, and the remaining treatments were at room temperature. Filters were air-dried. Labeling of probes, hybridization, and autoradiography were performed in accordance with standard methods [A. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983); T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)].
11. Methods of preparing high molecular weight yeast DNA in agarose plugs have been described [D. C. Schwartz and C. R. Cantor, *Cell* **37**, 67 (1984); G. F. Carle and M. V. Olson, *Methods Enzymol.* **155**, 468 (1987)]. For electrophoresis, the contour-clamped homogeneous electric field system was used [G. Chu, D. Vollrath, R. W. Davis, *Science* **234**, 1582 (1986)]. The electric field was 6.0 V/cm, the temperature 12° to 14°C, the running time 22 to 24 hours, and the running buffer was half-strength tris borate (Carle and Olson, above). For the gel in Fig. 2A, a ramped switching interval starting at 35 s and ending at 70 s was used, and for the gel in Fig. 2B, a constant switching interval of 70 s was used. The former conditions provide better resolution in the region most relevant for the analysis of typical YACs. The probe for factor IX was prepared by gel purifying the 1.0-kb Eco RI–Hind III insert in a factor IX cDNA clone whose insert spans nucleotides 1–1029, as numbered by M. Jaye *et al.* [*Nucleic Acids Res.* **11**, 2325 (1983)], and which recognizes all eight of the factor IX exons; the hybridization of this probe with Taq I–digested human DNA has been described [G. Camerino, M. G. Mattei, J. F. Mattei, M. Jaye, J. L. Mandel, *Nature* **306**, 701 (1983)]. The probe for PAI-2 was prepared by gel purifying the 1.9-kb full-length cDNA fragment described in (*5*), which had been subcloned into the Eco RI site of pGEM-4.

12. We acknowledge R. Rothstein for providing an unpublished yeast colony-screening protocol. M. A. Hutchinson, P. Dang, S. C. Silver, and M. Lackey provided technical assistance. J. L. Mandel provided the factor IX cDNA clone and J. E. Sadler provided the PAI-2 cDNA clone. R. Waterston made helpful suggestions on the manuscript. Supported by a grant from the James S. McDonnell Foundation to the Center for Genetics in Medicine, Washington University, St. Louis, MO, and by NIH grant GM40606. G.A.S. was supported by a National Research Service Award (HD07271) from the National Institute of Child Health and Human Development.

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Cl^- Channels in CF: Lack of Activation by Protein Kinase C and cAMP-Dependent Protein Kinase

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Secretory chloride channels can be activated by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase in normal airway epithelial cells but not in cells from individuals with cystic fibrosis (CF). In excised, inside-out patches of apical membrane of normal human airway cells and airway cells from three patients with CF, the chloride channels exhibited a characteristic outwardly rectifying current-voltage relation and depolarization-induced activation. Channels from normal tissues were activated by both cAMP-dependent protein kinase and protein kinase C. However, chloride channels from CF patients could not be activated by either kinase. Thus, gating of normal epithelial chloride channels is regulated by both cAMP-dependent protein kinase and protein kinase C, and regulation by both kinases is defective in CF.

THE APICAL Cl^- CONDUCTANCE OF airway epithelia controls transepithelial Cl^- secretion. The magnitude of Cl^- secretion, which is regulated by several secretagogues, determines the quantity of respiratory tract fluid (*1*). The apical Cl^- conductance of epithelia from patients with CF is abnormally low because of an inability of β-adrenergic agonists to increase the magnitude of the apical Cl^- conductance (*2*). This abnormality contributes to the production of thick mucous in CF patients.

Airway epithelia contain outwardly rectifying Cl^- channels that are activated through cAMP-dependent pathways (*3*). The purified catalytic subunit of cAMP-

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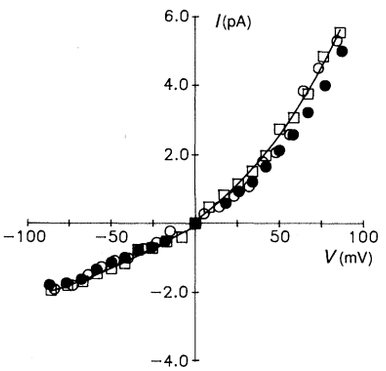

Fig. 1. Current-voltage relations (*I-V*) of Cl^- channels activated by PKC in normal human fetal tracheal cells and by depolarizing voltages both in adult normal and CF airway cells. The number of channels in a patch varied from one to four. Data were recorded by an EPC-7 patch-clamp amplifier (List Darmstadt, Federal Republic of Germany), filtered at 1 kHz (Frequency Devices, Haverhill, MA) digitized with a pulse-code modulator (Japanese Victor Corporation), and stored on video cassette recorder (Japanese Victor Corporation). Data were analyzed on a PDP 11/23 computer (Digital Equipment Corporation). The bath solution contained 150 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* EGTA, 5 m*M* Hepes, and 0.5 m*M* $CaCl_2$ (free Ca^{2+} was 1.2 μ*M*). The pipet contained 150 m*M* NaCl, 2 m*M* $CaCl_2$, and 5 m*M* Hepes. The *p*H of both solutions was adjusted to 7.3 with tris base. With 300 m*M* Cl^- in the bath, the reversal potential (15 mV) was close to that expected for the Cl^- concentration gradient (16.7 mV). Graph was drawn by Sigmaplot Version 3.1 (Jandel Scientific). ●, human fetus; ○, human adult; □, human CF.