imity to the blood stream endothelial cells are an obvious candidate for delivering therapeutic proteins systemically. Genetically engineered vascular grafts could also be used to target the delivery of a therapeutic protein to a specific organ or limb that is perfused by blood passing through the grafted artery. Specific applications might include the secretion of vasodilators or angiogenic factors to ischemic myocardium or the delivery of an antineoplastic agent to an organ riddled with metastatic tumor.

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Control of Gene Expression by Artificial Introns in *Saccharomyces cerevisiae*

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Artificial yeast introns that show cold-sensitive splicing have been constructed. These conditional introns can be inserted into a target gene as an "intron cassette" without disrupting the coding information, allowing expression of the gene to be cold sensitive. Insertion of these intron cassettes rendered the yeast *URA3* gene cold sensitive in its expression. The advantage of this intron-mediated control system is that any gene can be converted to a controllable gene by simple insertion of an intron.

ONLY A SMALL NUMBER OF GENES in *Saccharomyces cerevisiae* contain introns, which are usually found individually near the 5′ end of their genes (*1*). The role of such rare introns is not clear. The removal of an intron from the yeast actin gene did not affect its expression (*2*). Nevertheless, they do appear to have a regulatory function in some cases. For example, splicing of an intron in the yeast ribosomal gene *RPL32* is autogenously controlled by its product (*3*).

Each yeast intron contains three conserved sequences, GUAPyGU (Py, pyrimidine) at the 5′ donor end, UACUAAC at the branch point, and PyAG at the 3′ acceptor end (*4*). Base pairing between small nuclear RNAs (snRNAs) and GUAPyGU and UACUAAC sequences is thought to be important for efficient splicing (*5*). These consensus sequences are similar to those found in higher eukaryotes. However, the sequence requirement is more stringent in yeast than in higher eukaryotes (*6*). In addition, there are no conserved sequences in the flanking exons of yeast.

Heterologous introns have been successfully inserted into target genes in order to study mRNA splicing (*2, 3, 5, 7, 8*). The same strategy was also used to study Ty transposition (*9*) and activation at upstream sites (*10*). However, in these investigations, an intron was inserted along with its flanking exon sequences. These flanking sequences can cause inactivation of the target gene. Therefore, we have inserted an artificial intron totally devoid of flanking exon sequences and have used it to control the heterologous gene expression.

The intron consisted of 30 bp of the 5′ end of the yeast *RP51A* intron (*7*), 30 bp of the 3′ end of the yeast *S10* intron (*11*), and 44 bp of polylinker sequence of an M13 cloning vector (Fig. 1). This intron contains all the necessary sequences for splicing in yeast. In addition, we have placed Sna BI and Pvu II restriction sites at the ends. Since the Sna BI and Pvu II recognition sequences overlap with the three terminal bases at each intron end, the artificial intron can be cleaved out precisely as a "cassette" without any additional exon sequences. This intron fragment can be inserted into any gene without disrupting its coding information by cutting the gene with a blunt end–producing restriction enzyme and then inserting the intron fragment. Alternatively, if the restriction enzyme leaves a protruding 5′ terminus, the fragment can be treated at one end with single strand–specific nuclease and at the other end by the Klenow fragment of DNA polymerase I.

To show that the artificial intron can be inserted into a gene without disrupting its function, we inserted it into the *URA3* coding region at Eco RV, Sca I, or Stu I sites (*12*), which are located 186, 310, and 434 bp downstream of the start codon, respectively. These intron-containing *URA3* genes were located on a "2μ plasmid" construct (*13*). This construct was able to complement a *ura3-52* mutation on the yeast chromosome (*14*), which suggests that the artificial intron is spliced out precisely and

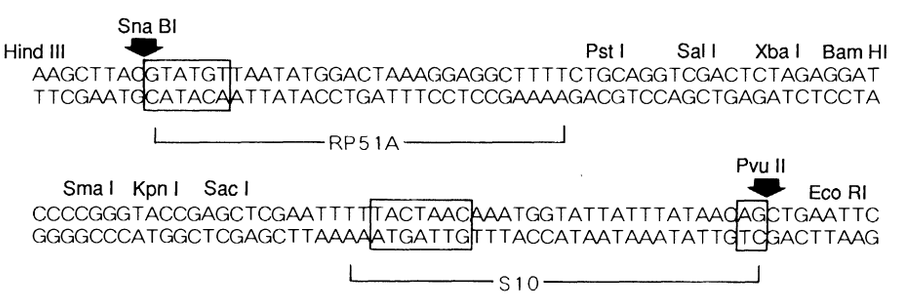

Fig. 1. Structure of the artificial intron. The 5′ end fragment and the 3′ end fragment were chemically synthesized, and the polylinker fragment (from Pst I to Sac I) was obtained from plasmid pUC18. The consensus sequences are shown in boxes. This intron was cloned in the Hind III–Eco RI site of pUC18 to generate pUC-AI. The 5′ end fragment of the yeast *RP51A* intron and the 3′ end fragment of the *S10* intron are indicated.

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Table 1. β-Galactosidase activity of the *PGK-lacZ* fusion gene with an intron cassette. See (*15–17, 21*) for details of the construction of the *PGK-lacZ* gene with and without the modified introns. Cells containing CEN plasmids were grown in minimal medium SD (*23*) supplemented with histidine and leucine for at least 20 generations at the indicated temperature. Assay for the β-galactosidase was performed as described (*24*). Each value represents the average of four experiments and the error was less than 30%. AI, artificial intron; AI-1 and AI-2, AI with inverted repeats; AI-3 and AI-4, cold-sensitive introns.

Construct	β-Galactosidase activity (units/mg protein)		
	36°C	23°C	16°C
None	750	860	1190
AI	870	920	1150
AI-1	7	6	8
AI-2	8	5	6
AI-3	120	21	11
AI-4	200	54	23

further implies that the artificial intron can be inserted anywhere in a target gene without disrupting its function.

To determine the efficiency and the accuracy of splicing, we inserted this intron into the *PGK-lacZ* fusion gene (*15*). The β-galactosidase activity produced by the intron-containing gene (*PGK-lacZ-AI*) was about the same as that produced by the intronless *PGK-lacZ* gene (Table 1). The level of mRNA produced by the *PGK-lacZ-AI* gene was also about the same, and a majority of the *lacZ* transcript was of the mature form (Fig. 2A), indicating that splicing of the artificial intron was efficient and accurate.

Base pairing between consensus sequences of an intron and snRNAs is important for the splicing reaction (*5*). Accordingly, we thought that introduction of a fragment complementary to one of the consensus sequences would be likely to interfere with this base pairing and thus prevent splicing. If so, by varying the length of the complementary fragments, we might be able to obtain a fragment that interferes with splicing at a low temperature but not at a high temperature. Splicing of the intron containing such a fragment would then become cold-sensitive.

To see the effect of an inserted fragment that can base pair with one of the consensus sequences, we have added a fragment that contained either the 5′ donor consensus sequence (GUAUGU) or the branch point sequence (UACUAAC) into the artificial intron in the inverted orientation. These two constructs contain 33-bp (*AI-1*) and 38-bp (*AI-2*) inverted repeats, respectively (*16, 17*). Yeast were transformed with plasmids containing the *PGK-lacZ* fusion gene plus one of these modified introns, and the level of β-galactosidase activity was measured. The level of β-galactosidase activity was about 1/100 of that of the parent (Table 1). Mature mRNA was not detected by primer extension (*14*). These results indicate that splicing is inhibited by an inverted repeat structure at the 5′ consensus region or the UACUAAC region. The inverted repeat structure itself was not responsible for this inhibition, since unrelated inverted repeats in the intron did not prevent splicing (*14*).

To obtain cold-sensitive introns, we made Bal 31 deletions of the *AI-1* and *AI-2* introns, starting either from the center of the inverted repeat (for *AI-1*) (*18*) or from the outside of the inverted repeat (for *AI-2*) (*19*). Introns with various sizes of inverted repeats were isolated and then inserted into the *PGK-lacZ* fusion gene. We looked for cold-sensitive introns by growing each yeast transformant on X-gal plates at either 36°C or 16°C (*20*). If the introns were spliced out, the cells would produce active β-galactosidase and form blue colonies; otherwise, white colonies would be observed. We found two deleted introns that led to the formation of blue colonies at 36°C and white colonies at 16°C, one from *AI-1*, the other from *AI-2*. We call these cold-sensitive introns *AI-3* (13-bp inverted repeat) and *AI-4* (17-bp inverted repeat), respectively (*21*). The β-galactosidase activities produced by these genes (Table 1) were clearly heat inducible, although even at the highest temperature the activities were 15 to 20% of those produced by the intronless gene. Analysis of the mRNAs by primer extension showed that these genes produce the mature size of mRNA at 36°C but not at 16°C (Fig. 2A). The possible RNA secondary structures of these introns are shown in Fig. 2B. In both cases the consensus sequence, GUAUGU or UACUAAC, is in a short hairpin structure. These short hairpin structures may be formed at 16°C (preventing splicing of the intron), but melt at 36°C (allowing the intron to be spliced out) in vivo. We have not ruled out the possibility that other cellular factors are involved.

We wanted to show that insertion of the

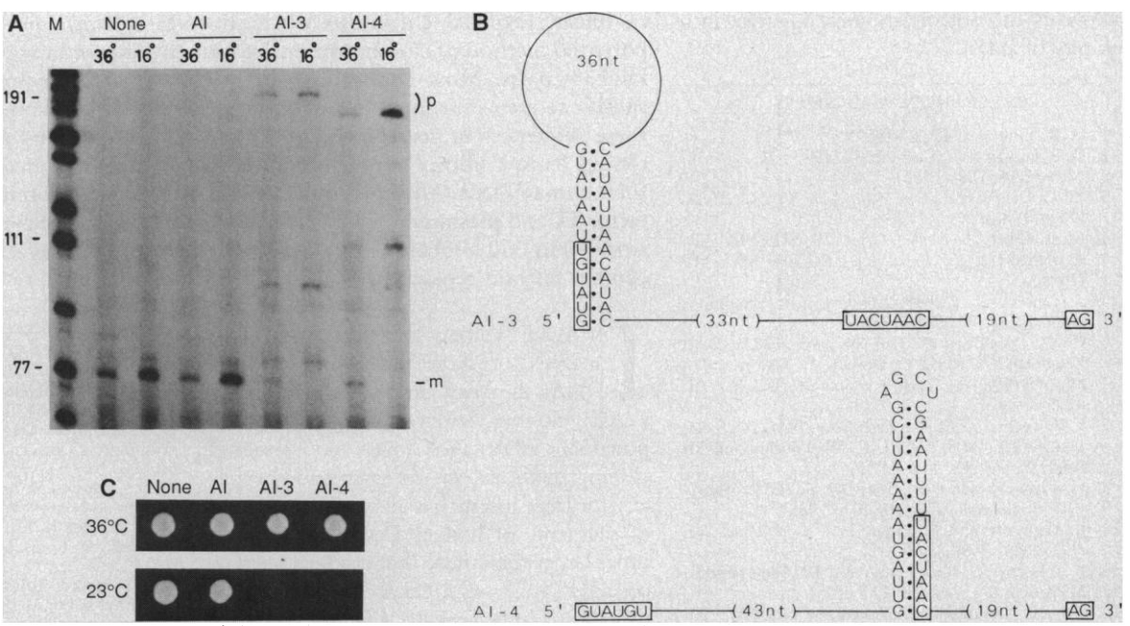

Fig. 2. The cold-sensitive introns. (**A**) Analysis of *PGK-lacZ* mRNA by primer extension. Cells were grown as described in Table 1. Total RNA was extracted and primer extension was performed as described (*25*). M13 sequencing primer [a 15-nucleotide oligomer (New England Biolabs)] was used as a primer. Precursor RNAs are indicated by p. Mature mRNA is indicated by m. pBR322 DNA cut with Hpa II and labeled with [α-^{32}P]dCTP and Klenow enzyme was used as a size marker (lane M). (**B**) Possible structure of the cold-sensitive introns. (**C**) Phenotype of the yeast strains NY6A (*MATa, ura3-52, leu2-3, leu2-112, his4-519*) containing the *URA3* gene with the *AI-3* or *AI-4* intron on 2μ plasmid (*13*). Cells were grown overnight at 23°C in minimal medium SD (*23*) supplemented with uracil and histidine, then spotted on SD plates supplemented with histidine (about 5000 cells in each spot). Plates were then incubated either at 36° or 23°C for 3 days.

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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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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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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