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DNA Sequence and Characterization of the S. cerevisiae Gene Encoding Adenylate Cyclase

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Summary

We have cloned *CYR1*, the S. cerevisiae gene encoding adenylate cyclase. The DNA sequence of *CYR1* can encode a protein of 2026 amino acids. This protein would contain a central region comprised of over twenty copies of a 23 amino acid repeating unit with remarkable homology to a 24 amino acid tandem repeating unit of a trace human serum glycoprotein. Gene disruption and biochemical experiments indicate that the catalytic domain of adenylate cyclase resides in the carboxyl terminal 400 amino acids. Elevated expression of adenylate cyclase suppresses the lethality that otherwise results from loss of *RAS* gene function in yeast. Yeast adenylate cyclase, made in E. coli, cannot be activated by added *RAS* protein.

Introduction

Cyclic nucleotides play an essential role in controlling many of the activities of eukaryotic and prokaryotic cells. In vertebrates, intracellular cAMP mediates many of the hormonal signals that regulate metabolism and growth. In the yeast S. cerevisiae, cAMP is required for growth (Matsumoto et al., 1982). The regulation of adenylate cyclase, the enzyme that converts ATP into cAMP, is therefore of central importance in cellular physiology and has been the subject of intensive research. In vertebrate cells, adenylate cyclase is regulated by guanine nucleotide binding, or "G" proteins (Gilman, 1984). In the yeast S. cerevisiae, adenylate cyclase is regulated by yeast *RAS* proteins, homologs of the proteins encoded by the mammalian *RAS* oncogenes, which also bind guanine nucleotides (DeFeo-Jones et al., 1983; Powers et al., 1984; Tamanoi et al., 1984; Temeles et al., 1984; Toda et al., 1985; Broek et al., 1985). To form a clearer picture of the interaction between *RAS* proteins and adenylate cyclase, and to learn more about the regulation of adenylate cyclase in general, we have cloned, sequenced, and begun some preliminary genetic and biochemical characterization of the S. cerevisiae gene encoding this adenylate cyclase. This effort was made possible by the work of Matsumoto and colleagues, who developed conditional lethal mutants of *CYR1*, the locus that encodes adenylate cyclase (Matsumoto et al., 1982, 1984).

Results

Cloning *CYR1*, the Gene Encoding Adenylate Cyclase

Several cyclic AMP requiring strains of yeast were developed by Matsumoto et al., 1982. Strains of one recessive complementation group, *cyr1*, lack adenylate cyclase activity. The strain AM110-4C, containing the *cyr1-2* allele, is temperature sensitive for growth and contains a thermolabile adenylate cyclase (Matsumoto et al., 1984). We used yeast strains carrying this allele to select genes enabling cells to grow at the restrictive temperature. For this purpose, we backcrossed AM110-4C with strains from our laboratory to derive the *ura3 cyr1-2* strain T50-3A (Table 1). T50-3A was transformed with DNA from a yeast genomic library contained on the YCP50 shuttle vector. YCP50 carries a yeast centromere (*CEN3*), yeast replication origin (*ARS1*), and the *URA3* marker as well as the pBR322 sequence. The library, constructed by Mark Rose (MIT), contains yeast DNA fragments, generated by partial cleavage with restriction endonuclease Sau 3A, which have been cloned into the unique Bam HI site of YCP50. After transformation of T50-3A with the YCP50 library, Ura$^+$ transformants were selected at the permissive temperature and were replica-plated at 35°C. Ten of the 10^4 Ura$^+$ transformants were able to grow at the higher temperature. DNAs from these transformants were used to transform E. coli to ampicillin resistance. Most of the resulting ampicillin-resistant E. coli strains contained two kinds of plasmids, p*CYR1-2* or p*CYR1*-11. Restriction endonuclease maps of these plasmids are shown in Figure 1. p*CYR1-2* and p*CYR1*-11 contain overlapping yeast DNA sequences. Upon transformation with either of these plasmids, all Ura$^+$ transformants of T50-3A were able to grow at the restrictive temperature.

Combined biochemical and genetic experiments indicated that we had indeed cloned adenylate cyclase and not another gene that suppressed the *cyr1-2* allele. First, we transformed the *cya*$^-$ E. coli strain CA8306 (Brickman et al., 1973) with p*CYR1-2* and p*CYR1*-11. This strain of E. coli lacks its own adenylate cyclase activity. CA8306 transformed with either p*CYR1-2* or p*CYR1*-11 contained significantly elevated levels of adenylate cyclase (data not shown, but see for example Tables 5 and 6 below). Second, *cyr1*-1 strains, which lack detectable levels of adenylate cyclase activity, regained adenylate cyclase activity (Table 2) when transformed with p*CYR1-2* or p*CYR1*-11. Third, we constructed a plasmid, p*cyr1::URA3*, in which the adenylate cyclase coding region was disrupted at its unique Xba I cleavage site by the insertion of the *URA3* gene (Figure 1). The Eco RI restriction endonuclease fragment flanking the disruption was then used to replace the wild-type *CYR1* locus by transforming a *ura3 bcy1* strain of yeast, T16-3B, according to the method of Rothstein, 1983. We chose as recipient the T16-3B strain, since the *bcy1* allele suppresses the lethality that otherwise results from loss of the adenylate cyclase gene (Toda et al., 1985). Southern blot analysis indicated that the *URA3* gene disrupted the locus corresponding to the adenylate cyclase coding region in most of the Ura$^+$ transformants (data not shown). Such transformants had negligible adenylate cyclase activity (Table 2).

Genetic experiments indicate that the adenylate cy-

Table 1. Yeast Strains Used in This Study

Strains	Genotypes
* JB-B	*MATα his3 leu2 ura3 ras2::LEU2*
* SP1	*MATa his3 leu2 trp1 ura3 ade8 can1*
* TS1	*MATa his3 leu2 trp1 ura3 ade8 can1 ras1::HIS3*
* AM18-5C	*MATα cyr1-1*
* AM110-4C	*MATα leu1 cyr1-2*
* AM238-3B	*MATα IAC1*
* T27-10D	*MATα leu2 his3 ura3 can1 bcy1 ras1::HIS3 ras2::LEU2*
† T50-3A	*MATα his3 leu2 trp1 ura3 cyr1-2*
† T43-6C	*MATa trp1 cyr1-1*
‡ T43-6C-A	*MATa trp1 cyr1-1* (p*CYR1-2*)
‡ T43-6C-B	*MATa trp1 cyr1-1* (p*CYR1-11*)
‡ T43-6C-C	*MATα trp1 cyr1-1* (YEP13-*CYR1-11*)
§ T16-3B	*MATa his3 leu2 ura3 bcy1*
§ T16-3B-A	*MATa his3 leu2 ura3 bcy1 cyr::URA3*
§ T16-3B-B	*MATa his4 leu2 ura3 bcy1 CYR1::HIS3*
§ T16-11A	*MATα his3 leu2 trp1 ura3 bcy1*
‖ T36-7C	*MATα his3 leu2 ura3 cyr1-1 bcy1*
# TK7	*MATa/α his3/his3 leu2/leu2 trp1/+ ura3/ura3 bcy1/bcy1 cyr1::URA3/+*
# TK8	*MATa/α his3/his3 leu2/leu2 ura3/ura3 bcy1/bcy1 cyr1::URA3/cyr1-1*
** TK4	*MATa/α his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 ade8/+ can1/+ ras1::URA3/+ ras2::LEU2/+*
** TK4-1	*MATa/α his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 ade8/+ can1/+ ras1::URA3/+ ras2::LEU2/+ CYR1::HIS3/+*
** TK4-1-2C	*MATa his3 leu2 trp1 ura3 CYR1::HIS3*
†† RS7-2D	*MATa his3 leu2 tyr1 ura3 ade2 IAC1*

* JB-B is a haploid segregant of a diploid between X2-2-4C and SX50-1C (Kataoka et al., 1984). SP1 and TS1 were previously described (Kataoka et al., 1985). AM18-5C (Matsumoto et al., 1984), AM110-4C and AM238-3B were obtained from Dr. K. Matsumoto at the Tottori University, Japan. T27-10D was previously described (Toda et al., 1985).

† T50-3A and T43-6C are segregants of diploid strains produced by mating SP1 with AM110-4C and AM18-5C, respectively.

‡ T43-6C-A,-B, and -C were produced by transformation with p*CYR1-2*, p*CYR1-11*, and YEP13-*CYR1-11*, respectively, and by selecting colonies that could grow on YPD plates without cAMP. T43-6C strain absolutely requires exogenously added cAMP for growth. Structures of p*CYR1-2* and p*CYR1-11* are shown in Figure 1, while that of YEP13-*CYR1-11* is described in Experimental Procedures. Extrachromosomal plasmids are listed in parentheses.

§ T16-3B and T16-11A are segregants of a diploid strain heterozygous for *bcy1* as described (Toda et al., 1985). T16-3B-A and -B were formed by transformation of a 4.8 kb Eco RI fragment of p*cyr1::URA3* or a 4.9 kb Eco RI fragment of p*CYR1::HIS3* and selecting Ura+ or His+ colonies, respectively.

‖ T36-7C is a segregant of a diploid that was formed by mating a *bcy1* strain with a *cyr1-1* strain in our laboratory.

Diploids TK7 and TK8 were produced by mating T16-3B-A with T16-11A or T36-7C, respectively, and picking diploid cells by a micromanipulator.

** TK4 is formed by a cross between JB-B and TS1. TK4-1 is produced by transformation of TK4 with the 4.9 kb Eco RI fragment of p*CYR1::HIS3*. TK4-1-2C is a haploid progeny of TK4-1.

†† This strain is a segregant of a diploid formed between AM238-3B and a haploid strain in our laboratory.

clase gene we have cloned corresponds to *CYR1*. For these experiments, we used the *bcy1* strain T16-3B-A in which the adenylate cyclase gene was disrupted by *URA3* in the manner described above. This strain was mated to the *bcy1* strain T16-11A, which contains the wild-type adenylate cyclase gene. The resulting diploid TK7 had nearly normal levels of adenylate cyclase activity as shown in Table 2. Therefore the *URA3* disruption of the adenylate cyclase gene is recessive. However, when T16-3B-A was mated with the *cyr1 bcy1* strain, T36-7C, the resulting diploid, TK8, had negligible adenylate cyclase activity (Table 2). Since the disrupted allele of the adenylate cyclase gene is recessive and since the *cyr1* allele is also recessive, the adenylate cyclase gene corresponds to *CYR1*.

The Nucleotide Sequence of *CYR1* and Its Encoded Amino Acid Sequence

The DNA sequence of *CYR1* was determined by the dideoxy method of Sanger et al. (1977) using [α-^{35}S]dATP as a substrate (Biggin et al., 1983). The sequencing scheme is shown in Figure 1. The nucleotide sequence and the predicted amino acid sequence of the encoded protein are shown in Figure 2. No consensus splice sites (Langford and Gallwitz, 1983) for yeast genes are seen in the one long open reading frame. If the first methionine in the open reading frame is used, the *CYR1* locus would encode a protein of 2026 amino acid residues. We found a stretch of very T-rich sequence, including poly(dT)$_{21}$, approximately 250 bp upstream from the first ATG in the open reading frame. Such a poly(dT) sequence has been observed in the 5′-flanking sequences of many yeast genes (Montgomery et al., 1980; Struhl, 1981; Bennetzen and Hall, 1982). These results support the assumption that the first ATG of the open reading frame encodes a true initiator methionine.

We have examined the predicted amino acid sequence of *CYR1* for homology with the predicted amino acid sequence encoded by the E. coli adenylate cyclase gene, recently published by Aiba et al., 1984. Using a computa-

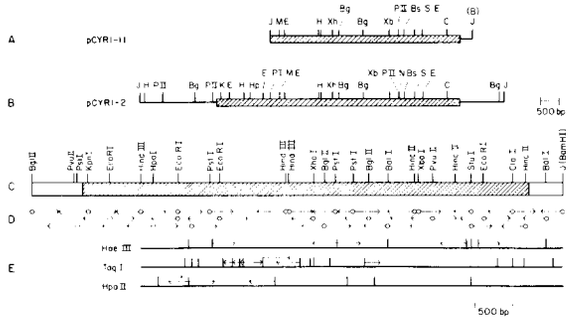

Figure 1. Structure and Sequencing Strategy of the Adenylate Cyclase Gene

(A and B) restriction cleavage maps of two plasmids, p*CYR1*-11 and p*CYR1*-2, respectively. Coding sequences for the *CYR1* gene are indicated by slashed bars. J represents a junction between an insert DNA and a Bam HI cleavage site of the vector YCP50 DNA. (C) an enlargement of the sequenced region of the *CYR1* gene and its fine restriction cleavage map. (D) strategy used for sequencing the *CYR1* gene using the restriction sites shown in C. Open circles and arrows indicate starting sites, directions, and ranges of sequencing by the M13-dideoxy sequencing method (Sanger et al., 1977). (E) cleavage map of the *CYR1* gene with respect to restriction endonucleases Hae III, Taq I, and Hpa II. Arrows represent the sequencing strategy using these cleavage sites. Abbreviations used are as follows: B, Bam HI; Bs, Bst EII; Bg, Bgl II; C, Cla I; E, Eco RI; H, Hind III; Hp, Hpa I; J, Junction; K, Kpn I; M, Mlu I; N, Nco I; PI, Pvu I; PII, Pvu II; Xb, Xba I; Xh, Xho I.

tional matching program (Goad and Kanehisa, 1982), no striking homologies were observed. We also examined homology between *CYR1* and several known ATP-binding proteins, various protein kinases, and ATPases. Again no striking homologies were observed.

The predicted *CYR1* sequence has considerable internal homology. This homology was readily detected using the above computational matching program. The internal homology domain spans about 600 amino acids, from position 710 to 1300. Inspection of this region indicates that it can be roughly organized into a set of tandem repeating units 23 amino acids long, as shown in Figure 3. When these units are aligned, a leucine-rich consensus sequence emerges, in which amino acid assignments at six positions are clear, and the amino acid character (neutral or aliphatic) is evident at four others. While reviewing recently published sequences of proteins with internal periodicity, we noted a strong resemblance between the 23 amino acid repeat of yeast adenylate cyclase and a 24 amino acid repeat found in a leucine-rich α_2 glycoprotein of human serum called LRG (Takahashi et al., 1985) (Figure 3). The function of LRG, which is a trace human serum protein, is unknown.

In yeast, as in mammalian cells, adenylate cyclase activity is found in the insoluble fraction of cells extracted without detergents (Liao and Thorner, 1980). It is likely, therefore, that yeast adenylate cyclase is membrane-bound. We calculated and plotted the Kyte-Doolittle hydropathy values (Kyte and Doolittle, 1982) for the predicted amino acid sequence of adenylate cyclase. We observe no hydrophobic region of sufficient length to resemble the simple membrane-spanning domains of the type seen in many transmembrane proteins (Kyte and Doolittle, 1982). Moreover, the sequences following the first methionine in the open reading frame do not have the signal sequence present in many secreted or integral membrane proteins (Perlman and Halvorson, 1983). However, as discussed above, yeast adenylate cyclase protein has a domain containing repeat units of a highly amphipathic character. We speculate that the repetitive occurrence of hydrophobic and hydrophilic amino acids at certain positions within this domain results in a regular folding pattern, creating a site that can imbed itself into membranes. Initial studies of adenylate cyclase expressed in E. coli are consistent with this idea (see below and Table 5).

Localizing the Catalytic Domain of *CYR1*

The adenylate cyclase protein predicted from DNA sequence analysis would be very large. However, it was im-

Table 2. Adenylate Cyclase Activities of Various Yeast Strains

Strain*	*CYR1* Locus†	Extrachromosomal *CYR1* Plasmid‡	Adenylate Cyclase Activities (units)§	
			Experiment 1	Experiment 2
T43-6C	*cyr1*-1		< 0.2	ND
T43-6C-A	*cyr1*-1	p*CYR1*-2	ND	41.9
T43-6C-B	*cyr1*-1	p*CYR1*-11	9.1	ND
T43-6C-C	*cyr1*-1	YEP13-*CYR1*-11	45.0	ND
T16-3B	*CYR1*		60.0	ND
T16-3B-A	*CYR1::HIS3*		1089	ND
T16-3B-B	*cyr1::URA3*		< 0.2	ND
TK4-1-2C	*CYR1::HIS3*		1043	4936
SP1	*CYR1*		ND	68.4
TK7	*CYR1/cyr1::URA3*		ND	31.1
TK8	*cyr1*-1/*cyr1::URA3*		ND	< 0.2

* Complete genotypes of the yeast strains are given in Table 1. TK7 and TK8 are diploid strains.
† The *cyr1*-1 allele encodes a defective adenylate cyclase. *cyr1::URA3* and *CYR1::HIS3* are *CYR1* gene disruptions described in the text.
‡ Extrachromosomal centromere plasmids p*CYR1*-2 and p*CYR1*-11 are described in the text. YEP13-*CYR1*-11 is a high copy 2 μ circle yeast plasmid constructed from p*CYR1*-11 as described in Experimental Procedures.
§ Crude membrane fractions were obtained from the log phase growing cells, and their adenylate cyclase activities were measured as described in Experimental Procedures. One unit of the activity is defined as 1 pmol cAMP formed in 1 min incubation with 1 mg membrane protein at 32°C under the standard assay condition. ND means not done.

Cell
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1 AGACCACTCGGCCAACCTCCATCTTATTTAAAAATCCTAGGAAATAGATGTTATATAAGTCATACATCATTGAAAACATCAAATACCTGTCCCTGCGTTG
101 TTCTACAGAAAAGCGCATTAGAGTAGGTCATACCCTTTAAAAAATGATTTTGTAAATGGGAGAAAGTAAACAAATATGCATTTCGGCTAGCTCCCAATGT
201 AAATAGCCATAAAAAAGGATAAAATGCCAGATTTAGAGTCAGTTCTCGTTCTTCTTCTTCTCTGTGTTCAATTGTTCAATTGCATTGTTAATCACTGAAA
301 TTCTATGTTTCAAATTGGAATGGCAGACTGAACTGAGACCTTCCTTACTTTCTCCTCTATCAAACCTAAATATTCAATCATAACCTTATTTTTTCACTTC
401 CTTTTTCTTACTTTTTTTTTTTTTTTTTTTTGTATTTTGGAAGGAAAGCAGGGGGGACTTACCATAAGAAAAGCGACTCTAAAAAAGTGTATTTTCTTC
501 CAAAGTTCAGTAAAGACAGCTGGGAAGTATAGTAACAGAGGAGAGAAAGCATAAGGAGACCTCTTGCTGCAGTTCACAACCAAAAATAGCATATCTTTAC
1 MetSerSerLysProAspThrGlySerGluIleSerGly
601 TATTGTCGCTAACCATTGCGAAACGAGCTAAAGCAACAGCAAACGAAATCCCTAGGTCGAAATGTCATCAAAACCTGATACTGGTTCGGAAATTTCTGGC
14 ProGlnArgGlnGluGluGlnGluGlnGlnIleGluGlnSerSerProThrGluAlaAsnAspArgSerIleHisAspGluValProLysValLysLysA
701 CCTCAGCGACAGGAAGAACAAGAACAACAGATAGAGCAGAGCTCACCTACGGAAGCAAACGATAGAAGCATTCATGATGAGGTACCAAAAGTGAAGAAGC
47 rgHisGluGlnAsnSerGlyHisLysSerArgArgAsnSerAlaTyrSerTyrTyrSerProArgSerLeuSerMetThrLysSerArgGluSerIleTh
801 GTCACGAACAAAATAGTGGTCACAAATCAAGAAGGAATAGCGCATATAGTTATTACAGCCCACGGTCGCTTTCTATGACCAAAAGCAGGGAGAGTATCAC
80 rProAsnGlyMetAspAspValSerIleSerAsnValGluHisProArgProThrGluProLysIleLysArgGlyProTyrLeuLeuLysLysThrLeu
901 TCCAAATGGTATGGATGATGTAAGTATTTCGAACGTGGAACATCCAAGGCCGACAGAACCGAAAATCAAAAGGGGTCCATATTTACTGAAGAAAACATTG
114 SerSerLeuSerMetThrSerAlaAsnSerThrHisAspAspAsnLysAspHisGlyTyrAlaLeuAsnSerSerLysThrHisAsnTyrThrSerThrH
1001 AGCAGTCTTTCAATGACGAGCGCGAATAGTACTCATGATGATAATAAAGACCACGGTTACGCTTTGAATTCATCCAAGACGCACAACTACACATCTACTC
147 isAsnHisHisAspGlyHisHisAspHisHisHisValGlnPhePheProAsnArgLysProSerLeuAlaGluThrLeuPheLysArgPheSerGlySe
1101 ATAACCATCATGACGGTCATCATGATCATCATCATGTTCAGTTTTTTCCCAATAGGAAGCCATCATTAGCGGAAACCCTATTCAAAAGGTTTTCAGGGTC
180 rAsnSerHisAspGlyAsnLysSerGlyLysGluSerLysValAlaAsnLeuSerLeuSerThrValAsnProAlaProAlaAsnArgLysProSerLys
1201 AAACAGTCACGATGGCAATAAGTCAGGAAAGGAAAGTAAAGTTGCTAACCTTTCCCTTTCAACGGTAAATCCTGCACCTGCTAATAGGAAACCTTCTAAA
214 AspSerThrLeuSerAsnHisLeuAlaAspAsnValProSerThrLeuArgArgLysValSerSerLeuValArgGlySerSerValHisAspIleAsnA
1301 GACTCCACTTTATCTAATCACTTGGCTGATAACGTGCCAAGCACTTTACGAAGGAAAGTGTCCTCATTGGTACGTGGTTCTTCCGTCCATGATATAAATA
247 snGlyIleAlaAspLysGlnIleArgProLysAlaValAlaGlnSerGluAsnThrLeuHisSerSerAspValProAsnSerLysArgSerHisArgLy
1401 ATGGTATTGCAGATAAACAGATTAGACCAAAGGCTGTTGCGCAATTAGAAAATACATTACATTCATCTGATGTTCCCAATAGCAAACGCTCGCACAGAAA
280 sSerPheLeuLeuGlySerThrSerSerSerSerSerArgArgGlySerAsnValSerSerMetThrAsnSerAspSerAlaSerMetAlaSerSerGly
1501 AAGCTTTCTGCTAGGCTCCACATCTTCTTCAAGCAGTAGAAGAGGTTCAAATGTCAGTTCAATGACTAACAGTGACAGTGCAAGTATGGCGACGTCGGGT
314 SerHisValLeuGlnHisAsnValSerAsnValSerProThrThrLysSerLysAspSerValAsnSerGluSerAlaAspHisThrAsnAsnLysSerG
1601 AGTCATGTTCTCCAACATAACGTATCTAATGTTTCTCCAACTACTAAAAGTAAGGACAGCGTTAACAGCGAATCCGCCGATCACACTAATAATAAATCCG
347 luLysValThrProGluTyrAsnGluAsnIleProGluAsnSerAsnSerAspAsnLysArgGluAlaThrThrProThrIleGluThrProIleSerCy
1701 AGAAAGTGACTCCAGAATATAATGAGAACATTCCGGAAAATTCTAACTCTGACAACAAACGCGAAGCCACAACGCCTACTATAGAAACACCCATTTCATG
380 sLysProSerLeuPheArgLeuAspThrAsnLeuGluAspValThrAspIleThrLysThrValProProThrAlaValAsnSerThrLeuAsnSerThr
1801 TAAACCATCCCTTTTCAGGCTAGATACAAACCTTGAGGATGTTACTGATATTACAAAGACGGTGCCACCCACCGCTGTCAATTCTACACTAAATTCTACA
414 HisGlyThrGluThrAlaSerProLysThrValIleMetProGluGlyProArgLysSerValSerMetAlaAspLeuSerValAlaAlaAlaAlaProA
1901 CACGGGACTGAGACTGCCTCACCCAAAACGGTGATCATGCCTGAAGGTCCTAGGAAGTCGGTGTCAATGGCTGATCCTCTCCGTCGCTGCCGCAGCACCTA
447 snGlyGluPheThrSerThrSerAsnAspArgSerGlnTrpValAlaProGlnSerTrpAspValGluThrLysArgLysLysThrLysProLysGlyAr
2001 ATGGTGAATTCACATCAACTTCCAATGATAGATCACAATGGGTAGCACCTCAAAGCTGGGATGTGGAAACCAAAAGGAAAAAAACAAAACCTAAAGGGAG
480 gSerLysSerArgArgSerSerIleAspAlaAspGluLeuAspProMetSerProGlyProProSerLysLysAspSerArgHisHisHisAspArgLys
2101 ATCGAAATCAAGAAGGTCAAGTATAGATGCTGATGAACTTGATCCCATGTCACCGGGGCCACCTTCAAAAAAAGACTCTCGTCATCATCACGATCGAAAG
514 AspAsnGluSerMetValThrAlaGlyAspSerAsnSerSerPheValAspIleCysLysGluAsnValProAsnAspSerLysThrAlaLeuAspThrL
2201 GATAACGAATCAATGGTCACTGCGGGTGACAGTAACTCAAGTTTTGTTGATATATGTAAAGAAAACGTTCCGAATGATAGCAAGACCGCACTCGATACTA
547 ysSerValAsnArgLeuLysSerAsnLeuAlaMetSerProProSerIleArgTyrAlaProSerAsnLeuAspGlyAspTryAspThrSerSerThrSe
2301 AACTTGTGAACCGCTTAAAAAGTAATTTGGCTATGAGTCCCCCAAGTATACGATATGCTCCATCAAATTTAGATGGGGACTACGACACGTCTTCCACTTC
580 rSerSerLeuProSerSerSerIleSerSerGluAspThrSerSerCysSerAspSerSerSerTyrThrAsnAlaTyrMetGluAlaAsnArgGluGln
2401 CTCATCTTTACCGTCCTCATCTATTAGTTCAGAACATACATCTTCCTGCAGCGATTCCTCTTCGTACACTAACGCGTATATGGAGGCCAACCGAGAGCAG
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2501 GATAATAAAACACCGATCCTGAATAAAACGAAATCGTATACCAAGAAATTTACATCCTCTTCGGTAAATATGAATTCACCAGATGGTGCCCAGAGTTCTG
647 lyLeuLeuLeuGlnAspGluLysAspAspGluValGluCysGlnLeuGluHisTyrTyrLysAspPheSerAspLeuAspProLysArgHisTyrAlaIl
2601 GATTATTACTACAAGATGAGAAGGACGATGAGGTCGAGTGCCAACTGGAACATTACTATAAAGATTTCAGTGATTTAGATCCAAAGAGGCACTATGCTAT
680 eArgIlePheAsnThrAspAspThrPheThrThrLeuSerCysThrProAlaThrThrValGluGluIleIleProAlaLeuLysIleLysPheAsnIle
2701 TCGTATATTCAATACTGATGACACTTTTACGACTCTCTCATGTACTCCAGCGACTACCGTCGAAGAGATAATACCTGCACTTAAAATAAAATTTAACATT
714 ThrAlaGlnGlyAsnPheGlnIleSerLeuLysValGlyLysLeuSerLysIleLeuArgProThrSerLysProIleLeuIleGluArgLysLeuLeuL
2801 ACAGCGCAAGGGAATTTTCAAATTTCCCTGAAGGTGGGAAAGTTGTCAAAAATTTTGAGACCAACTTCGAAACCTATTTTAATTGAAAGAAAACTTTTAC
747 euLeuAsnGlyTyrArgLysSerAspProLeuHisIleMetGlyIleGluAspLeuSerPheValPheLysPheLeuPheHisProValThrProSerHi
2901 TTTTGAATGGTTATCGAAAGTCAGACCCACTTCATATTATGGGTATAGAGGATTTAAGTTTTGTTTTTAAGTTTCTTTTCCATCCTGTCACACCTTCTCA
780 sPheThrProGluGlnGluGlnArgIleMetArgSerGluPheValHisValAspLeuArgAsnMetAspLeuThrThrProProIleIlePheTyrGln
3001 CTTTACTCCTGAACAAGAACAAAGAATAATGAGAAGCGAATTTGTTCACGTAGATTTAAGGAATATGGATCTCACTACACCTCCCATCATTTTTTACCAG
814 HisThrSerGluIleGluSerLeuAspValSerAsnAsnAlaAsnIlePheLeuProLeuGluPheIleGluSerSerIleLysLeuLeuSerLeuArgM
3101 CATACGTCAGAAATAGAAAGTTTAGACGTTTCTAATAACGCAAATATATTCCTACCTCTGGAGTTCATTGAAAGCTCGATTAAATTATTAAGTTTGAGAA
847 etValAsnIleArgAlaSerLysPheProSerAsnIleThrLysAlaTyrLysLeuValSerLeuGluLeuGlnArgAsnPheIleArgLysValProAs
3201 TGGTTAATATTAGAGCATCTAAATTTCCTTCCAATATCACTAAGGCGTATAAACTAGTATCTTTGGAATTACAGAGAAACTTCATAAGAAAAGTACCGAA
880 nSerIleMetLysLeuSerAsnLeuThrIleLeuAsnLeuGlnCysAsnGluLeuGluSerLeuProAlaGlyPheValGluLeuLysAsnLeuGlnLeu
3301 CTCAATCATGAAACTGAGTAATTTAACGATATTAAACCTTCAATGTAATGAGCTTGAAAGCCTACCGGCTGGATTTGTTGAACTGAAAAATCTGCAATTG
914 LeuAlaLeuSerSerAsnLysPheMetHisTyrProGluValIleAsnTyrCysThrAsnLeuLeuGlnIleAspLeuSerTyrAsnLysIleGlnSerL
3401 CTAGACTTGTCTTCAAACAAGTTCATGCACTACCCAGAAGTTATTAACTACTGCACCAATCTTTTACAAATAGACCTATCATATAATAAAATCCAAAGCT
947 euProGlnSerThrLysTyrLeuValLysLeuAlaLysMetAsnLeuSerHisAsnLysLeuAsnPheIleGlyAspLeuSerGluMetThrAspLeuAr
3501 TACCACAGTCCACTAAGTACCTAGTAAAGCTTGCGAAGATGAACCCTTCTCATAACAAACTAAATTTTATAGGCGACTTATCGGAAATGACAGATTTGAG
980 gThrLeuAsnLeuArgTyrAsnArgIleSerSerIleLysThrAsnAlaSerAsnLeuGlnAsnLeuPheLeuThrAspAsnArgIleSerAsnPheGlu
3601 GACGCTGAACCTAAGATATAACAGAATATCATCAATTAAGACAAATGCGTCTAACTTGCAGAACCTTTTTTTAACAGATAATAGAATTTCGAACTTTGAA
A

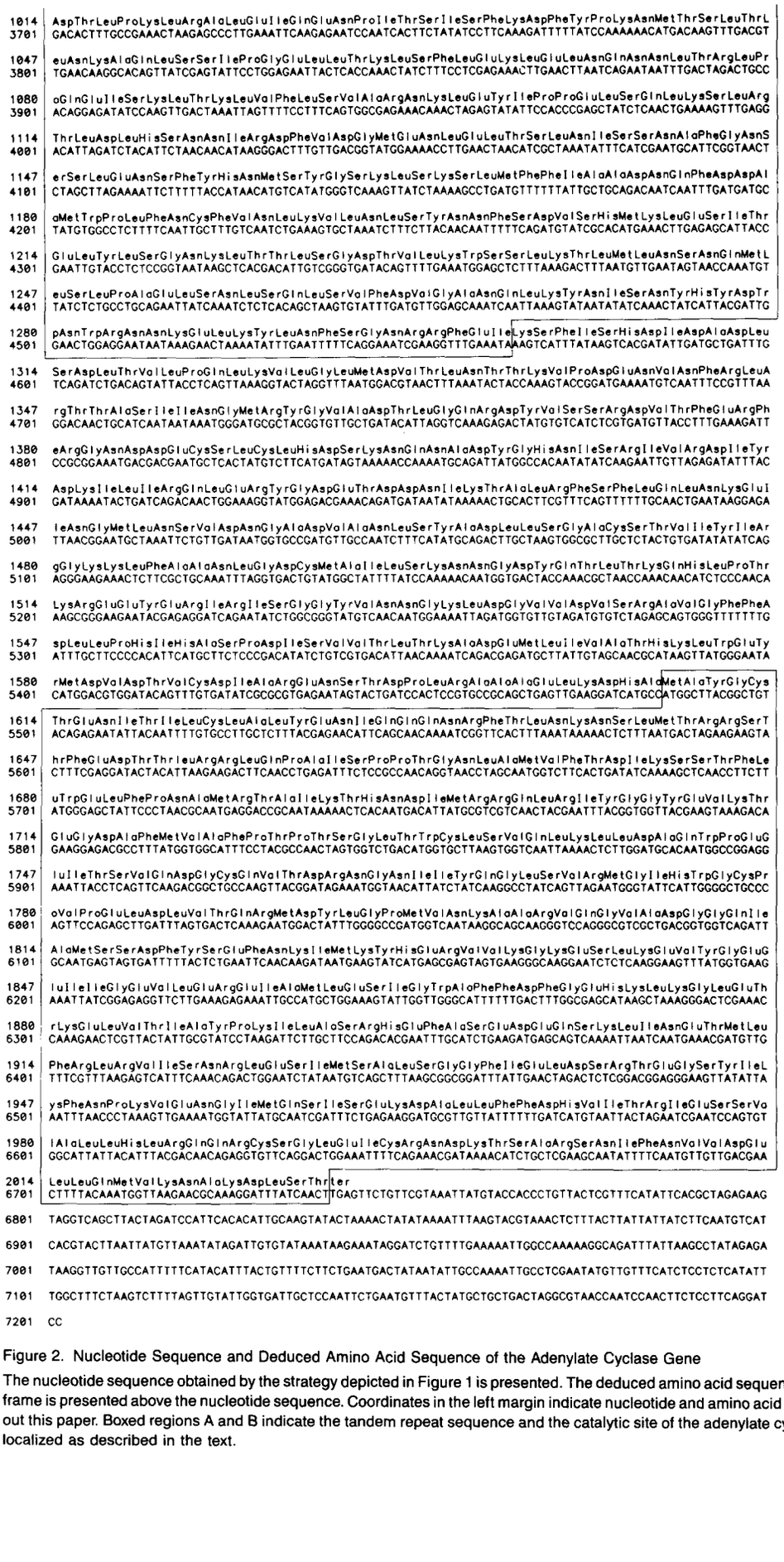

Figure 2. Nucleotide Sequence and Deduced Amino Acid Sequence of the Adenylate Cyclase Gene

The nucleotide sequence obtained by the strategy depicted in Figure 1 is presented. The deduced amino acid sequence of the one long open reading frame is presented above the nucleotide sequence. Coordinates in the left margin indicate nucleotide and amino acid positions and are used throughout this paper. Boxed regions A and B indicate the tandem repeat sequence and the catalytic site of the adenylate cyclase, respectively, which were localized as described in the text.

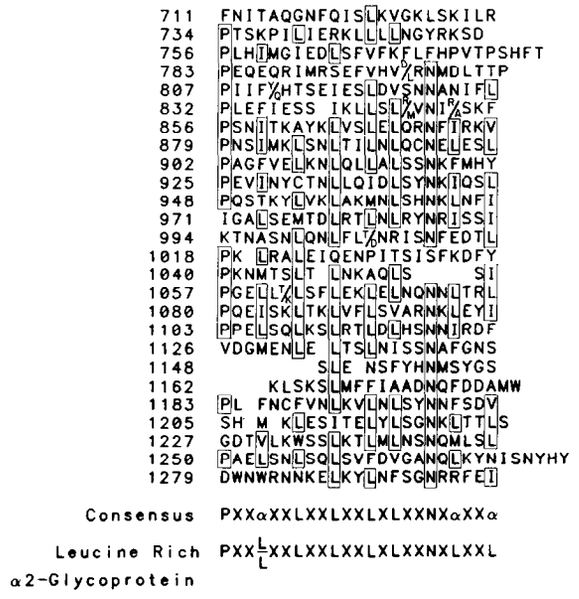

Figure 3. Structure of the Adenylate Cyclase Periodic Repeat

The sequence from amino acid position 711 to 1302 is indicated. Numbers on the left are coordinates for the amino acid position at the left of each line. Slashes indicate areas where two positions have been compressed, and blanks indicate the placement of gaps, which align this sequence to reflect the periodic structure. The consensus sequence for the periodic repeat is indicated, with α indicating one of the three aliphatic amino acids, valine, leucine, or isoleucine. The consensus sequence for the repeat of the α_2 leucine-rich glycoprotein of human serum is indicated at the bottom.

mediately evident that not all the coding region was needed to encode a protein with catalytic activity, since the plasmid p*CYR1*-11, which contains the carboxy-terminal three-fourths of the gene (beginning at amino acid residue 511), can complement *cyr1* yeast strains and can direct the synthesis of an adenylate cyclase activity in E. coli (data not shown, but see Table 5). Initial attempts at constructing adenylate cyclase genes disrupted by the insertion of auxotrophic markers led to the same conclusion. When the *URA3* marker was inserted at the Xba I restriction endonuclease site (amino acid residue 1540), the gene (*cyr1*::*URA3*) could no longer direct the synthesis of adenylate cyclase, as we discussed in a previous section (Table 2). However, yeast cells in which the adenylate cyclase gene was replaced by the *CYR1*::*HIS3* gene and in which the *HIS3* marker was inserted between the two Bgl II sites (amino acid positions 1116 and 1315, see Figure 1), had vastly elevated levels of adenylate cyclase activity (see Table 2).

To delineate more precisely the catalytic domain of *CYR1*, we constructed high copy extrachromosomally replicating plasmids containing the *LEU2* marker and new transcription units using the galactose-inducible *GAL10* promoter (St. John and Davis, 1981) and various fragments of the *CYR1* coding regions (Table 3). Most of these transcription units were constructed so that the first AUG encountered in a *GAL10*-promoted transcript would initiate translation in the proper reading frame. The plasmids were then used to transform a *leu2*$^-$ strain, T50-3A, containing the temperature-sensitive *cyr1*-2 allele. Leu$^+$ transformants were selected at both the permissive and nonpermissive temperatures on plates containing glucose. Glucose was used as a carbon source because the *GAL10* promoter is only weakly active under such conditions. Cells that overexpress adenylate cyclase grow poorly or not at all on galactose as the main carbon source (data not shown). We tested the ability of the constructs to complement *cyr1*-2 by comparing transformation efficiencies at the permissive and nonpermissive temperatures. The results of these experiments clearly show that the shortest fragment of *CYR1* coding region with complementing activity was the Nco I–Bam HI fragment, whereas the Bst EII–Bam HI fragment did not have this activity (Table 3). This showed that *GAL10* transcripts that would initiate translation at or before the methionine residue at amino acid position 1609 could complement the *cyr1*-2 strain. *GAL10* transcripts that initiate translation at or after the methionine residue at amino acid position 1669 could not complement *cyr1*-2. The ability of *GAL10* constructs to complement *cyr1*-2 correlates perfectly with their ability to produce adenylate cyclase activity when transformed into *cya*$^-$ E. coli strains (see Table 5). We interpret these results to mean that the catalytic domain of adenylate cyclase is contained within the carboxy-terminal 417 amino acids.

Analysis of the *CYR1* Transcription Unit

To determine whether the first AUG of the large open reading frame was a good candidate for the initiation of translation, we performed Northern blot analysis to determine the size of the transcript (or transcripts) from the *CYR1* gene. Poly(A)$^+$ RNA was prepared from log phase growing yeast cells, electrophoresed, and filter-blotted as described in Experimental Procedures. Poly(A)$^+$ RNA was prepared from three yeast strains as follows: SP1, RS7-2D, and TK4-1-2C. RS7-2D has the *IAC* mutation, which causes increased adenylate cyclase activity (Uno et al., 1982). TK4-1-2C has the *CYR1*::*HIS3* gene described in the previous section, and has 20- to 100-fold elevated levels of adenylate cyclase activity when assayed in the presence of manganese ions. SP1 contains the wild-type *CYR1* allele.

Two single-stranded RNA hybridization probes were used in this study, a 5′ probe including the first but not the second AUG of the open reading frame, and a 3′ probe spanning codons for amino acid positions 1602 and 1821 (Figure 4). One major 6.7 kb species in the RNA from SP1 and R27-2D hybridizes to both the 5′ and 3′ probes (Figure 4). The 6.7 kb RNA species is of sufficient length to include the entire open reading frame. We conclude from this that the entire open reading frame may indeed be used for the synthesis of adenylate cyclase. There was no discernible difference in the levels of *CYR1* mRNA content between the wild type (SP1) and the *IAC* mutant (RS7-2D). Hence the elevated level of adenylate cyclase activity in *IAC* strains is probably not caused by increased transcription from *CYR1*.

The 6.7 kb species is not seen in the RNA from TK4-1-2C. Instead, RNA from this strain contains high levels of a 2.7 kb RNA species, which hybridizes to the 3′ probe.

Table 3. Complementing Activity of Truncated *CYR1* Genes

Transforming Plasmid*	Amino Acid Position of the First Met in Frame*	Number of Yeast Transformants/μg DNA*			
		Experiment 1		Experiment 2	
		35°C	23°C	35°C	23°C
YEP51-*CYR1*-Sal I	1	NT‡	NT	156	462
YEP51-*CYR1*-Mlu I	607	NT	NT	220	416
YEP51-*CYR1*-Hind III	960	600	1820	96	328
YEP51-*CYR1*-Xho I	1129	795	2010	NT	NT
YEP51-*CYR1*-Bgl II	1328	510	2200	128	522
YEP51-*CYR1*-Xba I†	1569†	NT	NT	294	1240
YEP51-*CYR1*-Pvu II†	1609†	NT	NT	70	548
YEP51-*CYR1*-Nco I	1609	NT	NT	110	970
YEP51-*CYR1*-Bst EII	1669	NT	NT	0	710
YEP51-*CYR1*-Stu I	1773	NT	NT	0	944
YEP51-*CYR1*-Eco RI	1827	0	3500	0	1290
YEP51		0	2730	0	915

* The plasmids indicated were transfected into a *cyr1*-2 yeast strain, T50-3A, and Leu+ colonies were selected on plates lacking leucine at the nonpermissive (35°C) or the permissive (23°C) temperatures. The construction of the plasmids is described in Experimental Procedures. Positions of the first methionines in frame were determined from the nucleotide sequence of the *CYR1* gene shown in Figure 2.
† These plasmids have one extra ATG out of frame before the first ATG in frame.
‡ NT means not tested.

We estimate that the 6.7 kb transcript in normal cells is present at about one copy per wild-type cell. We estimate that the 2.7 kb RNA species is present in TK4-1-2C at about 100 copies per cell. Based on the size of this transcript and the site of insertion of *HIS3* (about 2.1 kb from the termination codon), we infer that this transcript may initiate within the *HIS3* fragment. The high copy number of this transcript may explain the enormously elevated adenylate cyclase activity we detect in this strain (Table 2).

Additional minor RNA species in SP1 and RS7-2D hybridize to either the 5′ or the 3′ probes, but none to both. Hybridization with the 5′ probe reveals 5.0 kb, 3.0 kb, and 1.2 kb species of RNA from SP1 and RS7-2D. None of these are of sufficient length to encompass the carboxy-terminal catalytic domain without splicing. However, no yeast consensus splicing sequences are observed in the DNA sequence of the open reading frame, and these species do not hybridize with the 3′ RNA probe. Most likely, these transcripts are prematurely terminated. In fact several consensus sequences for poly(A) addition (AATAAA) (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981) are found in coding regions, which is consistent with the observed lengths of the minor RNA species. It is known that the AATAAA sequence is not the only sequence requirement for poly(A) addition (Gil and Proudfoot, 1984; Conway and Wickens, 1985), but nevertheless poly(A) addition may occur in a small percentage of transcripts at these locations depending on their flanking sequences. It should be noted that some yeast genes do not have the AAUAAA sequence at their 3′ untranslated region and seem to use different sequences for recognition of the poly(A) addition site (Zaret and Sherman, 1982; Henikoff and Cohen, 1984).

Several minor species in the RNA from SP1 to RS7-2D hybridize with the 3′ probe. Of these, the most prominent are 5.1 kb, 4.6 kb, 3.7 kb, 3.2 kb, and 1.2 kb. The 3.7 kb and 3.2 kb species most likely represent a cross-hybridizing RNA, since the intensity of hybridization is a function of the stringency of hybridization conditions (data not shown). The other species may represent either specific RNA breakdown products or bona fide transcripts initiating from weak promoters within *CYR1* coding sequences. In the latter case, these species could potentially encode catalytically active molecules of adenylate cyclase lacking N-terminal domains.

CYR1::*HIS3* Suppresses Lethality Due to Loss of *RAS* Function

We previously demonstrated that *RAS* function is essential to vegetatively growing haploid yeast cells and demonstrated that intracellular cyclic AMP and the guanine nucleotide–magnesium ion stimulation of adenylate cyclase is completely dependent on *RAS* function (Toda et al., 1985; Broek et al., 1985). However, we do not know whether activation of adenylate cyclase is the only important function of *RAS*. To explore this question, we determined if the *CYR1*::*HIS3* gene, which greatly overexpresses adenylate cyclase catalytic activity, could suppress the lethality that normally results from loss of *RAS* function. An Eco RI fragment containing the *CYR1*::*HIS3* gene was transfected into a diploid yeast strain TK4, in which one copy each of the *RAS1* and the *RAS2* genes is disrupted by insertion of the *URA3* and the *LEU2* genes, respectively. Two of the resulting His+ transformants were shown by Southern blot analysis to contain one copy each of the wild-type *CYR1* gene and one copy of the *CYR1*::*HIS3* gene. One of these diploid strains was sporulated, and tetrads were dissected. The results shown in Table 4 clearly indicate that all of *ras1*⁻ *ras2*⁻ spores could germinate in the presence of the *CYR1*::*HIS3* gene, while no *ras1*⁻ *ras2*⁻ spores could germinate in the presence of the wild-type *CYR1* gene. Our genetic assign-

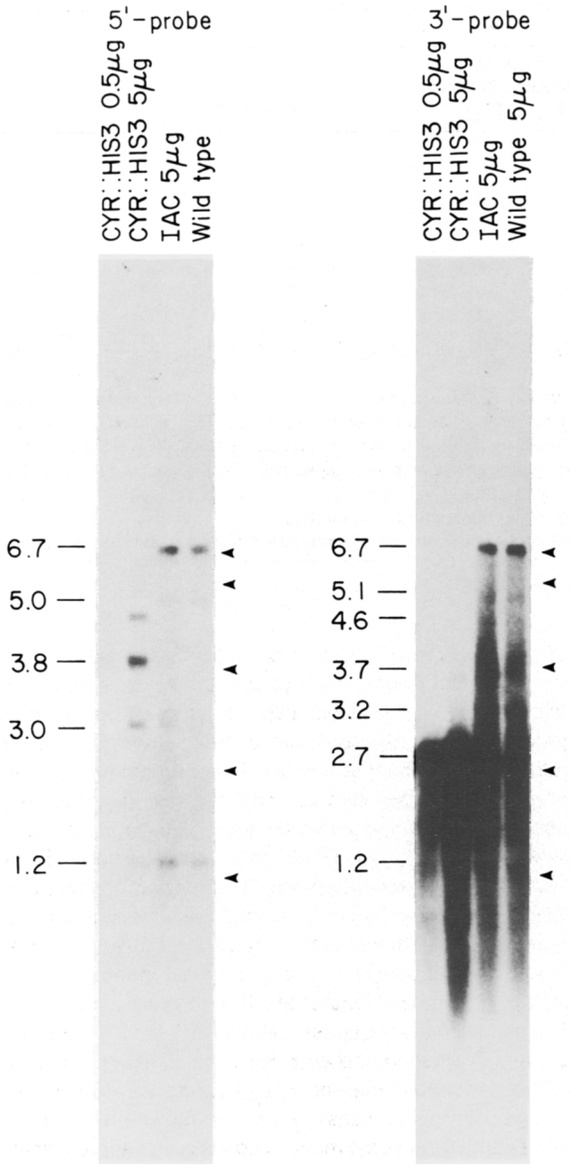

Figure 4. RNA Transcripts from the *CYR1* Locus

Poly(A)$^+$ RNA was prepared from the yeast strains indicated, and the indicated amounts were electrophoresed in a 1% agarose gel containing formaldehyde. The gel was then blotted to a nitrocellulose filter as described in Experimental Procedures. For the *CYR-1::HIS3*, *IAC*, and wild-type strains, TK4-1-C, RS7-2D, and SP1 were used (see Table 1 and text for their genotypes). As a 5′ probe spanning only the first AUG of the open reading frame, ^{32}P-labeled RNA synthesized from a pSP65 (Melton et al., 1984) clone containing a 750 bp Bgl II–Sac I fragment of the 5′ end of the *CYR1* gene cloned into the Bam HI–Sac I cleavage sites was used. As a 3′ probe, a 670 bp Pvu II–Eco RI fragment of the 3′ part of the *CYR1* gene was cloned into the Sma I–Eco RI cleavage sites of pSP65. These two pSP65 DNAs were cleaved by Sal I before RNA synthesis reaction (see Experimental Procedures). Numbers in the left margin represent molecular sizes of the bands in kilobases, which were estimated from DNA molecular size standards shown by the arrows in the right margin. The molecular sizes of the standard are 6.5 kb, 5.3 kb, 3.7 kb, 2.5 kb, and 1.1 kb from top to bottom.

ments of spores were confirmed by Southern blot analyses of DNAs (data not shown). Since the *CYR1::HIS3* gene produces vastly elevated levels of adenylate cyclase

Table 4. Viability of Haploid Progeny from a *ras1*$^-$*/RAS1 ras2*$^-$*/RAS2 CYR1/CYR1::HIS3* Diploid

Spore Genotype				Number of Spores	
RAS1	*RAS2*	*CYR1::HIS3*	*CYR*	Viable	Nonviable
+	+	+	–	4	0
+	+	–	+	9	1
+	–	+	–	8	0
+	–	–	+	6	0
–	+	+	–	9	0
–	+	–	+	5	0
–	–	+	–	7	0
–	–	–	+	0	7

A diploid TK4-1 (*leu2/leu2 his3/his3 ura3/ura3 ras1::URA3/RAS1 ras2::LEU2/RAS2 CYR1/CYR1::HIS3*) was sporulated, and tetrads were dissected. The diploid was formed as described in Table 1. The genotypes of haploid progenies were determined as described previously (Kataoka et al., 1985).

activity, our results indicate that overproduction of cAMP can suppress lethality due to loss of *RAS* function. Therefore, if *RAS* has any essential function in yeast besides stimulating adenylate cyclase, overexpression of adenylate cyclase can compensate for the loss of this function.

Properties of Yeast Adenylate Cyclase Expressed in E. coli

In crude yeast cell membranes lacking *RAS* proteins, adenylate cyclase has 50-fold higher activity when assayed in the presence of manganese ions than when assayed in the presence of magnesium ions (Toda et al., 1985; Broek et al., 1985). The activity of adenylate cyclase in such membranes is greatly enhanced in the presence of magnesium by the addition of guanine nucleotides and yeast *RAS* proteins purified from E. coli expression systems (Broek et al., 1985). Expressing the yeast adenylate cyclase gene in E. coli enables us to compare the enzymatic properties of yeast adenylate cyclase present in E. coli with that present in S. cerevisiae.

For our first experiments we assayed adenylate cyclase activity in extracts prepared from CA8306 *cya*$^-$ E. coli strains harboring the *GAL10* truncated *CYR1* genes described in Table 3. It is evident from Table 5 that, in contrast to the *cya*$^-$ E. coli host, many of the transformed E. coli strains expressed adenylate cyclase. In fact, those plasmids that direct the synthesis of adenylate cyclase in E. coli are precisely the ones that can complement the defective *cyr1* allele in yeast stains (Table 3). Transcription from the *CYR1* genes might proceed from within *CYR1*, the *GAL10* promoter, or adjoining plasmid sequences. E. coli transformed with different constructs express different levels of activity, but this may reflect variation in levels of transcription, translation, or protein stability as well as the intrinsic activity of the synthesized product.

We measured the adenylate cyclase activity in high speed supernatant and high speed pellet fractions from lysates of E. coli transformed with the various plasmids (Table 5). *CYR1* genes contained on the plasmids YEP51-*CYR1*-Sal I and YEP51-*CYR1*-Mlu I have the potential to encode the entire periodic amphipathic domain that was

Table 5. Adenylate Cyclase Activity of *CYR1* Genes in E. coli

Transforming Plasmid*	Specific Activity† of Adenylate Cyclase	Relative Activity‡ Associated with Pellet $P_{act}/(P_{act} + S_{act})$	Mn^{2+}/Mg^{2+}§
YEP51	< 10		
YEP51-*CYR1*-Sal I	1890	0.65	70
YEP51-*CYR1*-Mlu I	1520	0.58	66
YEP51-*CYR1*-Hind III	1840	0.28	47
YEP51-*CYR1*-Bgl II	660	0.23	45
YEP51-*CYR1*-Pvu II	4170	0.14	22
YEP51-*CYR1*-Nco I	1310	0.22	19
YEP51-*CYR1*-Bst EII	< 10		

* The plasmids indicated were transformed into E. coli strain CA8306, which lacks its own adenylate cyclase. See Table 3 and Experimental Procedures for more information about these plasmids.
† E. coli lysates were prepared, and unlysed cells were removed by centrifugation, creating supernatant 1 as described in Experimental Procedures. Supernatant 1 was fractionated into a high speed pellet and high speed supernatant as described in Experimental Procedures. The amount of protein in each fraction was determined. The total adenylate cyclase activity associated with each of the high speed pellets (P_{act}) and each of the high speed supernatants (S_{act}) was determined in the presence of manganese ions. The sum of these activities divided by the sum of the protein in the two fractions gives the specific activity of each strain in units of pmol cAMP/min/mg.
‡ The relative activity associated with the pellet was determined by dividing P_{act} by the sum of P_{act} and S_{act}.
§ The total amount of activity as assayed in manganese ions was divided by the total amount of activity as assayed in magnesium ions for each strain.

discussed previously. E. coli containing these plasmids have adenylate cyclase activity located predominantly in the pellet fraction. Adenylate cyclase activity in E. coli containing other plasmids is located predominantly in the supernatant fraction. These results suggest that this amphipathic domain either causes adenylate cyclase to aggregate or to localize to the membrane when synthesized in E. coli.

We compared ratios of manganese to magnesium ion dependent adenylate cyclase activity in lysates of E. coli transformed with various *CYR1* plasmids. It is apparent that all the *GAL10-CYR1* genes direct the synthesis of a catalytic activity that is many more times active in the presence of manganese ions than in the presence of magnesium ions. In this respect, yeast adenylate cyclase made in E. coli resembles the adenylate cyclase made in *RAS*-deficient S. cerevisiae. The *CYR1* genes contained on plasmids YEP51-*CYR1*-Pvu II and YEP51-*CYR1*-Nco I, which encode the smallest fragment of catalytically active adenylate cyclase, have the highest relative activity in the presence of magnesium, suggesting perhaps that some domain on the N-terminal side of the catalytic domain is inhibitory in the presence of magnesium.

In the yeast S. cerevisiae, the activity of adenylate cyclase is strongly dependent on *RAS* proteins and guanine nucleotides in the presence of magnesium. We wished to test this property of the yeast adenylate cyclase synthesized in E. coli. For this purpose, we constructed a transcription unit using the entire *CYR1* coding sequence, which can initiate from the *recA* promoter of E. coli contained on the plasmid pKS65 (A. Fujiyama, unpublished data). Transcription from the *recA* promoter can be rapidly induced by incubating E. coli with nalidixic acid (Shirakawa et al., 1984). CA8306 E. coli were transformed with the plasmid pKS-*CYR1* containing the *CYR1* coding region in the correct orientation relative to the *recA* promoter. Adenylate cyclase activity in the high speed pellet fractions of lysates was assayed under a variety of conditions. With the addition of nalidixic acid, the level of manganese ion dependent adenylate cyclase activity increases 40-fold, indicating that transcription is induced from the *recA* promoter (Table 6). Again we observe that the induced adenylate cyclase has low activity when assayed in the presence of magnesium ion. The magnesium-dependent activity is increased 2-fold by the addition of the yeast *RAS2* protein purified from E. coli, but the same 2-fold increase is observed when the *RAS2* protein is boiled or when similar amounts of bovine serum albumin are added. These studies are in marked contrast to the behavior of the adenylate cyclase of yeast membrane that lack *RAS* proteins (Toda et al., 1985; Broek et al., 1985). The magnesium-dependent level of activity of adenylate cyclase in these membranes is raised almost to the level of the manganese-dependent activity when *RAS2* protein and guanine nucleotides are present (Table 6). This induction of activity in yeast membranes is not observed when the *RAS2* protein is boiled, nor will bovine serum albumin serve. Thus, unlike the adenylate cyclase of yeast membranes lacking *RAS* proteins, the yeast adenylate cyclase synthesized in E. coli is not specifically responsive to *RAS2* protein. Similar studies were performed on E. coli transformed with the adenylate cyclase genes described in Table 3 and Table 5, and essentially similar results were obtained (data not shown).

Discussion

We have isolated *CYR1*, the adenylate cyclase gene from the yeast S. cerevisiae by complementation cloning using *cyr1* mutants. Evidence that this gene encodes adenylate cyclase is twofold. First, the gene we have isolated directs the synthesis of adenylate cyclase when transfected into an E. coli strain (CA8306) lacking its own endogenous adenylate cyclase. Second, it restores adenylate cyclase activity when transfected into *cyr1* mutants. Moreover, *CYR1* appears to be the only gene in yeast encoding

Table 6. *RAS* Dependence of Yeast Adenylate Cyclase Activity Synthesized in Yeast and in E. coli

Sources of Yeast Adenylate Cyclase		Mn^{2+}**	Mg^{2+}† Additional Components				
			None	GppNp‡	RAS2§ + GppNp	RAS2 (boiled)‖ + GppNp	BSA#
E. coli:	Uninduced**	7	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4
E. coli:	Induced††	290	5	4	10	10	9
Yeast (T27-10D)‡‡		65	< 0.4	< 0.4	51	< 1.0	< 1.0

All values presented are units of adenylate cyclase activity where one unit of activity is defined as the production of 1 pmol of cAMP per min per mg of protein. Duplicate samples containing 30 μg of protein were used for each adenylate cyclase assay (see Experimental Procedures).

* Adenylate cyclase assays were performed in the presence of 2.5 mM $MnCl_2$.

† Adenylate cyclase assays were performed in the presence of 2.5 mM $MnCl_2$. Additional components were delivered to the magnesium-dependent assays in 10 μl of buffer G (see Experimental Procedures).

‡ One microliter of 5 mM Gpp(NH)p was added to the assay.

§ Fifty picomoles of *RAS2* protein preincubated with 1 μl of 5 mM Gpp(NH)p was added to the assay.

‖ Same as (§), but the *RAS2* protein was heated to 100°C for 10 min then cooled and added.

Forty-five picomoles of bovine serum albumin (BSA) was added to the assay.

** Assays were performed on high speed pellet proteins from lysates of an uninduced culture of E. coli strain (CA8306) carrying the nalidixic acid-inducible plasmid pKS-*CYR1*. See Experimental Procedures for preparation of membranes and description of pKS-*CYR1*.

†† Same as (**), but the *recA* promoter was induced by addition of nalidixic acid to the culture medium (see Experimental Procedures).

‡‡ Membranes were prepared from the *RAS*-deficient yeast strain T27-10D. See Table 1 for a description of T27-10D and Experimental Procedures for preparation of yeast membranes.

adenylate cyclase, since cells in which the *CYR1* locus is disrupted with an auxotrophic marker fail to make measurable adenylate cyclase. Recently, Casperson et al., (1985) have reported cloning the adenylate cyclase gene from S. cerevisiae. The restriction map of their gene is in accord with ours.

We have completed the nucleotide sequence of the *CYR1* locus. This locus has one large open reading frame of 6078 bases with the potential to encode a very large protein of 2026 amino acids. Northern blot analysis indicates that the major transcript from this locus is about 6.7 kb, which likely includes the entire open reading frame. Analysis of the predicted amino acid sequence of the *CYR1* protein reveals a large domain of 600 amino acids that bisects the molecule. This domain can be organized into tandem repeating units that have a consensus sequence of 23 amino acids. We searched for similar structures in other proteins and have found only one other example: a trace human serum protein of unknown function, called LRG, which is comprised of a 24 amino acid repeating unit with an astonishingly similar consensus sequence (Takahashi et al., 1985). This similarity in structure may indicate an evolutionary conservation between the yeast and human proteins, or may reflect the coincidental and convergent evolution of a functional domain. In both cases, the repeat units are leucine rich and amphipathic.

Gene disruption experiments indicate that the carboxy-terminal 417 amino acids are sufficient for adenylate cyclase catalytic activity. What then is the function of the remainder of the molecule? The large middle region comprised of tandem amphipathic repeats may form a site for membrane attachment. Indeed, study of the localization of truncated molecules of adenylate cyclase synthesized in E. coli are consistent with this idea. The large N-terminal domain may have regulatory functions.

We have previously demonstrated that *RAS* proteins are essential for adenylate cyclase activity in S. cerevisiae. Cloning of the yeast adenylate cyclase has enabled us to study further *RAS* protein function. Indeed, we have now demonstrated that if *RAS* has any other functions, besides stimulating adenylate cyclase, required during germination or during vegetative growth in rich medium, these functions can be compensated by overexpression of adenylate cyclase.

We still do not know if *RAS* proteins act directly or indirectly on yeast adenylate cyclase. Experiments reported here demonstate that, unlike the adenylate cyclase found in S. cerevisiae, the S. cerevisiae adenylate cyclase made in E. coli does not respond to added *RAS* proteins. There are several plausible explanations for this observation. For example, the S. cerevisiae adenylate cyclase made in E. coli may be degraded or improperly modified or may not be in a correct conformation; or the *RAS* protein added to E. coli membranes may fail to undergo necessary modifications. An alternative explanation is that *RAS* proteins stimulate adenylate cyclase in yeast indirectly, through intermediate proteins, and therefore fail to stimulate the same adenylate cyclase made in E. coli because those intermediate proteins are lacking. This is the hypothesis that we favor, and it is consistent with genetic and yeast biochemical experiments in progress that suggest that *RAS* proteins act indirectly on adenylate cyclase. Since the yeast *RAS* and mammalian *ras* proteins are functionally homologous (Kataoka et al., 1985; DeFeo-Jones et al., 1985), knowledge of the immediate biochemical function of *RAS* in yeast may provide clues to the function of *ras* proteins in mammalian cells. Although we do not yet know the immediate biochemical function of *RAS* in yeast, study of the pathway by which *RAS* activates adenylate cyclase may lead to the discovery of this function.

Experimental Procedures

Yeast Strains, Growth Media, and Transformation

General genetic manipulation of yeast cells was carried out as de-

scribed (Mortimer and Hawthorne, 1969). Nomenclature of the genotypes and phenotypes was as described previously (Powers et al., 1984; Kataoka et al., 1984). Tetrad dissections and assignment of auxotrophic markers were performed as described (Kataoka et al., 1984, 1985). Transformation into yeast cells was carried out using lithium acetate (Ito et al., 1983).

Construction of the Truncated *CYR1* Genes Using the *GAL10* Promoter

Cloning and structure of p*CYR1*-11 and p*CYR1*-2 are described in the text and in Figure 1. In p*CYR1*-11, the *CYR1* gene was inserted in the opposite direction with respect to the tetracycline-resistance gene of YCP50 and could be isolated as a 5.3 kb fragment using Bam HI and Sal I. This fragment was cloned into Sal I–Bcl I-cleaved YEP51 (Broach et al., 1983). The resultant plasmid, YEP51-*CYR1*, was cleaved with Sal I, then cleaved completely by Mlu I, Hind III, Xho I, Pvu II, or Nco I, or partially by Xba I, Bst EII, Stu I, or Eco RI. The resulting DNA fragments of expected sizes containing the 3′ portion of the *CYR1* gene were isolated and were resealed by ligation either directly (Xho I) or after filling in with Klenow fragment of DNA polymerase I. These plasmids were designated YEP51-*CYR1*-XXX, where XXX is the appropriate restriction site used in construction. To produce YEP51-*CYR1*-Sal I, which has a complete coding sequence of the *CYR1* gene, we have introduced a Sal I cleavage site at the immediate 5′-flanking sequence of the first ATG by mutagenesis of GTCGAAATG to GTCGACATG using an oligonucleotide 5′-GATGACATGTCGACCTA-3′ and an M13mp8 derivative having a 1.78 kb Pst I subfragment of the *CYR1* gene (Zoller and Smith, 1983). Then a 1.6 kb Sal I–Pst I fragment was excised from the mutagenized M13 derivative and was inserted into YEP51-*CYR1* digested completely with Sal I and partially with Pst I to reconstruct the complete coding sequence.

Construction of E. coli Expression Plasmids of the *CYR1* Gene

pKS65, which contains the *recA* promoter derived from pKH502 (Shirakawa et al., 1984), contains a synthetic Shine-Dalgarno sequence and an Sph I cleavage site at its first methionine codon. This plasmid was prepared and kindly supplied by Dr. A. Fujiyama, University of Chicago (Fujiyama et al., unpublished data). The 1.6 kb Sal I–Pst I fragment of the mutagenized M13 clone described above was transferred to p*CYR1*-2, creating p*CYR1*-2(Sal). A 7.3 kb fragment containing the *CYR1* coding sequence was then excised by cleaving completely with Sal I and partially with Cla I, the cohesive ends were filled in with the Klenow fragment, and cloned into pKS65, which was cleaved with Sph I and treated with the Klenow fragment. We obtained the plasmid pKS-*CYR1*, which contained the complete *CYR1* insert in the correct orientation. The fidelity of the construction was checked by a recreation of a Sal I site at the junction between Sph I and Sal I.

Construction of Other Plasmids

YEP13-*CYR1*-11 was prepared by inserting a 5.2 kb Sph I–Bam HI fragment of p*CYR1*-11 into Sph I–Bam HI-cleaved YEP13 (Broach et al., 1979). The 5.3 kb Sal I–Bam HI fragment of the *CYR1* gene from p*CYR1*-11 was also cloned into Sal I–Bam HI-cleaved pUC8 (Viera and Messing, 1982). The resultant pUC8-*CYR1* was cleaved with Xba I and ligated with 1.2 kb Xba I fragment of the *URA3* gene (Botstein and Davis, 1981), excised from p*ras1*::*URA3* (Kataoka et al., 1984) to produce p*cyr1*::*URA3*. Similarly p*CYR1*::*HIS3* was constructed by cutting pUC8-*CYR1* with Bgl II, to delete a 0.6 kb fragment, and inserting a 2.0 kb Bam HI fragment of the *HIS3* gene (Struhl and Davis, 1980). For gene disruption experiments, suitable fragments were isolated and used for transformation of yeast cells as described (Rothstein, 1983). For construction of pSP65 derivatives, see Figure 4.

Preparation of E. coli Extracts

In all experiments the growth of E. coli was monitored by absorbance at 600 nm wavelength (A_{600}). All cultures were harvested at A_{600} = 0.6. CA8306 E. coli strains containing pKS65-derived plasmids were grown in 100 ml of L broth containing 50 mg/l ampicillin. Nalidixic acid was added to a final concentration of 40 μg/ml, to induce the *recA* promotor, and incubation was continued at 37°C for 1 hr. The induced and uninduced cultures were centrifuged and were washed in 50 ml of buffer C (50 mM Mes, pH 6.2; 1 mM β-mercaptoethanol; 0.1 mM EGTA; 0.1 mM $MgCl_2$; 1 mM PMSF; 1 μg/ml Leupeptin; and 1 μg/ml Pepstatin). The washed pellets were resuspended in 5 ml of buffer C, and cells were disrupted by sonication for four intervals of 30 sec with intermittent cooling. The disrupted cell suspensions was centrifuged at 1000 ×g for 10 min. The resulting supernatant was spun at 75,000 ×g for 1 hr to collect the crude membrane. The resulting crude membrane preparation was resuspended in buffer C containing 10% glycerol to a final protein concentration of 1 mg/ml.

CA8306 strains containing YEP51-derived plasmids were grown in 100 ml of L broth containing 50 mg/l ampicillin. Cells precipitated by centrifugation were washed with 20 ml buffer C. The washed pellet was resuspended in 2 ml of buffer C and were sonicated as above. Unbroken cells were removed by centrifugation at 4000 ×g for 10 min. The resulting supernatant was spun at 12,000 ×g for 15 min, yielding pellet 1 and supernatant 1. Pellet 1 was resuspended in 2 ml of buffer C followed by centrifugation at 12,000 ×g for 15 min, yielding pellet 2 and supernatant 2. Supernatants 1 and 2 were combined and centrifuged at 75,000 ×g, yielding pellet 3 and the high speed supernatant. Pellets 2 and 3 were resuspended as above and were combined; they are referred to as the high speed pellet in the text.

Adenylate Cyclase Assay

For all adenylate cyclase assays described here, 30 μg of protein was used per reaction. Adenylate cyclase assays and determinations of cAMP produced were carried out as previously described (Broek et al., 1985). Where indicated, 50 pmol of *RAS2* protein in 10 μl of buffer G (20 mM Tris-HCl, pH 7.4; 1 mM $MgCl_2$; 1 mM β-mercaptoethanol) was preincubated with 1 μl of 5 mM guanosine-5′(β,γ,-imino) triphosphate (GppNp) at 37°C for 30 min and was then added to the bacterial or yeast membranes using procedures previously described (Broek et al., 1985). Yeast membrane fractions and *RAS2* proteins were prepared as previously described (Broek et al., 1985).

RNA Preparation and Northern Blot Hybridization

Yeast cells were cultured in 1 l of YPD to the density of 1.5 × 10^7 cells/ml. After harvesting the cells at 0°C, we suspended the cell pellets in 5 volumes of 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 5 mM $MgCl_2$, and 10 mM vanadyl-ribonucleotide complex (Bethesda Research Lab). An equal weight of glass beads (diameter = 250–300 μm) was added, and the mixture was vigorously shaken over a vortex mixer for 3 min. After centrifuging the mixture, we extracted the supernatant with phenol:chloroform:isoamyl alcohol (25:24:1), and RNA was precipitated by ethanol. Poly(A)$^+$ RNA was obtained by using oligo(dT)-cellulose chromatograpy (Aviv and Leder, 1972), was electrophoresed in agarose gels containing formaldehyde, and was transferred to nitrocellulose filters as described (Lehrach et al., 1977).

Synthesis of labeled RNA probes from SP6 templates was performed as described (Melton et al., 1984) using [α-^{32}P]GTP (410 Ci/mmol, Amersham) and SP6 polymerase (Bethesda Research Lab). Hybridization and washing of the filters were carried out as described (Melton et al., 1984).

Other Materials and Methods

E. coli *cya*$^-$ strain CA8306 (Brickman et al., 1973) was obtained from Dr. A. Peterkofsky (NIH). MacConkey agar plates were prepared using a commercial mixture (Difco). Southern blot hybridization was carried out as described (Southern, 1975). All the plasmids used were purified by ethidium bromide–cesium chloride centrifugation (Tanaka and Weisblum, 1975). Sources of reagents and enzymes used in this study are as described previously (Powers et al., 1984; Kataoka et al., 1984).

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