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Isolation of a Maltase Structural Gene from *Saccharomyces carlsbergensis*

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The maltase structural gene *MAL6* of the yeast *Saccharomyces carlsbergensis* has been cloned by transformation of a maltose nonfermenting recipient strain with autonomously replicating chimeric recombinant plasmids. One recombinant plasmid, pMAL26, was shown by positive hybridization translation, as well as by Southern and Northern blot experiments, to carry the *MAL6* structural gene.

A monomeric α-glucosidase, maltase, is induced in the yeast *Saccharomyces carlsbergensis* upon the addition of maltose to the growth medium (21). The induction of maltase synthesis in yeast is dependent on any one of at least five unlinked loci (*MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*). Naumov, in analyzing the progeny of crosses between various naturally occurring maltose-negative and maltose-positive isolates of *Saccharomyces*, concluded that maltose fermentation requires the presence of two genes, termed *MALp* and *MALg*, and that each *MAL* locus was composed of these two linked genes (19, 20). A maltose-negative strain could arise if it lacked either a functional *MALp* or a functional *MALg* gene. A strain carrying *MALg* and *MALp*, either linked or at unlinked loci, allows the cell to utilize maltose as a carbon source. Strains having the genotype *mal1p MAL1g MAL2p mal2g*, for example, grow in maltose-containing medium, suggesting that the *MALg* or *MALp* gene product(s) or both act in *trans* (20). In fact, Naumov determined the genotype of the *MAL6* strain CB11 (the strain used in this study to prepare the clone bank) to be *mal1p MAL1g mal2p MAL2g MAL6p MAL6g* (20). That is, this maltose-positive strain contained three functional *MALg* genes and one functional *MALp* gene. Previously, ten Berge and co-workers had carried out extensive mutagenesis studies, using strain CB11 and selected mutants which were temperature sensitive for growth on maltose medium (26). None of these mutants, which mapped in the *MAL6* locus, possessed a thermolabile maltase. Moreover, nonconditional *mal6* mutants had basal (uninduced) levels of maltase activity. Naumov's analysis of ten Berge's *mal6* mutants indicated that they lacked a functional *MALp* gene (20). Thus, the simplest rationalization of the above observations was to ascribe a regulatory function to the *MALp* gene and to consider the *MALg* gene as encoding the maltase structural gene.

The transformation of appropriate yeast mutants with recombinant plasmid vectors which complement the defect in the mutants has facilitated the detection, cloning, and isolation of yeast genes (6, 18). This report describes the complementation of a maltase-negative strain by direct selection for maltose utilization after transformation by a pooled collection of autonomously replicating plasmid vectors, each containing a yeast nuclear DNA fragment.

MATERIALS AND METHODS

Bacterial and yeast strains. The following strains were used in this investigation: *Escherichia coli* K-12 strain RR101 (F⁻ *pro leuB thi lacY* Str^r *hsdR hsdM*); *S. carlsbergensis* CB11 (**a** *ade1 mal1p MAL1g mal2p MAL2g MAL6p MAL6g*), a maltose-fermenting strain; and *S. cerevisiae* JC27 (α *leu2–3 leu2–112 his4*), a maltose-nonfermenting strain derived from a cross between the Mal⁻ strain HF1066A (α *leu2–3 leu2–112 his4*) and strain CB11. The *mal* genotype of JC27 was determined by allele testing in crosses with CB11, a CB11 derivative carrying a *malp* gene and a number of *malp* and *malg* strains characterized and provided to us by G. Naumov. The results of this genetic analysis demonstrated that strain JC27 lacks a functional *MALp* gene but carries one or more functional *MALg* genes (data not shown).

Media. L medium (17) supplemented when required with 20 μg of chloramphenicol, ampicillin, or tetracycline per ml was used for culturing bacterial cells. Media for the growth of yeast cells were: YEPD (1% yeast extract, 2% peptone, 2% glucose) and SMal, the selective medium used to discriminate between Mal⁺ and Mal⁻ cells (0.7% yeast nitrogen base [Difco Laboratories] without amino acids, supplemented with 50 μg of leucine per ml, 50 μg of histidine per ml, and 2% maltose).

Plasmids. Two *E. coli*/*S. cerevisiae* hybrid plasmids, pYT11 and pYT14, were previously constructed in our laboratory. They consist of the *E. coli* plasmid pBR325 (4) joined to the yeast 2-μm DNA plasmid (14) at their

unique *Pst*I restriction enzyme cleavage sites. The two plasmids represent the two possible orientations of pBR325 relative to the 2-μm DNA plasmid. Both plasmids retain two drug resistance markers, Tet^r and Cam^r, coding respectively for resistance to tetracycline and chloramphenicol. Unique cleavage sites for the restriction endonucleases *Bam*HI and *Sal*I are present within the Tet^r gene and can be used for cloning purposes. By inserting a yeast gene (*LEU2*) into each plasmid, they were both tested and shown to be capable of transforming *leu2* yeast cells with high efficiency (data not shown). As is the case with similar hybrid plasmids containing the yeast 2-μm DNA replicon (2, 11), both plasmids behave in yeast as autonomously replicating extrachromosomal genetic elements exhibiting mitotic instability. The two plasmid vectors were used to construct a yeast genomic DNA library as described below.

Construction of a yeast genomic DNA library. Total DNA was isolated from the maltose-fermenting strain CB11 as previously described (8) and mechanically sheared into fragments of an average size of 15 kilobase pairs. Plasmid pYT11 and pYT14 DNAs, isolated as described below, were linearized by treatment with endonuclease *Bam*HI. Polydeoxythymidylate [poly(dT)], and polydeoxyadenylate [poly(dA)] homopolymers were added to the 3′ termini of, respectively, the yeast DNA and plasmid DNAs, as described elsewhere (22). Poly(dT)-tailed yeast DNA was annealed to poly(dA)-extended pYT11 and pYT14 DNA, and the mixture was used to transform *E. coli* RR101. Approximately 4,500 transformants of phenotype Cam^r Tet^s Amp^s were selected and combined into eight pools, each consisting of about 500 independent clones.

Isolation of plasmid DNA. Plasmid DNA was amplified in growing bacterial cultures by addition of spectinomycin (150 μg/ml). Extraction and purification of plasmid DNA were achieved by using the cleared lysate and CsCl-ethidium bromide density gradient centrifugation method of Guerry et al. (12).

Transformation of *E. coli* and yeast with plasmid DNA. Bacterial transformation of $CaCl_2$-treated cells was performed as described previously (7). For the transformation of Mal^- yeast cells to a Mal^+ phenotype, the procedure described by Hinnen et al. (13) was used. The medium for the selection of Mal^+ yeast transformants was SMal supplemented with 1 M sorbitol, 0.02% glucose, and 3% agar. When yeast transformation was performed with DNA isolated from clone pools, 100 μg of DNA was used per transformation. Transformation with individual recombinant plasmids was achieved with 1 to 5 μg of DNA.

Isolation of RNA. Yeast RNA was isolated by a modification of the method developed by Sripati and Warner (25). Cells were grown in YEP, supplemented with an appropriate carbon source, to early logarithmic phase (1×10^7 to 5×10^7 cells per ml). The cells were collected by centrifugation and washed with ice-cold sterile deionized water. Cells were resuspended at a density of 2 g (wet weight)/ml in LETS buffer, which contained 10 mM Tris-hydrochloride (pH 7.4)–10 mM EDTA–100 mM lithium chloride–0.2% sodium dodecyl sulfate (SDS). The cellular suspension was homogenized with glass beads (0.45 to 0.50 mm in diameter; 1:2 [vol/vol] beads-cell suspension) for 20 s with cooling in a Braun homogenizer. The cell lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was removed, adjusted to 0.1% (vol/vol) with respect to diethylpyrocarbonate, and placed on ice for 10 min. RNA was deproteinized by shaking with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) for 3 to 5 min. The mixture was centrifuged at 12,000 × *g* for 20 min, and the aqueous phase was removed. The organic phase was reextracted with an equal volume of LETS buffer, and after centrifugation (12,000 × *g* for 20 min), this aqueous phase was pooled with the first one. The combined aqueous phase was extracted by adding 1.5 volumes of redistilled, neutralized, water-saturated phenol and 0.5 volume of chloroform-isoamyl alcohol (24:1, vol/vol), mixing in a Vortex mixer (3 to 5 min), and centrifuging at 12,000 × *g* for 20 min. The RNA was precipitated by adding 0.08 volume of 5 M lithium chloride and 2 volumes of absolute ethanol and storing for a minimum of 5 h at −20°C.

Polyuridylate [poly(U)]-Sepharose was used for the preparation of $poly(A)^+$ RNA. Between 0.3 and 0.4 g of freeze-dried poly(U)-Sepharose was swollen in 30 to 40 ml of 1 M lithium chloride for 60 min. The Sepharose was washed on a sterile scintered-glass funnel with 40 ml of 0.1 M lithium chloride and suspended in 20 ml of the same salt solution. The slurry was poured into a small sterile column and washed with 20 ml each of L_4ETSF (40 mM lithium chloride, 10 mM EDTA, 10 mM Tris-hydrochloride [pH 7.5], 0.2% SDS, 5% deionized formamide), 90% formamide buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 0.2% SDS, 90% deionized formamide), and again with L_4ETSF buffer. Total RNA was dissolved in L_4ETSF buffer at a concentration of 500 μg/ml, heated to 60°C for 5 min, and rapidly cooled in an ice-water bath. The RNA sample was applied to the column and washed with the L_4ETSF buffer until no further optically absorbing (at 260 nm) material was eluted (usually 20 to 30 column volumes). $Poly(A)^+$ RNA was eluted from the column with 2 to 3 column volumes of 90% formamide buffer. The $poly(A)^+$ RNA was ethanol precipitated and stored at −20°C.

TABLE 1. Mal^+ transformation of yeast strain JC27 (Mal^-)[a]

Source of DNA used in transformation	No. of Mal^+ colonies obtained
Pool 1	580
Pool 2	25
Pool 3	40
Pool 4	315
Pool 5	0
Pool 6	0
Pool 7	0
Pool 8	0
No DNA	0

[a] Maltose-nonfermenting (Mal^-) strain JC27 was used as a recipient in transformation. JC27 was transformed with recombinant plasmid DNA prepared from each of the eight clone pools (see text). Mal^+ colonies appeared after 5 to 6 days of incubation at 30°C in SM medium.

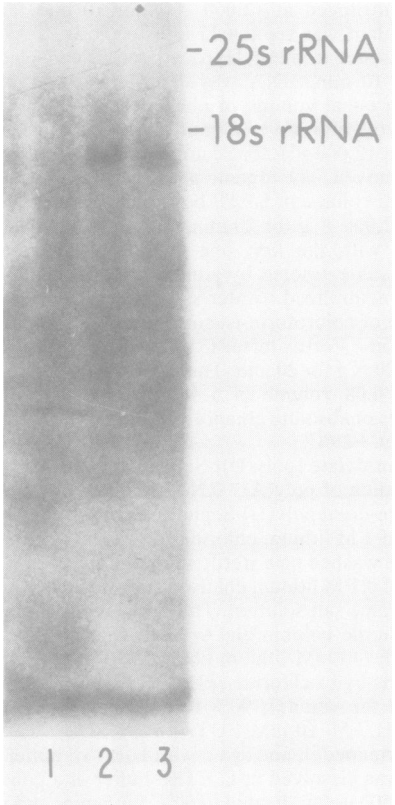

FIG. 1. Hybridization of radiolabeled pMAL26 DNA to fractionated RNA. A 25-μg portion of total RNA was denatured in a buffer containing 50 mM boric acid–5 mM sodium borate–10 mM sodium sulfate–1 mM EDTA–20 mM methyl mercuric hydroxide–20% glycerol, pH 8.2. Denatured RNA samples (10 μg each) were loaded on a 2.0% agarose gel in this buffer, except that glycerol was omitted and the methyl mercuric hydroxide concentration was decreased to 5 mM. The gel was run for 700 V-h, using borate buffer (50 mM boric acid, 5 mM sodium borate) with buffer circulation. The RNA was transferred to diazotized paper and hybridized with 10^5 cpm of nick-translated pMAL26 DNA per cm^2 (5×10^7 cpm/μg). (Lane 1) RNA prepared from uninduced cells of strain CB11; (lanes 2 and 3) RNA prepared from strain CB11, 1 and 2 h after the addition of maltose. The migration position of 18S and 25S rRNA's was determined by hybridizing the blot with nick-translated cloned yeast rDNA (3, 23).

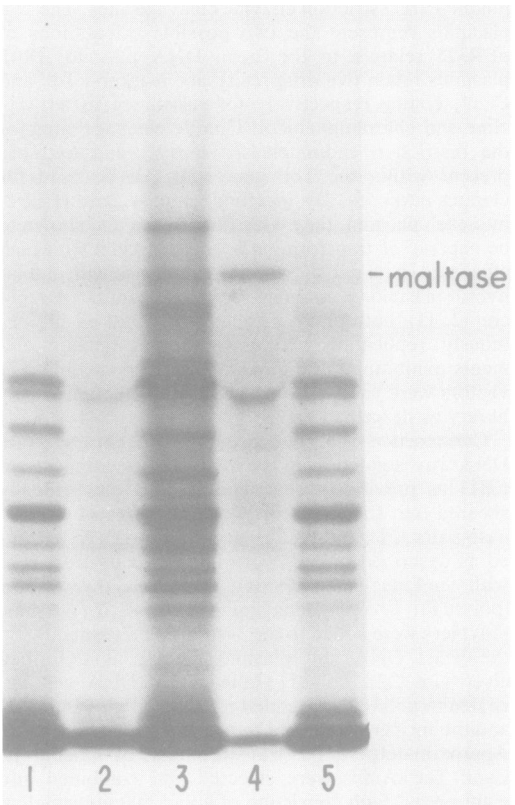

FIG. 2. Identification of maltase structural gene sequences on pMAL26 by positive hybridization translation. A 30-μg portion of polyadenylated RNA from maltose-grown cells of strain CB11 were hybridized to pMAL26 DNA which had been heated to 70°C to relax the supercoiled molecules. Hybridization was performed in a solution containing 0.1 M PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 7.8), 0.01 M EDTA, 0.4 M NaCl, and 70% formamide for 1 h at each of the following temperatures: 55, 52, 49, and 46°C. After cooling to room temperature, unhybridized RNA was partitioned from R-loops and duplex DNA by chromatography on Bio-Gel A 150 m, equilibrated with a buffer containing 0.01 M Tris-hydrochloride (pH 7.4)–0.8 M NaCl–0.001 M EDTA. The fractions containing R-loops were pooled and ethanol precipitated. RNA was melted from the hybrids by heating to 100°C for 3 min before translation in a yeast cell-free translation system (10). Approximately three-quarters of the translation reaction was immunoprecipitated with maltase antibody raised in rabbits to the purified enzyme. Total and immunoprecipitated translation products and controls were run on a 10% SDS-polyacrylamide gel (15) and autoradiographed (5). (Lane 1) Total R-looped RNA cell-free translation products; (lane 2) immunoprecipitated cell-free translation products of R-looped RNA; (lane 3) cell-free translation products from total CB11 polyadenylated RNA; (lane 4) immunoprecipitated total CB11 RNA cell-free translation products; (lane 5) endogenous cell-free translation products (no added RNA).

RESULTS AND DISCUSSION

To construct a clone bank from a donor yeast strain that carries the *MAL6p MAL6g* locus, randomly sheared fragments of DNA from strain CB11 were inserted by the poly(dA-dT) connector method (22) into the cloning vectors pYT11 and pYT14, which in turn were chimeras of

pBR325 (4) and the yeast 2-μm plasmid (14) joined at their unique *Pst*I restriction sites. The cloned yeast DNA was then amplified in *E. coli* RR101. The recombinant plasmid DNA isolated from pools of 500 *E. coli* (pYT) transformants was used to transform the Mal$^-$ strain JC27. Several pools gave higher numbers of Mal$^+$ yeast transformants than did other pools or controls lacking added DNA. These data are presented in Table 1.

Mal$^+$ colonies were identified as transformants and distinguished from possible revertants in the following manner: several Mal$^+$ colonies were picked from the original transformation plates and purified by streaking on SMal plates. Purified Mal$^+$ colonies were again picked, and the stability of their Mal$^+$ phenotype was tested after growth on nonselective medium (YEPD). Most of the Mal$^+$ colonies obtained with the DNA from pools 1, 2, 3, and 4 (Table 1) were found to be highly unstable during mitotic growth as they segregated mostly Mal$^-$ clones. This mitotic instability is an expected characteristic of transformants obtained with nonintegrating vectors such as pYT11 and pYT14 (11).

DNA prepared from several Mal$^+$ yeast transformants grown under selective conditions (SMal medium) was used to transform *E. coli* to chloramphenicol resistance (Cam^r) since each recombinant pYT DNA molecule still retained this drug resistance marker. Plasmid DNAs isolated from several individual *E. coli* Cam^r transformants were then tested for their ability to transform JC27 to the Mal$^+$ phenotype. The plasmid pMAL26 was subsequently identified by this approach. The CB11 genomic DNA fragment carried by pMAL26 thus complemented the defect in strain JC27 so as to allow the strain to grow with maltose as a carbon source.

It is unlikely that transformation to the Mal$^+$ phenotype was due to a suppressor carried by the plasmid since extracistronic suppression was not detected in genetic crosses between donor (CB11) and recipient (JC27) strains.

To determine whether pMAL26 contained any sequences complementary to the maltose-inducible RNA, pMAL26 DNA was hybridized to total cellular RNA that had been immobilized by the procedure of Alwine et al. (1). It had been shown previously that the maltase mRNA migrated with an apparent sedimentation coefficient of 17S (H. J. Federoff, Ph.D. thesis, Albert Einstein College of Medicine of Yeshiva University, New York, N.Y., 1979). Total RNA pre-

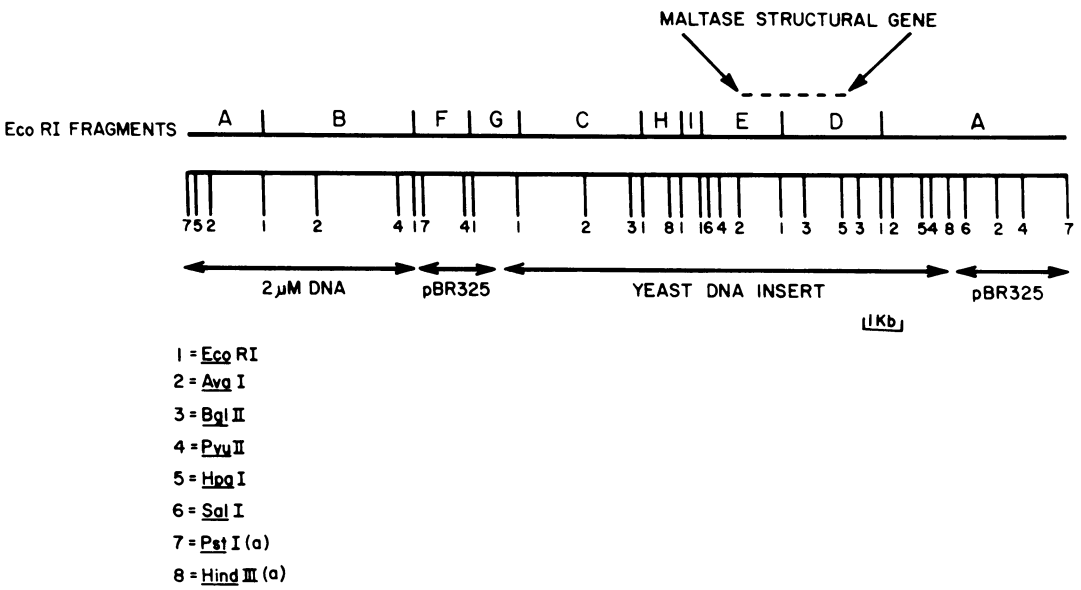

FIG. 3. Restriction map of plasmid pMAL26. Plasmid DNA was digested with the appropriate restriction endonucleases, using the reaction conditions suggested by the supplier (Bethesda Research Laboratories, Bethesda, Md.). When necessary, multiple digests were performed. In addition, several individual restriction fragments were purified from agarose gels (27) and subjected to restriction analysis. Restriction digests were analyzed on 0.8% agarose gels run in a buffer containing 40 mM Tris acetate (pH 7.8)–10 mM sodium acetate–1 mM EDTA. The approximate location of the structural gene for maltase was derived from the experiments and results described in the legent to Fig. 4. 1, *Eco*RI; 2, *Ava*I; 3, *Bgl*II; 4, *Pvu*II; 5, *Hpa*I; 6, *Sal*I; 7, *Pst*I (a); 8, *Hin*dIII (a). (a) indicates that not all *Pst*I and *Hin*dIII sites are shown.

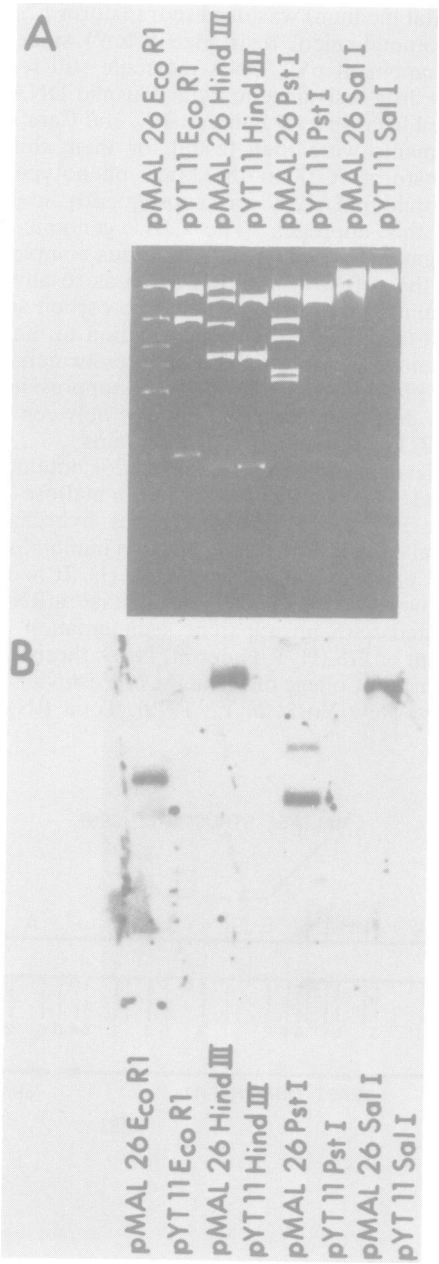

FIG. 4. Mapping the maltase structural gene in pMAL26 DNA. The DNAs of plasmids pMAL26 and pYT11 (parent vector) were digested with restriction enzymes, using conditions specified by the commercial supplier. DNA digests were fractionated on a 1% agarose gel and transferred to a nitrocellulose sheet (24). Polyadenylated RNA prepared from maltose-induced cells of strain CB11 was fractionated on a sucrose gradient, and the 17S region was collected. The RNA was fragmented in 40 mM Tris-hydrochloride, pH 9.0, at 80°C for 15 min, ice chilled, and then end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (16). Radioactivity labeled RNA was hybridized to the nitrocellulose sheet in modified Denhardt solution (9), which contained 5× SSC (1× SSC is 0.015 M sodium citrate, pH 7.0, plus 0.15 M sodium chloride), 50% formamide, 0.5% SDS, 10 μg of sheared denatured calf thymus DNA per ml, 10 mM sodium phosphate buffer (pH 6.8), 0.02% Ficoll (Pharmacia Fine Chemicals, Inc.), 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (fraction V; Sigma Chemical Co.) The hybridization was carried out at 45°C for 16 h. After extensive washing in hybridization solution, the nitrocellulose sheet was exposed to Kodak SB5 X-ray film in the presence of a Lightning-Plus (DuPont Corp.) intensifying screen. (A) Ethidium bromide stain pattern of the restriction fragments of pMAL26 and pYT11. (B) Autoradiogram of the nitrocellulose sheet bearing DNA transferred from the gel depicted in (A) after hybridization with ^{32}P-labeled 17S polyadenylated RNA. No hybridization was expected, or seen, in the control lanes containing only pYT11 DNA.

pared from induced and uninduced cells of strain CB11 was fractionated by electrophoresis on an agarose gel containing the denaturant methyl mercuric hydroxide and transferred to DBM paper. The immobilized RNA was then hybridized to ^{32}P-labeled pMAL26 DNA prepared by nick translation (23). The result shown in Fig. 1 indicates that the plasmid hybridized to a maltose-inducible RNA which migrated to the position expected for 17S RNA. This result indicated that the plasmid contained either all or part of a DNA sequence complementary to a maltose-inducible RNA species. To show that pMAL26 hybridized to a transcript which was indeed maltase mRNA, a positive hybridization-translation assay was performed. Supercoiled pMAL26 DNA was relaxed by heat treatment, denatured, and hybridized (under conditions which favor R-loop formation) to excess polyadenylated RNA prepared from maltose-grown cells of strain CB11. Specific R-loop and duplex DNA were partitioned from nonhybridized RNA by the method of Woolford and Rosbach (28). After the RNA was dissociated from the R-loops, it was translated in the presence of [^{35}S]methionine in a yeast cell-free protein-synthesizing system (10). The cell-free translation products were immunoprecipitated with maltase antibody, and the total immunoprecipitated cell-free translation products were displayed on a 10% SDS-polyacrylamide gel. The autoradiogram of this gel (Fig. 2) showed that immunoprecipitable maltase was synthesized in vitro from RNA complementary to pMAL26 DNA. Identical results were obtained with a wheat germ cell-free protein-synthesizing system (data not shown).

As a first step in determining the location of the maltase structural gene within the 11.4-kilobase genomic insert of pMAL26, a restric-

tion map was constructed (Fig. 3). Polyadenylated RNA prepared from maltose-induced cells of strain CB11 was fractionated by sucrose gradient centrifugation, and the maltase mRNA-enriched 17S region was collected. This 17S RNA fraction was then fragmented, radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, and used to hybridize to a Southern blot of an agarose gel containing various restriction enzyme digests of pMAL26 DNA. The ethidium bromide-stained gel and the autoradiogram of the hybridized blot are shown in Fig. 4A and B, respectively. Analysis of this experiment indicates that the maltase structural gene is contained within *Eco*RI fragments D and E. These fragments define a maximum coding region of 4.7 kilobase pairs. Hybridization-translation experiments by preparative R-looping with *Eco*RI fragment D have confirmed that it contains sequences complementary to maltase mRNA (data not shown).

Since the sequences complementary to the mature maltase mRNA are contained wholly within the DNA insert, the adjacent regions may contain other genes of the *MAL* locus. The observed complementation of *malp* strains by pMAL26 does not necessarily imply that the plasmid contains a functional *MALp* gene. The regulation of the plasmid-borne maltase structural gene may be different from that of the gene located at a chromosomal site. In addition, or alternatively, the cumulative effect of basal level RNA synthesis from a multicopy plasmid might produce enough maltase mRNA, and hence maltase, in the absence of a regulatory gene to allow the cells to utilize maltose. If these two possibilities do not hold, then it is likely that the genomic insert contains a *MALp* gene, specifically *MAL6p*, since the DNA was derived from strain CB11. Furthermore, if it is assumed that complementation of JC27 is due to a functional *MALp* gene on pMAL26, one cannot rigorously exclude the possibility that the cloned maltase-coding sequences are nonfunctional. These possibilities are currently being resolved by subcloning smaller fragments of the original 11.4-kilobase insert, determining the minimum size and DNA base sequences of the fragments which can complement *malp* and *malg* strains and finding their relationship to fragments which hybridize to maltase mRNA.

The availability of the cloned maltase structural gene will facilitate the study of the regulation of transcription and turnover of maltase mRNA in wild-type and mutant strains. The expression of chromosomal and plasmid-associated maltase genes will be compared under repressed and induced conditions. The cloned DNA should also be helpful in elucidating the organization of the *MAL6* locus.

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