Parsons, M. E., Owen, D. A. A., Ganellin, C. R., & Durant, G. J. (1977) *Agents Actions 7*, 31–37.
Steinberg, G. H., Eppel, J. G., Kandel, M., Kandel, S. I., & Wells, J. W. (1985a) *Biochemistry* (first paper of three in this issue).
Steinberg, G. H., Kandel, M., Kandel, S. I., & Wells, J. W. (1985b) *Biochemistry* (second paper of three in this issue).
Tran, V. T., Chang, R. S. L., & Snyder, S. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 6290–6294.
Wells, J. W., Cybulsky, D. L., Kandel, M., Kandel, S. I., & Steinberg, G. H. (1985) in *Advances in the Biosciences: Frontiers in Histamine Research* (Ganellin, C. R., & Schwartz, J.-C., Eds.) Vol. 51, pp 69–78, Pergamon Press, Oxford.

Diverse Properties of External and Internal Forms of Yeast Invertase Derived from the Same Gene†

Frederick K. Chu,* Kenji Takase,‡ Don Guarino, and Frank Maley
Laboratory of Biochemistry, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201
Received January 28, 1985

ABSTRACT: It has been shown by genetic analysis that the external and internal invertases from *Saccharomyces cerevisiae* share a common structural gene [Taussig, R., & Carlson, M. (1983) *Nucleic Acids Res. 11*, 1943–1954]. However, the only amino acid composition of these two forms of invertase reported to date has revealed extensive differences [Gascon, S., Neumann, N. P., & Lampen, J. O. (1968) *J. Biol. Chem. 243*, 1573–1577]. We have found from amino acid analyses of both enzymes and sodium dodecyl sulfate–polyacrylamide gel analysis of their cyanogen bromide peptides that they are most likely identical in their amino acid sequence. However, the invertases exhibit dramatically different physical properties, particularly in their stability. The most striking difference was in their renaturation following guanidine treatment where it was shown that inactivated external invertase could be renatured completely. Endo-β-*N*-acetylglucosaminidase H treated external invertase was restored to 40% of its original activity while internal invertase remained completely inactive. The observed differences may be attributed to the presence and absence of the oligosaccharide moiety in the external and internal invertases, respectively.

Yeast invertase (EC 3.2.1.26) exists in two forms (Gascon et al., 1968): a major glycosylated form (external) found mainly in the periplasmic space and secreted into the medium; and a minor nonglycosylated form (internal) found only in the cytosol. Of considerable interest are the structural and functional relationships between the external secreted invertase and its internal form. Although the two were shown to differ considerably in their amino acid compositions (Gascon et al., 1968), suggesting that they are products of different genes, recent genetic data indicate that their respective mRNA species are transcribed from a single gene, the SUC2 locus (Grossmann & Zimmermann, 1979; Rodriguez et al., 1981), but at different transcriptional starts so that translation of the mRNA for the external enzyme results in an amino-terminal signal peptide which is absent in the nascent internal peptide (Perlman & Halvorson, 1981; Carlson & Botstein, 1982; Taussig & Carlson, 1983). The signal peptide containing 19 amino acids is removed subsequently on transport of the external invertase to the periplasmic space, resulting in a subunit chain that is two amino acids (Ser-Met) longer than the corresponding internal invertase subunit (Carlson et al., 1983). However, a careful structural comparison of the mature forms of these two proteins has not been conducted and is necessary to establish that external and internal invertases are derived from the same gene.

Although external invertase contains up to 50% of its mass in the form of nine high-mannose oligosaccharide chains (Trimble & Maley, 1977), removal of the carbohydrate portion of the glycoenzyme with endo-β-*N*-acetylglucosaminidase H (Endo H)[1] (Tarentino & Maley, 1974) did not affect enzyme activity (Tarentino et al., 1974). Subsequent studies on the effects of Endo H deglycosylation of external invertase demonstrated not only that the invertase oligosaccharide moiety enhanced the enzyme's renaturation efficiency and stabilized its activity under a variety of conditions (Chu et al., 1978) but also that the carbohydrate component facilitated subunit interaction to form active oligomers (Chu et al., 1983). However, the use of Endo H in such studies raises possible objections in that removal of high-mannose oligosaccharide by Endo H is not quantitative unless the glycoprotein substrate is denatured by S-carboxymethylation (Trimble & Maley, 1977) or by boiling in sodium dodecyl sulfate (Chu & Maley, 1980). In addition, modifications which affect enzyme activity other than deglycosylation may occur, due to contaminating components such as proteases often found in Endo H prepa-

† This work was supported in part by National Institutes of Health Grant GM28816.
‡ Present address: Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba Science City, Yatabe, Ibaraki 305, Japan.

[1] Abbreviations: Endo H, endo-β-*N*-acetylglucosaminidase H; Con A, concanavalin A; WGA, wheat germ agglutinin; βMSH, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; UV, ultraviolet; CNBr, cyanogen bromide; PAGE, polyacrylamide gel electrophoresis; GdmCl, guanidinium chloride; CD, circular dichroism; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-6125$01.50/0

rations or associated in trace amounts with invertase. An example of the latter was seen with yeast carboxypeptidase Y (Chu & Maley, 1982). The native nonglycosylated form of invertase, however, presented us with the opportunity to examine the role of the carbohydrate moiety by direct comparison of its properties with those of the glycosylated form, without resorting to the use of Endo H to generate deglycosylated species.

In this paper, we present structural evidence that the protein moieties of the external and internal invertases are identical, confirming the genetic studies of Taussig & Carlson (1983) and Carlson et al. (1983) that they are products of the same gene. In addition, we show that the native glycosylated external form and the native nonglycosylated internal form exhibit different active subunit configurations and differ significantly in their stability.

Materials and Methods

Materials. *Saccharomyces cerevisiae* external invertase (β-fructofuranosidase, EC 3.2.1.26) was obtained from Boehringer-Mannheim Corp. as a dry powder containing about 10% enzyme by weight and purified to homogeneity as previously described (Trimble & Maley, 1977). DEAE-52 cellulose (microgranular) was from Whatman, Bio-Gel P-100 (100–200 mesh) from Bio-Rad, Ultrogel AcA 44 from LKB, and phenyl-Sepharose CL-4B from Pharmacia. Glycerol was from J. T. Baker Chemical Co. Lectins (concanavalin A and wheat germ agglutinin) were purchased from Boehringer-Mannheim and radiolabeled with $Na^{125}I$ (80–100 μCi at 13–17 mCi/μg from Amersham) as described elsewhere (Chu et al., 1981). Trizma base [tris(hydroxymethyl)aminomethane] was from Sigma Chemical Co.

Purification of Yeast Internal Invertase from FH_4C Strain. All steps were carried out at 4 °C or on ice. The purification was adapted from the scheme of Gascon & Lampen (1968) with extensive modifications.

(*A*) *Preparation of Cells and Lysate.* *S. cerevisiae* (FH_4C strain) was grown in YEP medium containing 1% glucose at 30 °C to late log phase (A = 1.5 at 600 nm) before harvest. About 500 g wet weight of cells was obtained from 40 L of culture. The cells were resuspended in 2 volumes (w/v) of buffer I (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 40 mM βMSH, and 1 mM PMSF) and broken by three 1-min pulses in a bead-beater (Biospec Products). The cell lysate was clarified by centrifugation at 30000g for 20 min (S-30).

(*B*) *Stepwise Precipitation.* Nucleic acids were precipitated from the S-30 fraction by incubating with 1% streptomycin sulfate for 1 h and removed by centrifugation. A 40–85% ammonium sulfate fraction (containing all the internal invertase but less than 10% of the external form originally in the S-30) was prepared from the streptomycin sulfate soluble fraction, dissolved in buffer II (10 mM Tris·HCl, pH 7.3, 1 mM βMSH, and 0.5 mM PMSF), and dialyzed exhaustively against the same buffer. The dialysate was adjusted to pH 4.9 by dropwise addition of 2 M acetic acid solution and gently stirred for 2 h. The precipitated protein was removed by centrifugation, and the supernatant was adjusted to pH 7.3 with 1 M Trizma base solution. At this stage, the relative amounts of internal to external invertase were about 1 to 10, respectively.

(*C*) *Column Chromatography.* The pH 4.9 supernatant was loaded onto a Whatman microgranular DE52 column (2 × 18 cm) and eluted with a 1-L linear gradient of 50–350 mM NaCl in 10 mM Tris·HCl, pH 7.3. External invertase eluted in the absence of NaCl whereas internal invertase did so at about 200 mM NaCl. The fractions containing the internal form were pooled, and protein in the pool was concentrated by ammonium sulfate (80%) precipitation. The resulting precipitate was dissolved in 1–2 mL of buffer III (10 mM Tris·HCl, pH 7.3, and 100 mM NaCl) and chromatographed in the same buffer on an Ultrogel AcA 44 column (1.5 × 98 cm). Internal invertase eluted as a sharp peak about two fractions (14 mL per fraction) later than the bovine serum albumin marker. Fractions containing internal invertase were pooled, made 20% in ammonium sulfate, and applied to a phenyl-Sepharose CL-4B column (1 × 4 cm) preequilibrated with 10 mM Tris·HCl, pH 7.3, containing 20% ammonium sulfate. The column was developed with 300 mL of a linear gradient of 20–0% ammonium sulfate in 10 mM Tris·HCl, pH 7.3. Internal invertase eluted in pure form at 5% ammonium sulfate. The fractions containing internal invertase were pooled, concentrated by Amicon filtration to about 1 mg/mL, then dialyzed against 10 mM sodium phosphate, pH 7.2, and stored frozen at −20 °C. No loss of activity was detected after 6 months of storage.

Analytical Gel Electrophoresis. SDS–polyacrylamide slab gel (5.5% for unboiled samples, 12.5% for boiled samples) electrophoresis was performed as described by Laemmli (1970). Detection of invertase activity after electrophoresis on a 5.5% gel was as described previously (Chu et al., 1983). The ability of the electrophoresed invertase protein bands to bind ^{125}I-labeled Con A or ^{125}I-WGA was tested by the lectin–gel overlay technique (Burridge, 1978; Chu et al., 1981). ^{125}I-Lectin binding was visualized by autoradiography using Kodak X-Omat R film.

Analytical Density Gradient Centrifugation. Sedimentation analysis with a Beckman SW50.1 rotor was carried out in 10–30% glycerol gradients prepared in 10 mM sodium acetate (pH 5) or 10 mM sodium phosphate (pH 8.5) containing 100 mM NaCl. Conditions of centrifugation and collection of fractions and their analysis were as described previously (Chu et al., 1983). Sedimentation markers used on parallel gradients were (molecular weights in parentheses) catalase (240 000), thyroglobulin (670 000), and denatured yeast ribosomal RNAs (5, 18, and 28 S).

Analytical Gel Filtration on Bio-Gel P-100. Purified internal invertase (0.1 mg or 400 units) was mixed with 2 mg each of appropriate protein size markers (molecular weights in parentheses), which included bovine serum albumin (135 000 and 68 000), ovalbumin (43 000), and cytochrome c (12 500). The protein mixture in a total volume of 3 mL of buffer III was chromatographed on a 3 × 85 cm Bio-Gel P-100 column (100–200 mesh) at 4 °C. Protein was assayed by measuring absorbance at 280 nm, and invertase activity was assayed colorimetrically by the method of Goldstein & Lampen (1975) with minor modification. Virtually all input invertase activity was recovered in a single peak. One unit of invertase is defined as the amount of enzyme that releases 1 μmol of glucose per minute from sucrose at 37 °C, when assayed at pH 5.

Spectrophotometric Studies. Circular dichroism profiles of various forms of invertase were obtained for both near- and far-UV regions as described previously (Chu et al., 1978). Tryptophan fluorescence measurements were made as described elsewhere (Chu et al., 1983).

Amino Acid and N-Acetylglucosamine Analyses. A Beckman Model 119 CL amino acid analyzer was used for the determination of amino acid composition and for *N*-acetylglucosamine (Plummer, 1976). For amino acid analysis, invertase samples hydrolyzed in 6 N HCl at 110 °C for 24, 48, and 72 h were used. Cysteine residues were quantitated as *S*-cysteic acid after hydrolysis in 6 N HCl at 110 °C in the

Table I: Purification of Internal Invertase from Yeast FH_4C

fraction	volume (mL)	total protein (A_{280})	total invertase act. (units)[a]	internal[b] invertase act. (units)	sp internal act. (units/A_{280})	recovery (%)	x-fold purification
(1) cell extract (S-30)	1880	225600	1220120	18302	0.082	100	1
(2) streptomycin sulfate (1%) supernatant	2220	210900	1198800	17982	0.085	98	1.04
(3) ammonium sulfate (40–80%) pptn	500	85500	54500	ND[c]	ND	ND	ND
(4) pH 4.9 supernatant	480	9600	52320	ND[c]	ND	ND	ND
(5) DE52 (200 mM NaCl) chromatography	95	80	9880	9880	124	54	1512
(6) Ultrogel AcA 44 chromatography	15	15.7	6475	6475	412	35	5024
(7) phenyl-Sepharose chromatography	55	2.5	4020	4020	1608	19	19610
(8) Amicon concentrate	1.5	2.2	3498	3498	1590	17	19390

[a] One unit of invertase is defined as the amount of enzyme that releases 1 μmol of glucose from sucrose at pH 5 at 37 °C per minute. [b] Internal invertase activity was estimated on the basis of the assumption that it constituted 1.5% of total cell invertase activity. [c] Internal invertase activity was not determined (ND) in these fractions as the exact amount of contamination by external invertase in them could not be conveniently determined.

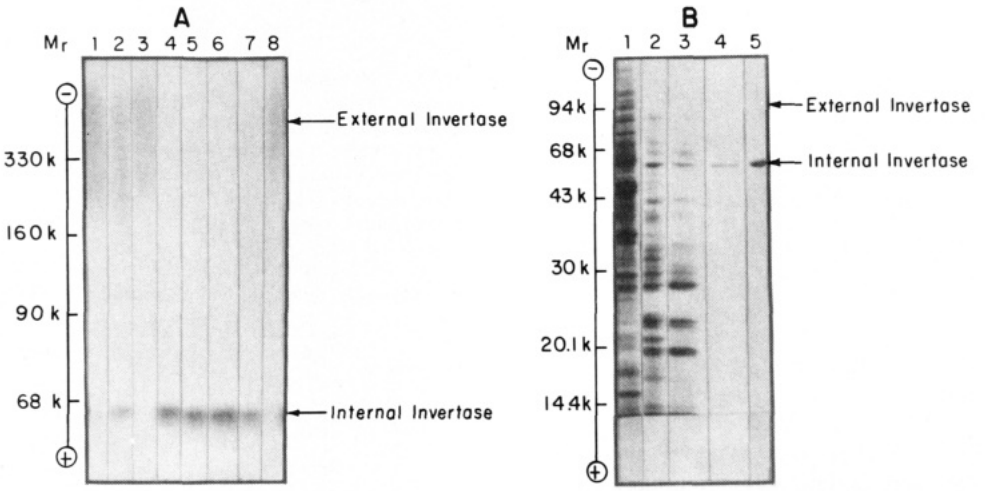


FIGURE 1: Purification of yeast internal invertase. (A) Separation of external and internal invertase activity during purification. Aliquots of each fraction containing 2 units of enzyme were electrophoresed without prior boiling on a 5.5% slab gel which was subjected to staining for invertase activity. The lanes are (1) S-30, (2) 40–80% ammonium sulfate fractions, (3) DE52 run-through, (4) DE52 200 mM NaCl pool, (5) Ultrogel AcA 44 pool, (6) phenyl-Sepharose 5% ammonium sulfate pool, (7) pure internal invertase, and (8) pure external invertase. Molecular weight markers were thyroglobulin (330 000), *E. coli* RNA polymerase subunits (160 000 and 90 000), and bovine serum albumin (68 000). (B) Degree of purity of internal invertase in selected fractions. Samples were boiled and electrophoretically analyzed on a 12.5% slab gel which was stained with Coomassie Blue for visualization of protein bands. The lanes are (1) S-30 (30 μg of protein); (2) DE52 200 mM NaCl pool (15 μg of protein), (3) Ultrogel AcA 44 pool (7.5 μg of protein), (4) phenyl-Sepharose 5% ammonium sulfate pool (0.5 μg of protein), and (5) purified internal invertase (1.5 μg of protein). The size markers were phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

presence of 0.2 M dimethyl sulfoxide for 23 h (Spencer & Wold, 1969). *N*-Acetylglucosamine was determined as glucosamine after hydrolysis in 2 N HCl at 110 °C for 18 h.

Other Methods. Cleavage of invertase polypeptide was effected with a 1000-fold molar excess of CNBr over methionine residues (10 per invertase subunit). Treatment of native or denatured external invertase with Endo H was as described previously (Chu et al., 1981). Denaturation was achieved by heating at 90 °C for 2 min in 0.5% SDS. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Carbohydrate was determined by the phenol–sulfuric acid method (Dubois et al., 1956) with mannose as standard.

Results and Discussion

Purification of Internal Invertase. Invertase, including both external and internal forms, constitutes about 0.3% of total yeast proteins of which about 0.005% is the internal form. It would therefore require a 20 000-fold purification, and as shown in the purification scheme (Table I), the final product was purified about 19 500-fold. Although the use of glusulase to generate spheroplasts would effectively remove the periplasmic external invertase, it was not employed because the internal form, being carbohydrate free, may be very susceptible to trace proteases in the glusulase preparation. However, ammonium sulfate fractionation of the cell lysate effectively removed 90% of the external form (Figure 1A, lane 2, and Table I). Subsequent DE52 column chromatography separated the internal form (Figure 1A, lane 4) eluting at 200 mM NaCl quantitatively from the remaining 10% of the external form, which did not bind to DE52 even in the absence of NaCl (lane 3). Gel filtration of the 200 mM NaCl DE52 pool on an Ultrogel AcA 44 column affected an additional 3.5-fold purification (Table I and Figure 1B, lane 3). Final and complete purification of internal invertase was achieved by differential elution with a 20–0% ammonium sulfate gradient on a phenyl-Sepharose column, where internal invertase eluted in a single sharp peak at 5% ammonium sulfate (Figure 1B, lane 4). A yield of 1 mg of pure internal invertase was obtained from about 500 g (wet weight) of cells and represented a 20 000-fold purification. This extremely low amount of internal invertase (0.005%) in yeast, requiring such a high degree of purification, has led us to increase the production of internal invertase in yeast by cloning the SUC2 gene modified for internal invertase production into a yeast shuttle vector (Williams et al., 1985).

Functional and Structural Similarities between the Internal and External Invertases. Purified internal invertase had a specific enzyme activity of 3600 units/mg of protein, similar to the pure external form (about 3500–4000 units/mg of

Table II: Amino Acid Composition of External and Internal Forms of Yeast Invertase

	calcd residues/60 000-dalton subunit[a]		
	external[b]	internal[b]	external[c]
aspartic acid	77	74	76
threonine	38	32	39
serine	43	43	47
glutamic acid	49	50	48
proline	29	31	26
glycine	31	32	30
alanine	28	31	29
half-cystine	3	3	2
valine	28	31	29
methionine	10	8	10
isoleucine	18	18	18
leucine	35	38	34
tyrosine	30	28	31
phenylalanine	35	35	36
histidine	6	7	4
lysine	24	25	25
arginine	13	12	13
tryptophan	ND[d]	ND[d]	16
N-acetylglucosamine	18	0	ND[d]

[a] This approximates the mass of the external invertase protein subunit (Trimble & Maley, 1977). [b] Average of at least two determinations. [c] According to the external invertase gene sequence from *S. cerevisiae* strain S288C (Taussig & Carlson, 1983). [d] Not determined.

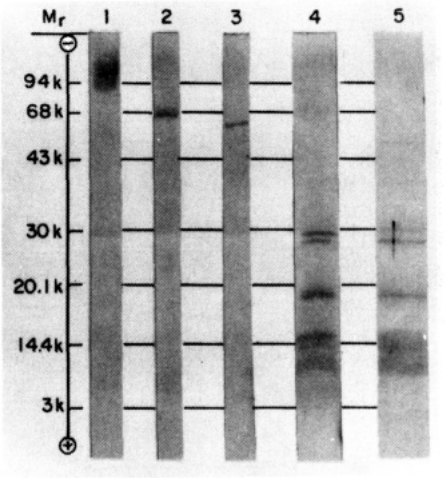


FIGURE 2: Cyanogen bromide peptides of the protein moiety of external and internal invertases. See Materials and Methods for exhaustive deglycosylation with Endo H and CNBr cleavage. Electrophoretic analysis was performed on a slab step gel containing 6.5 mL each of 17.5% acrylamide in the bottom and 15% acrylamide in the top. The lanes are (1) external invertase, (2) Endo H exhaustively deglycosylated external invertase, (3) internal invertase, (4) CNBr peptides of deglycosylated external invertase, and (5) CNBr peptides of internal invertase. The markers were as described in Figure 1B in addition to reduced insulin subunits (~3000).

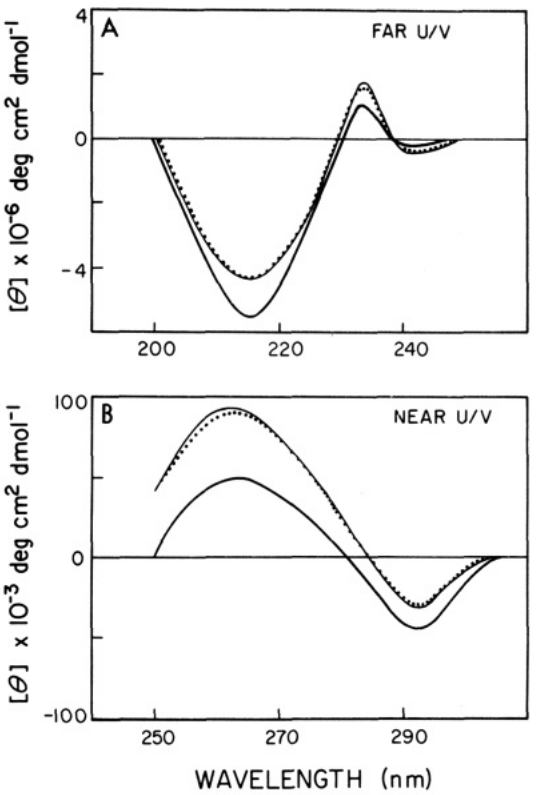


FIGURE 3: Circular dichroism spectra of external, Endo H treated external, and internal forms of invertase. The buffer used was 10 mM sodium phosphate, pH 7, and measurements were made with a Cary Model 16 CD apparatus. (A) Far-UV CD spectra measured at a protein concentration of 100–200 μg/mL with a 0.05-cm cell. (B) Near-UV CD spectra measured at a protein concentration of 300–400 μg/mL with a 1-cm cell. External invertase (fine line); Endo H treated external invertase (dotted line); internal invertase (bold line).

protein). The two forms exhibited almost identical amino acid compositions, within the limits of error of the analysis (Table II), in contrast to the amino acid compositions reported previously (Gascon et al., 1968). In addition, contrary to the same report that the internal form contained no cysteine residues (Gascon et al., 1968), our analysis revealed the presence of three cysteines per subunit for both the external and internal invertases. Recently, it was found that non-cysteine-containing proteins contribute a background to the cysteic acid region, which when subtracted yields a value closer to 2. Two cysteines are indicated in the deduced amino acid sequence of external invertase of yeast strain S288C (Taussig & Carlson, 1983) which shows a remarkably similar amino acid composition to our invertases purified from FH_4C cells (parent strain 303–67). The differences in the number of serine and histidine residues may be strain related.

Since the amount of pure internal invertase in our hands was too low for amino acid sequence analysis, CNBr cleavage was attempted to localize the methionine residues in the polypeptide backbone. To obtain a discrete CNBr peptide profile for external invertase, it was first denatured by heating in 0.5% SDS (Chu et al., 1981) and quantitatively deglycosylated with Endo H before CNBr cleavage. The resulting peptides from each form of invertase were electrophoretically analyzed in a 15–17.5% step polyacrylamide slab gel containing SDS. Although the CNBr cleavage was not quantitative, as evidenced by the presence of larger than expected peptides in the cleavage products, a comparison revealed remarkable similarities in the sizes of the CNBr peptides derived from each (Figure 2, lanes 4 and 5). It seems reasonable to assume, therefore, on the basis of amino acid analysis and CNBr peptide profiles, that external and internal invertases contain identical amino acid sequences. This belief was reinforced by the observed similarity in their respective circular dichroism spectra (Figure 3), a measurement of the three-dimensional folding of the polypeptide moiety, and by cross-immunoreactivity as detected in immunoprecipitation studies using antibody prepared against external invertase (unpublished experiments). Our structural data on the polypeptide moiety of each form of invertase therefore are in agreement with the genetic analysis (Grossmann & Zimmermann, 1979; Rodriguez et al., 1981; Perlman & Halvorson, 1981; Carlson & Botstein, 1982; Taussig & Carlson, 1983) indicating that the external and internal invertases are products of the same

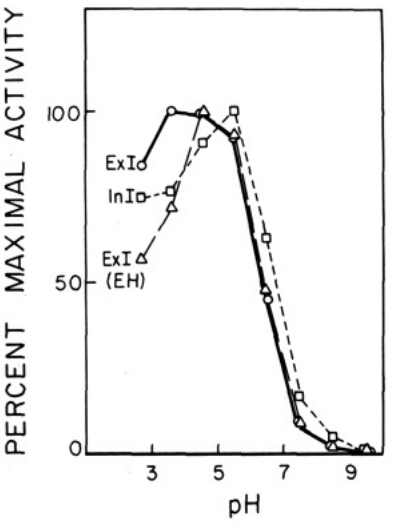
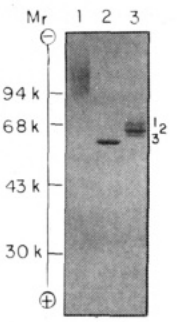


FIGURE 4: pH–activity profile of external, Endo H treated external, and internal forms of invertase. For sucrose hydrolysis (first step of the two-step method), 0.25 μg of invertase protein was incubated with 0.2 M sucrose in 20 mM of either sodium citrate (for below pH 6) or sodium phosphate (for pH 6 and higher) at 37 °C for 10 min. Released glucose was assayed as described by Goldstein & Lampen (1975). All assays were done in duplicate: (○) external invertase; (△) Endo H treated external invertase; (□) internal invertase.



AFFINITY FOR ^{125}I-ConA/WGA

INVERTASE / LECTIN	Ex I	Ex I (EH) BAND 1	Ex I (EH) BAND 2	Ex I (EH) BAND 3	In I
Con A	+	+	+	−	−
WGA	−	+	+	+	−
CARBOHYDRATE (%)	100%		10%		0%

FIGURE 5: Subunit size and lectin binding property of external, Endo H treated external, and internal forms of invertase. Duplicate samples (2 μg) of each invertase (heat denatured) were electrophoresed on a 12.5% polyacrylamide slab gel and stained for protein: (lane 1) external; (lane 2) internal; (lane 3) Endo H treated external invertase. The gel was subjected to the lectin–gel overlay procedure for detection of ^{125}I-labeled Con A and ^{125}I-WGA binding. The results are also tabulated in Figure 5. Percent carbohydrate represents the relative mannose content as assayed by the phenol–sulfuric acid method.

structural gene. The only difference in the two is the presence of a Ser-Met dipeptide on the amino terminus of external invertase which is a product of signal peptide cleavage (Perlman et al., 1982; Carlson et al., 1983) and which has been confirmed by us (Williams et al., 1985).

pH–Activity Profile of Various Forms of Invertase. It was previously shown by Gascon et al. (1968) that external and internal invertases shared identical pH–activity profiles. Since there are obvious differences between their internal invertase and ours, we performed a similar study with external and internal invertases, as well as with the Endo H treated external form containing 10% residual carbohydrate. As shown in Figure 4, external invertase exhibited a broad pH range for activity, peaking at about pH 4 (open circles), whereas internal invertase showed a sharp optimum at pH 5.5 (open squares). It is of interest to note that the 90% deglycosylated external form (Endo H treated) had a pH optimum of 4.5–5 (open triangles), intermediate between those for the fully glycosylated external and nonglycosylated internal forms. Since the difference between the external and internal forms is the presence and absence of carbohydrate, it appears that the carbohydrate moiety, though not required for enzyme activity, alters the pH optimum for enzyme activity.

Carbohydrate Content of External and Internal Invertase Subunits. Although external and internal invertases share an identical polypeptide moiety, only the former contains carbohydrate up to 50% of its mass in the form of nine high-mannose oligosaccharide chains, each linked to an asparagine residue by a di-*N*-acetylchitobiosyl core (Trimble & Maley, 1977). Internal invertase is devoid of mannose and *N*-acetylglucosamine residues, in contrast to the external form which contains 18 *N*-acetylglucosamine residues (Table II) in addition to mannose. Analysis of each form by SDS–PAGE showed the external subunit to be about 100 000 daltons (Figure 5, lane 1) and the internal subunit to be just under 60 000 daltons (lane 2). The Endo H deglycosylated external subunit (band 3 in lane 3) was, however, about 2000 daltons (the equivalent of nine *N*-acetylglucosamine residues) larger than the internal subunit. The subunit designated by band 1 (lane 3) contains two oligosaccharide chains, while that by band 2 contains one chain, with both band 1 and 2 oligosaccharides being resistant to Endo H (Chu et al., 1981). As explained earlier, this could be due to the folding of the polypeptide subunit (Trimble et al., 1983) and/or aggregation of subunits (Chu et al., 1983). The lectin (^{125}I-labeled Con A and ^{125}I-WGA) binding capacity of external and Endo H treated external invertase has also been described previously (Chu et al., 1981) and is presented here for comparison with that of the internal invertase (Figure 5, table). Although the mannose-rich external subunit bound Con A, it failed to bind WGA which has specific affinity for terminal *N*-acetylglucosamine residues. On treatment with Endo H, the completely deglycosylated form (band 3 in lane 3 of Figure 5) lost its Con A binding ability due to a complete loss of mannose residues. However, it then was able to bind WGA due to the nine exposed *N*-acetylglucosamines remaining on the polypeptide backbone. The internal invertase subunit did not bind either lecitin, confirming the absence of mannose and *N*-acetylglucosamine residues.

Aggregation Property of External and Internal Invertase Subunits. It was previously shown that native external invertase was oligomeric (mostly octamer, hexamer, and tetramer) and that, on deglycosylation with Endo H, it dissociated to a dimer (Chu et al., 1983). Dissociation to a monomer resulted invariably in the generation of an inactive species of external invertase. Additional evidence for the external monomer being intrinsically inactive was obtained from sedimentation analysis on 10–30% glycerol gradients at pH 8.5, a pH where sucrose is not hydrolyzed by external invertase (Figure 4). As shown in Figure 6, sedimentation analysis of external invertase at pH 5, optimum for enzyme activity, revealed its oligomeric nature (panel A, open circles) with all of the oligomers (dimer, tetramer, hexamer, octamer) equally active (closed circles). However, at pH 8.5, only a tetramer (shoulder), a major dimeric peak, and, interestingly, a minor monomeric peak were observed (Figure 6B, open circles). Analysis of the pH 8.5 collected peak fractions for enzyme activity at pH 5 showed that only the monomer was devoid of enzyme activity (closed circles).

These results are in contrast to those obtained with internal invertase which, as shown in the activity gel of fractions at various stages of purification (Figure 1A), indicate that internal invertase is active when present at an apparent monomeric size of about 60 000 daltons. To eliminate the possibility that the monomer, though active, might have been generated by the presence of SDS in the gel, purified internal

Table III: Functional Stability of External, Endo H Treated External, and Internal Forms of Yeast Invertase

		incubation conditions				% original activity[a]		
expt	test reagent	pH	temp (°C)	time (min)	enzyme concn	external	Endo H treated external	internal
1	4 M GdmCl	5.0	4	5	0.2 mg/mL	100	40	2
2	pH 2.9	2.9	4	60	0.5 μg/mL	100	81	39
3	pH 2.9	2.9	22	60	0.5 μg/mL	90	60	25
4	0.2% SDS	5.0	22	5	0.5 μg/mL	100	85	50
5	0.2% SDS	5.0	37	5	0.5 μg/mL	88	18	0

[a] After incubation, appropriate aliquots containing 0.25 μg of enzyme protein were assayed in duplicate by the two-step method (Goldstein & Lampen, 1975) for residual activity.

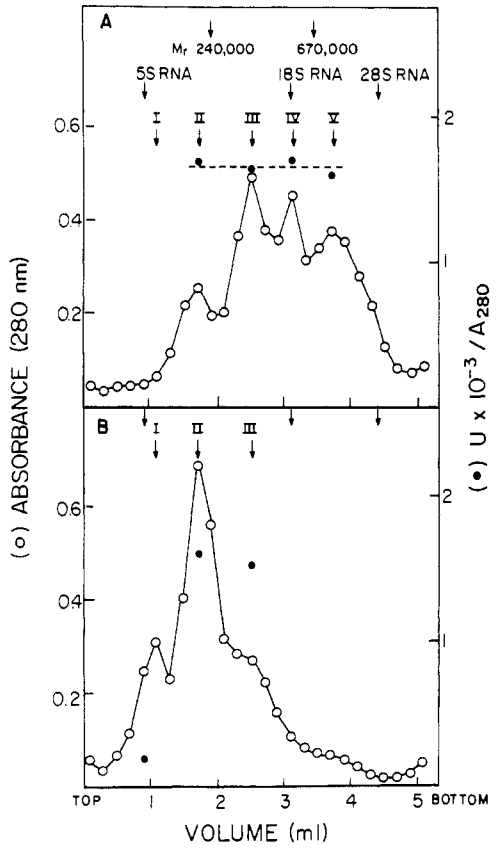


FIGURE 6: Sedimentation analysis of external invertase on 10–30% glycerol gradients. External invertase (500 μg in 100 μL) was subjected to glycerol density gradient centrifugation in 10 mM sodium acetate at pH 5 (A) or 10 mM sodium phosphate at pH 8.5 (B) containing 100 mM NaCl with a Beckman SW50.1 rotor at 49 000 rpm for 3.5 h at 22 °C. Fractions were collected and assayed for absorbance at 280 nm (O) as well as for specific invertase activity (●). Size markers were centrifuged on parallel gradients in the same buffer.

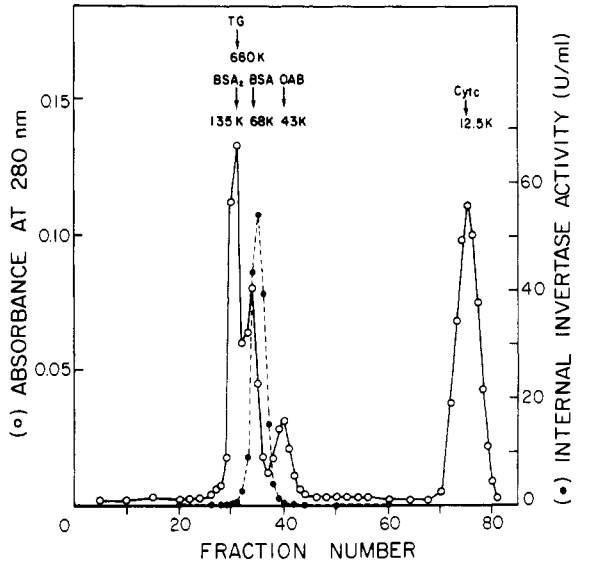


FIGURE 7: Bio-Gel P-100 column chromatography of purified internal invertase. See Materials and Methods for details. Column size markers were bovine serum albumin (135 000 and 68 000), ovalbumin (43 000), and cytochrome *c* (12 500). (O) A_{280}; (●) invertase activity.

invertase (400 units) was sized on a Bio-Gel P-100 column where dimeric and oligomeric forms of internal invertase, if present, should elute in the void volume. No invertase activity was detected in the fractions representing the void volume. The column fractions were assayed for invertase activity to determine the size of internal invertase, in relation to various coeluted protein size markers. As shown in Figure 7, a single active invertase peak eluting at about 60 000 daltons was observed in the absence of SDS. The column chromatography thus shows unequivocally that internal invertase exists in its native state as a monomer, in contrast to native oligomeric external invertase, which is inactive when converted to a monomer. The reason for this difference in the respective quarternary structures probably resides in the carbohydrate chains associated with the external form. How this is brought about at the molecular level is presently under investigation. It is not unreasonable to speculate that the presence of high-mannose oligosaccharide chains may induce subtle changes in external invertase, which are not detected by circular dichroism and which may facilitate subunit aggregation. Alternatively, the large oligosaccharide chains, by excluding water from the peptide backbone of external invertase, may enhance hydrophobic interactions among the subunits leading to an aggregated state.

Functional Stability of External and Internal Invertases. We have shown previously in in vitro deglycosylation studies that the carbohydrate moiety of the external invertase enhances its ability to renature as well as promotes both the stability of its enzyme activity (Chu et al., 1978) and the resistance of its polypeptide backbone to proteolysis (Brown et al., 1979). However, in these previous studies involving in vitro deglycosylation, we were unable to rule out instability as a result of possible proteolytic modifications. To unequivocally address the role of the carbohydrate moiety in invertase, we have compared native external invertase, Endo H treated external invertase, and native internal invertase for their ability to renature after unfolding in 4 M GdmCl, in addition to their stability at pH 2.9 and in 0.2% SDS. The procedures employed in these studies as well as the results are presented in Table III. Under conditions of unfolding (4 °C for 5 min at pH 5 at a protein concentration of 0.2 mg/mL in 4 M GdmCl), subsequent removal of GdmCl by dialysis resulted in the recovery of enzyme activity as follows: 100% for external, 40% for Endo H treated external, and 2% for internal invertase (Table III, experiment 1). It should be pointed out that incubation of internal invertase with Endo H did not affect its enzyme activity or polypeptide structure (data not shown).

It is interesting to note that the Endo H treated external invertase containing about 10% of the original amount of mannose (as assayed by the phenol–sulfuric acid method) was intermediate between the fully glycosylated external and the nonglycosylated internal forms in its ability to renature. In addition, stability studies at pH 2.9 at 4 and 22 °C (Table III, experiments 2 and 3, respectively) or in 0.2% SDS at 22 and 37 °C (Table III, experiments 4 and 5, respectively) revealed a similar relationship among external, Endo H treated external, and internal forms of invertase. The observed proportionality between the amount of attendant carbohydrate and the degree of functional stability is a finding that deserves further exploration.

In conclusion, structural characterization (amino acid analysis, CNBr peptide profile, CD spectra) of the protein moiety of external and internal forms of invertase reveals a close identity between the two and confirms the suggestion from genetic analysis that they are encoded in a single structural gene. A structure–function analysis of the glycosylated external and nonglycosylated internal invertases supports the notion that the carbohydrate moiety imparts functional stability to the protein moiety as well as facilitates renaturation (refolding) of the polypeptide. In addition, the carbohydrate in external invertase promotes subunit interaction either directly or indirectly, with the attendant result being the formation of active oligomers. Furthermore, the intracellular location of nonglycosylated internal invertase as well as the tunicamycin-induced inhibition of secretion of the external form (Babczinski & Tanner, 1978) suggests that glycosylation of the nascent external invertase polypeptide may be necessary for vectorial transfer of the external form through the secretory pathway in yeast as suggested by Novick et al. (1981) and Huffaker & Robbins (1983). Some of the functions of the carbohydrate moiety demonstrated for yeast invertase not only have been observed for other yeast glycoproteins such as carboxypeptidase Y (Chu & Maley, 1982) and acid phosphatase (Barbaric et al., 1984), which contain mainly high-mannose-type oligosaccharides, but also have been implicated in complex-type glycoproteins in higher eucaryotic cells (Elbein, 1981; Berger et al., 1982). Thus, it was shown that the carbohydrate in vesicular stomatitis virus G protein is involved in the renaturation of the polypeptide (Gibson et al., 1981), its intracellular stability, and its migration to the cell surface (Leavitt et al., 1977). In the case of collagen, the carbohydrate moiety was implicated in the organization of these molecules to form fibril structure (Morgan et al., 1970). Also, the presence of oligosaccharides is essential for the biological properties of some glycoproteins, e.g., rabbit IgG against sheep erythrocytes in cell-mediated cytotoxicity (Koide et al., 1977), in transport of glucose, uridine, and amino acid analogues in chick embryo fibroblasts (Olden et al., 1979), in hormone function (Sairam, 1980; Manjunath & Sairam, 1982; Kalyan & Bahl, 1983), and in viral infectivity (Schwarz et al., 1976; Stohrer & Hunter, 1979; Pizer et al., 1980). Finally, the attendant carbohydrate moiety was shown to stabilize the surface conformation of porcine pancreatic ribonuclease (Wang & Hirs, 1977) and to confer resistance against proteolysis to some glycoproetins such as the chick embryo fibroblast surface glycoprotein (Olden et al., 1978), Rous Sarcoma virus glycoproteins (Stohrer & Hunger, 1979), and mouse L-cell alkaline phosphatase (Firestone & Heath, 1981).

ACKNOWLEDGMENTS

We thank Drs. R. B. Trimble and R. J. Trumbly for many helpful discussions. Dr. Trimble's suggestion and demonstration of the usefulness of phenyl-Sepharose column chromatography in the purification of internal invertase are gratefully acknowledged.

Registry No. Invertase, 9001-57-4.

REFERENCES

Babczinski, P., & Tanner, W. (1978) *Biochim. Biophys. Acta 538*, 426–434.

Barbaric, S., Mrsa, V., Ries, B., & Mildner, P. (1984) *Arch. Biochem. Biophys. 234*, 567–575.

Berger, E. G., Buddecke, E., Kamerling, J. P., Kobata, A., Paulson, J. C., & Vliegenthart, J. F. G. (1982) *Experientia 38*, 1129–1258.

Brown, J. A., Segal, H. L., Maley, F., Trimble, R. B., & Chu, F. K. (1979) *J. Biol. Chem. 254*, 3689–3691.

Burridge, K. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4457–4461.

Carlson, M., & Botstein, D. (1982) *Cell (Cambridge, Mass.) 28*, 145–154.

Carlson, M., Taussig, R., Kustu, S., & Botstein, D. (1983) *Mol. Cell. Biol. 3*, 439–447.

Chu, F. K., & Maley, F. (1980) *J. Biol. Chem. 255*, 6392–6397.

Chu, F. K., & Maley, F. (1982) *Arch. Biochem. Biophys. 214*, 134–139.

Chu, F. K., Trimble, R. B., & Maley, F. (1978) *J. Biol. Chem. 253*, 8691–8693.

Chu, F. K., Maley, F., & Tarentino, A. L. (1981) *Anal. Biochem. 116*, 152–160.

Chu, F. K., Watorek, W., & Maley, F. (1983) *Arch. Biochem. Biophys. 223*, 543–555.

Dubois, M., Gilles, K. A., Hamilton, J. K., Revers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Elbein, A. D. (1981) *Trends Biochem. Sci. (Pers. Ed.) 6*, 219–221.

Firestone, G. L., & Heath, E. C. (1981) *J. Biol. Chem. 256*, 1404–1411.

Gascon, S., & Lampen, J. O. (1968) *J. Biol. Chem. 243*, 1567–1572.

Gascon, S., Neumann, N. P., & Lampen, J. O. (1968) *J. Biol. Chem. 243*, 1573–1577.

Gibson, R., Kornfeld, S., & Schlesinger, S. (1981) *J. Biol. Chem. 256*, 456–462.

Goldstein, A., & Lampen, J. O. (1975) *Methods Enzymol. 42*, 504–511.

Grossmann, M. K., & Zimmermann, F. K. (1979) *Mol. Gen. Genet. 175*, 273–279.

Huffaker, T. C., & Robbins, P. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7466–7470.

Kalyan, N. K., & Bahl, O. P. (1983) *J. Biol. Chem. 258*, 67–74.

Koide, N., Nose, M., & Muramatsu, T. (1977) *Biochem. Biophys. Res. Commun. 75*, 838–844.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Leavitt, R., Schlesinger, S., & Kornfeld, S. (1977) *J. Biol. Chem. 252*, 9018–9023.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Manjunath, P., & Sairam, M. R. (1982) *J. Biol. Chem. 257*, 7109–7115.

Morgan, P. H., Jacobs, H. G., Segrest, J. P., & Cunningham, L. W. (1970) *J. Biol. Chem. 245*, 5042–5048.

Novick, P., Ferro, S., & Schekman, R. (1981) *Cell (Cambridge, Mass.) 25*, 461–469.

Olden, K., Pratt, R. M., & Yamada, K. M. (1978) *Cell (Cambridge, Mass.) 13*, 461–473.

Olden, K., Pratt, R. M., Jaworski, C., & Yamada, K. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 791–795.
Perlman, D., & Halvorson, H. O. (1981) *Cell (Cambridge, Mass.) 25*, 525–536.
Perlman, D., Halvorson, H. O., & Cannon, L. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 781–785.
Pizer, L. I., Cohen, G. H., & Eisenberg, R. J. (1980) *J. Virol. 34*, 142–153.
Plummer, T. H., Jr. (1976) *Anal. Biochem. 73*, 532–534.
Rodriguez, L., Lampen, J. O., & Mackay, V. L. (1981) *Mol. Cell. Biol. 1*, 469–474.
Sairam, M. R. (1980) *Arch. Biochem. Biophys. 204*, 199–206.
Schwarz, R. T., Rohrschneider, J. M., & Schmidt, M. F. G. (1976) *J. Virol. 19*, 782–791.
Spencer, R. L., & Wold, F. (1969) *Anal. Biochem. 32*, 185–190.
Stohrer, R., & Hunter, E. (1979) *J. Virol. 32*, 412–419.
Tarentino, A. L., & Maley, F. (1974) *J. Biol. Chem. 249*, 811–817.
Tarentino, A. L., Plummer, T. H., & Maley, F. (1974) *J. Biol. Chem. 249*, 818–824.
Taussig, R., & Carlson, M. (1983) *Nucleic Acids Res. 11*, 1943–1954.
Trimble, R. B., & Maley, F. (1977) *J. Biol. Chem. 252*, 4409–4412.
Trimble, R. B., Maley, F., & Chu, F. K. (1983) *J. Biol. Chem. 258*, 2562–2567.
Wang, F.-F. C., & Hirs, C. H. W. (1977) *J. Biol. Chem. 252*, 8358–8364.
Williams, R., Trumbly, R., Trimble, R., & Maley, F. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1441.

Inhibition of Rabbit Globin mRNA Translation by Sequence-Specific Oligodeoxyribonucleotides†

Kathleen R. Blake, Akira Murakami, and Paul S. Miller*
Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205
Received December 28, 1984

ABSTRACT: Oligodeoxyribonucleotides 8–12 nucleotides in length whose sequences are complementary to the 5′ end, the initiation codon regions, or the coding regions of rabbit globin mRNA were tested for their ability to inhibit translation in a rabbit reticulocyte lysate and in a wheat germ extract. The oligomers interact specifically with their target mRNAs as shown by their ability to serve as primers with reverse transcriptase. In the reticulocyte lysate, oligomers complementary to the 5′ end or the initiation codon regions inhibit translation of both α- and β-globin mRNA, whereas oligomers complementary to the coding regions have little or no effect. This suggests that reticulocyte ribosomes are able to displace the oligomers from the mRNA during the elongation but not the initiation step of translation. In the wheat germ system, translation was effectively inhibited by all oligomers regardless of their binding site on the message. In contrast to their behavior in the reticulocyte system, the oligomers inhibited α- and β-globin synthesis in a specific manner. This observation suggests that control of α- and β-globin mRNA translation is coordinated in the reticulocyte lysate system but not in the wheat germ extract. The results of our studies indicate that oligodeoxyribonucleotides may be useful probes for studying control of mRNA translation in cell-free systems.

Messenger RNA translation can be specifically inhibited in vitro by hybridization to complementary DNA (cDNA).[1] This procedure, called hybridization arrest (Paterson et al., 1977), has been used to study the location and arrangement of adenovirus 2 genes within the viral genome and to analyze mRNA populations in mouse liver (Hastie & Held, 1978). Hybridization arrest has also been used to study the function of the 3′-noncoding region of globin mRNA in a cell-free translating system (Kronenberg et al., 1979).

Arrest of translation by complementary ribonucleic acids appears to occur naturally during osmoregulation of the Omp F protein of *Escherichia coli* (Mizuno et al., 1984). In this case, mRNA complementary to the 5′ end of Omp F mRNA is produced, and this complementary RNA inhibits translation of Omp F mRNA. Anti-sense mRNAs transcribed from plasmid DNA can be used to inhibit translation of specifically targeted mRNA in bacterial and mammalian cells. For example, Coleman et al. (1984) find reductions in the amount of Lpp protein or Omp C protein in *E. coli* cells transfected with plasmids that code for anti-sense Lpp mRNA or anti-sense Omp C mRNA. Production of Herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) is reduced dramatically in HSV-1-infected TK$^-$ mouse L cells which have been transformed by a plasmid which encodes anti-sense HSV-1 TK mRNA (Izant & Weintraub, 1984).

Oligodeoxyribonucleotides and oligodeoxyribonucleoside methylphosphonates have also been used to inhibit mRNA translation. A tridecanucleotide complementary to part of the 3′ and 5′ redundant sequence of Rous sarcoma virus (RSV) 35S RNA inhibits translation of the viral mRNA in a wheat

† This work was supported by a grant from the National Institutes of Health (GM 31927).

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; VSV, vesicular stomatitis virus; cDNA, complementary DNA; HSV-1, Herpes simplex virus type 1; TK, thymidine kinase; RSV, Rous sarcoma virus; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-6132$01.50/0