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A cDNA clone from Zea mays endosperm sucrose synthetase mRNA

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ABSTRACT

A cDNA clone for maize endosperm sucrose synthetase of 62o nucleotide pairs length was obtained by cloning double stranded DNA obtained from the total maize endosperm poly(A) RNA in pBR322, and identifying the appropriate clone by hybrid-promoted translation.

In Southern blotting to genomic BamHI-digested DNA, a single band only of ~2o Kb lights up, indicating that the sucrose synthetase gene is unique, or that closely linked copies are located on this DNA fragment.

INTRODUCTION

The first transposable genetic elements described and studied at the genetic level were the "controlling elements" in Zea mays (1-3). In the years to follow, the genetic analysis has been carried to a level still unsurpassed by any other system of transposable elements. Biochemical investigations, however, are still lacking. Such biochemical characterizations of transposable DNA elements have been carried out in bacteria (4-8), and more recently in a variety of eucaryotic organisms, including yeast (9, 1o), Drosophila (11-13), and retroviruses (14).

It would be most desirable to characterize the corn "controlling elements" biochemically, because much more is known about the detailed physiology and genetics than about any other of the eucaryotic transposable elements.

Among the several genes which have been mutated by the insertion of one of the controlling elements, gene Sh is a promising candidate for the biochemical investigation. Several insertions of the controlling element Ds at this locus are known (1). The product of the gene, sucrose synthetase, is an important enzyme

of starch metabolism, and is one of the major proteins of the developing endosperm (15, 16). The subunit of the enzyme has a molecular weight of 88.000 (17). It can be identified among the translation products of cell-free protein biosynthesis in rabbit reticulocyte lysates (18). We report here on the isolation and preliminary characterization of a cDNA clone containing parts of the sequence of sucrose synthetase mRNA which has been obtained by cloning cDNA in the multicopy plasmid pBR322.

MATERIAL AND METHODS

Maize endosperm mRNA was extracted from kernels that were harvested at the 23rd day after pollination, as described (18).

Maize rRNA. 23-day old kernels were homogenized in a buffer consisting of 200 mM Tris·HCl pH 8.5, 200 mM sucrose; 50 mM Mg-acetate; 60 mM KCl; 1 mM dithiothreitol (DTT); 2 mM 2'-(3') AMP in a Waring blendor, and filtered through four layers of cheese cloth. The supernatant was centrifuged twice for 20 min in a Sorvall centrifuge and layered on a sucrose cushion in the tubes of a SW27 rotor. The cushion consisted of 5 ml 60% sucrose and 15 ml 20% sucrose. The tubes were centrifuged at 3°C and 26.000 rpm for 5 h. The material collected at the interphase of the two concentrations was collected, phenolized twice, and precipitated with ethanol, and stored in 10 mM HEPES[2] pH 7.5; 1 mM EDTA[3]. RNA was labelled with ^{32}P at the 5'-termini according to (19), after purification on a 5 - 20% sucrose gradient for 16 h at 23.000 rpm in a SW27 rotor.

Maize DNA was prepared by a modified method based on procedures described (20, 21).

Unfertilized ears (size 5-6 cm) were stored at -20°C until use. They were lyophilized for 24 h and subsequently pulverized. The powder was resuspended in 160 mM EDTA, pH 8.0; 0.5% Na-lauroyl sarcosinate and proteinase K (Boehringer) at 100 µg/ml.

The suspension was digested at 33°C overnight, followed by removing undissolved material by centrifugation at 3000 x g for 5 min. The clear supernatant was extracted 4 times with a mixture of phenol, m-cresol and 8-hydroxyquinoline (100:14:0.1) for

3o min. Each time, an equal volume of chloroform and isoamylalcohol (24:1) was added before centrifugation for 1o min at 5ooo x g to achieve a better separation of the aqueous phase. The aqueous phase was dialyzed extensively against 5o mM Tris·HCl, pH 8.o; 1o mM EDTA and 1o mM NaCl. To 1.o ml of DNA solution, o.91 g CsCl were added, and the resulting solution was adjusted to 1oo µg/ml of ethidium bromide. The solution was centrifuged overnight in the V5oTi vertical rotor at 44.ooo rpm at 15°C in a Beckman ultracentrifuge. The fluorescent DNA band was recovered with a syringe fitted with a 1.5 mm needle, extracted with CsCl-saturated isopropanol to remove ethidium bromide, and dialyzed as above, yielding a DNA with an average size of ⩾7o Kb.

E.coli K12 C6oo $r_k^- m_k^-$ rec BC$^-$ (obtained from F.Rougeon, Paris) was used for transformation experiments.

pBR322 (obtained from H.Boyer, San Francisco) was used as a cloning vector.

Restriction enzymes were obtained from Boehringer, Mannheim, Biolabs (Beverly, Mass.), and BRL (Bethesda, Md), and used as specified by the suppliers.

Terminal nucleotidyl transferase was obtained from BRL.

Reverse transcriptase was obtained from Dr.Beard through the office of Program Resources and Logistics, Virus Cancer Program, National Cancer Institute.

The Klenow fragment of DNA polymerase I was obtained from Boehringer (Mannheim).

S1 nuclease was obtained from Sigma.

Radioactive nucleotides were obtained from Amersham/Buchler (Braunschweig).

Other chemicals were obtained from standard suppliers.

cDNA synthesis: Methods described in the literature (22, 23) were used with slight modifications. mRNA was converted to cDNA by an incubation in 1 ml of a reaction mixture containing 1 mM each of the 4 dXTPs (dGTP was α-labelled with ^{32}P, specific activity 1ooo Ci/mmole); 5o mM Tris pH 8.o; 1o mM $MgCl_2$; 1o mM DTT; 225 units AMV reverse transcriptase, and 5 µg oligo (dT). The mRNA was heated for 5 min at 65°C before addition to the incubation mixture and was incubated at 42°C for 6o min. The reaction was stopped by the addition of EDTA to 25 mM and NaOH to

o.15 M. RNA was hydrolysed at 65°C for 2o min, followed by neutralization with HCl and ethanol precipitation. The DNA was dissolved in 1o mM Tris pH 7.5, o.1 M NaCl, 1 mM EDTA and chromatographed on a o.8 x 22 cm column of Sephadex G5o. The void volume was collected, precipitated with ethanol and dissolved in 1oo µl of H_2O. The single stranded cDNA was heated to 7o°C for 5 min, followed by slow cooling to allow hairpin formation, incubated in 5oo µl of 3o mM Tris·HCl pH 7.5, 4 mM $MgCl_2$; 5 mM DTT, 1 mM each of the 4 dXTP (dATP ^{3}H-labelled); o.1 M NaCl, and 5o units of the Klenow fragment of DNA polymerase I. The mixture was incubated at 3o°C for 6o min, and the reaction was stopped by the addition of EDTA and ethanol precipitated. The precipitate was dissolved in Tris, NaCl, and EDTA as before, chromatographed on Sephadex G5o and ethanol precipitated. The DNA was then treated with S1 in 2oo µl of o.3 M NaCl; 3o mM Na-acetate, pH 4.5; 3 mM $ZnCl_2$ and 1ooo U S1-nuclease. The digestion was carried out at 37°C for 3o min, followed by 3 phenolizations, 4 extractions with ether and ethanol precipitation. The double stranded DNA was dissolved in 2oo µl o.1 M NaCl; 25 mM Tris·HCl, pH 7.5; and 2 mM EDTA. In order to enrich for the longer cDNA molecules, the preparation was centrifuged on a 5 - 2o% sucrose gradient in o.1 M NaCl; 25 mM Tris·HCl, pH 7.5; 2 mM EDTA in a SW4o rotor for 19 h at 38.ooo rpm at 4°C, and the leading fractions were pooled. Oligo (dC)·oligo(dG) tailing (24) was done in 14o mM K-cacodylate; 33 mM Tris·HCl, pH 7.2; o.1 M DTT; 1 mM $CoCl_2$; o.2 mM dCTP (dGTP); and 12 U terminal nucleotidyl transferase. The tailing of cDNA was carried out with dCTP, and pBR322 DNA, linearized with PstI, was tailed with dGTP. The incubation was carried out for 3o min at 37°C and was terminated by heating to 65°C for 5 min.

Annealing of the DNAs was carried out with 2o ng of the cDNA and 1oo ng pBR322 in 1oo µl of 1o mM EDTA; o.1 M NaCl by incubating at 7o°C for 1o min, slow cooling to 42°C and incubation at this temperature overnight. The DNA was precipitated by ethanol and dissolved in 1o mM Tris·HCl, pH 7.2; 1 mM EDTA.

Transformations were carried out as described by Cohen et al. (25).

Colony hybridizations were carried out as described by Grun-

stein and Hogness (26).

Plasmid DNA was prepared on a preparative scale as described (27).

Small scale DNA preparations were obtained from 1o ml cultures in M9 medium with o.5% glucose, o.2% glycerol and o.6% casamino acids (Difco), grown to A_{55o} of 1.o, and further incubated in the presence of chloramphenicol (15o µg/ml) overnight, followed by the addition of diethylpyrocarbonate to o.3% to kill the cells. The cells were then centrifuged, resuspended in 1.o ml of 1o mM Tris·HCl pH 7.5; 1 mM EDTA and were then transferred to an Eppendorf tube, centrifuged and resuspended in o.5 ml of 5o mM Tris.HCl pH 8.o; 5o mM EDTA; 15% sucrose and 1 mg/ml lysozyme. After incubation at room temperature for 1o min, 1o µl 1o% SDS[5] were added. The mixture was incubated at 7o°C for 1o min, cooled on ice and mixed with 5o µl of cold 5 M potassium acetate and incubated on ice for 2 h or longer, precipitated and centrifuged in a Sorvall centrifuge for 15 min at 15.ooo rpm at 4°C. The supernatant was recentrifuged, ethanol precipitated, dissolved in water, phenol extracted, followed by three ether extractions, reprecipitated with 3 vol of ethanol, and o.2 M NaCl (final conc.), and finally dissolved in 1oo µl 1o mM Tris·HCl pH 7.5; 1 mM EDTA. These preparations could be used without further purification for mRNA selection (H.Ploegh, pers.comm.).

RNA selection. DNA was denatured by o.33 M NaOH at room temperature for 1o min. The DNA is both nicked and denatured by this treatment and can be bound to nitrocellulose filters after neutralization with an equal amount of HCl and an excess of 2 M ammonium acetate. The total DNA of one of the small lysates described above was then applied under mild suction to a 3 x 3 mm field of a S & S BA85 filter (Schleicher & Schüll, Dassel) that had been boiled in water and stored in 2 M ammonium acetate. The filters carried an appropriate grid, and between two fields receiving DNA, one field was left empty. After applying the last DNA preparation, the filters were washed with 2 M ammonium acetate, followed by 4 x SSC, dried at room temperature, and baked at 8o°C for 2 h in a vacuum oven.

The DNA-containing fields of the filter were cut with a razor

blade, and up to 2o of these filters were prehybridized in 2oo µl of 5o% deionized formamide; o.1 m PIPES[4], pH 6.4; o.4 M NaCl; 1oo µg/ml poly(A) at 55°C for 1 h. Hybridization was carried out with 5o µg poly(A)-RNA in the same hybridization buffer for 3 h. The filters were then washed 1o times with 1 x SSC, containing o.5% SDS at 45°C, followed by 2 washes at room temperature with 2 mM EDTA. The filters were then separated into individual Eppendorf tubes, and the RNA was eluted by boiling for 3o s in 1oo µl H_2O, followed by quick cooling in ice. The filter was then removed, and the eluate was ethanol precipitated after the addition of NaCl to o.2 M and 5 µg calf liver tRNA (Boehringer). The precipitate was dried under vacuum and redissolved directly in the translation mixture.

The supernatants of the hybridization were saved. For translations experiments, aliquots corresponding to an initial amount of 1 µg poly(A) RNA were ethanol precipitated in the presence of 5 µg tRNA, and o.2 M NaCl, centrifuged, and the sediment washed once each with o.1 M NaCl in 65% ethanol and 7o% ethanol in water. The precipitate was then dried under vacuum, and redissolved directly in the translation mixture.

Translation, gel electrophoresis, and autoradiography were carried out, as described elsewhere (18).

Nick translation was done by a method described (28) with minor modifications.

Southern blotting was done according to (29), but the hybridization was in 5 x SSC at 68°C.

Containment: Experiments with live bacteria carrying cDNA clones from maize endosperm mRNA were carried out under L2Bl conditions according to the "Richtlinien für den Umgang mit neukombinierten DNA-Molekülen" of the "Bundesministerium für Forschung und Technologie".

RESULTS AND DISCUSSION

In a typical experiment, 36 µg mRNA were converted to 6oo ng double stranded DNA after S1 digestion. After sizing on the sucrose gradient and dC tailing 65 ng remained, of which 2o ng were used for the annealing. 8.5 ng were used for transformation and yielded 37 tetracyline-resistant colonies. In several of these

experiments, a total of 2oo transformants were obtained. Hybridization of plasmid DNA obtained from the transformants against ^{32}P-labelled rRNA showed that about 25% of the clones carry a DNA insert complementary to rRNA. This was unexpected, since rRNA does not contain poly(A) RNA and should not be able to bind oligo-dT primer for the reaction with reverse transcriptase. A similar observation was made, however, by Ohno et al. (23). The rDNA clones were excluded from the further study. A differential colony hybridization with mRNA preparations from wild type and from a sh/sh mutant gave no unambiguous results. We therefore tested the colonies individually for the ability to bind mRNA molecules which upon subsequent translation gave rise to one major protein band only in cell-free translation. The result of such an experiment is shown in fig. 1. It is seen that several clones were obtained, each of which selects a different mRNA which upon translation gives rise to one predominant protein band in subsequent gel electrophoresis. The molecular weight of the proteins translated from the mRNAs selected varies between less than 2o.ooo and 1oo.ooo Mr. One of these clones (pKS5oo) selects RNA the translation of which yields a predominant band upon subsequent gel electrophoresis that migrates at the position of sucrose synthetase. In fig. 2, the selection with pKS5oo DNA is shown to indeed select sucrose synthetase mRNA, as judged from precipitation with antibodies. In addition to sucrose synthetase, a few other faint bands of lower molecular weight are detected in these autoradiographs upon prolonged exposure. Most of them show a faint reaction with the antiserum and are probably degradation products or products of incomplete translation. Two bands are not at all detected after precipitation of the primary antigen-antibody complex with anti-rabbit immunoglobulin antiserum. These two bands, however, migrate at a position of the gel, where bands are distorted due to the presence of an excess of unlabelled heavy chains of the antiserum used for the precipitation. Two weakly precipitated bands have been seen in this position on a gel, when the translation products of total RNA were reacted with anti-sucrose synthetase, and then precipitated with staphylococcal protein A, which has a lower molecular weight than heavy chains (data not shown). It is therefore possible that even these

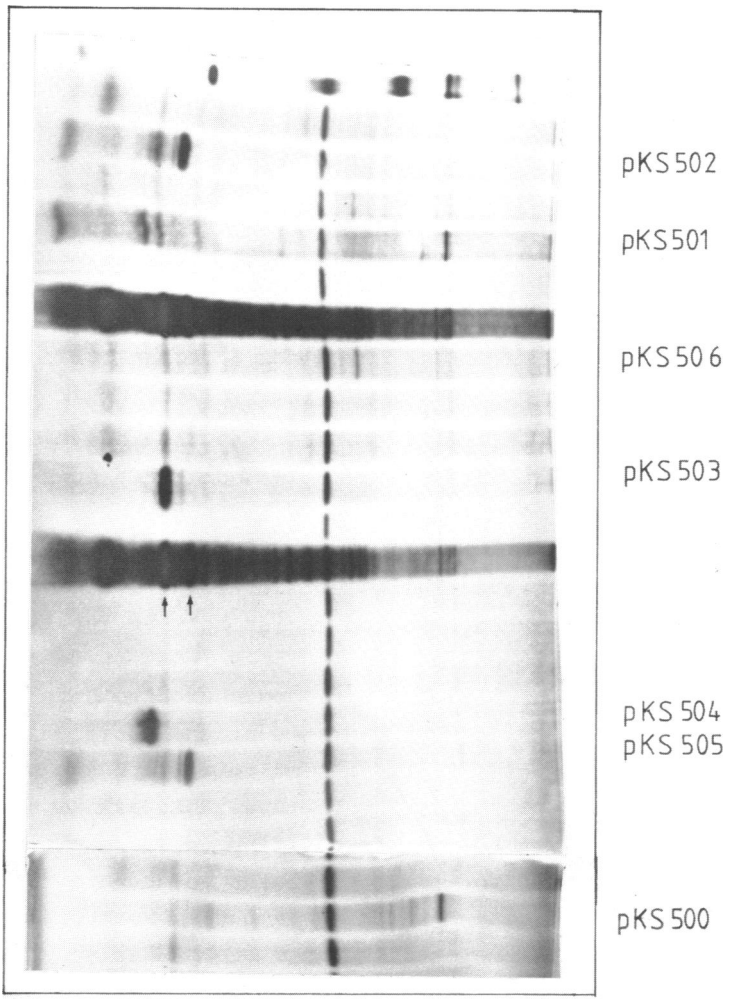

Fig. 1: Electrophoresis of the translation products of RNA samples selected by different cDNA clones. lane 1: molecular weight markers; lanes 2 - 5: RNAs selected by different clones; lane 6: RNA eluted from a filter without DNA ; lane 7: translation products of the supernatant of the hybridization; lanes 8 -11: RNAs selected by different clones; lane 12: no DNA; lane 13: supernatant of the hybridization; lanes 14 - 23: RNAs selected by different clones. Lane 22 is the translation product of RNA selected with pKS5oo-DNA. The predominant band comigrates with sucrose synthetase.

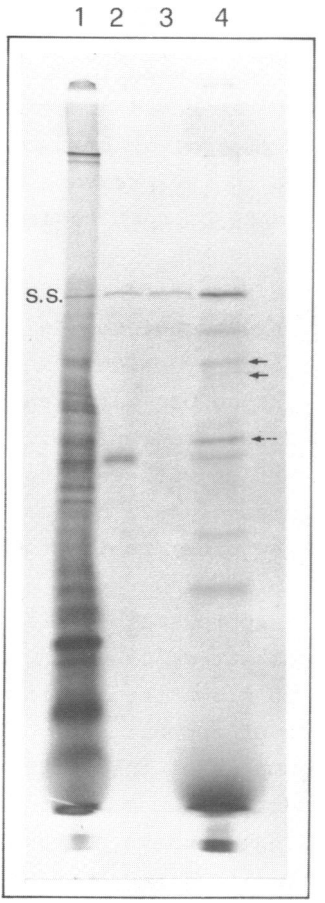

Fig. 2: Electrophoresis of translation products selected with the sucrose synthetase clone. Lane 1: translation of total poly(A)-RNA; lane 2: immunoprecipitation of 1; lane 3: immunoprecipitation of lane 4; lane 4: translation of RNA selected on pKS5oo DNA.
The letters ss denote sucrose synthetase. The broken arrow shows an endogenous band that is observed even after incubation of the reticulocyte lysate in the absence of added RNA. The solid arrows show two bands that have not been seen after antibody precipitation. These migrate in the region, where in the antibody precipitation an excess of unlabelled heavy chain is found, as can be seen from the indentation of the adjacent lane 1.

bands are incomplete sucrose synthetase molecules. Alternatively, it cannot be excluded presently that they are the translation products of an mRNA molecule unrelated to sucrose synthetase mRNA. This message might then be selected either by sequence similarity to the insert carried by pKS5oo, or by forming a hybrid with sucrose synthetase mRNA due to the presence of repeated sequences on these mRNAs. The presence of such sequences has recently been reported (3o-32).

As the minor bands are significantly fainter than the band of sucrose synthetase, we assume that the latter is the predominant product of the mRNA selected by pKS5oo DNA.

Double digestion of pKS5oo DNA with restriction endonucleases HindIII and PvuII yields two fragments with a total size of 494o nucleotides. Double digestion with PstI and HindIII yields three fragments with a total size of 492o nucleotides. These digestions and experiments with a few other restriction enzymes show the following:

1) The insert size is approximately 62o nucleotides.

2) pKS5oo has two PstI sites, of which one is at or very near the PstI site expected to flank the insert. The second PstI site is located at a distance of approximately 12o nucleotides from the first one at the terminus of the insert. The PstI site expected to flank the insert at the other terminus (near the EcoRI site of pBR322) is missing. We have often observed the loss of PstI sites from our clones obtained by dG:dC tailing, possibly due to unknown nuclease contaminations in the enzymes used.

3) HindIII, EcoRI, AvaI, BamHI, and Sal_I do not cleave within the insert. HindII cleaves once, between the PstI cleavage sites.

Maize DNA was digested with BamHI, separated on o.7% agarose gels and blotted to nitrocellulose filters as described in Methods.

Nick-translated pKS5oo DNA (3o x $1o^6$ cpm/µg) was hybridized to the filters for 4 h. An autoradiograph is shown in fig. 3. It is seen that a single band of 2o kb lights up with the probe, indicating that sucrose synthetase is either a unique gene, as was already indicated by the genetic evidence (1), or else that several copies of a sucrose synthetase gene are closely linked on the fragment hybridizing to pKS5oo DNA.

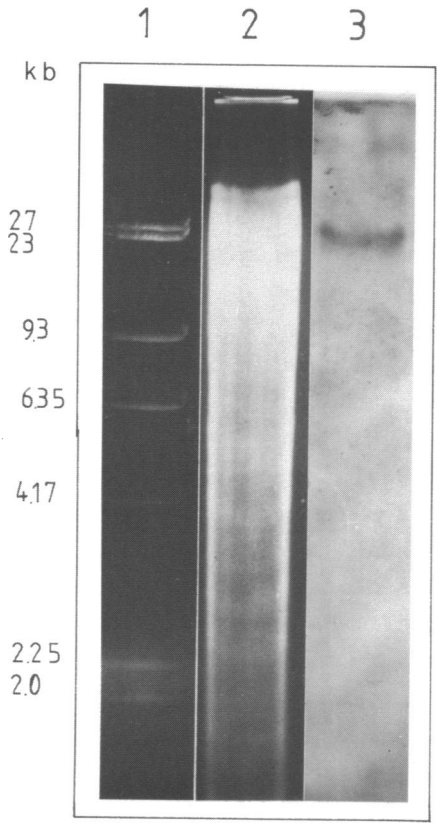

Fig. 3: Hybridization of pKS 5oo DNA to BamHI-digested DNA from wild type maize. Lane 1: HindIII-digested λ-DNA, stained with ethidium bromide; lane 2: BamHI-digested maize DNA, stained with ethidium bromide; lane 3: hybridization of pKS5oo DNA with the DNA shown in lane 2.

The DNA was obtained from the Sh/Sh strain 78o 475, kindly provided by B.Burr (Brookhaven, N.Y.). An identical picture was obtained with DNA from the German variety "Badischer Saatmais", indicating that no major DNA polymorphism at this locus is present between different sources (data not shown).

The finding that pKS5oo DNA hybridizes to one DNA fragment only supports our assumption that the cDNA selects only sucrose synthetase mRNA and that the minor bands observed among the products of hybrid-selected translation are not encoded by mRNA mo-

lecules that are unrelated to sucrose synthetase mRNA.

In summary, we have obtained a 62o base pair cDNA clone in pBR322 that can serve as a probe for the isolation both of genomic DNA (and possibly controlling element DNA adjacent to the Sh gene in sh mutants) and of undegraded mRNA for sucrose synthetase (and possibly mRNA precursors).

The isolation of a similar cDNA clone for sucrose synthetase and its hybridization to genomic DNA has been reported by F.Burr and B.Burr (33).

A further point deserving attention is the failure to select a message encoding the protein of molecular weight 44.ooo which is precipitated from the translation product of 23 day endosperm mRNA by the antiserum against su crose synthetase (fig. 2, lane 3). Though this protein is efficiently precipitated by the antiserum and thus must share antigenic determinants with sucrose synthetase, it does not seem to be encoded by a message complementary to our cDNA clone. This is in agreement with our data on the fractionation of mRNA on polyacrylamide gels, where this protein is synthesized by an mRNA species of lower molecular weight than sucrose synthetase mRNA (18). Since cDNA clones carrying an insert of a size smaller than the mRNA molecule from which it was transcribed are preferentially derived from the 3'-termini of mRNA molecules, it is conceivable that the mRNA encoding the 44.ooo molecular weight protein does not contain this 3'-terminus, either because it is the product of premature termination, or because it is a degradation product. We will pursue this question further.

In addition to the sucrose synthetase cDNA clone, we have isolated a few other clones, as is seen from fig. 1. Some of these seem to be zein clones. We have not done experiments to show the identity of the bands labelled by the arrows with prezein, but the similarity to the prezein protein described by Wienand et al. (34) is apparent. Other clones correspond to proteins of a molecular weight of 53.ooo and 1oo.ooo, respectively. We have no indication yet for the identity of these proteins.

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FOOTNOTES

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[2] HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid).

[3] EDTA (ethylenediamine tetraacetate).

[4] PIPES (Piperazine-N, N'- bis (2-ethane sulfonic acid)).

[5] SDS (sodium dodecylsulfate).

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