Vol. 92, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1980 Pages 563-569

FAITHFUL AND EFFICIENT TRANSLATION OF VIRAL AND CELLULAR EUKARYOTIC mRNAs IN A CELL-FREE S-27 EXTRACT OF SACCHAROMYCES CEREVISIAE

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Received November 30,1979

SUMMARY: The preparation of yeast spheroplast 27,000 x g supernatant /S-27/ which initiates translation of endogenous and exogenous mRNAs is described. The activity of this protein synthesis system is comparable to that of the most efficient cell-free extracts currently in use. The yeast S-27 system is able to carry out faithful translation of distinct eukaryotic mRNAs into proteins as large as 180,000 daltons.

INTRODUCTION

Low genetic complexity and extensive knowledge of genetics of yeast Saccharomyces cerevisiae make it an ideal eukaryote for the study of control mechanisms operating at transcription and translation levels. Studies of the mechanism and regulation of protein synthesis in yeast have long been hampered by the lack of a cell-free protein synthesis system able to initiate translation of natural mRNAs /1,2/. Gąsior et al. /3/ have recently reported that using a modified 100,000 x g supernatant /"S-100"/ of yeast spheroplasts it is possible to obtain translation of exogenous mRNAs. Removal of polysomes seemed to be necessary for obtaining the yeast system able to initiate translation in vitro. In this work we describe the preparation of an active S-27 protein synthesis extract from yeast spheroplasts. This system translates endogenous and exogenous mRNAs with activity approaching that of the most efficient in vitro systems described so far, like the rabbit reticulocyte lysate or wheat germ extract. It is shown that the

Abbreviations used: S-27, 27,000 x g supernatant; SDS, sodium dodecyl sulphate; DTT, dithiothreitol; m^7GMP, 7-methylguanosine-5'-monophosphate; TMV, tobacco mosaic virus; PVX, potato virus X; AMV, alfalfa mosaic virus; TYMV, turnip yellow mosaic virus.

0006-291X/80/020563-07$01.00/0

S-27 spheroplast extract is able to carry out faithful translation of several distinct mRNAs originating from cells and viruses of higher eukaryotes.

MATERIALS AND METHODS

Source of mRNAs. TMV RNA and rabbit globin mRNA were obtained as described /4/. PVX RNA was prepared by phenol-SDS extraction of purified PVX particles /5/. TYMV RNA and AMV RNA 4 were the generous gifts from Drs A.L.Haenni, Paris and L.Van Vloten-Doting, Leiden, respectively.

Preparation of yeast spheroplast extract. Cultures of S.cerevisiae SBTD /diploid, arg⁻ strain, obtained from Dr.H.Baranowska of this Institute/ were grown on YPG /1% yeast extract, 1% bactopeptone, 2% glucose/ at 28°C and collected at the density of 1.0 unit at 660 nm. Yeast spheroplasts were prepared essentially according to Hutchinson and Hartwell /6/. Cells obtained from 1 l of original culture were washed with water, resuspended in 50 ml of 1.0 M sorbitol and 1 ml of β-glucuronidase /Sigma/ was added. After incubation for 1 hr at 20°C the resulting spheroplasts were collected by centrifugation at 2,000 x g for 5 minutes, washed 3 times with cold 1.2 M sorbitol, resuspended in 60 ml of YM-5 medium /6/ containing 0.4 M $MgSO_4$ and incubated for 30 min at 20°C. The incubated spheroplasts were pelleted and washed once with cold 1.2 M mannitol. Final spheroplast pellet was carefully drained and resuspended in buffer A /30 mM Hepes-KOH, pH 7.4, 100 mM K acetate, 2 mM Mg acetate, 2 mM DTT/ containing 8.5% mannitol /1 ml of buffer per g of pellet/. Spheroplasts were disintegrated by 10-12 strokes in the Dounce homogenizer /Kontes/ with a tightly fitting pestle. The lysate was centrifuged for 15 min at 27,000 x g and the upper 2/3 of the supernatant were collected avoiding the lipid layer floating at the top. Aliquots of this S-27 extract were stored at -80°C without considerable loss of activity during several weeks.

Cell-free translation. The yeast protein synthesis assays contained /per 100 µl volume/ 60 µl of S-27 extract / 1.0 mg of protein/ and the following components added at the indicated concentrations: 20 mM Hepes-KOH, pH 7.4, 200 mM K acetate, 1.5 mM DTT, 0.5 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate /Na_2 salt/, 10 µg of creatine phosphokinase, 20 µM each of 19 unlabeled aminoacids and /^{14}C/leucine /0.8 µCi, 300 Ci/mol, New England Nuclear/ or /^{35}S/methionine /9.7 µCi, 760 Ci/mmol, Amersham/. Temperature and time of incubation were 20°C and 100 min except when stated otherwise. Aliquots were withdrawn for estimation of aminoacid incorporation into protein by the Whatman 3MM paper disc method /7/. ^{14}C counting efficiency was 62%.
Translation in a wheat germ extract and in micrococcal nuclease-treated rabbit reticulocyte lysate was performed with /^{35}S/ methionine as described previously /4/. Isotope dilution experiments were performed similarly as in ref.8. Electrophoresis in polyacrylamide-SDS slabs and autoradiography were described /4,7/.

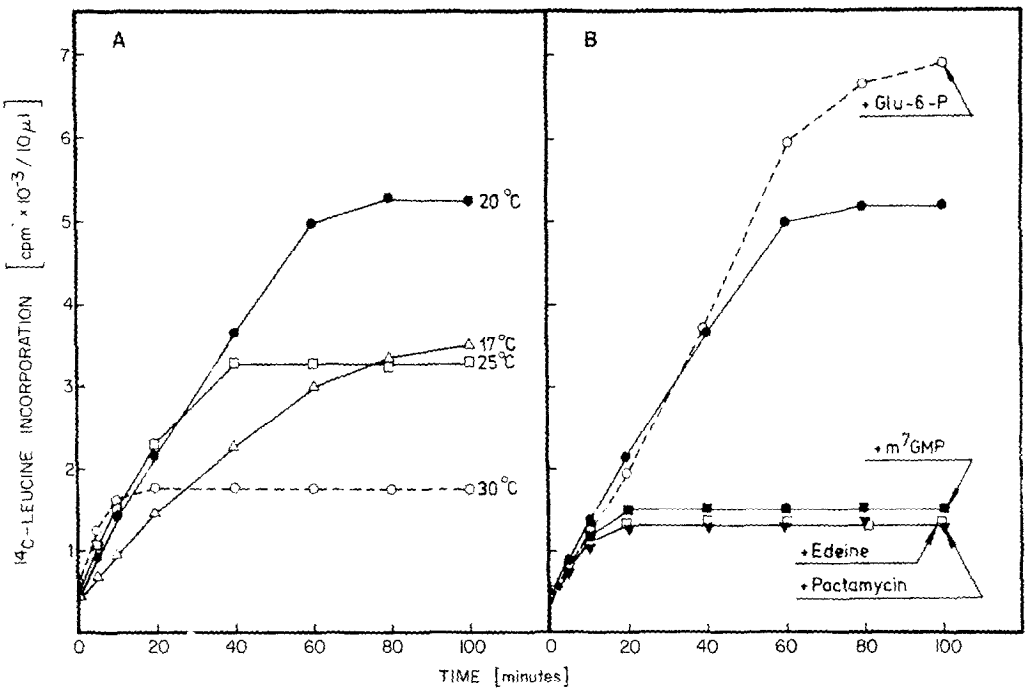

Fig.1. Time course of protein synthesis at different temperatures /A/ and in the presence of initiation inhibitors or glucose-6-phosphate /B/. In /B/ incubations were carried out at 20°C with the following additions: /■/ 0.5 mM m^7GMP /P.L. Biochemicals/, /□/ 1.2 µM edeine /Calbiochem/, /▼/ 1.2 µM pactamycin /Upjohn Co./, /o/ 2 mM glucose-6-phosphate /BDH/ and /●/ none.

RESULTS AND DISCUSSION

Similarly as in Gąsior et al. /3/ yeast spheroplasts were used for preparation of a cell-free extract. The spheroplast S-27 fraction, supplemented with an energy source, aminoacids and the proper ions, efficiently translated endogenous yeast mRNA present in the system. Incorporation of /^{14}C/leucine into protein was linear for 60 min when assayed at 20°C /Fig.1A/. At 25°C or 30°C it was merkedly reduced, possibly due to the more rapid hydrolysis of mRNA at higher temperatures /9/.

Inhibitors of initiation of protein synthesis /10/ like edeine, pactamycin or m^7GMP strongly inhibited translation in yeast extract /Fig.1B/, indicating that the system actively initiates synthesis of new polypeptides coded by endogenous mRNA. In the presence of initiation inhibitors aminoacid incorporation into protein stopped after 10-20 min, a time required for completion of preinitiated polypeptides. It was ascertained

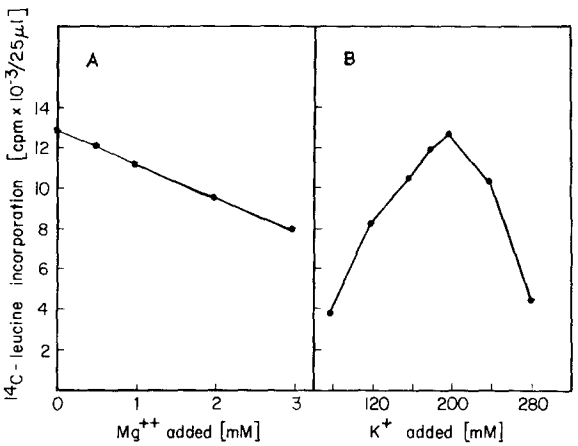

Fig.2. Effect of increasing concentrations of Mg acetate /A/ and K acetate /B/ on protein synthesis in yeast spheroplast extracts. Incubations were carried out with 200 mM K acetate added in /A/ and without addition of Mg acetate in /B/.

that edeine /1.2 µM/, pactamycin /1.2 µM/ or m^{7}GMP /0.5 mM/ do not affect the elongation process; none of these compounds inhibited translation of polyU in yeast extract /assayed at 10 mM Mg acetate/ by more than 10% /data not shown, see also ref. 1 and 3/. As in other cell-free protein synthesis systems /11, 12/ addition of glucose-6-phosphate to the yeast extract resulted in a 25-30% increase in protein synthesis /Fig.1B/. Addition of polyamines, cAMP or hemin was without effect.

Spheroplast extracts contained sufficient amounts of Mg^{++} ions to support the translation process and addition of exogenous Mg acetate resulted in a decrease of protein synthesis /Fig.2A/. The highest level of aminoacid incorporation was obtained with 200 mM K acetate added to the system /Fig.2B/. Taking into account the monovalent ions contributed by the yeast extract and other components of the assay /$\gtrsim$ 100 mM/, this result indicates that the yeast system as described here requires much higher concentrations of K^{+} than other eukaryotic systems /4,11/. Consistently, translation of endogenous mRNA in the extract dialysed against buffer A /see Materials and Methods/ containing 15% glycerol occurred optimally at 300-320 mM K acetate /unpublished results/.

The proteins synthesized in the yeast S-27 extract in the presence of different exogenous viral or cellular mRNAs were analysed by polyacrylamide gel electrophoresis. With endogenous yeast mRNA as a template about 20-25 distinct protein bands

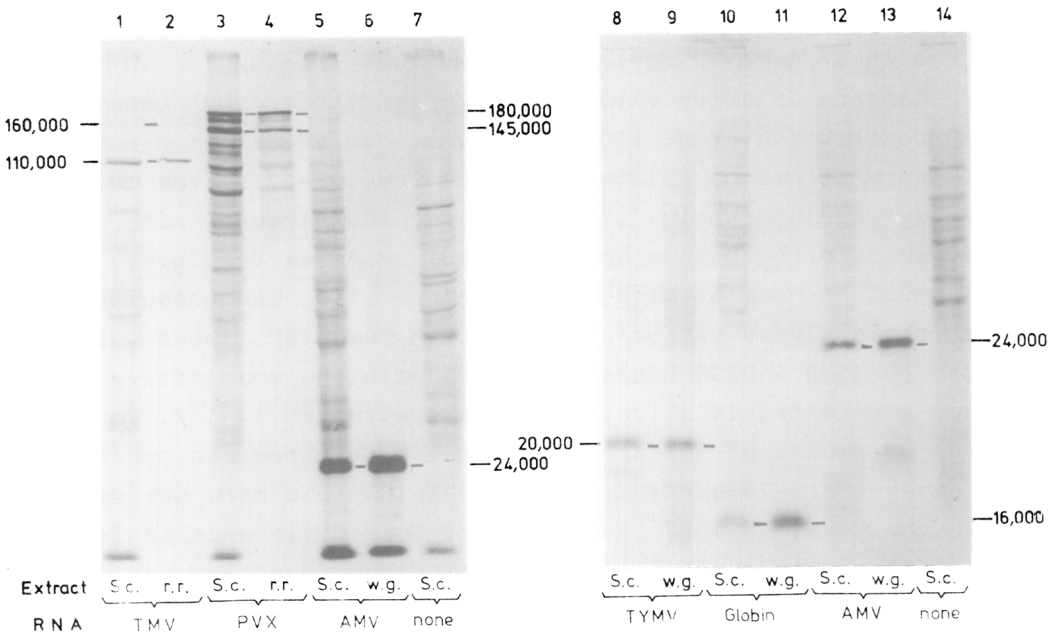

Fig.3. Polyacrylamide gel electrophoresis of proteins synthesized by yeast spheroplast extracts in response to endogenous and exogenous mRNAs. [^{35}S]methionine was used as the labeled aminoacid and 2 mM glucose-6-phosphate was present in yeast translation assays. Translation in wheat germ and rabbit reticulocyte extracts was performed with [^{35}S]methionine as described previously /4/. Incubations /25 µl/ with yeast extract contained the following mRNAs: /1/ TMV RNA, 7 µg; /3/ PVX RNA, 3 µg; /5/ and /12/ AMV RNA 4, 0.5 µg; /8/ TYMV RNA, 4 µg; /10/ rabbit globin mRNA, 0.5 µg. Incubations in reticulocyte lysates /25 µl/ contained: /2/ TMV RNA, 2.0 µg; /4/ PVX RNA, 1.3 µg. Wheat germ extracts /25 µl/ contained: /6/ and /13/ AMV RNA 4, 0.5 µg; /9/ TYMV RNA, 3.0 µg; /11/ globin mRNA, 2.0 µg. Aliquots of translation assays were subjected to electrophoresis in 10% /slotes 1-7/ or 12% /slotes 8-14/ polyacrylamide-SDS slab gels.

/MW 30,000 - 170,000/ were observed /Fig.3 - 7,14/. Addition of TMV RNA to the yeast system resulted in the appearance of two additional protein bands corresponding to 110,000 and 165,000-MW proteins coded by TMV RNA in the reticulocyte lysate /Fig.3 - 1,2, ref.13/. Similarly, PVX RNA stimulated the synthesis of 145,000 and 180,000-MW polypeptides /Fig.3 -3/ which are the main translation products of PVX RNA in the reticulocyte lysate and wheat germ extract /Fig.3 - 4, ref.5/. Among other mRNAs assayed for activity in the yeast system were: AMV RNA 4 which codes for a coat protein of MW 24,000 /ref.13/, TYMV RNA preparation coding for coat protein of MW 20,000 /ref.13/, and rabbit globin mRNA coding for globin chains of MW 16,000. In all instances the products synthesized in

the yeast extract comigrated with the respective proteins made in the wheat germ or reticulocyte systems /Fig.3/

Isotope dilution experiments /8/ indicated that yeast S-27 contributes about 130 μM leucine into translation mixture. Hence it was calculated that the yeast S-27 system incorporates 750-1050 pmoles of leucine per 100 μl assay with either endogenous or both endo and exogenous mRNAs as templates; similar values were obtained when incorporation was measured with the dialysed extract /unpublished results/. These values approach 1500 - 8000 pmoles obtained with the most active wheat germ extracts or reticulocyte lysates /8,14-18/. In terms of pmoles of leucine incorporated into protein per 100 μl assay the S-27 extract is about 25-100 fold more active than the yeast "S-100" system /3,9/. This difference is less pronounced when comparing $/^{14}C/$leucine incorporation per mg of protein. The aminoacid polymerization rate in yeast S-27 system is 0.7 aa/sec /the completion of TMV RNA-coded 110,000-MW protein requires 25 min at 20°C, results not shown/ and also closely resembles the rates calculated for the reticulocyte lysate or wheat germ extract at 20°C /ref.19,20/.

In summary we find that removal of polysomes /3/ is not a prerequisite for the preparation of a yeast system capable of carrying out the initiation reaction. It is not obvious why previous attempts /1-3/ to prepare active S-27 fraction from yeast were not successful. The explanation may possibly lay in the different ionic conditions used for preparation of the extract or translation. Alternatively it could be due to the different yeast strains employed. The S-27 system as described here is able to translate efficiently a variety of exogenous natural mRNAs into proteins as large as 180,000 daltons. The availability of different protein synthesis mutants of yeast /e.g. see ref.21 and 22/ make this system a potentially useful tool in studies of mechanism and regulation of translation. Removal of endogenous protein synthesis by pretreatment with micrococcal nuclease /3,18/ could make the yeast S-27 system still more versatile.

ACKNOWLEDGEMENTS: We thank Drs T.Biliński, E.Gąsior and J.Rytka for many helpful discussions. This investigation was carried out as Project 09.7. of the Polish Academy of Sciences, and profited also from the partial support of the Agricultural Research Service, U.S.Department of Agriculture /FG-Po-334/.

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