Volume 335, number 2, 273–276 FEBS 13338 December 1993

Internal ribosome entry site of encephalomyocarditis virus RNA is unable to direct translation in *Saccharomyces cerevisiae*

A.G. Evstafieva*, A.V. Beletsky, A.V. Borovjagin, A.A. Bogdanov

A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russian Federation

Received 12 October 1993

To evaluate the potential of the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) to promote efficient expression of foreign genes in the yeast, *S. cerevisiae*, we have constructed *E. coli*–yeast shuttle vectors in which the EMCV 5′ non-coding region was fused to the reporter gene, human prothymosin α. Efficiency of translation of corresponding RNA transcripts in mammalian cell-free systems was highly dependent on the sequence context and/or position of the initiation codon. No translation of these IRES-dependent mRNAs occurred in *S. cerevisiae*.

Translation; Internal initiation; Encephalomyocarditis virus; Prothymosin α; *Saccharomyces cerevisiae*

1. INTRODUCTION

Picornaviruses, such as encephalomyocarditis virus (EMCV), are small animal RNA viruses. Initiation of translation of their RNA utilizes an unusual cap-independent binding of ribosomes to a specific internal sequence within the 5′ non-coding region which is therefore known as the internal ribosome entry site (IRES) (reviewed in [1]). The EMCV 5′ non-coding region directs efficient translation of reporter genes in mammalian cells and has been incorporated into expression vectors used in higher eukaryotes [2–4]. We decided to find out whether it is possible to use the EMCV IRES for efficient expression of foreign genes in yeast. Mechanisms of translation in yeast cells are thought to be functionally similar to those of mammals [5], yet no conclusive evidence has been obtained concerning the potential of yeast to utilize internal initiation of translation [6,7].

Here the EMCV 5′ non-coding region was fused to a human prothymosin α reporter gene and tested for its ability to direct translation in *S. cerevisiae*. We demonstrate that although such RNA transcripts are efficiently translated in mammalian cell-free systems, no translation of these mRNAs occurs in *S. cerevisiae*.

*Corresponding author. Fax: (7) (095) 939 3181.

Abbreviations: E. coli, Escherichia coli; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; *S. cerevisiae*, *Saccharomyces cerevisiae*; PAAG, polyacrylamide gel; nt(s), nucleotide(s); PAAG, polyacrylamide gel(s); PCR, polymerase chain reaction; PTB, 'pyrimidine tract binding protein'.

2. MATERIALS AND METHODS

Plasmid pHT15, containing human prothymosin α cDNA in the *Sma*I site of pUC19, was kindly provided by A. Vartapetian and used in PCR with primers (5′-dATGTCAGACGCAGCCGTAGA-3′) and (5′-dCCCGGATCCTAGTCATCCTCGTC-3′).

To obtain pEMCHT1, -2 and -3 (Fig. 1), plasmid pTE1 [8] containing EMCV cDNA (nts. 315–1,165) downstream of the T7 promoter was cleaved with the restriction endonuclease, *Bal*I, at nucleotide +4 of the EMCV coding sequence, incubated with Klenow fragment of *E. coli* DNA polymerase I in the presence of dCTP and dATP followed by nuclease S1 treatment to eliminate the initiation codon, and ligated with human prothymosin α cDNA. The primary structure of prothymosin α cDNA and its junction with the EMCV leader was determined by DNA sequence analysis [9]. To obtain pEMCHT4, plasmid pTE1 was digested with *Nco*I, filled in with Klenow fragment and ligated with human prothymosin α cDNA. pYeEMCHT3 and pEMCHT4 were constructed by insertion of the small *Bam*HI–*Bam*HI fragment from pEMCHT3 and -4 into the *Bam*HI site of pYeDP1/8-2 kindly provided by D. Pompon [10]. Plasmid pYeHT1 was constructed by insertion of the *Bam*HI–*Kpn*I fragment from pHT15 into pYeDP1/8-2.

Plasmids pYeEMCHT1–4 linearised with *Pst*I and blunt-ended with T4 DNA polymerase were used in T7 RNA polymerase reactions as described previously [8]. The transcripts were analyzed in denaturing 4% PAAG. In vitro translation in Krebs2 cell extracts were carried out as in [8], and in HeLa cell extracts as in [11]. Transformation of *S. cerevisiae* cells 2805 and Sky 594 was performed as described in [12]. For induction, cultures grown in liquid glucose-containing SD medium were washed twice with SD medium containing galactose instead of glucose, resuspended in this medium and grown overnight at 30°C. Preparation of total yeast RNA and Northern blot analysis were done according to [13] and [14], respectively. An α-^{32}P-labeled prothymosin α cDNA probe (specific activity 10^8 cpm/μg) was prepared by random priming [15]. Prothymosin α was isolated from yeast cells by phenol (100°C) and phenol–chloroform extraction in the presence of SDS followed by ethanol precipitation (essentially as described previously [16]). Protein labelling with the ^{125}I Bolton and Hunter reagent was described in [16].

3. RESULTS AND DISCUSSION

3.1. *Translation of prothymosin α mRNAs containing the EMCV leader sequence in mammalian cell-free systems*

Human prothymosin α DNA was inserted downstream of the EMCV leader sequence in the derivative of pTZ18R. Prothymosin α was chosen as a reporter gene in this study because of the unique property of the 13 kDa acidic protein that it encodes which is retained in the aqueous phase upon phenol deproteinization of cell lysates [17]. This protein can be visualized after gel electrophoresis by prior iodination or by staining with Methylene blue or Coomassie blue. The EMCV leader sequence (bases 315–833) is known to contain all the necessary signals for internal initiation of translation in mammalian cells [8,18].

Three different constructs were obtained, termed pEMCHT1, -2 and -3, respectively, which differ in the sequence context and location of the prothymosin α initiation codon with respect to the position of the authentic EMCV initiation codon, AUG-11 (Fig. 1). The sequence context of the prothymosin α initiation codon in pEMCHT3 corresponds to the yeast initiator region consensus sequence, (5′-AA/UAAUGUCU-3′) [5] except at position +6.

Expression of prothymosin α from RNA transcripts of pEMCHT1, -2 and -3 was analysed in two mammalian in vitro translation systems. Both mouse Krebs 2 and human HeLa cell lysates were able to translate T7 transcripts of pEMCHT3, in which the initiation codon has a favorable context (A) at position −3. pEMCHT1 and pEMCHT2 transcripts contain U at position −3

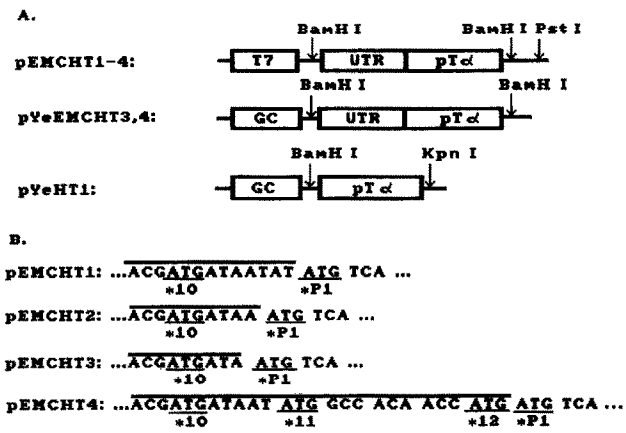

Fig. 1. (A) Schematic diagrams of plasmids used in this study. T7, T7 RNA polymerase promoter; GC, yeast Gal10–Cyc1 promoter; UTR, EMCV 5′ untranslated region; pTα, human prothymosin α cDNA. (B) Sequence junctions between the EMCV leader and prothymosin α cDNA. The EMCV leader sequence is highlighted by a thick black line above the sequence. The EMCV AUGs are underlined and labeled *10, *11 and *12; the prothymosin α initiation codon is underlined and labeled *P1.

and were not translated in either system (Fig. 2A,B). This result is consistent with the previously described observation that efficient IRES-mediated initiation of translation at EMCV AUG-11 in the mammalian system exhibits a requirement for A in position −3 [19] similar to cap-dependent translation.

In the work of Davies and Kaufman [19] the A → U substitution at position −3 of AUG-11 resulted in an approximately fourfold lower utilization of this triplet as a start codon. In our work the presence of U in position −3 inhibited in vitro translation completely.

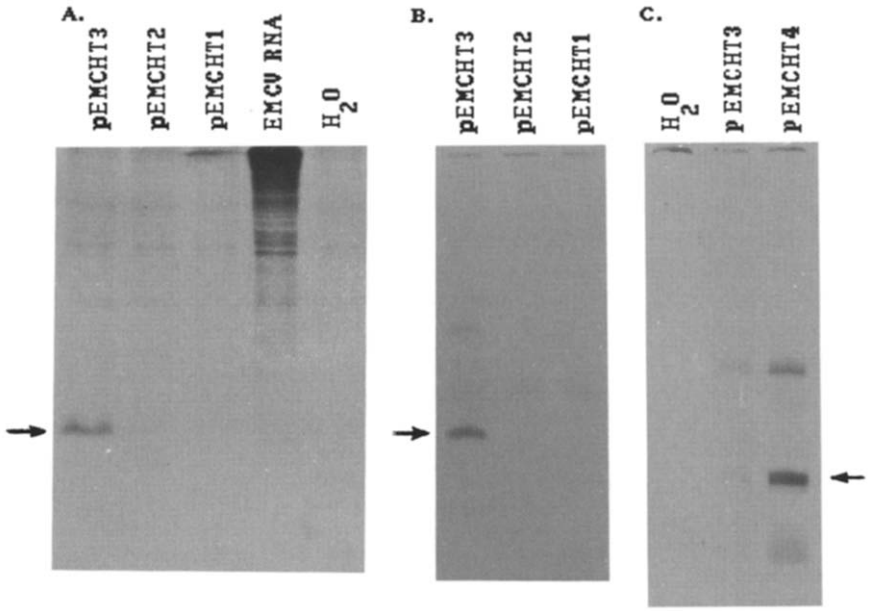

Fig. 2. Translation of T7 transcripts derived from the respective plasmids in Krebs 2 (A,C) and HeLa (B) cell extracts (H_2O–no RNA added). Translations were performed in the presence of [^{14}C]Glu (A) or [^{35}S]Met (B,C). The samples were analysed on 17.5% SDS-PAAG in A, or were phenol–chloroform extracted, ethanol precipitated, and fractionated in 8% PAAG in B and C. The position of prothymosin α is indicated by an arrow.

This discrepancy could be explained by the unfavorable context downstream of the start codon in RNAs transcribed from pEMCHT1, -2 and -3 (which contain U at position +4 instead of the G presented in EMCV). The presence of G at position +4 is an important determinant of initiation codon recognition in the mammalian (but not in the yeast) system [5]; therefore its requirement for efficient IRES-mediated initiation cannot be excluded. In order to optimize the context we constructed pEMCHT4 that retained the EMCV leader sequence up to EMCV AUG-12 followed by the prothymosin α start codon (Fig. 1). Since EMCV AUG-12 is able to initiate translation efficiently in the mammalian system [19], three potential in-frame initiation codons exist in pEMCHT4 RNA: the authentic EMCV initiation codon (AUG-11) containing G at position +4, EMCV AUG-12, and the prothymosin α start codon. Initiation of translation from EMCV AUG-11 of pEMCHT4 RNA was expected to result in prothymosin α containing the additional sequence, Met-Ala-Thr-Thr-Met, at its N-terminus. These residues are not charged or hydrophobic and therefore would not be expected to change the unique property of prothymosin α to partition to the aqueous phase during phenol extraction. This consideration is important because a simple procedure elaborated for isolation of prothymosin α from yeast cells is based on this property.

As expected, pEMCHT4 transcripts were efficiently translated in the Krebs2 cell-free system (Fig. 2C). The translation product was identical to prothymosin α, as judged both by its electrophoretic mobility and by its retention in the aqueous phase during phenol extraction. The specific initiation codon utilized on translation of pEMCHT4 RNA was not identified. The upper band (Fig. 2C) seems to be peptidyl-tRNA since it was sensitive to RNAses (data not shown).

3.2. *Translation of prothymosin α mRNAs containing EMCV leader sequence in yeast cells*

Since prothymosin α synthesis was detected in mammalian in vitro systems only a translation of pEMCHT3 and pEMCHT4 RNAs, EMCV leader–prothymosin α cDNA fusions from both plasmids were transferred into the shuttle vector pYeDP1/8-2 [10] downstream of the hybrid yeast Gal10–Cyc1 promoter. *S. cerevisiae* cells were transformed with the resulting plasmids, pYeEMCHT3 and pYeEMCHT4. After induction of transcription with galactose both RNA and putative prothymosin α species isolated from these cells were analyzed. Yeast cells transformed with pYeHT1, which contains prothymosin α cDNA directly fused to the Gal10–Cyc1 promoter sequences of pYeDP1/8-2, were used as a control to evaluate cap-dependent translation.

To determine whether prothymosin α cDNA had been transcribed we performed Northern blot analysis using a prothymosin α-specific cDNA probe. Prothymosin α-specific transcripts were detected in yeast cells

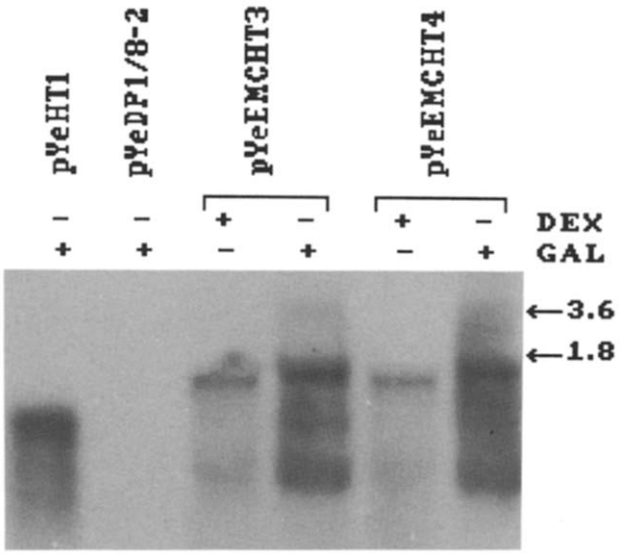

Fig. 3. Northern analysis of total RNA from the transformed yeast cultures grown on glucose without induction (DEX) or induced with galactose (GAL). The blot was probed with ^{32}P-labeled prothymosin α cDNA. RNA size markers (in kilobases) are shown on the right.

transformed with pYeHT1, pYeEMCHT3 and pYeEMCHT4, but not pYeDP1/8-2 used as a control (Fig. 3). These RNA species had the expected electrophoretic mobility.

To determine whether prothymosin α-specific RNAs were translated, prothymosin α was isolated from yeast cells and subjected to electrophoretic analysis. Although the sequence context of the prothymosin α initiation codon in pYeHT1-encoded mRNA (5′-CCCCAUGUCA-3′) is rather unfavorable for yeast [5], prothymosin α synthesis was detected in yeast cells transformed with pYeHT1, but not with pYeEMCHT3 or pYeEMCHT4 (Fig. 4A). To increase the sensitivity of this analysis, the putative prothymosin α was labeled with the ^{125}I Bolton and Hunter reagent and fractionated on denaturing PAAG (Fig. 4B). Even when the film was over-exposed no evidence for the EMCV IRES-mediated prothymosin α synthesis could be obtained (Fig. 4C). As a control, 1% of the radio-iodinated translation product isolated from an equivalent amount of pYeHT1-transformed yeast cells was loaded and prothymosin α was visualized as an intense band (Fig. 4C, lane 1). The other ^{125}I-labeled bands in lanes 2 and 3 (Fig. 4C) appeared to be tRNA and non-protein contaminants, since they were not sensitive to proteinase K treatment (data not shown).

Thus we have shown that *S. cerevisiae* cells do not translate EMCV IRES-containing prothymosin α mRNAs with even 0.5% of the efficiency of cap-dependent translation. This fact cannot be explained by the presence of the translational inhibitor recently detected in yeast cell lysates by Coward and Dasgupta [6], since it was shown to inhibit translation in HeLa cell extracts of RNAs containing poliovirus IRES, but not that of

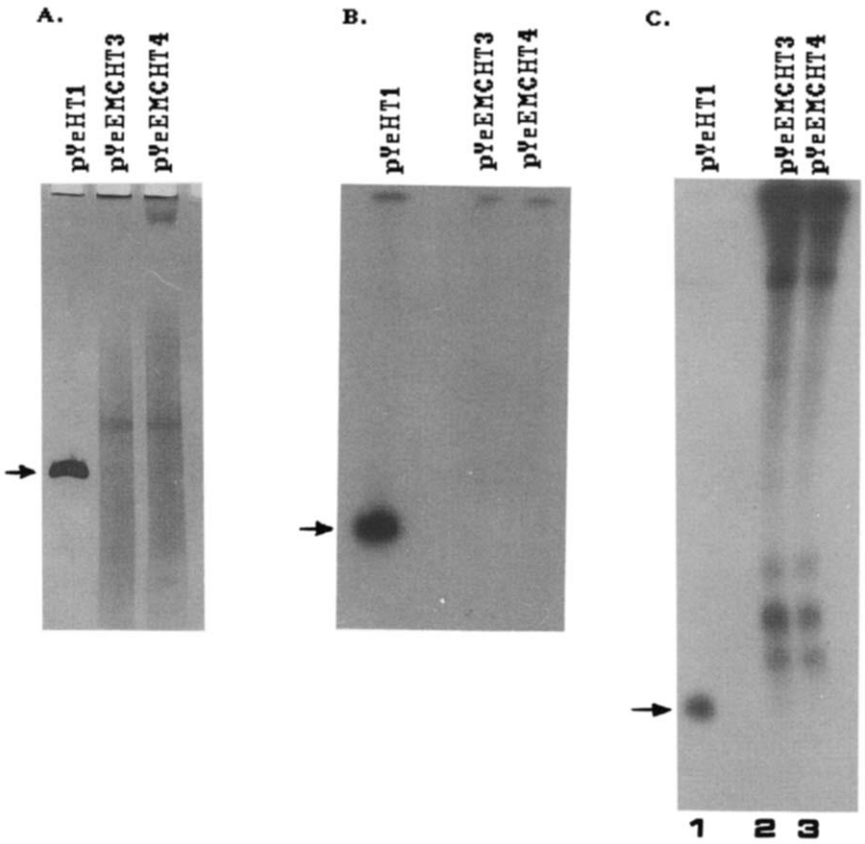

Fig. 4. In vivo translation assays. Unlabeled (A) and ^{125}I-labeled (B and C) putative prothymosin α species isolated from induced yeast cells transformed with the respective plasmids were fractionated in 8% PAAG. In A the gel was stained with Methylene blue, in B the gel was exposed to X-ray film for 3 h, in C the gel was exposed for 70 h. The samples isolated from equivalent amounts of yeast cells were loaded in all the lanes except in lane 1 of (C) in which 1% of the corresponding sample was loaded. The position of prothymosin α is indicated by an arrow.

EMCV. It is more likely that yeast cells are deficient in specific factor(s) involved in internal initiation of translation, at least as mediated by the EMCV IRES. It is interesting to note that p58/PTB, a factor found in Krebs2 and HeLa cell extracts that specifically binds to the 5′ untranslated region of EMCV RNA [20] and seems to play an essential role in internal initiation of translation [21], has not been found in extracts of yeast cells (I.N. Shatsky, personal communication).

Acknowledgements: We thank I.N. Shatsky and A.B. Vartapetian for helpful discussions and critical reading of the manuscript. This work was supported in part by grants from the Russian 'Frontiers in Genetics' and 'Genetic and Cellular Engineering' Research Programs.

REFERENCES

[1] Jackson, R.J., Howell, M.T. and Kaminski, A. (1990) Trends Biochem. Sci. 15, 477–483.
[2] Elroy-Stein, O., Fuerst, Y.R. and Moss, B. (1989) Proc. Natl. Acad. Sci. USA 86, 6126–6130.
[3] Venema, H., Rijnbrand, R., Heijnen, L., Horzinek, M.S. and Spaan, W.J.M. (1991) Gene 108, 201–210.
[4] Adam, M.A., Ramesh, N., Miller, A.D. and Osborne, W.R.A. (1991) J. Virol. 65, 4985–4990.
[5] Cigan, A.M. and Donahue, T.F. (1987) Gene 59, 1–18.
[6] Coward, P. and Dasgupta, A. (1992) J. Virol. 66, 286–295.
[7] Altman, M., Blum, S., Pelletier, J., Sonenberg, N., Wilson, T.M.A. and Trachsel, H. (1990) Biochim. Biophys. Acta 1050, 155–159.
[8] Evstafieva, A.G., Ugarova, T.Yu., Chernov, B.K. and Shatsky, I.N. (1991) Nucleic Acids Res. 19, 665–671.
[9] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[10] Cullin, C. and Pompon, D. (1988) Gene 65, 203–217.
[11] Pestova, T.V., Hellen, C.V.T. and Wimmer, E. (1991) J. Virol. 65, 6194–6204.
[12] Rech, E.L., Dobson, M.J., Davey, M.R. and Mulligan, B.J. (1990) Nucleic Acids Res. 18, 1313–1317.
[13] Schmitt, M.E., Brown, T.A. and Trumpower, B.L. (1990) Nucleic Acids Res. 18, 3091–3092.
[14] Membrane transfer and detection methods, Amersham International plc, 1985.
[15] Feinberg, A.P. and Vogelstein, V.A. (1983) Anal. Biochem. 132, 6–11.
[16] Vartapetian, A.B., T.N. Makarova, E.V. Koonin, V.I. Agol and A.A. Bogdanov (1988) FEBS Lett. 232, 35–38.
[17] Sburlati, A.R., Manrow, R.E. and Berger, S.L. (1990) Protein Expression Purif. 1, 184–190.
[18] Duke, G.M., Hoffman, M.A. and Palmenberg, A.C. (1992) J. Virol. 66, 1602–1609.
[19] Davies, M.V. and Kaufman, R.J. (1992) J. Virol. 66, 1924–1932.
[20] Borovjagin, A.V., Evstafieva, A.G., Ugarova, T.Yu. and Shatsky, I.N. (1990) FEBS Lett. 261, 237–240.
[21] Hellen, C.V.T., Witherell, C.W., Schmid, M., Shin, S.H., Pestova, T.V., Gil, A. and Wimmer, E. (1993) Proc. Natl. Acad. Sci. USA 90, 7642–7646.