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Direct introduction and transient expression of capped and non-capped RNA in *Saccharomyces cerevisiae*

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ABSTRACT

We report the introduction of functional RNA molecules into yeast spheroplasts. Plasmids containing the firefly luciferase coding region were transcribed to yield RNAs suitable for introduction into yeast cells and direct assay of their translation products. The 5′ noncoding regions of the RNAs were derived either from the 5′ noncoding regions of firefly luciferase, poliovirus, or yeast virus-like-particle (VLP) L-A or M1 RNAs. Capped and non-capped mRNAs were made by T7 RNA polymerase-directed transcription and introduced into yeast spheroplasts. The peak time of luciferase transient expression from introduced RNAs was 2–4 h after their introduction. In contrast, transient expression of luciferase from a non-replicative, luciferase-encoding plasmid introduced into the cells was maximal at 16 h. For capped mRNAs, luciferase activity increased linearly with transcript amount for both yeast and human (HeLa) cells. Although non-capped luciferase mRNAs were expressed more efficiently following introduction into yeast than into HeLa cells, the 5′ noncoding sequences from yeast double-stranded (ds)RNA VLP RNAs conferred no greater apparent cap-independence than non-VLP RNA sequences in this transient expression assay. The RNA transient expression system will allow the study of translation of capped and non-capped RNAs in yeast cells and of the replicative cycle of yeast virus-like RNA genomes.

INTRODUCTION

A wide variety of methods is available to introduce nucleic acids into cells of many organisms. In most cases, DNA has been used because of its stability and the capacity to replicate autonomously or integrate into the host genome. There are fewer systems in which exogenous functional RNA has been introduced into cells. In eukaryotic systems, direct introduction of RNA molecules into cells provides advantages over DNA transfection methods when studying certain biological functions. For example, RNA transient expression allows potentially variable nuclear events to be ignored, which include transcription, RNA splicing, and RNA transport to the cytoplasm. Infectious viral RNA molecules (1–5), viral-derived, non-replicative RNA molecules, and non-viral, non-replicative RNA molecules (6, 7) have all been successfully introduced into mammalian cells.

Transformation of yeast cells with DNA plasmids is a routine procedure for molecular biological manipulations of the organism. Yeast cells are surrounded by a cell wall composed principally of glucans and mannoproteins (8). Yeast cells can be made competent for DNA uptake either by treatment with lithium acetate (9) or by converting them to spheroplasts by enzyme treatment (10–13). Recently, procedures have been developed for transforming intact yeast cells by electroporation (14, 15).

We are interested in studying the cis- and trans-acting factors required for the establishment of infection and the *in vivo* replication of certain dsRNA VLPs of yeast (16–19). That is, most strains of *Saccharomyces cerevisiae* contain cytoplasmic, noninfectious, virus-like particles (VLPs) that are cytoplasmically inherited and that contain linear dsRNA molecules (20–23; reviewed in16–19). The major families of yeast viruses are L-A, L-BC, M, T and W, of which L-A and M1 have received the most experimental attention. The L-A VLPs contain a single copy of a 4.6-kb dsRNA (L-A dsRNA) and the M1 VLPs contain one or two copies of a 1.8-kb dsRNA (M1 dsRNA) enclosed in an icosahedral capsid. L-A dsRNA encodes the 80-kDa major capsid protein and a 180-kDa minor capsid protein with single-stranded (ss)RNA binding activity and probably with RNA-dependent RNA polymerase activity as well (18, 24); both the major and minor proteins are found in the icosahedral capsid. M1 dsRNA is encapsidated in particles encoded by L-A; that is, M1 is a satellite RNA which can exist only in cells that have L-A dsRNAs.

In this paper, we describe the parameters for introducing RNA into yeast spheroplasts. The RNAs introduced are single-stranded mRNAs that have the firefly luciferase coding sequence located downstream from its own 5′ noncoding region or the 5′ noncoding sequences of L-A or M1 yeast VLP RNA. Our results

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indicate that both m^{7}GpppG-capped and non-capped mRNAs can be transiently expressed from RNA introduced directly into yeast cells. Since transformation is defined as a process in which nucleic acid is introduced into a cell, resulting in a heritable change, the introduction of RNA into the cell is better defined as transient expresssion when no infection results and no heritable change occurs.

MATERIALS AND METHODS

DNA plasmids

T7−5′ncPolio-LUC (Figure 1) contains the promoter for T7 RNA polymerase followed by the entire 5′ noncoding region of poliovirus and the firefly luciferase coding region (LUC) (25). A unique PvuII restriction site is located 15 bp upstream from the T7 promoter element, and two NcoI restriction sites are located in the poliovirus 5′-noncoding region, one at position 388 and the second at 741. The ATG used as the translational start signal is located within the second NcoI site.

T7−5′ncLuc-LUC is similar to T7−5′ncPolio-LUC, except the 5′-noncoding region contains nucleotides 30 to 51 of the firefly luciferase 5′-noncoding region and there is no NcoI site associated with the translational start codon ATG. The LA- and M1-LUC.EXP ('luciferase expresson') plasmids were made by replacing sequences from the PvuII site to the second NcoI site of T7−5′ncPolio-LUC with a smaller DNA fragment containing a PvuII site, T7 promoter sequences, the 5′ noncoding region of either L-A or M1, and an NcoI site. The replacement PvuII-NcoI fragments were made by annealing two complementary synthetic oligonucleotides. The LA-LUC.EXP plasmid contains the 5′ terminal 32 base pairs of L-A, including the 29-base 5′-noncoding region. M1-LUC.EXP contains the 5′ terminal 13 bases that constitute its 5′-noncoding region. In wild-type M1, the ATG start codon immediately follows the 5′ non-coding sequence, but in this construct the first two C nucleotides of the NcoI site used for cloning are present between the M1 5′ non-coding region and the ATG.

pDO432 contains the cauliflower mosaic virus (CaMV) 35S RNA promoter and the coding region for firefly luciferase cDNA in pUC19 (26); it was obtained from Dr. D.Ow (Plant Gene Expression Center, Albany, CA). When introduced into yeast, the luciferase sequence in pDO432 has been shown to be effeciently transcribed in the nucleus from the CaMV promoter (26); no sequences are present on plasmid pDO432 to confer replication ability in yeast.

RNA transcripts

T7 RNA polymerase (Promega)-directed *in vitro* RNA transcription was done with HpaI-linearized LUC.EXP plasmids in the presence and absence of the 5′ cap analog P^1-5′-(7-methyl)-guanosine-P^3-guanosine triphosphate (m7GpppG; Boehringer Mannheim) for 2 h at 37°C (27, 28). The RNA transcripts from LA- and M1 LUC.EXP are estimated to be 2356 and 2342 nucleotides, respectively. To estimate the amount of RNA obtained from transcription reactions, 1−5 μl samples of the transcription reactions were subjected to electrophoresis in a 1.0% (wt/vol) agarose gel containing 0.5% (wt/vol) sodium dodecyl sulfate and 1 μg/ml ethidium bromide. The fluorescent intensities of the product RNA bands were compared with those of individual bands of a 0.25−9.4-kb RNA ladder containing 0.5 μg of each RNA species (BRL). We estimate the resulting RNA concentrations to be correct to within 50%.

Introduction of nucleic acids into yeast cells

The introduction of both RNA and DNA into yeast spheroplasts was done with strain 2889 (2601-Δ-9), *ura3 his5 mkt1* [L-A-o] [M1-o], obtained from R.B.Wickner (National Institutes of Health, Bethesda, MD). This strain contains neither L-A nor M1 VLPs. Introduction of RNA and DNA was performed according to the procedure described for transformation with DNA (11) with minor modifications. In brief, cells were grown in 50 ml of YAPD (1% Difco yeast extract, 0.05% adenine hemisulfate, 2% Bacto peptone, 2% glucose) at 30°C, with shaking at 250 rpm, to a density of 3×10^7 cells/ml. After this point all solutions used were RNase-free. The cells were collected by centrifugation, washed with 20 ml of water, then with 20 ml of 1 M sorbitol, and were resuspended in 20 ml of SCEM (1 M sorbitol, 0.1 M sodium citrate pH 5.8, 10 mM EDTA, 30 mM 2-mercaptoethanol). Two thousand five hundred units of lyticase (Sigma) in 250 μl of SCEM were added and the cells were incubated at 30°C with occasional mixing. The production of spheroplasts was followed by measuring the decrease in A_{800} of a 10-fold dilution of cells in water. When the A_{800} value had decreased by 90% (about 30 min), the spheroplasts were collected by low-speed centrifugation, washed by resuspension in 20 ml of 1 M sorbitol, and then in 20 ml of STC (1 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$). The spheroplasts were collected by centrifugation and resuspended in 2 ml of STC. The

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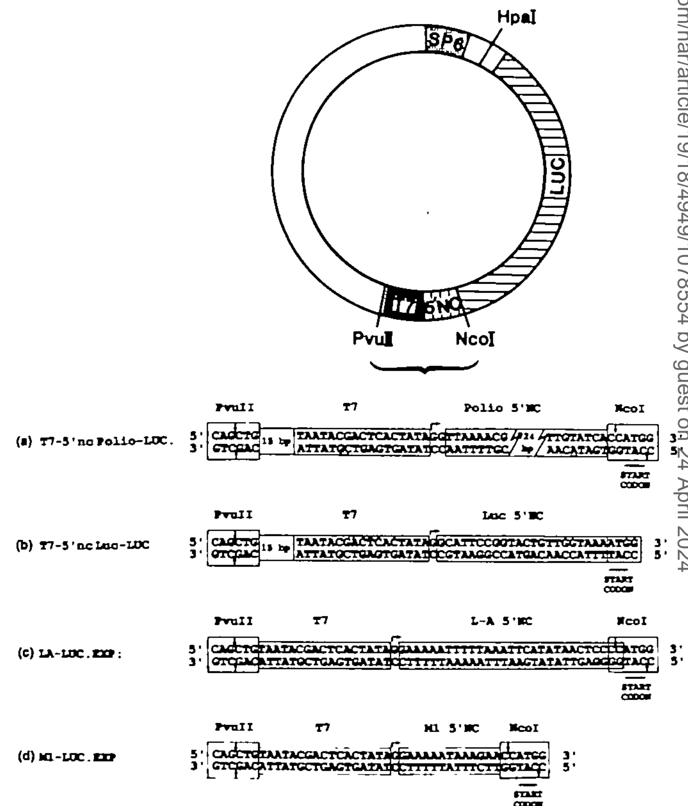

Figure 1. Structures of the plasmids from which firefly luciferase transcripts with various 5′-noncoding sequences were made *in vitro*. (a) T7−5′ncPolio-LUC (b) T7−5′ncLuc-LUC (c) LA-LUC.EXP and (d) M1-LUC.EXP ('luciferase expresson') plasmids. Key: T7 = T7 promoter; 5′NC = 5′ noncoding region; LUC = firefly luciferase cDNA; SP6 = SP6 promoter, → = transcription start for T7 RNA polymerase.

spheroplasts were either used immediately or frozen after the addition of DMSO to 15% (vol/vol).

For the introduction of nucleic acids into spheroplasts, 100 μl samples of spheroplasts were mixed with 20 μl samples either of the *in vitro* RNA transcription mixtures (containing approximately 1 μg of template DNA and 2 μg of RNA), with equivalent amounts of the DNA plasmid alone, or with 20 μl samples of the RNA transcriptions mixtures treated with DNase 1. For DNase 1 treatments, 60 units of RNase-free DNase 1 (Boehringer-Mannheim) were added to 20 μl of the transcription mixes; incubation at 37°C was continued for 40 min. Analysis of the products of such a reaction by agarose gel electrophoresis revealed that DNase 1 treatment under these conditions reduced the amount of intact DNA to undetectable levels: at least a 20-fold reduction (data not shown). Nucleic acid samples were incubated with spheroplasts for 10 min at room temperature, after which 1 ml of PEG solution (20% filter-sterilized polyethylene glycol 8000 (Aldrich), 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$) was added. After 10 min further incubation at room temperature, the spheroplasts were collected by low-speed centrifugation and resuspended in 1 ml of SOS (1 M sorbitol, 6.5 mM $CaCl_2$, 1/3×YAPD). After transfer to glass tubes, the spheroplasts were incubated at 30°C with shaking at 250 rpm for varying periods of time. For luciferase assays, the spheroplasts were transfered to a 1.5 ml tube (Eppendorf), collected by centrifugation, and resuspended in 150 μl of luciferase extraction buffer (LEB: 0.1 M potassium phosphate pH 7.8, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride; 25). Acid-washed glass beads (0.15 g of 425–600 μm: Sigma) were added and the mixture was placed on ice for 5 min. The samples were vortexed for a total of 2 min each for four 30 sec intervals, with ice treatment between each interval. The extracts were centrifuged for 20 min, and 150 μl of the supernatant was transfered to a fresh tube for luciferase assay.

Introduction of RNA into HeLa cells

Transfection of RNA molecules into human HeLa cells using diethylaminoethyl-dextran (DEAE-dextran) was performed as described (29). Varying amounts of the same T7 transcription reactions used in the yeast transient expression experiments were diluted to 40 μl with transcription buffer (27), mixed with 435 μl TS buffer (29) and 25 μl of freshly-prepared 10 mg/ml DEAE-dextran. The 500 μl mixtures were placed on one 100 mm Petri dish of subconfluent HeLa cells as described (29). Incubations under medium were continued for 1 h to allow luciferase expression. HeLa cell extracts for assaying luciferase activity were prepared as described (25).

Luciferase assays

Samples of the extracts from yeast or HeLa cells were diluted into 400 μl of luciferase assay buffer (LAB: 25 mM potassium phosphate pH 7.8, 8 mM $MgCl_2$, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol; 30) plus 1 μl of 100 mM ATP. The tube was placed in a luminometer (Analytical Luminescence Laboratory Monolight 2010), and the luciferase-catalyzed reaction was initiated by the injection of 100 μl of 1 mM D-luciferin (Sigma). Luciferase was assayed in 5, 20 and 80 μl of cell extracts and the light unit values (corrected for background values of extracts prepared from non-transfected spheroplasts taken through the same experimental conditions) are presented as the equivalent for the total extract volume of 150 μl.

RESULTS

Experimental System

The experimental system involves the measurement of firefly luciferase activity in cell extracts after introducing into yeast spheroplasts mRNA made by the transcription of the DNA plasmids shown in Figure 1 by T7 RNA polymerase. Samples of the *in vitro* transcription reaction mixtures were used directly for RNA introduction. The effectiveness of the introduction of RNA into cells was determined by subsequently measuring luciferase activity in cell extracts.

From each experiment introducing nucleic acids into spheroplasts, 150 μl of extract was obtained. Luciferase activity was linear over a range of extract concentrations, using from 5 to 80 μl of extract (data not shown). The values used in the following experiments were each determined using data from all 3 concentrations of cell extract.

Time Course of Luciferase Expression

The time course of luciferase expression following introduction of RNA into yeast spheroplasts was determined, and found to differ greatly from the time course of transient DNA expression.

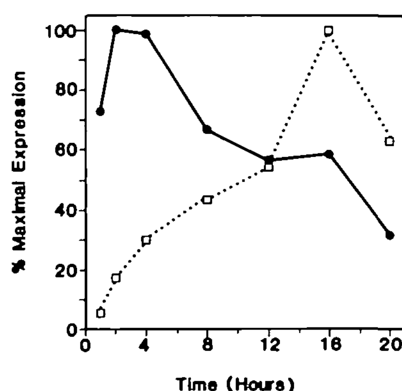

Figure 2. Time course for transient luciferase expression in yeast of 2 μg of mRNA transcribed from LA-LUC.EXP (●) and of 20 μg of the DNA plasmid pDO432 (□). For LA-LUC.EXP and pDO432, the maximum light unit values obtained were 13,940 and 50,150, respectively; those values were each set to 100% for comparison of relative light unit values. The values for 2 samples of untreated spheroplasts were 216 and 336 light units. From a standard curve of light unit versus luciferase concentration (United States Biochemical recombinant firefly luciferase), the LA-LUC.EXP and pDO432 maximum light unit values correspond to 4.8 and 17.3 units of luciferase, respectively.

Table 1. Luciferase activity present in yeast extracts after the introduction of *in vitro* transcription reaction mixtures into yeast cells is the result of mRNA, not DNA[a].

Source of introduced nucleic acid	Light Units From LUC.EXP L-A	M1
Total transcription reaction	23,137	57,640
Transcription reaction without T7 RNA polymerase	263	207
DNase-treated transcription reaction	12,477	28,436

[a] Nucleic acids from the indicated source were introduced into spheroplasts of yeast strain 2889. Luciferase activity was measured in extracts prepared after 16 h. The light unit value for untreated spheroplasts was 205; the buffer alone gave a value of 162 light units.

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A transcription reaction mixture using LA-LUC.EXP (Fig. 1) as the template was performed in the presence of the 5′-cap analog, m^{7}GpppG was used to transfect spheroplasts, samples were taken from 1 to 20 hours after RNA introduction, cell extracts were prepared and luciferase activities were determined. In parallel, the transient expression of luciferase from pDO432, a nonreplicating DNA plasmid encoding luciferase, was monitored in the same batch of spheroplasts.

Figure 2 shows a representative luciferase time course experiment. For the LA-LUC.EXP transcripts, the peak of transient luciferase expression was at 2–4 h after RNA introduction. By contrast, the peak of transient luciferase expression for plasmid pDO432 was consistently much later, at 16 h after introduction of the DNA.

The direct introduction of RNA shown in Figure 2 was performed with total transcription reaction mixtures that contain both plasmid DNA and the mRNA transcripts. That the DNA in the transcription reaction mixtures was not responsible for the luciferase activity measured in the cell extracts was shown in two ways: 1) by experiments with reaction mixtures from which T7 RNA polymerase was omitted; and 2) by experiments using reaction mixtures treated with RNase-free DNase I. For these experiments, luciferase activity was measured 16 hours after exposure of spheroplasts to the reaction mixtures, at the time of maximal DNA expression (Fig. 2). The results shown in Table 1 indicate clearly that the observed transient expression of luciferase derives from expression of the mRNA and not from DNA. Luciferase activity was at control levels for the reaction mixtures lacking T7 RNA polymerase. Furthermore, luciferase activity was not abolished by DNase I treatment of the transcription reactions. The 50% decrease in luciferase activity after DNase I treatment of the transcription reaction may well result from a modest amount of RNA degradation or from a reduced efficiency of RNA introduction or transient expression in the absence of 'carrier' DNA. Most importantly, the luciferase activity observed in the transient expression experiments shown in Table 2 clearly resulted from expression of the introduced RNA molecules.

Table 2. Accumulation of translation product (in light units) of capped and noncapped luciferase mRNAs with different 5′-noncoding regions in yeast and HeLa cells[a]

Source of RNA[b]	Yeast Capped	Yeast Noncapped	HeLa Capped	HeLa Noncapped
LA-LUC.EXP[c]	23,137	1877	2140	192
M1-LUC.EXP[c]	57,640	1659	1597	195
T7–5′ncPolio-LUC[c]	13,879	1422	6792	2207
T7–5′ncLuc-LUC[c]	4732	2127	1333	160
LA-LUC.EXP[d]	1304	383		
T7–5′ncPolio-LUC[d]	1496	213		
T7–5′ncLuc-LUC[d]	3477	928		

[a] Yeast spheroplasts were treated with 20 μl of transcription reaction; HeLa cells were transfected with 10 μl. The control light unit value for untreated yeast spheroplasts was 183 and that for mock-transfected HeLa cells was 183.
[b] The plasmids from which firefly luciferase mRNAs were made for introduction into yeast cells are described in Figure 1. For transcription, the plasmids were linearized with HpaI at the 3′ end of the luciferase cDNA.
[c] Yeast spheroplasts were of strain 2889.
[d] Yeast spheroplasts were of strain 9933-13A (a *his3D200 ura3-52 trp1D1 leu2-3,112*, VLP genotype unknown; obtained from G.R.Fink, Whitehead Institute for Biomedical Research).

Effects of RNA capping and RNA concentration on the efficiency of transient expression from RNA

All eukaryotic cellular mRNAs, and most viral mRNAs, are modified at their 5′ ends with a 7-methylguanylate cap structure (31). The 5′ m^{7}GpppG cap is recognized by cap-binding proteins, which direct the binding of 40S ribosomal subunits to the mRNAs. Some viral mRNAs, including poliovirus RNA, certain animal and plant viral RNAs, and VLP RNAs L-A and M1 do not bear 5′ m^{7}GpppG cap structures. These RNAs must be translated by a cap-independent mechanism. For poliovirus RNA translation, evidence has been obtained that translation initiation occurs by internal binding of ribosomes to a specific sequence of the 5′-noncoding region (32). The question arises whether yeast VLP RNAs can be translated without a 5′ m^{7}GpppG cap, and if they have specific sequences conferring cap-independent translatability that can be detected by an RNA transient expression assay.

Various concentrations of RNA were introduced into yeast spheroplasts, and luciferase activity in yeast extracts prepared from cells 4 h later was determined. The results of transient expression of m^{7}GpppG-capped and uncapped LA-LUC.EXP and M1-LUC.EXP transcripts into yeast spheroplasts are shown in Figure 3a. The LA-LUC.EXP and M1-LUC.EXP m^{7}GpppG-capped messages both showed levels of expression only slightly less than that observed in HeLa cells (next section), and luciferase activity increased linearly with transcript amount.

When non-capped LA-LUC.EXP and M1-LUC.EXP mRNAs were introduced into yeast cells, significant luciferase activity was also seen (Figure 3a). The levels of transient expression of luciferase from non-capped LA-LUC.EXP and M1-LUC.EXP RNAs, while significant, were clearly lower than the luciferase levels following the introduction of m^{7}GpppG-capped RNAs. The ratios of the slopes of the best-fit lines in Figure 3a of the expression of non-capped vs. m^{7}GpppG capped mRNAs were

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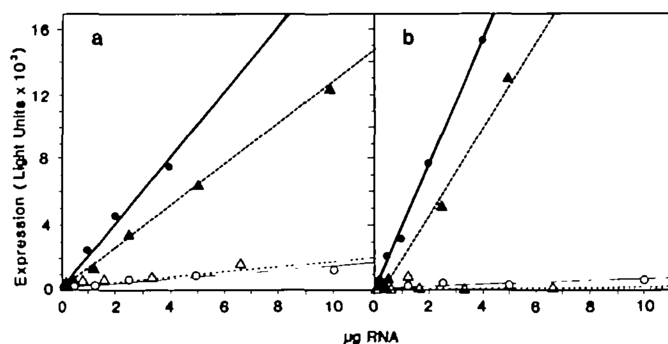

Figure 3. Luciferase activity versus amount of RNA introduced into yeast strain 2889 and human HeLa cells. (a) Light units obtained following introduction of RNA into yeast as a function of amount of capped and noncapped RNA. The same preparation of spheroplasts was used for each sample. ●: m^{7}GpppG capped LA-LUC.EXP transcripts; ○: non-capped LA-LUC.EXP transcripts; ▲: m^{7}GpppG capped M1-LUC.EXP transcripts; △: non-capped M1-LUC.EXP transcripts. The average value of several buffer blanks in this experiment was 158 light units; this value was subtracted from the values shown. Untreated spheroplasts gave an average value of 183 light units in this experiment. (b) Light units obtained following introduction of RNA into HeLa cells as a function of amount of capped and noncapped RNA are reported. ●: m^{7}GpppG capped LA-LUC.EXP transcripts; ○: non-capped LA-LUC.EXP transcripts; ▲: m^{7}GpppG capped M1-LUC.EXP transcripts; △: non-capped M1-LUC.EXP transcripts. The value for untreated HeLa cells (154 light units) was the same as for the buffer blank; this was subtracted from the values shown.

5.6% and 9.1% for LA-LUC.EXP and M1-LUC.EXP RNAs, respectively. Whether the lower translation of non-capped RNAs following introduction into yeast resulted from the cap-dependence of translational initiation in yeast or greater stability of capped mRNAs has not yet been determined. What was surprising by comparison to mammalian cells (next section) was that non-capped mRNAs were expressed at levels as high as those observed in Figure 3a.

The results of introducing the same preparations of m^7GpppG-capped and non-capped LA-LUC.EXP and M1-LUC.EXP RNAs into human HeLa cells are shown in Figure 3b. Confirming the results of others (33, 34), capped luciferase-containing RNAs were efficiently translated in HeLa cells following transfection with DEAE-dextran (29). The dependence of the transient expression of introduced mRNAs on the presence of a 5′ m^7GpppG cap is shown, in this experiment, by the 1.4% and 0.3% activities of non-capped LA-LUC.EXP and M1-LUC.EXP RNAs, respectively, as compared with their capped counterparts. Again, the lower expression of non-capped mRNAs could be due to a number of reasons. For mammalian cells, however, the cap-dependence of RNA transient expression mimics the effects of a 5′ cap structure in *in vitro* translation systems, and is probably not due to differences in RNA stability, but instead, to a cap-independent mechanism of translation (33).

It is clear from comparison of Figures 3a and 3b that non-capped RNAs bearing the LA and M1 5′ noncoding regions were expressed more efficiently in yeast cells than in HeLa cells. The possibility exists that translational initiation in yeast is intrinsically less cap-dependent than in mammalian cells. Of course, other possibilities, such as the presence of cytoplasmic capping activities in yeast cells or increased stability of non-capped RNAs in yeast, must be considered as well. Our experiments have not yet addressed these issues, so quantitative conclusions about the relative translational efficiencies of capped and uncapped RNAs cannot be drawn. What is important here is that both capped and non-capped RNAs can be translated following direct introduction into yeast cells.

The transient expression of noncapped RNAs following introduction into yeast cells is not specifically conferred by L-A or M1 5′-noncoding sequences

Table 2 shows the results of introducing capped and noncapped mRNAs transcribed from the four different luciferase-encoding plasmids LA-LUC.EXP, M1-LUC.EXP, T7−5′ncPolio-LUC, and T7−5′ncLuc-LUC into yeast cells (see Figure 1). The data show that RNAs containing all four noncoding regions are expressed in yeast, and neither poliovirus, M1 nor LA sequences confers a special cap-independence in either of two yeast strains.

DISCUSSION

In conclusion, we have demonstrated that capped and non-capped functional mRNA molecules can be successfully introduced into yeast cells. Although the presence of a 5′ m^7GpppG cap on the transfected RNA molecules greatly improves the expression of protein from those molecules, noncapped RNA molecules can also be transiently expressed. This system for the direct introduction of RNA will be useful for investigating various biological functions in yeast, including the genetic control of the replication of yeast virus-like RNA genomes, and the stability and translational regulation of RNA molecules in yeast cells.

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