Enzymic and Nonenzymic Translocation by Yeast Polysomes. Site of Action of a Number of Inhibitors[†]

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ABSTRACT: The enhancement in peptidyl[^{3}H]puromycin formation by yeast polysomes, observed at high potassium concentrations (1 M KCl), measures the nonenzymic translocation of peptidyl-tRNA. It takes place at 37 °C but not at 0 °C. Neither GTP nor elongation factor 2 nor –SH ribosomal groups are required for the reaction to take place. It is suggested that, at high potassium concentrations, a conformational change of the ribosome occurs resulting in the positioning of the 3' end of the peptidyl-tRNA on the donor site of the peptidyl transferase center located on the 60S ribosomal subunit. The potassium-induced nonenzymic translocation is inhibited by cycloheximide and pederine but unaffected by cryptopleurine, emetine, and tubulosine. Both the enzymic and the nonenzymic translocations by polysomes from a cycloheximide-resistant yeast mutant are resistant in vitro to cycloheximide, but sensitive to pederine. These results suggest that, although cycloheximide and pederine have a similar overall effect, they act at different sites on the 60S ribosomal subunit. The enzymic translocation catalyzed by polysomes from a cryptopleurine-resistant yeast mutant is resistant to inhibition by cryptopleurine, emetine, and tubulosine but sensitive to cycloheximide and pederine. Since resistance to cryptopleurine is due to an alteration on the 40S ribosomal subunit, these results suggest that emetine and tubulosine also act on this site. The implications of these results on the mechanisms of the nonenzymic translocation are discussed in the text.

The elongation of the polypeptide chain in protein synthesis takes place through the repetition of cyclic events involving the binding of the aminoacyl-tRNA, peptide bond formation, and translocation. One of the less understood steps in this process is the translocation of the peptidyl-tRNA from the A ribosomal site to the P site, which takes place in the presence of EF 2 and GTP. We refer to this process as the enzymic translocation. More recently a simplified model system has been devised to study the translocation of the peptidyl-tRNA under high potassium concentration and in the absence of EF 2 and GTP (the nonenzymic translocation) (Blobel and Sabatini, 1971; Baliga et al., 1973; van der Mast and Bloemers, 1973; Barbacid et al., 1975; Carrasco et al., 1976).

The fact that cycloheximide inhibits the enzymic and nonenzymic translocation of the peptidyl-tRNA on eukaryotic polysomes (Barbacid et al., 1975), whereas tubulosine and emetine have only an inhibitory effect on the enzymic reaction (Carrasco et al., 1976), indicated that the translocation inhibitors acting on the ribosome do not share a common mechanism of action. Protein syntheses carried out by polysomes from a yeast mutant able to grow in the presence of cycloheximide are resistant to the action of this antibiotic in vitro (Jiménez et al., 1972) but sensitive to emetine and tubulosine (Carrasco et al., 1976), further suggesting a different site of action of cycloheximide and the two drugs emetine and tubulosine, which were initially proposed as analogues of cycloheximide (Grollman, 1966, 1967).

We have now studied the mechanism by which nonenzymic translocation takes place by eukaryotic polysomes. Furthermore, studies on enzymic and nonenzymic translocation by polysomes from a wild strain of *Saccharomyces cerevisiae* and derived mutants resistant to cycloheximide or cryptopleurine have allowed us to locate with precision the site and mechanisms of action of the inhibitors of translocation which act on eukaryotic ribosomes.

Materials and Methods

Wild type haploid *Saccharomyces cerevisiae* strain Y_{166} and the spontaneous SR_{17} (resistant to cycloheximide) and CRY_6 (resistant to cryptopleurine) mutants have been described elsewhere (Jiménez et al., 1972; Grant et al., 1974).

The preparation of yeast polysomes, a partially purified yeast supernatant fraction containing the elongation factors EF 1 and EF 2 (crude supernatant), [^{14}C]Phe-tRNA, rabbit reticulocyte ribosomes, and the purified reticulocyte elongation factors EF 1 and EF 2 was as previously described (Jiménez et al., 1975; Carrasco et al., 1976). The assay systems for poly(U)-directed polyphenylalanine synthesis, endogenous mRNA programmed polypeptide synthesis, and enzymic and nonenzymic peptidyl-tRNA translocation by yeast polysomes have also been described (Jiménez et al., 1975; Barbacid et al., 1975; Carrasco et al., 1976). Otherwise, specific conditions concerning each experiment are indicated in the legends of figures and tables. The source of the inhibitors used is given elsewhere (Carrasco et al., 1976).

Results

Characteristics of the Nonenzymic Translocation. The time course of peptidyl[^{3}H]puromycin formation by yeast polysomes in the presence of 1 M KCl indicates that the reaction has reached completion after only 5 min of incubation at 37 °C (Figure 1). It also shows the temperature dependence of the reaction since the nonenzymic translocation does not take place at 0 °C. This result suggests that the high potassium induced enhancement of peptidyl[^{3}H]puromycin formation is not due to a direct effect on the peptidyl transferase since the puromycin reaction by eukaryotic ribosomes and polysomes does occur at 0 °C in different assay systems studied (Barbacid et al., 1975; Vázquez et al., 1969; Silverstein, 1969). Furthermore, the reaction is strongly inhibited by cycloheximide,
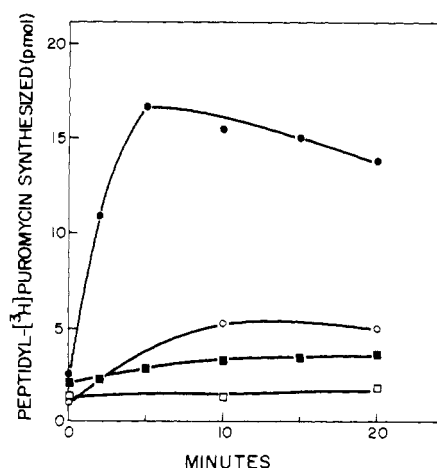
FIGURE 1: The effect of temperature and cycloheximide on the nonenzymic translocation. Reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl$_2$, 1 M KCl, 0.88 μM wild type polysomes, and 40 μM [^{3}H]puromycin were incubated at the indicated temperature. At the indicated times, 40-μL samples were withdrawn, precipitated with 1 mL of 10% trichloroacetic acid, and processed to estimate radioactivity as described by Carrasco et al. (1976). (● — ●) Control at 37 °C; (■ — ■) control at 0 °C; (O — O) + cycloheximide at 37 °C; (□ — □) + cycloheximide at 0 °C.

TABLE I: Reversion of the Nonenzymic Translocation by Lowering Potassium Concentration or Addition of Either Cycloheximide or Pederine.[a]

Preincubation (10 min at 37 °C)		Incubation (2 min at 37 °C)		Peptidyl-[^{3}H]puromycin formed (pmol)
KCl concn	Inhibitor	KCl concn	Inhibitor	
Expt 1: The Inhibitor Tested Was Cycloheximide (1 mM)				
Low	—	Low	—	5.49
Low	—	High	—	16.26
High	—	Low	—	4.61
High	—	High	—	13.43
High	+	High	+	4.15
High	—	High	+	4.78
Expt 2: The Inhibitor Tested Was Pederine (0.1 mM)				
Low	—	Low	—	6.15
Low	—	High	—	31.30
High	—	Low	—	5.74
High	—	High	—	27.71
High	+	High	+	4.65
High	—	High	+	5.97

[a] Reaction mixtures contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl$_2$, 45 pmol of wild type yeast polysomes, and KCl as required. Low and high concentrations of potassium were 80 mM and 1 M, respectively. The final volumes were 10 and 100 μL for preincubation and incubation mixtures, respectively. After the first incubation the tubes were placed on ice, and the required adjustments were made and, immediately after the addition of 60 μM [^{3}H]puromycin, the second incubation was carried out. Reactions were stopped by addition of 10% trichloroacetic acid and the samples processed to estimate peptidyl[^{3}H]puromycin formation following the methods already described (Bucher and Skogerson, 1976). Experiments 1 and 2 were performed with different preparations of polysomes.

which is widely accepted as a specific inhibitor of translocation acting at the level of the 60S ribosomal subunit and does not directly affect peptide bond formation (Vázquez, 1974 (review)). Therefore it appears that the high potassium concentration induces a change in ribosomal conformation favoring the positioning on the donor site of the peptidyl transferase
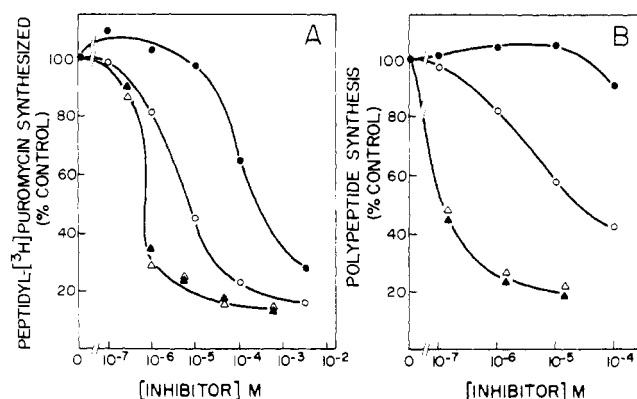

FIGURE 2: The effects of cycloheximide and pederine on ribosomes from a wild type and a cycloheximide-resistant mutant. (A) Nonenzymic translocation by cycloheximide-sensitive (Y$_{166}$) and -resistant (SR$_{17}$) polysomes: the effects of cycloheximide and pederine. Fifty-microliter reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl$_2$, 1 M KCl, 0.88 μM polysomes, and 40 μM [^{3}H]puromycin were incubated for 5 min at 37 °C and the reaction was stopped by adding 1 mL of 10% trichloroacetic acid. Samples were filtered, washed, and dried, and radioactivity was estimated as described under Figure 1. In the untreated controls, 8.45 and 6.69 pmol of peptidyl[^{3}H]puromycin were synthesised by Y$_{166}$ and SR$_{17}$ polysomes, respectively. (O — O) Y$_{166}$ polysomes + cycloheximide; (■ — ■) SR$_{17}$ polysomes + cycloheximide; (△ — △) Y$_{166}$ polysomes + pederine; (▲ — ▲) SR$_{17}$ polysomes + pederine. (B) Polypeptide synthesis by cycloheximide-sensitive and -resistant ribosomes: the effects of cycloheximide and pederine. Fifty-microliter reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 12.5 mM MgCl$_2$, 80 mM KCl, 1 mM ATP, 0.05 mM GTP, 4 mM creatine phosphate, 2 μg of creatine phosphokinase, 1 mM dithiothreitol, 0.075 mM [^{14}C]phenylalanine (Amersham, 180 mCi/mmol), 125 μg of yeast tRNA, about 2 mg/mL partially purified supernatant enzymes, and 10.7 pmol of polysomes were incubated for 20 min at 30 °C and then precipitated with 1 mL of 10% trichloroacetic acid. Samples were processed to estimate radioactivity as described under Figure 1. In the untreated controls, 5.24 and 4.77 pmol of [^{14}C]phenylalanine were incorporated by Y$_{166}$ and SR$_{17}$ polysomes, respectively. (O — O) Y$_{166}$ polysomes + cycloheximide; (● — ●) SR$_{17}$ polysomes + cycloheximide; (△ — △) Y$_{166}$ polysomes + pederine; (▲ — ▲) SR$_{17}$ polysomes + pederine.

center, of the 3' end of the peptidyl-tRNA attached to polysomes. The potassium-induced translocation appears to be a reversible process, because simply by lowering the potassium concentration or by addition of pederine or cycloheximide once translocation has taken place the peptidyl-tRNA becomes unreactive with puromycin, hence presumably indicating that it is located again in the A ribosomal site (Table I). As might be expected, fusidic acid does not inhibit the nonenzymic translocation since it does not interact with the ribosome in the absence of EF 2 and GTP (Willie et al., 1975). N-Ethylmaleimide is known to inhibit the enzymic translocation by blocking the sulfhydryl groups of EF 2 (Skogerson and Moldave, 1968). However, the sulfhydryl groups of the yeast ribosome do not appear to be relevant for the nonenzymic translocation since this reaction is not affected by N-ethylmaleimide (Table II).

The Effects of Inhibitors on Ribosomes from Wild Type and Cycloheximide- and Cryptopleurine-Resistant Yeast Strains. Both cycloheximide and pederine were powerful inhibitors of the nonenzymic translocation as shown in Table I. These results might suggest that both drugs have a similar site of action on the eukaryotic 60S ribosomal subunit. Therefore, we further studied their action on both enzymic and nonenzymic translocation and polypeptide synthesis by polysomes from the wild type strain (Y$_{166}$) and the cycloheximide-resistant mutant (SR$_{17}$). The results obtained in these assays show that polysomes from the SR$_{17}$ strain are sensitive to pederine (Figure 2) and strongly suggest that pederine and cyclohexi-

TABLE II: Fusidic Acid and N-Ethylmaleimide on the Nonenzymic Translocation.[a]

| Conditions | Peptidyl[^{3}H]puromycin formation[b] | |
	pmol	% control
Control	13.97 (11.59)	100 (100)
+ fusidic acid (1 mM)	12.30 (9.92)	88 (86)
+ N-ethylmaleimide (10 mM)	14.60 (12.22)	105 (105)
+ pederine (0.2 mM)	2.38 (0)	17 (0)

[a] Reaction mixtures (50 μL) contained 50 mM Tris-HCl buffer (pH 7.4), 1 M KCl, 12.5 mM MgCl$_2$, 40 μM [^{3}H]puromycin, and 0.62 μM wild type yeast polysomes. Incubations were carried out at 37 °C for 2 min and were stopped by adding 1 mL of 10% trichloroacetic acid. Samples were processed to estimate radioactivity following the methods already described (Carrasco et al., 1976). [b] Figures within parentheses are obtained by subtracting the values of peptidyl[^{3}H]-puromycin synthesized in the presence of pederine. These values correspond to peptidyl-tRNA initially bound to the donor site (Barbacid et al., 1975).

TABLE III: The Effect of Inhibitors on the Enzymic Translocation by Wild Type (Y$_{166}$) and Cryptopleurine-Resistant (CRY$_6$) Polysomes.[a]

| | Peptidyl[^{3}H]puromycin formation | | | |
| | Y$_{166}$ polysomes | | CRY$_6$ polysomes | |
Conditions	pmol	% control	pmol	% control
	Experiment 1			
Control	3.99	100	4.59	100
+ cryptopleurine (2 μM)	2.11	53	4.77	104
+ cryptopleurine (20 μM)	0.05	1	1.77	39
+ tubulosine (50 μM)	1.23	31	3.70	81
+ tubulosine (0.5 mM)	0.50	12	2.51	55
+ emetine (0.1 mM)	1.95	50	4.64	101
+ emetine (1 mM)	0.18	4	2.04	44
	Experiment 2			
Control				
+ cycloheximide (20 μM)	2.52	72	2.60	70
+ cycloheximide (0.1 mM)	1.46	41	1.60	43
+ pederine (1 μM)	2.63	74	2.86	77
+ pederine (10 μM)	1.28	36	1.67	45

[a] Fifty-microliter reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 80 mM KCl, 12.5 mM MgCl$_2$, 5 mM GTP, 40 μM [^{3}H]puromycin; 1 mM dithiothreitol; 0.6 μM polysomes, and 2 mg/mL of partially purified supernatant enzymes were incubated for 1 min at 30 °C and the reaction was stopped by adding 1 mL of 10% trichloroacetic acid. The cited amounts of peptidyl[^{3}H]puromycin synthesized have been obtained after subtracting controls values in the absence of supernatant enzymes (3.8 and 3.06 pmol of peptidyl[^{3}H]puromycin for Y$_{166}$ and CRY$_6$ polysomes, respectively).

mide have different binding sites on the eukaryotic ribosomes.

Emetine and tubulosine at concentrations that totally inhibit the translocation of peptidyl-tRNA do not have any effect on the nonenzymic translocation (Carrasco et al., 1976) as it has also been found in the present work for cryptopleurine (results not shown). These results indicate differences in the mode and possibly the site of action of translocation inhibitors. To further locate the site of action of these inhibitors we have studied their effects on poly(U)-directed polyphenylalanine synthesis and enzymic translocation by ribosomes from wild type (Y$_{166}$) and cryptopleurine-resistant (CRY$_6$) yeast strains. There is a single

TABLE IV: Polyphenylalanine Synthesis by Wild Type (Y$_{166}$) and Cryptopleurine-Resistant (CRY$_6$) Ribosomes. The Effects of Inhibitors.[a]

| | Polyphenylalanine synthesis | | | |
| | Y$_{166}$ | | CRY$_6$ | |
Conditions	pmol	% control	pmol	% control
Control	350.90	100	427.80	100
+ cryptopleurine (20 μM)	170.60	50	342.75	78
+ tubulosine (0.1 mM)	41.37	12	332.75	78
+ emetine (1 mM)	112.72	32	378.95	89
+ cycloheximide (2 μM)	173.60	49	209.10	49
+cycloheximide (20 μM)	147.37	42	166.78	39
+ cycloheximide (0.2 mM)	108.80	31	122.20	29
+ pederine (0.2 μM)	175.70	50	227.28	53
+ pederine (2 μM)	29.63	8	31.62	7
+ pederine (20 μM)	19.42	6	22.93	5

[a] Fifty-microliter reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 80 mM KCl, 12.5 mM MgCl$_2$, 1 mM dithiothreitol, 1 mM ATP, 0.05 mM GTP, 0.075 mM [^{14}C]phenylalanine (Amersham, 33 mCi/mmol), 4 mM creatine phosphate, 2 μg of creatine phosphokinase, 125 μg of yeast tRNA, about 2 mg/mL crude supernatant, and 10.7 pmol of high salt washed ribosomes were incubated for 30 min at 30 °C and then 1 mL of 10% trichloroacetic acid was added.

nuclear mutation in CRY$_6$ which is expressed in the 40S ribosomal subunit as also observed with other cryptopleurine-resistant mutants (Grant et al., 1974; Bucher and Skogerson, 1976). Enzymic translocation by Y$_{166}$ polysomes is sensitive to cryptopleurine, tubulosine, emetine, cycloheximide, and pederine as previously reported (Vázquez, 1974; Grollman and Jarkovsky, 1975 (reviews)), whereas CRY$_6$ polysomes are resistant to cryptopleurine, and tubulosine and emetine are sensitive to cycloheximide and pederine (Table III). Cycloheximide and pederine inhibit similarly the poly(U)-directed synthesis of polyphenylalanine by the Y$_{166}$ and CRY$_6$ high salt washed ribosomes. On the other hand, the ribosomes from Y$_{166}$ are more sensitive to cryptopleurine, tubulosine, and emetine than those from the CRY$_6$ mutant resistant to cryptopleurine (Table IV). These results strongly suggest that cryptopleurine, emetine, and tubulosine might have not only a similar mechanism of action but a common or overlapping binding site on the 40S ribosomal subunit which certainly differs from the action and binding site of cycloheximide and pederine.

Since crytopleurine, emetine, and tubulosine inhibit the enzymic translocation, their effects on polyphenylalanine synthesis might be dependent on the concentration of EF 2. This has been shown previously for tubulosine (Carrasco et al., 1976) and is extended here for cryptopleurine and emetine (Figure 3). A similar observation was reported by other workers with cryptopleurine (Bucher and Skogerson, 1976). Thus, the inhibitory effects of the three drugs are diminished by increasing the concentrations of elongation factor 2.

Discussion

The study of many biochemical processes has been greatly facilitated by the availability of drugs which specifically inhibit the individual steps in the process. For the study of the translocation step in protein synthesis in eukaryotes, several inhibitors are available that could be classified in distinct cate-
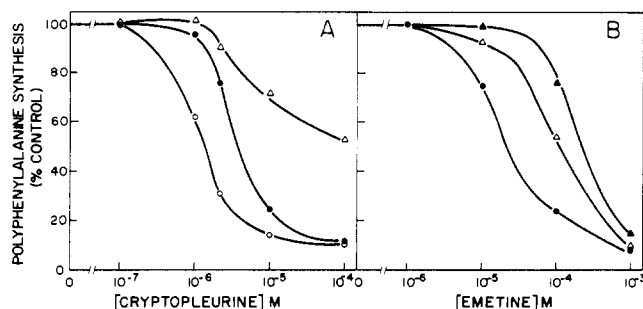
FIGURE 3: The effects of cryptopleurine and emetine on polyphenylalanine synthesis by reticulocyte ribosomes: dependence on EF 2 concentration. One-hundred-microliter reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 60 mM KCl, 11 mM MgCl$_2$, 9 mM 2-mercaptoethanol, 12.3 pmol of reticulocyte ribosomes, 0.5 mM GTP, 6 nCi of [^{14}C]Phe-tRNA (sp act. 463 Ci/mol), 5 μg of poly(U), 9 μg of EF 1, EF 2 as required, and emetine or cryptopleurine as indicated were incubated at 37 °C for 15 min, precipitated with 1 mL of 10% trichloroacetic acid, and processed to estimate radioactivity as described under Figure 1. Additions of EF 2 were as follows: (O — O) 6 μg of EF 2/mL (incorporation in the control was 1.04 pmol of [^{14}C] phenylalanine); (● — ●) 12 μg of EF 2/mL (incorporation in the control was 1.83 pmol); (△ — △) 30 μg of EF 2/mL (incorporation in the control was 3.85 pmol); (▲ — ▲) 48 μg of EF 2/mL (incorporation in the control was 4.44 pmol).

gories. Fusidic acid can inhibit translocation in vitro by sequestering on free ribosomes the EF 2 available for translocation (Carrasco and Vázquez, 1973), whereas diphtheria toxin enzymically modifies the EF 2 rending it inactive for translocation (Vázquez, 1974 (review)). The translocation inhibitors which directly interact with the ribosome can be now classified in two different groups according to the present study. One group is comprised of pederine and cycloheximide, which act on the large ribosomal subunit and are powerful inhibitors of both the enzymic (Vázquez, 1974)) and the nonenzymic translocation (Table I). Their mechanism of action could be interpreted as if they anchored the peptidyl-tRNA in the A ribosomal site, stabilizing the binding of this substrate at that position and hence freezing the polysomes. However, their binding site on the 60S subunit cannot be exactly the same because cell-free extracts derived from the mutant strain SR$_{17}$ resistant to cycloheximide are sensitive to pederine (Figure 2). The second group includes cryptopleurine, emetine, and tubulosine that probably act at the level of the 40S ribosomal subunit. Thus, enzymic translocation by cryptopleurine-resistant polysomes is resistant to cryptopleurine, emetine, and tubulosine (Table III). Genetic and biochemical studies of the CRY$_6$ mutant have shown that the resistance to these three drugs is the consequence of a single nuclear mutation and is expressed in the 40S ribosomal subunit (Grant et al., 1974; L. Sánchez, personal communication). The fact that increasing concentrations of EF 2 reverse the block of translocation by these inhibitors (Figure 3) suggests that they could compete with the interaction of EF 2 with the ribosome. Consequently their binding site on the smaller ribosomal subunit must be located at the interface with the 60S subunit in close relation with the EF 2 binding site (Bucher and Skogerson, 1976).

The nonenzymic translocation can be interpreted as the movement of the 3' end of the peptidyl-tRNA from the acceptor to the donor site of the peptidyl transferase center. This movement is induced by potassium possibly by changing the conformation of this center and increasing the affinity of the 3' end of peptidyl-tRNA for the P site. Alternatively, the high potassium concentration may cause a loose binding of the 3'

end of peptidyl-tRNA, so that this can shift easily between the donor and acceptor sites of the peptidyl transferase. The reversal of nonenzymic translocation by pederine and cycloheximide (Table I) could result from a stabilization by these drugs of the binding of the 3' end of peptidyl-tRNA to the acceptor site. In addition, these results suggest that translocation of peptidyl-tRNA induced by high potassium concentration is not mediated by detachment of peptidyl-tRNA from the ribosome into the buffer and subsequent binding to the P site (Bloebel and Sabatini, 1971). There is no evidence that nonenzymic translocation may extend to the position of the peptidyl-tRNA in contact with the 40S ribosomal subunit and to the mRNA. Moreover, the fact that cryptopleurine, emetine, and tubulosine act most probably at the same site of the 40S subunit and inhibit enzymic translocation, but are without effect on the nonenzymic one, argues against this possibility.

Acknowledgments

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