Ca\textsuperscript{2+} Ions Inhibit Messenger Ribonucleic Acid Degradation, but Permit Messenger Ribonucleic Acid Transcription and Translation in Deoxyribonucleic Acid-coupled Systems from *Escherichia coli*

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**SUMMARY**

DNA-directed formation of RNA and protein was compared in subcellular systems in the absence or presence of Ca\textsuperscript{2+} ions. In the absence of Ca\textsuperscript{2+}, messenger RNA was unstable, and protein and RNA accumulation tended to stop after incubations of 10 to 15 min at 37\textdegree. In contrast, in the presence of Ca\textsuperscript{2+} mRNA was protected against decay, but when puromycin was used to block protein synthesis, mRNA still degraded rapidly.

The implication that a number of ribonucleases are directly inhibited by Ca\textsuperscript{2+} ions was confirmed for two purified *E. coli* enzymes, the endonuclease RNase III and the exonuclease RNase II. Thus, Ca\textsuperscript{2+} ions provide an effective way to dissociate in *viva* the physiological coupling of mRNA formation and translation from its degradation.

In the absence of Ca\textsuperscript{2+}, a number of features of mRNA metabolism in *vivo* could be reproduced. Chemical decay of *E. coli* and T4-specific mRNA was studied. As in *vivo*, decay of both types of mRNA was exponential. Also, the half-lives (2.25 min for *E. coli* and 4.45 min for T4 mRNA) were of the same magnitude and in the same ratio as in *vivo*.

Furthermore, the response of mRNA metabolism to antibiotics was comparable to that in *vivo*; when protein synthesis was blocked by the addition of chloramphenicol, new mRNA was protected against decay, but when puromycin was used to block protein synthesis, mRNA still degraded rapidly.

In growing cells of *Escherichia coli*, the synthesis and function of messenger RNA such as that for the trp operon (1-3) is closely followed by its degradation (4-5). The recent development of DNA-coupled systems makes it possible to analyze relationships between these processes under conditions that may be similar to those in the cell.

Completely purified components have been used in *vitro* to transcribe specific mRNA species from T4 DNA (6-7), \lambda DNA (8-9), T7 DNA (10-11), pX 174 DNA (12), and *E. coli* DNA (13-14). Purified components have also been used to translate selected mRNAs, such as R17 phage RNA (15) and T4 mRNA (16). However, the synthesis of inducible and repressible bacterial enzymes has been reported only with systems containing cell extracts, notably that developed by Lederman and Zubay (17). This *in vitro* system included a number of unique components not added in any other reported *in vitro* system. Here we report that the most critical of these is Ca\textsuperscript{2+} ions and that the major effect of Ca\textsuperscript{2+} ions is to inhibit ribonuclease function. The effect of Ca\textsuperscript{2+} ions on mRNA stability was also observed with the other widely used DNA-coupled system, that developed by Gold and Schweiger (18) for T4 DNA-directed synthesis of functional T4 enzymes. Several purified *E. coli* RNases were also tested and were inhibited by Ca\textsuperscript{2+} ions.

While the translation yields of the DNA-coupled systems is low (see under "Discussion"), systems incubated without added Ca\textsuperscript{2+} can mimic many of the features of chemical decay of mRNA in *vivo*. These include exponential decay rates of phage and bacterial mRNA similar to those observed in *vivo* (19) and strong inhibition of degradation in presence of chloramphenicol (20-21), but rapid degradation in presence of puromycin (22-23).

**MATERIALS AND METHODS**

**Coupled Cell-free Systems**—*E. coli* K12 strain 514 (17) was used in all of the studies reported here. We have also used *E. coli* K12 strains D10 and AB301 with essentially the same results.

Growth of cells and preparation of the S-30 extracts were described by Zubay *et al.* (24). Modifications suggested by Wetekam *et al.* (25) were followed. Preincubation of the S-30 extract was according to Nirenburg (26) as modified by Wetekam *et al.* (25). After dialysis against buffer 111 (three changes for a total of 6 hours), aliquots of the S-30 extract were frozen and stored in liquid nitrogen.

The incubation conditions for the Zubay coupled system are the same as described (24), except for the deletion of various coenzymes. Essentially similar changes have been made by Wete-

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was collected by centrifugation, washed once with 3 ml of 100% sodium acetate, pH 5.0. The solution was extracted once in the cold with phenol saturated with 0.1 grade), and 150 fig of Chagrin Falls, Ohio) and allowed to sit at 4° for 20 min. The acid suspensions were incubated for 10 min at 90°, then allowed to cool to 4°. Precipitates were collected on glass fiber filters and counted in a Packard Tri-Carb scintillation counter with a toluene-based counting fluid.

For a second type of coupled system, with preparations of ribosomes and supernatant protein fraction freed of contaminating nucleic acids, the cells were grown and fractions prepared according to Gold and Schweiger (18). The standard conditions for T4 DNA-directed translation and transcription in this system included magnesium acetate at 11 mm, trNA at 1 mg per ml, ribosomes at 3 mg per ml, supernatant protein at about 3 mg per ml, and T4 DNA at 30 to 50 fig per ml; these conditions are similar to those of O'Farrell and Gold (29). [3H]UTP was used at 50 PCi per mmole and 0.5 mM. Reactions (0.5 or 1.0 ml) were prepared, and aliquots (0.025 ml) were removed at the times indicated and precipitated with trichloroacetic acid as above.

Preparation of DNA—Phase T4 was purified from lysates (5 to 10 liters) by polyethylene glycol precipitation (80), followed by equilibrium sedimentation in a CsCl step gradient (31). DNA was isolated by alcohol precipitation and gentle shaking at room temperature, followed by ethanol precipitation and "spooling" out of the DNA. The DNA was resuspended in a low salt buffer (0.001 M NaCl, 0.001 M EDTA, and 0.001 M Tris-HCl, pH 7.5) (32).

E. coli DNA was purchased from General Biochemicals and phenol extracted twice before ethanol precipitation and "spooling" out. [3H]dUTP DNA was a gift from Dr. David Kennell and was prepared by published procedures (33).

**TABLE I**

Effect of DNA synthesis on *β*-galactosidase synthesis—The data shown in Table I show a typical requirement for Ca++ as described by Zubay et al. (24), for *β*-galactosidase synthesis directed by φ30dλ DNA. In the extracts reported here, the requirement for Ca++ was essentially absolute; in several other high efficiency extracts, the requirement for Ca++ was less marked, but its effects

<table>
<thead>
<tr>
<th><strong>Incubation system</strong></th>
<th><strong>Experiment 1</strong></th>
<th><strong>Experiment 2</strong></th>
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<tbody>
<tr>
<td><strong>β-Galactosidase formed</strong></td>
<td>Complete system</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>No Ca++ (&lt;0.1 x 10^-8)</td>
<td>&lt;0.1 x 10^-9</td>
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<tr>
<td></td>
<td>No DNA</td>
<td>&lt;0.1 x 10^-9</td>
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<tr>
<td></td>
<td>Plus 100 µg per ml of chloramphenicol</td>
<td>&lt;0.1 x 10^-9</td>
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<tr>
<td></td>
<td>Plus 20 µg per ml of rifampicin</td>
<td>&lt;0.1 x 10^-9</td>
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* Refers to the composition of the incubation mixture in the DNA-coupled system.
on mRNA stability (see below) were still observed. A number of other special additives originally recommended (24), including pyridoxine-HCl, NADP+, FAD, and p-aminobenzoic acid, have proven to be unnecessary in every extract tested. The levels of synthesis regularly achieved (Table I) are comparable to the highest reported in the literature. The magnesium acetate concentration required for maximal rate of synthesis of ß-galactosidase was in the range of 12 to 15 mM, similar to that reported by Zubay et al. (24). Similar simplified reaction mixtures have been used by Wetekam et al. (25) for the synthesis of gal operon-specific enzymes, and by Dottin and Pearson (27), and Pouwels and Van Rotterdam (28), for the synthesis of trp operon-specific enzymes.

Nascent RNA and Protein Accumulation in Presence and Absence of Ca2+

In an attempt to determine a role of Ca2+ in DNA expression, [14C]amino acids or [3H]ribonucleotide triphosphates were incorporated into the system, and possible effects on mRNA formation, function, or metabolism were assayed.

Fig. 1 and 2 show the relative effects of Ca2+ on the synthesis of RNA and protein, with phage T4 or E. coli DNA as template.

![Fig. 1. Effect of Ca2+ on RNA transcription and protein synthesis directed by T4 DNA in the Zubay DNA-coupled system. Cell-free incubations were carried out in the presence or absence of 7 mM CaCl2. At 2 min 100 μg per ml of rifampicin were added to some of the reactions. Aliquots (0.025 ml) were removed and assayed for [3H]UTP incorporation or [14C]leucine incorporation. Two such experiments are shown for each of RNA synthesis (A, C) and protein synthesis (B, D). ○—○, no CaCl2, no rifampicin; △—△, 7 mM CaCl2, 100 μg per ml of rifampicin; 10 min; △—△, 7 mM CaCl2, 100 μg per ml of rifampicin at 2 min.](http://www.jbc.org/)

There is some variability from extract to extract, and trials with two different extracts are shown to indicate the range.

For these and other DNA templates tested (gal and λprp), there was sometimes a lag and slower initial rate of both RNA and protein synthesis in the presence of Ca2+ ion compared to its absence (for example, Fig. 1, C and D). However, the over-all amount of RNA and protein accumulated was always substantially increased in the presence of Ca2+. As exemplified in Figs. 1 and 2, after 15 min of incubation the net amount of new RNA in the presence of Ca2+ was 2- to 3-fold greater with all the DNA templates used. Protein synthesis in the presence of Ca2+ continued at a constant rate for 20 to 45 min, compared to 10 to 15 min in its absence, and the levels of protein formed were thereby much increased.

Thus, in spite of an apparent decrease in initial rates of RNA and protein synthesis, Ca2+ ions promoted continued accumulation of RNA and protein. Further studies showed that this was in great part attributable to a stabilization of mRNA.

Relative RNA Stability in Presence and Absence of Ca2+

Since it was repeatedly noticed that the T4 RNA formed was far more stable in the presence of Ca2+ ion, the increase in protein synthesis and RNA accumulation might result from comparable levels of synthesis, with an inhibition of degradation of mRNA in the presence of Ca2+. To test this notion, RNA formation was directed by T4 DNA in the presence or absence of Ca2+ ions, and in replicate samples rifampicin was added after 2 min to block further initiation of transcription (37). The stability of the RNA and the accumulation of protein was then followed (Fig. 1). The stabilization of the RNA in the presence of Ca2+ appeared complete. Breakdown to acid-soluble products was undetectable during 30-min incubations in repeated trials with extracts from three strains (also, see Part B, below).

Comparable though less extreme results were obtained with E. coli DNA as the template for RNA synthesis. Two experiments are depicted in Fig. 2. Here the balance of rapid synthesis and degradation produced an apparent plateau level of new RNA in the mixtures incubated without added Ca2+. However, rapid mRNA breakdown in the absence of Ca2+ was evident when rifampicin was added to limit synthesis. In contrast,
when RNA synthesis was limited by rifampicin in the presence of Ca\(^{2+}\), different extracts showed none or a small amount of RNA degradation over a 20- to 30-min incubation period.

Endonuclease Action is also Inhibited by Ca\(^{2+}\) Ions—The assays in Figs. 1 and 2 measured acid-precipitable RNA. Thus, they showed that RNA formed in the presence of Ca\(^{2+}\) was not degraded to small oligonucleotides. However, E. coli extracts contain activity capable of cleaving T4 mRNA endonucleolytically (38-39). To test whether such endonucleolytic cleavages were also inhibited in the presence of Ca\(^{2+}\), the size of T4 mRNA was measured. Synthesis was once again limited by rifampicin and the RNA extracted at intervals and analyzed by polyacrylamide gel electrophoresis. No reduction in the size of T4 mRNA transcripts was detected even 22 min after rifampicin addition (Fig. 3). Thus, little if any endonuclease was active in the presence of Ca\(^{2+}\) ions.

Extension to Other Coupled Systems—In another powerful DNA-directed coupled system, that developed by Gold and Schweiger (18), fractionated components are recombined in the in vitro reaction. Background levels of RNA and protein formed in the absence of added DNA are negligible compared to those in the Zubay system, and in this case the amount of protein formed in the presence and absence of Ca\(^{2+}\) were rather comparable (data not shown). Nevertheless, we have found effects of Ca\(^{2+}\) on RNA metabolism similar to those observed in the Zubay system (Fig. 4). Again, a lower rate of synthesis was observed in the presence of Ca\(^{2+}\), but 2-fold more RNA was accumulated (Fig. 4) in a form stable even in the presence of rifampicin (data not shown).

RNase II and RNase III Activities are Inhibited by Ca\(^{2+}\)—The results of Figs. 1 to 4 suggested most simply that many ribonucleases are inhibited by Ca\(^{2+}\). To test this suggestion directly, we assayed two of the major activities known in E. coli, with the use of a highly purified preparation of the exonuclease RNase II and the endonuclease RNase III in the presence or absence of Ca\(^{2+}\). The enzyme assays were carried out in the buffer solution of the Zubay coupled system, which contains more Mg\(^{2+}\) and a buffer of higher pH than the standard RNase II assay buffer of Singer and Tolbert (40). In this buffer, with a saturating level of poly(A) as substrate, RNase II was completely inhibited by the 7 mM Ca\(^{2+}\) present (Fig. 5). The observed rate of hydrolysis in the absence of added Ca\(^{2+}\) was 13 μmoles of 5'AMP per mg of enzyme per hour. The rate of hydrolysis in the presence of Ca\(^{2+}\) was undetectable (less than 0.13 μmole of 5'AMP hydrolyzed per mg of protein per hour), in other words, an effective inhibition greater than 99%. In comparable experiments, we found that the action of RNase III on poly(I)-[\(^{3}H\)]poly(C) was also inhibited >99% in assays containing Ca\(^{2+}\) ions.

**FIG. 3** (left). Acrylamide gel electrophoresis of T4 mRNA synthesized in vitro. Standard reaction mixtures of the Zubay T4 DNA-directed coupled system were incubated at 37° with [\(^{3}H\)]UTP (6 Ci per mmole, 300 μCi per ml) for 8 min (○—○) or 30 min (O—O) in the presence of 7 mM Ca\(^{2+}\). At 8 min 100 μg per ml of rifampicin were added to a portion of the reaction mixture, and incubation continued for an additional 22 min (△—△). [\(^{3}H\)]RNA was prepared as under "Materials and Methods" and fractionated by electrophoresis in acrylamide gels for 2 hours. The RNA recovered from each gel was normalized (about ±10 to 15%) to the trichloroacetic acid-precipitable material present in a 0.005-ml aliquot of the reaction taken at 8 min and 30 min. [\(^{14}C\)]uniformly labeled 23S and 16S RNA were extracted from *Escherichia coli* and included as markers in each RNA sample (arrows).

**FIG. 4** (center). Effect of Ca\(^{2+}\) on transcription directed by T4 DNA in the Gold and Schweiger coupled system. Cell-free incubations were carried out in the presence or absence of 7 mM CaCl\(_2\). Aliquots (0.020 ml) were removed and assayed for [\(^{3}H\)]UTP incorporation. ○—○, no CaCl\(_2\); △—△, 7 mM CaCl\(_2\). Fig. 5 (right). RNase II activity is inhibited by Ca\(^{2+}\). RNase II assays were carried out as under "Materials and Methods," in the absence or presence of 7 mM Ca\(^{2+}\). The substrate concentration was 1 × 10\(^{-4}\) M poly(A) (4 mCi per mmole) (1 × 10\(^{-4}\) mole of phosphate); protein concentration was 15 μg per ml, with 1000-fold purified RNase II. Aliquots (0.05 ml) were removed at the indicated times and precipitated in 0.2 ml of 95% ethanol. ○—○, no Ca\(^{2+}\); O—-O, 7 mM Ca\(^{2+}\).
I I I 1 1 1
! 5 10 IS 20 25 30
MINUTES
FIG. 6. Synthesis and degradation of RNA synthesized in vitro from Escherichia coli or T4 DNA in the Zubay system. A, cell-free incubations with [3H]UTP were carried out with E. coli DNA (100 µg per ml) or T4 DNA (50 µg per ml) as templates. At 2 min 100 µg per ml of rifampicin were added to block further initiation of RNA synthesis. The synthesis and degradation of the [3H]RNA were followed by removing 0.030-ml aliquots at the times indicated and precipitated with trichloroacetic acid. A--&
2 4 6 6 IO 12 14
MINUTES
B. mRNA Decay in Absence of Ca2+

The fate of mRNA synthesized in the absence of Ca2+ has been further detailed.

Exponential Decay of mRNA—When T4 DNA-directed reactions were studied, the content of newly formed mRNA reached a maximum and then declined (Fig. 1). However, this breakdown showed no obvious relation to the kinetics of protein synthesis in various trials (e.g., cf. Fig. 1, A and B), and as Zubay et al. (24) have pointed out, components other than mRNA are usually limiting in these systems. Thus, this “spontaneous” breakdown may or may not be related to mRNA metabolism in vitro. Therefore, to try to ensure that mRNA content was limiting for protein synthesis, RNA synthesis was limited by the addition of rifampicin.

In reaction mixtures like those of Figs. 1 and 2, RNA synthesis was started for 2 min at 37°. Rifampicin (100 µg per ml) was then added to limit further initiation of RNA chains more than 98% (37). The subsequent decay of the pulse-labeled RNA was then followed more extensively than in the preliminary trials of Figs. 1 and 2.

Consistent results for mRNA decay were obtained with extracts of high efficiency, and only extracts that showed at least 1 amino acid residue incorporated into protein per codon of RNA synthesized have been used (see under “Materials and Methods”). Fig. 6A shows the relative rates of synthesis and decay of mRNA transcribed in vitro from T4 and E. coli DNA. The data were replotted on a semilogarithmic plot in Fig. 6B. Both types of mRNA display exponential decay curves similar to those seen in vivo experiments (19), with half-lives of 2.25 min for E. coli and 4.45 min for T4 mRNA. (Since the T4 mRNA decay curve may not be at a plateau value at 30 min, the curve may give a slight underestimate of the mRNA half-life.)

The half-lives were determined with several different extracts from two strains of E. coli. The rate of breakdown of T4 mRNA was always about half that of E. coli RNA, although the respective half-lives were sometimes slightly longer than those shown in Fig. 6B.

An even slower exponential decay rate, with a half-life of 8 min, was observed for the degradation of T4-specific mRNA in the coupled system prepared according to Gold and Schweiger (18) (Fig. 7); however, in those extracts transcription of E. coli and other DNA templates was not efficient enough to permit an accurate determination of decay rates for comparison purposes.

Inhibition of Breakdown of New mRNA by Addition of Chloramphenicol—The breakdown of mRNA was substantially inhibited by addition of chloramphenicol, but not by addition of puromycin. Sample results with the modified Zubay-type system are shown in Fig. 8 for T4 mRNA. Chloramphenicol treatment prolonged the half-life about 2-fold, to 11 min, while puromycin treatment did not change the half-life of 4.5 min observed in its absence. In our trials with the Gold and Schweiger-type coupled system treated with chloramphenicol, T4 mRNA was degraded with a half-life of approximately 15 min (Fig. 7), a stabilization of about 2-fold.

In contrast to the results with newly formed mRNA, preformed labeled polynucleotides added to the system were very rapidly degraded, even in the presence of chloramphenicol. For example, \( 2 \times 10^{-6} \) M [3H]poly(A) was rendered totally ethanol soluble within 2 min, even in a reaction mixture in which the soluble proteins and ribosomes were diluted 1:10 from the normal levels in the coupled system (0.65 mg per ml versus 6.5 mg per ml).

DISCUSSION

Since Ca++ is totally dispensible for E. coli growth (41) and is at very low or zero levels in cells (42), it must have a function or

1 Unpublished data.
ducible observed β-galactosidase synthesis in a modified, par-
not been excluded. Furthermore, Kung et al. (46) have repro-
effects of Ca2+ on RNA polymerase or translation have certainly
system, other factors must also be involved. More complex
rationalize much of the power of the Zubay coupled
not required in intrinsic cell physiology.

Ca2+ is known to fail as a cofactor for many of the reactions
require Mg2+ or Mn2+ (44); the list includes a variety of nu-
involved in mRNA degradation; they are tested primarily as
equally major ribonucleases.)

As a result of these distinctive properties, addition of Ca2+
ions provides a way to permit mRNA transcription and transla-
tion in the absence of bulk RNA degradation. The mechanism
nucleation inhibition is as yet not shown, but perhaps this is the
reason that Schumacher and Ehring (45), using the Zubay cou-
system that contains Ca2+ ions, have found a functional
lifetime of gal operon mRNA much longer than it is in vivo. In
a preliminary report by Lederman and Zubay, apparent relative
can vary independently
that in vivo (47). Nevertheless, perhaps because translation
yields and chemical stability of mRNA can vary independently
many features of mRNA metabolism in vivo can be imitated
when the DNA-coupled system is incubated with rifampicin in
the absence of Ca2+ ions. mRNA decay is extensive and ex-
ponential, and the half-lives of 2.25 and 4.45 min for E. coli and
early mRNA at 37° (Fig. 6) are to be compared with 2.2 and
8 min observed at 30° in vivo (19). Several laboratories have
reported 3 to 4 min at 37° for the half-life of T4 mRNA in vivo
(49–51).

Concerning results with the Gold-Schweiger system, the 8-
min chemical half-life observed when RNA formation was
limited by rifampicin (Fig. 7) agrees well with the estimate of 7
to 8 min when transcription was halted by actinomycin D (52).
Thus, as in whole cells, the characteristic decay rates of T4 and
coli mRNA species are different and seem to be determined by
their structure (cf. Fig. 6 and Refs. 19 and 53), possibly by 5’-
terminal sequences (21, 54–55).

The inhibition of mRNA breakdown by chloramphenicol (Fig. 7)
corroborates earlier findings with whole cells (20, 56) and with
polyribosomes incubated in vitro (67, 58). Also as in vivo (21–23),
addition of puromycin blocks protein synthesis but does not
prolong mRNA survival (Fig. 8). Ribosomes blocked by chlor-
amphenicol action may protect mRNA, while puromycin would
promote release of the ribosomes (31). Consistent with this no-
tion is the relative instability of free RNA added to extracts
(see Part B under "Results"). As has been discussed elsewhere
(58–60), the detailed enzymatic basis of the mRNA decay re-
ains unknown, but study of early events in the DNA-coupled
system, possibly in the presence of varying levels of Ca2+ ions,
may further the analysis.
Ca$^{2+}$ Ions Inhibit Messenger Ribonucleic Acid Degradation, but Permit Messenger Ribonucleic Acid Transcription and Translation in Deoxyribonucleic Acid-coupled Systems from *Escherichia coli*

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