

Applications of TNT™ Coupled Transcription/Translation Systems

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TNT System-based production of [³⁵S]eIF-4alpha for functional studies

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Co-expression of multiple NF-KB subunits using the TNT™ System

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Since the mid-1970s, researchers have been able to identify and characterize eukaryotic genes utilizing *in vitro* translation extracts. The two most popular systems, rabbit reticulocyte lysate and wheat germ extract, initially were used to translate purified natural mRNAs (1,2). In the mid-1980s, the translation *in vitro* of specific cloned genes was made possible by the use of phage promoters and RNA polymerases to generate large amounts of specific RNAs (3). While *in vitro* transcription and translation of specific cloned genes is a powerful technique, the process of *in vitro* RNA transcription can be time-consuming, and RNase-free technique must be employed to prevent degradation of the RNA.

The TNT Coupled Transcription/Translation Systems couple the process of phage transcription (T3, T7 or SP6) with translation in a eukaryotic cell-free system, either reticulocyte lysate or wheat germ extract (4-6). The resulting TNT Systems offer a number of advantages which previously had been restricted to coupled prokaryotic systems, such as E. coli S30 systems (7-9). The modest amounts of either

supercoiled or linear DNA needed to program the TNT reactions are simple to produce. By removing RNA transcription as a separate step, the in vitro translation products are produced in much less time, in significantly greater amounts, and with greater consistency in expression levels.

These are just some of the possible applications of the TNT Coupled Transcription/Translation Systems:

- Increased protein expression (4)
- Mutagenesis screening (10)
- Co-expression of multiple genes (11)
- Expression for protein-protein and protein-nucleic acid binding studies (11,12)
- Expression of gene fragments for epitope/domain characterization (13)

The following two articles illustrate a few of these applications. We encourage you to explore additional applications of the TNT Systems to your research and contact us with your ideas and results.

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TNT™ System-based production of [³⁵S]eIF-4alpha for functional studies

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The TNT Coupled Reticulocyte Lysate System has simplified the preparation of ³⁵S-labeled initiation factor eIF-4alpha for functional studies, resulting in 2- to 3-fold higher incorporation levels and greater batch to batch consistency.

Elucidating the role of eIF-4 in translational control

The research interest in this laboratory is the translational regulation of gene expression and, in particular, the regulation of activity of eukaryotic protein synthesis initiation factors. We have found that translation of *in vitro* transcripts of cloned initiation factor genes is a useful method for production of [³⁵S]methionine-radiolabeled initiation factors. These ³⁵S-labeled factors are used to track the association of initiation factors with other components of the translational machinery.

Using this method, we have demonstrated that the sea urchin inhibitor of eIF-4 function inhibits the initiation sequence *after* mRNA binding to the small ribosomal subunit, but *before* 60S subunit joins. Binding of this inhibitor leads to a significant sequestration of the small pool of eIF-4alpha (the alpha subunit of eIF-4). Experiments of this type require high levels of [³⁵S]eIF-4alpha, since the factor could be distributed over many gradient fractions.

We have recently used this approach in attempting to elucidate the mechanism of action of a developmentally regulated inhibitor of eIF-4 function found in unfertilized sea urchin eggs. eIF-4 is the protein synthesis initiation factor that promotes mRNA interaction with the small ribosomal subunit. Reconstitution experiments suggest that the target of the sea urchin inhibitor is the small (alpha) subunit of eIF-4, which binds to the 5' cap structure of mRNA. We have used [³⁵S]methionine-radiolabeled eIF-4alpha to track the point in the initiation sequence at which the sea urchin inhibitor blocks eIF-4 function. To perform these experiments, we needed to produce [³⁵S]eIF-4alpha in an mRNA-dependent rabbit reticulocyte cell-free translation system and add this to non-nuclease-treated reticulocyte lysate incubated in the presence or absence of the sea urchin inhibitor. The block in the initiation sequence was monitored by sucrose density gradient fractionation of the initiation intermediates, followed by SDS-

PAGE and fluorography to visualize the location of [³⁵S]eIF-4alpha.

Higher incorporation and improved consistency with the TNT System

Use of the TNT Coupled Reticulocyte Lysate System to produce [³⁵S]eIF-4alpha has routinely given us 2- to 3-fold higher incorporation than a standard reticulocyte lysate translation system, which requires *in vitro* transcripts. Furthermore, use of the TNT System has increased the consistency of [³⁵S]eIF-4alpha production.

Table 1. Comparison of [³⁵S]eIF-4alpha Production With TNT Coupled Reticulocyte Lysate and Separate Transcription and Translation Reactions.

Sample #	Separate Transcription and Translation (cpm)	TNT Coupled Transcription/ Translation (cpm)
1	37,603	274,090
2	113,220	228,328
3	118,932	269,002
4	76,388	256,442
5	70,430	239,350

Table 1 compares the production of [³⁵S]eIF-4alpha in a standard translation system using *in vitro* transcripts (1) and in the TNT Reticulocyte Lysate System (2). Using the TNT System, incorporation into [³⁵S]eIF-4alpha was consistently higher and there was little day-to-day variation. In addition to the increased levels of incorporation and consistency observed, the TNT System considerably lightened the experimental load, since linearized DNA and *in vitro* RNA transcripts did not have to be produced as separate steps.

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Co-expression of multiple NF-KB subunits using the TNT™ System

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Promega's TNT Wheat Germ Coupled Transcription/Translation System was used for in vitro protein-protein interaction studies of subunits of the transcriptional activator NF-KB. Multiple genes can be expressed simultaneously in TNT Extract. Furthermore, these in vitro-translated proteins were shown to be capable of protein-protein interactions by immunoprecipitation and gel shift assays.

Role of the NF-KB family in signal transduction

Proteins in the NF-KB family act as tertiary messengers, transducing signals from the environment to the nucleus of the cell. NF-KB resides in the cytoplasm as an inactive complex consisting of heterodimeric DNA-binding subunits sequestered by an inhibitor (I-kappa-B). When an appropriate signal is received, I-kappa-B is thought to be phosphorylated and then dissociate from the DNA-binding subunits, allowing the subunits to translocate to the nucleus and bind their target genes.

Purified human NF-KB was initially described by Baltimore and his colleagues as a heterodimer of two major subunits, p50 and p65 (1), whose sequences showed high homology with the proto-oncogene *c-rel*. The p50 subunit (known as NF-KB1) is translated as a larger precursor polypeptide (p105 in humans), while p65 is translated as a mature product (2,3,11-13). A related NF-KB subunit, p55 (known as NF-KB2), also is translated as a larger precursor (p98 or p100) (6,7,10,13).

Recently, an additional pathway by which NF-KB mediates signaling has been identified. In this pathway, the NF-KB precursor proteins p105 and p98 can form heterocomplexes with both p65 (RelA) and c-Rel. This results in the retention of these heterocomplexes in the cytoplasm (5,6). When p105 or p98 is proteolytically processed to yield p50 or p55, the DNA binding subunits are released from the heterocomplexes and are free to translocate to the nucleus or to interact with I-kappa-B.

Attempts to express NF-KB subunit genes *in vitro*

To study NF-KB protein-protein associations *in vitro*, we decided to produce these proteins simultaneously *in vitro* from cloned genes. Very little full-length product was made, however, when we expressed the proteins using standard transcription reactions followed by translation in Rabbit Reticulocyte Lysate or Wheat Germ Extract. When two or more genes were expressed together, the translation yields were even lower. Different transcription reactions produced RNAs that varied in their translation efficiency, which further complicated the production of proteins in defined ratios for binding studies.

Both the TNT Rabbit and Wheat Germ Transcription/Translation Systems allowed us to express multiple genes easily in the same reaction mixture. Large proteins such as p105 and p98 were produced

efficiently at yields high enough for our functional studies. The TNT Wheat Germ System was selected for the work presented here, since it lacks endogenous NF-KB DNA binding activity.

Simultaneous production of multiple proteins in the TNT System

Figure 1 illustrates the expression of multiple genes.* In Panel A, p98 and c-Rel were expressed separately and together in TNT Wheat Germ reactions driven by T7 RNA Polymerase. When expressed alone, p98 is more efficiently translated than c-Rel. The level of c-Rel expression is noticeably reduced when co-expressed with p98. Such a reduction is common when co-expressing multiple proteins and is attributable, in part, to the lower amount of template DNA added for each gene.

*At this time, Promega can not guarantee that all lots of TNT Reticulocyte Lysate and Wheat Germ Extract will support translation by multiple RNA polymerases. We are currently working to refine the TNT Systems so that they will function consistently with SP6, T3 or T7 TNT RNA Polymerase.

In Panel B, we demonstrate that multiple proteins can be expressed simultaneously from different promoters in the same TNT reaction. In this case, TNT-qualified T3 and SP6 RNA Polymerase were added to a T7 TNT Wheat Germ System along with T7, T3 or SP6 promoter constructs as indicated. This allows great flexibility in designing experiments for co-expression of multiple genes. If the genes are cloned into vectors containing dual, opposed promoters (such as Promega's pGEM[®] Vectors), often the template must be linearized before use to prevent the production of both sense and anti-sense RNA from a given construct.

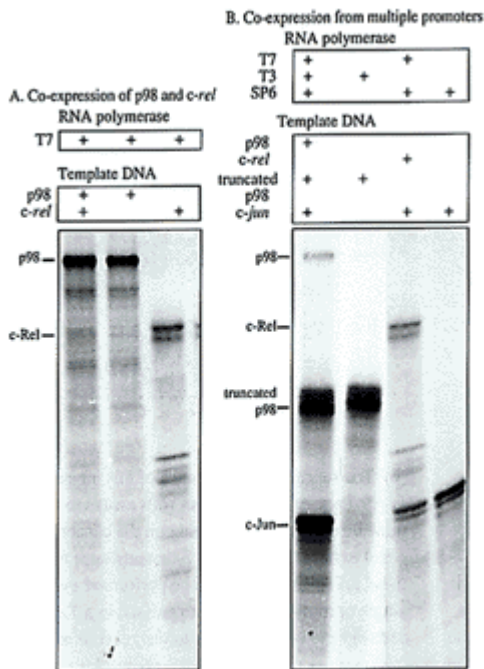


Figure 1. Co-expression of multiple proteins in the TNT Wheat Germ Extract System. The indicated recombinant proteins were produced in 25µl TNT Wheat Germ Extract reactions containing equivalent amounts of template DNA, with the total always equal to 1.5µg of DNA. One exception to this was c-Jun, for which only 0.1µg of DNA was used per reaction. The truncated p98 gene was linearized with *Xho* I, generating a polypeptide with a mobility of about 62kDa. TNT reactions were performed as described in reference 6. Where

multiple polymerases were used, 1µl of each polymerase was added to each reaction. Two equal aliquots (4µl) were taken from each reaction. One aliquot was analyzed by SDS-PAGE (10% gel) and detected by autoradiography for 8 hours at -70°C as shown in [Figure 1](#). The other aliquot was used for the immunoprecipitations shown in [Figure 2](#). **Panel A:** Demonstration of the relative expression levels of p98 and c-Rel that were used for subsequent immunoprecipitation ([Figure 2](#), lane 3). **Panel B:** Illustration of expression of multiple genes from different promoters in the same TNT reaction.

Protein-protein association assay performed in TNT Wheat Germ Extract

We employed immunoprecipitation to demonstrate that the p98 and c-Rel proteins are capable of interacting *in vitro*. In the experiment shown in [Figure 2](#), a c-Rel-specific antibody was used to immunoprecipitate c-Rel from TNT reactions expressing either p98, c-Rel or both of these proteins. p98 was co-precipitated when expressed in the presence of c-Rel (lanes 1-3), but not when expressed alone (lane 5).

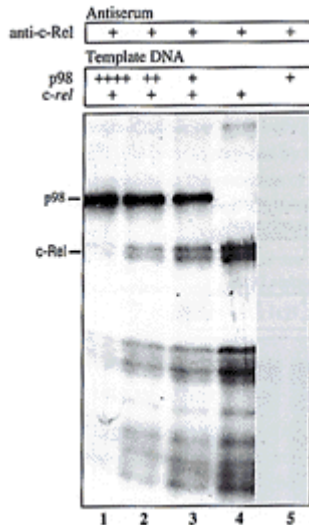


Figure 2. Protein-protein binding studies using proteins co-expressed in the TNT Wheat Germ System. ³⁵S-labeled c-Rel and p98 were produced in 25µl TNT Wheat Germ Extract reactions containing 1.5µg of *c-rel* template DNA (in lanes 1-4) and 1.0µg, 0.5µg, 0.25µg or 0.25µg of p98 template DNA (in lanes 1, 2, 3 or 5, respectively). A 4µl aliquot from each reaction was analyzed by immunoprecipitation with anti-c-Rel antiserum as described in reference 6 and then analyzed on a 10% SDS-polyacrylamide gel. For the gel shown in this figure, lanes 1-3 were loaded so as to contain equivalent amounts of p98. The immunoprecipitation results were detected by autoradiography for 16 hours at -70°C.

The consistent expression levels obtained with the TNT Systems were a distinct advantage for these studies, allowing co-expression of multiple proteins predictably in the specific ratios for binding studies. The three TNT reactions shown in lanes 1-3 of [Figure 2](#) contained different relative amounts of the p98 and *c-rel* DNA templates. The gel loading was adjusted so each lane contained an equivalent amount of p98.

Based upon our previous work (4,6), we expected to observe that as the ratio of p98 to c-Rel was

increased, more c-Rel would be associated with p98, and thus less c-Rel would be required to co-precipitate a given amount of p98. This was, in fact, the result we obtained. Note that where p98 DNA was expressed in great excess over *c-rel* DNA, very little c-Rel was visible in the immunoprecipitation (lane 1). Since the anti-c-Rel antibody exhibits no affinity for p98 alone (lane 5), the presence of p98 in lane 1 was due entirely to its interaction with c-Rel.

Gel shift assay performed using the TNT System

Recently, we demonstrated that p98 sequesters c-Rel in the cytoplasm, thereby acting as an I-kappa-B (6). The functional significance of this interaction *in vitro* was examined by gel shift assays to determine the effect of p98 binding on the capacity of c-Rel to bind DNA. [Figure 3](#) demonstrates that these gel shift assays could be performed using single or multiple proteins expressed in a TNT reaction and added directly to the gel shift assay.

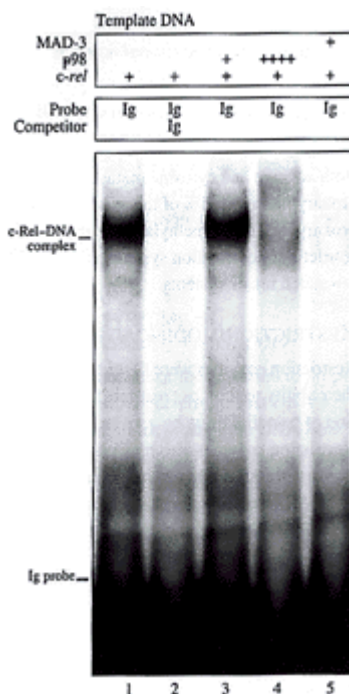


Figure 3. Gel shift assays using proteins produced in the TNT Wheat Germ System. c-Rel, p98 and MAD-3 proteins were produced in unlabeled 25 μ l TNT Wheat Germ Extract reactions containing 1.0 μ g of *c-rel* template DNA (lanes 1-5), 0.25 μ g or 1.0 μ g of p98 template DNA (lanes 3 and 4, respectively), and 1.0 μ g of MAD-3 template DNA (lane 5). The relative expression levels of each protein were estimated using a parallel set of TNT reactions containing [³⁵S]methionine. Gel shift assays were performed as described in reference 4. Based on the data from the ³⁵S-labeled reactions, the volume of unlabeled TNT reaction products added to each gel shift reaction was adjusted to contain a constant amount of c-Rel (from 2-8 μ l). Each reaction also contained 40pg of ³²P-labeled Ig enhancer probe DNA (8). In lane 2, the unlabeled competitor Ig probe was added in 50-fold excess (2ng). The gel shift reactions were analyzed by electrophoresis on a 5% polyacrylamide gel and detected by autoradiography for 24 hours at -70°C.

c-Rel bound specifically to the immunoglobulin kappa light chain enhancer probe (lanes 1 and 2) (8). As

expected, this binding could be inhibited by adding *in vitro*-translated MAD-3 (I-kappa-B-alpha, a 37kDa member of the I-kappa-B family) (9) to the c-Rel binding reaction (lane 5).

When c-Rel was co-translated with a small amount of p98, very little inhibition of c-Rel binding to DNA was observed (lane 3). However, when a 4-fold higher ratio of p98:c-*rel* DNA was used in the TNT System co-expression and the binding reaction was then performed using an amount of c-Rel equivalent to that in lane 3, no c-Rel-DNA complex was observed (lane 4). Thus, p98 acts as an I-kappa-B *in vitro* by preventing c-Rel from binding DNA. This *in vitro* interaction is similar to the ability of p98 to retain c-Rel in the cytoplasm *in vivo* (6).

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Editor's note: The p55 NF-KB subunit described in this article is functionally analogous to the p49 NF-KB subunit supplied by Promega. The p50 subunit mentioned in this article also is available from Promega.

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