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Biochemistry

Escherichia coli thioredoxin: A subunit of bacteriophage T7 DNA polymerase*

(DNA synthesis/TsnC protein/*tsnC* mutant)

DAVID F. MARK AND CHARLES C. RICHARDSON

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **T7 DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) is composed of an 84,000 dalton protein specified by the gene *5* of the phage and a 12,000 dalton protein (TsnC protein) specified by the *tsnC* gene of *E. coli* [Modrich, P. & Richardson, C. C. (1975) *J. Biol. Chem.* 250, 5515–5522]. Both proteins are necessary for T7 DNA polymerase activity and for the replication of T7 DNA. The TsnC protein is identical to thioredoxin of *E. coli* by the following criteria: (*1*) Homogeneous preparations of both proteins have TsnC and thioredoxin activity. (*2*) Both proteins show similar stability to heat. (*3*) They have identical mobilities, corresponding to a molecular weight of 12,000, on polyacrylamide gels containing sodium dodecyl sulfate. (*4*) Their amino-acid compositions are indistinguishable. (*5*) Antibody prepared against thioredoxin inhibits TsnC activity. (*6*) TsnC protein isolated from purified T7 DNA polymerase has thioredoxin activity. In addition, preparations of T7 DNA polymerase itself exhibit thioredoxin activity and are partially inhibited by antibody to thioredoxin.**

The synthesis of bacteriophage T7 DNA *in vivo* requires the products of the viral genes *1*, *2*, *3*, *4*, *5*, and *6* (2–4). In addition, T7 DNA replication is dependent on at least two components provided by the host. Chamberlin (5) has isolated two classes of *Escherichia coli* mutants, *tsnB* and *tsnC*, which are unable to support the growth of the phage. Both mutants are killed by T7 phage infection. Although all of the phage genes are expressed as judged by gel electrophoretic analysis of radioactively labeled proteins synthesized after infection, T7 DNA replication terminates prematurely in the infected *tsnB* hosts, and there is no detectable DNA synthesis in the infected *tsnC* hosts (5). The functions of the *tsnB* and *tsnC* gene products were unknown, however, since *E. coli* mutants defective in either gene were able to grow normally.

Recently we reported that extracts of the infected *tsnC* mutants are deficient in T7 DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) activity (6), an enzyme previously thought to be the product of a single gene, gene 5 of the phage (7). Extracts prepared from uninfected *E. coli* $tsnC^+$ cells restored normal levels of the phage DNA polymerase to the infected cell extracts and provided a source for the purification to homogeneity of TsnC protein responsible for this complementation. The TsnC protein has a molecular weight of 12,000 and is unusually heat stable. The T7 DNA polymerase itself is composed of two subunits, one specified by the *tsnC* gene of *E. coli*, and the other specified by gene 5 of the phage (8). The *E. coli* TsnC protein functions by combining with the T7 gene 5 protein to become a subunit of the phage-induced polymerase.

In this paper we show that the TsnC protein is, in fact, thioredoxin (9), an *E. coli* protein involved in the synthesis of deoxyribonucleotides (10). As shown in Fig. 1, thioredoxin can provide the reducing power in the form of two cysteine residues for the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates in a reaction catalyzed by ribonucleoside diphosphate reductase. In the process the two cysteine residues of thioredoxin are oxidized to cystine, and the reduced form of thioredoxin is regenerated by the action of thioredoxin reductase at the expense of one molecule of TPNH. The identity of TsnC protein as thioredoxin provides an additional role for this protein in DNA metabolism since, as a host-specified subunit of phage T7 DNA polymerase, it is essential for the replication of T7 DNA. In addition, our data raise questions concerning the direct participation of components of nucleotide precursor pathways in the process of DNA replication. Furthermore, an absolute requirement for thioredoxin in nucleotide reduction must be questioned in view of the existence of nonlethal mutations affecting this protein. A preliminary report of this work has been presented (11).

MATERIALS AND METHODS

Enzymes and Proteins. *E. coli* TsnC protein was the Sephadex G-75 fraction (Fraction VI) of Modrich and Richardson (6). *E. coli* thioredoxin and thioredoxin reductase were gifts from Dr. Neal Brown. T7 DNA polymerase was the DNA-cellulose pool (Fraction VI) of Modrich and Richardson (8), while T4 DNA polymerase was purified according to Goulian *et al.* (12). Gene 5 protein was the phosphocellulose passthrough fraction previously described (6). TsnC protein, thioredoxin, and T4 and T7 DNA polymerases were judged to be greater than 90% pure by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (13). Antiserum prepared against homogeneous *E. coli* thioredoxin (14) was a gift from Dr. Arne Holmgren.

Materials. Salmon sperm DNA (Sigma) was denatured according to Grippo and Richardson (7). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and TPNH were obtained from P. L. Biochemicals. Unlabeled nucleotides were from Schwarz Bioresearch, and [^{3}H]dTTP was from New England Nuclear. Ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine were from Canalco. Sodium dodecyl sulfate was from Pierce, and acrylamide was from Bio-Rad. DEAE-cellulose (DE52) from Whatman was prepared according to the recommendations of the manufacturer.

Enzyme Assays. *E. coli* TsnC activity was determined by Method B of Modrich and Richardson (6), which measures

Abbreviations: DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); thioredoxin-$(SH)_2$, reduced form of thioredoxin; thioredoxin-$(S)_2$, oxidized form of thioredoxin.

* This is Paper VII in a series entitled "Bacteriophage T7 Deoxyribonucleic Acid Replication *In Vitro*." The previous paper is ref. 1.

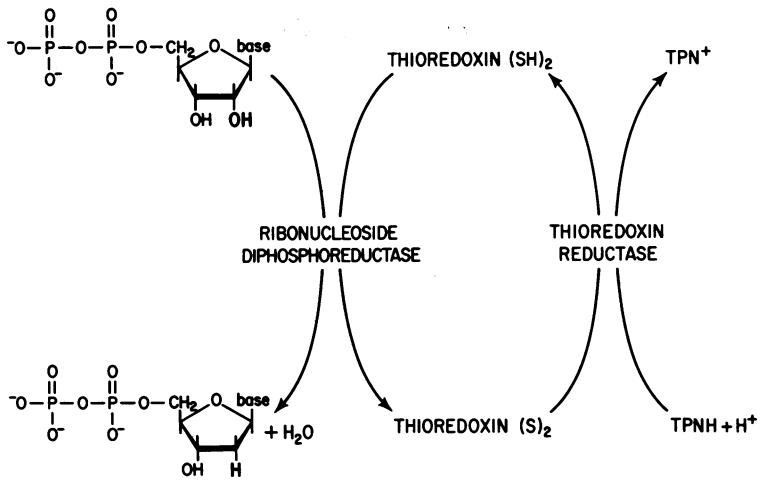

FIG. 1. Scheme for the reduction of ribonucleotides to deoxyribonucleotides.

the ability of TsnC protein to restore T7 DNA polymerase activity to the T7 gene 5 protein partially purified from T7-infected *E. coli* 7004 *tsnC*. One unit of TsnC activity is defined as the amount resulting in the incorporation of 10 nmol of total nucleotide into acid-insoluble form under the conditions of the assay.

T7 and T4 DNA polymerase activities were determined as previously described (7, 12) except that heat-denatured salmon sperm DNA was used in both assays.

Thioredoxin activity was determined by Method 2 of Laurent *et al.* (9). This assay measures the reaction between DTNB and the thiol groups in thioredoxin-$(SH)_2$ to form a yellow product and thioredoxin-$(S)_2$. Thioredoxin-$(SH)_2$ is regenerated by thioredoxin reductase and TPNH. Thus the rate of color formation at 412 nm measures the amount of thioredoxin present in the reaction mixture. A Gilford-Beckman recording spectrophotometer was used for these rate measurements. One unit of thioredoxin activity is defined as the amount resulting in a change in absorbance at 412 nm of 1.0 per minute under the conditions of the assay. The enzymes were diluted, when necessary, with 0.05 M Tris·HCl buffer (pH 7.6), 1 mg/ml of bovine serum albumin, and 0.05 mM dithiothreitol.

Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed according to the method of Weber and Osborn (13) except that the samples were denatured and reduced at 70° for 20 min. The gels were stained for protein with Coomassie brilliant blue (15).

RESULTS

In considering the role of the TsnC protein in the host, the low molecular weight and extreme heat stability of the protein suggested a relationship to other known *E. coli* proteins with similar properties. Among these the *E. coli* DNA binding protein (16) and the host factor for the QB replicase (17) failed to show TsnC activity (8). However, by a number of criteria the 12,000 dalton heat-stable thioredoxin of *E. coli* is identical to TsnC protein. We are grateful to Dr. Neal Brown for pointing out similarities between the two proteins and for providing us with homogeneous thioredoxin.

Electrophoretic analysis

The molecular weight of 12,000, previously determined for the denatured and reduced TsnC protein (6), is the same as that of *E. coli* thioredoxin (9). As shown in Fig. 2, the denatured and reduced TsnC protein and thioredoxin do in fact have the same electrophoretic mobility when analyzed under identical conditions in 5% polyacrylamide gels containing sodium dodecyl sulfate.

TsnC and thioredoxin activities

The nearly homogeneous preparations of the TsnC protein and of thioredoxin have TsnC and thioredoxin activities of equal magnitude (Table 1). Thus both proteins are equally active in combining with the T7 gene 5 protein to form an active polymerase. Similarly, both proteins are recognized and reduced by *E. coli* thioredoxin reductase.

Amino-acid composition

The amino-acid compositions of TsnC protein and of thioredoxin are indistinquishable (Table 2), each protein having

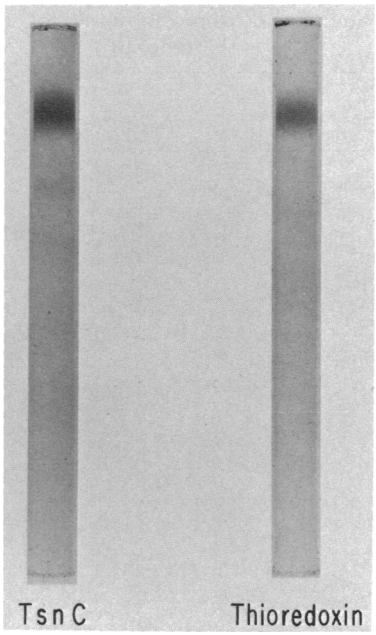

FIG. 2. Polyacrylamide gel electrophoresis of TsnC protein and thioredoxin. Purified samples of TsnC protein (12 μg) and thioredoxin (15 μg) were reduced and denatured, applied to 5% polyacrylamide gels containing sodium dodecyl sulfate and subjected to electrophoresis, and the gels were stained as described in *Materials and Methods*. The major band in the gel containing TsnC protein, representing 90% of the Coomassie-positive material, has been shown previously to be the TsnC protein.

Table 1. Activities of TsnC protein and thioredoxin

Protein	TsnC activity, units/mg	Thioredoxin activity, units/mg
TsnC	9857	9.6
Thioredoxin	8315	13.9

Units and assay procedures for both TsnC protein and thioredoxin are described under *Materials and Methods*. Thioredoxin activity was measured after dialysis against 0.02 M Tris·HCl (pH 7.6), 0.25 mM EDTA, to remove 2-mercaptoethanol or dithiothreitol which were present in the TsnC protein or thioredoxin preparations.

one residue of methionine, arginine, and histidine, and the two residues of half-cystine characteristic of thioredoxin. The molecular weights of the two proteins, as calculated from their amino-acid compositions, are 11,273 for the TsnC protein and 11,360 for thioredoxin. Both the amino-acid analysis and the molecular weight estimates for these two proteins coincide closely with the previously published results (8, 18).

Inhibition of TsnC activity by antibody to thioredoxin

Antibody prepared against homogeneous preparations of thioredoxin (14) inhibits the ability of preparations of both TsnC protein and thioredoxin to form an active T7 DNA polymerase in the presence of gene 5 protein (Table 3). The amount of antibody used in the experiment shown in Table 3 was sufficient to inhibit TsnC activity by greater than 95%. In another experiment, however, an amount sufficient to inhibit TsnC activity of thioredoxin by 60% inhibited TsnC protein only 34%. The inhibition observed in these studies does not arise from destruction or inhibition of some other component of the DNA polymerase reaction, since T4 DNA polymerase is fully active in the presence of the antibody (Table 3).

Table 2. Amino-acid composition of TsnC protein and thioredoxin

Amino acid	TsnC	Thioredoxin
Lysine*	10	10
Histidine*	1	1
Arginine*	1	1
Tryptophan*	2	2
Aspartic acid	15	15
Threonine	6	6
Serine	3	4
Glutamic acid	8	8
Proline	5	5
Glycine	9	9
Alanine	11	11
Half cystine†	2	2
Valine	5	5
Methionine†	1	1
Isoleucine	7	7
Leucine	12	12
Tyrosine	2	2
Phenylalanine	4	4
Total	104	105
Molecular weight	11,273	11,360

* Protein samples were hydrolyzed in methane sulfonic acid containing 0.2% tryptamine.

† Performic acid oxidation of the samples before hydrolysis in 5.7 M HCl.

Table 3. Inhibition of DNA polymerase activity by antibody to thioredoxin

Additions	DNA polymerase activity, nmol [^{3}H]dTTP incorporated: − antibody	+ antibody
Gene 5 protein		
+ Thioredoxin	0.43	0.005
+ TsnC protein	0.41	0.010
+ Boiled T7 DNA polymerase	0.10	<0.01
T7 DNA polymerase	0.45	0.23
T4 DNA polymerase	0.40	0.40

The ability of TsnC protein or thioredoxin to restore T7 DNA polymerase activity to a partially purified preparation of gene 5 protein (TsnC activity) was measured as described under *Materials and Methods*. Purified thioredoxin (0.25 μg) and TsnC protein (0.25 μg) were incubated either in the presence or absence of 50 μg of partially purified serum protein containing antibody to thioredoxin in a total volume of 0.10 ml containing 0.02 M Tris·HCl (pH 7.6), 0.10 M NaCl, 0.25 mM EDTA for 5 min at 0°. Then 0.02 ml of the antibody-thioredoxin or antibody-TsnC protein mixture were added to the standard reaction mixture for measuring polymerase activity. TsnC activity of boiled T7 DNA polymerase in the presence or absence of antibody was determined in an identical manner, except that 0.5 μg of boiled DNA polymerase (6) and 20 μg of antibody protein were incubated together. T7 and T4 DNA polymerase activities in the presence or absence of antibody were determined in an identical manner except that 0.20 μg of purified T7 or T4 DNA polymerase and 20 μg of antibody protein were incubated together, and 0.01 ml of the polymerase-antibody mixture was added to the standard reaction mixture for measuring polymerase activity.

Association of thioredoxin with purified T7 DNA polymerase

In a previous report (8) it was shown that TsnC protein is present in purified T7 DNA polymerase in one-to-one stoichiometry with T7 gene 5 protein. Thus, when purified T7 DNA polymerase was chromatographed on DEAE-cellulose, TsnC activity, measured after heating column fractions to 100°, eluted together with the polymerase activity at 0.21 M NaCl. However, if the polymerase was heated to 100° prior to chromatography, the TsnC activity eluted at 0.1 M NaCl, the salt concentration at which TsnC protein from uninfected *E. coli* elutes from DEAE-cellulose (8). Similarly, as shown in Fig. 3, when the T7 DNA polymerase is heated to 100° prior to chromatography on DEAE-cellulose, both TsnC and thioredoxin activities elute together at 0.1 M NaCl. Thus, thioredoxin is present in T7 DNA polymerase.

The presence of thioredoxin in purified T7 DNA polymerase can be demonstrated directly without prior heat denaturation of the enzyme. As shown in Table 4, T7 DNA polymerase has intrinsic thioredoxin activity in the DTNB assay. A similar amount of gene 5 protein, devoid of polymerase activity, has no detectable thioredoxin activity. The specific thioredoxin activity is 0.78 unit/mg of DNA polymerase. Since the molecular weight of thioredoxin is 12,000, it represents only 13% of the molecular weight of the 96,000 dalton polymerase. The specific activity of the thioredoxin subunit is therefore 6.2 units/mg, in reasonable agreement

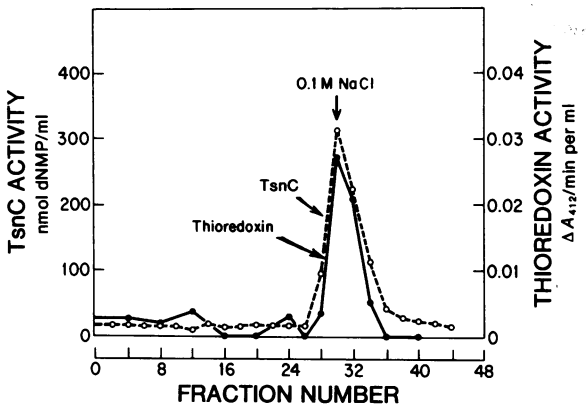

FIG. 3. Association of thioredoxin with purified T7 DNA polymerase. T7 DNA polymerase (0.4 mg) in 0.5 ml of 0.02 M potassium phosphate (pH 7.4), 0.5 mM dithiothreitol, 0.2 mM EDTA, 50% (vol/vol) glycerol was heated at 100° for 2 min, chilled on ice, and then centrifuged at 15,000 × *g* for 15 min. The supernatant fluid was then adsorbed to a 2.5 ml DEAE-cellulose column equilibrated with 0.02 M Tris·HCl (pH 7.6), 0.25 mM EDTA. After washing with 2.5 ml of the same buffer, the protein was eluted with a 20 ml linear gradient of NaCl (0.0–0.3 M) in the same buffer. Fractions of 0.5 ml were collected. TsnC and thioredoxin activities were determined as described under *Materials and Methods*.

with the values found for purified TsnC protein and thioredoxin (Table 1).

The polymerizing activity of T7 DNA polymerase is also inhibited by antibody prepared against thioredoxin (Table 3). The addition of antibody to the standard polymerase assay, or incubation of antibody with polymerase prior to addition to the reaction mixture, gave a maximal inhibition of approximately 50%. Addition of a 10-fold excess of antibody did not increase this inhibition. By comparison, the T4 DNA polymerase is not inhibited under these conditions (Table 3). Examination of the kinetics of the polymerization revealed that the effect of the antibody was to decrease the rate of the polymerization at all times during a 30-min incubation (data not shown). In contrast to the 50% inhibition observed with purified T7 DNA polymerase, a preparation of T7 DNA polymerase reconstituted from purified gene 5 protein and thioredoxin could be completely inhibited (greater than 95%) by antibody.

DISCUSSION

Bacteriophage T7 DNA polymerase is composed of two subunits, the 84,000 dalton gene 5 protein specified by the phage, and the 12,000 dalton TsnC protein provided by the host (6, 8). The evidence presented in this communication identifies the TsnC protein as *E. coli* thioredoxin. The specificity of the thioredoxin–gene 5 association is illustrated by the inability of the thioredoxin coded by phage T4 (20, 21) to interact with gene 5 protein to form an active T7 DNA polymerase (unpublished results). This result is not surprising, however, in view of the immunological and structural differences in the two thioredoxins (14, 22).

The efficiency of forming an active DNA polymerase from T7 gene 5 protein and thioredoxin *in vitro* (6), combined with the detailed structural data on thioredoxin (19), makes this an ideal system for studying functional protein–protein interactions. Thioredoxin forms dimeric molecules in the crystalline state (19) and it has been postulated that these interactions might be of physiological significance, perhaps constituting the sites involved in subunit interaction

Table 4. Thioredoxin activities of T7 DNA polymerase and T7 gene 5 protein

	Amount added, units	Thioredoxin activity, units × 10^3
T7 DNA polymerase	12	6.2
Gene 5 protein	9	<0.1

T7 DNA polymerase and gene 5 protein were assayed for thioredoxin activities in the DTNB assay as described under *Materials and Methods*. The amount of thioredoxin activity is expressed as the total amount of activity found in the sample assayed. The amounts of DNA polymerase and gene 5 protein assayed for thioredoxin activity are given as units of each activity as measured in their respective assays. Both proteins were diluted into 0.05 M Tris·HCl (pH 7.6), 1 mg/ml of bovine serum albumin, 0.5 mM dithiothreitol. The protein preparations were dialyzed to remove reducing agents prior to thioredoxin assays as described in Table 1.

with the gene 5 protein. Our finding that T7 DNA polymerase itself has reducing activity suggests that the active reducing site of the molecule is not involved in subunit interaction. Unfortunately the partial inhibition of T7 DNA polymerase by antibody prepared against thioredoxin does not provide information on the interaction of the two subunits. The partial inhibition could arise from conformational changes in the thioredoxin subunit resulting in alteration in the "native format antigenic determinants" (23) of the molecule. Alternatively, there could be two classes of polymerase molecules, one having a conformation rendering the antigenic site on thioredoxin more readily available to antibody and resulting in an inactive polymerase–antibody complex. In this regard it is interesting that 35% of T7 DNA polymerase reconstituted from thioredoxin and gene 5 protein *in vitro* has chromatographic properties distinct from T7 DNA polymerase synthesized *in vivo* (8). Hopefully, the ability to obtain defined peptide fragments of thioredoxin, which can be reconstituted to form active molecules (23), will provide more precise information on the specific sites involved in the subunit interaction.

The presence of thioredoxin as a subunit in T7 DNA polymerase, an enzyme essential for the replication of T7 DNA (7), constitutes an example of a direct role of a component involved in nucleotide precursor synthesis in DNA replication. Recently evidence has been presented that, in T4-infected cells and in regenerating liver, a number of enzymes, including those important in nucleotide metabolism, may function as a complex together with enzymes of DNA replication (24, 25). Thus thioredoxin, possessing a number of potential sites for interaction with other proteins (19) could serve as a key component in maintaining such a hypothetical complex.

The possibility must also be considered that T7 DNA polymerase *in vivo* has a ribonucleotide reducing activity in addition to its polymerase activity. The reduction of nucleotides at the polymerization site could enhance the efficiency of reduction and polymerization, or it could function as a control mechanism. A more remote possibility is that T7 DNA polymerase could play a role in a hypothetical *in situ* reduction of ribonucleotide primers at the moment of initiation of DNA replication.

The thioredoxin-deficient mutant *E. coli* 7004 *tsnC* can grow normally, and the only phenotypical difference be-

tween it and its intact parent is the failure of the mutant strain to support the growth of phages ϕ2, T3, and T7 (5). In fact, the *E. coli* 7004 mutant has no detectable thioredoxin activity in assays using thioredoxin reductase or ribonucleotide reductase, and immunoassay for thioredoxin in the mutant cells reveals less than 1% of the levels present in wild-type *E. coli* (Arne Holmgren, personal communication). The immunoassay could of course reflect a change only in the antigenic sites of the thioredoxin, leaving the residual protein function intact. It is possible that the normal cell requires only minute amounts of thioredoxin in order to synthesize nucleotide precursors since, as indicated in Fig. 1, thioredoxin is constantly recycled. However, if such a small amount of thioredoxin were sufficient *in vivo*, it would be difficult to explain why 3000 to 5000 molecules are normally present per cell (6). Perhaps there is another as yet undiscovered *in vivo* function for thioredoxin which would justify the synthesis of so many molecules. The possibility must be considered that there are cofactors other than thioredoxin, either known in other capacities or as yet undetected, which can supply reducing power to ribonucleotide reductase.

We are grateful to Dr. Neal Brown for helpful discussions and for the generous gift of thioredoxin and thioredoxin reductase. For providing us with information of his own results prior to this publication and for a gift of antibody to thioredoxin we thank Dr. Arne Holmgren. We are grateful to Dr. Chun-Yen Lai of the Roche Institute of Molecular Biology for performing the amino-acid analyses on thioredoxin and TsnC protein. This investigation was supported by U.S. Public Health Service Grant AI-06045, Grant no. NP-1E from the American Cancer Society, Incorporated, and National Institutes of Health Research Career Development Award GM13, 534 to C.C.R.

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