Region 3.2 of the σ Subunit Contributes to the Binding of the 3’-Initiating Nucleotide in the RNA Polymerase Active Center and Facilitates Promoter Clearance during Initiation*

Received for publication, March 13, 2006, and in revised form, May 3, 2006
Published, JBC Papers in Press, May 10, 2006, DOI 10.1074/jbc.C600060200
Andrey Kulbachinskiy1 and Arkady Mustaev†

From the †Institute of Molecular Genetics, Russian Academy of Sciences, Moscow 123182, Russia, the †Waksman Institute, Rutgers University, Piscataway, New Jersey 08854, and the †Public Health Research Institute, Newark, New Jersey 07103

Region 3.2 of the RNA polymerase σ subunit forms a loop that protrudes toward RNA polymerase active center and partially blocks RNA exit channel. To provide some insights into the functional role of this region, we studied a deletion variant of the Escherichia coli σ subunit that lacked amino acids 513–519 corresponding to the tip of the loop. The deletion had multiple effects on transcription initiation including: (i) a significant decrease in the amount of short abortive RNAs synthesized during initiation, (ii) defects in promoter escape, (iii) loss of the contacts between the σ subunit and the nascent RNA during initiation and, finally, (iv) dramatic increase in the Km value for the 3’-initiating nucleotide. At the same time, the mutation did not impair promoter opening and the binding of the 5’-initiating purine nucleotide. In summary, our data demonstrate an important role of σ region 3.2 in the binding of initiating substrates in RNA polymerase active center and in the process of promoter clearance.

The σ subunit of bacterial RNA polymerase (RNAP) plays the key role in promoter-specific initiation of RNA synthesis. The σ subunit is involved in multiple processes during initiation including initial promoter recognition, DNA melting, abortive synthesis, and promoter clearance (1). Recent progress in structural studies of RNAP together with the bulk of biochemical data allowed the creation of an integral picture of transcription initiation and a proposal of functional roles for individual domains of the σ subunit. In RNAP holoenzyme, the σ subunit occupies the upstream part of the main RNAP channel. Two DNA binding domains of σ, involved in the recognition of the −10 and −35 promoter elements, are formed by σ conserved regions 2 and 4 and interact with a coiled-coil element of the β subunit and the flap domain of the β subunit, respectively (2–5). The promoter recognition domains of σ are connected by a flexible linker formed by conserved region 3.2 (amino acids 498–526 in Escherichia coli numbering). A hairpin-like loop from region 3.2 protrudes toward the active center of RNAP and occupies a part of the RNA exit channel (Fig. 1). This led to several predictions about the functional role of this region. First, the region 3.2 loop was proposed to directly participate in the binding of the 5’-initiating nucleotide at the i-site but instead contributes to the binding of the 3’-substrate at the i+1 site of RNAP active center.

EXPERIMENTAL PROCEDURES

Protein Purification—E. coli core RNAP bearing a hexahistidine tag at the C terminus of the β subunit was purified as described (13, 14). The mutant rpoD gene encoding the Δ513–519 σ subunit was obtained by standard PCR mutagenesis methods. Both wild type and mutant rpoD genes were cloned between the NdeI and EcoRI sites of the pET28 vector and overexpressed in the E. coli BL21(DE3) strain. The resulting proteins contained a six-histidine tag at the N terminus. Bacterial pellet from 1 liter of cell culture was resuspended in 25 ml of lysis buffer (20 mM Tris-HCl, pH 7.9, 500 mM KCl, 0.1% Tween-20, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5% glycerol) and sonicated. Supernatant obtained after centrifugation was loaded onto a 5-ml chelating HiTrap column (GE Healthcare) charged with Ni2+ and equilibrated with the same buffer. The column was washed with buffer containing 35 mM imidazole, the σ subunit was eluted with 200 mM imidazole and precipitated with ammonium sulfate. The pellet was dissolved in 15 ml of buffer containing 40 mM Tris-HCl, pH 8.3, 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, and DEAE ion-exchange chromatography was performed as described (13).

Transcription in Vitro—DNA fragments bearing the sequences of either wild type or consensus T7A1 promoters (from −85 to +53 nucleotides relative to the starting point of transcription) were obtained by PCR from synthetic oligonucleotide templates. Holoenzyme RNAP was prepared by incubating the core polymerase (100 nm) and either the wild type or the mutant σ subunit (500 nm) in transcription buffer (40 mM Tris-HCl, pH 7.9, 40 mM KCl, 10 mM MgCl2) for 5 min at 37 °C. The DNA template was added (30 nm), and the samples were incubated for 5 min at 37 °C. Nucleotide substrates were added (25 μM concentration of each NTP with addition of [α-32P]GTP, 3000 Ci/mmol, PerkinElmer Life Sciences) and transcription was proceeded for 5 min. The CpA primer was present at 50 μM, when indicated. RNA products were separated on 20% denaturing polyacrylamide gel and analyzed by phosphorimaging. Apparent Km values for the initiating substrates were measured on the wild type T7A1 promoter; reactions contained 100 nm core RNAP, 500 nm σ subunit, and 10 fmol DNA in 10 μl of the transcription buffer. One of the two initiating nucleotides (ATP or UTP) was taken at 1 mM (with the addition of the corresponding α-32P-labeled nucleotide), while the concentration of the other was varied from 1 μM to 6 mM. The samples were incubated for 1 min at 37 °C, and the reaction was stopped with 10 μl of solution containing 8 mM urea and 20 mM EDTA. Dinucleotide product was separated from mononucleotides on 30% polyacrylamide gel and quantified with PhosphorImager (Amersham Biosciences). The data were fit to the hyperbolic equation \[ V = V_{max}[NTP]/(K_m + [NTP]) \] (where V is the observed rate of the reaction, and V max is the maximum analogue in a segment between amino acids 508 and 561 containing regions 3.2 and 4.1. (8). However, no experiments were done demonstrating the functional role for this region in substrate binding. Second, region 3.2 was proposed to play an important role in the process of promoter escape (3, 6, 7, 9). As this region blocks the path for RNA exit (Fig. 1), the elongating RNA transcript must either displace it from the RNA exit channel thus promoting σ dissociation or dissociate itself from the complex and be released as an abortive product. Thus, direct competition between the elongating RNA transcript and region 3.2 may be one of the major causes for abortive initiation. In support of this, deletion of the C-terminal part of σ including region 3.2 was shown to lead to a significant decrease in the amount of abortive products synthesized during initiation from an extended –10 promoter (6). However, the deletion also removed σ conserved region 4 that led to multiple defects during initiation and complicated interpretation of the experimental data. The role of region 3.2 in promoter escape has not been directly studied. Intriguingly, a finger-like domain of the eukaryotic general transcription factor TFII-B was found to occupy a similar position at the RNA exit channel of RNAP II and was proposed to clash with the growing RNA during initiation (10). This indicates that prokaryotic and eukaryotic RNAPs may share a common mechanism of abortive synthesis and promoter escape (11, 12).

In this work, we show that, in agreement with structural predictions, displacement of region 3.2 by nascent RNA is required for efficient promoter escape. At the same time, we demonstrate that σ region 3.2 is not involved in the binding of the 5’-initiating nucleotide at the i-site but instead contributes to the binding of the 3’-substrate at the i+1 site of RNAP active center.

1 The abbreviations used are: RNAP, RNA polymerase; nt, nucleotide(s).
[α-32P]GTP that allowed to label RNA products longer than 4 nucleotides (see the initial transcribed sequence of T7A1 on Fig. 2A). RNAP containing the wild type σ57 subunit was active on both promoters independently of the presence of the primer dinucleotide (Fig. 2A, lanes 1–4). In contrast, RNAP containing the mutant σ subunit was essentially inactive in the absence of the initiating primer (Fig. 2A, lanes 5 and 6, and Fig. 2C). KMnO4 footprinting demonstrated that the efficiency of promoter melting and the size of transcription bubble in the open promoter complex were the same for wild type and mutant RNAPs (Fig. 2B). Thus, inability of the mutant polymerase to initiate transcription could not be explained by abnormalities in promoter melting. The activity of the mutant RNAP was greatly stimulated in the presence of the initiating dinucleotide CPA (Fig. 2A, lanes 7 and 8, and Fig. 2C). This suggested that the defect of the mutation may be in the binding of initiating substrates and the first phosphodiester bond formation.

To test this hypothesis, we determined apparent Km values for the first (ATP, bound at the i-site) and the second (UTP, bound at the (+1)-site) initiating NTPs for wild type and mutant RNAPs in a reaction of dinucleotide synthesis on the T7A1 promoter (Fig. 3). We found that the deletion of amino acids 513–519 from region 3.2 did not impair the binding of the 5′-initiating nucleotide (ATP). In fact, the mutant had even lower Km value for ATP than the wild type polymerase (450 ± 200 μM versus 920 ± 300 μM for wild type RNAP) (Fig. 3). This observation is consistent with the hypothesis that the RNA polymerase initiation site, specific for purine nucleotides, is present in the core RNAP and neither the DNA template nor the σ subunit is required for its formation (16). At the same time, the mutation had dramatic effect on Kd for the second substrate (UTP) which was increased by about two orders of magnitude (250 ± 70 μM versus 3.3 ± 0.6 μM for wild type RNAP) (Fig. 3). From this, we conclude that inability of the mutant RNAP to start RNA synthesis from mononucleotides can be attributed to the defect in the binding of 3′-initiating NTP in the RNAP active center.

To test whether region 3.2 could be involved in direct contacts with initiating nucleotides, we performed a cross-linking experiment using a γ-phosphate-modified initiating ATP analogue (Fig. 4A) (8). The alkylating group of the reagent has a broad specificity and can react with any nucleophilic amino acid side chain (17). Prior to the cross-linking, the aldehyde group of the analogue was reduced with NaBH4 that resulted in a significant increase of the reactivity of the alkylating group (17). Previously, this reagent was shown to cross-link between amino acids 508 and 561 of the RNAP subunit (8) as well as to bind at the NADFDGD-motif of the active site (red) and the F-bridge helix of the β subunit (dark green).

rate at indefinite concentration of NTP) using GraFit software (Erithacus Software).

**KmO4 Footprinting**—The promoter DNA fragment used in the footprinting experiments contained a EcoRI site at position –81 relative to the starting point of transcription. DNA was treated with EcoRI and labeled at 5′-end of the template promoter strand with Klenow enzyme (Amersham Biosciences) and [α-32P]dATP. Holoenzymes containing either wild type or mutant σ subunits were incubated with the labeled promoter fragment (10 nm) in 10 μl of the transcription buffer for 10 min at 37 °C. 1 μl of 20 mM KMnO4 was added, and the reaction was stopped after 15 s by addition of 5 μl of stop-solution containing 1 μl β-mercaptoethanol and 1 μl sodium acetate, pH 4.8. DNA was processed as described (15) and analyzed on 10% denaturing polyacrylamide gel.

**Cross-linking Experiments**—Cross-linking of RNAP subunits with the initiating ATP analogue in the open promoter complex was performed as described (8). The structure of the ATP analogue is shown on Fig. 4A. Prior to cross-linking, the aldehyde group of the reagent was reduced with 10 mM NaBH4 for 10 min at 25 °C. Open complexes formed by either wild type or mutant RNAP on the wild type T7A1 promoter were immobilized on nickel-nitritotriacetic acid-agarose (Qiagen) through a hexahistidine tag present at amino acids 513–519, which correspond to the tip of the loop formed lacking amino acids 513–519, which correspond to the tip of the loop formed by this region (amino acid sequence . . . 512-GDDDSHLG-520 . . . , the deleted residues are bold underlined) (Fig. 1). The activity of RNAP containing the mutant σ subunit was studied in an in vitro transcription test. Two variants of a T7A1 promoter were used as templates. The first one was the wild type T7A1 promoter; the second (T7A1c) was based on the sequence of T7A1 but contained two nucleotide changes in the –10 element bringing it to the consensus (TATAAT instead of GACTACT in the wild type promoter). Transcription was performed either in the absence or in the presence of a dinucleotide primer (CPa) that was complementary to the –1 and +1 nucleotides of the template promoter strand. The reaction was supplemented with [α-32P]GTP that allowed to label RNA products longer than 4 nucleotides (see the initial transcribed sequence of T7A1 on Fig. 2A). RNAP containing the wild type σ57 subunit was active on both promoters independently of the presence of the primer dinucleotide (Fig. 2A, lanes 1–4). In contrast, RNAP containing the mutant σ subunit was essentially inactive in the absence of the initiating primer (Fig. 2A, lanes 5 and 6, and Fig. 2C). KMnO4 footprinting demonstrated that the efficiency of promoter melting and the size of transcription bubble in the open promoter complex were the same for wild type and mutant RNAPs (Fig. 2B). Thus, inability of the mutant polymerase to initiate transcription could not be explained by abnormalities in promoter melting. The activity of the mutant RNAP was greatly stimulated in the presence of the initiating dinucleotide CPA (Fig. 2A, lanes 7 and 8, and Fig. 2C). This suggested that the defect of the mutation may be in the binding of initiating substrates and the first phosphodiester bond formation.

To test this hypothesis, we determined apparent Km values for the first (ATP, bound at the i-site) and the second (UTP, bound at the (+1)-site) initiating NTPs for wild type and mutant RNAPs in a reaction of dinucleotide synthesis on the T7A1 promoter (Fig. 3). We found that the deletion of amino acids 513–519 from region 3.2 did not impair the binding of the 5′-initiating nucleotide (ATP). In fact, the mutant had even lower Km value for ATP than the wild type polymerase (450 ± 200 μM versus 920 ± 300 μM for wild type RNAP) (Fig. 3). This observation is consistent with the hypothesis that the RNA polymerase initiation site, specific for purine nucleotides, is present in the core RNAP and neither the DNA template nor the σ subunit is required for its formation (16). At the same time, the mutation had dramatic effect on Kd for the second substrate (UTP) which was increased by about two orders of magnitude (250 ± 70 μM versus 3.3 ± 0.6 μM for wild type RNAP) (Fig. 3). From this, we conclude that inability of the mutant RNAP to start RNA synthesis from mononucleotides can be attributed to the defect in the binding of 3′-initiating NTP in the RNAP active center.

To test whether region 3.2 could be involved in direct contacts with initiating nucleotides, we performed a cross-linking experiment using a γ-phosphate-modified initiating ATP analogue (Fig. 4A) (8). The alkylating group of the reagent has a broad specificity and can react with any nucleophilic amino acid side chain (17). Prior to the cross-linking, the aldehyde group of the analogue was reduced with NaBH4 that resulted in a significant increase of the reactivity of the alkylating group (17). Previously, this reagent was shown to cross-link between amino acids 508 and 561 of the RNAP subunit (8) as well as to bind at the NADFDGD-motif of the active site (red) and the F-bridge helix of the β subunit (dark green).

**RESULTS AND DISCUSSION**

**Role of σ Region 3.2 in NTP Binding**—To clarify the role of region 3.2 during transcription initiation, we generated a deletion variant of the σ57 subunit lacking amino acids 513–519, which correspond to the tip of the loop formed by this region (amino acid sequence . . . 512-GDDDSHLG-520 . . . , the deleted residues are bold underlined) (Fig. 1). The activity of RNAP containing the mutant σ subunit was studied in an in vitro transcription test. Two variants of a T7A1 promoter were used as templates. The first one was the wild type T7A1 promoter; the second (T7A1c) was based on the sequence of T7A1 but contained two nucleotide changes in the –10 element bringing it to the consensus (TATAAT instead of GACTACT in the wild type promoter). Transcription was performed either in the absence or in the presence of a dinucleotide primer (CPa) that was complementary to the –1 and +1 nucleotides of the template promoter strand. The reaction was supplemented with
Role of $\sigma$ Region 3.2 in Abortive Initiation and Promoter Escape—Transcription on the mutant T7A1cons promoter resulted in the synthesis of a large number of short RNA transcripts up to 16 nucleotides in length (Fig. 2A, lane 2). Control experiments demonstrated that these RNAs were not associated with transcribing RNAP and therefore represented real abortive products (data not shown). In this property, the T7A1cons promoter is similar to the semisynthetic T5 N25antiDSR promoter (20). However, in the case of T5 N25antiDSR the appearance of long abortive products was associated with unfavorable initially transcribed sequence, while in the case of T7A1cons it is most likely a result of strong interactions between RNAP and the core promoter region. T7A1cons was therefore used as a model promoter to compare the processes of abortive synthesis and promoter escape by the wild type and mutant RNAPs.

The reaction was performed in the presence of the CpA primer, i.e. at conditions when both RNAPs were active. The efficiency of the full-length RNA synthesis by wild type RNAP on T7A1cons was about 2-fold lower than on the wild type promoter indicative of the problems in promoter clearance (Fig. 2A, lanes 3 and 4, and Fig. 2C). The defect in promoter clearance became more pronounced in the case of mutant RNAP that synthesized four times less of the full-length transcript on the consensus promoter as compared with the wild type template (Fig. 2A, lanes 7 and 8, and Fig. 2C). Analysis of the abortive products synthesized on the T7A1cons promoter in the presence of CpA.

FIGURE 2. Activity of RNAP containing either the wild type (WT) or $\Delta$S13–519 $\sigma^{70}$ subunits. A, results of transcription on the wild type T7A1 (W) and T7A1cons (C) promoters. For each $\sigma$, transcription was performed either in the absence or in the presence of a CpA primer with all four NTPs present at 25 $\mu$M. The initial transcribed sequence of T7A1 (starting from the 5'-end of the primer) is shown below the figure. The length of RNA products is indicated on the right. 5–7-mer RNAs are not resolved on the gel and appear as a single band. RO, the full-length (runoff) RNA transcript. B, permanganate footprinting on the template strand of the wild type T7A1 promoter in open complexes formed by wild type (lane 3) and mutant RNAPs (lane 4). The sample on lane 2 contained no RNAP. Lane 1 represents an A$^\cdot$G cleavage sequence marker. The sequence of the promoter region between nucleotides −13 and +3 is shown below the gel, the −10 promoter element is boxed. The modified thymine nucleotides in the melted promoter region are indicated on the right of the gel and below the promoter sequence. C, the efficiency of RO synthesis by RNAPs containing the wild type and mutant $\sigma$s on T7A1 and T7A1cons promoters (quantitation of data from A). The activity for each reaction is shown in percent of the activity of the wild type RNAP on the wild type T7A1 in the absence of the primer. D, the efficiency of abortive synthesis on the T7A1cons promoter by RNAPs containing the wild type and mutant $\sigma^{70}$ subunits. For each RNA product, the ratio of the radioactivity in the corresponding band on the gel to the radioactivity in the RO band is shown.

FIGURE 3. The effect of the deletion in $\sigma$ region 3.2 on the binding of initiating NTPs. Shown are data from a representative experiment on $K_m$ measurements for the ATP (triangles) and UTP (circles) initiating nucleotides in the reaction of dinucleotide synthesis on the wild type T7A1 promoter. The rate of abortive synthesis is shown in picomoles of pppApU synthesized per minute per sample. The reaction was performed with either the wild type (open symbols) or mutant RNAP (closed symbols). The lines represent non-linear fits of the data (see “Experimental Procedures” for details).
revealed that the mutant RNAP produced much lower amounts of short 5–7-nt transcripts relative to the wild type polymerase (Fig. 2, A and D). At the same type, the ratio of longer abortive transcripts (8–16 nt) to the full-length RNA was severalfold higher for the mutant enzyme than for wild type RNAP (Fig. 2D). This indicated that although removal of \( \sigma \) region 3.2 stabilized short RNAs in the RNAP active center, it resulted in the increase of the amount of longer transcripts and led to a general defect in promoter escape. Our data are in agreement with the results of Hernandez et al. (21) who demonstrated that mutations in positions 504 and 506 of \( \sigma^{32} \) region 3 decreased the number of short abortive RNAs (4–8 nt) but stimulated synthesis of longer (9–10 nt) abortive transcripts. In general, these results suggest that direct competition between \( \sigma \) region 3.2 and growing RNA during initiation facilitates promoter clearance, probably by weakening the contacts of the \( \sigma \) subunit with core RNAP or/and the non-template DNA strand. This is consistent with the conclusions made from structural consideration (6, 7).

The increase in the amount of long abortive transcripts in the case of the mutant \( \sigma \) subunit is likely explained by steric clashing of growing RNA with the C-terminal part of the \( \sigma \) subunit further upstream of the RNAP active center (see Fig. 1). Remarkably, the longest abortive transcript that was synthesized in agreement with the results of Hernandez et al. (21) who studied initiation of primer RNA synthesis on the single stranded DNA promoter, region 3.2 of E. coli RNA polymerase (23). Although core RNAP possessed certain defects in the binding of the 3' initiating nucleotide and the first phosphodiester bond formation but is not absolutely required for the subsequent cycles of nucleotide addition. This demonstrates that RNAP can use different mechanisms for initiation of RNA synthesis on different kinds of templates.

Acknowledgments—We thank N. Zenkin, V. G. Nikiforov, and L. Minakhin for helpful discussions and I. Artsimovitch for reading the manuscript.

REFERENCES
1. Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. (1998) Cold Spring Harb. Symp. Quant. Biol. 63, 141–155
2. Campbell, E. A., Muzzin, O., Chlenov, M., Sun, J. L., Olson, C. A., Weinman, O., Trester-Zedlitz, M. L., and Darst, S. A. (2002) Mol. Cell. 9, 527–539
3. Vassylyev, D. G., Sekine, S., Laptenko, O., Lee, J., Vassylyeva, M. N., Borukhov, S., and Yokoyama, S. (2002) Nature 417, 712–719
4. Murakami, K. S., Masuda, S., Campbell, E. A., Muzzin, O., and Darst, S. A. (2002) Science 296, 1285–1290
5. Kuznedelov, K., Minakhin, L., Niedziela-Majka, A., Dove, S. L., Rogulja, D., Nickels, B. E., Hochschuld, A., Heyduk, T., and Severinov, K. (2002) Science 295, 855–857
6. Murakami, K. S., Masuda, S., and Darst, S. A. (2002) Science 296, 1280–1284
7. Young, B. A., Gruber, T. M., and Gross, C. A. (2002) Cell 109, 417–420
8. Severinov, K., Fenyo, D., Severinova, E., Mustaev, A., Chait, B. T., Goldfarb, A., and Darst, S. A. (1994) J. Biol. Chem. 269, 20826–20828
9. Murakami, K. S., and Darst, S. A. (2003) Curr. Opin. Struct. Biol. 13, 31–39
10. Bushnell, D. A., Westover, K. D., Davis, R. E., and Kornberg, R. D. (2004) Science 303, 983–988
11. Roeger, H., Bushnell, D. A., Davis, R., Griesenbeck, J., Lorch, Y., Straattan, J. S., Westover, K. D., and Kornberg, R. D. (2005) FEBS Lett. 579, 899–903
12. Pal, M., Ponticelli, A. S., and Luse, D. S. (2005) Mol. Cell. 19, 101–110
13. Borukhov, S., and Goldfarb, A. (1993) Protein Expression Purif. 4, 503–511
14. Kasslev, M., Martin, E., Polyakov, A., Severinov, K., Nikidovor, V., and Goldfarb, A. (1993) Gene (Amst.) 130, 9–14
15. Minakhin, L., and Severinov, K. (2003) J. Biol. Chem. 278, 29710–29718
16. Narayshkina, T., Mustaev, A., Darst, S. A., and Severinov, K. (2001) J. Biol. Chem. 276, 13308–13313
17. Severinov, K., Mustaev, A., Severinova, E., Kozlov, M., Darst, S. A., and Goldfarb, A. (1995) J. Biol. Chem. 270, 29428–29432
18. Artsimovitch, I., Patlan, V., Sekine, S., Vassylyeva, M. N., Hosaka, T., Ochi, K., Yokoyama, S., and Vassylyev, D. G. (2004) Cell 117, 299–310
19. Artsimovitch, I., Vassylyeva, M. N., Svetlov, D., Svetlov, V., Perederina, A., Igarrashi, N., Matsugaki, N., and Vassylyev, D. G. (2005) Cell 122, 351–363
20. Hsu, L. M., Vo, N. V., and Chamberlin, M. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11588–11592
21. Hernandez, V. J., Hsu, L. M., and Cashel, M. (1996) J. Biol. Chem. 271, 18775–18779
22. Nickels, B., Garrity, S. J., Melker, V., Minakhin, L., Severinov, K., Ebright, R. H., and Hochschild, A. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4488–4493
23. Zenkin, N., and Severinov, K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4396–4400

\(^3\) N. Zenkin, personal communication.