Supplementary Discussion

Discussion of prior work on NCIN hypothesis.

An early study reported incorporation of NAD^+ into initial products by *E. coli* RNAP *in vitro*[34]. However, the work involved only promoter-independent transcription on the homopolymer template poly(dA-dT):poly(dA-dT), did not directly demonstrate the identity of the initial products, and did not demonstrate the ability of the initial products to be extended to yield longer products[34]. Subsequent studies attempted, but failed, to detect the incorporation of NAD^+, NADH, and dpCoA into RNA during transcription initiation by *E. coli* RNAP[4,5]. However, a technical issue with assay conditions used in these studies--i.e., the use of ATP concentrations able to out-compete, and make undetectable, transcription initiation by NAD^+, NADH, and dpCoA--may render the negative results in this study non-determinative. Here, we re-examine the NCIN hypothesis using an assay of promoter-dependent transcription, directly assessing the identity of the initial products, directly assessing the ability of the initial products to be extended to yield longer products, and using assays that do not contain ATP concentrations able to out-compete NAD^+, NADH, and dpCoA.

Discussion of crystal structures of RPo-pppApC, RPo-NAD^+pC, RPo-dpCoApC.

A crystal structure of a substrate complex for transcription initiation, consisting of RPo, ATP as initiating nucleotide, and CMPcPP as extending nucleotide, previously has been reported[16,17]. However, no crystal structure of an initial product complex for transcription initiation previously has been reported. The crystal structure of the initial product complex obtained herein for intiation with ATP and CTP (RPo-pppApC) shows ordered density for all non-hydrogen atoms of pppApC (Figure 4a, left). The pppA residue of pppApC base pairs to the

DNA template strand in the RNAP active-center "i-1 site" and interacts, through its α-phosphate, with the same RNAP residues that interact with the γ-phosphate of the initiating nucleotide in the substrate complex (βQ688 and βH1237; residues numbered as in *E. coli* RNAP; [16,17] and, through its β- and γ-phosphates, with βR529 and βK1242 (Figure 4a, right). The pC residue of pppApC base pairs with the DNA template strand in the RNAP active-center "i site," making interactions, through its sugar and phosphate, with the RNAP catalytic Mg^{2+} ion [Mg^{2+} (I)], and RNAP residues, notably βK1065 and βK1073, that are identical to those made by the sugar and α-phosphate of the initiating nucleotide in the substrate complex (Figure 4a, right; [16,17]. Strikingly, the organization of the RNAP active center enables the same RNAP residues (βQ688 and βH1237) to interact with the α-phosphate of the initiating nucleotide in the product complex and the γ-phosphate of the initiating nucleotide in the substrate complex and enables the same RNAP residues (βK1065 and βK1073) to interact with the α-phosphate of the extending nucleotide in the product complex and the α-phosphate of the initiating nucleotide in the substrate complex.

The crystal structure of the initial product complex obtained upon initiation with NAD^+ and CTP (RPo-NAD^+pC) shows ordered density for all non-hydrogen atoms of NAD^+pC (Figure 4b, left). The NAD^+ residue of NAD^+pC base pairs to the DNA template strand in the RNAP "i-1 site," making interactions through its base and phosphates identical to those in the product complex for initiation with ATP (Figures 4a-b, right), and making additional interactions through its nicotinamide ribonucleoside moiety with RNAP βD516 and βH1237 (Figure 4b, right). The pC residue of NAD^+pC base pairs to the DNA template strand in the RNAP active-center "i site," making the same interactions as in the product complex for initation with ATP (Figures 4a-b, right).

The crystal structure of the initial product complex obtained upon intiation with dpCoA and CTP (RPo-dpCoApC) shows ordered electron density for the adenosine diphosphate moiety and two atoms of the pantetheine moiety of the dpCoA residue and for all atoms of the pC residue (Figure 4c, left). The dpCoA residue of dpCoApC base pairs to the DNA template strand in the RNAP "i-1 site," making interactions through its base and phosphates identical to those in the product complex for initiation with ATP (Figures 4a,c, right). The first two atoms of the pantetheine moiety project into a space having sufficient volume to accommodate the pantetheine moiety in multiple conformational states; terminal atoms of the pantetheine moiety are disordered, suggesting conformational heterogeneity (i.e., flexibility). The pC residue of dpCoApC base pairs to the DNA template strand in the RNAP active-center "i site," making the same interactions as in the product complex for initation with ATP (Figures 4a,c, right).

Discussion of structural basis of specificity at promoter position -1.

The crystal structures of product complexes in Figure 4 demonstrate NCIN-mediated initiation but do not explain the specificity of NAD^+-mediated initiation for an A:T base pair at promoter position -1. A definitive explanation for the specificity at promoter position -1 must await determination of an NAD^+-containing substrate complexes (i.e., RPo-NAD^+-NTP). However, model building suggests that, in an NAD^+-containing substrate complex, the NAD^+ nicotinamide moiety can be positioned to form a "pseudo-base pair" with template-strand T at position -1, forming two H-bonds with Watson-Crick H-bonding atoms of the template-strand thymine base at position -1 and stacking on the NAD^+ adenine base.

Supplementary Table 1. Oligonucleotides used to generate templates for transcription assays.

Name	Description	Sequence (5' to 3')
JB 221	For cloning NudC- XbaI forward primer	CAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAATGGATCGTATAATTGAAAAATTAGATCACGGC
JB 222	For cloning NudC- NotI reverse primer	GTGCTCGAGTGCGGCCGCGCTGCCGCGCGGCACCAGCTCATACTCTGCCCGACACATCGCCACCGT
JB 224	*gadY* template (-65 to +35) oligo used in Figures 1b, 2a and 2b	AGCGTATAGCTTATGTTTATAAAAAAATGGCTGATCTTATTTCCAGTAAAAGTTATATTTAACTTACTGAGAGCACAAAGTTTCCCGTGCCAACAGGGAG
JB 228	Forward primer for amplification of *T7A1* template	GATTAATTTAAAATTTATCAAAAAGAGTATTGAC
JB 229	Reverse primer for amplification of *T7A1* template	TCGTTGGGATGGCTA
JB 230	Forward primer for amplification of *gadY* template	AGCGTATAGCTTATG
JB 231	Reverse primer for amplification of *gadY* template	CTCCCTGTTGGCACGGGAAAC
JB 232	*N25* template (-65 to +35) oligo used in Figure 2b	ATCCGTCGAGGGAAATCATAAAAAATTTATTTGCTTTCAGGAAAATTTTTCTGTATAATAGATTCATAAATTTGAGAGAGGAGTTTAAATATGGCTGGTT
JB 233	Forward primer for amplification of *N25* template	ATCCGTCGAGGGAAATCATAAAAAATTTATTTGC
JB 234	Reverse primer for amplification of *N25* template	AACCAGCCATATTTAAACTCCTC
JB 235	*T7A1*(+2C,+3T) template (-65 to +35) oligo used in Figure 2b	GATTAATTTAAAATTTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCCaCTGAGAGGGACACGGCGAATAGCCATCCCAACGA
JB 244	*gadY*(+1G) template (-65 to +35) oligo used in Figure 2a	AGCGTATAGCTTATGTTTATAAAAAAATGGCTGATCTTATTTCCAGTAAAAGTTATATTTAACTTGCTGAGAGCACAAAGTTTCCCGTGCCAACAGGGAG
JB 281	Forward primer for amplification of *rnaI* templates in Figures 1b, 2a and 2b	CGAGGTATGTAGGCGGTGCTACAG
JB 288	*rnaI* template (-65 to +112, A-less) oligo used to make rnaI (-65 to +35) and (-65 to +112) templates in Figures 1b, 2a and 2b	CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACTTGTTTTGGTGTCTGCGCTCCTCCTTGCCTGTTTCCTCGGTTCTTTGTGTTGGTTGCTCTGTGTTCCTTCGTTTTTCCGCCCTGCTTGGCGGTTTTTTCGTTTTCTGTGC
JB 251	Reverse primer (+112) for amplification of rnaI (-65 to +112) A-less template used in Figure 1b	GCACAGAAAACGAAAAAACCGCCAAGCAGG

JB 287	*rnaI* (+1G) template (-65 to +112, A-less) oligo used to make *rnaI* (+1G) (-65 to +35) template used in Figure 2a	CGAGGTATGTAGGCGGTGCTACAGAGTTCTT GAAGTGGTGGCCTAACTACGGCTACACTAG AAGAGCTTGTTTTGGTGTCTGCGCTCCTCCT TGCCTGTTTCCTCGGTTCTTTGTGTTGGTTGC TCTGTGTTCCTTCGTTTTTCCGCCCTGCTTGG CGGTTTTTTCGTTTTCTGTGC
JB 269	Reverse primer to amplify *rnaI* (-65 to +35) and *rnaI* (+1G) (-65 to +35) templates used in Figures 2a and 2b	AACAGGCAAGGAGGAGCGCAG
JB 270	*N25* (+2C, +3T) template (-65 to +35) oligo used in Figure 2b	ATCCGTCGAGGGAAATCATAAAAAATTTATT TGCTTTCAGGAAAATTTTTCTGTATAATAGA TTCACTAATTTGAGAGAGGAGTTTAAATATG GCTGGTT
CK01639	Bubble template, template strand	TGAAGTCTTGTGTGGTCCTGAGAAAGTGTTG AGATCCATGACAGAAAGATTAATAATTGTA TGACTATTTATACGCGTCCTGT
CK01621	Bubble template, non-template strand (carries 5' biotin)	Biotin/ACAGGACGCGTATAAATAGTCATACA ATTATTAATCTTTCACGATCTTTCCTCAACAC TTTCTCAGGACCACACAAGACTTCA
JB 293	P_{rnaI} forward primer to amplify JB313 for cloning pJB89	GCCAGTCTAGACGAGGTATGTAGGCGGTGC TACAG
JB 303	MazF-mt3 cut site reverse cloning primer for cloning pJB89 and pJB91	CTATTCGGATCCAAGGAACG
JB 313	P_{rnaI} (+43 MazF-mt3 cut site) PCR template for cloning pJB89	CGAGGTATGTAGGCGGTGCTACAGAGTTCTT GAAGTGGTGGCCTAACTACGGCTACACTAG AAGAACAGTATTTGGTATCTGCGCTCTGCAC GATGGGTTAATTCGTTCCTTGGATCCGAATA G
JB 306	P_{T7A1} forward primer to amplify JB315 for cloning pJB91	GCCAGTCTAGAGATTAATTTAAAATTTATCA AAAAGAGTATTGAC
JB 315	P_{T7A1} (+43 MazF-mt3 cut site) PCR template for cloning pJB91	GATTAATTTAAAATTTATCAAAAAGAGTATT GACTTAAAGTCTAACCTATAGGATACTTACA GCCACAGTATTTGGTATCTGCGCTCTGCACG ATGGGTTAATTCGTTCCTTGGATCCGAATAG
JB 367	*rnaI (-1C)* template (-65 to +112, A-less) oligo used to make *rnaI (-1C)* (-65 to +35) template used in Figure 2c	CGAGGTATGTAGGCGGTGCTACAGAGTTCTT GAAGTGGTGGCCTAACTACGGCTACACTAG AAGCACTTGTTTTGGTGTCTGCGCTCCTCCTT GCCTGTTTCCTCGGTTCTTTGTGTTGGTTGCT CTGTGTTCCTTCGTTTTTCCGCCCTGCTTGGC GGTTTTTTCGTTTTCTGTGC