Supplementary Information

Structural basis of template strand deoxyuridine promoter recognition by a viral RNA polymerase

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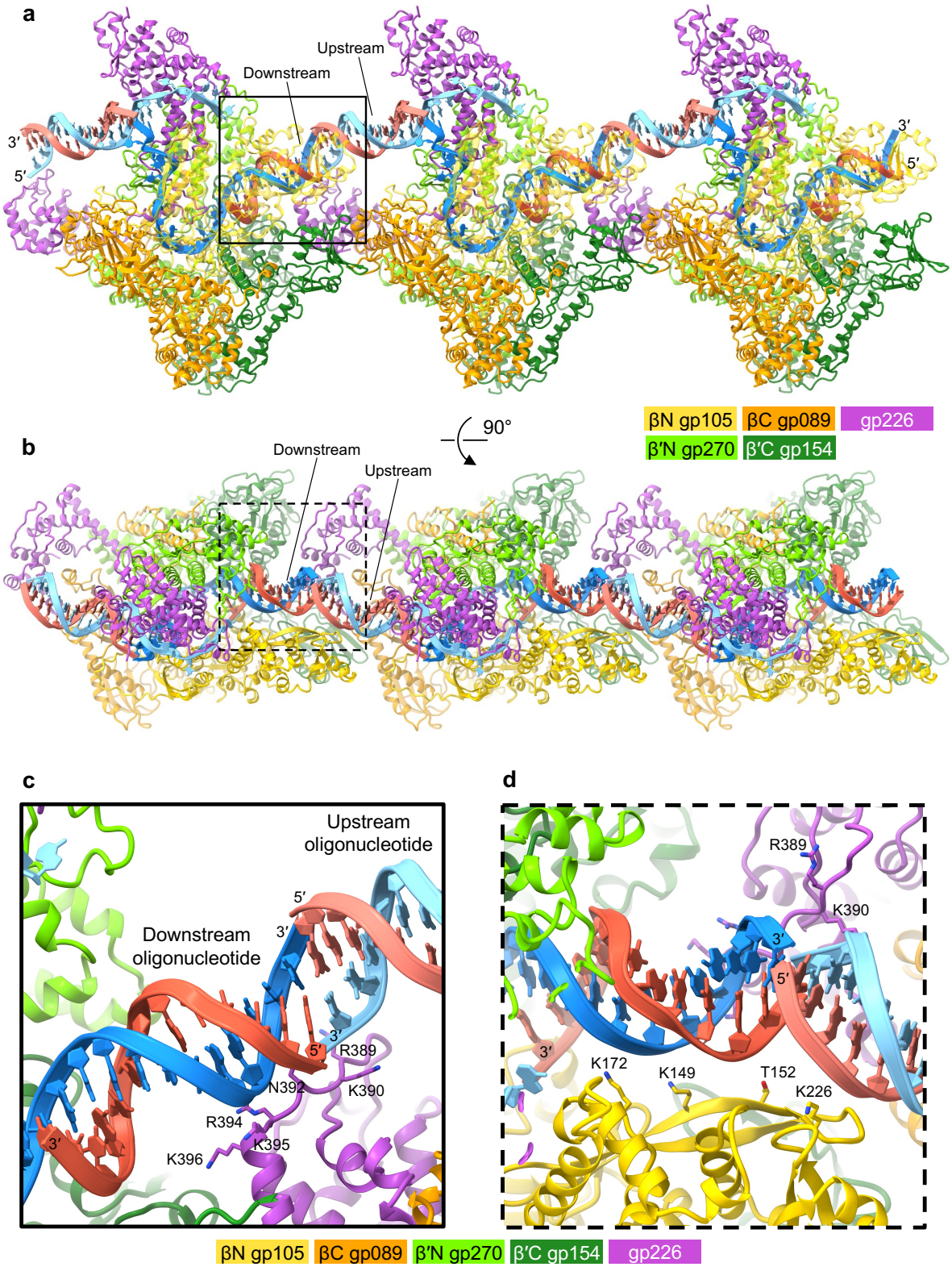
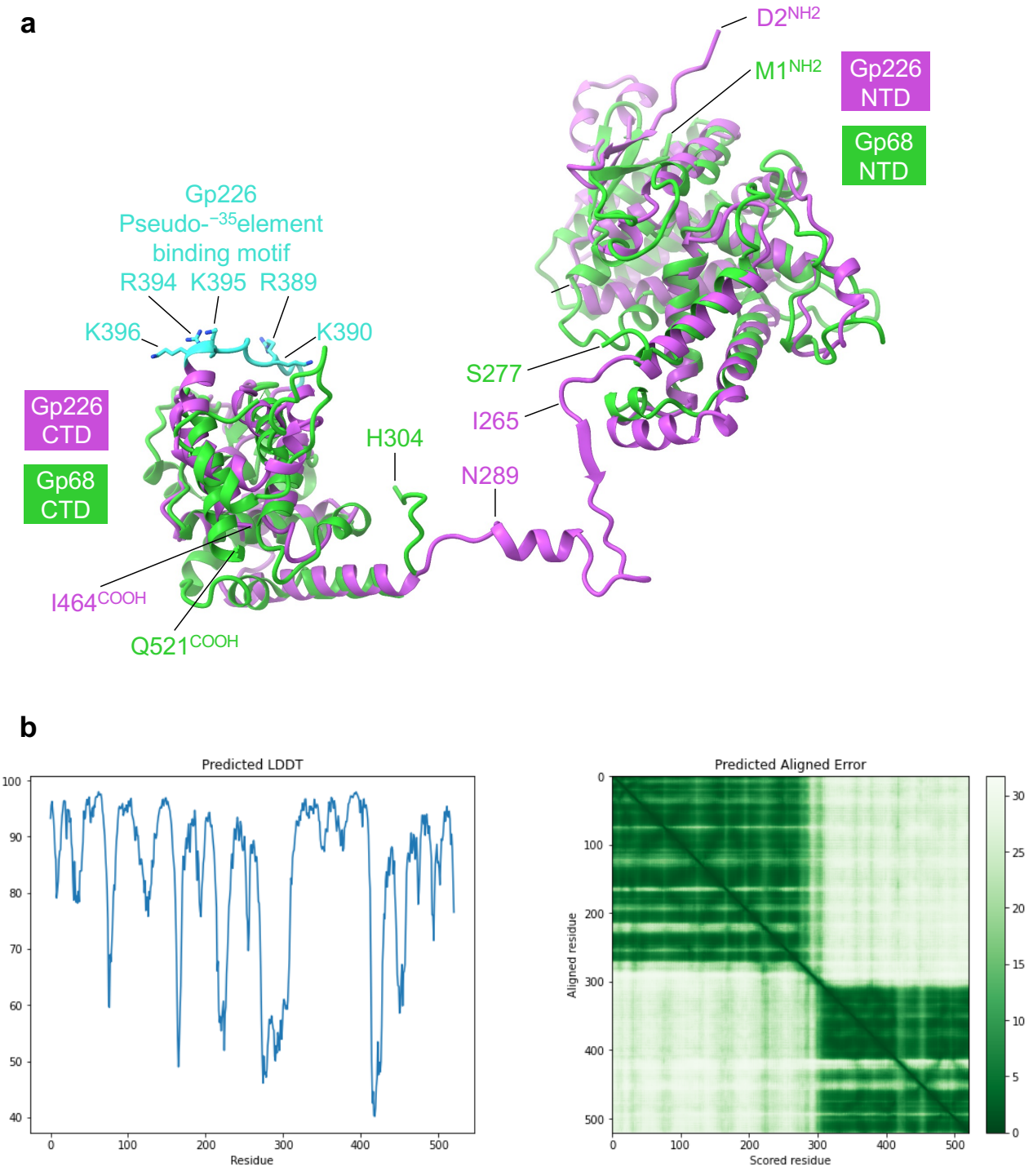


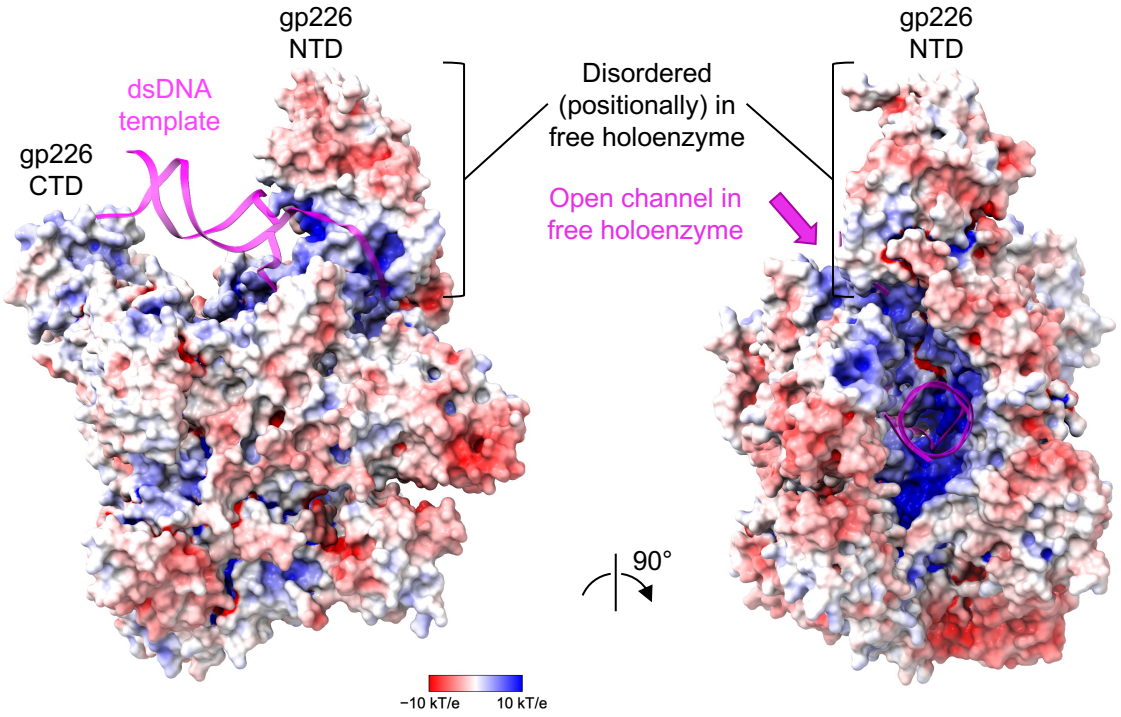
Supplementary Figure 1. AR9 nvRNAPs and up- and downstream oligonucleotides form a train *in crystallo*.

a and **b**, Two orthogonal views of three nvRNAPs demonstrating the peculiar crystal packing.

c and **d**, Two areas of interest indicated with a solid and dashed line square in panels **a** and **b** that show details of pi-pi stacking interactions between the ends of the up- and downstream oligonucleotides. Residues most proximal to the DNA are shown in a stick representation and labeled. In both panels, the color code is as in **Fig. 2a**.

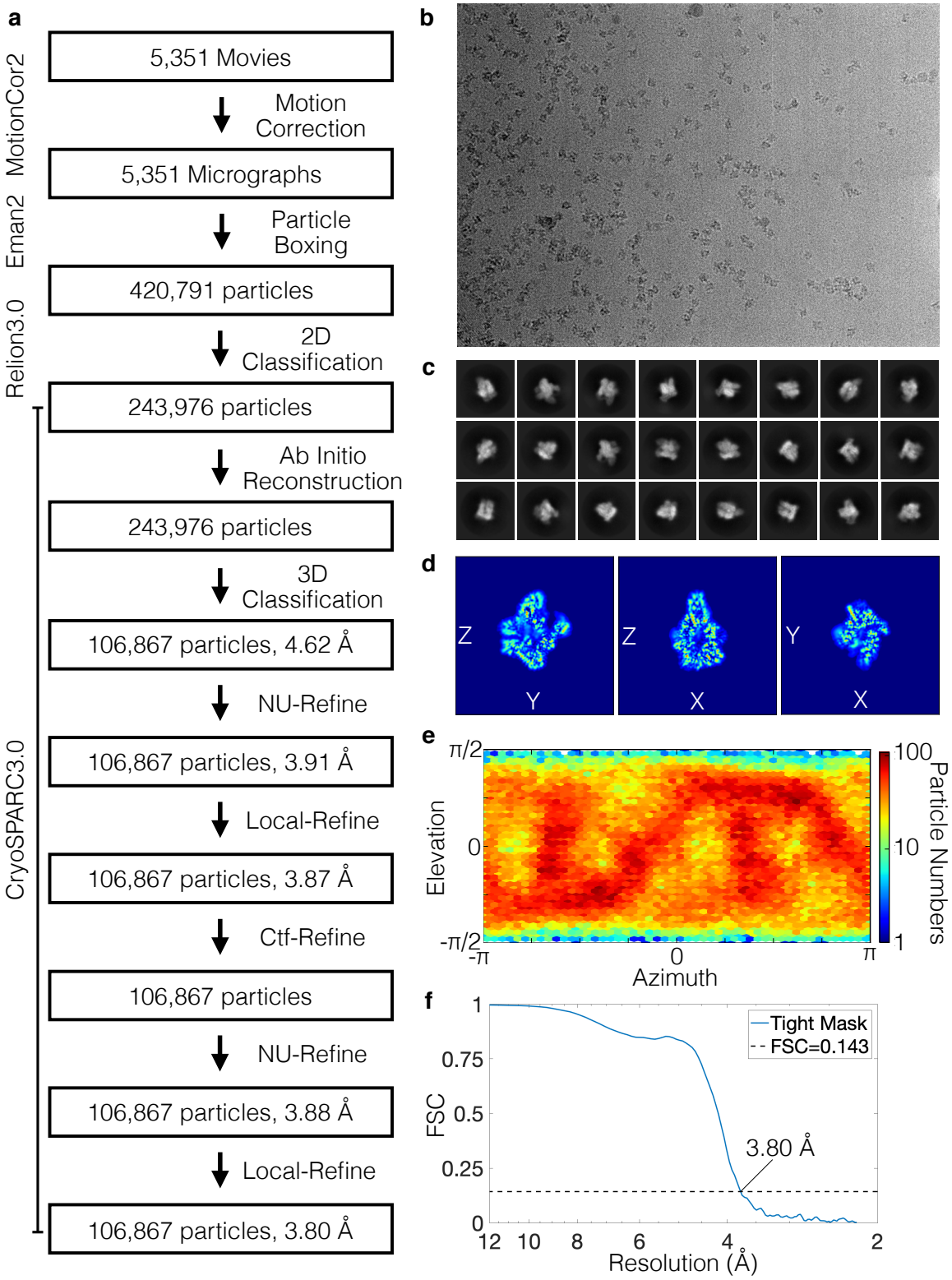


Supplementary Figure 2. Comparison of AR9 gp226 and phiKZ gp68 structures.
a, Superposition of phiKZ gp68 NTD and CTD onto gp226. The structure of gp68 NTD is predicted by AlphaFold Colab using default parameters. The structure of gp68 CTD is taken from the cryo-EM structure of phiKZ nvRNAP holoenzyme (PDB code 7OGP). The orientation of AR9 gp226 is as in **Extended Data Fig. 6**. Residue identities and numbers are given at strategic locations.
b, Quality factors of the gp68 model created by AlphaFold Colab. The entire sequence of gp68 was used. The accuracy of the gp68 NTD model (residues 1-277) can be evaluated by comparing the AlphaFold model of the gp68 CTD (residues 304-521) with its cryo-EM structure. The latter two can be superimposed with an RMSD of 1.2 Å for 191 equivalent Cα atoms (out of 203 ordered in the cryoEM structure).



Supplementary Figure 3. Distribution of electrostatic potential on the surface of the AR9 nvRNAP promoter complex.

The DNA (colored magenta) was excluded from the calculations. The orientation of the molecule is as in **Fig. 2a**.



Supplementary Figure 4. Cryo-EM image processing workflow for AR9 nvRNAP promoter complex.
a, Schematic illustrating image processing jobs, software, and numbers of micrographs/particles from motion correction to the final map.
b, Representative micrograph after motion correction.
c, 2D class averages representing 244k particles.
d, Three cross sections of the final map in the Z-Y, Z-X and Y-X planes.
e, 2D histogram (heat map) of particles based on their orientations (according to their azimuth and elevation).
f, Fourier shell correlation (FSC) curve of the tightly masked map, with a nominal resolution of 3.8 Å.

Figure 1c

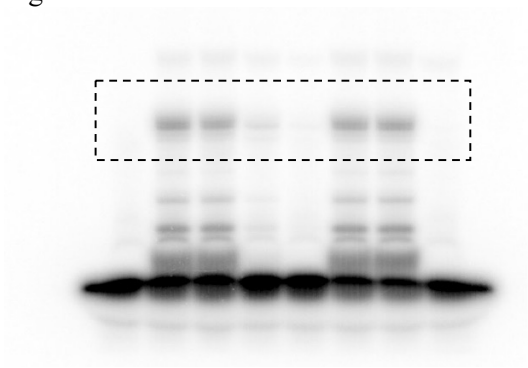


Figure 1d

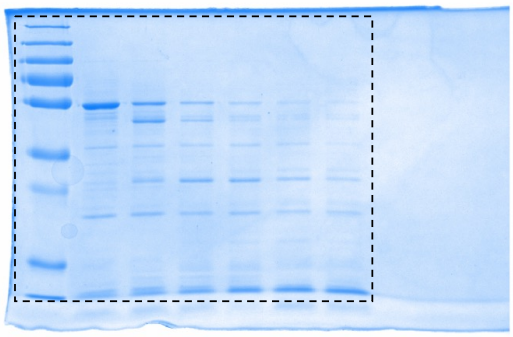


Figure 8b

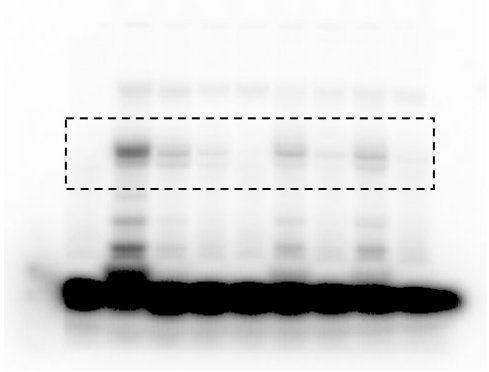


Figure 8f

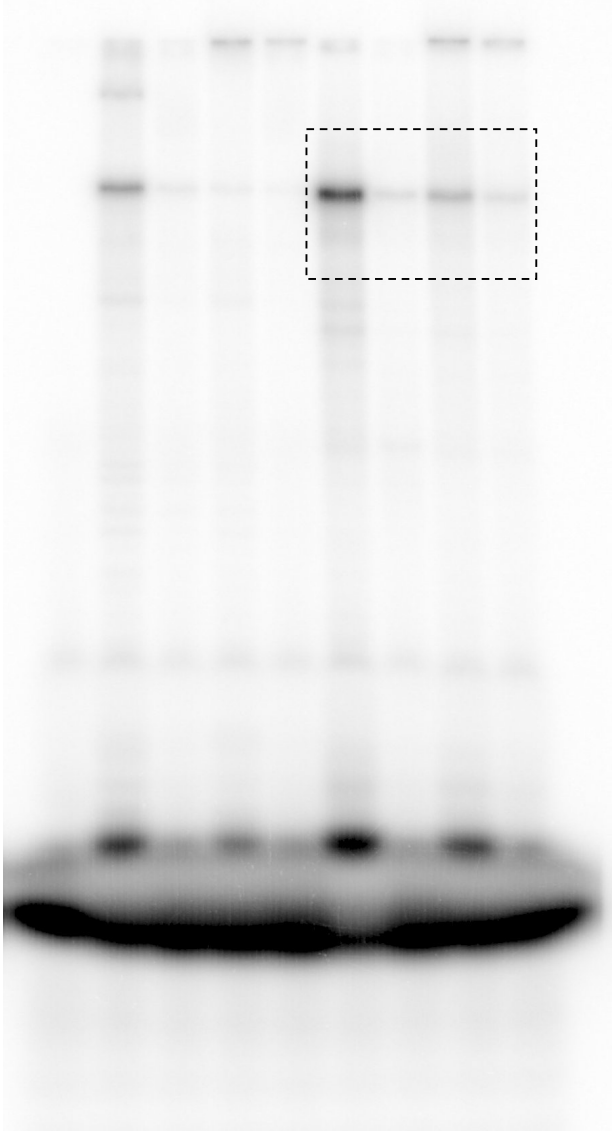


Figure 8d, top panel

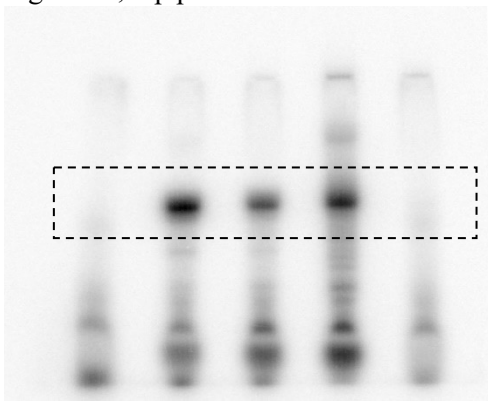
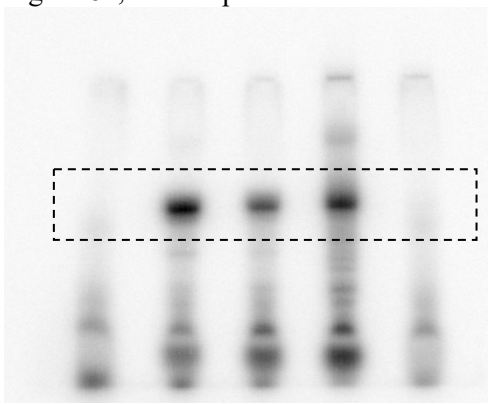


Figure 8d, bottom panel



Supplementary Figure 5. Source images of the gels used in main text figures with the cropped areas marked by dashed lines.

Supplementary Table 1. X-ray data collection and refinement statistic.

Dataset name and type	AR9 nvRNAP core Standard unit cell Native (PDB code: 7S00)	AR9 nvRNAP core Standard unit cell Thimerosal (Hg) derivative	AR9 nvRNAP core Standard unit cell Ta_6Br_{12} derivative	AR9 nvRNAP core Large unit cell Thimerosal (Hg) derivative	AR9 nvRNAP Promoter complex Native (PDB code: 7S01)
Number of molecules in the asymmetric unit	2	2	2	8	1
Data collection					
Beamline	APS 21-ID-G	APS 21ID-F	ALS 5.0.2	APS 21-ID-D	APS 21-ID-D
Detector	Rayonix MX-300	Rayonix MX-300	Dectris Pilatus3 6M 25Hz	Dectris Eiger 9M	Dectris Eiger 9M
Wavelength (Å)	0.97857	0.97872	1.25515	1.0050	0.91840
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	C2
Cell dimensions					
a, *b*, *c* (Å)	112.86, 166.27, 307.22	113.43, 169.51, 308.30	112.77, 171.51, 309.76	171.24, 231.78, 592.45	176.93, 110.46, 222.38
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 98.52, 90.00
Resolution (Å)	50.0-3.30 (3.50-3.30)*	50.0-3.60 (3.82-3.60)	50.0-4.53 (4.81-4.53)	50.0-3.79 (4.02-3.79)	50.0-3.38 (3.58-3.38)
R_{merge} (%)	12.3 (124.2)	18.2 (201.2)	20.6 (172.2)	20.9 (138.0)	15.1 (101.1)
I / σI	10.07 (1.17)	8.77 (1.13)	6.75 (1.07)	6.22 (1.02)	7.91 (1.13)
Completeness (%)	98. 6 (98.7)	99.7 (98.5)	99.6 (98.3)	99.4 (99.7)	95.3 (85.7)
Redundancy	4.55 (4.20)	6.95 (6.87)	6.25 (6.44)	6.30 (6.18)	3.33 (3.40)
$CC_{1/2}$	99.8 (42.1)	99.8 (54.8)	99.7 (47.1)	99.5 (52.4)	99.2 (65.0)
Refinement					
Resolution (Å)	49.4 – 3.30				50.0 – 3.40
No. reflections	86,075				56,728
R_{work} / R_{free}	0.2180 / 0.2580				0.2379 / 0.2921
No. atoms					
Protein	33,892				21,749
Ligand/ion	2 (Zn^{2+})				1,500 (DNA) / 96 (ions)
Water	0				0
B-factors (Å^2)					
Protein	154.65				126.15
Ligand/ion	154.85				239.32 (DNA) / 157.88 (ions)
Water	NA				NA
R.m.s. deviations					
Bond lengths (Å)	0.003				0.002
Bond angles (°)	0.57				0.462
Validation					
MolProbity score	1.46				1.34
Clashscore	8.48				6.24
Poor rotamers (%)	0.03				0.00
Ramachandran plot					
Favored (%)	98.30				98.07
Allowed (%)	1.70				1.93
Disallowed (%)	0.00				0.00

*Values in parentheses are for the highest resolution shell.

Supplementary Table 2. Cryo-EM data collection and refinement

	AR9 nvRNAP promoter complex (EMDB-24763)	AR9 nvRNAP holoenzyme (EMDB-24765)
Data collection and processing		
Magnification	80,000	130,000
Voltage (kV)	300	300
Electron exposure (e^{-}/Å^2)	43.7	43.2
Defocus range (μm)	−1 to −4	−1 to −3
Pixel size (Å)	1.09	1.08
Symmetry imposed	C1	C1
Initial particle images (no.)	420,791	227,577
Final particle images (no.)	106,876	104,471
Map resolution (Å)	3.8	4.4
FSC threshold	0.143	0.143
Map resolution range (Å)	2.5-5.5	3.1-6.1

Supplementary Table 3. Oligonucleotides used in the study.

PCR primers used for site-directed mutagenesis of gp226 (Figure 8b, 8d, 8f)		
Mutation	*Oligonucleotide name*	*5′-3′ sequence*
gp226 V206G	gp226-V206X-rev	aacatctgagtactttgtctcgaagacgcga
	gp226-V206G-dir	gagacaaagtactcagatgttggaatctggacgtaccttaaaaac
A^5 mutant (gp226 R389A, K390A, R394A, K395A, K396A)	gp226-R389X-rev	cacatttggtgcgatagcgcaggataaaatctc
	gp226-A-for	gctatcgcaccaaatgtggccgcgatgaacaacgctgcagcattagttgataaaatcatc
gp226 Y246A	gp226_Y246A_F	atctccgcgcttgacgttgtgatcaaacagaaattg
	gp226_Y246A_R	gtcaagcgcggagattaccgagctattgtgtttc
gp226 S245E	gp226_S245E_F	gtaatcgaataccttgacgttgtgatcaaacag
	gp226_S245E_R	aaggtattcgattaccgagctattgtgtttcaac
PCR primers used to create a plasmid for expression of the tagless version of AR9 nvRNAP core		
	Oligonucleotide name	*5′-3′ sequence*
	nvRNAP-tag-free-for-66.5	tttaactttaagaaggagatataccatggggaaaaaattatcgttaatcgatttcaac
	nvRNAP-tag-free-rev-72.1	cagcagcggtttctttaccagactcgagttatttttcatc
DNA oligonucleotides used for structure determination of AR9 nvRNAP promoter complex		
	Oligonucleotide name	*5′-3′ sequence*
	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand all U	ctccaatatgtgatataatatauguuuattg
DNA templates used to examine the dependence of *in vitro* transcription activity of the AR9 nvRNAP on the position and number of T bases in the promoter (Figure 1c)		
Name of the template in the figure	*Oligonucleotide name*	*5′-3′ sequence*
all U	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand all U	ctccaatatgtgatataatatauguuuattg
(-12)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-12)	ctccaatatgtgatataatatauguutattg
(-11)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-11)	ctccaatatgtgatataatatauugutuattg
(-10)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-10)	ctccaatatgtgatataatatauugtuuattg

(-8)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-8)	ctccaatatgtgatataatatautguuuattg
(-7)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-7)	ctccaatatgtgatataatatatuguuuattg
all T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand all T	ctccaatatgtgatataatatattgtttattg
DNA templates used to examine the *in vitro* transcription activity of the AR9 nvRNAP gp226 V206G mutant (Figure 8b)		
Name of the template in the figure	*Oligonucleotide name*	*5′-3′ sequence*
all U	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand all U	ctccaatatgtgatataatatauuguuuattg
(-11)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-11)	ctccaatatgtgatataatatauugutuattg
(-10)T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand T(-10)	ctccaatatgtgatataatatauugtuuattg
all T	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand all T	ctccaatatgtgatataatatattgtttattg
DNA templates used to examine the *in vitro* transcription activity of the AR9 nvRNAP gp226 Y246A and gp226 S245E mutants (Figure 8d)		
Name of the template in the figure	*Oligonucleotide name*	*5′-3′ sequence*
ds DNA	[-16;+16] non-template strand	caataaacaatatattatatcacatattggag
	[-16;+16] template strand all U	ctccaatatgtgatataatatauuguuuattg
fork DNA	[+3;+16] non-template strand	atcacatattggag
	[-16;+16] template strand all U	ctccaatatgtgatataatatauuguuuattg
PCR primers that were used for PCR amplification of genomic DNA fragments to examine the *in vitro* transcription activity of the A^5 mutant (Figure 8f)		

Name of the template in the figure	*Oligonucleotide name*	*5′-3′ sequence*
[-60;+80] DNA	P077-for-UP-60-63.4	taatcctcctacttatctagtctataattaattgttg
	P077-rev-ROff-80-61.9	attgcttcattaaacataaatgaagactc
[-16;+80] DNA	P077-for-UP-16-60.4	caataaacaatatattatatcacatattggagg
	P077-rev-ROff-80-61.9	attgcttcattaaacataaatgaagactc

Supplementary Table 4. Summary of MD simulation configurations and results.

Free energy term	Simulation Name	Window Number	Window Steps ($\times 10^3$)	Equilibration Steps per Window ($\times 10^3$)	Total Time (ns)	Energy Value (kcal/mol)
	DNA-RNAP Alchemical	400	40	8	64	705.7 ± 2.3
	DNA (bulk water) Alchemical	400	150	30	240	693.0 ± 0.4
It is a sum of seven energy terms with a combined (propagated) error	DNA-RNAP Constraint: r	20	600	120	48	0.3 ± 0.0
	DNA-RNAP Constraint: ϕ	20	60	12	4.8	0.3 ± 0.0
	DNA-RNAP Constraint: θ	20	300	60	24	0.9 ± 0.4
	DNA-RNAP Constraint: χ	20	60	12	4.8	0.2 ± 0.0
	DNA-RNAP Constraint: ψ	20	60	12	4.8	1.0 ± 0.0
	DNA-RNAP Constraint: ξ	20	60	12	4.8	0.9 ± 0.0
	DNA-RNAP Constraint: RMSD	20	600	120	48	3.3 ± 1.4
	DNA (bulk water) Constraint: RMSD	19	10,000	2,000	760	12.7 ± 0.1